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MT1-MMP cleaves Dll1 to negatively regulate Notch signaling to maintain normal B cell development

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1st Editorial Decision 02 September 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are provided below. As you can see below, the referees appreciate the analysis reporting on the role of MT1-MMP mediated Dll1 cleavage regulates Notch signaling during B cell development. However they also raise many concerns that would have to be addressed in order to consider publication in the EMBO Journal. Both referees #1 and 2 want more in vivo data to support the described mechanism, while referee #3 finds that important controls are missing and that much further data would be needed to support the conclusions drawn. In other words, a very extensive revision would be needed in order to consider the suitability of the study for publication here. Should you be able to address the concerns raised in full, by the inclusion of additional data, we would be willing to consider a revised version. Acceptance of your paper will be dependent upon persuading the referees that you have provided a sufficient amount of new data to answer all their criticisms. I would like to add that it is EMBO Journal policy to allow a single round of revision only and it is therefore essential that you completely answer the points raised if you wish the manuscript ultimately to be accepted. I also recognize that to carry out all the requested experiments will take additional time and efforts. What I can offer is to extend the revision time to 6 months if that is helpful.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript, the authors describe a new function for the membrane-bound matrix metalloproteinase MT1-MMP to cleave and inactivate the Notch ligand Delta-like1 (Dll1) within bone marrow stromal cells, thereby suppressing Notch signaling in the bone marrow microenvironment. Studying MT1-MMP knockout mice, they report suppression of B cell development in the bone marrow, without emergence of ectopic T cells. This was associated with increased accumulation of active Notch in bone marrow progenitors and elevated Hes5 but not Hes1 expression. Through transfer of MT1-MMP-deficient progenitors into wild-type mice, they demonstrate rescue of the phenotype, consistent with a non-cell-autonomous effect. In cocultures of wild-type progenitors with MT1-MMP-deficient stromal cells, they again show suppression of B cell development and this is rescued in the presence of the gamma secretase inhibitor DAPT.

Furthermore, MT1-MMP-deficient stromal cells inhibited myotube formation in cocultured C2C12 cells in a DAPT-sensitive manner, consistent with their increased ability to trigger Notch signaling. Finally, they used transfection assays in 293 cells and mass spectrometry to demonstrate that MT1-MMP can cleave Dll1 at a specific site, producing a ca. 30 kDa presumably inactive fragment. These are interesting results since low levels of Notch signaling have previously been reported to occur in bone marrow hematopoietic progenitors despite the presence of Notch ligands in the bone marrow stroma and Notch receptors in the progenitors. Previous results from Maeda and colleagues have revealed a cell-autonomous suppression of Notch signaling in hematopoietic progenitors by the transcriptional repressor LRF/Zbtb7, through mechanisms that remain to be clarified (Maeda et al., Science 2007). The current paper describes an additional mechanism operating in the ligand-expressing cells that also suppresses Notch signaling via ligand inactivation. This prevents Notch-mediated suppression of bone marrow B cell development. Thus, several distinct mechanisms appear to cooperate in order to suppress Notch signaling in bone marrow hematopoietic progenitors.

Specific comments:

1) In the absence of LRF/Zbtb7, Notch-mediated suppression of B cell development happens both in the fetal liver and in the bone marrow. Have the authors examined B cell development in MT1MMP-deficient fetal liver?
2) The authors argue that enhanced Notch signaling in MT1-MMP-deficient mice is sufficient to suppress B cell development but not to trigger ectopic T cell development. If this is the case, it is unclear why LRF/Zbtb7 is not able to suppress this low level of Notch signaling. Can the authors comment on that?
3) The authors show that only Dll1 and not Dll4 is expressed in BM stromal cells. However, Dll4 could be expressed in other MT1-MMP-expressing cell types in the marrow, for example in endothelial cells that could be important for the hematopoietic niche. Thus it would be informative if the data presented in fig. 7 could be extended to Dll4 to evaluate of it is also a putative target of the protease.
4) The authors present several pieces of correlative evidence using cocultures approaches to document increased potential to trigger Notch signaling by the MT1-MMP-deficient stromal cells. However, a definitive evidence that suppression of B cell development is mediated by Notch in vivo is lacking. This is important since this is the central claim of the paper and since MT1-MMP-deficient mice might have other structural bone abnormalities that could impair B cell lymphopoiesis independently of Notch signaling. This could be addressed by inhibiting Notch in vivo either genetically or pharmacologically.
5) Fig. 1: pre-pro-B cells are not convincingly defined in the plots. Use of AA4.1 as a marker for

developing BM B cells would be useful.
6) Fig. 2: unclear if compensation has been correctly used.
7) Fig. S1; are dotted lines isotype control? Showing CD45.1 vs. CD45.2 in different channels would be easier to read if available.
8) Fig. S3: should be referred to in the Results section rather than in the discussion.

Referee #2 (Remarks to the Author):

Matrix metalloproteases are involved in the breakdown of extracellular matrix during normal (physiological) and abnormal (disease) conditions. There are two subfamilies of matrix metalloproteases, based on whether they are membrane type or secreted. This work focuses on the role of MT1-MMP, which is a membrane type matrix metalloprotease, in vivo and in vitro using bone marrow stromal cells from MT1-MMP knockout mice.

The Notch signaling pathway plays important and well-described roles in the immune system. Proteolytic cleavage of the full-length Notch heterodimer is initiated by ADAM family proteases. The authors of this manuscript are interested in another metalloprotease, MT1-MMP, which is expressed by bone marrow stromal cells (BMSCs), and they focus on the role of MT1-MMP in Notch ligand, specifically Dll1, processing in the BMSCs, and its effects on regulating Notch signaling, and thus lymphocyte development within the bone marrow.

In general the authors have taken a logical, stepwise approach to investigating the role of MT1-MMP processing of Dll1. The study initially includes examining B cell development from MT1-MMP-deficient mice, and they show that there is a clear defect in B cell development in vivo. The authors then show expression patterns of MT1-MMP in BMSCs and hematopoietic cells (HPCs and B cells). The defect in B cell development is attributed to an increase in Notch signaling as indicted by the presence of intracellular Notch protein and the Notch target gene Hes5 by quantitative PCR in vivo. In vitro assays also suggest that MT1-MMP is affecting Notch signaling. Finally the authors show the direct interaction with and processing of Dll1 by MT1-MMP as different than that of ADAM10, which adds an elegant molecular insight to this work.

Major issues:
1). The authors conclude that loss of MMP14 (MT1-MMP) leads to increased Dll1 signaling in the bone marrow, leading to a Notch-dependent reduction in B cell differentiation from HPCs. However, this causal link is only addressed in vitro, and it would be important to show that B cell differentiation could be rescued in mice in which Notch signaling is attenuated, such as in an Notch1+/- mouse (or Hes5-/- if this is the key Notch target gene responsible for the observed phenotype), such that loss of MMP14 in Notch1+/- mice will still give rise to a normal set of B cells, or to a lesser drop in B cell numbers.
2). The authors likely overstate their conclusions from the BM chimera experiments, and to really establish that no effects are seen in HPCs from MMP14-/- mice, they should do a competitive reconstitution BM chimera, by using 50:50 cells from WT and MMP14-/- BM HPCs, and then determining if equal contribution is seen in the reconstituted mice.
3). The defect observed in B cell development is not absolute, and although the authors suggest and discuss the possibility that the increased Notch signals are sufficient to block B cell differentiation, but of a reduced strength of signal for promoting T cell development in the bone marrow, are there any other MMP homologues expressed in the BMSCs, if so, are these compensating for the absence of MT1-MMP? Can this be addressed by using a double deficient mouse? This possibility should at the very least be discussed.
4). Did the authors check other Notch target genes such as Deltex1 in Figures 4 and 5? Or is Hes5 the likely target at this level of Notch signaling, if so, then breeding to a Hes5-/- mouse may be an important experiment to consider.

Minor issues:
1). Gene names need to be italicized. MT1-MMP should be referred to as MMP14 at least once in the abstract, introduction, and title as well.
2). Figure 1F shows DN subsets as 80%, for both WT and MT1-MMP-/- mice, it does not state in the legend that these subsets are gated on CD4-CD8- populations, and therefore this percentage is misleading, and perhaps could be omitted from the graph?

3). Bar graphs in figures 4, 5 and 6 do not have any y axis labels and there are no molecular weight markers in figures3, 4, 5, 7 and supplemental figure 3.
4). Page 4, the authors state: "Therefore there must be mechanisms negatively regulating Notch signaling to maintain normal B cell development." Although the meaning is understood, the use of the word "must" seems over the top.
5). Page 13, when making reference to the Dll4 expression and requirements in the thymus for T cell development, the authors should also cite the paper from Hozumi et al. (J. Exp. Med. 2008, 205:2507).
6). Supplemental figure 3, when showing the percentages for the CD44 v CD25 quadrants, the authors should denote the relative %'s within the DN subset, rather than showing the % of total.
7). There are several grammatical, tense and spelling mistakes throughout the manuscript. In addition, the authors have not abbreviated acronyms at the earliest mention in the manuscript.

Referee #3 (Remarks to the Author):

The manuscript by Jin et al. reports that Delta like 1 (Dll1), a ligand for Notch receptor, is a substrate for membrane-type 1 matrix metalloproteinase (MT1-MMP). The cleavage of Dll1 by MT1-MMP in bone marrow stromal cells (BMSCs) downregulates Notch signaling in neighboring hematopoietic progenitor cells (HPCs) and maintains normal B cell development in bone marrow. There are major problems with the experimental design, lack of controls, and data interpretation, which preclude publication of the manuscript.

Major problems:

1. In transplantation experiments shown in Fig. 2, bone marrow from wild-type mice or MT1-MMP deficient mice was transplanted into lethally irradiated wild-type mice. Since B cell differentiation was comparable in these two cases, the authors conclude that the abnormal B cell differentiation in MT1-MMP deficient mice is not cell autonomous and it is a consequence of the defective niche. However, to directly demonstrate a role of MT1-MMP present in stromal cells on B cell differentiation, a reciprocal transplantation should be done, i.e., bone marrow from wild type mice should be transplanted into lethally irradiated wild type mice or MT1-MMP deficient mice.

2. There are multiple problems with results shown in Fig. 7. In the co-immunoprecipitation experiment in Fig 7A, it is not known whether the Dll1 band represents the full-length Dll1 or the C-terminal fragment of Dll1. Similarly, in Fig. 7C, it is not clear whether Jag1 band represents the full-length of the cleaved protein. In Fig. 7B, the entire length of the gel spanning the region from 30 kDa (the size of CTF) to 100 kDa (roughly the size of full-length Dll1) should be shown. The identity of the Dll1-FL and Dll1-CTF bands should be confirmed by using siRNA to Dll1. When 293hN1 cells are cultured on HeLa cells expressing Dll1 and either active or inactive MT1-MMP, control lanes are needed for 293hN1 cells only and HeLa cells only. Since anti-beta actin antibody detects actin from both 293hN1 and HeLa cells, a different approach is needed to confirm an equal 293hN1 cell density.

3. The fact that MT1-MMP deficient BMSCs inhibit myotube formation and that this inhibition occurs in a Notch-dependent manner (Fig. 6) is not well documented. The difference in cell morphology of C2C12 cells cultured on wild-type BMSCs or MT1-MMP deficient BMSCs in Fig. 6A, in the presence of DMSO, is not evident. Most importantly, the interpretation of the results of the experiments studying the effect of DAPT on C2C12 differentiation is erroneous. DAPT, by inhibiting gamma-secretase and inhibiting activation of Notch, stimulates cell differentiation. If the reduced differentiation of C2C12 cells cultured on MT1-MMP deficient BMSCs was dependent on Notch, DAPT should completely abolish the difference between MT1-MMP deficient BMSCs and wild-type BMSCs. This is obviously not the case, as Fig. 6B,C shows still a significant difference in Mlc2 and Myog (markers of C2C12 differentiation) between wild-type and MT1-MMP deficient BMSCs, in the presence of DAPT. DAPT simply appears to shift the levels of Mlc2 and Myog upward in all conditions, which is consistent with the inhibitory effect of Notch on C2C12 differentiation. In fact, this experiment suggests that the difference between the effect of MT1-MMP deficient BMSCs and wild-type BMSCs on C2C12 differentiation, if any, can NOT be rescued by DAPT.

4. When determining the potential cleavage site of Dll1 by MT1-MMP in vitro, the Dll1 peptide is incubated with the recombinant catalytic form of MT1-MMP, following the analysis of reaction products by mass spectroscopy. This experiments lacks essential controls. The incubations need to be performed in the presence of the catalytic form of MT1-MMP, in the presence of a catalytically inactive mutant of MT1-MMP, and in the absence of MT1-MMP, and the reaction products need to be compared.

5. Again, when determining the cleavage site of Dll1 by MT1-MMP in intact cells, cells need to be co-transfected with Dll1 and catalytically active MT1-MMP, catalytically inactive MT1-MMP, and an empty vector, and N-terminal sequencing of the C-terminal Dll1 fragment obtained in these three conditions should be performed and compared. The raw data need to be shown.

6. In Fig. 5A, control lane with HPCs only is needed. Despite the fact that wild-type HPCs appear not to have active Notch1 in the experiment shown in Fig. 4A, control conditions with HPCs only are needed in the experiment shown in Fig. 5A (as this experiment is different from that one shown in Fig. 4A).

7. The sizes of molecular weight markers should be indicated in each immunoblot. Without the indicated sizes, it is impossible to confirm the authenticity of active Notch1 or Dll1. Currently, the positions of the molecular weight markers, originally marked with a pen, are visible, but they are totally useless when left unlabeled.

Minor points:

8. Anti-cleaved Notch1 antibody used in this study detects a neoepitope in Notch1 (Val1744) generated after gamma-secretase cleavage, and thus it is specific for active Notch1. Active Notch1 comprises the cytoplasmic domain (NICD1) and a fragment of the transmembrane region, and it is different from NICD1. Thus, it is not correct to refer the active Notch1 as NICD1 (in the text and in figures).

9. Lehar et al, 2005, is not the primary reference for the inhibitory effect of Notch on C2C12 myoblast differentiation.

1st Revision - authors' response 01 March 2011

Referee #1 (Remarks to the Author):

In this manuscript, the authors describe a new function for the membrane-bound matrix metalloproteinase MT1-MMP to cleave and inactivate the Notch ligand Delta-like1 (Dll1) within bone marrow stromal cells, thereby suppressing Notch signaling in the bone marrow microenvironment. Studying MT1-MMP knockout mice, they report suppression of B cell development in the bone marrow, without emergence of ectopic T cells. This was associated with increased accumulation of active Notch in bone marrow progenitors and elevated Hes5 but not Hes1 expression. Through transfer of MT1-MMP-deficient progenitors into wild-type mice, they demonstrate rescue of the phenotype, consistent with a non-cell-autonomous effect. In cocultures of wild-type progenitors with MT1-MMP-deficient stromal cells, they again show suppression of B cell development and this is rescued in the presence of the gamma secretase inhibitor DAPT. Furthermore, MT1-MMP-deficient stromal cells inhibited myotube formation in cocultured C2C12 cells in a DAPT-sensitive manner, consistent with their increased ability to trigger Notch signaling. Finally, they used transfection assays in 293 cells and mass spectrometry to demonstrate that MT1-MMP can cleave Dll1 at a specific site, producing a ca. 30 kDa presumably inactive fragment. These are interesting results since low levels of Notch signaling have previously been reported to occur in bone marrow hematopoietic progenitors despite the presence of Notch ligands in the bone marrow stroma and Notch receptors in the progenitors. Previous results from Maeda and colleagues have revealed a cell-autonomous suppression of Notch signaling in hematopoietic progenitors by the transcriptional repressor LRF/Zbtb7, through mechanisms that remain to be clarified (Maeda et al., Science 2007). The current paper describes an additional mechanism operating in the ligand-expressing cells that also suppresses Notch signaling via ligand inactivation. This prevents Notch-mediated suppression of bone marrow B cell development. Thus, several

distinct mechanisms appear to cooperate in order to suppress Notch signaling in bone marrow hematopoietic progenitors.
We are grateful for the very positive comments and suggestions from this reviewer. The manuscript has been significantly improved following his/her suggestion.

Specific comments:
1) In the absence of LRF/Zbtb7, Notch-mediated suppression of B cell development happens both in the fetal liver and in the bone marrow. Have the authors examined B cell development in MT1MMP-deficient fetal liver?
In MT1-MMP deficient fetal liver, we did not observe defects in either the B cell development (Page 6 and Supplementary Figure 2) or notch signaling (Supplementary Fig. 9B). This could be due to restricted expression of MT1-MMP.

2) The authors argue that enhanced Notch signaling in MT1-MMP-deficient mice is sufficient to suppress B cell development but not to trigger ectopic T cell development. If this is the case, it is unclear why LRF/Zbtb7 is not able to suppress this low level of Notch signaling. Can the authors comment on that?
Obviously LRF did not suppress the enhanced Notch signaling in MT1-MMP deficient mice. LRF is suggested to suppress Notch signaling in bone marrow, though the underlying mechanism remains unknown (Maeda et al, 2007). In normal bone marrow where LRF functions, Dll1 induced Notch signaling is suppressed by MT1-MMP. Therefore predominant Notch signaling is triggered by the ligands other than Dll1. LRF may suppress Notch signaling induced by other ligands. On the other hand, loss of MT1-MMP may disturb LRF function.

3) The authors show that only Dll1 and not Dll4 is expressed in BM stromal cells. However, Dll4 could be expressed in other MT1-MMP-expressing cell types in the marrow, for example in endothelial cells that could be important for the hematopoietic niche. Thus it would be informative if the data presented in fig. 7 could be extended to Dll4 to evaluate of it is also a putative target of the protease.
We have now evaluated Dll4 cleavage by MT1-MMP. Original Fig.7 has been rearranged to become Fig.8. The co-transfection of MT1-MMP and Dll4 in HEK293 cells showed that Dll4 was not cleaved by MT1-MMP (Page 15 and Figure 8F).

4) The authors present several pieces of correlative evidence using cocultures approaches to document increased potential to trigger Notch signaling by the MT1-MMP-deficient stromal cells. However, a definitive evidence that suppression of B cell development is mediated by Notch in vivo is lacking. This is important since this is the central claim of the paper and since MT1-MMP-deficient mice might have other structural bone abnormalities that could impair B cell lymphopoiesis independently of Notch signaling. This could be addressed by inhibiting Notch in vivo either genetically or pharmacologically.
This is a very good suggestion and we have followed the suggestion and performed the in vivo experiment using Notch inhibitor DAPT. We have generated stromal specific MT1-MMP knockout mice to test the Notch signaling and B cell development. Administration of Notch inhibitor DAPT into the conditional MT1-MMP KO mice significantly rescued the B cell development defect caused by MT1-MMP deletion (Page 13 and Fig. 7).

5) Fig. 1: pre-pro-B cells are not convincingly defined in the plots. Use of AA4.1 as a marker for developing BM B cells would be useful.
We have followed the suggestion to use another set of markers including AA4.1. Similar results were observed (Page 6 and Supplementary Fig. 1).

6) Fig. 2: unclear if compensation has been correctly used.
We have reset the gating so that two $B220^{+}$ populations were separated ($B220^{high}$, $B220^{low}$). It has been shown that the $B220^{high}$ population represents the recruited mature B cells. The $B220^{low}$ population represents the bone marrow derived lineage B cell precursors (Masuzawa et al, 1994).(page 7-8 and Fig. 2)

7) Fig. S1; are dotted lines isotype control? Showing CD45.1 vs. CD45.2 in different channels would be easier to read if available.

Supplementary Fig. 1 has been rearranged as Supplementary Fig. 4. The dotted lines are isotype controls. It is stated in the figure legend (Supplementary Fig 4). CD45.1 and CD45.2 we used were in the same channel. We agree different channels are better.

8) Fig. S3: should be referred to in the Results section rather than in the discussion.
The description of Supplementary Fig. 3 has been referred to the results (Page 7).

Referee #2 (Remarks to the Author):

Matrix metalloproteases are involved in the breakdown of extracellular matrix during normal (physiological) and abnormal (disease) conditions. There are two subfamilies of matrix metalloproteases, based on whether they are membrane type or secreted. This work focuses on the role of MT1-MMP, which is a membrane type matrix metalloprotease, in vivo and in vitro using bone marrow stromal cells from MT1-MMP knockout mice.

The Notch signaling pathway plays important and well-described roles in the immune system. Proteolytic cleavage of the full-length Notch heterodimer is initiated by ADAM family proteases. The authors of this manuscript are interested in another metalloprotease, MT1-MMP, which is expressed by bone marrow stromal cells (BMSCs), and they focus on the role of MT1-MMP in Notch ligand, specifically Dll1, processing in the BMSCs, and its effects on regulating Notch signaling, and thus lymphocyte development within the bone marrow.

In general the authors have taken a logical, stepwise approach to investigating the role of MT1-MMP processing of Dll1. The study initially includes examining B cell development from MT1-MMP-deficient mice, and they show that there is a clear defect in B cell development in vivo. The authors then show expression patterns of MT1-MMP in BMSCs and hematopoietic cells (HPCs and B cells). The defect in B cell development is attributed to an increase in Notch signaling as indicted by the presence of intracellular Notch protein and the Notch target gene Hes5 by quantitative PCR in vivo. In vitro assays also suggest that MT1-MMP is affecting Notch signaling. Finally the authors show the direct interaction with and processing of Dll1 by MT1-MMP as different than that of ADAM10, which adds an elegant molecular insight to this work.
We appreciate the reviewer's comments and encouragement and also thank for the constructive suggestion in making this manuscript better.

Major issues:
1). The authors conclude that loss of MMP14 (MT1-MMP) leads to increased Dll1 signaling in the bone marrow, leading to a Notch-dependent reduction in B cell differentiation from HPCs. However, this causal link is only addressed in vitro, and it would be important to show that B cell differentiation could be rescued in mice in which Notch signaling is attenuated, such as in an Notch1+/- mouse (or Hes5-/- if this is the key Notch target gene responsible for the observed phenotype), such that loss of MMP14 in Notch1+/- mice will still give rise to a normal set of B cells, or to a lesser drop in B cell numbers.
This is a very good suggestion! To save time, we have performed the in vivo experiment using Notch inhibitor DAPT instead of Notch mutant mice. We tested the consequence of inhibition of Notch signaling in stromal-specific MT1-MMP KO mice by administration of Notch inhibitor DAPT. Inhibition of Notch signaling by DAPT significantly rescued the B cell development defect caused by MT1-MMP deletion (Page13 and Fig. 7).

2). The authors likely overstate their conclusions from the BM chimera experiments, and to really establish that no effects are seen in HPCs from MMP14-/- mice, they should do a competitive reconstitution BM chimera, by using 50:50 cells from WT and MMP14-/- BM HPCs, and then determining if equal contribution is seen in the reconstituted mice.
We agree that competitive reconstitution will provide a stronger evidence for non-cell autonomous defect in B cell development in MT1-MMP deficient bone marrow. However, the expression pattern of MT1-MMP supports our statement. Anyway, following this reviewer's suggestion, we have softened our statement from original manuscript "… the abnormal B cell development in MT1-MMP deficient mice **is not** resulted from intrinsic…" to "…the abnormal B cell development in MT1-MMP deficient mice **is likely not** resulted from intrinsic…" to avoid overstatement (see revised manuscript page 8).

3). The defect observed in B cell development is not absolute, and although the authors suggest and discuss the possibility that the increased Notch signals are sufficient to block B cell differentiation, but of a reduced strength of signal for promoting T cell development in the bone marrow, are there any other MMP homologues expressed in the BMSCs, if so, are these compensating for the absence of MT1-MMP? Can this be addressed by using a double deficient mouse? This possibility should at the very least be discussed.
It has been reported that Dll1 is cleaved by some membrane metalloproteinases other than MT1-MMP, such as ADAM9 and ADAM10 (Dyczynska et al, 2007; Six et al, 2003). The expression of ADAM9 and ADAM10 were examined in BMSCs and both are expressed in BMSCs (Supplementary Fig. 8). This is consistent with the existence of Dll1-CTF in MT1-MMP deficient BMSCs (Fig. 8B). The cleavage by ADAMs may partially compensate for the absence of MT1-MMP and minimize the activation of Notch signaling in MT1-MMP deficient mice. We have added this to the discussion in the revised manuscript (Page 19).

4). Did the authors check other Notch target genes such as Deltex1 in Figures 4 and 5? Or is Hes5 the likely target at this level of Notch signaling, if so, then breeding to a Hes5-/- mouse may be an important experiment to consider.
We checked the Notch signaling regulator Deltex1. The mRNA level was similar between wild-type and MT1-MMP deficient HPCs (Page10 and Fig. 4B). It is likely that Hes5 is the target of this level Notch signaling because inhibiting Notch signaling by DAPT restores the Hes5 expression in the in vivo rescuing experiment.

Minor issues:
1). Gene names need to be italicized. MT1-MMP should be referred to as MMP14 at least once in the abstract, introduction, and title as well.
We have followed the suggestion and revise the manuscript accordingly.

2). Figure 1F shows DN subsets as 80%, for both WT and MT1-MMP-/- mice, it does not state in the legend that these subsets are gated on CD4-CD8- populations, and therefore this percentage is misleading, and perhaps could be omitted from the graph?
We have now stated that DN is CD4CD8 double negative in the figure legend (Page 29).

3). Bar graphs in figures 4, 5 and 6 do not have any y axis labels and there are no molecular weight markers in figures3, 4, 5, 7 and supplementary figure 3.
We have now included y axis labels in figures 4, 5 and 6. Molecular weight markers have been shown in the immunoblots in the revised manuscript.

4). Page 4, the authors state: "Therefore there must be mechanisms negatively regulating Notch signaling to maintain normal B cell development." Although the meaning is understood, the use of the word "must" seems over the top.
The sentence has been replaced by “These studies indicate that Notch signaling might be negatively regulated to maintain normal B cell development.”(Page 4)

5). Page 13, when making reference to the Dll4 expression and requirements in the thymus for T cell development, the authors should also cite the paper from Hozumi et al. (J. Exp. Med. 2008, 205:2507).
This paper is now cited (Page15).

6). Supplementary figure 3, when showing the percentages for the CD44 v CD25 quadrants, the authors should denote the relative %'s within the DN subset, rather than showing the % of total.
The percentages in the CD44 v CD25 quadrants (Supplementary Figure 3C) use the relative percentages within the DN subset in the revised manuscript.

7). There are several grammatical, tense and spelling mistakes throughout the manuscript. In addition, the authors have not abbreviated acronyms at the earliest mention in the manuscript.
We have carefully checked the errors and corrected them accordingly.

Referee #3 (Remarks to the Author):

The manuscript by Jin et al. reports that Delta like 1 (Dll1), a ligand for Notch receptor, is a substrate for membrane-type 1 matrix metalloproteinase (MT1-MMP). The cleavage of Dll1 by MT1-MMP in bone marrow stromal cells (BMSCs) downregulates Notch signaling in neighboring hematopoietic progenitor cells (HPCs) and maintains normal B cell development in bone marrow. There are major problems with the experimental design, lack of controls, and data interpretation, which preclude publication of the manuscript.
We thank this reviewer for his/her very critical comments and suggestions in clarifying several technique issues in the revised manuscript.

Major problems:

1. In transplantation experiments shown in Fig. 2, bone marrow from wild-type mice or MT1-MMP deficient mice was transplanted into lethally irradiated wild-type mice. Since B cell differentiation was comparable in these two cases, the authors conclude that the abnormal B cell differentiation in MT1-MMP deficient mice is not cell autonomous and it is a consequence of the defective niche. However, to directly demonstrate a role of MT1-MMP present in stromal cells on B cell differentiation, a reciprocal transplantation should be done, i.e., bone marrow from wild type mice should be transplanted into lethally irradiated wild type mice or MT1-MMP deficient mice.
We agree that the reciprocal transplantation will provide additional evidence for non-cell autonomous effect. However, we did show that MT1-MMP is expressed in BMSCs, but not in HPCs. Together with the reconstitution experiments, these data should be sufficient to indicate that the B cell differentiation defect in MT1-MMP deficient mice is likely not cell autonomous, but due to the defective niches. To avoid overstatement, we have softened the conclusion by adding “likely” in the statement.

2. There are multiple problems with results shown in Fig. 7. In the co-immunoprecipitation experiment in Fig 7A, it is not known whether the Dll1 band represents the full-length Dll1 or the C-terminal fragment of Dll1. Similarly, in Fig. 7C, it is not clear whether Jag1 band represents the full-length of the cleaved protein. In Fig. 7B, the entire length of the gel spanning the region from 30 kDa (the size of CTF) to 100 kDa (roughly the size of full-length Dll1) should be shown. The identity of the Dll1-FL and Dll1-CTF bands should be confirmed by using siRNA to Dll1. When 293hN1 cells are cultured on HeLa cells expressing Dll1 and either active or inactive MT1-MMP, control lanes are needed for 293hN1 cells only and HeLa cells only. Since anti-beta actin antibody detects actin from both 293hN1 and HeLa cells, a different approach is needed to confirm an equal 293hN1 cell density.
Fig.7 has been rearranged to Fig. 8.
The Dll1 band in Fig 8A represents the full-length Dll1. The Jag1 band in Fig. 8C also represents the full-length protein.
The entire length of the blot showing all Dll1 bands in BMSCs was presented in Supplementary Fig. 5.
Dll1 knocking down in BMSCs was done in Fig. 8B and Supplementary Fig. 5.
During the co-culture of 293hN1 on HeLa cells, the 293hN1 cells and Hela cells were collected separately, because 293hN1 cells can be easily detached while HeLa cells attached the dishes tightly. Therefore, the immunoblot showed the proteins in 293hN1 cells only or HeLa cells only.

3. The fact that MT1-MMP deficient BMSCs inhibit myotube formation and that this inhibition occurs in a Notch-dependent manner (Fig. 6) is not well documented. The difference in cell morphology of C2C12 cells cultured on wild-type BMSCs or MT1-MMP deficient BMSCs in Fig. 6A, in the presence of DMSO, is not evident. Most importantly, the interpretation of the results of the experiments studying the effect of DAPT on C2C12 differentiation is erroneous. DAPT, by inhibiting gamma-secretase and inhibiting activation of Notch, stimulates cell differentiation. If the reduced differentiation of C2C12 cells cultured on MT1-MMP deficient BMSCs was dependent on Notch, DAPT should completely abolish the difference between MT1-MMP deficient BMSCs and wild-type BMSCs. This is obviously not the case, as Fig. 6B,C shows still a significant difference in Mlc2 and Myog (markers of C2C12 differentiation) between wild-type and MT1-MMP deficient BMSCs, in the presence of DAPT. DAPT simply appears to shift the levels of Mlc2 and Myog upward in all conditions, which is consistent with the inhibitory effect of Notch on C2C12 differentiation. In fact, this experiment suggests that the difference between the effect of MT1-MMP

deficient BMSCs and wild-type BMSCs on C2C12 differentiation, if any, can NOT be rescued by DAPT.
We have noticed that 1μM DAPT did not completely rescue the defect of C2C12 differentiation on MT1-MMP deficient BMSCs. The partially rescue of the defect is most likely due to the partial inhibition of Notch by DAPT at that concentration. We have repeated the experiment using 10 μM DAPT. As shown in Fig 6B, the difference in mRNA level of Mlc2 and Myog between C2C12 cells on MT1-MMP deficient BMSCs and C2C12 on wild-type BMSCs was completely abolished (Page 12 and Fig. 6B). Therefore, Notch inhibitor DAPT can rescue the myotube formation defect on MT1-MMP deficient BMSCs, suggesting that defective C2C12 myotube formation on MT1-MMP deficient BMSCs is mediated by enhanced Notch signaling.

4. When determining the potential cleavage site of Dll1 by MT1-MMP in vitro, the Dll1 peptide is incubated with the recombinant catalytic form of MT1-MMP, following the analysis of reaction products by mass spectroscopy. This experiment lacks essential controls. The incubations need to be performed in the presence of the catalytic form of MT1-MMP, in the presence of a catalytically inactive mutant of MT1-MMP, and in the absence of MT1-MMP, and the reaction products need to be compared.
We have now included the controls in the incubations of Dll1 polypeptide with the catalytic form of MT1-MMP. One is to introduce EDTA which will inhibit MT1-MMP activity. Another one is Dll1 polypeptide only. When comparing the products of these reactions, only active MT1-MMP generated the cleaved product at 2015.9. (Supplementary Figure 6).

5. Again, when determining the cleavage site of Dll1 by MT1-MMP in intact cells, cells need to be co-transfected with Dll1 and catalytically active MT1-MMP, catalytically inactive MT1-MMP, and an empty vector, and N-terminal sequencing of the C-terminal Dll1 fragment obtained in these three conditions should be performed and compared. The raw data need to be shown.
Following reviewer's suggestion, we have performed the experiments by co-transfecting Dll1 with either full-length wild-type MT1-MMP, or inactive MT1-MMP or empty vector. Dll1 was then purified by immunoprecipitation. As shown in Supplementary Fig. 7A, only wild-type MT1-MMP generated significant Dll1 C-terminal fragment. This band was then subjected to Edman N-terminal sequencing. The raw data of N-terminal sequencing result has been shown in Supplementary Figure 7B.

6. In Fig. 5A, control lane with HPCs only is needed. Despite the fact that wild-type HPCs appear not to have active Notch1 in the experiment shown in Fig. 4A, control conditions with HPCs only are needed in the experiment shown in Fig. 5A (as this experiment is different from that one shown in Fig. 4A).
The control lane with wild-type HPCs without co-culture is now shown in Fig. 5A.

7. The sizes of molecular weight markers should be indicated in each immunoblot. Without the indicated sizes, it is impossible to confirm the authenticity of active Notch1 or Dll1. Currently, the positions of the molecular weight markers, originally marked with a pen, are visible, but they are totally useless when left unlabeled.
Molecular markers have been included in the immunoblots.

Minor points:
8. Anti-cleaved Notch1 antibody used in this study detects a neoepitope in Notch1 (Val1744) generated after gamma-secretase cleavage, and thus it is specific for active Notch1. Active Notch1 comprises the cytoplasmic domain (NICD1) and a fragment of the transmembrane region, and it is different from NICD1. Thus, it is not correct to refer the active Notch1 as NICD1 (in the text and in figures).
It has been reported that the gamma-secretase cleavage releases the cytoplasmic domain of Notch receptor (NICD), which do not contain the transmembrane domain (Brou et al, 2000; Mumm et al, 2000; Schroeter et al, 1998). Therefore, the anti-cleaved Notch1 antibody used in this study detects NICD1.

9. Lehar et al, 2005, is not the primary reference for the inhibitory effect of Notch on C2C12 myoblast differentiation.
We thank for reminding us this error and the primary reference is now cited (Page 12).

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2nd Editorial Decision 29 March 2011

Thank you for submitting your revised manuscript to the EMBO Journal. Your revised version has now been seen by the original referees # 1 and 2 and their comments are provided below. As you can see both referees appreciate the introduced changes and support publication here. Referee #1 has a few remaining issues that should be addressed before publication here. These last issues should not involve too much additional work to address.

Thank you for submitting your interesting work to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors provide significant new information based on new experiments and on some clarifications. The manuscript is improved as a result. In particular, use of DAPT in vivo to rescue the B lymphopoietic defect of MT1-MMP-deficient mice is an important point. However, there are still some results that are technically suboptimal as detailed below.

Specific comments:

Fig 7 (rescue by DAPT in vivo) is an important addition. However, Fig 7D should show percentages. Information about the number of mice analyzed should be given in fig. 7F (only information about number of experiments is given).

Figure S2. In principle this is a nice addition, however the quality of flow cytometry results is not optimal. In particular, CD19 expression is not convincingly documented, and the B220 stain only shows low level positivity. CD19 should be the gold standard for B cells and double staining for B220 and CD19 should be more clearly documented. Alternatively, some fetal B1 B cell precursors express CD19 and no or low levels of B220 (Montecino-Rodriguez et al., Nat Immunol 2006). It would be acceptable to identify those as well as a readout. Altogether, the data are suggestive that no impairment in B cell lymphopoiesis is observed in MT1-MMP-deficient fetal liver, but definitive conclusions cannot be drawn on the basis of the data shown.

Reviewer #3, point 8: although this is a semantic point, the question about NICD1 vs. active Notch1 is not addressed correctly. The authors are not correct in their statement that active Notch1 as identified by the Val 1744 only contains cytoplasmic elements.

Referee #2 (Remarks to the Author):

The authors have done an excellent job in addressing all my initial concerns, and the manuscript is now much improved, and no other issues need to be addressed.

2nd Revision - authors' response 31 March 2011

Point-to Point Response to reviewers' comments:

Referee #1 (Remarks to the Author):

The authors provide significant new information based on new experiments and on some clarifications. The manuscript is improved as a result. In particular, use of DAPT in vivo to rescue the B lymphopoietic defect of MT1-MMP-deficient mice is an important point. However, there are still some results that are technically suboptimal as detailed below.

Specific comments:

Fig 7 (rescue by DAPT in vivo) is an important addition. However, Fig 7D should show percentages. Information about the number of mice analyzed should be given in fig. 7F (only information about number of experiments is given).

The percentages are now showed in Fig. 7D. The number of mice is included in Fig. 7F legend.

Figure S2. In principle this is a nice addition, however the quality of flow cytometry results is not optimal. In particular, CD19 expression is not convincingly documented, and the B220 stain only shows low level positivity. CD19 should be the gold standard for B cells and double staining for B220 and CD19 should be more clearly documented. Alternatively, some fetal B1 B cell precursors express CD19 and no or low levels of B220 (Montecino-Rodriguez et al., Nat Immunol 2006). It would be acceptable to identify those as well as a readout. Altogether, the data are suggestive that no impairment in B cell lymphopoiesis is observed in MT1-MMP-deficient fetal liver, but definitive conclusions cannot be drawn on the basis of the data shown.
We agree that the flow cytometry may not be perfect. However, we didn't see any significant difference in B220 positive B cell populations in fetal livers, suggesting that no significant impairment in B cell lymphopoiesis is observed in MT1-MMP deficient fetal liver. In addition, Notch signaling doesn't show any difference between wild-types and mutants, further indicating that loss of MT1-MMP in fetal liver does not significantly affect B lymphopoiesis through Notch signaling, as it does in bone marrow. We therefore have followed the reviewer's suggestion to soften the conclusion accordingly (see page 6).

Reviewer #3, point 8: although this is a semantic point, the question about NICD1 vs. active Notch1 is not addressed correctly. The authors are not correct in their statement that active Notch1 as identified by the Val 1744 only contains cytoplasmic elements.
As suggested, we now use active Notch1 (aNotch1) instead of NICD1 in the revised manuscript

Referee #2 (Remarks to the Author):

The authors have done an excellent job in addressing all my initial concerns, and the manuscript is now much improved, and no other issues need to be addressed.

