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Nardilysin and ADAM proteases promote gastric cancer cell growth by activating intrinsic cytokine signaling via enhanced ectodomain shedding of TNF-α

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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.)

1st Editorial Decision 23 September 2011

Thank you for the submission of your manuscript to EMBO Molecular Medicine. It has now been seen by three referees whose comments are shown below. All reviewers find the manuscript of potential interest but raised significant concerns. While their reports are explicit, I would just highlight the most critical concerns here:

- More causative relationships should be established and all three referees recommend using siRNA and inhibitors/neutralizing antibody to strengthen your conclusions, as indicated (ref#1 points 1-2 and 3b, ref#2 point 4, ref#3 point 6)

- The contribution of precursor pro-TNF should be ruled out (ref#1, points 3a and 3c)

- A certain number of points and statements need to be clarified and further discussed, as suggested by the referees. Particularly, clinical information regarding samples used should be provided (ref#3 point1)

- A co-localization experiment would be desirable to show that ADAM17 and NDRDc are found at the same place in the cell, at the same time (ref#2 point 3)

Given the balance of these evaluations, I would like to give you the opportunity to revise your manuscript, with the understanding that the referee concerns must be fully addressed and that acceptance of the manuscript would entail a second round of review, within the space and time

constraints outlined below. Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Also, the length of the revised manuscript may not exceed 60,000 characters (including spaces) and, including figures, the paper must ultimately fit onto optimally ten pages of the journal. You may consider including any peripheral data (but not methods in their entirety) in the form of Supplementary information.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Other Remarks):

This manuscript reports an association of nardilysin, a modulator of ecto-domain shedding, with gastric cancer, and it addresses whether and how the protein may be promoting gastric cancer cell growth. These findings appear to be novel and are clearly of substantial interest and importance. However, some of the conclusions regarding the mechanism of action of nardilysin require further supporting data.

The evidence of higher average serum concentration in gastric cancer patients versus controls and the detection by immunohistochemistry in gastric cancer tissue appear to be significant. The authors proceed to a series of experiments with gastric cancer cell lines in which nardilysin is reduced by siRNA. These experiments suggest that lower nardilysin correlates with slower proliferation, less TACE activity and processing of TNF, and less secretion of IL-6 (and other cytokines). They present evidence that the greater amount of IL-6 in the medium of the parental cells causes greater STAT3 activation and the more rapid proliferation of these cells. Seeking to explain the higher IL-6 level in the parental cultures, they present evidence that these cells have greater activation of NFkB and that this difference is due to a higher level of TNF in the medium, e.g., they find that neutralizing anti-TNF antibodies reduced the level of IL-6 message in the parental cells, and that adding TNF to cultures of the nardilysin knock-down cells increases IL-6 message and growth of the cells. Finally, they show that the knock-down cells have less cyclin D1, c-Myc and Bcl-2 mRNA than the parental cells, and that anti-TNF or -IL-6 or TAPI (an inhibitor of ecto-domain shedding) reduce expression of these genes. (There is also a section on growth of tumors due to implanting parental versus nardilysin-knockdown cells in vivo.)

From these results the authors conclude that nardilysin contributes to the proliferation of gastric cancer cells by increasing the shedding of TNF, which then activates NFkB and hence increases IL-6 expression, which induces expression of cyclin D1, c-Myc and Bcl-2 mRNA.

While parts of this argument are well-supported, there are some significant gaps in the evidence:

1. The heart of the argument is that the greater amount of TNF released by the parental cells accounts for their more rapid proliferation, yet the authors don't show that TNF knockdown or simply inclusion of a neutralizing antibody against TNF slows the proliferation of these cells. One of these experiments is essential, and the latter is very easy to do.

2. A related weakness is that they don't show whether knockdown of TACE and/or ADAM-10 (the other major sheddase) affects growth of a gastric cancer cell line. I think that this is obligatory to support the argument that reduced ADAM-mediated shedding is responsible for the slower growth

of nardilysin-deficient cells. Knockdown of these two proteins has been reported in other papers, so it is do-able.
The paper does show that TAPI reduced proliferation of parental cells over a period of 30 hours-Fig. S2B-but TAPI is an inhibitor of many metalloproteases and could have nonspecific effects, i.e., not due to its effect on shedding. At the least, the authors should show that TAPI or TIMP-3 reduces soluble TNF levels without reducing the level of cell-associated TNF after 30 hours. But this experiment would still be less definitive than knockdown of the sheddases.

3. There are weaknesses in the evidence that TNF shedding is greater in the parental than the nardilysin knockdown cells:
a. The authors do not address the possibility that the knockdown cells release less TNF because they express less of the precursor, rather than because they shed less efficiently. They say in the Discussion that they "investigated whether TNF- mRNA expression changes after NRDc knockdown, but TNF- mRNA did not show a reproducible unidirectional change". It's not clear what that means, but in any event the critical issue is whether there was less precursor TNF protein in the knockdown cells. That can be readily determined by lysing the cells in the presence of a protease inhibitor cocktail and doing a western blot or ELISA-preferably both since the ELISA could detect processed TNF. Determining the relative level of pro-TNF in the different cell lines is essential before concluding that there's a difference in shedding efficiency.
b. Fig. 2E shows about 30% less TACE activity (TACE is the major TNF-releasing enzyme) in the knockdown cells. But the authors do not validate the assay by showing that a TACE inhibitor eliminates the signal. In fact, Fig. S2A-right panel shows only modest inhibition by TAPI, albeit with lysates of cells that apparently had been treated with the inhibitor prior to lysis. The authors need to add TAPI or TIMP-3 directly to the lysate prior to the assay, and determine whether the activity is eliminated.
c. They show that when FLAG-tagged pro-TNF is over-expressed in parental and knockdown cells, there's less cell-associated remnant in the knockdown lysates. This finding is suggestive but doesn't answer whether the endogenous precursor is processed to mature TNF to a lesser extent. The best way to address this issue is to determine the ratio of soluble TNF in the medium to cell-associated pro-TNF (see comment [a] above).

4. The authors don't show that adding TNF to the nardilysin-knockdown cells actually does increase NFkB activation, even though they assay for NFkB activation state in other experiments.

5. They don't show that adding IL-6 to the knockdown cells actually does increase STAT3 activation, even though they assay for STAT3 activation in other experiments.

Less major comments:
6. As noted above, it is incorrect to call TAPI an ADAM-protease inhibitor, since it also inhibits virtually all of the MMPs and possibly other metalloproteases.

7. Is there any validation of the specificity of the anti-TACE antibody used for the western shown in Fig.2E?

8. Do human cells respond to mouse IL-6? If they do, it's not clear why the nardilysin knockdown cells form smaller tumors in the mice since apparently mouse IL-6 is expressed around the tumors.

Referee #2 (Comments on Novelty/Model System):

This is a solid piece of work, which is very interesting. The point that the catalytic activity of Nardilysin is not needed for ADAM17 activation should be more emphasized and could be the starting point of more experiments to elucidate the molecular details of this protein-protein interaction. In this respect the quality and location of the protein-protein interaction with ADAM17 should be addressed.

Referee #2 (Other Remarks):

Nardilysin is a metalloprotease, which enhances shedding of membrane proteins by stimulating the

activity of ADAM proteases. The authors demonstrate that Nardilysin is upregulated in gastric cancer. Knockdown of Nardilysin suppressed tumor growth in vitro and in vivo. This effect could be reverted by treatment of cells with recombinant TNF or IL-6 protein. In gastric cancer cells, NRDc promotes shedding of pro-TNF , which stimulates expression of NF B-regulated multiple cytokines such as IL-6, which in turn activated STAT3. The authors speculate that gastric cancer cell growth is maintained by activation of a TNF -NF B and IL-6-STAT3 axis and that these signals are positively influenced by Nardilysin through the induction of TNF shedding.

This is an interesting report on the biologic activity of the metalloprotease Nardilysin. There are, however, some points the authors might want to address.

Major points:
1. The authors should mention in the introduction that several mouse models of hypomorphic ADAM17 show strongly enhanced susceptibility to dextran sodium sulfate induced colitis.
2. Recently, the role of IL-6 and IL-11 has been addressed in animal models of gastric cancer and it turned out that possibly IL-11 plays a dominant role in this disease. In IL-11R-/- but not in IL-6-/- mice, gastic tumor growth was severely compromised. These reports need to be mentioned and discussed.
3. The cell biology of Nardilysin seems to be somewhat controversial. Since the interaction of Nardilysin with ADAM17 is important in this study, this point needs to be explained to the reader. Do the authors have data demonstrating that Nardilysin and ADAM17 meet in the ER or golgi? If Nardilysin is exported from the cell via a non-classic pathway, do the two proteins co-localize on the cell surface of gastric cancer cells?
4. On p9 (bottom) the authors state that '... ADAM protease activity is necessary for the maintenance of gastric cancer cell growth.' This is a strong statement, which needs to be supported by an ADAM17 knockdown experiment.
5. In Fig. S4 the authors show that sIL-6R do not change upon Nardilysin knockdown. The authors need to confirm that there is no change in alternative splicing of the IL-6R mRNA, which also results in generation of a soluble form of the IL-6R.
6. In Fig. 5A, the authors demonstrate cleavage of the TNF protein. The size of the cleaved TNF protein of 17 kDa seems to differ between the experiments. Have the authors observed this in all experiments? In this case, this band should be analyzed by mass spectroscopy.
7. On p13 the authors state: ' Taken together with the result that endogenous secretion of soluble TNF- was reduced in the NRDc-KD cells (Fig 3D), this result demonstrated that NRDc expression is essential for processing of pro-TNF- to a mature ligand.' This is not true; the authors only demonstrate a reduction of TNF shedding upon Nardilysin knockdown, not an abrogation.
8. The authors favor a chain of events leading from Nardilysin to activation of ADAM17 to TNF shedding to IL-6 secretion to STAT3 activation, which eventually leads to growth of cancer cells. The critical point is the first step, namely the interaction and activation of ADAM17 by Nardilysin. What kind of interaction is this? The authors have shown earlier that the enzymatic activity of Nardilysin is not needed for the activation of ADAM17. This point needs to be explained and discussed in some detail. In this respect it might be helpful to describe the Nardilysin protein in more detail. What kind of protein is this? The metalloprotease domain is predicted to be relatively small; what are the other domains?
9. On p18 the authors write: 'In mice experiments, several reports demonstrated that forced expression of the constitutively active form of gp130, a coreceptor for IL-6 family cytokines such as IL-6 or IL-11, resulted in spontaneous gastric tumorigenesis by STAT3 activation (Judd et al, 2004; Tebbutt et al, 2002).' This is an incorrect statement. The mice used by Judd et al (2004) and Tebutt et al (2002) carry a single tyrosine to phenylalanine point mutation in gp130, which leads to a blockade of the ras/map kinase pathway together with a blockade of the SOCS3 negative feedback loop. Consequently these mice show higher STAT3 activation upon gp130 stimulation. These were the mice in which high incidence of gastric cancer has been observed (see point 2).

Minor points:
1. Some references in the text are incompletely formatted.
2. Is Nardilysin also expressed in non-malignant gastric tissue? What about the expression and role of this protein in other tissues?
3. Fig. S4B should be labeled with 'sIL-6R'.
4. There are a couple of typographical and grammatical errors, which need to be corrected.

Referee #3 (Comments on Novelty/Model System):

The observation that Nardilysin can regulate shedding events independent of its enzyme activity has potential implications in understanding how ADAM/BACE proteolytic activity is controlled and maybe dysregulated in different diseases. This manuscript is the first to address the relationship between nardilysin expression and gastric cancer. In gastric tumor cells in vitro, this study demonstrates that nardilysin controls TNFa shedding and downstream NF-kB signaling which subsequently activates IL-6 transcription and STAT3 signaling required for tumor cell growth. These are novel findings and provide insights that should stimulate further studies into understanding how nardilysin regulates ADAM/BACE activity (which is currently not defined). However, the relationship between the in vitro data and nardilysin expression in advanced gastric cancer is only correlative. As stated below, it would be helpful to have more specific information regarding the nardilysin expression in different staged gastric tumors with more clinical staging etc. Importantly, the in vitro studies show only a partial rescue with IL-6 in gastric cells that have had nardilysin knockdown. Additional information on the IL-6R/gp130 receptor signaling, TNFR and ADAM status and levels of IL-6 protein in conditioned medium after neutralizing TNFa antibody treatment or after addition of exogenous TNFa would strengthen the manuscript (see comments below). The model should also qualify that other signaling pathways besides IL-6 are likely to contribute to the response.

Referee #3 (Other Remarks):

Nardilysin (NRDc) is a zinc peptidase that selectively cleaves dibasic residues. Recent studies have implicated NRDc in regulating the proteolytic activity of several ADAM proteins as well as the maturation and proteolytic activity of BACE1. The Nishi lab has shown that shedding of several ADAM substrates (HB-EGF, TNFa, NRG-1 and APP) can be modulated by changes in NRDc expression. In addition, this same group has shown that the regulation of ADAM/BACE enzyme activity by NRDc requires direct protein-protein interactions but does not require NRDc proteolytic activity. However, the exact mechanism by which NRDc regulates ADAM/BACE enzyme activity has not been defined. In this manuscript, for the first time, Kanda et al, examine the expression of NRDc in human gastric tumors and demonstrate a critical role for NRDc in gastric tumor cell growth in vitro and in xenograft models. In several human gastric cancer cell lines, NRDc enhances constitutive TNFa shedding which stimulates NF-kB transcription leading to an increase in IL-6 production and subsequent STAT3 activation and growth promotion. These findings are correlated with elevated NRDc protein expression in serum and tumor cells from patients with advanced gastric cancer suggesting that NRDc may be involved in human gastric cancer.

Points.

1. In Fig 1, the authors examine NRDc expression in serum and tumor cells from patients with advanced gastric cancer. It would be helpful if more information was provided regarding the staging and metastatic status of these patients with advanced gastric cancer and how serum levels correlate with tumor burden. In addition, IHC analysis of NRDc expression in tumors from patients with early Stage I gastric cancer would be insightful as to the significance of increased NRDc expression in advanced gastric cancer and whether NRDc expression correlates with gastric cancer development, progression or both.

2. In Fig 3, NRDc knockdown in gastric cancer cells reduces IL-6 levels in conditioned medium from 80ng/ml to 20ng/ml. However, in Fig 4F and G the addition of recombinant IL-6 (50ng/ml) only partially rescues the growth responses of NRDc knockdown cells. As mentioned by the authors in the discussion regarding the xenograft tumor growth responses, it would appear that other factors are likely to be contributing to the in vitro growth response as well. The presentation of results and discussion should reflect the partial responsiveness to IL-6 more clearly and that other cytokines (IL-8 and IL-1 etc) or signaling pathways maybe contributing.

3. Likewise in Fig 3, NRDc knockdown in gastric cancer cells reduces TNFa levels in conditioned

medium from 60pg/ml to 35pg/ml. However, in Fig 4H the addition of recombinant TNFa requires ~80-fold higher levels (5ng/ml) to restore a growth response in NRDc knockdown cells. If TNFa is the essential cytokine signaling pathway involved, why does it require such high exogenous TNFa levels to achieve this response? ADAM17 is also responsible for TNFR1 and TNFR2 shedding. The status of TNFR1 and TNFR2 expression in gastric cell lines is not defined. What is the effect on the cell surface expression and changes in shedding of TNFR1 and TNFR2 upon modulation of NRDc expression in the gastric cells? Does the requirement for high exogenous TNFa levels reflect changes in the ratio of cell surface TNFR1/2 signaling and/or membrane-anchored TNFa to soluble TNFa signaling?

4. In gastric cancer cells, neutralizing antibodies to TNFa inhibit IL-6 transcription whereas in NRDc knockdown cells, treatment with exogenous recombinant TNFa restores IL-6 transcription. In Fig 5, what are the IL-6 protein levels in the CM under these different treatment conditions? Do the changes in IL-6 protein levels in CM correlate with the responses observed in Fig 4?

5. Like TNFa shedding, ADAM17 is responsible for IL-6 receptor shedding in human cells. The authors show that soluble IL-6 receptor levels in conditioned are not altered upon modulation of NRDc expression. Are cell surface levels of IL-6 receptor changed upon reduction of NRDc expression? For either cell surface IL-6R/gp130 signaling or IL-6/IL-6R transisgnaling, one would expect changes gp130 receptor activation (phosphorylation) to correlate with changes in IL-6 expression upon modulation of NRDC expression. What is the status of gp130 receptor activation?

6. While the authors have previously reported that ADAM17 protein levels do not appear to change in other cell systems, it is important to eliminate this possibility in the gastric cancer cell lines. What is the expression of ADAM17 mature and precursor forms in gastric cancer cell lines upon NRDc modulation? The authors use the relatively broad spectrum metalloprotease inhibitor TAPI-1 to demonstrate the relationship between metalloprotease activity and cytokine transcription/production. Does knockdown of ADAM17 (or other ADAM) recapitulate this response?

Minor points.

1. In Fig 2A, western blot for siRNA knockdown of NRDc in MKN-45 cells needs to be presented.

2. In Fig 5A western blotting shows a decrease TNFa remnant but no concomitant increase its transmembrane-bound form.

3. Fig 5F and 5G are mis-labeled in Figure legend.

1st Revision - authors' response 18 December 2011

Authors' response to reviewers

We thank the editor and all the referees for finding interest in our paper, and appreciate their critical evaluations that helped us to improve our manuscript. We answered almost all the concerns raised by the reviewers as described below. Especially, we focused on the following issues: (1) NRDc promotes TNF-a secretion by enhancing ectodomain shedding of the precursor form of TNF-a (pro-TNF-a), not by increasing pro-TNF-a expression either at the mRNA or at the protein level. (2) Knockdown of ADAM17 or ADAM10, two representative ADAM proteases, provided some biological effects similar to those induced by NRDc RNAi. The referees' comments are shown in italics.

Referee #1 (Other Remarks):

...While parts of this argument are well supported, there are some significant gaps in the evidence:

1. The heart of the argument is that the greater amount of TNF released by the parental cells accounts for their more rapid proliferation, yet the authors don't show that TNF knockdown or

simply inclusion of a neutralizing antibody against TNF slows the proliferation of these cells. One of these experiments is essential, and the latter is very easy to do.

We also think this concern is essential. To address this, we added the experiment using anti-TNF-a neutralizing antibody, which significantly suppressed the proliferation of both TMK-1 and MKN-45 cells (Fig 5J and Fig S5F of Supporting information), confirming the involvement of autocrine TNF-a signalling in the growth regulation of gastric cancer cells.

2. A related weakness is that they don't show whether knockdown of TACE and/or ADAM-10 (the other major sheddase) affects growth of a gastric cancer cell line. I think that this is obligatory to support the argument that reduced ADAM-mediated shedding is responsible for the slower growth of nardilysin-deficient cells. Knockdown of these two proteins has been reported in other papers, so it is do-able.
The paper does show that TAPI reduced proliferation of parental cells over a period of 30 hours-Fig. S2B-but TAPI is an inhibitor of many metalloproteases and could have nonspecific effects, i.e., not due to its effect on shedding. At the least, the authors should show that TAPI or TIMP-3 reduces soluble TNF levels without reducing the level of cell-associated TNF after 30 hours. But this experiment would still be less definitive than knockdown of the sheddases.

We agree with the referee that we should demonstrate whether knockdown of representative ADAM proteases phenocopies several biological effects induced by NRDc gene knockdown in gastric cancer cells. Gene silencing of ADAM17 or ADAM10 (or both) by siRNA recapitulated many of the changes observed in cells in which NRDc was knocked down; that is, (1) attenuated cell growth (Fig 2E), (2) impaired TNF-a secretion (without affecting *TNFA* transcription) and NF-kB transcriptional activity (Fig 6A), and (3) reduced mRNA expressions of several cytokines and growth-related genes downstream of NF-kB and/or STAT3 (Fig 6B and E). Taken together with the results of TAPI-1-treatment experiments already shown in the initial manuscript, we think these results support the hypothesis proposed by us that NRDc promotes gastric cancer cell growth by enhancing sheddase activity of ADAM proteases.

3. There are weaknesses in the evidence that TNF shedding is greater in the parental than the nardilysin knockdown cells:
a. The authors do not address the possibility that the knockdown cells release less TNF because they express less of the precursor, rather than because they shed less efficiently. They say in the Discussion that they "investigated whether TNF-a mRNA expression changes after NRDc knockdown, but TNF-a mRNA did not show a reproducible unidirectional change". It's not clear what that means, but in any event the critical issue is whether there was less precursor TNF protein in the knockdown cells. That can be readily determined by lysing the cells in the presence of a protease inhibitor cocktail and doing a western blot or ELISA-preferably both since the ELISA could detect processed TNF. Determining the relative level of pro-TNF in the different cell lines is essential before concluding that there's a difference in shedding efficiency.

We apologize for the unclear description in the original manuscript regarding TNF-a mRNA expression in the NRDc-KD cells. As shown in the original manuscript, TNF-a secretion into CM was reduced in the NRDc-KD clones (Fig 3D). To exclude the possibility that this is due to the decreased transcription of *TNFA* gene, we carefully reevaluated whether NRDc modulates the mRNA and protein expression of the precursor form of TNF-a (pro-TNF-a) in gastric cancer cells. As shown in Fig S5A, mRNA expression of TNF-a was slightly increased (TMK-1 cells) or almost unchanged (MKN-45 cells) when NRDc was transiently knocked down by RNAi. We next investigated the mRNA level of TNF-a in the NRDc-KD stable TMK-1 cells. One stable clone (NRDc-KD #1) showed an elevated expression of TNF-a mRNA, while mRNA level in another clone (NRDc-KD #2) was almost comparable to that in the control cells (Fig 5A). A similar expression pattern was obtained at the protein level when cell lysates were subjected to ELISA for TNF-a (Fig 5B, left panel). This ELISA kit could recognize both the precursor (pro-TNF-a) and soluble form (sTNF-a) of TNF-a; so the lysates were analyzed by Western blotting using an antibody binding to the ectodomain of TNF-a protein. Indeed, a band of approximately 27 kDa (presumably representing pro-TNF-a) was observed, while 17 kDa sTNF-a was not detected in the same membrane (Fig 5B, right panels). Taken together with the result that shedding of

overexpressed FLAG-pro-TNF-a was reduced in the NRDc-KD cells compared with the control cells (Fig 5C), these results demonstrated that decreased secretion of TNF-a from the NRDc-KD cells were due to the reduced ectodomain shedding of pro-TNF-a protein, rather than to the impaired expression of pro-TNF-a mRNA or protein in the NRDc-KD cells.

b. Fig. 2E shows about 30% less TACE activity (TACE is the major TNF-releasing enzyme) in the knockdown cells. But the authors do not validate the assay by showing that a TACE inhibitor eliminates the signal. In fact, Fig. S2A-right panel shows only modest inhibition by TAPI, albeit with lysates of cells that apparently had been treated with the inhibitor prior to lysis. The authors need to add TAPI or TIMP-3 directly to the lysate prior to the assay, and determine whether the activity is eliminated.

Following this suggestion, we added the experiments in which cell lysates of TMK-1 cells were incubated with increasing doses of TAPI-1 in vitro, followed by the TACE activity analysis. Although values were corrected by subtracting the fluorescence of the blank, inhibition of fluorescence induction by TAPI-1 remained limited (Fig S2B, right panel), similarly to the TAPI-1 treatment in intact cells (Fig S2B, left panel). Given that ADAM17 siRNA induced more strong reduction of TACE activity in TMK-1 cells (Fig S2C of Supporting information), one possibility is that some proteases in the cell lysates, which bound to ADAM17 and were not inhibited by TAPI-1 cleaved the substrate.

c. They show that when FLAG-tagged pro-TNF is over-expressed in parental and knockdown cells, there's less cell-associated remnant in the knockdown lysates. This finding is suggestive but doesn't answer whether the endogenous precursor is processed to mature TNF to a lesser extent. The best way to address this issue is to determine the ratio of soluble TNF in the medium to cell-associated pro-TNF (see comment [a] above).

Please see our response to the comment 3a of referee #1.

4. The authors don't show that adding TNF to the nardilysin-knockdown cells actually does increase NFkB activation, even though they assay for NFkB activation state in other experiments.

As expectedly, recombinant TNF-a treatment restored the NF-kB transcriptional activity in NRDc-KD cells in a dose-dependent manner (Fig 5F).

5. They don't show that adding IL-6 to the knockdown cells actually does increase STAT3 activation, even though they assay for STAT3 activation in other experiments.

We additionally performed this experiment, demonstrating the recovery of STAT3 transcriptional activity in NRDc-KD cells by IL-6 stimulation (Fig 4F).

Less major comments:
6. As noted above, it is incorrect to call TAPI an ADAM-protease inhibitor, since it also inhibits virtually all of the MMPs and possibly other metalloproteases.

Following this recommendation, TAPI-1 is referred to as "sheddase (MMPs and ADAMs) inhibitor" in this revised manuscript.

7. Is there any validation of the specificity of the anti-TACE antibody used for the western shown in Fig.2E?

We appreciate this suggestion. In the original manuscript, we used the anti-ADAM17 (TACE) goat polyclonal antibody (C-15, purchased from Santa Cruz Biotechnology). Western blotting

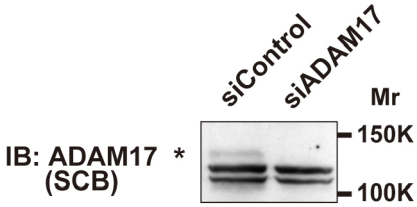

Figure R1

analysis with this antibody showed three bands between 100 and 150 KDa in the TMK-1 cell lysates. To test the specificity of this antibody, ADAM17 was knocked down by siRNA. As shown in Fig R1, a faint 130 kDa band (marked with asterisk) almost disappeared by ADAM17 RNAi, while the other two bands (which migrated more rapidly) remained unchanged. It was suggested that this 130 kDa band corresponds to the precursor form of ADAM17 and the others are non-specific.

Alternatively, we applied the rabbit polyclonal anti-ADAM17 antibody from Cell Signaling Technology. This antibody detected 130 and 100 kDa bands in the TMK-1 cell lysates, and intensities of both bands were strongly reduced by ADAM17 knockdown (Fig S2C of Supporting information). Therefore, it is likely that these 130 and 100 kDa bands represent the precursor and mature form of ADAM17 protein, respectively.

8. Do human cells respond to mouse IL-6? If they do, it's not clear why the nardilysin knockdown cells form smaller tumors in the mice since apparently mouse IL-6 is expressed around the tumors.

It seems to be a controversial issue whether human cells respond to mouse IL-6 (Chiu, CP et al. (1988) PNAS 85: 7099-7103). To address the reviewer's question, we quantified human and mouse IL-6 proteins in the xenografted tumors using species-specific ELISA kits. As expectedly, the concentration of human IL-6 was about 100-fold higher than that of mouse IL-6, suggesting that human IL-6 secreted from the tumor cells was predominantly involved in the activation of STAT3 signaling in this xenograft model (Fig 7D).

Referee #2 (Other Remarks):

...This is an interesting report on the biologic activity of the metalloprotease Nardilysin. There are, however, some points the authors might want to address.

Major points:
1. The authors should mention in the introduction that several mouse models of hypomorphic ADAM17 show strongly enhanced susceptibility to dextran sodium sulfate induced colitis.

Following this recommendation, these mouse models are mentioned in the Introduction section of the revised manuscript (page 6, line 18).

2. Recently, the role of IL-6 and IL-11 has been addressed in animal models of gastric cancer and it turned out that possibly IL-11 plays a dominant role in this disease. In IL-11R-/- but not in IL-6-/- mice, gastic tumor growth was severely compromised. These reports need to be mentioned and discussed.

In our experiments using human gastric cancer cell lines, treatment with anti-IL-6 neutralizing antibody suppressed the cell growth as well as mRNA expressions of growth-related and anti-apoptotic genes (Fig 4C and 6D). Furthermore, antibody array experiment demonstrated a strong signal of IL-6 in the CM from TMK-1 cells, while signal of IL-11 was hardly detected (Fig 3C). Therefore, this discordance may be caused by the differences in species (human and mouse) or characters of individual gastric tumor. This speculation has been added to the Discussion section (page 19, line 16).

3. The cell biology of Nardilysin seems to be somewhat controversial. Since the interaction of Nardilysin with ADAM17 is important in this study, this point needs to be explained to the reader. Do the authors have data demonstrating that Nardilysin and ADAM17 meet in the ER or golgi? If Nardilysin is exported from the cell via a non-classic pathway, do the two proteins co-localize on the cell surface of gastric cancer cells?

To address this question, we performed immunocytochemical analysis for determining the subcellular distributions of endogenous ADAM17 (a known ER protein) and NRDc proteins. Both proteins expressed diffusely in the cytoplasm of PMA-treated AGS gastric cancer cells. These proteins colocalize at the plasma membrane rather than in the cytoplasm (Fig 1E), supporting the

speculation that NRDc is secreted via non-classical (non-ER/Golgi) pathway, which is not determined at present, and binds to ADAM proteases (ADAM17 etc.) on the cell surface.

4. *On p9 (bottom) the authors state that '... ADAM protease activity is necessary for the maintenance of gastric cancer cell growth.' This is a strong statement, which needs to be supported by an ADAM17 knockdown experiment.*

We added several RNAi experiments in which ADAM17 and/or ADAM10 were knocked down, which demonstrate the involvement of ADAM proteases in maintaining intrinsic cytokine signalling and gastric cancer cell growth (please see our comments to referee #1, point 2).

5. In Fig. S4 the authors show that sIL-6R do not change upon Nardilysin knockdown. The authors need to confirm that there is no change in alternative splicing of the IL-6R mRNA, which also results in generation of a soluble form of the IL-6R.

We additionally performed RT-PCR experiments for quantifying the mRNA levels of the full-length form of IL-6R and the splice variant form coding sIL-6R. As shown in Fig S4D, mRNA of both forms increased in NRDc-KD cells compared with the control cells, which seems to be a compensatory mechanism for reduced efficiency of ectodomain shedding of IL-6R in NRDc-KD cells. This result is mentioned in the Results section (page 13, line 16).

6. In Fig. 5A, the authors demonstrate cleavage of the TNFa protein. The size of the cleaved TNF-a protein of 17 kDa seems to differ between the experiments. Have the authors observed this in all experiments? In this case, this band should be analysed by mass spectroscopy.

With regard to this question, we show another result of Western blotting experiment, in which cytosolic remnant form of overexpressed TNF-a (16 kDa) showed a similar mobility on SDS-PAGE between control and NRDc-KD TMK-1 cells (Fig 5C).

7. On p13 the authors state: ' Taken together with the result that endogenous secretion of soluble TNF-a was reduced in the NRDc-KD cells (Fig 3D), this result demonstrated that NRDc expression is essential for processing of pro-TNF-a to a mature ligand.' This is not true; the authors only demonstrate a reduction of TNF-a shedding upon Nardilysin knockdown, not an abrogation.

We removed this sentence from the manuscript.

8. The authors favour a chain of events leading from Nardilysin to activation of ADAM17 to TNF-a shedding to IL-6 secretion to STAT3 activation, which eventually leads to growth of cancer cells. The critical point is the first step, namely the interaction and activation of ADAM17 by Nardilysin. What kind of interaction is this? The authors have shown earlier that the enzymatic activity of Nardilysin is not needed for the activation of ADAM17. This point needs to be explained and discussed in some detail. In this respect it might be helpful to describe the Nardilysin protein in more detail. What kind of protein is this? The metalloprotease domain is predicted to be relatively small; what are the other domains?

By using cell-based shedding assay and *in vitro* peptide cleavage assay for substrates such as HB-EGF, APP, and TNF-a, we have demonstrated that the metalloendopeptidase activity of NRDc is not required for the enhancement of ADAMs activity (Nishi et al, 2006; Hiraoka et al, 2007 and 2008). Enhancement of the peptide cleavage of TNF-a by the recombinant enzymatically-inactive (E>A) mutant NRDc might be one of the most definite evidence for that (Hiraoka et al, 2008). In terms of the binding, we demonstrated the direct interaction of NRDc and recombinant TACE protein, which has only the extracellular domain by pull-down assay (Nishi et al, 2006). We have also revealed that the mutant form of TACE lacking its transmembrane domain and cytoplasmic tail makes a complex with NRDc in cell-based co-precipitation assay (data not shown). Given that (1) there is no overlapped localization in the intracellular space between NRDc (in the cytosol) and TACE (on the

conventional secretory pathway) and (2) NRDc binds to the extracellular domain of TACE, the interaction of these two proteins occurs, most probably, on the cell surface. This conclusion is also supported by the fact that PMA, a general activator for ectodomain shedding, enhanced cell surface expression of NRDc (Nishi et al, 2006), co-localization of NRDc and TACE on the cell surface (Fig 1E) and the complex formation of NRDc and TACE (Nishi et al, 2006).

NRDc has an inverted zinc-binding motif, HXXEH, compared to the HEXXH motif found in metallopeptidases of clan MA. NRDc is thereby classified in the inverzincin/M16 family of metalloendopeptidases. NRDc has no well-characterized domain structures other than M16 domain and two M16 inactive sites (Fig R2). NRDc is characterized by an insertion of a highly acidic stretch within M16 domain, which is located upstream of the HXXEH pentapeptide. While the acidic stretch is responsible for the direct binding of many proteins, such as HB-EGF, p42IP4/centaurin-alpha and polyamine, the stretch is dispensable for its enzymatic activity. While there is no information about tertiary structure of NRDc, crystal structure of insulysin (IDE; insulin degrading enzyme), a member of M16 family and the closest mammalian homologue having 34% amino acid identity, has been reported. IDE in complex with its substrates revealed that the N- and C-terminal domains of IDE form an enclosed cage just large enough to encapsulate the substrate, resembling clam shells, with two bowl-shaped halves connected by a flexible hinge (Shen, Y. et.al. *Nature* 2006, *443*, 870-4). As the residues involved in substrate binding and interaction between N- and C-terminal domains are well conserved between IDE and NRDc, NRDc might have a similar 'clam shell'. Characterization of NRDc protein in the Introduction section was revised in the current manuscript (please see page 6, line 5 from bottom).

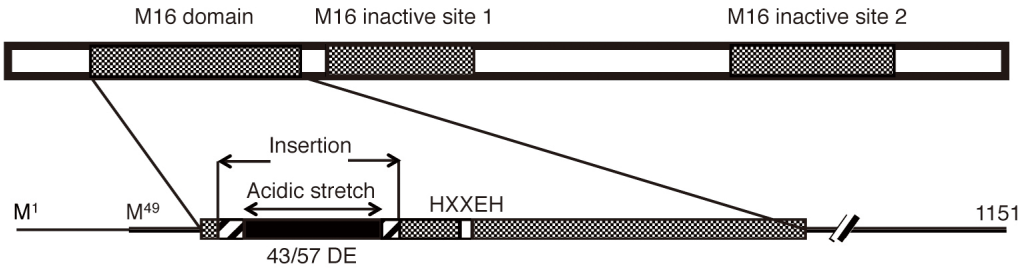

Figure R2 Domain structure of human NRDc

9. On p18 the authors write: 'In mice experiments, several reports demonstrated that forced expression of the constitutively active form of gp130, a coreceptor for IL-6 family cytokines such as IL-6 or IL-11, resulted in spontaneous gastric tumorigenesis by STAT3 activation (Judd et al, 2004; Tebbutt et al, 2002).' This is an incorrect statement. The mice used by Judd et al (2004) and Tebutt et al (2002) carry a single tyrosine to phenylalanine point mutation in gp130, which leads to a blockade of the ras/map kinase pathway together with a blockade of the SOCS3 negative feedback loop. Consequently these mice show higher STAT3 activation upon gp130 stimulation. These were the mice in which high incidence of gastric cancer has been observed (see point 2).

We corrected the statement concerning the *gp130*^{Y757F} knock-in mouse model following this advice (please see page 19, line 16).

Minor points:
1. Some references in the text are incompletely formatted.

All the incompletely formatted references we recognized were corrected.

2. Is Nardilysin also expressed in non-malignant gastric tissue? What about the expression and role of this protein in other tissues?

We added a qRT-PCR result demonstrating that NRDc mRNA was detected also in the adjacent non-cancerous tissue, while the expression level was one-half of that in the cancer epithelium (Fig

1C). IHC analysis demonstrated that NRDc was expressed predominantly at the plasma membrane in non-malignant gastric foveolar epithelium (Fig 1B), raising the possibility that NRDc has biological roles to some extent in non-neoplastic cells. As described in the Discussion section, another group has recently reported that NRDc is highly expressed in invasive breast cancer tissues and promotes the proliferation of breast cancer cells (Choong et al, 2011), suggesting that upregulated NRDc enhances cell growth in a wide variety of human cancers.

3. Fig. S4B should be labeled with 'sIL-6R'.

This labeling has been added to the Fig S4C of Supporting information.

4. There are a couple of typographical and grammatical errors, which need to be corrected.

We have checked the manuscript carefully and corrected all the errors we found.

Referee #3 (Other Remarks):

Nardilysin (NRDc) is a zinc peptidase that selectively cleaves dibasic residues. Recent studies have implicated NRDc in regulating the proteolytic activity of several ADAM proteins as well as the maturation and proteolytic activity of BACE1. The Nishi lab has shown that shedding of several ADAM substrates (HB-EGF, TNFa, NRG-1 and APP) can be modulated by changes in NRDc expression. In addition, this same group has shown that the regulation of ADAM/BACE enzyme activity by NRDc requires direct protein-protein interactions but does not require NRDc proteolytic activity. However, the exact mechanism by which NRDc regulates ADAM/BACE enzyme activity has not been defined. In this manuscript, for the first time, Kanda et al, examine the expression of NRDc in human gastric tumors and demonstrate a critical role for NRDc in gastric tumor cell growth in vitro and in xenograft models. In several human gastric cancer cell lines, NRDc enhances constitutive TNFa shedding which stimulates NF-kB transcription leading to an increase in IL-6 production and subsequent STAT3 activation and growth promotion. These findings are correlated with elevated NRDc protein expression in serum and tumor cells from patients with advanced gastric cancer suggesting that NRDc may be involved in human gastric cancer.

Points.

1. In Fig 1, the authors examine NRDc expression in serum and tumor cells from patients with advanced gastric cancer. It would be helpful if more information was provided regarding the staging and metastatic status of these patients with advanced gastric cancer and how serum levels correlate with tumor burden. In addition, IHC analysis of NRDc expression in tumors from patients with early Stage I gastric cancer would be insightful as to the significance of increased NRDc expression in advanced gastric cancer and whether NRDc expression correlates with gastric cancer development, progression or both.

In our IHC analysis, NRDc was highly expressed also in stage I gastric cancer (data not shown). It was difficult to evaluate the difference of NRDc expression quantitatively among patients with different clinical stages by IHC, because the sensitivity of anti-NRDc antibody we used in IHC experiments is relatively high. Alternatively, gastric cancer patients were subdivided into stage I/II and stage III/IV in the ELISA experiment for serum NRDc concentration. Although the number of stage I/II patients was relatively small, NRDc tended to be expressed more highly in stage III/IV gastric cancer patients. We speculate that serum NRDc level correlates with the whole-body tumor burden rather than with the local invasiveness of gastric cancer.

2. In Fig 3, NRDc knockdown in gastric cancer cells reduces IL-6 levels in conditioned medium from 80ng/ml to 20ng/ml. However, in Fig 4F and G the addition of recombinant IL-6 (50ng/ml) only partially rescues the growth responses of NRDc knockdown cells. As mentioned by the authors in the discussion regarding the xenograft tumor growth responses, it would appear that other factors are likely to be contributing to the in vitro growth response as well. The presentation of

results and discussion should reflect the partial responsiveness to IL-6 more clearly and that other cytokines (IL-8 and IL-1 etc) or signalling pathways maybe contributing.

First, we apologize for the mislabelling of IL-6 concentration in Fig 3D; exact concentrations of IL-6 are 10-fold lower than those in the initial manuscript. We agree with the referee that partial growth recovery of NRDc-KD gastric cancer cells by relatively high-dose IL-6 treatment suggest that other cytokines or growth factors downstream of NRDc and NF-kB might be involved in the growth-promoting function of NRDc. This speculation was mentioned both in the Results and Discussion sections (Please see page 13, line 4 and page 21, line 8, respectively).

3. Likewise in Fig 3, NRDc knockdown in gastric cancer cells reduces TNFa levels in conditioned medium from 60pg/ml to 35pg/ml. However, in Fig 4H the addition of recombinant TNFa requires ~80-fold higher levels (5ng/ml) to restore a growth response in NRDc knockdown cells. If TNFa is the essential cytokine-signalling pathway involved, why does it require such high exogenous TNFa levels to achieve this response? ADAM17 is also responsible for TNFR1 and TNFR2 shedding. The status of TNFR1 and TNFR2 expression in gastric cell lines is not defined. What is the effect on the cell surface expression and changes in shedding of TNFR1 and TNFR2 upon modulation of NRDc expression in the gastric cells? Does the requirement for high exogenous TNFa levels reflect changes in the ratio of cell surface TNFR1/2 signaling and/or membrane-anchored TNFa to soluble TNFa signaling?

It is not clearly determined why apparently high dose of recombinant TNF-a protein was required for the recovery of cell growth of NRDc-KD cells, but one possible explanation is that it is due to the relative instability of exogenous recombinant protein (produced in *E. coli*), compared with the constitutive autocrine secretion of endogenous TNF-a. We agree with the referee that it should be investigated if shedding of TNF receptors is modulated by NRDc. We focused on the shedding of TNF-R1, because TNF-R2 is dominantly expressed in leukocytes and endothelial cells. As shown in Fig S6A, secretion of sTNF-R1 into the culture medium was reduced in the NRDc-KD cells compared with the control cells, while protein expression of TNF-R1 in cell lysates were almost unchanged (Fig S6B of Supporting information). These data seem to suggest the involvement of NRDc in the ectodomain shedding of TNF receptors.

4. In gastric cancer cells, neutralizing antibodies to TNFa inhibit IL-6 transcription whereas in NRDc knockdown cells, treatment with exogenous recombinant TNFa restores IL-6 transcription. In Fig 5, what are the IL-6 protein levels in the CM under these different treatment conditions? Do the changes in IL-6 protein levels in CM correlate with the responses observed in Fig 4?

To address this, we performed additional ELISA experiments. First, incubation with TNF-a neutralizing antibody reduced IL-6 protein level in the culture medium of the WT TMK-1 cells (Fig 5H). Second, recombinant TNF-a treatment restored the IL-6 secretion from NRDc-KD TMK-1 cells (Fig 5I). Consistent with the decreased IL-6 protein expression in CM, TNF-a antibody treatment attenuated the growth of gastric cancer cells (Fig 5J), as in the case of IL-6 neutralizing antibody treatment (Fig 4C).

5. Like TNFa shedding, ADAM17 is responsible for IL-6 receptor shedding in human cells. The authors show that soluble IL-6 receptor levels in conditioned are not altered upon modulation of NRDc expression. Are cell surface levels of IL-6 receptor changed upon reduction of NRDc expression? For either cell surface IL-6R/gp130 signaling or IL-6/IL-6R transisgnaling, one would expect changes gp130 receptor activation (phosphorylation) to correlate with changes in IL-6 expression upon modulation of NRDC expression. What is the status of gp130 receptor activation?

As described above, we analyzed the change in mRNA levels of full-length and splice variant forms of IL-6R after gene silencing of NRDc (please see our comments to Referee #2, point 5). To examine the role of NRDc in gp130 activation, we first performed IP-WB experiment, in which gp130 in cell lysates were immunoprecipitated with anti-gp130 rabbit polyclonal antibody (from Chemicon/Millipore), followed by Western blotting with anti-phosphotyrosine antibody (4G10, Upstate/Millipore). However, we could not detect the phosphorylated gp130 in steady-state TMK-1 cells using this method. Alternatively, gp130 phosphorylation status was analyzed using two ELISA

kits, which recognize total and tyrosine-phosphorylated gp130 in cell lysates, respectively. As shown in Fig S4A, the ratio of phospho- to total gp130 was slightly reduced or almost unchanged in the NRDc-KD cells compared with the control cells, indicating that tyrosine phosphorylation of gp130 is not markedly modulated by NRDc knockdown, at least in the steady-state culture condition.

6. While the authors have previously reported that ADAM17 protein levels do not appear to change in other cell systems, it is important to eliminate this possibility in the gastric cancer cell lines. What is the expression of ADAM17 mature and precursor forms in gastric cancer cell lines upon NRDc modulation? The authors use the relatively broad-spectrum metalloprotease inhibitor TAPI-1 to demonstrate the relationship between metalloprotease activity and cytokine transcription/production. Does knockdown of ADAM17 (or other ADAM) recapitulate this response?

As mentioned in our response to referee #1, point 7, we used anti-ADAM17 rabbit polyclonal antibody for Western blotting in this revised manuscript. This antibody detected 130 and 100 kDa bands, presumably corresponding to the precursor and mature form of ADAM17, respectively (Fig S2C of Supporting information). Interestingly, intensity of 100 kDa band was reduced in NRDc-KD TMK-1 cells, while both 130 and 100 kDa bands were recognized in the control cells in the same way as in WT cells (Fig 2D). We previously demonstrated that *in vitro* sheddase activity of recombinant ADAM17 (mature form) was enhanced by direct binding to recombinant NRDc (Nishi et al, 2006). Taken together, at least in TMK-1 cells, the possibility was raised that NRDc enhances ADAM protease activity by promoting the prodomain processing of ADAM17 as well as by direct binding, but further investigation is required.

Minor points.

1. In Fig 2A, western blot for siRNA knockdown of NRDc in MKN-45 cells needs to be presented.

We added the Western blotting analysis of NRDc knockdown also in MKN-45 cells (Fig 2A, right panels).

2. In Fig 5A western blotting shows a decrease TNFa remnant but no concomitant increase its transmembrane-bound form.

The reason for this apparent discrepancy is unclear, but one possible explanation is that excess amount of pro-TNF-a is degraded by an undetermined mechanism before processing with ectodomain shedding. We added the result of densitometric analysis for quantifying relative expression of the remnant form of TNF-a to that of the precursor form (Fig 5C).

3. Fig 5F and 5G are mislabelled in Figure legend.

We corrected this mislabelling.

2nd Editorial Decision 04 January 2012

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed final report. As you will see the reviewer is now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

The referee has raised a certain number of points that need to be addressed in the final manuscript. Please modify the main text as suggested providing a track-changes document and a final document that will be used for publication.

Could you also modify the references formatting by not italicizing the journal names and not bolding the issue number.

Table 1 should be relabelled "Table I".

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #2 (Comments on Novelty/Model System):

The majority of the points raised by the reviewers has been addressed and have definitely improved the quality of the manuscript

Referee #2 (Other Remarks):

Nardilysin is a metalloprotease, which enhances shedding of membrane proteins by stimulating the activity of ADAM proteases. The authors demonstrate that Nardilysin is upregulated in gastric cancer. Knockdown of Nardilysin suppressed tumor growth in vitro and in vivo. This effect could be reverted by treatment of cells with recombinant TNF or IL-6 protein. In gastric cancer cells, NRDc promotes shedding of pro-TNF , which stimulates expression of NF B-regulated multiple cytokines such as IL-6, which in turn activated STAT3. The authors speculate that gastric cancer cell growth is maintained by activation of a TNF -NF B and IL-6-STAT3 axis and that these signals are positively influenced by Nardilysin through the induction of TNF shedding.

Most of the points raised by this reviewer have been satisfactorily addressed. There are, however, some points the authors need to correct.

Points:
1. The authors (reviewer #2; point 2) address the issue of IL-6 species specificity in an incorrect manner (see also reviewer #1; point 8). They write: 'It seems to be a controversial issue whether human cells respond to mouse IL-6 (Chiu, CP et al. (1988) PNAS 85: 7099-7103)'. This is not a correct statement. In the mentioned publication, the authors write on p7101: ' Interestingly, human HepG2 cells only responded to human IL-6 but not to mIL-6-containing cell supernatants (data not shown)'. The fact that murine IL-6 does not act on human cells has also been found by the van Snick group, who molecularly cloned murine IL-6 around the same time. It should be noted, however, that human IL-6 acts on both, human and murine cells. This needs to be mentioned when discussing the results shown in Fig. 7. are discussed (p19).
2. The current wording: 'Furthermore, experiments using a neutralizing antibody or recombinant protein demonstrated the profound implication of endogenously secreted IL-6 in STAT3 activation in gastric cancer cells. This discrepancy may arise from the difference between species (human and mouse), and cytokine secretion profile may differ among individual human gastric cancers' is not exact and should be changed accordingly.
3. In response 3 to reviewer #1 the authors write that NRDc and ADAM17 are expressed diffusely in the cytoplasm..... This is an incorrect statement. While it might be true for NRDc, it cannot be valid for ADAM17, which is a type I membrane protein and therefore cannot be expressed diffusely in the cytoplasm. The authors probably be mean to say that the staining obtained with the antibody is localized diffusely in the cytoplasm.
4. The sentence on p20, line 6 should rather read: 'In those reports, it seems that IL-6, which is produced by infiltrating immune cells and stimulates epithelial cells in a paracrine manner, has a

pivotal role in the early stage of inflammation-associated carcinogenesis' since stromal cells do not infiltrate.

3rd Revision - Authors' Response 08 January 2012

Referee #2 (Other Remarks):

...Most of the points raised by this reviewer have been satisfactorily addressed. There are, however, some points the authors need to correct.

Points:
1. The authors (reviewer #2; point 2) address the issue of IL-6 species specificity in an incorrect manner (see also reviewer #1; point 8). They write: 'It seems to be a controversial issue whether human cells respond to mouse IL-6 (Chiu, CP et al. (1988) PNAS 85: 7099-7103)'. This is not a correct statement. In the mentioned publication, the authors write on p7101: ' Interestingly, human HepG2 cells only responded to human IL-6 but not to mIL-6-containing cell supernatants (data not shown)'. The fact that murine IL-6 does not act on human cells has also been found by the van Snick group, who molecularly cloned murine IL-6 around the same time. It should be noted, however, that human IL-6 acts on both, human and murine cells. This needs to be mentioned when discussing the results shown in Fig. 7. are discussed (p19).

We apologize for our misunderstanding of the results regarding mouse IL-6 activities on human cells shown in the indicated paper (Chiu et al, 1988). We referred to this report when we discussing the results of xenograft experiments and the involvement of IL-6 in tumour development (page 18, line 3).

2. The current wording: 'Furthermore, experiments using a neutralizing antibody or recombinant protein demonstrated the profound implication of endogenously secreted IL-6 in STAT3 activation in gastric cancer cells. This discrepancy may arise from the difference between species (human and mouse), and cytokine secretion profile may differ among individual human gastric cancers' is not exact and should be changed accordingly.

We are afraid that what the term “discrepancy” means was unclear. We rewrote the sentences as follows: Furthermore, our experiments using a neutralizing antibody or recombinant protein demonstrated the implication of autocrine IL-6 signalling in STAT3 activation in human gastric cancer cells. Whether IL-6 or IL-11 is responsible for the STAT3 activation in gastric tumour cells may differ between species, or even among individual human gastric cancers (page 19, line 4 from bottom).

3. In response 3 to reviewer #1 the authors write that NRDc and ADAM17 are expressed diffusely in the cytoplasm..... This is an incorrect statement. While it might be true for NRDc, it cannot be valid for ADAM17, which is a type I membrane protein and therefore cannot be expressed diffusely in the cytoplasm. The authors probably be mean to say that the staining obtained with the antibody is localized diffusely in the cytoplasm.

We think that the term “cytoplasm” refers to the entire area between the plasma membrane and nuclear envelope. Thus, “cytoplasm” seems to include both the cytosol and membranous organelles such as endoplasmic reticulum. However, as suggested by the referee, our statement might be confusing, so we changed the sentence in the main text as follows: In AGS cells, immunocytochemical analysis demonstrated that NRDc and ADAM17 colocalized at the plasma membrane but not in the cytoplasm, while both molecules were immunostained throughout the cytoplasm (page 8, line 3 from bottom).

4. The sentence on p20, line 6 should rather read: 'In those reports, it seems that IL-6, which is produced by infiltrating immune cells and stimulates epithelial cells in a paracrine manner, has a pivotal role in the early stage of inflammation-associated carcinogenesis' since stromal cells do not infiltrate.

We corrected this sentence according to this reviewer's suggestion as follows: In those reports, it seems that IL-6, which is produced by stromal or immune cells and stimulates epithelial cells in a paracrine manner, has a pivotal role in the early stage of inflammation-associated carcinogenesis (page 20, line 8).