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XIAP inhibits autophagy via XIAP-Mdm2-p53 signaling

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 06 February 2013

Thank you for submitting your research manuscript (EMBOJ-2012-84319) to our editorial office. It has now been seen by three referees and their comments are provided below.

All reviewers appreciate the concept of your study and acknowledge a potential interest to a wide audience. Nevertheless, they do raise a number of important concerns, and emphasize that a substantial revision of the manuscript will be required. I would like to highlight that following the suggestions of reviewer #1, the physiological relevance of your observations needs to be addressed. Furthermore, additional markers of autophagy should be examined throughout your analysis to strengthen your conclusions. In addition, it will also be crucial to rule out that the effects of XIAP on tumorigenesis are mediated by its anti-apoptotic functions. Finally, the XIAP-Mdm2 interaction data should be strengthened based on the constructive comments provided by referee #3.

Given the overall evaluations, I would like to provide you with the opportunity to revise your manuscript, with the understanding that the referees' concerns must be fully addressed by additional experimentation where necessary, and that acceptance of the manuscript will entail validation with a subset of the referees. I should add that it is our policy to allow only a single round of major revision and that it is therefore important to address all criticism at this stage. Please do not hesitate to contact me if you would like to further discuss the required revisions.

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

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

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

General Remarks
This manuscript would like to show that

XIAP inhibits autophagy
XIAP inhibits autophagy via the Mdm2-p53 pathway
XIAP is a novel E3 ligase for Mdm2
XIAP regulates serum starvation-induced autophagy
XIAP-modulated autophagy is associated with the tumorigenicity

The authors use a range of systems including knock-out XIAP deficient HCT116 cells, XIAP knock-down, endogenous immunoprecipitation and biochemical assays to demonstrate that XIAP is a ubiquitin E3 ligase for MDM2. Decreased levels of MDM2 result in increased p53. In XIAP depleted cells therefore MDM2 goes up, p53 goes down and autophagy is increased.

Finally they show that XIAP knock-out HCT116 cells grow more slowly in nude mice and that reconstitution with a phosphorylation defective mutant of XIAP does not revert this phenotype in knock-out cells but reconstitution with a wild type or phospho mimic does. And remarkably that a diverse set of human tumors have consistently higher levels of XIAP and p53 and lower levels of LC3 lipidation.

The main issue is the physiological relevance. XIAP knock-out mice are phenotypically normal and do not suffer from increased tumorigenesis. To all intents and purposes this is a study that shows that HCT XIAP knock-out cells have increased autophagy and tumorigenetic potential, but it does not address whether this is a generic XIAP knock-out phenomenon. I think for such an important claim (and a p53/MDM2/XIAP link would be highly cited) an analysis of real XIAP knock-out cells is called for.

Finally I think that other markers of autophagy should be examined throughout, eg p62 accumulation etc. LC3 lipidation on its own is not convincing enough.

Specific Remarks

Figure 1 should do the knock-out with embelin, to see whether embelin is on target.

All the background of the autorads is the same, and monochrome/monotone, should show the originals.

Other changes than LC3 conversion p62?

Figure 2 In Tasdemir Nutlin and RITA inhibited autophagy because they prevented degradation of p53, this is consistent with these results but it would be good to see a dose response to Nutlin.

Figure 3D I never seen XIAP H467A expressed to the same levels as wt. Are the same amounts of construct transfected? DKOs (p53/MDM2 described in text pg 8 for 3G but first used in 3D.

Figure 3E and H. What antibodies were used? If his antibodies then this does not prove that either MDM2 or p53 are ubiquitylated it could be p53 or mdm2 interacting proteins. Therefore the experiment needs to be done in denaturing conditions using His-tagged ubiquitin. Then probe with p53 or mdm2 antibodies.

Figure 4B&C total AKT levels need to be shown.

Figure 5 Tumor size in B & C does not clearly correspond with the pictures in 5A. For example, Mouse 2 and 3 in SA (Panel A3) have large tumors on both sides, as large as the right tumor in mouse 2 of the knock-outs reconstituted with XIAP (Panel A2), yet the tumor pictures in B do not correspond (Panel B2 compared Panel B3). Nor for XIAP knock-out compared to wild type: mouse 6 in Panel A1 has a large tumor on the right side but this appears to be one of the smallest tumors in Panel B1.

Referee #2 (Remarks to the Author):

Huang, Wu et al.' manuscript "IAP, more than Inhibitor of Apoptosis Protein: X-linked IAP (XIAP) inhibits autophagy via XIAP-Mdm2-p53 signaling" is a detailed and well-written work.

The authors study the role of XIAP in the physiological inhibition of autophagy via an Mdm2-p53-dependent mechanism. They also point out the importance of XIAP-dependent autophagy inhibition in tumorigenesis. Evidence that their findings are relevant from mice to human tumors are also provided. Overall, the manuscript is very interesting but there is one major concern that needs to be addressed.

The authors demonstrate really well and with different approaches that XIAP reduction results in increased autophagy. They further show that XIAP binds and regulates Mdm2 stability through its ubiquitin E3-ligase activity. Mdm2 ubiquitination and degradation results in turn in p53 stabilization and, consequently, in autophagy induction. Furthermore, the effect of XIAP on autophagy inhibition is Mdm2/p53 dependent and it requires phosphorylation of Ser87, fundamental for Mdm2 binding.

The described signaling cascade seems to happen just in the cytoplasm and should not affect p53 transcriptional nuclear functions (hence also activation of apoptotic genes). The main known function of XIAP though is its anti-apoptotic function.
We cannot exclude that the effects on tumorigenesis seen in the xenograft mouse model studies (and the correlated studies in human tumors) could be the result of the combined XIAP functions on autophagy and apoptosis.
Since the authors have tried to assess the relative role of the different XIAP phosphorylation mutants in tumorigenesis, they could also test the effect of the apoptosis-incompetent mutant shown in Fig 1, XIAP-D148A/W310A.

On a minor note, it could be useful having the LC3 processing shown in the different WB figures expressed as graphs of the ratio LC3 II/ LC I over actin. Sometimes the increase in the processing does not look very clear and having the ratio would help discriminate between LC3 processing and LC3 overall induction.

Referee #3 (Remarks to the Author):

It is commonly accepted that the ubiquitin ligase XIAP is a major regulator of apoptosis. In this manuscript, Huang et al. report that XIAP is also a regulator of autophagy by targeting the ubiquitin ligase Mdm2 for degradation, thereby inducing p53 activity. Furthermore, the authors report that XIAP binds to Mdm2 and that this interaction, and subsequent ubiquitylation and degradation of Mdm2, depend on the phosphorlyation status of XIAP. Finally, analysis of about 40 primary tumors derived from different tissues revealed that about 30 percent of these show decreased signs of autophagy and that this correlates with increased levels of XIAP and p53 levels. The notions that

XIAP is a regulator of autophagy and that XIAP acts as a ubiquitin ligase for Mdm2 are of potential interest to a wide audience. As my expertise with respect to autophagy is limited, I will not further comment on this issue (the effect of knockdown of XIPA expression and ectopic expression of XIAP on the apoptotic rate of the respective cells should be determined in at least some of the experiments to substantiate the notion that the effects observed are not due to alterations in the cell cycle/apoptotic profile). However, the data concerning the interaction of XIAP with Mdm2 are rather preliminary/not convincing.

1. Fig. 3C and Fig. S2B: In both experiments, Mdm2 levels at time 0 do not appear to significantly differ between HCT116 cells and HCT116 cells null for XIAP or with a knockdown of XIPA expression (as would be expected if XIAP plays a major role in Mdm2 turnover; or are Mdm2 mRNA levels different in the different cell lines?). Furthermore, levels of Mdm2 should be quantified and SD values indicated (i.e. how reproducible are the data?).

2. Fig. 3D: Does the XIAP mutant (H467A) still bind to Mdm2?

3. Fig. 3E: I assume that the Western blot analysis was performed with an anti-ubiquitin antibody. If this assumption is correct, it would be highly desirable to provide further evidence that the smear indicated as ubiquitylated Mdm2 indeed represents ubiquitylated forms of Mdm2 (e.g., His-tagged ubiquitin was coexpressed (for what reason?). So, why not perform a Ni-pulldown and blot against Mdm2 or perform an anti-HA IP and probe with anti-Mdm2 antibody?).

4. Fig. 3F and Fig. S2D: If the Western blot analysis was performed with an anti-ubiquitin antibody (in general, the experiments are poorly described), it is mandatory to provide direct evidence that the bands observed indeed represent ubiquitylated Mdm2 (rather than ubiquitylated forms of XIAP, for example).

5. Fig. 3H: If I understand correctly, the experiment was performed in the presence of a proteasome inhibitor. This may explain why levels of Mdm2 are not affected in the presence of XIAP. However, if the main effect of XIAP on p53 levels is via destabilization of Mdm2 (and this obviously does not happen under the conditions used), why are levels of ubiquitylated p53 decreased in the presence of XIAP and MG132 (if it is p53; see comment 3)? Does binding of XIAP (to Mdm2) interfere with the Mdm2-p53 interaction?

6. Fig. 1G: Does the XIAP mutant used bind to Mdm2 and affect its half-life?

Other comments

7. Fig. S1D: Is it known from the literature that tumor-derived p53 mutants interfere with autophagy induction? If not, this requires more attention. Furthermore, it would be helpful if transfection efficiencies were indicated (since the effect seems to be rather dramatic).

8. Fig. 3D. Does the XIAP mutant still bind Mdm2? If not, what conclusion can be drawn from this experiment?

9. Fig. 5D: In the absence of data concerning the p53 status in the different tumors, the significance of the data is limited (i.e. the increased in p53 levels may be due to mutation of the p53 gene rather than to an increase in XIAP levels).

1st Revision - authors' response 24 March 2013

Response to Referee #1:

The authors use a range of systems including knock-out XIAP deficient HCT116 cells, XIAP knock-down, endogenous immunoprecipitation and biochemical assays to demonstrate that XIAP is a ubiquitin E3 ligase for MDM2. Decreased levels of MDM2 result in increased p53. In XIAP depleted cells therefore MDM2 goes up, p53 goes down and autophagy is increased. Finally they show that XIAP knock-out HCT116 cells grow more slowly in nude mice and that reconstitution with a phosphorylation defective mutant of XIAP does not revert this phenotype in knock-out cells but reconstitution with a wild type or phospho mimic does. And remarkably that a diverse set of human tumors have consistently higher levels of XIAP and p53 and lower levels of LC3 lipidation. The main issue is the physiological relevance. XIAP knock-out mice are phenotypically normal and do not suffer from increased tumorigenesis. To all intents and purposes this is a study that shows that HCT XIAP knock-out cells have increased autophagy and tumorigenetic potential, but it does not address whether this is a generic XIAP knock-out phenomenon. I think for such an important claim (and a p53/MDM2/XIAP link would be highly cited) an analysis of real XIAP knock-out cells is called for.

To address the reviewer's concern, we have got the XIAP KO MEFs and matching XIAP wild-type MEFs from Prof. David Vaux (Walter and Eliza Hall Institute of Medical Research, Australia). We found that compared to XIAP wild-type MEFs, XIAP KO MEFs indeed expressed increased levels of Mdm2 and decreased levels of p53, and exhibit enhanced conversion of LC3-I to LC3II (Supplementary Figure S2D). This finding strongly supports our conclusion that XIAP inhibits autophagy via regulating the Mdm2-p53 pathway.

Finally I think that other markers of autophagy should be examined throughout, eg p62 accumulation etc. LC3 lipidation on its own is not convincing enough.

According to the reviewer's comments, we used an electron microscope to evaluate autophagic vesicle formation in XIAP wide-type HCT116 cells and XIAP KO HCT116 cells. As shown in Figure 1C, XIAP deficiency led to a dramatic increase in autophagic vesicle formation. This finding reinforces the important role of XIAP in the regulation of basal autophagy.

In fact, at the beginning of this project, we had examined the levels of p62 in XIAP wild-type and XIAP KO HCT116 cells. It turned out that levels of p62 were unexpected increased upon XIAP depletion, although XIAP KO HCT116 cells exhibited enhanced autophagy compared with XIAP wild-type HCT116 cells. This finding confused us for a while. But now we know that XIAP acts as an E3 ubiquitin ligase of p62 and regulates p62 function. We are currently wrapping up this story. Therefore, we did not use p62 accumulation as an autophagic marker in this study.

Specific Remarks

Figure 1 should do the knock-out with embelin, to see whether embelin is on target.

As suggested by the reviewer, we examined the effect of Embelin on LC3 conversion in XIAP KO HCT116 cells. As shown in Supplementary Figure S1D, Emblin failed to increase conversion of LC3-I

to LC3-II in XIAP KO HCT116 cells. This data suggests that the enhancing effect of Embelin on autophagy is through targeting of XIAP.

All the background of the autorads is the same, and monochrome/monotone, should show the originals.

As we mentioned in the Materials and Methods section of the manuscript, visualized images were obtained using ImageQuart ^{TM} LAS-4000 mini (GE Fujifilm), which allowed us to get the low-background images.

For the reviewer's reference, we have provided the original images for Figure 1 as Supplementary Figure S14.

Other changes than LC3 conversion p62?

We have showed that XIAP deficiency led to a dramatic increase in autophagic vesicle formation (Figure 1C). Additionally, we have found that XIAP can act an E3 ubiquitin ligase of p62 and regulates p62 function (please see the above response).

Figure 2 In Tasdemir Nutlin and RITA inhibited autophagy because they prevented degradation of p53, this is consistent with these results but it would be good to see a dose response to Nutlin.

In Supplementary Figure S2J, we have showed that Nutlin 3 inhibited autophagy in XIAP KO HCT116 cells in a dose-dependent manner.

Figure 3D I never seen XIAP H467A expressed to the same levels as wt. Are the same amounts of construct transfected? DKOs (p53/MDM2 described in text pg 8 for 3G but first used in 3D.

The reviewer is correct. In order to better compare the effects of XIAP and its H467 mutant on the regulation of Mdm2 and p53, we had adjusted the amounts of XIAP and XIAP H467A expressing plasmids used for transfection to ensure expression levels of XIAP and XIAP H467A were similar. During the study, XIAP H467A expressing plasmid was always used less than XIAP expressing plasmid. This information has been provided in the Figure legends section of the revised manuscript.
In the revised manuscript, the information about p53 and Mdm2 double knockout MEFs has been presented in the right order.

Figure 3E and H. What antibodies were used? If his antibodies then this does not prove that either MDM2 or p53 are ubiquitylated it could be p53 or mdm2 interacting proteins. Therefore the experiment needs to be done in denaturing conditions using His-tagged ubiquitin. Then probe with p53 or mdm2 antibodies.

We appreciate the reviewer's critical comments. We should emphasize that all the previous *in vivo* ubiqitination experiments were performed in denaturing condition (Please see the Materials and Methods section of the previous manuscript). Under this condition, most of protein-protein interactions should be disrupted (Tang et al. 2006 ***Nature Cell Biol.*** 8: 855-862).

To make our conclusions more persuasive, we have re-performed all the ubiquitination experiments as the reviewer suggested. In these experiments, cell lysates were denatured before proteins conjugated to His-ubiquitin were pulled down by Ni^{2+}-NTA beads. The beads-bound proteins and total cell lysates (TCL) were analyzed by western blotting with anti-Mdm2 or anti-p53 antibody. The results consistently showed that wide type XIAP, but not its H467A mutant increased Mdm2 polyubiquitination and decreased p53 polyubiquitination (Supplementary Figures S3E and S5B).

Figure 4B&C total AKT levels need to be shown.

In the revised manuscript, total AKT levels have been shown in Figures 4B and 4C.

Figure 5 Tumor size in B & C does not clearly correspond with the pictures in 5A. For example, Mouse 2 and 3 in SA (Panel A3) have large tumors on both sides, as large as the right tumor in mouse 2 of the knock-outs reconstituted with XIAP (Panel A2), yet the tumor pictures in B do not correspond (Panel B2 compared Panel B3). Nor for XIAP knock-out compared to wild type: mouse 6 in Panel A1 has a large tumor on the right side but this appears to be one of the smallest tumors in Panel B1.

We had performed the tumor xenograft experiments very carefully. We did notice that some tumor-bearing mice had purulent fluid around the tumors, possibly due to the infection. That is the reason why the sizes of some excised tumors shown in Figure 5B were not perfectly matched with the mice-bearing tumors shown in Figure 5A.

Response to Referee #2

Huang, Wu et al.' manuscript "IAP, more than Inhibitor of Apoptosis Protein: X-linked IAP (XIAP) inhibits autophagy via XIAP-Mdm2-p53 signaling" is a detailed and well-written work. The authors study the role of XIAP in the physiological inhibition of autophagy via an Mdm2-p53-dependent mechanism. They also point out the importance of XIAP-dependent autophagy inhibition in tumorigenesis. Evidence that their findings are relevant from mice to human tumors are also provided. Overall, the manuscript is very interesting but there is one major concern that needs to be addressed. The authors demonstrate really well and with different approaches that XIAP reduction results in increased autophagy. They further show that XIAP binds and regulates Mdm2 stability through its ubiquitin E3-ligase activity. Mdm2 ubiquitination and degradation results in turn in p53 stabilization and, consequently, in autophagy induction. Furthermore, the effect of XIAP on autophagy inhibition is Mdm2/p53 dependent and it requires phosphorylation of Ser87, fundamental for Mdm2 binding. The described signaling cascade seems to happen just in the cytoplasm and should not affect p53 transcriptional nuclear functions (hence also activation of apoptotic genes). The main known function of XIAP though is its anti-apoptotic function. We cannot exclude that the effects on tumorigenesis seen in the xenograft mouse model studies (and the correlated studies in human tumors) could be the result of the combined XIAP functions on autophagy and apoptosis. Since the authors have tried to assess the relative role of the different XIAP phosphorylation mutants in tumorigenesis, they could also test the effect of the apoptosis-incompetent mutant shown in Fig 1, XIAP-D148A/W310A.

We appreciated the reviewer's constructive comments. As suggested by the reviewer, we have used a xenograft mouse model to determine the tumorigenecity of HCT116 XIAP KO cells stably expressing XIAP D148A/310A caspase-binding mutant. As shown in supplementary Figure S9, when XIAP D148A/310A was reconstituted into HCT116 XIAP KO cells, their tumorigenecity was greatly improved. These data indicate that the inhibition of apoptosis contributes little if any to the tumor-promoting effect of XIAP.

On a minor note, it could be useful having the LC3 processing shown in the different WB figures expressed as graphs of the ratio LC3 II/ LC I over actin. Sometimes the increase in the processing does not look very clear and having the ratio would help discriminate between LC3 processing and LC3 overall induction.

As the reviewer suggested, the protein bands on the blots were qualified using Gel-Pro analyzer software (Rockville, MD, USA). The ratio of LCII/LC3I to actin for the indicated Figures has been provided in supplementary Figures S1A-B, S1E-H, S2A, S2E-G and S2I.

Response to Referee #3

It is commonly accepted that the ubiquitin ligase XIAP is a major regulator of apoptosis. In this manuscript, Huang et al. report that XIAP is also a regulator of autophagy by targeting the ubiquitin ligase Mdm2 for degradation, thereby inducing p53 activity. Furthermore, the authors report that XIAP binds to Mdm2 and that this interaction, and subsequent ubiquitylation and degradation of Mdm2, depend on the phosphorlyation status of XIAP. Finally, analysis of about 40 primary tumors derived from different tissues revealed that about 30 percent of these show decreased signs of autophagy and that this correlates with increased levels of XIAP and p53 levels. The notions that XIAP is a regulator of autophagy and that XIAP acts as a ubiquitin ligase for Mdm2 are of potential interest to a wide audience. As my expertise with respect to autophagy is limited, I will not further comment on this issue (the effect of knockdown of XIPA expression and ectopic expression of XIAP on the apoptotic rate of the respective cells should be determined in at least some of the experiments to substantiate the notion that the effects observed are not due to alterations in the cell cycle/apoptotic profile). However, the data concerning the interaction of XIAP with Mdm2 are rather preliminary/not convincing.

The reviewer is right. To exclude the possibility that XIAP promotes tumorigenesis via inhibiting apoptosis, we have used a xenograft mouse model to determine the tumorigenecity of HCT116 XIAP KO cells stably expressing XIAP D148A/W130A caspase-binding mutant. We found that when XIAP D148/W310A was reconstituted into HCT116 XIAP KO cells, their tumorigenecity was markedly improved (Supplementary Figure S9). These data indicate that the inhibition of apoptosis contributes little if any to the tumor-promoting effect of XIAP.

The interaction of XIAP H467 mutant with Mdm has been shown in the Supplementary Figure S4C. Also, XIAP D148A/D310A still retained the Mdm2-binding ability (Supplementary Figure S4A).

1. Fig. 3C and Fig. S2B: In both experiments, Mdm2 levels at time 0 do not appear to significantly differ between HCT116 cells and HCT116 cells null for XIAP or with a knockdown of XIPA expression (as would be expected if XIAP plays a major role in Mdm2 turnover; or are Mdm2 mRNA levels different in the different cell lines?). Furthermore, levels of Mdm2 should be quantified and SD values indicated (i.e. how reproducible are the data?).

We apologized for causing confusions for the reviewer. For the previous Figures 3C and S2B, amounts of cell lysates were adjusted to achieve similar expression levels of Mdm2 at time 0. This information has been provided in the legends of the corresponding Figures. We have also qualified the protein bands on the blots using Gel-Pro analyzer software (Rockville, MD, USA). The ratio of Mdm2 to actin was then calculated. The data are represented as mean±SD from three independent experiments (Supplementary Figures S3B and S3D).

2. Fig. 3D: Does the XIAP mutant (H467A) still bind to Mdm2?

We have confirmed the interaction of XIAP mutant H467A with Mdm2 by performing an immunoprecipitation assay (Supplementary Figure S4C).

3. Fig. 3E: I assume that the Western blot analysis was performed with an anti-ubiquitin antibody. If this assumption is correct, it would be highly desirable to provide further evidence that the smear indicated as ubiquitylated Mdm2 indeed represents ubiquitylated forms of Mdm2 (e.g., His-tagged ubiquitin was coexpressed (for what reason?). So, why not perform a Ni-pulldown and blot against Mdm2 or perform an anti-HA IP and probe with anti-Mdm2 antibody?).

We should emphasize that all the previous ubiqitination experiments were performed in denaturing condition (Please see the Materials and Methods section of the previous manuscript). Under this condition, most of protein-protein interactions should be disrupted (Tang et al. 2006 ***Nature Cell Biol.*** 8: 855-862).

To make our conclusions more persuasive, we have re-performed all the ubiquitination experiments as the reviewer suggested. In these experiments, cell lysates were denatured before proteins conjugated to His-ubiquitin were pulled down by Ni^{2+}-NTA beads. The beads-bound proteins and total cell lysates (TCL) were analyzed by western blot with anti-Mdm2 or anti-p53 antibody as indicated. The results consistently showed that wide type XIAP, but not its H467A mutant increased Mdm2 polyubiquitination and decreased p53 polyubiquitination (Supplementary Figures S3E and S5B)

4. Fig. 3F and Fig. S2D: If the Western blot analysis was performed with an anti-ubiquitin antibody (in general, the experiments are poorly described), it is mandatory to provide direct evidence that the bands observed indeed represent ubiquitylated Mdm2 (rather than ubiquitylated forms of XIAP, for example).

According to the reviewer's comments, we have re-performed the *in vitro* ubiquitination experiments. This time, the purified proteins were incubated with E1, E2 and Ub in a total 20ul *in vitro* ubiquitination reaction buffer at 37 for 2h. The reaction mix was analyzed by Western blotting using anti-Mdm2 antibody. As shown in the revised Figure 3F, ubiquitination of both wild-type Mdm2 and Mdm2 C464A mutant was strongly enhanced by wild-type but not H467A mutant XIAP. Additionally, XIAP was shown to promote Mdm2 C464A ubiquitination in a dose-dependent fashion (Supplementary Figure S5A). The previous Figure 3F and Figure S2D have been replaced by new figures (Figure 3F and supplementary Figure S5A) in the revised manuscript.

5. Fig. 3H: If I understand correctly, the experiment was performed in the presence of a proteasome inhibitor. This may explain why levels of Mdm2 are not affected in the presence of XIAP. However, if the main effect of XIAP on p53 levels is via destabilization of Mdm2 (and this obviously does not happen under the conditions used), why are levels of ubiquitylated p53 decreased in the presence of XIAP and MG132 (if it is p53; see comment 3)? Does binding of XIAP (to Mdm2) interfere with the Mdm2-p53 interaction?

We apologize again for the confusion we created due to the incomplete description of Figure 3H. Actually, the experiment was performed in the following way: $p53^{-/-}Mdm2^{-/-}$ MEF cells were transfected with the indicated plasmids. 24 h after transfection, cells were treated with 20μM MG-132 for additional 4 h. Cell lysates were denatured before immunoprecipitation with anti-Flag antibody. The immunoprecipitates were analyzed by Western blotting using anit-ubiquitin antibody. We have

revised the description of Figure 3H.

To make our conclusion more persuasive, we have re-performed this experiment as the reviewer suggested. This time, cell lysates were denatured before proteins conjugated to His-ubiquitin were pulled down by Ni^{2+}-NTA beads. The beads-bound proteins and total cell lysates (TCL) were analyzed by Western blot with anti-p53 antibody. This result further confirmed that wide type XIAP, but not its H467A mutant decreased p53 polyubiquitination (Supplementary Figure S5B).

6. Fig. 1G: Does the XIAP mutant used bind to Mdm2 and affect its half-life?

To address the reviewer's question, we have performed an immunoprecipitation assay and a half-life experiment. The results showed that XIAP D148A/D310A mutant was still able to interact with Mdm2 and decrease Mdm2's half-life (Supplementary Figures S4A and S4B). We have also qualified the protein bands on the blots using Gel-Pro analyzer software (Rockville, MD, USA). The ratio of Mdm2 to actin was then calculated. The data are represented as mean±SD from three independent experiments (Supplementary Figure S4B).

Other comments

7. Fig. S1D: Is it known from the literature that tumor-derived p53 mutants interfere with autophagy induction? If not, this requires more attention. Furthermore, it would be helpful if transfection efficiencies were indicated (since the effect seems to be rather dramatic).

It has recently been shown that wild type p53 as well as tumor-derived p53 mutants inhibit autophagy (Tasdemir et al. (2008) ***Nature Cell Biol.*** 10: 676-87 and Morselli et al. (2008) ***Cell Cycle*** 7: 3056-3061). Consistent with these reports, we also showed that tumor-derived p53 mutants can inhibit autophagy (Supplementary Figure S2H).

Transfection of HCT116 cells by lipofectamine 2000 (Invitrogen, USA) was performed according to the manufacturer's instruction, which gives～85% transfection efficiency. This has been indicated in the Materials and Methods section of the revised manuscript.

8. Fig. 3D. Does the XIAP mutant still bind Mdm2? If not, what conclusion can be drawn from this experiment?

The interaction of XIAP H467A mutant and Mdm2 has been confirmed by co-immunoprecipitation experiments (Supplementary Figure S4C).

9. Fig. 5D: In the absence of data concerning the p53 status in the different tumors, the significance of the data is limited (i.e. the increased in p53 levels may be due to mutation of the p53 gene rather than to an increase in XIAP levels).

We agree with the reviewer's point that the increased p53 levels may be partly due to mutation of the *p53* gene. We currently can not totally exclude this possibility, since determination of each p53 gene sequence in every tumor sample is technically not feasible. This has been included in the revised

manuscript. However, based on the data presented in this manuscript, autophagy inhibition mediated by the XIAP-Mdm-2-p53 pathway will likely play an important role in promoting tumor formation.

Additional correspondence 23 April 2013

Thank you again for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees and both raise issues concerning the quality of the provided data sets. As you might be aware, The EMBO Journal encourages the publication of source data, i.e. the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures, with the aim of making primary data more accessible and transparent to the reader. Given the hesitations expressed by the referees, I would like to kindly ask you to provide the source data upfront for quality assurance. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. Please send a ZIP archive containing these individual files via reply mail.

I will refrain from making a decision on your manuscript pending our assessment of the source data. However, I would like to stress that at the very minimum an extensive reformatting of your manuscript will be required, because the majority of the data added in response to the original referees' comments needs to be included in the main body of the text and not added merely as supplemental figures.

Additional correspondence 23 April 2013

Attached please find the original images for all the figures shown in the previous revised manuscript. We understand the reviewers' concerns regarding the qualities of our figures. We should emphasize that 1) all the original images were obtained using ImageQuart TM LAS-4000 mini (GE Fujifilm), which allowed us to get the images with low background; 2) The specificities of antibodies we used in this study are very high; 3) The original images shown in PowerPoint format have not been modified in Photoshop or any software.

In our Western blot experiments, we did not use fluorescent protein marker. After transferring proteins from SDS-PAGE to nitrocellulose membrane, the membrane was pre-stained with Ponceau S for visualizing the marker proteins. The positions of the marker proteins were labeled by punching holes on the membrane using a tip. The blot was then incubated with antibodies and proteins were visualized by using ImageQuart TM LAS-4000 mini.

As the editor suggested, we have included the majority of the data added in response to the reviewers' comments in the main body of the text. The new version of manuscript is also attached.

Thanks very much again for all the constructive suggestions and I am looking forward to hearing from you soon.

2nd Editorial Decision 02 May 2013

Thank you for your patience while we re-reviewed your revised manuscript and assessed your source data. As you can see from the comments provided below, Reviewer #1 is generally supportive of publication, but stresses that you need to provide natural looking images for all western blots. I appreciate that you used a lumino-imaging analyzer to obtain the scans of your western blots. However, you do need to ascertain that identical standard settings for brightness and contrast are used during all image capture.

Referee 3 is not entirely convinced that - in contrast to Figure 2B - the data presented in Figure 3C demonstrate that knock-down of XIAP significantly affects Mdm2 levels in the absence of CHX. However, we find that future studies will have to show the physiological importance of this regulation. In contrast, attending to her/his request for additional controls in Fig 3F and 3H is in our

view a crucial prerequisite to publication. In addition, you did not yet address if binding of XIAP to Mdm2 interferes with the ability of Mdm2 to bind to p53.

I would like to mention once again that it is our policy at The EMBO Journal to allow only a single major round of revision. Nevertheless, given the interest of your findings, I will return your manuscript to you once more for the necessary amendments. However, failure to address all criticism now will preclude publication in The EMBO Journal.

Please do not hesitate to contact me should any points require further clarification!

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I have looked over the manuscript as carefully as I can and the authors appear to have addressed my concerns reasonably well. There is still the issue that almost all the auto-rads look completely artificial. I do not care what program they are using the background and the bands should not look as monochromatic as they do, the authors need to show the default filter or whatever it is to see the "natural" picture. As long as they can provide natural looking, unmanipulated scans I think this should be published.

Referee #3 (Remarks to the Author):

As for the original manuscript, I will limit myself to commenting on the potential interaction of XIAP with Mdm2.

1. While skimming thru the revised manuscript, it appeared to me that the ubiquitylation patterns observed in the experiments to Fig. 3E and Fig. 3H for Mdm2 and p53, respectively, are rather similar (which is unexpected, since the migration behavior of p53 and Mdm2 in SDS-PAGE is rather different), reinforcing my concerns about the method used for detection of ubiquitylated forms of Mdm2/p53 (I apologize for not having brought this up in my original comments but it obviously escaped my attention). Indeed, in response to the referees' comments, the authors used another method for detecting ubiquitylated forms of Mdm2 and p53, and the respective patterns are quite different to the original ones. Besides the notion that this does not increase my confidence in the original data, the current Figs. 3E and 3H need to be replaced by the new data (and it must be indicated how often the respective experiments were performed).

2. Fig. 3C and Fig. S3B: In response to comment 1, the authors state "amounts of cell lysates were adjusted to achieve similar expression levels at time 0". Just by eyeballing, however, actin levels (i.e. loading control) look rather similar indicating that similar amounts of lysate were analyzed. The quantitation shown in Fig. S3B appears to support this conclusion (similar amounts of lysate were analyzed). In fact, the ratio of Mdm2 to Actin at time 0 in control cells and in si-XIAP cells is rather similar (1.0 vs. 1.2) indicating that Mdm2 levels are hardly affected by knockdown of XIAP expression. Is this difference indeed statistically significant? The same line of arguments holds true for XIAP KO cells. In contrast, in the experiment to Fig. 2B, Mdm2 levels in wild-type cells and XIAP KO cells appear to be significantly different. How come?

3. Fig. 3F and Fig. S5A: In response to my original comments, the in vitro ubiquitylation experiments were monitored by Western blotting against Mdm2. If this is the case, the running position of the non-modified form of Mdm2 needs to be indicated. Furthermore, controls in the absence of Mdm2 need to be performed (to provide evidence for the specificity of the antibody used). As is, I am not convinced that the smear observed indeed represents ubiquitylated Mdm2 (e.g., in Fig. S5A, I assume that the fastest migrating band represents the non-modified form of

Mdm2. Why does the intensity of this band increase with increasing amounts of XIAP?).

4. Fig. 3H, Fig. S5B: Going through the experimental details, I would assume that most, if not all, of the ubiquitylated forms of p53 observed are generated upon addition of MG132 (otherwise one would not need to add MG132). Thus, I am still puzzled about the rather dramatic difference observed in the absence and presence of XIAP, since Mdm2 levels are fully rescued in the presence of MG132 (if they are decreased by XIPA). Thus, to appreciate the results, Mdm2 levels and ubiquitylated forms of p53 need to be determined before and upon addition of MG132. Furthermore, I am still wondering if binding of XIAP to Mdm2 interferes with the ability of Mdm2 to bind to p53).

5. Fig. S4B: The half-life of ectopically expressed Mdm2 does not appear to be affected, or is only mildly affected by the XIAP mutant (levels at time 0 are different but the slope of the decrease appears to be rather similar).

2nd Revision - authors' response 12 May 2013

Response to Referee #1

I have looked over the manuscript as carefully as I can and the authors appear to have addressed my concerns reasonably well. There is still the issue that almost all the auto-rads look completely artificial. I do not care what program they are using the background and the bands should not look as monochromatic as they do, the authors need to show the default filter or whatever it is to see the "natural" picture. As long as they can provide natural looking, unmanipulated scans I think this should be published.

We understand the reviewer’s concerns regarding the qualities of our figures. We should mention that during capture of the previous image, the nearly identical standard setting for brightness and contrast were used. This detailed information has been provided in the Materials and Methods section of the revised manuscript.

Response to Referee #3

As for the original manuscript, I will limit myself to commenting on the potential interaction of XIAP with Mdm2.

1. While skimming thru the revised manuscript, it appeared to me that the ubiquitylation patterns observed in the experiments to Fig. 3E and Fig. 3H for Mdm2 and p53, respectively, are rather similar (which is unexpected, since the migration behavior of p53 and Mdm2 in SDS-PAGE is rather different), reinforcing my concerns about the method used for detection of ubiquitylated forms of Mdm2/p53 (I apologize for not having brought this up in my original comments but it obviously escaped my attention). Indeed, in response to the referees' comments, the authors used another method for detecting ubiquitylated forms of Mdm2 and p53, and the respective patterns are quite different to the original ones. Besides the notion that this does not increase my confidence in the original data, the current Figs. 3E and 3H need to be replaced by the new data (and it must be indicated how often the respective experiments were performed).

As suggested by the reviewer, the previous Figures 3E and 3H have been replaced. We have indicated that the experiments have been successfully repeated three times in the corresponding Figure legends.

2. Fig. 3C and Fig. S3B: In response to comment 1, the authors state "amounts of cell lysates were adjusted to achieve similar expression levels at time 0". Just by eyeballing, however, actin levels (i.e. loading control) look rather similar indicating that similar amounts of lysate were analyzed. The quantitation shown in Fig. S3B appears to support this conclusion (similar amounts of lysate were analyzed). In fact, the ratio of Mdm2 to Actin at time 0 in control cells and in si-XIAP cells is rather similar (1.0 vs. 1.2) indicating that Mdm2 levels are hardly affected by knockdown of XIAP expression. Is this difference indeed statistically significant? The same line of arguments holds true for XIAP KO cells. In contrast, in the experiment to Fig. 2B, Mdm2 levels in wild-type cells and XIAP KO cells appear to be significantly different. How come?

We apologize for the confusion that has caused to the reviewer. Given the strong effect of XIAP on Mdm2 expression levels (Figures 2A and 2B), we have to adjust amounts of lysates from XIAP knockdown cells and control cells to achieve similar expression levels of Mdm2 at time 0. However, to confirm the knockdown efficiency of XIAP in these experiments, the same amounts of lysates from XIAP knockdown cells and control cells were used to determine the levels of XIAP and Actin (Figure 3C). The detailed information has been provided in the corresponding Figure legends. The half-life experiments have been repeated more than three times.

3. Fig. 3F and Fig. S5A: In response to my original comments, the in vitro

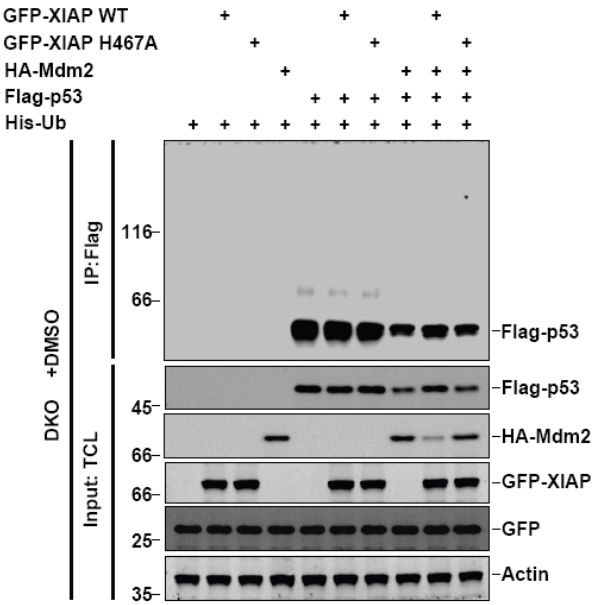
ubiquitylation experiments were monitored by Western blotting against Mdm2. If this is the case, the running position of the non-modified form of Mdm2 needs to be indicated. Furthermore, controls in the absence of Mdm2 need to be performed (to provide evidence for the specificity of the antibody used). As is, I am not convinced that the smear observed indeed represents ubiquitylated Mdm2 (e.g., in Fig. S5A, I assume that the fastest migrating band represents the non-modified form of Mdm2. Why does the intensity of this band increase with increasing amounts of XIAP?).

According to the reviewer's comments, two additional controls have been added in the *in vitro* ubiquitination assay. The previous Figure 3F has been replaced by the new data. The results consistently showed that XIAP but not its enzyme-inactive mutant promoted uibiquitination of both Mdm2 and Mdm2 C464A *in vitro*.

We thank the reviewer for raising his/her concerns about the data shown in Figures 3F and S5A. We were indeed puzzled by these data and we thought that the non-modified form of Mdm2 was quickly degraded during the assay even in the absence of XIAP. Now we know the possible reason is that the incubation time for the previous *in vitro* ubiquitination experiments may be too long. This was supported by the new data shown in the revised Figure 3F. When we decreased the incubation time from the past 2 h to the present 1 h, the non-modified form of Mdm2 was showed up (Revised Figure 3F).

4. Fig. 3H, Fig. S5B: Going through the experimental details, I would assume that most, if not all, of the ubiquitylated forms of p53 observed are generated upon addition of MG132 (otherwise one would not need to add MG132). Thus, I am still puzzled about the rather dramatic difference observed in the absence and presence of XIAP, since Mdm2 levels are fully rescued in the presence of MG132 (if they are decreased by XIPA). Thus, to appreciate the results, Mdm2 levels and ubiquitylated forms of p53 need to be determined before and upon addition of MG132. Furthermore, I am still wondering if binding of XIAP to Mdm2 interferes with the ability of Mdm2 to bind to p53).

Given that ubiquitinated forms of p53 are quickly degraded by proteasome, MG132 has to be added to the cells to accumulate ubiquitinated species (Tang et al. Nature Cell Biology 8: 855-862; Zhang et al. Nucleic Acid Res. 38: 6544-54). According to the reviewer's comments, we performed an *in vivo* ubiquitination assay in the absence of MG132. Without addition of MG132, ubiquitinated forms of p53 are barely detectable (Please see the following Figure). As pointed out by the reviewer, Mdm2 levels in XIAP-overexpressing cells were fully rescued 4 hours after treatment with MG132 (Figure 3H). However, levels of mdm2 should be always lower in XIAP-overexpressing cells than in control or XIAP H467A-overexpressing cells before Mdm2 levels in these cells were finally equalized by MG132 treatment. This is the reason why dramatic difference in p53 ubiquitination was observed in the absence and presence of XIAP (Figure 3H and Figure S5B).



($p53^{-/-}Mdm2^{-/-}$ MEF cells were transfected with the indicated plasmids. 24 h after transfection, cell lysates were denatured before immunoprecipitation with anti-Flag antibody.)

To address whether binding of XIAP to Mdm2 interferes with the ability of Mdm2 to interact with p53, we performed a co-immunoprecipitation experiment. The result showed that XIAP did not obviously affect the Mdm2-p53 interaction (Figure S5C).

5. Fig. S4B: The half-life of ectopically expressed Mdm2 does not appear to be affected, or is only mildly affected by the XIAP mutant (levels at time 0 are different but the slope of the decrease appears to be rather similar).

The blots shown in the Figure S4B are representative of three independent experiments. We agree with the reviewer's point that the half-life of Mdm2 is mildly affected by XIAP D148A/W310A, but this effect was consistently observed. We have indicated this mild effect of XIAP D148A/W310A on Mdm2 half-life in the revised manuscript.