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A synthetic lethal screen identifies FAT1 as an antagonist of caspase-8 in extrinsic apoptosis

Michael Boutros

Corresponding author: Dominique Kranz, Deutsches Krebsforschungszentrum

Review timeline:

Submission date:	16 May 2013
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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.)

Editors: Anke Sparmann and Thomas Schwarz-Romond

1st Editorial Decision 21 June 2013

Thank you for submitting your research manuscript entitled "The atypical cadherin FAT1 acts as a direct antagonist of caspase-8 to inhibit extrinsic apoptosis" (EMBOJ-2013-85686) to our editorial office. It has now been seen by three referees and their comments are provided below. I apologize for the delay in reaching a decision, which was caused by one delayed report.

All reviewers appreciate your study and are in general supportive of publication in The EMBO Journal. Nevertheless, they do raise several important concerns, which will require significant revision as detailed below. Given these evaluations, I would like to give you the opportunity to revise your manuscript, with the understanding that the referees concerns must be addressed by additional experimentation where necessary, and that acceptance of the manuscript is likely to entail validation with a subset of the referees.

Specific issues

Referee #1

Pt.1) suggests important control experiments, that need to be provided. Especially the rescue experiment with FAT1 that is not targeted by siRNA is critical and recommended by referee #2 as well. We encourage the use of cells with conditionally targeted Fat1 alleles. However, should it

prove difficult to obtain these cells, we are open to discuss this experiment further.

Pt.2) can be addressed by textual revision.

In contrast, Pt.3) to 7) should be addressed experimentally

Ref #2 provides constructive suggestions that should be considered. As mentioned, Pt.3 needs to be addressed experimentally.

Ref #3
Pt.1 entails moderating your conclusions to accurately reflect the data.

Pt.2 raises an interesting question regarding the generality versus specificity of your observations and should be addressed.

Please consider minor concern 1. and implement to other textual changes required.

I would like to add that it is our policy to allow only a single major round of revision and that it is therefore important to address all criticism at this stage. Please do not hesitate to contact me should any particular point require further clarification.

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.

REFEREE REPORTS:

Referee #1:

Ligation of death receptors (members of the TNF receptor family with an intra-cellular death domain) can trigger apoptosis, but under certain conditions can also elicit production of inflammatory cytokines, in part through activation of the NF-kB and JNK/AP-1 signaling pathways. The induction of apoptosis by death receptors is strictly dependent on caspase-8 (in humans caspase-10 may compensate) and its adaptor, FADD. The activation of caspase-8 and caspase-8 mediated downstream apoptotic signaling are both subject to stringent control, for example by FLIP proteins that act within the death inducing signaling complex (DISC) at the plasma membrane or XIAP, which inhibits downstream effector caspases, such as caspases -3 and -7.

In this manuscript Kranz and Boutros present results that indicate that the transmembrane protein FAT1 can negatively regulate death receptor mediated apoptosis but has no impact on death receptor mediated activation of NF-kB signaling with consequent production of inflammatory cytokines and chemokines. Many of the data shown are of good quality and the conclusions are of potentially far-reaching interest. There are, however, several problems with the data shown:

Major issues:

1) Figure 1: the authors state that FAT1 siRNA #4 has only minimal impact on FAT1 protein levels and they therefore conclude that the finding that this siRNA greatly enhances TRAIL induced apoptosis must be due to an off target effect. Of course, by stating this, it becomes equally possible

that the effects of the other siRNA on TRAIL induced apoptosis may also be due to off target effects. If this is the case, then all conclusions of the paper will be invalid.
There are a number of experiments that the authors can and should do to address this problem:
a) they should show effects of all individual siRNAs on FAT1 protein expression individually.
b) they should test whether re-expression of FAT1 in FAT1 knockdown cells (using an expression vector of FAT1 that is not targeted by the FAT1 siRNAs) will restore normal sensitivity to TRAIL in cell lines (this should be done using at least three cell lines).
c) they should use mice with loxP targeted FAT1 alleles and an inducible (e.g. tamoxifen inducible) or lineage specific Cre transgene to demonstrate in primary (non-transformed cells) that loss of FAT1 increases death receptor induced apoptosis.

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3) Figure 3E: it is good to see that the authors examined whether FAT1 knockdown can affect apoptosis induced by death receptor independent stimuli (Dox and CPT). However, the cells used here are very poorly responsive to these chemotherapeutic drugs with only 10-20% loss of viability with both drugs. To make the conclusion that FAT1 knockdown only affects death receptor induced apoptosis but not apoptosis mediated by the intrinsic (mitochondrial) apoptotic pathway, the authors should perform this experiment also in cell lines that are considerably more sensitive to these drugs (reaching 70-90% loss of viability).

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5) Figure 5A: the data on caspae-8 processing would be more convincing if the authors did first a pulldown of active caspases (e.g. using beads conjugated with zVADfmk-biotin) and then probed the captured material (only active caspases but not inactive caspases) with antibodies to caspase-8 and caspase-3.

6) Figure 5B: this experiments lacks the essential control of all conditions in the absence of TRAIL.

7) Figure 6: the authors should show data on the impact of FAT1 knockdown on survival of the glioblastoma derived cell lines, not only impact on caspase processing.

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1) the authors discuss both XIAP and survivin as inhibitors of apoptosis. Only XIAP inhibitos apoptosis; multiple elegant studies from the labs of David Vaux and Tak Mak using several species have shown that survivin is a regulator of mitotic division and has no role in apoptosis. This needs to be corrected.

2) the text contains many problems with English language and needs editing.

3) the authors write that caspase-8 needs to be cleaved to become active. While such cleavage does occur, elegant work from Guy Salvesen et al and David Wallach et al has shown that caspase-8 can become fully active by a conformational change (induced by its adaptor FADD) that does not need proteolytic cleavage. This should be mentioned in the Introduction.

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The paper by Kranz and Boutros describes results of a screen for siRNAs that enhance cell killing by sub-lethal levels of TRAIL. Among the hits, the investigators identified FAT1, a very large cadherin.

To confirm and characterize this finding, they showed that knock-down of FAT1 could increase the sensitivity of cells to CD95 ligation as well as TRAIL, and that antibodies to caspase 8 could co-ip FAT1 in both resting and TRAIL-treated cells.

I found the data to be very interesting, scientifically sound, and convincing.

I have only some minor optional suggestions and specific comments.

1. It would be nice to show in a clonagenic assay that knock-down of FAT1 will reduce the number of cells that can form clones after treatment with TRAIL (and does not merely increase the rate of TRAIL induced cell death.

2. Why do you think the amount of FAT1 i.p.ed by anti-caspase 8 increased after TRAIL treatement? (Fig. 5c compare 0 and 15 min.)

3. Can you protect the cells with a mutant form of FAT1 that has been altered so that its message is not bound by one or more of the FAT1 siRNAs?

4. Fig. 5a coud be re-drawn so it's possible to see all of the lines.

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The present article by Dominique Kranz and Michael Boutros identifies the cadherin FAT1 as a novel inhibitor of Caspase 8 activation and extrinsic pathway of apoptosis. By using a genome-wide siRNA screening for synthetic lethal interactions with death-receptor mediated apoptosis, the authors identified FAT1 as a new protein with an important role in sensitizing neuroblastoma cells to TRAIL-induced apoptosis.

MAJOR concerns:

1. Despite showing that:
- FAT1 and Caspase 8 co-immunoprecipitate
- knockdown of FAT1 induces an increase of Caspase 8 at the DISC and Caspase 8 cleavage/activation (in the presence of TRAIL)
- knockdown of FAT1 induces the formation of a high molecular weight complex which includes Caspase 8
the authors do not undoubtedly prove that FAT1 and Caspase8 directly interact. Caspase 8 and FAT1 co-immunoprecipitation prove that they can form a complex, not necessary that they directly interact. Moreover, in the Figure 6, the authors show that FADD levels increase at the DISC when FAT1 levels are downregulated (after TRAIL stimulation), in the same way as Caspase 8 does, suggesting that another model could be that FAT1 may prevent the FADD incorporation into the DISC (or may prevent both Caspase 8 and FADD to participate in DISC formation after TRAIL stimulation).

2. Is this phenomenon and interaction specific to neuroblastoma cells or it occurs in other cells as well?

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2. Results (p9): correct "activate procaspase-8 und subsequently to induce apoptosis". Please correct "und".
3. Figure 6A: Despite that the low-adherent cells have several fold less FAT1 than adherent cells, TRAIL-induced caspase activation is significantly lower. I find it surprising. Can the authors find an explanation ?
4.Methods section:
- Please correct everywhere "according manufacturer's instructions" with "according to (the) manufacturer's instructions" (p15, 16, 17 - 4 times)
- add a space for "24channel" (p15), ""384well" (p16)
- "over night" (p17) should be one word
- use "DISC-immunoprecipitations" instead of "DISC-immunprecipitations"
5. Table S4: Please provide the exact catalog number for each of the antibodies in the Table. Several of the companies mentioned have more than one antibody for the detection of a particular protein. As an example, Abcam has ab8229 and ab8226 for the detection of beta-actin. The reader has to know exactly what reagents were used for each experiment.

1st Revision - authors' response 30 October 2013

We thank for the overall positive evaluation. Please find below point-by-point responses on the reviewers' comments. To ease readability, we are repeating the reviewer comments. Our responses are marked in *italics.*

In particular, we have newly introduced the following data and changes:

- We present clonogenicity assays showing that FAT1 depletion decreases long-term clonogenic survival that can be rescued by simultaneous knockdown of caspase-8 (NEW Figure 5D).
- We included the control blots without TRAIL treatment (Figure 5B) showing that high-molecular weight complexes of caspase-8 are only detected in the absence of FAT1 upon TRAIL stimulation.
- We performed cell viability assays in patient derived glioblastoma cell lines showing that FAT1 knockdown also sensitizes primary cell lines for extrinsic apoptosis (NEW Figure 6C).
- We generated mutant cells lines lacking *FAT1* or *caspase-8* expression by CRISPR/Cas9-genome engineering (NEW Figure 7A). We demonstrate that deficiency of *caspase-8* renders cells completely resistant towards death-receptor mediated apoptosis, whereas loss of *FAT1* increased the susceptibility towards TRAIL-induced apoptosis (NEW Figure 7B and 7C).
- We implemented changes into our model taking into account that FAT1 impedes caspase-8 recruitment to the DISC either by direct interaction or indirect interaction via the adaptor protein FADD (NEW Figure 8).
- Clarification of the presentation of the qPCR analysis (Supplementary Figure S1A).
- We show that the siRNA-mediated FAT1 sensitivity effect is not restricted to glioblastoma cell lines presenting additional data on osteosarcoma, hepatocellular and cervical carcinoma cell lines (NEW Supplementary Figure S1B).

Referee #1:

Ligation of death receptors (members of the TNF receptor family with an intra-cellular death domain) can trigger apoptosis, but under certain conditions can also elicit production of

inflammatory cytokines, in part through activation of the NF-kB and JNK/AP-1 signaling pathways. The induction of apoptosis by death receptors is strictly dependent on caspase-8 (in humans caspase-10 may compensate) and its adaptor, FADD. The activation of caspase-8 and caspase-8 mediated downstream apoptotic signaling are both subject to stringent control, for example by FLIP proteins that act within the death inducing signaling complex (DISC) at the plasma membrane or XIAP, which inhibits downstream effector caspases, such as caspases -3 and -7.

In this manuscript Kranz and Boutros present results that indicate that the transmembrane protein FAT1 can negatively regulate death receptor mediated apoptosis but has no impact on death receptor mediated activation of NF-kB signaling with consequent production of inflammatory cytokines and chemokines. Many of the data shown are of good quality and the conclusions are of potentially far-reaching interest. There are, however, several problems with the data shown:

We thank for the overall positive evaluation and we are now providing additional experiments to address the problems as shown in detail below.

Major issues:

1) Figure 1: the authors state that FAT1 siRNA #4 has only minimal impact on FAT1 protein levels and they therefore conclude that the finding that this siRNA greatly enhances TRAIL induced apoptosis must be due to an off target effect. Of course, by stating this, it becomes equally possible that the effects of the other siRNA on TRAIL induced apoptosis may also be due to off target effects. If this is the case, then all conclusions of the paper will be invalid.
There are a number of experiments that the authors can and should do to address this problem:
a) they should show effects of all individual siRNAs on FAT1 protein expression individually.
b) they should test whether re-expression of FAT1 in FAT1 knockdown cells (using an expression vector of FAT1 that is not targeted by the FAT1 siRNAs) will restore normal sensitivity to TRAIL in cell lines (this should be done using at least three cell lines).
c) they should use mice with loxP targeted FAT1 alleles and an inducible (e.g. tamoxifen inducible) or lineage specific Cre transgene to demonstrate in primary (non-transformed cells) that loss of FAT1 increases death receptor induced apoptosis.

To clarify the presentation of the knockdown efficiency of Suppl.Fig. S1A we changed the axis labelling. As indicated by Suppl Fig. S1A the mRNA levels of FAT1 were reduced to 90% for siRNAs #1, #2 and#3 but not for siRNA #4.

The individual knockdown of the siRNAs on the protein level is presented in Figure 2C. It reflects similar knockdown efficiency as observed on the mRNA level. It also shows that siRNAs#1,#2,#3 increased caspase cleavage after TRAIL addition similar to the pooled siRNA. Based on this data we conclude that siRNA#4 acts partially through an off-target effect. However, three independent siRNAs reduced FAT1 levels and showed an apoptosis sensitivity effect, hinting towards a specific FAT1-dependent effect.

Since FAT1 has a huge size of 15kb, it was not possible to generate a transfectable full-length FAT1 expression construct. For this reason, we tested a vector that lacked most of the huge extracellular part (most of the cadherin-like repeats) in rescue experiments. However, double transfection with siRNA and FAT1-TRUNC (lacking the extracellular cadherin-repeats) plasmid rendered the cells extremely sensitive towards apoptosis even without death ligand stimulation. Therefore, we were not able to draw any conclusions from these experiments. Furthermore, we are also not confident that the N-terminal deletion construct would be able to appropriately compensate for the loss of the wild-type protein and might have additional "side" phenotypes.

To address the question in a different way, we generated FAT1 and caspase-8 knockout cell lines with the CRISPR/Cas9-system that has been recently used for genome editing in human cell lines (Cong et al, 2013; Mali et al, 2013). We obtained two different clones for each knockout (Figure 7A). As expected caspase-8 knockout cells were completely resistant towards TRAIL-induced apoptosis (Figure 7B and 7C). By contrast, FAT1 knockout cells were more sensitive towards TRAIL-induced apoptosis as indicated by increased caspase cleavage (Figure 7B) and decrease in cell survival (Figure 7C).

Taken together, these results show that loss of FAT1 enhances sensitivity towards death-receptor mediated apoptosis.

2) Figure 3: I am surprised that death receptor induced NF-kB signaling and induction of expression of chemokines and cytokines is normal in FAT1 knock-down cells. Given that FAT1 knockdown increases the death of these cells when stimulated with TRAIL, FasL or TNFa, I would have expected to see a reduction in expression of these target genes (given that dead cells will not be able to synthesize these mRNAs). The authors should explore this and provide an explanation.

Activation of NF-κB-dependent inflammatory and survival pathways by TRAIL has been reported in previous studies (recently reviewed in (Azijli et al, 2013).

Furthermore, despite their difference in the sensitivity towards TRAIL-induced apoptosis, it has been previously shown that TRAIL-resistant and TRAIL sensitive cell lines upregulate chemokines on the mRNA level and secrete e.g. CXCL2, CXCL8, CCL2, CCL4 and CCL20 in a NF-κB-dependent manner (Tang et al, 2009). These reports and our data indicate that although undergoing apoptosis death ligands can still induce the expression of NF-κB-dependent inflammatory genes. It should be noted that most cells are resistant to apoptosis induced by TNFα alone (Sugarman et al, 1985), however, we observed that depletion of FAT1 sensitized cells for TNFα-induced apoptosis (Figure 3B). The mechanism for this difference is not yet understood.

3) Figure 3E: it is good to see that the authors examined whether FAT1 knockdown can affect apoptosis induced by death receptor independent stimuli (Dox and CPT). However, the cells used here are very poorly responsive to these chemotherapeutic drugs with only 10-20% loss of viability with both drugs. To make the conclusion that FAT1 knockdown only affects death receptor induced apoptosis but not apoptosis mediated by the intrinsic (mitochondrial) apoptotic pathway, the authors should perform this experiment also in cell lines that are considerably more sensitive to these drugs (reaching 70-90% loss of viability).

We chose the concentration of chemotherapeutic drugs on purpose because we observed that FAT1 knockdown increased the sensitivity towards death-receptor mediated apoptosis. For this reason, we intended to address the sensitivity towards intrinsic apoptosis stimuli in a similar manner. If the concentrations were increased to achieve 70-90% reduced viability in the control that would enable us to observe a sensitivity effect.

4) Figure 4A: the impact of the caspase-8 siRNA on caspase-8 protein expression levels is not impressive. It is therefore difficult to reach solid conclusions from the experiment shown. Also, the authors should present data on the impact of caspase-8 knockdown on cell survival after TRAIL treatment (with or without FAT1 knockdown).

We agree that the siRNA of caspase-8 shows only moderate knockdown on protein level. This could be due to high stability of the protein in this cell line. But as presented in Figure 4A knockdown of caspase-8 did not neither result in caspase3-cleavage nor PARP-cleavage indicating that the loss of caspase-8 renders the cells insensitive towards apoptosis. Accordingly, caspase-8 knockout cells generated by CRISPR/Cas9 genome engineering are completely resistant towards death-receptor mediated apoptosis (Figure 7B and 7C).

As pointed out by the reviewer we performed clonogenicity assays to investigate the long-term surivival of these cells. We show in Figure 5D that knockdown of FAT1 decreased the amount of surviving colonies after TRAIL stimulation, but this effect was rescued by simultaneous knockdown of FAT1 and Caspase-8. As expected cells depleted of caspase-were resistant towards TRAIL treatment. This suggests that loss of FAT1 also reduces the long-term survival of cells after TRAIL stimulation.

5) Figure 5A: the data on caspae-8 processing would be more convincing if the authors did first a pulldown of active caspases (e.g. using beads conjugated with zVADfmk-biotin) and then probed the captured material (only active caspases but not inactive caspases) with antibodies to caspase-8 and caspase-3.

The intention was not to show the processing of caspase-8 in Figure 5A. In the previous Figure 2B, we showed increased caspase-8 cleavage upon TRAIL treatment and FAT1 knockdown. Additionally, immunoprecipitation of caspase-8 showed increase of cleavage fragment p43/41 and p18 only in FAT1 depleted cells after TRAIL stimulation (Figure 5C).

In Figure 5A we demonstrate that upon knockdown of FAT1 and TRAIL treatment caspase-8 cleavage products p43/p41 are localized in the membrane fraction, whereas p18 is found in the cytoplasmic fraction. It has been previously shown that the p18 fragment is released from the DISC (in the membrane) into the cytoplasm after death ligand stimulation (Hughes et al, 2009).

Furthermore, Figure 5A complements Figure 5B, where we show that combination of siRNA-FAT1 and TRAIL treatment results in the formation of high-molecular weight complexes containing caspase-8.

6) Figure 5B: this experiments lacks the essential control of all conditions in the absence of TRAIL.

We repeated the experiment and provide now the blots in the absence and presence of TRAIL (Figure 5B). As shown before, the formation of caspase-8 high molecular weight complexes are only present upon FAT1 knockdown and after TRAIL stimulation.

7) Figure 6: the authors should show data on the impact of FAT1 knockdown on survival of the glioblastoma derived cell lines, not only impact on caspase processing.

As suggested by the reviewer, we performed cell viability assays in both cell lines NCH89 and NCH342 (Figure 6D). In agreement to the increased caspase activity, FAT1 knockdown impaired survival of primary derived glioblastoma cell lines after TRAIL stimulation.

Minor issues:

1) the authors discuss both XIAP and survivin as inhibitors of apoptosis. Only XIAP inhibits apoptosis; multiple elegant studies from the labs of David Vaux and Tak Mak using several species have shown that survivin is a regulator of mitotic division and has no role in apoptosis. This needs to be corrected.

Thank you for the comment. We rephrased the sentence and replaced "anti-apoptotic proteins" by "BIR-domain-containing proteins".

2) the text contains many problems with English language and needs editing.

We are sorry for the inconvenience caused and we believe that we have corrected the mistakes.

3) the authors write that caspase-8 needs to be cleaved to become active. While such cleavage does occur, elegant work from Guy Salvesen et al and David Wallach et al has shown that caspase-8 can become fully active by a conformational change (induced by its adaptor FADD) that does not need proteolytic cleavage. This should be mentioned in the Introduction.

We thank for the comment and included the references in the revised manuscript.

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1. It would be nice to show in a clonagenic assay that knock-down of FAT1 will reduce the number of cells that can form clones after treatment with TRAIL (and does not merely increase the rate of TRAIL induced cell death.

As suggested by the reviewer, we performed clonogenic assays upon FAT1 and Caspase-8 knockdown. In Figure 5D we show that depletion of FAT1 reduced the number of surviving colonies after TRAIL stimulation. However, the effect was rescued by simultaneous knockdown of caspase-8.

This suggests that loss of FAT1 also reduces the long-term survival of cells after TRAIL stimulation.

2. Why do you think the amount of FAT1 i.p.ed by anti-caspase 8 increased after TRAIL treatment? (Fig. 5c compare 0 and 15 min.)

The small increase of FAT1 after 15min might indicate that there is an increased interaction between caspase-8 and FAT1 after TRAIL stimulation. However, since this effect was variable in different biological replicates it could also suggest that this effect might arise from experimental variations.

3. Can you protect the cells with a mutant form of FAT1 that has been altered so that its message is not bound by one or more of the FAT1 siRNAs?

Since FAT1 has a huge size of 15kb, it was not possible to generate a transfectable full-length FAT1 expression construct. For this reason, we tested a vector that lacked most of the huge extracellular part (most of the cadherin-like repeats) in rescue experiments. However, double transfection with siRNA and FAT1-TRUNC plasmid rendered the cells extremely sensitive towards apoptosis even without death ligand stimulation. Therefore, we were not able to draw any conclusions from these experiments. Furthermore, we are also not confident that the N-terminal deletion construct would be able to appropriately compensate for the loss of the wild-type protein and might have additional "side" phenotypes.

To address the question in a different way, we generated FAT1 and caspase-8 knockout cell lines with the CRISPR/Cas9-system that has been recently used for genome editing in human cell lines (Cong et al., Mali et al.). We obtained two different clones for each knockout (Figure 7A). As expected caspase-8 knockout cells were completely resistant towards TRAIL-induced apoptosis

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4. Fig. 5a could be re-drawn so it's possible to see all of the lines.

We re-drawn the lines as suggested.

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- knockdown of FAT1 induces an increase of Caspase 8 at the DISC and Caspase 8 cleavage/activation (in the presence of TRAIL)

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the authors do not undoubtedly prove that FAT1 and Caspase8 directly interact. Caspase 8 and FAT1 co-immunoprecipitation prove that they can form a complex, not necessary that they directly interact. Moreover, in the Figure 6, the authors show that FADD levels increase at the DISC when FAT1 levels are downregulated (after TRAIL stimulation), in the same way as Caspase 8 does, suggesting that another model could be that FAT1 may prevent the FADD incorporation into the DISC (or may prevent both Caspase 8 and FADD to participate in DISC formation after TRAIL stimulation).

Thank you for the comment. We agree that the co-immunoprecipitation could also reflect an interaction between FAT1 and the complex of FADD and caspase-8. Within this model, FAT1 could inhibit the incorporation of either proteins alone or together into the DISC. Accordingly, we rephrased our conclusions in the discussion of the revised manuscript and changed the model to include both hypothesis (Figure 8).

2. Is this phenomenon and interaction specific to neuroblastoma cells or it occurs in other cells as well?

To address this question we performed synthetic lethality assays in other cell types. We transfected different cell lines with control and FAT1 siRNAs, treated them with TRAIL and measured cell viability (Supplementary Figure S2). We observed that cervix carcinoma cells (HeLa), osteosarcoma

cells (U2OS) and hepatocellular carcinoma cells (HepG2) are sensitized towards TRAIL-induced apoptosis upon depletion of FAT1. These results suggest that this sensitivity effect is not restricted to glioblastoma cells.

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1. Although not critical, it would be helpful if the authors would overexpress FAT1 in a cell line with low levels of endogenous FAT1 and show that the resistance to TRAIL-induced apoptosis is increasing.

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Taken together, these results show that loss of FAT1 enhances sensitivity towards death-receptor mediated apoptosis.

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As suggested we changed labelling of the axis.

2. Results (p9): correct "activate procaspase-8 und subsequently to induce apoptosis". Please correct "und".

Thanks for pointing this out, we corrected it in the revised manuscript.

3. Figure 6A: Despite that the low-adherent cells have several fold less FAT1 than adherent cells, TRAIL-induced caspase activation is significantly lower. I find it surprising. Can the authors find an explanation ?

It has been previously shown that cancer cells such as glioblastomas can acquire adhesion-mediated apoptosis resistance (Westhoff & Fulda, 2009). Adhesion-mediated apoptosis resistance is mediated by cell-extracellular matrix or cell-cell interactions. Previous data indicated the alternation between cell-extracellular matrix and cell-cell adhesion in glioblastoma cells (Westhoff et al, 2008). Furthermore, within this study it was shown that in the absence of fibronectin the cells

switched to the alternative form of adhesion-mediated apoptosis resistance and only inhibiting both pathways sensitized cells for death-receptor mediated apoptosis. Although the molecular mechanism has not been identified yet, several survival pathways such as PI3K/Akt and MAPK/ERK have been implicated in the adhesion-mediated apoptosis resistance (Westhoff & Fulda, 2009).

Importantly, we show that depletion of FAT1 could still sensitize non-adherent cells towards death-receptor mediated apoptosis suggesting that the sensitivity effect is independent from cell-extracellular matrix interactions and supporting the previous known function of FAT1 and its role in cell-cell interactions.

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Thanks for the comment, we included now the catalogue numbers.

REFERENCES

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Editorial Correspondence 05 November 2013

Thank you very much for the revised study.

One of the original referees commented on the modifications and is supporting publication of your study.

Before formal acceptance though, please notice that The EMBO Journal encourages the presentation of source data, particularly for electrophoretic gels/blots, with the aim to make primary data more accessible and transparent to the reader. This entails un-cropped/unprocessed scans for KEY data of published work. We would be grateful for one PDF-file per figure with such information. These will be linked online as supplementary "Source Data" files.

Further, I would be grateful for a short SYNOPSIS, summarizing in 2 up to 5 bullet points the major conclusions on your study.

Overall, please allow me to congratulate you to your work. I look forward to receiving relevant source data/a synopsis soon and confirm that the editorial office will be in touch with necessary paperwork related to formal acceptance within the next couple of days.

Referee Report:

The authors have responded adequately to all my requests and comments. I believe that this paper is now suitable for publication in The EMBO Journal.