THE EMBO JOURNAL

Manuscript EMBO-2011-77876

FBF represses the Cip/Kip cell cycle inhibitor CKI-2 to promote self-renewal of germline stem cells

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Review timeline:

Submission date:	20 April 2011
Editorial Decision:	14 May 2011
Revision received:	23 June 2011
Editorial Decision:	07 July 2011
Revision received:	08 July 2011
Accepted:	11 July 2011

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 14 May 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees express interest in your work and are broadly in favour of publication here, pending satisfactory revision. The most critical point here is that raised by both referees 1 and 2 regarding the need for better markers to demonstrate the rescue of the fbf1/2- phenotype upon loss of cki2. Regarding referee 2's point 4, I would encourage you to perform the requested experiment, but it would certainly not be necessary to try and identify alternative cki2 regulators that may be important at the L4 stage. I hope that addressing the concerns raised should be relatively straightforward, but please don't hesitate to get in touch if you have any questions or comments about the revision.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

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

Best wishes,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This study addresses the integration of cell cycle control with cell fate decisions, specifically the choice between mitosis and meiotic entry in the C. elegans germ line. The work also addresses the question of how cell cycle regulation is coordinated with stem cell maintenance. The authors use genetic and molecular methods to examine the role of the cyclin-dependent kinase inhibitors, CKI-1 and CKI-2, in the germ line. They demonstrate that repression of CKI-2 is important for maintenance of germline proliferation and, conversely, expression of CKI-2 is important for meiotic entry. They demonstrate that CKI-2 repression occurs via activity of the FBF translational regulator, and also implicate MEX-3 as an additional repressor of cki-2 expression. Their genetic and molecular data are compelling. In addition, they demonstrate physical binding of FBF-2 to the putative FBF binding elements in the cki-2 3' UTR. Their data lead to a model whereby cyclin E/CDK-2 activity promotes mitosis in the distal germ line, and up regulation of CKI-2 as cells move proximally leads to repression of cyclin E/CDK-2 activity and, consequently, entry into meiosis.

Data in this paper are consistent with two other recent studies implicating cyclin E/ CDK2 activity as important for maintenance of germline proliferation by Jeong et al. (2011) and Fox et al. (2011). (The authors cite the former study; they should also cite the latter, which was published in the current issue of Development 138, 2223-2234.) Cyclin E/CDK2 appears to be regulated via multiple mechanisms, one of which is revealed by the Kalchhauser et al study. This information is particularly important because it provides a direct link between a well-studied positive regulator of germ cell proliferation, FBF, and a well-studied inhibitor the cell cycle, CKI.

Specific suggestions

While they authors provide multiple types of data to substantiate the regulation of cki-2 by FBF, it would be reassuring to see indirect immunofluorescence data showing expression of CKI-2 in the distal fbf-1 fbf-2 germ line.

The authors' genetic data indicate that cki-2 is not the sole target of FBF activity, although it appears to be a very important one. Fig. 5 indicates that 89% of one day-old fbf-1 fbf-2 cki-2 adult germlines contain mitotic cells. In order to think about the relative importance of cki-2 as an FBF target, it would be informative to know more about the degree of suppression. What is the size of the mitotic region in these animals: how does it compare with wild type, and is it retained in older adults?

Another consideration is that the authors rely on nuclear morphology - as opposed to visualization of marker proteins via indirect immunofluorescence - to identify mitotic and meiotic germ cells. The resolution of photos available to reviewers is too small for this reviewer to evaluation the extent to which nuclear morphology is completely normal. Better quality images should be provided. In addition, the authors should consider providing additional evidence that the distal germ cells, e.g., in fbf-1 fbf-2 cki-2 adults, are mitotic.

Referee #2 (Remarks to the Author):

The central, significant finding of this paper is that translational repression of the cell cycle inhibitor

CKI-2 (a p21 homolog) is required for maintenance of stem cells in the C. elegans germ line. Specifically, the authors provide evidence that the PUF RNA binding proteins FBF-1/2 repress cki-2 mRNA directly, and that this repression is essential for germline stem cell (GSC) maintenance. This finding is significant as it provides a mechanism for control of stem cell maintenance that is tied to cell cycle regulation in vivo, which is poorly understood for any stem cell system. Some results in support of this finding are convincing. In particular, specific and high-affinity binding of FBF-2 to cki-2 FBEs is rigorously demonstrated. In addition, the authors demonstrate that a region of the cki-2 3'UTR carrying these FBEs is essential for repression in the GSC region of the germ line. The authors also present evidence that CKI-2 repression is functionally important for FBF control of GSG maintenance. However, this critical finding needs additional support and/or description to be convincing. Additionally, the role of FBF's in cki-2 repression through the cki-2 3'UTR needs a more quantitative analysis, and some more information. These points are detailed below. Should these issues be resolved, this paper represents a significant and interesting finding in our understanding of GSC control mechanisms.

1. The most important support for the critical role of FBF-mediated cki-2 repression in GSC maintenance is the restoration of GSC's in fbf-1;fbf-2 mutants upon loss of cki-2, which is shown in Fig. 5. However, the criteria used to identify germ cells as stem cells or other kinds of cells is not convincing as described. Essentially, GSCs are identified by DAPI staining, and it is not at all clear what criteria were used to identify these nuclei as GSC's. First, the authors need to specify what the DAPI morphology of GSCs are, and how this supports these nuclei as stem cells. More convincing would be some independent test to show an increase in GSCs in fbf-1;fbf-2;cki-2 animals. For example, is there an increase in numbers of mitotic nuclei, marked by metaphase/anaphase morphology or phospho-Histone H3 staining, as was done in other studies? Or, is there an increase in total germ cell number in these mutants compared to fbf-1;fbf-2 gonads, as would be expected if GSC maintenance is rescued? Likewise, what criteria disinquishes pachytene and spermatocyte DAPI staining from GSCs? For sperm nuclei, nomarski microscopy can be used to confirm these as sperm; was this done?

2. A related, more minor issue to point 1 is that Figure 5B is unclear. Does the figure show the percentage of gonads that have any GSCs versus those that have none (i.e. only sperm or some combo of pachytene, spermatocytes, plus sperm)? Clarification in the figure or in the figures legend is needed.

3. The authors show images in figure 3 suggesting that loss of fbf-1 and fbf-2 de-represses the gfp-cki2 reporter in the distal tip region. However, this de-repression is modest if not weak. It seems important to quantify these images, either by measuring the ratio of GFP pixel intensity in distal tip nuclei to meiotic nuclei in the same gonad, or by quantifying the percentage of gonads with undetectable distal GFP after comparing wild type to mutant at the same exposure settings (no information on whether exposures were matched is presented, and should be).

4. To explain the modest effect of FBFs on cki-2 repression in L4 animals (point 3), the authors suggest that cki-2 is also repressed by some other unknown factor at the L4 stage when FBFs are not essential for stem cell maintenance. This raises the question of whether FBFs are the only repressors at the adult stage, or if other repressors contribute throughout development. This question seems important and interesting because this other putative repressor could be the major regulator, even though this scenario would not negate a role for FBF control of cki-2 in GSC maintenance (assuming issues in point 1 are resolved). Given the partial effects of fbf-1;fbf-2 at L4, one simple and easy test of this idea is to examine the gfp-cki2(FBE deletion) mutant transgene in wild type L4 gonads. If this FBE deletion completely de-represses GFP expression at the L4 stage as it does in adults, it would suggest that another factor contributes to repression through this same 3'UTR region and could function with FBFs throughout development. If instead the FBE mutant reporter is partially repressed at the L4 stage but fully de-repressed in adults, it would support another factor repressing cki-2 at L4 through a different RNA element and that FBFs are most critical in adults.

Referee #3 (Remarks to the Author):

This manuscript shows that the FBF translational repressors control translation of the cyclin/CDK inhibitor CKI-2, which acts on Cyclin E/CDK2 to block the G1-S transition, in the germline stem

cells of C. elegans. Previous work from this lab and the Kimble lab showed that regulation of Cyclin E protein levels are critical for controlling the transition from stem cell division and maintenance to the onset of meiosis and differentiation in the germline. It was shown that GLD1 represses Cyclin E translation and that Cyclin E in turn promotes degradation of GLD1. In this paper the authors present experiments leading to the conclusions that the redundant Pumilio family members fbf1 and fbf2 are needed for full repression of a reporter with the CKI-2 3'UTR, that FBF binding elements in the 3'UTR are required for repression of the reporter in the germline stem cells, that the FBF proteins can bind the 3'UTR, and that mutation of cki-2 suppresses germ cell loss of fbf1,2 mutants. The experiments are clear, well presented, and compelling, but in two cases additional data are needed. The authors examine the effect of mutations of fbf1 and 2 solely on the 3'UTR reporter and observe only partial derepression in the germline stem cells. They should show whether these mutations affect the protein levels of CKI-2 itself in the germline, using the antibody that works for germline staining and was used in the first experiment to show absence of the protein in wild-type germline stem cells. This would indicate whether other regions of the CKI-2 mRNA could function in regulation by the FBF proteins. The authors showed that after one day of adulthood, germline stem cells were retained in the fbf1,2 cki-2 triple mutant, indicating suppression of the fbf1,2 phenotype by removal of cki-2. To support this conclusion more strongly it would be helpful to show whether this persists after the first day. The authors need to state explicitly in this paper whether the cki-2 allele they are using is a null.

1st Revision - authors' response 23 June 2011

I am happy to say that we have been able to address all concerns. I am hoping that you and our reviewers will be satisfied and that we will be able to go ahead with publishing. Please find our point-by-point response below.

Editor’s remarks

The most critical point here is that raised by both referees 1 and 2 regarding the need for better markers to demonstrate the rescue of the fbf1/2- phenotype upon loss of cki2.

To quantify stem cell rescue in fbf-1 fbf-2 cki-2 animals, we employed markers that are commonly used to assay proliferation vs. differentiation in the worm germ line. To assay proliferation, we used antibodies against H3Ser10 phosphorylation, which marks condensed chromosomes during mitosis. To follow the entry into meiosis, we used antibodies against HTP-3, a HORMA-domain protein, which localizes to the axes of meiotically synapsed chromosomes, and which is absent from stem cells. Finally, sperm were assayed based on the characteristic ‘dot’-like appearance on DAPI-stained nuclei. This data is presented in Figure 5.

Regarding referee 2's point 4, I would encourage you to perform the requested experiment, but it would certainly not be necessary to try and identify alternative cki2 regulators that may be important at the L4 stage.

This has been done, as explained in our response to Referee 2, and is presented in Figure 3B.

Referee #1

Data in this paper are consistent with two other recent studies implicating cyclin E/ CDK2 activity as important for maintenance of germline proliferation by Jeong et al. (2011) and Fox et al. (2011). (The authors cite the former study; they should also cite the latter, which was published in the current issue of Development 138, 2223-2234.)

The report by Fox et al.(2011) was published when our manuscript was under review. We have now

included this citation.

While they authors provide multiple types of data to substantiate the regulation of cki-2 by FBF, it would be reassuring to see indirect immunofluorescence data showing expression of CKI-2 in the distal fbf-1 fbf-2 germ line.

Expression of CKI-2 in the distal fbf-1 fbf-2 germ line is now shown in Figure 5A.

The authors' genetic data indicate that cki-2 is not the sole target of FBF activity, although it appears to be a very important one. Figure 5 indicates that 89% of one day-old fbf-1 fbf-2 cki-2 adult germlines contain mitotic cells. In order to think about the relative importance of cki-2 as an FBF target, it would be informative to know more about the degree of suppression. What is the size of the mitotic region in these animals: how does it compare with wild type, and is it retained in older adults?
Another consideration is that the authors rely on nuclear morphology - as opposed to visualization of marker proteins via indirect immunofluorescence - to identify mitotic and meiotic germ cells. The resolution of photos available to reviewers is too small for this reviewer to evaluation the extent to which nuclear morphology is completely normal. Better quality images should be provided. In addition, the authors should consider providing additional evidence that the distal germ cells, e.g., in fbf-1 fbf-2 cki-2 adults, are mitotic.

We have now used markers for mitotic cells (phospho-Histone H3, H3Ser10p), meiotic cells (HTP-3), and sperm (dot-like appearance of DAPI-stained sperm nuclei), to quantify the rescue of stem cell loss at 12h-intervals over two days (Figure 5). Using these markers, we confirmed that proliferation is restored in fbf-1 fbf-2 cki-2 animals to about half of the wild-type level. The proliferative capacity is retained beyond day 2 of adulthood, and declines with age at a similar rate as in the wild type (Figure 5).

Referee #2 (Remarks to the Author):

More convincing would be some independent test to show an increase in GSCs in fbf-1;fbf-2;cki-2 animals. For example, is there an increase in numbers of mitotic nuclei, marked by metaphase/anaphase morphology or phospho-Histone H3 staining, as was done in other studies?

Using phospho-Histone H3 (H3Ser10p) staining, we confirmed that distal germ cells proliferate in fbf-1 fbf-2 cki-2 animals. They also do not express HTP-3, a meiotic marker (Figure 5).

Or, is there an increase in total germ cell number in these mutants compared to fbf-1;fbf-2 gonads, as would be expected if GSC maintenance is rescued?

The fbf-1 fbf-2 cki-2 animals produce visibly higher numbers of germ cells (including sperm) when compared to fbf-1 fbf-2 animals. Indeed, this is most likely due to the rescue of stem cells proliferation, as shown and quantified in Figure 5.

The authors need to specify what the DAPI morphology of GSCs are, and how this supports these nuclei as stem cells. Likewise, what criteria disinquishes pachytene and spermatocyte DAPI staining from GSCs? For sperm nuclei, nomarski microscopy can be used to confirm these as sperm; was this done?

We have confirmed the identity of distal cell types using markers for mitotic cells (H3Ser10p) and meiotic cells (HTP-3). We rely on DAPI to identify sperm, since sperm nuclei display a very characteristic dot-like appearance by DAPI staining. We have explained this appearance now in the legend to Figure 5.

A related, more minor issue to point 1 is that Figure 5B is unclear. Does the figure show the percentage of gonads that have any GSCs versus those that have none (i.e. only sperm or some combo of pachytene, spermatocytes, plus sperm)? Clarification in the figure or in the figures legend is needed.

We hope that we were able to clarify in the Figure 5 legend that each category represents the fraction of gonads containing the indicated cell type in the distal region: "Fractions of gonads that in the distalmost part contain: stem cells (light blue), meiotic cells (intermediate blue), or sperm (dark blue), measured at 12h intervals after the larval-to-adult molt."

It seems important to quantify these images, either by measuring the ratio of GFP pixel intensity in distal tip nuclei to meiotic nuclei in the same gonad, or by quantifying the percentage of gonads with undetectable distal GFP after comparing wild type to mutant at the same exposure settings (no information on whether exposures were matched is presented, and should be).

We have measured fluorescence intensities of the wild type cki-2 3'UTR reporter in distal nuclei of fbf-1 fbf-2 / + and fbf-1 fbf-2 animals from images taken at the same exposure settings (Figure 3A) and have stated the use of identical exposure times in the Methods section ("All images subject to direct comparison were taken at identical exposure times."). Distal GFP signal in fbf-1 fbf-2 animals is 1.24 fold higher (which is highly significant, p=1.298e-05) than in fbf-1 fbf-2 / + animals (Figure 3A). Reporter de-repression in fbf-1 fbf-2 animals is fully penetrant (all animals show de-repression).

Given the partial effects of fbf-1;fbf-2 at L4, one simple and easy test of this idea is to examine the gfp-cki2(FBE deletion) mutant transgene in wild type L4 gonads. If this FBE deletion completely de-represses GFP expression at the L4 stage as it does in adults, it would suggest that another factor contributes to repression through this same 3'UTR region and could function with FBFs throughout development. If instead the FBE mutant reporter is partially repressed at the L4 stage but fully de-repressed in adults, it would support another factor repressing cki-2 at L4 through a different RNA element and that FBFs are most critical in adults.

We observed that FBEs 1-4 are required for regulation not only in the adult, but also in wild type L4 larvae (Figure 3B). Thus, full repression in L4 larvae appears to require an additional factor(s) besides FBF, but FBE1-4 are critical for regulation throughout germline development.

Referee #3 (Remarks to the Author):

They should show whether these mutations affect the protein levels of CKI-2 itself in the germline, using the antibody that works for germline staining and was used in the first experiment to show absence of the protein in wild-type germline stem cells.

We have performed CKI-2 immunofluorescence experiments in fbf-1 fbf-2 mutants and detect CKI-2 in the distal fbf-1 fbf-2 germ line (Figure 5A).

The authors showed that after one day of adulthood, germline stem cells were retained in the fbf1,2 cki-2 triple mutant, indicating suppression of the fbf1,2 phenotype by removal of cki-2. To support this conclusion more strongly it would be helpful to show whether this persists after the first day.

We have quantified the suppression using antibodies recognizing mitotic (H3Ser10p) and meiotic (HTP-3) cells, and found that stem cells are indeed maintained beyond day 2 of adulthood in fbf-1 fbf-2 cki-2 animals. By this time, fbf-1 fbf-2 animals have lost stem cells (Figure 5B).

The authors need to state explicitly in this paper whether the cki-2 allele they are using is a null.

In the revised version, we state in Results and in Method sections that cki-2(ok2105) behaves as a genetic null: "In both cki-2(ok2105) mutants and cki-2(RNAi) animals, CKI-2 protein is essentially absent (Figure 1B-C; unpublished observation)." and "Unless stated otherwise, all mutations are, or phenocopy, null alleles."). This is based on the observation that cki-2(RNAi) and cki-2(ok2105) behave identically in wild type and mutant backgrounds. Also, the cki-2(ok2105) deletion leaves only exon 1 (of 5) intact and entirely removes the Cyclin-dependent kinase inhibitor domain (Figure 2B).

2nd Editorial Decision 07 July 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-77867R1. It has now been seen again by referees 1 and 2, whose comments are enclosed below. As you will see, both find your responses to the previous round of review satisfactory, and now fully support publication. I am therefore pleased to be able to tell you that we can accept your manuscript for publication here. There are just a few little issues to be addressed from the editorial side first.

- Please can you merge all the Supplementary files into a single PDF?
- In figure 3A and S1, the statistical details are incomplete: for all figures where statistical significance is presented, we need to know the 'n' number, the statistical test used and what the error bars represent.

If you can just make these final changes, we should then be able to accept the study without further delay.

Many thanks for choosing EMBOJ for publication of this study, and congratulations on a fine piece of work!

Best wishes,
Editor

The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Kalchhauser and colleagues have submitted a revised version of their ms describing FBF repression of CKI-2 in the C. elegans germ line. As I outlined in my original review of this study, the authors address the mechanism whereby cell cycle regulation and stem cell maintenance are coordinated. They demonstrate that FBF, a translational regulator known to promote germ cell proliferation, represses expression of the cyclin-dependent kinase inhibitor, CKI-2. This repression allows cyclin E/CDK-2 activity to remain high, in turn allowing stem cells to proliferate. The authors' data lead to a model whereby up regulation of CKI-2 as cells move proximally leads to repression of cyclin E/CDK-2 activity and, consequently, entry into meiosis. In the revised version, the authors do a very good job of addressing the reviewers' comments, primarily by including additional data and by clarifying some of the data presented in the original submission. Figures 3 and 5 are much improved. I judge the ms to be ready for publication.

Referee #2 (Remarks to the Author):

The authors have alleviated the most important issues with more convincing new data and modifications, and I recommend publication.

2nd Revision - authors' response 08 July 2011

We have now assembled the Supplement into a single PDF file.

Also, we have added all requested statistical information:

- Figure S1D:
In the figure legend, we explain that "Error bars = SEM, n = 3 (see Material and Methods)." and accordingly in Material and Methods, "50 gonads per strain were dissected for each of three replicates [...]"

- Figure 3A:
In the figure legend, we explain that "(n = number of examined gonads, error bars = SEM). P-value = 1.298e-05 (see Material and Methods)" and accordingly in Material and Methods, "The p-value was calculated by t-test in R.".

Please preserve figures in their entirety as CMYK and avoid converting any figures or figure parts to black-and-white.

