THE EMBO JOURNAL

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L1CAM Regulates DNA Damage Checkpoint Response of Glioblastoma Stem Cells through NBS1

Lin Cheng, Qiulian Wu, Zhi Huang, Olga Guryanova, Weinian Shou, Jeremy Rich, Shideng Bao

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(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.)

Additional correspondence 03 August 2010

Thank you for submitting your research manuscript for our consideration, and please excuse the slight delay in getting back to you with an editorial decision. We have in the meantime received the comments of three referees, which I am enclosing below. As you will see, referees 1 and 2 raise several questions and concerns but are overall favorable regarding possible publication in The EMBO Journal. Referee 3, on the other hand, has a number of more substantive criticisms related to a lack of sufficient understanding of molecular mechanisms and causalities underlying your current findings. Given that most of these points appear to be well-taken, and that they come from a trusted referee in the DNA damage & cancer field who is well familiar with the journal and its standards, I would not want to disregard these concerns and simply overrule this position, even though I understand that a full mechanistic elucidation may be beyond the scope of the current submission. Nevertheless, the manuscript would clearly be a stronger candidate for an EMBO J paper if at least some further mechanistic insight could be added, and before taking a final editorial decision here, I would therefore like to invite you to carefully consider the below comments, and to provide us with a response letter detailing how you might be able to improve the study to address each of the major criticisms of the referees, especially referee 3. Such a response outline would be very helpful for us to estimate the potential for successful revision and thus to decide whether inviting a revised version of the manuscript may be warranted. Please, let me know should you have any further questions in this regard.

Looking forward to hearing from you and reading your response

Yours sincerely,

Editor
The EMBO Journal

Referee 1 (comments to the authors):

In this manuscript the authors address the differential radiation response of glioblastoma stem cells (GSCs) as compared to their non-stem counterparts. This group has previously shown that glioblastoma stem cells are more resistant to radiation than non-stem cells, and that this resistance is accompanied by preferential activation of the DNA damage checkpoint response. In this paper the authors expand upon their previous studies and determine a role for L1CAM in this increased resistance and checkpoint activation in glioblastoma stem cells.

First the authors use the radiomimetric drug neocarzionstatin (NCS) to induce DNA damage and assess the expression of L1CAM after insult in glioblastoma stem cells. The authors find an increase in protein and RNA expression of LICAM in GSCs 24-36 hours post treatment with NCS. While the authors clearly show that DNA damage induces L1CAM expression, they should explain why they chose to use NCS instead of IR and should describe whether this expression is also increased following IR.

Next the authors confirmed that GSCs had preferential activation of checkpoint responses post IR, and using shRNA lentiviral techniques they were able to show that L1CAM regulates this activation in response to DNA damage due to both IR and NCS. GSCs infected with shL1CAM showed attenuation of phosphorylated checkpoints post IR and NCS. Non-stem cells had lower levels of L1CAM and reduction in this had little or no effect on checkpoint activation. Additionally, ectopic expression of L1CAM in GSCs increased phosphorylation of checkpoint proteins. Taken together all of this data shows that L1CAM regulates checkpoint activation in GSCs and leads to increased DNA repair and survival. One wonders whether overexpression of L1CAM in non-stem cells, which are low in L1CAM expression, affects checkpoint activation and resistance. If such were the case, then the differences between GSC and non stem cell counterparts (at least in this respect) could be based on differential regulation of L1CAM expression. If not, then the differences are more complex. Either answer would be fine, but the question is probably worth answering.

Further the authors examine whether knockdown of L1CAM influences DNA repair in GSCs using H2AX and the comet assay. Reduced expression of L1CAM delayed resolution of H2AX in GSCs and this was also confirmed with the comet assay. One question is whether L1CAM has a role in mediating damage in these cultured cells in the absence of radiation. That is, is there any difference in DNA damage in cells infected with shL1CAM under baseline conditions?

The authors also examined how L1CAM expression affects radiation sensitivities. GSCs that were infected with shL1CAM showed reduced sphere formation efficiency and size and further showed sensitivity to radiation; shL1CAM GSCs that were irradiated formed no spheres. Thus L1CAM mediates checkpoint response, DNA damage repair, and radiation resistance in GSCs. It is not inherently obvious why L1CAM shRNA reduces sphere size. One might predict that inhibition of the checkpoint would actually promote proliferation in those cells that escape radiation-induced toxicity. Does shL1CAM affect cell cycle? Does it have an influence on the cell cycle of nonirradiated cells? What is the effect of shL1CAM on total cell numbers in the cultures under irradiated and non-irradiated conditions?

In general, the authors clearly and precisely show a link between L1CAM expression and preferential checkpoint activation, increased DNA repair, and ultimately survival in GSCs. They also confirm that L1CAM functions through Nbs1. Overall this study is

thorough, well organized and clearly lays out an argument for the relationship between L1CAM, Nbs1 and preferential radiation resistance in GSCs. To my knowledge this is the first study to clearly describe a mechanism responsible for a preferential DNA damage response in GSCs.

Referee 2 (comments to the authors):

The manuscript by S. Bao and colleagues deals with a important issue in the field of glioblastoma (GBM) biology and cancer stem cells (GSCs) physiology, that is the investigation of the molecular mechanisms underpinning the resilience of GSCs to radiation, particularly as mediated by L1CAM/CD177. In their work, the authors show that that L1CAM (CD171, which is differentially expressed in GSCs regulates DNA damage checkpoint responses and radiosensitivity of GSCs. When they target L1CAM by RNA interference, they observed an attenuated DNA damage checkpoint activation and repair, and sensitization of GSCs to radiation. Conversely, overexpression of L1CAM resulted in enhanced DNA damage checkpoint activation. The authors furthered their investigation by showing that L1CAM regulates expression of
Nbs1, a critical component of the MRN complex (Mre11-Rad50-Nbs1), which in turn activates ATM kinase and early checkpoint response. Ectopic expression of Nbs1 in GSCs was show tp rescue the reduced checkpoint activation and radioresistance resulted from L1CAM knockdown, demonstrating that L1CAM functions through Nbs1 to regulate DNA damage checkpoint responses. The authors concluded that L1CAM augments DNA damage checkpoint activation and radioresistance of GSCs through Nbs1-ATM axis.

This is an excellent manuscript from a group with a solid record in this area of investigation. The experimental design is well conceived, the quality of the data is most satisfactory and the conclusion follow from them, thus standing on solid experimental and logical ground.

I only have a few minor suggestions that I wish to be taken care of.
Page 7, 3rd paragraph. The authors need to expand their data on CD133 cells and L1CAM expression. They state that "...L1CAM is co-expressed with CD133 in GSC tumorspheres". The statement is somewhat "suspended" and it is unclear what this observation, evident in figure 1C, is intended to mean. The role of CD133 as a stem cell marker has now been challenged by multiple groups, while it is emerging that the expression of the antigen AC133 may be related to specific cell functional state of GBM cells rathr than to their GSCs identity. The authors should comment on what the expression of L1CAM appears to be in the CD133- counterpart in their cultures, also commenting on the relative frequencies of CD133+/CD133- and the cells unsed in this study. I recommend to include FACS analysis data on this and to clarify that CD133 labeling is not construed here as identifying GSCs.

Referee 3 (comments to the authors):

This paper by Cheng et al reports that L1CAM, a cell surface molecule coexpressed on CD133+ glioblastoma stem cells (GSCs) acts as a transducer of the DNA damage response. Knockdown of L1CAM in GSCs attenuates the DNA damage response, inhibits DNA repair and causes radiosensitisation, whereas L1CAM overexpression enhances the DNA damage response. L1CAM knockdown coincides with NBS1 downregulation, identifying NBS1 as a potential transducer of the DNA damage signal. The experiments are largely well controlled, using multiple cell lines and multiple DNA damage response markers to demonstrate the effect of L1CAM on the DNA damage response. However, mechanistic clarifications would need to be made prior to publication.

Major points:

1. Lack of Mechanistic Insight

No clue is given as to how L1CAM regulates NBS1 levels. This really needs to be understood in at least outline for publication in EMBO J.

2. Specificity of L1CAM's role in the DNA damage response.
The experiments presented do not unambiguously demonstrate a role for L1CAM in the DNA damage response. As the authors discuss, the mechanistic link from the membrane-bound L1CAM to the nuclear DNA damage response is still unclear. In particular:

3. In the author's previous characterisation of L1CAM knockdown in GSCs, L1CAM downregulation was shown to retard cell growth (also figures 4 and 6 of this manuscript) and to induce the cdk inhibitor p21WAF1. The immunoblots in Figure 2AB would benefit from being accompanied by cell cycle profiles, as the lesser DNA damage response in L1CAM cells may be explained merely by a lower proliferative rate, rather than a direct mechanistic link between L1CAM and the DNA damage response.

4. It is also not fully proven that NBS1 mechanistically links L1CAM to the DNA damage response. While NBS1 downregulation is observed upon L1CAM knockdown, this is a correlation rather than a demonstration of cause and effect. The authors should show that knockdown of NBS1 can phenocopy knockdown of L1CAM. The authors attempt to address this link by showing that NBS1 overexpression rescues the DNA damage response defect in L1CAM knockdown cells. However, two questions remain regarding NBS1 overexpression:
i) The blots should include total NBS1 (+- FLAG) in order to show the levels of endogenous and exogenous NBS1. The greater DNA damage response may be an artefact of non-physiological expression levels of NBS1 and would explain the observed hyperactivation of Chk2 and ATM (Fig6A lanes 5,6)
ii) why is the L1CAM shRNA unable to downregulate the exogenous NBS1 (Fig6A) but downregulates endogenous NBS1 in Fig 5A?

Minor points:

1. Figure 4 and Figure 6B should be shown as a dose response curve over a range of IR treatments.

2. To control for off-target effects, multiple independent shRNAs should be used. The methods section mentions 2 shRNAs, but only one is shown each time. The full data needs to be shown.

3. Some graphs are mislabelled. For example, Figure 3B quantitation is for 1 and 24 hours, not 0 and 24 hours. Figure 5C is time after lentiviral transduction, not time after NCS treatment.

Additional correspondence 09 August 2010

Thank you very much for allowing us to respond to the referee comments. We have carefully considered the concerns and suggestions raised by the reviewers. We think that most concerns and questions are addressable. We agree with you and the referee #3 that it is important to further understand the molecular mechanism underlying the L1CAM-mediated regulation on NBS1 expression, although we have demonstrated that L1CAM regulates checkpoint activation through NBS1. We have been working on this critical issue for a while. The good news is that we now have the outline for the potential mechanism associated with the L1CAM-mediated regulation on NBS1 (please see the attached response letter). Our recent new data and several important publications from other groups allow us to propose a signaling pathway mediated by the nuclear translocation of the cytoplasmic domain of L1CAM (L1-ICD) to regulate NBS1 expression through c-Myc (please see Fig. 1 in the response letter), although we are still working on the issue and try to elucidate the pathway in more details. Meantime, we are starting to perform the suggested experiments and

address other concerns raised by the reviewers.

We will be very grateful if you are willing to give us the opportunity to address the reviewer concerns and consider a revised manuscript for the publication in EMBO J. I am looking forward to hearing from you soon.

1st Editorial Decision 10 August 2010

Thank you for providing a tentative response to the referee comments on your manuscript, EMBOJ-2010-75245. I have read it with great interest, and I am pleased to hear that you seem to be in a good position to address the key concerns raised in these reports. We shall therefore be happy to consider a manuscript revised along the discussed lines further for publication, and I am herewith sending you the link for submitting your revision. Please, do not hesitate to get back to me should there be any further questions.

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

Yours sincerely,

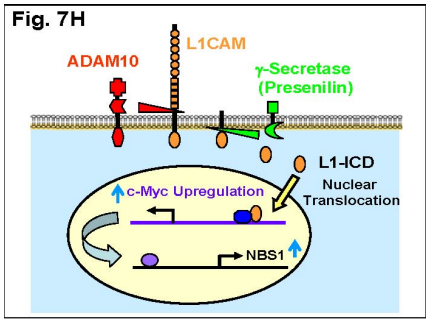
Editor
The EMBO Journal

———

1st Revision - authors' response 17 November 2010

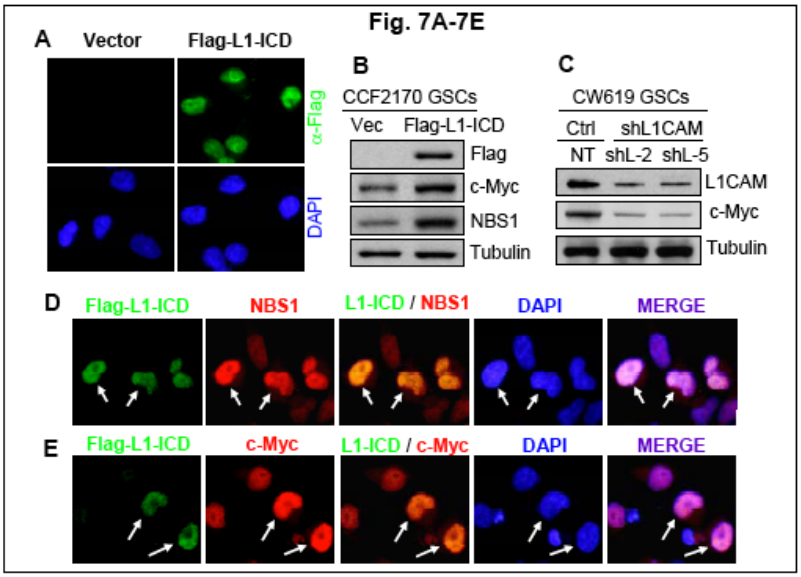
Response to the main issue:
"... even though I understand that a full mechanistic elucidation may be beyond the scope of the current submission. Nevertheless, the manuscript would clearly be a stronger candidate for an EMBO J paper if at least some further mechanistic insight could be added....."

Response: We agree with the editor and the reviewer #3. Although we have demonstrated that L1CAM regulates checkpoint activation through NBS1 in glioma stem cells (GSCs), it is important to further understand how L1CAM transduces a signal into nuclei to up-regulates NBS1 expression. We have been working on this critical issue for a while, and now we have the outline for the mechanistic link between L1CAM-mediated surface signaling and the NBS1 up-regulation in nuclei. Several pieces of new data we obtained recently and a number of related publications from other groups allow us to propose a signaling pathway mediated by the nuclear translocation of the L1CAM intracellular (cytoplasmic) domain (L1-ICD) to regulate NBS1 expression through c-Myc (illustrated in Fig. 7H).

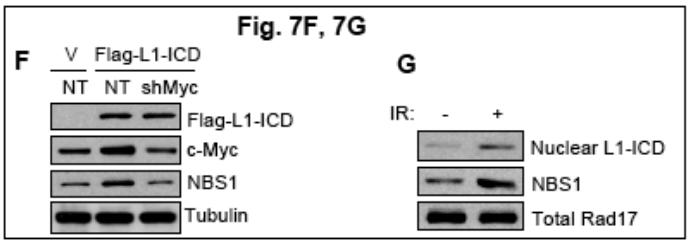


Although we have not fully elucidated all signaling nodes in this L1CAM-mediated signaling pathway, this signaling model is supported by the following publications and our recent data:

(1). A recent study by Dr. Altevogt's group demonstrated that the L1CAM intracellular domain (L1-ICD, 28 kDa) can be released from the membrane-bound L1CAM through specific cleavages by ADAM10 and Presenilin/ -secretase and then translocated into nuclei to regulate gene expression (Riedle et al., Nuclear translocation and signaling ofL1-CAM in human carcinoma cells requires ADAM10 and presenilin/ -secretase activity. Biochem. J. 420: 391-402, 2009).
(2). We recently found that expression of the intracellular domain of L1CAM (Flag-L1-ICD) up-regulated c-Myc and NBS1 expression in GSCs (Fig. 7B, 7D, 7E), and confirmed that Flag-L1-ICD indeed entered nuclei (Fig. 7A, 7D, 7E).



(3). An early study demonstrated that NBS1 expression is directly up-regulated by c-Myc at transcriptional level (Chiang et al., c-Myc directly regulates the transcription of the NBS1 gene involved in DNA double-strand break repair. J. Biol. Chem. 278:19286-19291, 2003).
(4). We found that L1CAM knockdown also reduced c-Myc expression in GSCs (Fig. 7C, above), suggesting that L1CAM may indirectly regulate NBS1 expression through c-Myc.
(5). A recent publication showed that c-Myc is also required for the ATM-dependent checkpoint activation (Guerra et al., Myc is required for activation of the ATMdependent checkpoints in response to DNA damage. PLoS One. 5:e8924, 2010).
(6). We recently demonstrated that c-Myc knockdown attenuated the L1-ICD-induced NBS1 expression in GSCs (Fig. 7F, below), suggesting that L1-ICD indirectly upregulates NBS1 through c-Myc.
(7). In addition, we found that radiation increased nuclear L1-ICD and induced NBS1 in GSCs (Fig. 7G), suggesting a L1-ICD-mediated nuclear signaling in response to radiation and possible other stresses or stimulations.

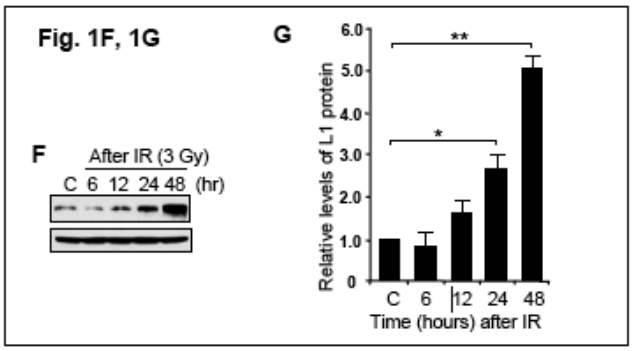


Our current new data suggest that nuclear translocation of the L1CAM intracellular domain (L1-ICD) mediates L1CAM signaling from cell surface into nuclei to up-regulate NBS1 expression via c-Myc. Thus, we have determined the mechanistic link from membrane-bound L1CAM to nuclear regulation of NBS1 expression and checkpoint response in GSCs. We hope these additional data have addressed the major concern raised by reviewer #3.

Responses to reviewer's comments:
Referee #1

Referee #1: 1. .....First the authors use the radiomimetric drug neocarzionstatin (NCS) to induce DNA damage and assess the expression of L1CAM after insult in glioblastoma stem cells. The authors find an increase in protein and RNA expression of LICAM in GSCs 24-36 hours post treatment with NCS. While the authors clearly show that DNA damage induces L1CAM expression, they should explain why they chose to use NCS instead of IR and should describe whether this expression is also increased following IR.

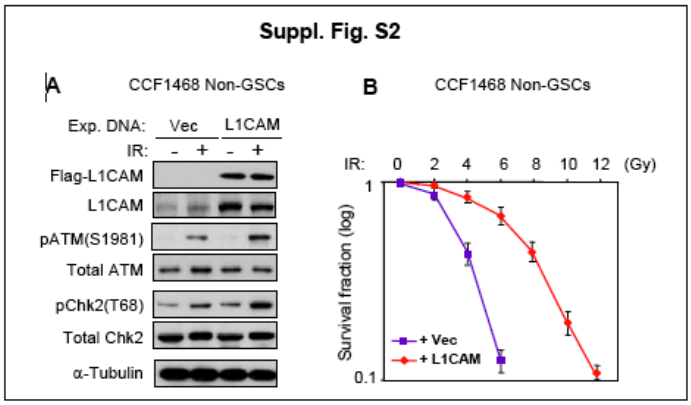
Response: We thank the reviewer for raising this point. We have examined the effect of IR on L1CAM expression and found that IR (3 Gy) also induced L1CAM expression in GSCs. We have included this data (Fig. 1F-1G) in the revised manuscript. This result is similar to that obtained with NCS treatment. As both NCS and IR induce DNA doublestrand breaks, NCS has been widely used to study DNA damage checkpoint response.



Referee #1: 2. ..... Taken together all of this data shows that L1CAM regulates checkpoint activation in GSCs and leads to increased DNA repair and survival. One wonders whether overexpression of L1CAM in non-stem cells, which are low in L1CAM expression, affects checkpoint activation and resistance. If such were the case, then the differences between GSC and non stem cell counterparts (at least in this respect) could be based on differential regulation of L1CAM expression. If not, then the differences are more complex. Either answer would be fine, but the question is probably worth answering.

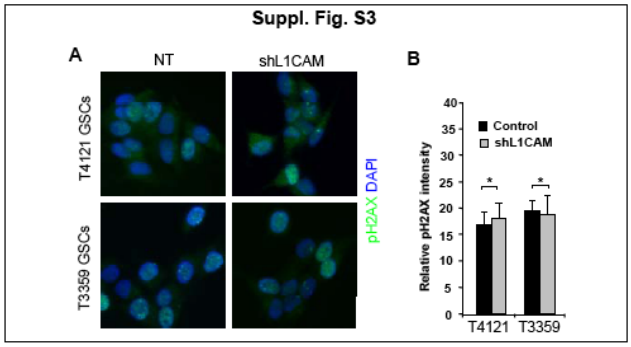
Response: We thank the reviewer for raising this interesting point. We have done the suggested experiment and found that overexpression of L1CAM in non-stem cancer cells also increased checkpoint activation and radioresistance, while the basal and activating phosphorylation levels of

checkpoint proteins in non-stem cancer cells are generally lower than that in the matched GSCs. We have included these data as supplementary figures (Suppl. Fig. S2) in the revised manuscript.



Referee #1: 3. .....Further the authors examine whether knockdown of L1CAM influences DNA repair in GSCs using H2AX and the comet assay. Reduced expression of L1CAM delayed resolution of H2AX in GSCs and this was also confirmed with the comet assay. One question is whether L1CAM has a role in mediating damage in these cultured cells in the absence of radiation. That is, is there any difference in DNA damage in cells infected with shL1CAM under baseline conditions?

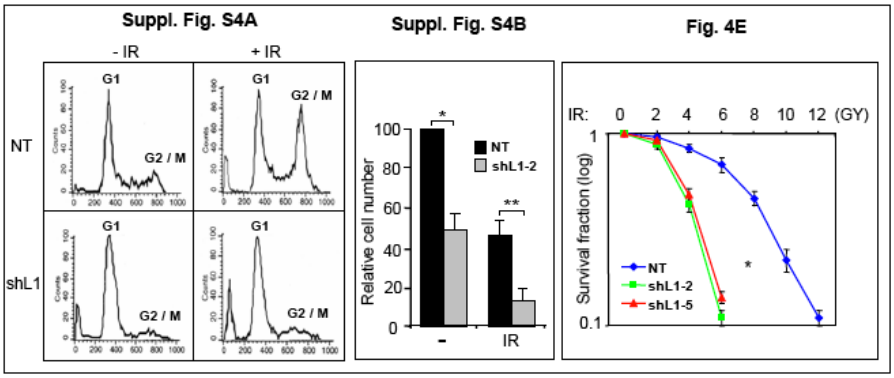
Response: We thank the reviewer for raising this interesting question. We have examined the effect of L1CAM knockdown on DNA damage in GSCs in the absence of radiation or NCS treatment, and found that L1CAM knockdown showed little effect on endogenous DNA damage in GSCs under baseline conditions without induction of DNA damage. We now include this data as a supplementary figure (Suppl. Fig. S3) in the revised manuscript.



Referee #1: 4. .....The authors also examined how L1CAM expression affects radiation sensitivities. GSCs that were infected with shL1CAM showed reduced sphere formation efficiency and size and further showed sensitivity to radiation; shL1CAM GSCs that were irradiated formed no spheres. Thus L1CAM mediates checkpoint response, DNA damage repair, and radiation resistance in GSCs. It is not inherently obvious why L1CAM shRNA reduces sphere size. One might predict that inhibition of the checkpoint would actually promote proliferation in those cells that escape radiation- induced toxicity. Does shL1CAM affect cell cycle? Does it have an influence on the cell cycle of nonirradiated cells? What is the effect of shL1CAM on total cell numbers in the cultures under irradiated and non-irradiated conditions?

Response: We appreciate these interesting questions. From our previous study and recent new data, we know that L1CAM knockdown also down-regulates c-Myc and Olig2 transcription factors, which may explain why L1CAM shRNA alone can reduce cell

growth and sphere size in the absence of radiation. Under the radiation-induced DNA damage, inhibition of checkpoint activation by L1CAM knockdown allows more cells without repair of damaged DNA to enter cell cycle, which will eventually result in cell death and reduced cell viability. It is well recognized that the activation of checkpoints primarily plays cytoprotective roles. However, in the situation with prolonged DNA damage that is not repaired, cells will undergo apoptosis. Although inhibition of the checkpoint would promote entering cell cycle of those cells that escape radiation-induced cell cycle arrest in the initial cycle after IR, daughter cells carrying damaged DNA will trigger apoptosis and reduce cell viability. Nevertheless, we have re-examined the effect of L1CAM knockdown on cell cycles and total cells numbers in GSCs under irradiated and non-irradiated conditions using FACS analysis, and we confirmed that L1CAM knockdown increased G1 arrest in the non-irradiated GSCs but reduced G2 arrest and cell death in the irradiated GSCs (Suppl. Fig. S4A). A dose response study with a range of IR treatment showed that L1CAM knockdown rendered GSCs more sensitive to IR and reduced cell survival (Fig. 4E). Cell viability analysis showed that L1CAM knockdown in GSCs reduced cell survival and proliferation even in the condition without IR (Suppl. Fig. S4B). These additional data may explain why L1CAM knockdown reduced neurosphere size in the absence of radiation.



Referee #1: 5. In general, the authors clearly and precisely show a link between L1CAM expression and preferential checkpoint activation, increased DNA repair, and ultimately survival in GSCs. They also confirm that L1CAM functions through Nbs1. Overall this study is thorough, well organized and clearly lays out an argument for the relationship between L1CAM, Nbs1 and preferential radiation resistance in GSCs. To my knowledge this is the first study to clearly describe a mechanism responsible for a preferential DNA damage response in GSCs.

Response: We are very grateful for these positive comments by the reviewer and his/her excitement about our work.

Referee #2

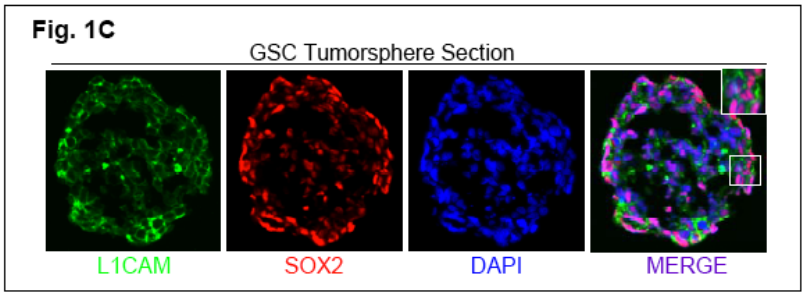
Referee #2: ...This is an excellent manuscript from a group with a solid record in this area of investigation. The experimental design is well conceived, the quality of the data is most satisfactory and the conclusion follows from them, thus standing on solid experimental and logical ground.

Response: We thank the reviewer for his/her kind words and enthusiasm on our manuscript.

Referee #2: 1. I only have a few minor suggestions that I wish to be taken care of. Page 7, 3rd paragraph. The authors need to expand their data on CD133 cells and L1CAM expression. They state that "...L1CAM is co-expressed with CD133 in GSC tumorspheres". The statement is somewhat "suspended" and it is unclear what this observation, evident in figure 1C, is intended to mean. The role of CD133 as a stem cell marker has now been challenged by multiple groups, while it is emerging that the expression of the antigen AC133 may be related to specific cell functional state of

GBM cells rathr than to their GSCs identity. The authors should comment on what the expression of L1CAM appears to be in the CD133- counterpart in their cultures, also commenting on the relative frequencies of CD133+/CD133- and the cells unsed in this study. I recommend to include FACS analysis data on this and to clarify that CD133 labeling is not construed here as identifying GSCs.

Response: We thank the reviewer for this helpful suggestion. We regret the confusion regarding GSC identity in the previous manuscript. We usually identify GSC populations by functional characterizations (self-renewal by neurosphere formation, multi-potency by differentiation induction, and tumorigenic potential by in vivo tumor formation), not just by CD133 expression. We agree with the reviewer that CD133 is not a good marker to identify GSCs and use of CD133 as a single GSC marker has been challenged by several studies. Thus, we replaced original Fig. 1C with new L1CAM and SOX2 staining of neurosphere section, as SOX2 is a well-defined marker for GSCs and other stem cells. We confirmed that cells expressing SOX2 also express L1CAM in the GSC neurosphere sections (Fig. 1C). Following the suggestion by the reviewer, we also used FACS analysis to examine L1CAM expression in CD133- and CD133+ cells, and found that 0.42-1.23% of CD133- cells express L1CAM and 64-91% of CD133+ cell express L1CAM with variation in different GBM samples. We have not used CD133 labeling for the identity of GSCs in the revised manuscript.



Referee #3
Referee #3: ....The experiments are largely well controlled, using multiple cell lines and multiple DNA damage response markers to demonstrate the effect of L1CAM on the DNA damage response.

Response: We thank the reviewer for his/her nice words on our manuscript.

Referee #3: 1. Lack of Mechanistic Insight
No clue is given as to how L1CAM regulates NBS1 levels. This really needs to be understood in at least outline for publication in EMBO J.

Response: We thank the reviewer for raising this critical concern shared by the editor.
We agree with the reviewer that it is important to understand how L1CAM mediates signaling into nuclei to up-regulate NBS1 expression. We have obtained several pieces of new data (Figure 7) demonstrating that a signaling pathway mediated by the nuclear translocation of the L1CAM intracellular (cytoplasmic) domain (L1-ICD) regulates NBS1 expression through c-Myc. We have addressed this major concern in details in the first section of this response letter (please see page 1-3 of this rebuttal letter).

Referee #3: 2. Specificity of L1CAM's role in the DNA damage response.
The experiments presented do not unambiguously demonstrate a role for L1CAM in the DNA damage response. As the authors discuss, the mechanistic link from the membranebound L1CAM to the nuclear DNA damage response is still unclear:

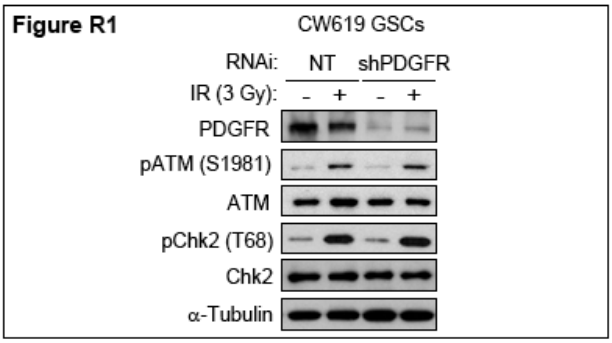
Response: We appreciate the reviewer concern. As detailed in the response (page 1-3) to the major issue, we have elucidated the mechanistic link from the membrane-bound L1CAM to nuclear regulation of NBS1 that is a key component of MRN complex in activating the ATM-dependent checkpoints. Our additional new data and the recent publications support that

nuclear translocation of the L1CAM intracellular domain (L1- ICD) play a critical role to transduce nuclear signaling of L1CAM to regulate c-Myc and NBS1 expression. Thus, the regulation of checkpoint activation by L1CAM is mediated through a specific signaling pathway, although L1CAM has other roles in GSCs.

Referee #3: 3. In the author's previous characterisation of L1CAM knockdown in GSCs, 1CAM downregulation was shown to retard cell growth (also figures 4 and 6 of this manuscript) and to induce the cdk inhibitor p21WAF1. The immunoblots in Figure 2AB would benefit from being accompanied by cell cycle profiles, as the lesser DNA damage response in L1CAM cells may be explained merely by a lower proliferative rate, rather than a direct mechanistic link between L1CAM and the DNA damage response.

Response: We thank the reviewer for this insightful suggestion. We have performed the FACS to analyze the cell cycle profiles in GSCs expressing shL1CAM or NT shRNA in non-irradiated or irradiated conditions (Suppl. Fig. S4). L1CAM knockdown in GSCs appeared to increase G1 arrest in the non-irradiated condition and reduced G2 arrest in response to irradiation (IR), which is consistent with that L1CAM knockdown mainly reduced ATM and Chk2 activation in GSCs in response to IR. Although replication checkpoint response in S phase is closely associated with cell proliferative rate, DNA damage checkpoint activation in response to DNA double-strand breaks induced by IR or NCS is generally independent of cell proliferative rate. For example, PDGFR knockdown in GSCs also reduced cell proliferative rate, but PDGFR knockdown did not affect checkpoint activation in response to radiation-induced DNA damage (see Figure R1 below, not shown in the revised manuscript). Thus, even L1CAM knockdown in GSCs reduced cell proliferation, the lower proliferate rate itself is unlikely to contribute to the reduced ATM and Chk2 activating phosphorylations in response to DNA damage (DSBs) induced by IR. We concluded that the effect of L1CAM knockdown on DNA damage checkpoint activation in response to IR or NCS is specific.

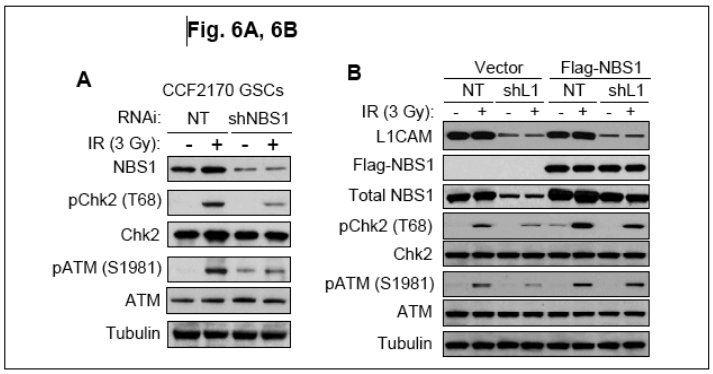
Figure R1



Referee #3: 4. ....The authors should show that knockdown of NBS1 can phenocopy knockdown of L1CAM. The authors attempt to address this link by showing that NBS1 overexpression rescues the DNA damage response defect in L1CAM knockdown cells. However, two questions remain regarding NBS1 overexpression:
i) The blots should include total NBS1 (+- FLAG) in order to show the levels of endogenous and exogenous NBS1. The greater DNA damage response may be an artefact of non-physiological expression levels of NBS1 and would explain the observed hyperactivation of Chk2 and ATM (Fig6A lanes 5,6)
ii) why is the L1CAM shRNA unable to downregulate the exogenous NBS1 (Fig6A) but downregulates endogenous NBS1 in Fig 5A?

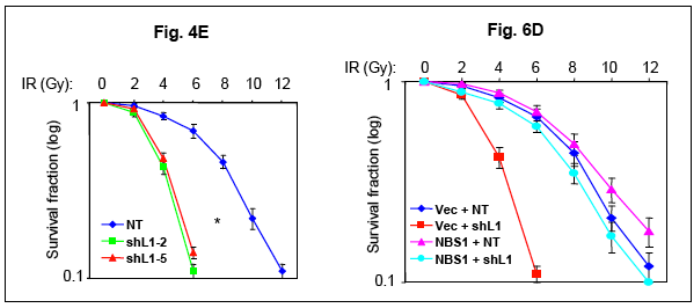
Response: We appreciate the helpful suggestions. We have performed the suggested experiment and demonstrated that NBS1 knockdown in GSCs indeed phenocopied L1CAM knockdown on suppressing checkpoint activation (Fig. 6A, below), although many studies have shown that NBS1 is required for checkpoint activation in response to IR-induced DNA damage and the maintenance of cellular viability after radiation.
i) We have included the total NBS1 in Fig. 6B (previous Fig. 6A) to show the relative levels of endogenous and exogenous NBS1 in the revised manuscript. The levels of exogenous NBS1 were roughly similar to that of endogenous NBS1, indicating a

physiological expression of the exogenous NBS1 in this rescue experiments.
ii) We demonstrated that L1CAM regulates endogenous NBS1 expression through c-Myc that functions on endogenous NBS1 promoter (Chiang et al, J. Biol. Chem. 278:19286-19291, 2003). However, the expression of the exogenous NBS1 is driven by a CMV promoter. Thus, it is understandable why the L1CAM shRNA is unable to down-regulate the exogenous NBS1 but down-regulate endogenous NBS1.



Minor points:
Referee #3: 5. Figure 4 and Figure 6B should be shown as a dose response curve over a range of IR treatments.

Response: We thank the reviewer for the suggestion. We have performed the suggested experiments and shown the dose response curve over a range of IR treatment (0, 2, 4, 6, 8, 10 and 12 Gy) in Fig. 4E and Fig. 6D in the revised manuscript.



Referee #3: 6. To control for off-target effects, multiple independent shRNAs should be used. The methods section mentions 2 shRNAs, but only one is shown each time. The full data needs to be shown.

Response: We agree with the reviewer. We have used two L1CAM shRNAs in most experiments and obtained the similar results. We have included data with two shRNA in most figures (Fig. 2C; Fig. 3B, 3D; Fig. 4C-4E; Fig. 7C) in the revised manuscript.

Referee #3: 7. Some graphs are mislabelled. For example, Figure 3B quantitation is for 1 and 24 hours, not 0 and 24 hours. Figure 5C is time after lentiviral transduction, not time after NCS treatment.

Response: We regret the mislabeling in the figures. We have corrected the mistakes in current figures in the revised manuscripts.

2nd Editorial Decision 06 December 2010

Thank you for submitting your revised manuscript for our consideration. It has now been reassessed by two of the original referees, as well as by a new fourth reviewer with expertise in intramembrane proteolysis and ICD signaling, whom we asked specifically to comment on the new data on L1CAM cleavage and nuclear regulation of NBS1 expression. I am pleased to inform you that the two original referees have no further criticisms, and also referee 4 considers the new data valid in principle, while raising however one specific experimental issue that remains to be addressed (see also comments below): the use of a gamma-secretase inhibitor to directly confirm the proposed chain of signaling events. In this respect, I would not expect you to repeat all functional assays in the presence of the inhibitor, but simply to conduct a repeat of the experiment in Figure 7G with an additional lane using IR and a gamma-secretase inhibitor to test whether upregulation of 'Nucl L1-LCD' and 'NBS1' levels upon IR would indeed depend on presenilin activity as proposed. Pending the outcome of this straightforward additional experiment, we should then be able to ultimately consider the final version of the study for publication. When sending a re-revised manuscript, please make sure to also include a brief 'author contribution' statement at the end of the manuscript text. Please, do not hesitate to contact me should you have any questions regarding this re-revision, or should any unforeseen difficulties arise with these last additional experiments.

I am looking forward to hearing from you.

Yours sincerely,

Hartmut Vodermaier, PhD
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors have thoroughly addressed my concerns and, I believe, those of other reviewers.This strong manuscript has now been made even better.

Referee #4 (Remarks to the Author):

The explanation in the response to the referees makes sense. In any event, I would like to see one experiment that could prove the full picture: if the authors would use in their system a gamma-secretase inhibitor and show that the signaling via L1CAM does not longer occur, than they have direct proof for the proposed mechanism (this would result in the blockage of L1CAM intracellular domain by inhibiting presenilin). I did not check whether this experiment was included in their final paper, but it is basically an easy and straight forward experiment.

2nd Revision - authors' response 03 January 2011

Response to Editor’s Comments:

"...and also referee 4 considers the new data valid in principle, while raising however one specific experimental issue that remains to be addressed (see also comments below): the use of a gamma-secretase inhibitor to directly confirm the proposed chain of signaling events. In this respect, I

would not expect you to repeat all functional assays in the presence of the inhibitor, but simply to conduct a repeat of the experiment in Figure 7G with an additional lane using IR and a gamma-secretase inhibitor to test whether upregulation of 'Nucl L1-LCD' and 'NBS1' levels upon IR would indeed depend on presenilin activity as proposed. Pending the outcome of this straightforward additional experiment, we should then be able to ultimately consider the final version of the study for publication."

Response: We really appreciated the insightful suggestion by the editor and the referee #4. We have performed the suggested experiment, and confirmed that the gamma-secretase inhibitor (DAPT) attenuated the radiation-induced nuclear translocation of L1-ICD and NBS1 expression (Fig. 7G). This additional piece of data demonstrates that radiation-induced upregulation of nuclear L1-ICD and NBS1 expression indeed depends on gamma-secretase (presenilin) activity, and further supports the proposed mechanism of L1CAM signaling to regulate NBS1 and checkpoint response (Fig. 7H).

"When sending a re-revised manuscript, please make sure to also include a brief 'author contribution' statement at the end of the manuscript text."

Response: We have added a brief ‘author contributions’ statement at the end of the manuscript text (page 27).

Response to Reviewer’s Comments:

Referee #1: The authors have thoroughly addressed my concerns and, I believe, those of other reviewers.This strong manuscript has now been made even better.

Response: We are very grateful for the kind comments and enthusiasm on our revised manuscript.

Referee #4: The explanation in the response to the referees makes sense. In any event, I would like to see one experiment that could prove the full picture: if the authors would use in their system a gamma-secretase inhibitor and show that the signaling via L1CAM does not longer occur, than they have direct proof for the proposed mechanism (this would result in the blockage of L1CAM intracellular domain by inhibiting presenilin). I did not check whether this experiment was included in their final paper, but it is basically an easy and straight forward experiment.

Response: We thank the reviewer for the helpful suggestion and comment. We have performed the suggested experiment. Please see the response to the editor above.

