Author's response to reviews

Title: BAG-1 haplo-insufficiency impairs lung tumorigenesis

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Author’s response to reviews: see over
Dear BioMed Central Editorial Team,

Thank you for the comments to our manuscript “BAG-1 haplo-insufficiency impairs lung tumorigenesis”. We would like to address the criticism as follows:

**Reviewer 1**

**Major Comments:**

1. *...difference in the level of Bag-1 must be re-examined...*
   We have repeated the quantitative Western blots and analysed additional SP-C C-Raf BXB mice either homozygous or heterozygous for BAG-1. For clarity of presentation, we show only one protein dosage (40µg) per animal. The new figure unambiguously shows the difference in the BAG-1 protein expression between four BAG-1\(^{+/+}\) and four BAG-1\(^{+/−}\) mice as well as equal protein loading (GAPDH immune blot).

2. *...a better description of the knock-out strategy is required...*
   Additional information has been added to the text (page 4):

   To inactivate the BAG-1 gene, we constructed a vector where exons 1 and 2 are replaced with a neomycin resistance gene. A phage clone with a 15-kb genomic insert from mouse strain 129/Sv spanning all seven exons of BAG-1 was identified and characterised using standard methods. The targeting construct contained 1.1-kb from the BAG-1 locus upstream of the neomycin resistance gene of plasmid pPNT and 6-kb downstream. The upstream arm of 1.1kb is located 5’to the start codon in the first exon of BAG-1 and the 3’arm of 6 kb is located downstream of exon 2. The mutation was introduced into embryonic stem cells by homologous recombination. Positive clones were identified by Southern blot analysis. Germline transmitting chimaeras were obtained and bred to C57BL/6 mice. Further details will be described elsewhere.

   *...a better description of the knock-out results is required...*
A comprehensive description of the BAG-1\textsuperscript{-/-} phenotype is subject of another manuscript. The new version of the manuscript reads now (page 5):

To inactivate the BAG-1 gene, exons 1 and 2 were replaced with a neomycin resistance gene. This strategy was chosen to disrupt the expression of all known isoforms of BAG-1 which are generated by alternate translation initiation of a single mRNA; the start codons are present in exons 1 and 2. Western blot analysis of liver protein extracts of BAG-1 deficient embryos showed the complete loss of all BAG-1 protein isoforms. Embryos homozygous for this allele died at midgestation at around E13.5, but the heterozygous animals (BAG-1\textsuperscript{+/—}) are normal. A comprehensive description of the BAG-1\textsuperscript{-/-} phenotype is subject of another manuscript.

3. ...to help understand which Bag-1 isoform is doing what...
Questions concerning specific roles of the different BAG-1 isoforms were not addressed in these experiments. Indeed, as the referee points out correctly, these questions cannot be solved with the knock-out mouse strain employed here, as both isoforms of BAG-1, p50 and p32, are absent in protein extracts of knock-out embryos and reduced in the lungs of BAG-1 heterozygous mice. A coresponding sentence has been added to the text (page 8):

Questions concerning specific roles of the different BAG-1 isoforms were not addressed with this BAG-1 deficient mouse as both isoforms of BAG-1, p50 and p32 are absent in protein extracts of knock-out embryos.

4. The scheme in Fig. 4 is a bit premature ....
The reason for this figure was to make clear the two possibilities how BAG-1 might protect from apoptosis, either via direct action on Raf or indirectly as a co-chaperone of heat shock proteins.

Minor Comments: NONE

Reviewer 2

Major Comment: NONE

Minor Comments:
1. ...addressed the significance of BAG-1 in other disease states...
Another setting where BAG-1 has a physiological role is the heart, where up-regulation of BAG-1 after ischemia rescues cells from apoptosis.

2. *...to see in more detail the design and generation protocol of the mouse...*

Additional information has been added to the text (page 4):

To inactivate the BAG-1 gene, we constructed a vector where exons 1 and 2 are replaced with a neomycin resistance gene. A phage clone with a 15-kb genomic insert from mouse strain 129/Sv spanning all seven exons of BAG-1 was identified and characterised using standard methods. The targeting construct contained 1.1-kb from the BAG-1 locus upstream of the neomycin resistance gene of plasmid pPNT and 6-kb downstream. The upstream arm of 1.1kb is located 5´to the start codon in the first exon of BAG-1 and the 3´arm of 6 kb is located downstream of exon 2. The mutation was introduced into embryonic stem cells by homologous recombination. Positive clones were identified by Southern blot analysis. Germline transmitting chimaeras were obtained and bred to C57BL/6 mice. Further details will be described elsewhere.

3. *... a difference in intensity of the H&E staining and maybe a brief explanation...*

The difference in the intensity of the two lung sections derives mainly from the observation that the adenoma cells have a tendency to stain more intensively than normal lung cells. New text has been added (page 6):

The histological picture emphasizes the difference in adenoma formation between a representative SP-C C-RafBxB/BAG-1+/+ and SP-C C-RafBxB/BAG-1+/- lung. The difference in the staining intensity of the two lung sections derives mainly from the observation that the adenoma cells have a tendency to bind more intensively hematoxylin and eosin compared to normal lung cells.

4. *How does the BAG-1 staining compare in adenomas with one or two BAG-1 alleles?*

There was no obvious difference in the BAG-1 immunohistochemistry of SP-C C-RafBxB/BAG-1+/+ and SP-C C-RafBxB/BAG-1+/- lungs. New text has been added (page 6):

There was no obvious difference in the BAG-1 immunohistochemistry of SP-C C-RafBxB/BAG-1+/+ and SP-C C-RafBxB/BAG-1+/- lungs.
5. How might BAG-1 regulate C-Raf activity? Some text is stated on page 9:
Experiments dealing with this question are currently ongoing.

6. ...designing a molecular therapy for BAG-1...
New text has been added (page 9):
One way to reduce BAG-1 expression is through use of RNA interference-based gene silencing, in particular as BAG-1 overexpression has been observed in human tumours [11]. Drugs that bind to the ATP binding site of Hsc70/Hsp70 might also be expected to be effective as they would inhibit the interaction of BAG-1 with the ATPase domain of heat shock proteins. Such new specific BAG-1 inhibitors may be identified, aided by the known three-dimensional structure of the BAG domain [18,19].

7. ...question slightly the quality of the western blots shown in Fig. 2a...
We have repeated the quantitative Western blots (Fig. 2a) and analysed additional SP-C C-Raf BXB mice either homozygous or heterozygous for BAG-1. For clarity of presentation, we show only one protein dosage (40µg) per animal. The new figure unambiguously shows the difference in the BAG-1 protein expression between four BAG-1+/+ and four BAG-1+/− mice as well as equal protein loading (GAPDH immune blot).

We hope that you find the revised manuscript acceptable for publication and look forward to hearing from you.

Best regards