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ROS-Mediated Amplification of AKT/mTOR Signaling Pathway Leads to Myeloproliferative Syndrome in Foxo3-/- Mice

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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 05 July 2010

Thank you very much for submitting your research paper for consideration to The EMBO Journal editorial office. Three expert referees initially agreed to assess merits and suitability of your paper for our journal. I did receive two rather consistent reports by now. Given that I have no indication from the third referee to provide comments despite multiple chasers, I am thus making a decision based on the comments of these two scientists to prevent further unnecessary delay and enable efficient proceedings for your study. As you will see, ref#1 raises some concerns that might need some further experimentation. S/he mostly refers to a better integration of the mTOR/rapamycin versus the Lnk-effects and requests specificity controls for NAC on ROS, respective Lnk-expression. Further, evaluation of Lnk-protein, including modulation upon NAC as well as Foxo3 re-expression would be beneficial to confirm the circuitry you propose. Rather more importantly, ref#2 demands to specifically discuss phenotypic differences between your and earlier Foxo3-abrogation studies and potential explanations for the distinct phenotypes. Given the overall relatively positive assessment, we are happy to offer you the chance to thoroughly revise the current paper during our usual timeframe of a single round of revision. Please be reminded that it is EMBO_J policy to allow a single round of revision only and that the ultimate decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript.

Yours sincerely,

Editor

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REFEREE REVIEWS

Referee #1 (Remarks to the Author):

This is an interesting study that brings together several threads including ROS, foxo3, myeloproliferation, and Lnk. The main findings are that ROS accumulate in Foxo3 hematopoietic progenitor cells, resulting in myeloproliferation. This effect is reduced by NAC and the phenotype is the result of ROS-medated repression of Lnk, a negative regulator of cytokine signalling. The study is reasonably well-executed although some issues need to be addressed. The findings are of broad interest. Perhaps the greatest weakness is that the role of mTOR signalling is not well-integrated into the rest of the study, nor is the significance of the rapamycin effects vis a vis Lnk. Specific comments follow-

pg. 7 The statement that Foxo3 mice have reduced RBCs but are not anemic is confusing. It has been shown that Foxo3 RBCs have a short half-life and the mice are slightly anemic. This suggests that the observed increase in Ter119 erythroid precursors is compensatory. The authors should discuss these issues and include RBC counts/hematocrit data in Table 1.

Fig 4/5. Can the authors show that NAC results in decreased ROS per the assays shown in Fig. 3? This would exclude the possibility that NAC has some non-specific effects. Along these lines, have the authors performed experiments where cells were stressed by treatment with ROS-inducing agents? Do such treatments result in Lnk repression?

pg. 14. The authors need to show that the Lnk protein is reduced, not just the mRNA and also that NAC treatment results in increased Lnk protein (again, not just mRNA).
The studies with enforced retroviral-Lnk are interesting, but the authors need to show that enforced expression of Foxo3 in Foxo3-/- hematopoietic progenitors results in Lnk induction.

Referee #2 (Remarks to the Author):

The manuscript by Yalcin et al. describes a myeloproliferative syndrome in mice lacking Foxo3a, and defines the molecular mechanism in detail. They show that ROS levels are elevated in myeloid progenitors and this correlates with elevated signaling through AKT and mTOR. Aspects of the cellular and molecular phenotype can be corrected by treatment in vivo with either rapamycin or the ROS scavenger NAC. An important observation is that the Foxo3a-deficient myeloid progenitors have a specific reduction in mRNA expression of Lnk, a negative regulator of cytokine signaling. They show that re-expression of Lnk corrects the expansion of Foxo3a-deficient progenitors in a CFU-S assay. While the results build on and are consistent with previous literature, the findings are important and novel. Furthermore, the experiments are generally thorough and well controlled.

I have one substantive criticism. In the Discussion, the authors mention the 2004 paper from Lin et al. that reported a lymphoproliferative defect in Foxo3a-gene trap mice. The text implies that the similarities of the present result support the general conclusions. In fact, the observations are quite different and need to be explained. Is the different cellular mechanism (T lymphoid vs. myeloid) a result of the different targeting strategies? Furthermore, have the authors actually investigated whether their strain exhibits T lymphoproliferation and autoimmune infiltrates? This is particularly important considering the data on extramedullary hematopoiesis. Are those really hematopoietic progenitors in the splenic red pulp and liver, or could they be activated T cells?

I have some other minor points that should be addressed:
1. Some of the graphs show representative experiments. This is OK, but statistical convention dictates that intra-assay replicates should not be depicted with error bars.
2. In Figure 3, the FACS histograms show clearly that there is an increase in the fraction of ROS-high cells, rather than a general increase in ROS in all cells. Therefore, I suggest that the data be re-plotted as "% ROS-hi" cells. The authors might also want to discuss the presence of two distinct populations (ROS-lo and ROS-hi) that exist in both WT and Foxo3a-deficient, and how that impacts

the interpretation of the molecular mechanism.
3. Near the bottom of page 9, the authors begin the AKT/mTOR data analysis with "To our surprise". This is not necessary and probably not warranted, since increased ROS might indeed be expected to increase activity of AKT/mTOR through inhibition of phosphatases.
4. In many of the bar graphs (Fig. 4C and Fig. 6), it is hard to figure out which groups are being compared statistically and which p values match which comparisons. I would guess that there are two brackets denoting two comparisons, but it looks like one long bracket with three different possible comparisons.
5. There are a couple of redundant sentences on p. 12 in the description of cell cycling and apoptosis data.

Additional Correspondence 06 July 2010

I eventually received a report from the third referee that I enclose below for your information. Contrasting the rather more positive referees #1 and #2, these comments emphasize the need to illustrate and possibly explain phenotypic differences to earlier FOXO-depletion studies. Also, working out the proposed mechanism (as outlined already in the initial decision letter as similarly broad up by ref#1 and #2) seems key to experimentally strengthen your current proposal and thus the novelty of your study.

All in all, I am happy to maintain our current decision and look very much forward to your revised version.

with best regards

REFEREE REVIEW

The study by Yalcin et al. studies the role of FoxO3 in the hematopoietic system by analysing mice deficient for this protein. Their data suggest that loss of FoxO3 causes myeloproliferative disease with splenomegaly associated with an increased number of hematopoietic progenitors. These progenitors appear to be hypersensitive to cytokines and show increased ROS levels, increased AKT-mTOR signalling and a relative deficiency of Lnk, a negative regulator of cytokine receptor signalling. These defects can be rescued by the treatment with NAC. The role of FoxO3 in the hematopoietic system has already been reported in several papers including Tothova et al., 2007, Cell; Miyamaoto et al., Cell Stem Cell 2007 to name the most important ones. The current paper by Yalcin et al., is disappointing, as it does not add any significant additional information. Moreover, the authors in part over interpret their primary data. Thus this study should be reported in a more specialized journal.

Specific comments:
- The effects on the myeloid compartment (increased cell numbers and colony forming cells) is at odds with the study by Miyamoto et al 2007. The authors should comment on this.
- While the authors always refer to "general" cytokine signalling, they only tested IL-3 and moreover this is only examined in cultured cells.
- The only novel aspect is the part exploring a possible link to the Lnk kinase, however these data remain very preliminary.
- the paper is hard to read and would profit from editing by a native English speaker

1st Revision - authors' response 12 October 2010

We would like to thank the reviewers for their thoughtful comments that guided us in our revision and have enhanced the clarity and strengthened and significantly improved the manuscript.

Figures 4C right panel, 6D right panel, 7B and 7C, 8D, 9B and 9C and Supplemental Figures 7, 8, 9 and 10 are all new.

General Comments:

(1) A better integration of the mTOR/rapamycin versus the Lnk-effects.
We have now performed experiments (Figures 8D, 9C and Supplemental Figures 7 and 9) to address the impact of Lnk on AKT/mTOR signaling pathway and the effect of mTOR on Lnk expression and have found that Lnk expression reduced the activation of AKT/mTOR signaling in response to IL-3. In addition, we found that treatment of bone marrow with the mTOR inhibitor rapamycin induced the expression of Lnk.

(2) Request for specificity controls for NAC on ROS:
These controls are now included in Figures 4C and 6D in addition to Figure 3.

(3) Western blot of Lnk expression:
We have been able to show reduced expression of endogenous Lnk in Foxo3-/- bone marrow cells as compared to wild type controls (Figure 7B) using commercial antimouse Lnk polyclonal antibodies. We also show that NAC treatment increases Lnk protein expression (Figure 9B) in addition to Lnk mRNA (Figure 9A) in Foxo3-/- cells.

(4) Phenotypic differences between our and earlier Foxo3-abrogation studies and potential explanations for the distinct phenotypes:
We have summarized the comparison differences in the bottom of page 19/top of page 20 and in the first response to referee #2: consistent with a previous report (Dejean et al, 2009), we did not find any T cell activation or lymphoproliferation at the steady state in Foxo3-/- mice.

Response to Referees:

Referee #1:

"Perhaps the greatest weakness is that the role of mTOR signalling is not wellintegrated into the rest of the study, nor is the significance of the rapamycin effects vis a vis Lnk."

This concern has now been addressed in two sets of experiments:

First we investigated the effect of Lnk, a negative regulator of cytokine receptor signaling, on mTOR phosphorylation. We found that overexpression of Lnk significantly reduces the IL-3-induced phosphorylation of AKT, mTOR and S6 ribosomal protein (an S6K1 substrate) in primitive myeloid progenitor cells (Figure 8D, Supplemental Figure 7). Next, we evaluated the effect of mTOR inhibitor rapamycin, on Lnk. In vitro treatment of bone marrow cells with rapamycin more than doubled Lnk expression (Figure 9C). These findings suggest that mTOR signaling represses Lnk expression and that there is a cross regulation between Lnk and mTOR. This might be mediated by rapamycinmediated reduction of ROS levels in Foxo3-/- primitive myeloid progenitor cells (Supplemental Figure 9).

Specific comments

pg. 7 The statement that Foxo3 mice have reduced RBCs but are not anemic is confusing. It has been shown that Foxo3 RBCs have a short half-life and the mice are slightly anemic. This suggests that the observed increase in Ter119 erythroid precursors is compensatory. The authors should discuss these issues and include RBC counts/hematocrit data in Table 1.

For the sake of clarity and to avoid any confusion we have now removed "in the absence of anemia". We had previously shown (Marinkovic et al., JCI, 2007) that

despite shortened RBC lifespan, Foxo3 mice have normal hemoglobin concentrations and therefore do not meet the definition of anemia that is a reduction in oxygen-carrying capacity of the blood. We attributed this condition as the reviewer pointed out, to a compensatory mechanism. We had reported RBC counts and hematocrits data in (Marinkovic et al., JCI, 2007) that we have discussed in the first section of results reported in the present manuscript (bottom of page 6).

Fig 4/5. Can the authors show that NAC results in decreased ROS per the assays shown in Fig. 3? This would exclude the possibility that NAC has some nonspecific effects. Along these lines, have the authors performed experiments where cells were stressed by treatment with ROS-inducing agents? Do such treatments result in Lnk repression?

The reduction of ROS after NAC treatment was shown only in Figure 3 but was indeed verified for most experiments. We now report the results for Fig4 and Fig 6D as well (we had not measured ROS in experiments reported in Figure 5). In addition, to induce ROS as a stressor, we treated bone marrow cells with hydrogen peroxide and measured its effect on Lnk expression (Supplemental Figure 8). These experiments showed that hydrogen peroxide reduces expression of Lnk, although these results not surprisingly were dependent on concentrations of H2O2.

pg. 14. The authors need to show that the Lnk protein is reduced, not just the mRNA and also that NAC treatment results in increased Lnk protein (again, not just mRNA).
The studies with enforced retroviral-Lnk are interesting, but the authors need to show that enforced expression of Foxo3 in Foxo3-/- hematopoietic progenitors results in Lnk induction.

We were able to show that Lnk protein is reduced in Foxo3-/- bone marrow cells (Figure 7B). In addition, treatment with NAC increased the expression of Lnk protein (Figure 9B) in addition to Lnk mRNA (Figure 9A).

Please note that to detect endogenous Lnk protein in primary bone marrow cells using available commercial anti-Lnk antibodies, we had to use over 100 g of protein. That is perhaps why most reports on Lnk expression in the bone marrow (Bersenev et al, 2010; Takaki et al, 2002; Tong & Lodish, 2004; Velazquez et al, 2002) require either overexpressing recombinant Lnk or immunoprecipitation of endogenous Lnk without being able to verify the efficiency of immunoprecipitation..

We have now performed experiments in which Foxo3 was overexpressed in primitive hematopoietic cells and have shown an increase in Lnk mRNA expression (Figure 7C). Given the limited material in these experiments (primitive bone marrow cells transduced with Lnk), we could not perform Western blot analysis of Lnk.

Referee #2:

I have one substantive criticism. In the Discussion, the authors mention the 2004 paper from Lin et al. that reported a lymphoproliferative defect in Foxo3a-gene trap mice. The text implies that the similarities of the present result support the general conclusions. In fact, the observations are quite different and need to be explained. Is the different cellular mechanism (T lymphoid vs. myeloid) a result of the different targeting strategies? Furthermore, have the authors actually investigated whether their strain exhibits T lymphoproliferation and autoimmune infiltrates? This is particularly important considering the data on extramedullary hematopoiesis. Are those really hematopoietic progenitors in the splenic red pulp and liver, or could they be activated T cells?

We thank the reviewer for pointing out this awkward statement. In our comparison with the Lin s data we were referring to Foxo3 being the main regulator of progenitors in the

hematopoietic system. We do agree with the reviewer that the phenotype is totally different. Similar to (Dejean et al, 2009), we did not find any T cell activation at the steady state in young Foxo3-/- mice. The circulating lymphocyte number in Foxo3-/- peripheral blood was not increased as compared to controls (Table 1). The frequency and total number of Foxo3-/- T lymphocytes in the bone marrow was similar to controls and although the total number of splenocytes has increased, the frequency of CD3, CD4 and CD8 T cells in the Foxo3-/- spleen and their differentiation were normal and we have not seen thymic abnormalities in Foxo3-/- mice (Figure 1, Supplemental Figure 1, Supplemental Figure 10 and data not shown). There is a minimal depletion of marginal zone lymphocytes in Foxo3-/- spleen where the T-cell regions are retained (Figure 1A). The red pulp and liver of young Foxo3-/- mice contain myeloid cells as analyzed by our pathologist collaborator Dr. Sellers of Albert Einstein School of Medicine (Figure 1A and Supplemental Figure 2). In contrast, there is an increase of myeloid progenitors in the spleen of young (10-12 week old) Foxo3-/- mice that consist of granulocytic macrophage colony-forming cells (Figures 1-2, Supplemental Figures 1 and 10) accompanied with increased neutrophils in Foxo3-/- peripheral blood (Table 1). Thus consistent with a previous report, we have concluded that young Foxo3-/- mice do not exhibit a T lymphoproliferation and do not have any lymphocytic infiltrations of organs at the steady state (Dejean et al, 2009). The T lymphoproliferation seen in Foxo3 gene-trap mice (Lin et al, 2004) may be due, as has been suggested by (Dejean et al, 2009) to the mixed strain, or to the strategy used to generate these mice. We don t know whether there are myeloid abnormalities in the Foxo3 gene-trap mice. We have now discussed this in our manuscript (bottom of page 19 and top of page 20).

I have some other minor points that should be addressed:
1. Some of the graphs show representative experiments. This is OK, but statistical convention dictates that intra-assay replicates should not be depicted with error bars.

We agree with this statement of the reviewer which has been discussed in length in an article in JCB by (Cumming et al, 2007). As recommended by Cumming et al., in our in vivo experiments “n” “refers to independent results obtained from individual” wild type and Foxo3-/- mice subjected to various manipulations. “The n does not refer to multiple measurements on one individual in a single condition or multiple measurements of the same sample”. Thus we showed the results with error bars according to (Cumming et al, 2007). In our in vitro experiments, n represents the number of experiments and we have shown the mean of multiple experiments. We have now made this clear in legends.

2. In Figure 3, the FACS histograms show clearly that there is an increase in the fraction of ROS-high cells, rather than a general increase in ROS in all cells. Therefore, I suggest that the data be re-plotted as "% ROS-hi" cells. The authors might also want to discuss the presence of two distinct populations (ROS-lo and ROS-hi) that exist in both WT and Foxo3a-deficient, and how that impacts the interpretation of the molecular mechanism.

We have measured ROS high (ROS-hi) cells in all our experiments according to the gate shown in Figure 3. We have now made this clear in Figure 3 and also Figures 4C and 6D as well as Supplemental Figure 9. We have discussed ROS lo versus hi and have reported the results accordingly (First paragraph page 9 as well as in last sentence of the second paragraph page 16).

3. Near the bottom of page 9, the authors begin the AKT/mTOR data analysis with "To our surprise". This is not necessary and probably not warranted, since increased ROS might indeed be expected to increase activity of AKT/mTOR through inhibition of phosphatases.

We were referring to the impact of ROS on primary cell signaling that was surprising to us. We have now modified this sentence.

4. In many of the bar graphs (Fig. 4C and Fig. 6), it is hard to figure out which

groups are being compared statistically and which p values match which comparisons. I would guess that there are two brackets denoting two comparisons, but it looks like one long bracket with three different possible comparisons.

In Figure 4C there are three groups with three brackets; all panels of Figure 6 (but Figure 6D) have two groups with two brackets. We have now changed the position of the brackets to make this clear.

5. There are a couple of redundant sentences on p. 12 in the description of cell cycling and apoptosis data.

We have now corrected this.

Referee #3

The role of FoxO3 in the hematopoietic system has already been reported in several papers including Tothova et al., 2007, Cell; Miyamaoto et al., Cell Stem Cell 2007 to name the most important ones. The current paper by Yalcin et al., is disappointing, as it does not add any significant additional information. Moreover, the authors in part over interpret their primary data. Thus this study should be reported in a more specialized journal.

In contrast to the referee s assertion Tothova et al reported that there was no hematopoietic abnormalities in Foxo3 conditional knock out mice whereas while Miyamoto et al. reported that Foxo3 null hematopoietic stem cell compartment was abnormal they could not find any abnormalities in Foxo3-/- hematopoietic progenitor compartment. We discussed this in bottom of page 19 and top of page 20.

Specific comments:

- The effects on the myeloid compartment (increased cell numbers and colony forming cells) is at odds with the study by Miyamoto et al 2007. The authors should comment on this.

We have explained these results in the bottom half of page 19 and in (Yalcin et al, 2008).

While the authors always refer to "general" cytokine signalling, they only tested IL-3 and moreover this is only examined in cultured cells.

We have tested signaling by Epo and G-CSF in addition to IL-3 and results are similar to the ones with IL-3 (see Supplemental Figure for Referees; reported for Epo in Blood (ASH Annual Meeting Abstracts), Nov 2008; 112: 3870). Concerning “signaling being examined in cultured cells”:
Mice were treated in vivo with NAC for several days before primary cells were isolated and starved in culture for two hours and stimulated with IL-3 (that is a common approach for interrogating cytokine receptor signaling).

The only novel aspect is the part exploring a possible link to the Lnk kinase, however these data remain very preliminary.

Our additional experiments (Figure 8D, Figure 9B and 9C, and Supplemental Figures 7 and 9) further confirm our initial findings and strongly suggest that Lnk adaptor protein (and not kinase) is a negative regulator of AKT and mTOR signaling. Furthermore, Our data also suggest that mTOR negatively regulates Lnk in Foxo3-/- cells. Together these findings indicate that Lnk is regulated by ROS and there is a cross-talk between Lnk and mTOR signaling pathway.

the paper is hard to read and would profit from editing by a native English speaker

We thank the reviewer for this comment and have had the paper reviewed by a native English speaking colleague (Mitch Weiss, M.D.-Ph.D., University of Pennsylvania).

References

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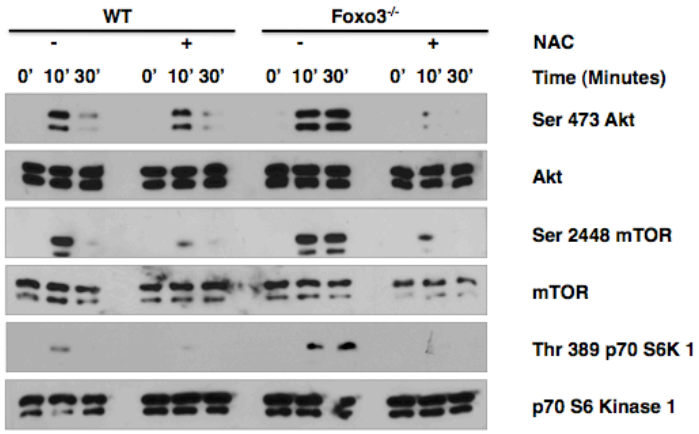
Supplemental Figure For Referees



A) Western blot analysis of phosphorylation of signaling proteins in lineage negative bone marrow cells isolated from wild type and Foxo3$^{-/-}$ mice. Mice were administered daily with NAC (100mg/kg) or PBS *in vivo* for 3 days. Lineage negative cells were starved from serum and cytokines for 2 hours followed by Epo stimulation (10 U/ml) for the indicated time points (0, 10 and 30 minutes).

Additional Correspondence 25 October 2010

One of the original referees has just come back with only positive remarks about the revisions on your paper.

Correspondingly, the editorial office will soon be in touch for official acceptance and necessary post-acceptance paperwork.

Congratulations to a fine piece of work.

yours truly

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