THE EMBO JOURNAL

Manuscript EMBO-2010-76443

Fis1 and Bap31 bridge the mitochondria/ER interface to establish a platform for apoptosis induction

Ryota Iwasawa, Anne-Laure Mahul-Mellier, Christoph Datler, Evangelos Pazarentzos and Stefan Grimm

Corresponding author: Stefan Grimm, Imperial College London

Review timeline:

Submission date:	30 May 2010
Editorial Decision:	28 June 2010
Resubmission:	05 November 2010
Editorial Decision:	23 November 2010
Revision received:	06 December 2010
Additional correspondence	07 December 2010
Reply	07 December 2010
Accepted:	08 December 2010

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 28 June 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while all three referees consider the study as interesting in principle it becomes clear that the study is not sufficiently developed to justify publication of the study here at this stage of analysis. I will not repeat all their individual points of criticism here, but one major issue is that considerably stronger data on the physiological significance of your findings including loss-of-function experiments would need to be provided. Second, the functional interplay between Fis1, Bap31 and caspase-8 activation needs to be addressed in considerably more mechanistic depth. Clearly, the referees point to major shortcomings in respect to key aspects of the experimental evidence provided. Furthermore, extensive further experimentation is needed and the outcome of these experiments cannot be predicted at this point. The amount and nature of the additional work required does thus not lie within both the scope and the time frame (3 months) of a single revision and it is our policy to allow for a single round of revision only. I therefore see little choice, but to come to the conclusion that we cannot offer publication of the study here at this point.

Still given the interest expressed by the referees in principle we would not exclude to consider a new submission on the same topic should future studies allow you to strengthen the study considerably along the lines suggested by the reviewers and to develop the study further both in terms of its physiological significance as well as at the mechanistic level. To be completely clear, however, I would like to stress that if you wish to send a new manuscript this will be treated as a new

submission rather than a revision and will be evaluated again at the editorial level and reviewed afresh (involving our original referees again if available at the time of resubmission), also with respect to the literature and the novelty of your findings at the time of resubmission. At this stage of analysis, though, I am sorry to have to disappoint you.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this manuscript by Iwasawa et al. the authors describe their findings suggesting that a molecular interaction between mitochondrial fission protein Fis1 and ER localized Bap31 contribute to activation of Fis1 overexpression-induced apoptosis. These findings also hint at mechanism in which Fis1 overexpression stimulates caspase8-dependent cleavage of Bap31 and therefore conveys a proapoptotic signal from the mitochondria to the ER. Furthermore, it is also shown that procaspase8, Fis1, and Bap31 form a molecular complex that might be required for apoptosis activation. In addition, it appears that Fis1-overexpression induces changes in calcium homeostasis that could also contribute to cell death.

Overall, the conclusions reached by the authors are mostly based on the Fis1 overexpression experiments. To reveal Fis1 role in the apoptosis more convincingly analyzes of endogenous Fis1, as well as Fis1 downregulation approaches are needed. Also, most of the data is rather correlative and additional studies are needed to strengthen the mechanistic link between Fis1, Bap31 and caspase8 activity (see below). Furthermore, some of the data is not very clear and therefore not convincing (see below).

Specific remarks:

1. The significance of the findings shown in the Figure 1 is not clear. Although, the data indicate that Fis1 overexpression stimulates Bap31 cleavage, the physiological importance of this event should be strengthened. For example, the authors should show the effect of Fis1 downregulation on Bap31 cleavage in other modes of apoptosis in which Bap31 is cleaved (for example, ActD or etoposide, as shown in the Figure 4 of this work). In addition, in Figure1 blots of Fis1 should be shown in all Fis1 overexpression experiments.

2. The data showing a colocalization/spatial association of Bap31 and Fis1 (Figure 2) is not very informative. Unless the authors show that other mitochondrial outer membrane proteins (e.g. Tom20, VDAC) have less overlap with Bap31, and vice versa the ER associated proteins (e.g. calnexin) with Fis1, these data merely corroborate a known fact that ER and mitochondria are close to each other.

3. Figure 4 demonstrates that processing of procaspase8 is associated with molecular interaction of this protein with Fis1 early in apoptosis. Is Fis1 required for this event? Furthermore, to gain more mechanistic insights into Fis1/Bap31 complex the authors should also determine whether Fis1 is required for Bap31/procaspase8 binding. The IP control should be also included in Figure 4D.

4. The data showing Fis1-induced changes in calcium homeostasis have already been published by others (Frieden et al. J Biol Chem. 2004;279(21):22704-14); this needs to be appreciated in the manuscript.

5. There are two potential mechanisms of Fis1-induced cell death described here. One depends on activation of caspase8, and one on changes in calcium homeostasis that lead to mitochondrial permeability transition (as described earlier by Alirol et al. MBoC. 2006; 17(11):4593-4605). Although, the data suggest that mitochondrial permeability might be a result of caspase8-dependent cleavage of Bap31, this need to be established more convincingly. Does chemical inhibition of caspase8 affect mitochondrial steps in apoptosis (e.g. cytochrome c release and Bax/Bak activation)? Does bongkrekic acid affect Fis1 induced caspase8 processing and subsequent apoptosis?

6. Overall the quality of co-immunoprecipitation data is a little bit troubling (e.g. high background, and non-specific binding). Therefore, the authors are encouraged to supplement these results with some alternative approaches. For example to reveal that the analyzed proteins indeed form molecular complexes the gel filtration assay could be applied.

Referee #2 (Remarks to the Author):

In this manuscript, Iwasawa et al propose that the mitochondrial fission protein Fis1 and the ER protein Bap31 form a functional pathway for triggering apoptosis. The results presented are potentially interesting, however further studies are required to establish this novel pathway.

Specific comments:

1) The authors conclude that Fis1's activity at the ER is independent of its mitochondrial fission activity. Thus, it is important to demonstrate that inhibiting mitochondrial fission does not affect Fis1's activity at the ER. In addition, the authors should indicate how Fis1 activates the "ER pathway" without initiating mitochondrial fission.

2) The authors conclude that Fis1's ability to induce ER calcium release depends on Bap31 cleavage to generate the p20 product. The authors should demonstrate that a Bap31 non-cleavable mutant (expressed in cells in which endogenous Bap31 was knocked down) blocks Fis1-induced calcium release and apoptosis.

3) The co-IP's studies demonstrating the interactions between Bap31-Fis1-caspase-8 are not convincing (Figs 4D and 4E). These studies should be improved. In addition, all Western blots should be accompanied by molecular weight markers.

Referee #3 (Remarks to the Author):

In this study by Iwasawa et al, cell death initiated by the mitochondrial fission protein Fis1 (or via stress signals for which Fis1 down regulation confers some resistance) is shown to depend on the ER protein Bap31. Further, they conclude that Fis1-Bap31 form an inter-organelle complex which can recruit procaspase-8, resulting in its activation and cleavage of Bap31, release of ER calcium stores, and calcium-mediated activation of mitochondrial death pathways.

Overall, the results are of potential importance but a more detailed investigation of the effects of Fis1 and Bap31 knock down on indirect influences on cell death (perhaps due to enhanced mitochondrial fusion which can be cytoprotective and changes in ER homeostasis, respectively) as opposed to a model involving a procaspase-8 activation platform. Also, only a single co-immunoprecipitation experiment employing endogenous proteins leads to the conclusion that such a platform exists, and this needs to be validated by additional approaches.

Specific comments.

The overall methodology and figure legends were too abbreviated and some experimental results were difficult to interpret as a result.

I do not understand Fig. 2 or the significance of the fluorescence overlap. For example, what about comparisons of ER and mito outer membrane proteins that would not be expected to interact?

For the critical Fig. 4D, there was not control (non- or pre-immune)

Fig. 5. Not clear how cytosolic calcium levels determined or validated.

Resubmission 05 November 2010

We very much appreciate the helpful comments from the reviewers of our manuscript "Fis1 and Bap31 bridge the mitochondria/ER interface to establish a platform for apoptosis induction".

In order to integrate the new data (see below) and present them in the most appropriate way we have split the old figure 4 into figure 3 and 4. Also, figure 2 was put into the supplementary data file in response to the referees' queries (below). We have also extended the Introduction a bit to stress the importance of caspaseactivating complexes (page 3, lines 2-9).

In the following we would like to specifically address every individual point raised by the reviewers. We have adopted the numbering and the wording used by the referees (in italics below).

Referee #1 raised the following concerns:

1.) the authors should show the effect of Fis1 downregulation on Bap31 cleavage in other modes of apoptosis in which Bap31 is cleaved (for example, ActD or etoposide ..

We have now inserted the new figure 3C (left panel) in which we present a Western blot showing a reduction of the cleavage of the Bap31-EYFP fusion protein when Fis1 was downregulated and the cells treated with etoposide. This experiment is now also mentioned in the text on page 8, lines 14-16.

...in Figure1 blots of Fis1 should be shown in all Fis1 overexpression experiments

We have now inserted in all relevant experiments of Figure 1 the control blots for the expression of Fis1. In addition, we show the expression of Bax, tBid, and Bap31. The loading was controlled by detecting GAPDH.

2.) The data showing a colocalization/spatial association of Bap31 and Fis1 (Figure 2) ... merely corroborate a known fact that ER and mitochondria are close to each other

Our intention with these experiments was to show that in our cellular system Fis1 and Bap31 are in close proximity but we acknowledge the referee's point that these data are confirming previous results and we have now put them into the supplementary data file as figure S2. We have also removed the former figures S2A,B,C, which showed the same localisation in a single cell. The former figures S2D,E are now figures S5A,B.

3.) ... processing of procaspase8 is associated with molecular interaction of this protein with Fis1... Is Fis1 required for this event?

We have now inserted the new figure 3C (right panel) in which we present the consequences on caspase-8 activity (which is the result of its procession) when Fis1 was downregulated and the cells treated with etoposide. In line

with the reduced procession of Bap31-EYFP (same figure, left panel) under these conditions, we observed a significantly reduced capsase-8 activity. This is now mentioned in the text on page 8, lines 18-19.

… determine whether Fis1 is required for Bap31/procaspase8 binding.

In the new figure 4E we now show that the association of endogenous caspase-8 with endogenous Bap31 is reduced when Fis1 was downregulated by RNAi and the cells were treated with etoposide or actinomycin D. These experiments are now mentioned in the text on page 9, lines 18-22.

The IP control should be also included in Figure 4D.

We have now included the IP control in the new figure 4A (which replaces the former figure 4D).

4.) (Frieden et al. J Biol Chem. 2004;279(21):22704-14)… needs to be appreciated

This reference was now included in the discussion (page 17, lines 3-4).

5.) Does chemical inhibition of caspase8 affect mitochondrial steps in apoptosis (e.g. cytochrome c release and Bax/Bak activation)?

Owing to the potential problems with the specificity of caspase-8 inhibitors we downregulated this gene with RNAi. We have now included the new figure 7G in which we recorded the changes of ΔΨm and the percentage of cells with activated Bax. In line with our model, caspase-8 inhibition led to a reduction of Bax positive cells and to an attenuated loss of ΔΨm upon transfection of Fis1. These results are now mentioned on page 13, lines 14-16.

Does bongkrekic acid affect Fis1 induced caspase8 processing and subsequent apoptosis?

The new figure 7E shows that the PT-pore inhibitor bongkrekic acid could not affect caspase-8 activation, when the cells were treated with etoposide or actinomycin (left panels) or when Fis1 was transfected (right panel). These experiments are now mentioned in the text on page 13, lines 11-13. We would like to emphasise that we had shown the effect on apoptosis already in the previous submission (Figure 7C).

6.) …the quality of co-immunoprecipitation data is a little bit troubling … to reveal that the analyzed proteins indeed form molecular complexes the gel filtration assay could be applied.

Several co-immunoprecipitations were repeated such as those in figures 2B and 4C. We have now included the new figure 4B in which we analysed the elution profile of Fis1, Bap31 and caspase-8 with and without treatment with actinomycin D in whole cell lysates. The data indicate that a small amount of the total Bap31 and Fis1 proteins elute together (fractions 39 and 40). Upon treatment of the cells with actinomycin D the bulk of the Fis1 protein changes its distribution, while the majority of the Bap31 and the caspase-8 proteins remain unchanged. Hence, we believe that only a small amount of the total Fis1, caspase-8 and Bap31 proteins constitute the ARCosome. This is now mentioned in the Discussion (page 18, lines 4-7) and the experiments are described in the text on page 9, lines 5-9. Please note that with the novel Figure 4E we now shown another co-immunoprecipitation with endogenous proteins and hence provide additional confirmation of the complex.
Referee #2 raised the following points:

1. ... demonstrate that inhibiting mitochondrial fission does not affect Fis1's activity at the ER. ... indicate how Fis1 activates the "ER pathway" without initiating mitochondrial fission.

In the new figure 8A,B,C we have now addressed the effect of caspase inhibition on mitochondria fission. We found that Fis1 could still induce fission in the presence of zVAD, which inhibits caspases and hence Bap31 cleavage. This excludes that fission is downstream of ARCosome activation, which relies on caspase-activation (Figs 3, 7). In order to assess whether mitochondrial fission is upstream of the ARCosome we studied the temporal correlation of fission with caspase-activation upon treatment of the cells with etoposide (new figure 8D,E) and found that at a time point when etoposide leads to Bap31 cleavage (Fig. 3B), fission was still unaffected. These experiments separate the signalling pathway described in our study from mitochondrial fission and are mentioned in the Results on page 13, line 16-23 and page 14, lines 1-3, in the Discussion (pages 16, lines 10-19), and in the M&M section (page 22, lines 20-22 and page 23, lines 1-2).

2. ... demonstrate that a Bap31 non-cleavable mutant (expressed in cells in which endogenous Bap31 was knocked down) blocks Fis1-induced calcium release and apoptosis.

We have now inserted the new figure 6C in which we expressed a noncleavable Bap31 construct that is not targeted by the RNAi sequence against Bap31 (supplementary figure S6) and observed a reduction of the cytosolic Ca2+ increase when Fis1 was transfected. We have also inserted the new figure 7F in which we assess the dissipation of $\Delta\Psi$m for apoptosis measurement upon Fis1 transfection, which turned out to be equally inhibited when the non-cleavable Bap31 construct was expressed in Bap31 knockdown cells. Both experiments are now mentioned in the text on page 12, line 1-3 and page 13, lines 13-14.

3. The co-IP's ... (Figs 4D and 4E) ...should be improved.

We have repeated the experiment in figure 4E (now figure 4C). We have now also inserted a new exposure of the same blot for the IPs of figure 4D (the new figure 4A).

all Western blots should be accompanied by molecular weight markers.

We have now included the sizes of the respective proteins in all Western blots of figure 1.

Referee #3 made the following remarks:

... more detailed investigation of the effects of Fis1 and Bap31 knock down on indirect influences on cell death (perhaps due to enhanced mitochondrial fusion which can be cytoprotective and changes in ER homeostasis ..

In the novel figure S4 we show the results from additional experiments on the ER homeostasis when Bap31 was knocked-down and examined the expression levels of ER stress indicators (CHOP, BiP, AFT4, ATF6, and XBP-1), which are otherwise upregulated during ER stress. We have not found any changes when Bap31 was reduced (new Figure S5). This is now mentioned in the text on page 8, line 22-23. We have now also investigated the effect of Fis1 downregulation on fission and observed an increase of the mitochondrial network. This is now presented in the new figure S7 and mentioned in the text, page 13, lines 21-22. There is no indication that the reduction of Fis1 has a cyto-protective effect under our conditions. When Fis1 was downregulated the basal apoptosis rate (2.8+/-0.68) was even slightly increased to (5.5 +/-4.2).

These data were from figure 3C. This is now mentioned in the text on page 8, lines 16-17.

In general, the sum of all experiments, rather than only the knock-down results on Fis1, led us to our conclusions on the function of the ARCosome. We have included a sentence in the discussion to make it clear that all our experiments taken together suggest the existence of the ARCosome rather than individual experiments (page 18, line 8).

only a single co-immunoprecipitation experiment …. this needs to be validated by additional approaches.

As mentioned in the remarks for referee #1, query #6 we performed a gel filtration experiment in which we observed the co-elution of small amounts of the total Bap31 and Fis1 proteins of whole cell lysates. We have also seen a redistribution of the Fis1 protein to higher molecular weight fractions while the elution profiles of Bap31 and caspase-8 remain largely constant. Hence, we believe that only a small amount of the total Bap31 and Fis1 proteins participate in this complex. This experiment is now shown in figure 4B, mentioned in the text on page 9, lines 5-9, and discussed on page 18, lines 4-7. We would like to stress that with figure 4E we have now performed another coIP in which we see the same complex formation and demonstrated that it is dependent on Fis1.

His/her specific comments:

… overall methodology and figure legends were too abbreviated

We have now substantially expanded the Materials and Methods section and provide a more thorough description of the constructs under "Plasmid vectors and transfection" with each primer listed that we used, more details on how we measured apoptosis ("Quantification of apoptosis"), including the composition of the lysis buffer. We also describe in more detail how we performed "Immunoprecipitations and immunoblotting" with the new antibodies for the additional experiments, and we give more information on how we constructed the viral vectors ("Viral production and stable cell lines"). We also added new paragraphs on the Caspase-8- and Bax-activity assays and the gel filtration/size-exclusion chromatography, which were used for the additional experiments.

For the figures we have likewise added additional details such as on the concentrations of the compounds used (Fig. 4, 7), the densitometory analysis (Fig. 4D), the calculation of the Ca^{2+} concentration and the times of the cell harvests (Fig 6). If the figure legends do not provide enough space to cover the details we make reference to the Materials and Methods section such as for the gel filtration experiment (Fig. 4B).

.. Fig. 2 … significance of the fluorescence overlap

We have now put the fluorescence study in the supplementary data (see also our response to query 2 from referee #1).

.. Fig. 4D, there was not control (non- or pre-immune)

We have now included this control (see also our response to referee #1, query 3).

Fig. 5. Not clear how cytosolic calcium levels determined

We have now added more detail to the legend of figure 5A in which we now

mention the dye that we use as well as that the measurements were calculated relative to the control-transfected cells. We now also give more information on the calcium measurements in the relevant M&M section.
We would like to thank the referees for their comments that improved the quality of our article. We have worked very hard and made every effort to address them with numerous additional experiments, new figures (8 sub-figures, the completely new figure 8, 2 revised figures, as well as 3 new supplementary figures), and many modifications of the manuscript.

We very much hope that our study is now found acceptable for publication in the *EMBO Journal*.

2nd Editorial Decision 23 November 2010

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I would like to ask you to address the minor issues suggested by referee 2 including the removal of figure 4B (see below). Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have adequately addressed all my comments

Referee #2 (Remarks to the Author):

Iwasawa et al have now submitted a significantly revised and greatly improved ms on their findings involving mitochondrial Fis1 and ER Bap31, and their role in propagating mitochondrial stress signals to the ER, resulting in release of ER Ca+ and feedback sensitization of mitochondria to execute cell death. The experiments elucidating functional relationships between the various components of this pathway - Fis1, Bap31, pocaspase-8 - are convincing. Experiments elucidating physical associations have also been improved and are comparable to the standards of top journals. And at least based on these criteria are convincing. In an attempt to further extend the evidence for inter-organellar interactions between Fis1 and Bap31, the authors chose to examine the potential for overlapping elution of cell extracts from gel filtration columns. It is impossible to utilize this approach and derive anything meaningful. Fig 4B should be removed from the paper. Given the improvements in co-IPs, I am less concerned about secondary assays. However, should the authors decide to pursue this question either for this or a follow on study, a biochemical approach would be to isolate intact MAMs or post-nuclear heavy membranes (mitochondria with associated ER), and screen with a library of chemical cross linking agents. While there is no guarantee that adducts will form (depends on the nature and accessibility of target functional groups), signals would be convincing. Alternative approaches include combined live cell imaging and FRET.

Minor points.

1. A brief mention of the normal physiological role of Bap31 in regulating ER protein traffic should

be made in the Intro.

2. Page 8 bottom and Fig. S4 - change the reference to ER homeostasis, to ER stress response.

3. p.15. Procaspase-8L represents a 59 (not 74 as stated) aa extension of the procaspase-8 isoform procaspase-8a.

Referee #3 (Remarks to the Author):

The manuscript has been significantly improved and I believe it is now appropriate for the publication without any additional work.

1st Revision - authors' response 06 December 2010

1.) It is impossible to utilize this approach (i.e. gel filtration assay) *and derive anything meaningful. Fig 4B should be removed from the paper.*

We have removed figure 4B from the manuscript. The corresponding sentences in the text, in the figure legend, and the experimental procedure in the Materials and Methods section were likewise removed. As a result, the previous figure 4C is now figure 4B, similarly the previous figures 4D and 4E are now figures 4C and 4D, respectively. These changes are reflected in the main text on page 9, lines 10, 12 and 14.

2.) A brief mention of the normal physiological role of Bap31 in regulating ER protein traffic should be made in the Intro.

We now mention the physiological role of this protein in the text on page 4, lines 17-23.

3.) Page 8 bottom and Fig. S4 – change the reference to ER homeostasis, to ER stress response.

These changes are now implemented (page 8, line 23).

4.) … Procaspase-8L represents a 59… aa extension of the procaspases-8 isoform proccaspase-8a.

This is now changed in the text on page 14, line 12.

Additional correspondence 07 December 2010

Thank you for sending us your amended manuscript. Prior to acceptance there is one editorial issue that needs further attention. At acceptance stage we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication in all cases where such processing has been performed according to our editorial policies. Also, suitable control lanes need to be present. In the case of figure S1B we do not think that our criteria are fully met. The PARP blot does not show consecutive lanes, but the GAPDH control blot does. I would therefore like to kindly ask you to explain the matter and to provide us with the original scans for this panel. Also, it should be explained in the figure legend that all lanes come from the same gel and that the PARP lanes and the control lanes correspond to

each other. Please let us have a suitably amended version of the supplementary material together with the primary scans via e-mail as soon as possible.

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation.

Yours sincerely,

Editor
The EMBO journal

Reply 07 December 2010

Many thanks for drawing our attention to Figure S1B.
We have now recovered the information on how these blots were assembled.
We first had a figure that comprised the PARP cleavage and also
contained Bid and Procaspase-3 cleavage after 48 hours (figure A in the
attached doc file).

The GAPDH in the bottom blot referred to those latter two caspase
substrates. We had not included a loading control for PARP as the
loading was equally uniform. This is demonstrated in figure B in the
attached doc file in which we show the complete blots of PARP and the
loading for GAPDH.

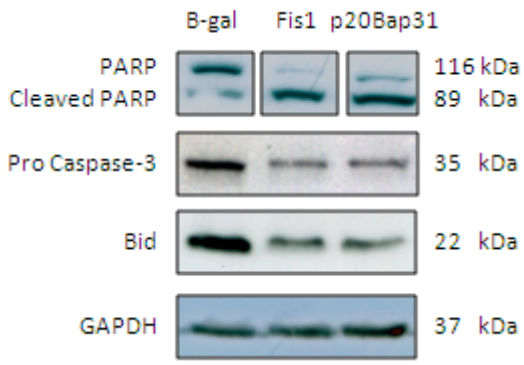
For various reasons we later took out the Bid and the procaspase-3
cleavage blots, which led to the wrong combination of the GAPDH and the
PARP cleavage blots.

We have now reassembled the correct blots and say in the figure that
they are from the same blot. The new supplementary data file is
attached.

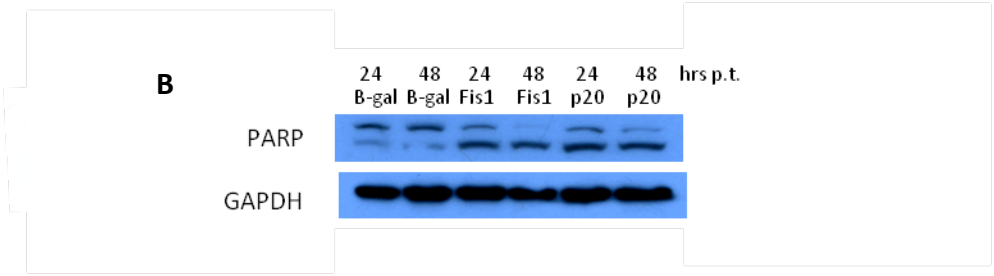
Please do let me know if anything is still unclear.

Many thanks again for pointing out this mistake.

A


B-gal
Fis1
p20Bap31
PARP
Cleaved PARP
116 kDa
89 kDa
Pro Caspase-3
35 kDa
Bid
22 kDa
GAPDH
37 kDa

B


24 B-gal
48 B-gal
24 Fis1
48 Fis1
24 p20
48 p20
hrs p.t.
PARP
GAPDH