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Tumor growth inhibition and anti-metastatic activity of a mutated furin-resistant Semaphorin 3E isoform

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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 05 August 2011

Thank you for the submission of your manuscript "Tumor growth inhibition and anti-metastatic activity of a mutated furin-resistant Semaphorin 3E isoform" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, Reviewers 1 and 2 highlight that the anti-angiogenic/vascular effect of the peptide should be investigated in greater detail to provide mechanistic insight. Importantly, both reviewers 2 and 3 feel that evaluation of preclinical parameters like longterm toxicity should be provided. Of note, reviewer 2 points out that the investigation of a possible combination therapy would increase the translational impact of the study.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged otherwise with the editor.

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:

In this manuscript Casazza et al describe the mechanism of action and the putative therapeutic potential of an uncleavable full-length form of Semaphorin 3E, demonstrating its anti-angiogenic, anti-tumor and anti-metastatic efficacy in several models of cancer.
The study is extensive, properly designed and developed, and significantly contributes to the understanding of Sema3E biology, but also sheds light on the potential usage of a recombinant mutant molecule as a therapeutic drug to block tumor growth, tumor progression and tumor dissemination in the form of distant metastasis.

Nevertheless, several issues need to be clarified and further documented in order for this manuscript to gain the significance and relevance to be published.

1. While the section describing the inhibition of prometastatic signaling by uncleaved Sema3E antagonizing mature p61-Sema3E is strongly determined, the section describing the anti-angiogenic effects in HUVEC is much weaker. Uncl-Sema3E is used in the all evaluations (Fig.3), but in order to robustly document that anti-angiogenic function of this mutant form is "unaffected" or is a "partial agonist", at least a p61-Sema3E control should be used in these experiments.

2. The transgenic pancreatic model study contributes with relevant data to further describe the anti-angiogenic and anti-tumor effects, but does not show any reduction in tumor spread/dissemination and metastasis formation. On the other hand, the studies in 4T1 tumors, A549 tumors and LLC tumors clearly document an anti-tumor effect together with inhibition of metastasis formation. These discordant results should be further clarified to unravel the cause of this discrepancy. One possible hypothesis could be related to the fact that Plexin-D1 expression is needed for the anti-metastatic effects of Uncl-Sema3E (Supl.Fig.7), but the RIP-Tag2 model shows tumor-cell expression of Plexin-D1 but the treatment does not exert an anti-metastatic effect. More data is needed to clarify this discrepancy.

3. Determination of alterations in 'in vitro' growth rate are included for MDA-MB-435 cells. The data shown for 3 days in culture seems to show a relevant tendency to decrease cell growth in Uncl-Sema3E. This should be evaluated for longer time, and maybe using other more specific parameters (proliferation, apoptosis...) in order to further solidify the paracrine / stromal contribution to this effect.
Moreover, similar analysis should be done for all other tumor models (RIP-Tag2, 4T1, A549 and LLC) (maybe only as supplementary data).

4. The strong statement in the abstract that that Uncl-Sema3E is effective even in anti-VEGF resistant tumors is a misunderstanding. First, while the term "resistance" is often misused in the field to refer to a tumor model that does not respond to these therapies, the proper term for these type of tumors should be "non-responsive" or "refractory". This way there is less room for misunderstandings.
On the other hand, this is a strong statement based only on the use of one single tumor cell line that has been shown to be partly-refractory to anti-VEGF therapy. While this is a relevant result to document that Uncl-Sema3E and anti-VEGF are completely independent pathways, it is not sufficiently substantiated with data in order to put it as a hard conclusion (and should not be in the abstract either).
Moreover, the anti-VEGF treatment study is much more than a "positive control" of anti-vascular, anti-tumor and pro-metastatic drug. The way it is shown in the figures, it poses an intriguing comparison between the two treatments and opens to speculation about the effects of a combined treatment of anti-VEGF together with Uncl-Sema3E. Although this is not the focus of this manuscript, this data poses several other questions that are out of scope for this study.

Minor Comments:

- Quality of Fig.8C and F is very poor and the graphs should be re-done with proper error bars. (current ones seem to be artificially shortened!)
- Toxicity results from recombinant Uncl-Sema3E are very relevant, but maybe more informative Before the results, and not after.
- What is an "intervention trial"? This expression is used in p8 and p11 and should be clarified.
- Past and present tense are mixed in different paragraphs in the abstract and the main text.

Referee #2:

Casazza and co-workers have explored the role of Semaphorin 3E processing on tumor progression and metastasis. The authors identified uncleaved Sema3E as a potential anti-cancer therapeutic. Uncl-Sema3E acted anti-angiogenic and inhibited metastatic dissemination in different tumor models by blocking the pro-metastatic effect of processed p61-Sema3E. Uncl-Sema3E binds to PlexinD1 receptor on tumor cells and thereby prevents the binding of p61-Sema3E resulting in less tumor cell dissemination. In contrast to anti-angiogenic (sFlk1) therapy, the systemic delivery of uncl-Sema3E did not promote tumor invasion and metastasis making uncl-Sema3E a promising therapeutic agent. This is a very strong, clearly presented and largely convincing manuscript building on previously published data showing that processed p61-Sema3E induces metastatic foci (Christensen, 2005). The present study confirms that the processing of semaphorins determines its function on endothelial and tumor cells. Furthermore, this study is in line with a recent publication by the same authors showing that systemic delivery of Sema3A prevents tumor angiogenesis and metastasis (Casazza, 2011). As such, the straightforwardness of the manuscript's experimental program is somewhat counterbalanced by the manuscript's limited novelty in light of the previously published work. Despite these limitations, the reviewer acknowledges the beauty of the present study, but would nevertheless encourage the authors to dig deeper to shed additional mechanistic insights into the observed phenomena (most notably: analysis of vascular phenotype). This should enable the authors to generate findings that would go beyond the expected. From a translational perspective, the authors are strongly encouraged to include preclinical experiments that are in line with the way anti-angiogenic therapy is use (most importantly combination therapy).

Taken together, this manuscript would benefit from some revisions, which should involve a number of additional experiments. Below list of comments is lengthy, but this reviewer would consider it most important that the authors i.) characterize the vascular phenotype upon uncl-Sema3E treatment in some more detail and ii.) that they pursue at least some combination therapies with chemotherapeutic regimen.

Specific comments:

1. The authors claim that tumor cell produced uncl-Sema3E competes with processed p61 for the binding to PlexinD1. In order to confirm that the overexpression of uncl-Sema3E in different tumor cell lines does not interfere with endogenous Sema3E production, secretion and processing, the authors should include Western blots of secreted Sema3E in conditioned media of tumor cells as well as cell lysates.

2. Is there a correlation between endothelial cell apoptosis and uncl-Sema3E overexpression in MDA-MB-435 tumors as in the RipTag2 model? If yes, which molecular mechanism would explain uncl-Sema3E-induced apoptosis?

3. The authors should provide information about the fraction of uncleaved and cleaved Sema3E in the circulation of mice treated with Sema3E-wt cDNA constructs.

4. How does uncl-Sema3E act on endogenous p61-Sema3E? Just by blocking the binding to PlexinD1 or dimerization of p61 and uncl-Sema3E?

5. Does uncl-Sema3E affect EMT of tumor cells?

6. The vessel phenotype needs to be analyzed mechanistically in greater detail. The cellular endothelial cell experiments are barely state-of-the-art. If endothelial cells act as the vascular target of Sema3E, what is the authors' explanation for the observed pericyte dropout phenotype? This issue is mechanistically among the most critical aspects of the manuscript: The pericyte phenotype and the accumulation of hypoxia argue that uncl-Sema3E is not leading to vessel normalization. In turn, the buildup of intratumoral hypoxia may be among the most important invasion promoting microenvironmental factors. Thus, if uncl-Sema3E negatively impacts distant metastasis, why has it no effect on local invasion of tumor cells in lymph node and liver (Fig. 6E)?

7. Related to above comment: Based on a more detailed analysis of the anti-vascular effect, how would uncl-Sema3E combine with chemotherapy? Admittedly, such experiments would involve significant additional experimentation. Yet, given the moderately low mechanistic advance of the manuscript (in light of above mentioned previous publications on WT and p61 Sema3E), such additional experiments would strongly strengthen the manuscript to make its translational ambition more compatible with the state-of-the-art of contemporary clinical use of combined anti-angiogenic and anti-tumorigenic therapy.

8. What are the molecular effects of uncl-Sema3E on the well-known signaling pathways (GTPases, Arf, integrins) in endothelial cells?

9. The authors are encourage to study in some detail eventual adverse effects of the preclinical use of uncl-Sema3E. Most notably, is there any effect on coagulation or blood pressure upon long term treatment of mice?

Minor comments:

1. Please include experimental confirmation that furin cannot cleave uncl-Sema3E.

2. Fig2 E/F: The quality of the lung images should be improved.

3. Page 6: 4T1, 168-FARN were selected for their high endogenous levels of Sema3E. Protein data of Sema3E should be included in the supplemental data

4. Figure 3A: The quality of the picture is poor.

5. Figure 3B: It is suggested to include life-cell imaging to show that uncl-Sema3E induces cell plasticity changes. Alternatively, a lower magnification image could be included.

6. Does uncl-Sema3 inhibit VEGF/FGF induced sprouting angiogenesis?

7. Spelling mistake in Fig 9A.

8. Figure 6E: Error bars are missing.

Referee #3:

Casazza and co-workers report on the use of an uncleavable form of Semaphorin 3E (uncl-Sema3E) to compete with the cleaved form of Sema3E (p61) for the binding to Plexin D1/ErbB2 and thereby to repress metastatic dissemination and at the same time to retain Sema3E's ability to repress tumor angiogenesis in a plexin D1-dependent manner. In a comprehensive survey of transplantation and transgenic mouse models of cancer, the authors demonstrate an impressive therapeutic potential of uncl-Sema3E in preventing tumor outgrowth and metastasis formation.
Overall, the manuscript is presented in an impressively concise manner, the experimental approaches are state-of-the-art and have been thoughtfully designed and in most parts are well controlled. Finally, with its careful interpretation of the experimental results, the manuscript provides exciting new insights into an interesting novel approach of anti-cancer therapy.

There are, however, a few points that need clarification:

Page 5, the last sentence of the page seems misleading: Figure 1D does not show an evaluation of ErbB2 tyrosine phosphorylation, rather it displays the analysis of Erk phosphorylation.

Figure 4E is not showing reduced micro vessel density at early stages of tumor development, as stated in the text. Rather it shows blood vessel coverage with smooth muscle cells (rather than "pericytes" which are in most cases SMA-negative. NG2 would be an appropriate marker for pericytes). The figure is mentioned for both purposes.... The data on the first point are not shown anywhere.

Figure 5C: It is unfortunate that p61 has not been included in the experiment shown here. With the negative result obtained, it is even more important to employ a positive control for the demonstration that the experimental conditions have been correct. A historical reference (Casazza et al., 2010) can not serve as a control in an actual experiment. The same remark is true for the experiment shown in Suppl. Figure 6.

Suppl. Figure 4B and Figure 6E need clarification: Uncl-Sema3E is compared to sunitinib in affecting liver metastasis, yet the mock-treated (control) animals have not been analyzed. They should be included.
Moreover, based on the profound effects on primary tumor growth, an appropriate quantification of liver metastasis incidence vs. size vs. metastatic index (metastatic size per primary tumor volume) is required. The difference in the size of metastatic nodules in uncl-Sema3E-treated animals could be due to a repression of tumor angiogenesis and not so much to a repression of tumor cell dissemination. Along these lines, staging and grading of the primary tumors should be assessed to show the effects of uncl-Sema3E (and sunitinib) on tumor cell invasion.

Since the Rip1Tag2 mice have been treated at the end of their life expectancy, it is also important to mention how many mice have died during the treatment, and whether the analyzed mice have been actually selected for surviving mice which in Rip1Tag2 mice correlates with reduced insulin expression = less differentiation = increased tumor cell invasion and metastasis.

Figure 8: The reference to the panels is wrong in the text (LLC and 4T1 are exchanged).

How is sFlk1 increasing metastasis in LLC, when there is no anti-angiogenic effect as determined by a lack of changes in micro vessel density and tumor growth. Is tumor hypoxia still increased in the LLC transplanted, sFlk1-treated mice? Based on the recent findings that anti-angiogenic therapy can increase tumor malignancy, this is an interesting observation that actually could be relevant for the interpretation of the results shown here.

Since the manuscripts touches upon initial toxicity issues of treatment with uncl-Sema3E and various applications in preclinical models, together arguing for a development of this therapy approach for clinical use, it seems appropriate to provide some basic data on tissue distribution and longterm toxicity (as opposed to the acute toxicity shown here), for example in the heart, liver, kidney and endocrine organs.

As a side comment:
Besides its previous publication in a respected journal, one wonders about the actual impact of the hydroporation method on the well-being and experimental behavior of mice. I.v. injection of 2.5 ml of solution within 7 seconds seems a rather severe approach....

1st Revision - Authors' Response 16 November 2011

REVIEWER #1

We are grateful to the Reviewer for stating that our study is *"extensive, properly designed and developed, and significantly contributes to the understanding of Sema3E biology*" as well as "*sheds light on the potential usage of a recombinant mutant molecule as a therapeutic drug to block tumor*

growth, tumor progression and tumor dissemination". Our response to the specific requests for clarification follows below.

1.

Reviewer: The section describing the inhibition of pro-metastatic signaling by uncleaved Sema3E antagonizing mature p61-Sema3E is strongly determined; but *the section describing the anti-angiogenic effects in HUVEC is much weaker. In order to robustly document that the anti-angiogenic function of Uncl-Sema3E mutant is "unaffected"* compared to the processed form of the semaphorin*, a p61-Sema3E control should be used in these experiments.*

We have reported previously that the anti-angiogenic activity of p61-Sema3E fragment is functionally comparable to that of the wild type processable form of the semaphorin (Casazza et al., 2010). Uncl-Sema3E, which we focus on in this study, actually represents a stable "precursor" form of the semaphorin. It was not known previously whether this isoform could have any activity of its own. We show here for the first time that non-processed Sema3E binds PlexinD1 and triggers analogous functional effects in endothelial cells as reported for the proteolytic fragment p61. However, the properties of the two isoforms diverge when their effects on tumor cells are compared.

Following the reviewer's suggestions, we have compared side-by-side in this revised manuscript the effects of Uncl-Sema3E with the effects of p61-Sema3E in all the experiments that were previously included in the old Fig. 3 of the initial submission. These new data indicate that both the uncleaved precursor and the proteolytic mature p61 fragment of Sema3E display similar effects on endothelial cells in-vitro. In fact, they both repel endothelial cells inhibit cell migration (new Fig. 4A), inhibit endothelial tube formation (new Fig. 4B), and sprouting angiogenesis as determined using a 3D in-vitro assay (Laib et al. 2009) (new Fig. 4C).

We have also performed in vivo experiments in order to compare the anti-angiogenic and tumor suppressing activity of Uncl-Sema3E and p61-Sema3E in mice. These results are shown in the revised Figures 5 and 6; they are consistent with our findings in vitro, and indicate that both uncleaved precursor and p61 fragment of Sema3E are similarly endowed of anti-angiogenic activity in vivo. Again, we have added to the experiments shown in the initial version additional analyses of tumor samples. In particular, we have assessed tumor vessel density (Fig. 5D), vessel coverage by $NG2^+$ pericytes (new Fig. 5E), vessel perfusion (with fluorescent Lectin injected in the circulation; new Fig. 5F), and activated-$Caspase3^+$ apoptotic vessels (Fig. 6B); moreover we labeled tumor hypoxic areas with pimonidazole (Fig. 6A). In all of these experiments Uncl-sema3E and p61-Sema3E produced similar effects.

2.

Reviewer: The transgenic pancreatic model study contributes with relevant data to further describe the anti-angiogenic and anti-tumor effects, but does not show any reduction in tumor spread/dissemination and metastasis formation. On the other hand, the studies in 4T1 tumors, A549 tumors and LLC tumors clearly document an anti-tumor effect together with inhibition of metastasis formation. More data is needed to clarify this discrepancy.

The RipTag2 transgenic pancreatic tumor model was selected for its particular relevance to the study of tumor angiogenesis. However, this is not a good model for the study of metastatic dissemination, as a small number of metastases develop to distant organs before the mice die due to hyperinsulinemia and primary tumor burden. For instance, over a large number of previous experiments, we have observed an incidence of approx. 2 cases of liver metastasis for every 10 control untreated mice (analyzed at the 14^{th} week, shortly before death). Upon treatment with anti-angiogenic molecules blocking VEGF signaling, liver metastases have been found to increase in this model, consistent with what we observed in Sunitinib-treated mice in our study. In contrast, mouse treatment with Uncl-Sema3E produced a strong anti-angiogenic and tumor suppressing effect that was not associated with a significant increase in the occurrence of liver metastasis. Prompted by the reviewer, we decided to focus on this particular aspect by revising the analysis of liver metastasis in our experiments. First of all, by extending the number of analyzed cases, we confirmed that the incidence of liver metastasis in untreated control RipTag2 mice is only 10-20%, consistent with previous literature (Paez-Ribes et al., 2009). By analyzing the livers of Uncl-Sema3E treated mice, we found that 2 out of 14 (approx. 14%) and contained small and isolated metastatic foci (see Suppl.

Fig. 10B). Considering the anecdotal finding of metastasis in these mice, in order to assess whether there is any statistically significant difference from untreated controls, we should have performed many more experiments treating RT2 mice with an amount of purified Uncl-Sema3E far beyond our reach. On the contrary, the scenario was dramatically different in Sunitinib-treated mice, which were frequently carrying numerous liver metastasis (5 out of 8 cases, approx. 63%), consistent with previous literature (Paez-Ribes et al., 2009). Notably, the occurrence of distant metastasis by hematogenous dissemination is the main cause of death for cancer patients, therefore avoiding increased metastatic spreading is a major issue in cancer therapy and a potential caveat when treating non-metastatic patients with anti-angiogenic drugs.

Although the RipTag2 mice could not be used as a relevant model to demonstrate the anti-metastatic activity of Uncl-Sema3E in vivo, we however further analyzed RT2 primary tumors by applying recognized histo-pathological criteria to classify them into "encapsulated" non-invasive (IT), microinvasive (IC1) and highly invasive (IC2)(Lopez and Hanahan, 2002; Paez-Ribes et al., 2009) On these bases, we found that, while Sunitinib-treatment strongly induced the invasive phenotype (consistent with previous reports), Uncl-Sema3E significantly reduced tumor invasiveness (Fig. 7E).

We have furthermore assessed the presence of tumor cell foci in peri-pancreatic lymph-nodes, in the different experimental conditions. Islet tumor cells commonly infiltrate peri-pancreatic lymph nodes due to their direct access to lymphatic vessels in the exocrine tissue surrounding endocrine tumors (which are not limited by a basement membrane). Notably, upon Sunitinib treatment, cancer cell spreading to lymph nodes was strongly increased (Suppl. Fig. 10A); in contrast, tumor shrinkage induced by Uncl-Sema3E was not associated with increased tumor dissemination through lymphatics (Suppl. Fig. 10A). In sum, although RipTag2 mice are not an ideal model to study the metastatic process, we found that the treatment with Uncl-Sema3E coupled a strong tumor-suppressing activity with a block of local invasiveness and of the tendency to form distant metastasis.

Notably, in order to further address the question raised by the Reviewer, we have also performed new experiments to assess the inhibitory effect of Uncl-Sema3E during the vessel extravasation step in the metastatic process, which we have previously shown to be crucially controlled by p61-Sema3E (Casazza et al, 2010). In new Fig. 2E, we show that Uncl-Sema3E strongly reduces the ability of highly aggressive carcinoma cells to extravasate from the circulation and form initial metastatic seeds in the lungs, displaying a symmetrical opposite effect to p61. Notably, we show that Uncl-Sema3E overexpression has no effect on cancer cell viability (Suppl. Fig. 3A-B); moreover the assay above focuses on an early event (48 hours after tumor cell dissemination) which precedes any angiogenic switch associated with the growth of metastatic foci. Thus, these data further indicate the ability of Uncl-Sema3E to directly interfere with Sema3E-driven cancer cell migration, extravasation and metastatic spreading in mice.

3.

Reviewer: The data shown for 3 days in culture seems to show a relevant tendency to decrease cell growth in Uncl-Sema3E. This should be evaluated for longer time. Moreover, similar analysis should be done for all other tumor models (RIP-Tag2, 4T1, A549 and LLC) (maybe only as supplementary data).

It should be underlined that the slight difference in cell growth noticed by the Reviewer in Fig. 3A of the initial submission was not statistically significant. However, following reviewer's suggestion, we have now extended our analysis, not only for MDA-MB435, but also including A549 and 4T1 cancer cells overexpressing Uncl-Sema3E. The experiments were performed in serum deprivation conditions (0.5% FBS), in order to reveal even a weak inhibitory activity, and the analysis was extended for at least 5 days of growth in culture (Fig. 5A and Suppl. Fig. 3A-B). In addition, we stained multiple tumor samples with Ki67 marker to evaluate cancer cell proliferation in vivo, in the presence of Uncl-Sema3E (Fig. 6C and Suppl. Fig. 9A). In all the above experiments, Uncl-Sema3E did not directly affect cancer cell proliferation, consistent with our hypothesis that the paracrine activity in endothelial cells leading to reduced angiogenesis in vivo is likely responsible for the tumor suppressing activity of Uncl-Sema3E in mice. Notably, these data are consistent with our previous findings concerning other Sema3E isoforms (Casazza et al., 2010).

4.

The reviewer criticized our statement that '*Uncl-Sema3E is effective even in anti-VEGF resistant tumors'*, since *'the term "resistance" is often misused in the field to refer to a tumor model that does not respond to these therapies'* while *'the proper term for these type of tumors should be "non-responsive" or "refractory"'*.

We are grateful to the reviewer for this recommendation, and we have modified the text accordingly throughout the manuscript. Moreover, we thank the reviewer for pointing out that investigating the potential effects of a combined treatment of anti-VEGF together with Uncl-Sema3E, although intriguing, is out of the scope of this study.

- As a minor comment, the reviewer criticized the quality of graphs shown in Fig. 8C and 8F of the initial manuscript. We apologize for this problem, due to unexpected conversion problems into the PDF format of those Excel-generated graphs. This has now been solved in the new revised Figure 9 (including these data).
- The reviewer asked for clarification concerning the use of the expression "intervention trial" in page 8 and 11. We found that the term "intervention trial" is used in biomedical research to indicate a protocol aimed at testing experimentally whether a certain medical intervention or drug administration (our case) is effectively blocking or improving an ongoing pathological state. This is opposed to a "prevention trial", e.g. pre-treating with Uncl-Sema3E before tumor transplantation. Such terms are frequently applied in studies using the RipTag2 model, thus we felt that their use was also appropriate in our case. We have however modified the wording in the two statements on page 8 and 11.
- We have moreover amended manuscript text in a few other aspects, as suggested by the reviewer.

Cited References

Casazza et al. (2010) J Clin Invest 120(8):2684-98.
Laib et al. (2009) Nat Protoc. 4(8):1202-15.
Lopez and Hanahan (2002) Cancer Cell 1(4):339-53.
Pàez-Ribes (2009) Cancer Cell 15(3):220-231.

REVIEWER #2

We thank the reviewer for acknowledging *'the beauty of the present study'*. The reviewer also asked to '*dig deeper to shed additional mechanistic insights into the observed phenomena (most notably: analysis of vascular phenotype)',* as well as he/she encouraged to include further preclinical experiments, such as combination therapy approaches. Answers to the specific comments follow below.

1.

Reviewer: In order to confirm that the overexpression of uncl-Sema3E in different tumor cell lines does not interfere with endogenous Sema3E production, secretion and processing, the authors should include Western blots of secreted Sema3E in conditioned media of tumor cells as well as cell lysates.

Detecting endogenous Sema3E protein by Western blotting is a challenging task because no commercial antibody is satisfactorily performing in this application. We have used in the past a

proficient polyclonal antiserum produced by the group of Dr. Lukanidin, but this not available any longer, and several subsequent efforts to produce analogous tools have not been very successful. In order to address reviewer's request, we focused on 4T1 cancer cells which display the highest endogenous levels of Sema3E (almost totally converted into p61 fragment, see Casazza et al 2010). We collected the conditioned medium of control cells and cells overexpressing uncl-Sema3E (the same used in our experiments shown in Fig. 1 and 2). Conditioned media (CM) were spin-filter concentrated 1000-times and subjected to immunoblotting analysis by applying all available antibodies on the market. In order to include a specificity control, we analyzed in parallel the (equally concentrated) conditioned medium of 4T1 cells subjected to Sema3E knock down by RNAi (that we described previously in Casazza et al. 2010). As shown in new Suppl. Fig. 2A, by using a new polyclonal anti-Sema3E antibody produced by R&D (cat. AF3239), a specific band of the expected molecular weight was detected at comparable levels in the medium of EV-control and Uncl-Sema3E overexpressing cells, whereas it was absent in Sema3E-depleted cells. Notably, the expression of endogenous Sema3E in cell lysates was below our detection capability (not shown), likely because it was impossible to concentrate or immunopurify these samples before analysis. Thus our data suggest that Uncl-Sema3E overexpression is not impairing p61 production and release in the conditioned medium of tumor cells. Notably, further experiments requested by the Reviewer ruled out that Uncl-Sema3E may associate in a complex with p61 inside the cells or in solution (see Suppl. Fig. 2B-C).

2.

Reviewer: Which molecular mechanism would explain uncl-Sema3E-induced endothelial cell apoptosis?

We have previously reported an increased apoptotic index in blood vessels of tumors overexpressing p61-Sema3E (Casazza et al., 2010), moreover other semaphorins have been implicated in apoptosis. In particular, we and others showed that Sema3A can elicit endothelial cell apoptosis in vitro and in vivo (Guttmann-Raviv et al., 2007; Maione et al., 2009; Reidy et al, 2009; Casazza et al., 2011). Notably, several studies have shown that Sema3A-dependent inhibition of endothelial cells in vitro and angiogenesis in vivo depends on a negative regulation of integrin-mediated cell-substrate adhesion. Moreover, a recent study demonstrated that Sema3E can trigger integrin-beta1 turnover, inhibiting endothelial cell-substrate adhesion (Sakurai et al., 2009). It is well known that endothelial cells are strictly dependent on these consensus signals mediated by the extracellular matrix for adhesion, migration and survival (Cheresh and Stupack, 2008). We have therefore analyzed the regulation of integrin-dependent signaling in endothelial cells in response to Uncl-Sema3E, and found that five minutes after stimulation, integrin-β1 activation is reduced and focal adhesions are disassembled in HUVEC (Fig. 3B-C). This is accompanied by the loss of actin stress fibers (Fig. 3B), and by major cytoskeletal rearrangements known as the semaphorin-dependent "collapsing" effect (Fig. 3A)(see Barberis et al., 2004). Consistent with the inhibition of integrin-mediated adhesion, we found that the tyrosine phosphorylation of focal adhesion kinase (FAK) was decreased in response to Uncl-Sema3E (Fig. 3D). Integrin-dependent FAK activation is known to upregulate MAPK signaling, and it was previously shown that this pathway is crucially required to sustain growth and survival of endothelial cells (e.g. see Eliceiri et al., 1998; Lu and Rounds, 2011). Notably, we found that Uncl-Sema3E rapidly decreased MAPK phosphorylation in endothelial cells (new Fig. 3D) and induced the activation of Caspase-3 (new Fig. 3E-F), a major mediator of cellular apoptosis that we also found activated in vessels of Uncl-Sema3E treated tumors in mice (Fig. 6B and Suppl. Fig. 9C). Consistent with what observed for p61 (Casazza et al., 2010), Uncl-Sema3E did not trigger any of these "inhibitory" effects in the cancer cells analyzed in our study (not shown); this is likely explained by differences in the signaling receptor complex, including the adaptor molecule Rnd2, between endothelial and cancer cells (Casazza et al., 2010).

3.

The reviewer asks to provide information about the fraction of uncleaved and cleaved Sema3E isoforms in the circulating blood of mice treated with Sema3E-wt cDNA constructs. Unfortunately, it is not currently possible to acquire this kind of information due to technical issues. In fact, the ELISA assay (from R&D Systems) that we used to specifically measure Sema3E levels in the blood does not discriminate between the two isoforms (p87 and p61); nor we could manage to apply any of

the available commercial antibodies for detecting Sema3E in raw mouse sera by western blotting; moreover, these antibodies do not work in immunoprecipitation experiments.

Notably, previous studies in cancer cells demonstrated that wild type Sema3E is readily converted into the pro-metastatic p61 fragment by furin-like proteases (Christensen et al., 2005; Casazza et al., 2010). New data shown in this revised version (Fig. 4, 5 and 6) demonstrate that both unprocessed Uncl-Sema3E and the p61-Sema3E fragment are similarly capable to inhibit endothelial cell migration, tubular morphogenesis in vitro and angiogenesis in vivo. These findings suggest that the commonly observed proteolytic cleavage of Sema3E is not implicated in regulating the anti-angiogenic activity of this secreted semaphorin, while instead it unleashes its pro-metastatic activity.

4.

Reviewer: How does uncl-Sema3E act on endogenous p61-Sema3E? Just by blocking the binding to PlexinD1 or dimerization of p61 and uncl-Sema3E?

We have demonstrated in Figure 2A-B that uncl-Sema3E is competent for binding PlexinD1, and it competes with p61 inhibiting receptor binding by 50% when added at equal molar concentration. We have moreover demonstrated in Fig. 1C-D that uncl-Sema3E is unable to induce PlexinD1 association with ErbB2, as well as its tyrosine phosphorylation and downstream signaling pathway. This is likely explained by the fact that uncl-Sema3E retains a bulky 25kDa Ig-like domain (not required for PlexinD1 binding) that is removed by proteolytic processing.

Following the reviewer's suggestion, we now tested the possibility that Uncl-Sema3E may block p61 activity by physically coupling with it in solution. Results of pull down experiments shown in Suppl. Fig. 2B-C seem to rule out this hypothesis, since p61 did not co-purify with Uncl-Sema3E from the conditioned media of cells co-expressing both Sema3E isoforms; moreover, the two purified molecules did not specifically associate in solution. Thus our data are consistent with an antagonistic role of Uncl-Sema3E on p61 pro-metastatic signaling due to competition for PlexinD1 receptor binding and ErbB2 transactivation.

5.

Reviewer: Does uncl-Sema3E affect EMT of tumor cells?

We have carefully investigated this issue by assessing the level of Epitelial-Mesenchymal Transition markers (such as E-cadherin, N-cadherin and vimentin) in the Uncl-Sema3E overexpressing cells that revealed reduced metastatic behavior compared to controls. However, neither A459, nor 4T1 cells displayed any significant change of EMT markers upon Uncl-Sema3E overexpression (Suppl. Fig. 2C-E). Moreover, we tested the effect of treating control cells with exogenous purified Uncl-Sema3E and could detect no significant change in EMT markers, either in protein or mRNA analyses (Suppl. Fig. 2C-E); instead, our positive control TGFbeta was very effective in modifying EMT markers in A549 cells, associated with striking morphological changes that were never observed in response to uncl-Sema3E (not shown). Notably, 4T1 cells are well known for carrying high expression of transcription factors driving EMT (such as Twist); however, Uncl-3E had no effect on these parameters, suggesting that its anti-metastatic activity is not due to a change in EMT regulation. We are aware of a recent report suggesting that Sema3E expression correlates with EMT in ovarian carcinoma cells; however, when we analyzed Sema3E-depleted 4T1 cells (by RNAi technology) that we previously showed to become much less metastatic (Casazza et al, 2010), we did not observe any change in the levels of EMT markers (not shown). In sum, we cannot find any evidence that Uncl-Sema3E is controlling EMT of cancer cells, while we show that it antagonizes ErbB2 activation by endogenous p61-Sema3E, a major pathway driving invasion and metastasis.

6.

Reviewer: The vessel phenotype needs to be analyzed mechanistically in greater detail.

Sema3E is an established factor regulating angiogenesis. Similar to other secreted semaphorins, it exerts a repelling activity on endothelial cells. Its role in developmental

angiogenesis is complex and relatively well characterized in different systems and animal models (Tamagnone and Mazzone, 2011). We have shown previously that cancer cells release Sema3E at levels which are not significantly effective in regulating tumor angiogenesis (Casazza et al, 2010). However, Sema3E overexpression in experimental tumor models strongly inhibited tumor angiogenesis (Casazza et al, 2010), recapitulating the developmental function of this molecule. In this study, we demonstrated that uncl-Sema3E is comparable to other Sema3E isofoms for its anti-angiogenic activity in vitro and in vivo. The vascular phenotype associated with uncl-Sema3E stimulation is due to inhibition of endothelial cell adhesion to the extracellular matrix (Fig. 3B-C and Suppl. Fig. 4 and 5), associated with reduced intracellular signaling and increased cellular apoptosis (Fig. 3D-F) and reduced migration in 2D and 3D morphogenetic assays (Fig. 4A-C); moreover, it is characterized by reduced vascular density and reduced functionality (perfusion) in vivo (e.g. Fig. 5D-F, Fig. 7B-C and Suppl. Fig. 6). Notably, uncl-Sema3E treated tumor vessels contain apoptotic cells and lack normal pericyte coverage. Vessel mural cell recruitment is driven by endothelial cells and it is likely to be deficient in apoptotic vessels. Moreover, following reviewer's suggestions, we investigated a potential direct role of Uncl-Sema3E in the regulation of pericytes recruitment. As the reviewer states, elucidating this issue is complicated by the lack of suitable pericyte cell lines to perform mechanistic experiments in vitro. However, by studying primary human perictyes derived from retina, we found that they express PlexinD1 (although at lower levels compared to endothelial cells) and observed that uncl-Sema3E can act as a repelling factor for these cells in vitro in PlexinD1-dependent manner (Suppl. Fig. 7B), potentially consistent with a direct effect on pericytes in tumor vessels in vivo. Moreover, we found that endothelial cells exposed to Uncl-Sema3E downregulated their production of PDGF-B (Suppl. Fig. 7A), which is a major factor recruiting mural cells to vessels (see Jain and Booth, 2003; von Tell et al, 2006). Thus, in addition to its inhibitor effect on endothelial cell adhesion, migration and survival, Uncl-Sema3E might interfere with pericyte recruitment to vessels resulting in vessel destabilization.

7.

Reviewer: how would uncl-Sema3E combine with chemotherapy?

We would like to defer this specific request for further complex pre-clinical experiments with uncl-Sema3E. The reviewer explains his/her request with the need to compensate for the "moderately low mechanistic advance" of our study. Actually, we would like to better underscore the novelty of our study, especially in view of new data included in this revised version. In fact, previous studies had reported that Sema3E is subject to proteolytic cleavage, releasing the fully active fragment p61 (Christensen et al, 2005; Casazza et al, 2010), and it was not known whether the uncleaved precursor had any activity of its own. Its functional characterization is therefore the first major contribution of this manuscript. Moreover, we demonstrated that uncl-Sema3E can block the pro-metastatic activity of p61-Sema3E while retaining its anti-angiogenic function. We have therefore described a how a single molecule can concomitantly block two important mechanisms sustaining tumor progression. On one hand, uncl-Sema3E efficiently blocks angiogenesis, because it is capable to bind and activate PlexinD1 in endothelial cells. Moreover, uncl-Sema3E can block the pro-metastatic activity of processed Sema3E (p61), which is the reason why otherwise it would not be safe to apply wild type Sema3E to treat cancer. In fact, we have shown previously that Sema3E is mostly converted to p61 in tumors (Casazza et al, 2010), likely because furin-like pro-convertases are commonly up-regulated in cancer cells and their expression was positively correlated with invasiveness and metastasis (Bassi et al, 2005). Thus, while cancer cells may convert a larger fraction of precursor-Sema3E into pro-metastatic p61, the treatment with uncl-Sema3E should tilt the balance in the opposite direction by competing for receptor binding. Importantly, we show that uncl-Sema3E is as effective as VEGF-targeted drugs in inhibiting tumor growth but, unlike anti-VEGF treatments, does not fuel metastasis. Moreover, our data indicate that the antiangiogenic activity of uncl-Sema3E is not simply due to an inhibition of VEGF-signaling, since it can effectively suppresses the growth of tumors refractory to VEGF-blocking drugs. The mechanistic explanation based on our experimental evidence is that uncl-Sema3E impairs integrin-based endothelial cells adhesion to the extracellular matrix, cell migration and cel survival. Thus, we do not agree that the manuscript only provides a "moderately low mechanistic advance".

The Reviewer asks to try in vivo experiments to test the effects of combining chemotherapeutics with uncl-Sema3E. However, we showed that uncl-Sema3E inhibits tumor development in several stringent pre-clinical models -very effectively- by itself, and that by i.p. injection it inhibits the development of preformed tumors by about 60%. Addition of chemotherapy

makes sense if the primary effect is small, which is not the case here. Because instead uncl-Sema3E can very efficiently achieve the dual effect of inhibiting tumor angiogenesis and tumor growth, as well as metastatic progression, as a single molecule, we are not convinced of the relevance of mixing up its activity with that of other molecules; neither VEGF-blocking drugs (which do not seem to be more effective and may allow cancer cells to escape from primary tumor and metastasize); nor with classical chemotherapy, which might be additive to uncl-Sema3E in reducing tumor growth and is more likely to produce toxic effects. For these reasons, we feel that chemotherapy combination experiments are out of the scope of this manuscript, which is the description of a new type of dual-activity molecule potentially that is potentially applicable to block cancer progression. and we ask the reviewer to assess our revised manuscript beyond this specific issue.

8.

Reviewer: What are the molecular effects of uncl-Sema3E on the well-known signaling pathways in endothelial cells?

The intracellular signaling pathway triggered by Sema3E in endothelial cells, responsible for the inhibition of migration and angiogenesis, is partly understood. We have previously demonstrated that Rnd2 binding to PlexinD1 and subsequent R-Ras inactivation are crucial events in this pathway (Casazza et al. 2010). Moreover, a recent publication underlined the role of Arf6 activation in endothelial cells to induce integrin turnover and functional inactivation, in response to Sema3E (Sakurai et al., 2009). Based on the data shown in this revised version of the manuscript, we can conclude that uncl-Sema3E is not different from other Sema3E isoforms inasmuch it binds to PlexinD1 receptor (Fig. 2A-B), it requires Rnd2 expression (Suppl. Fig. 5B) and it triggers integrin-inhibitory signals in endothelial cells (Fig. 3B-C and Suppl. Fig. 4 and 5A). In particular, in response to Uncl-Sema3E, focal adhesions are disassembled in endothelial cells, FAK phosphorylation is decreased (Fig. 3D), as well as it is the activation of the downstream MAPK pathway (Fig. 3D). The latter finding is entirely novel and particularly relevant, since it typically associates with the anoikis of endothelial cells, which can explain the direct pro-apoptotic effect of uncl-Sema3E observed in our study (Fig. 3E-F).

9.

The reviewer encourages *to study in some detail eventual adverse effects of the preclinical use of uncl-Sema3E.*

We found that the systemic treatment of mice with Uncl-Sema3E does not affect blood counts, or functional and liver parameters, nor it interferes with the growth of newborn mice (Suppl. Fig. 13A-B). Moreover, now we show that that its local or systemic delivery does not significantly affect liver or kidney histology (Suppl. Fig. 12). These experiments reflected the effects of uncl-Sema3E delivery for up to 25 days. Notably, the goal of our study was to provide proof of principle that a modified semaphorin can be the prototype of new molecular tools concomitantly interfering with tumor growth and metastatic progression. Thus, we feel that the request for additional "long-term" cytotoxicity experiments, assessing with statistical significance the incidence of any potential adverse effects is out of the scope of the present study, and more suited for the preclinical validation of a new drug developed by a pharmaceutical company. Moreover, similar experiments would necessarily require large amounts of purified protein, and we do not have the manpower and resources for large scale production and purification of hundreds of milligrams of Uncl-Sema3E that would be required for such long-term experiments on a sufficient number of mice. For these reasons, we are presently unable to fully address the reviewer's requests on this issue.

<u>Answers to additional minor comments:</u>

1. The reviewer asked for evidence that Uncl-Sema3E recombinant protein is actually uncleavable by proteolytic enzymes. It should be said that Uncl-Sema3E construct was first reported by Christensen et al. (2005) and it was then demonstrated that it does not undergo proteolytical cleavage by furins, unlike the wildtype protein. In our study, we have also used an Fc-conjugated

Uncl-Sema3E construct that behaves in the same manner, as documented in Figure R2 at the end of the comments for this Reviewer.

2. The reviewer asked for better quality macroscopic images of lungs with metastatic foci (for Fig. 2F). It is actually difficult to choose small representative images that at the same time show the full picture of the lungs and the detail of metastatic foci, which often have different sizes and tend to confluence in highly metastatic samples. However, in Fig. 2F, now we show two enlarged and better resolution macroscopic lung images (we could show more and larger images into Supplemental material, if relevant). Moreover, we show in revised Fig. 2E representative images of new experiments with fluorescent-labeled cancer cells colonizing the lungs as early as 48 hours after injection in the circulation (also in this case, we could show wide fields in into Suppl. material, if required).

3. We have discussed above the difficulty to detect endogenous Sema3E levels by Western blotting. Moreover, unfortunately, a batch of polyclonal antibodies produced by the group of E. Lukanidin and used in the past to perform this expression analysis (Christensen et al, 2005), is not any more available. However, in response to Reviewer's request, we could confirm by Quantitative-PCR analysis that Sema3E expression levels in 168-FARN carcinoma cells are over 10000-folds lower compared to those seen in 4T1. Moreover, Sema3E expression levels for the different cell lines used in this study are shown in revised Suppl. Fig. 1.

4. The Reviewer criticized the quality of microscopic images of COS cells "collapsing" in response to Uncl-Sema3E (old Fig. 3A). The presented phenotype is that typically seen in cells undergoing semaphorin-induced collapsing response, the morphological and mechanistic details of which have been previously discussed (Barberis et al., 2004). Here we purely intended to report the fact that uncl-Sema3E is fully active in a classical collapsing assay in COS cells overexpressing the receptor PlexinD1. Cell morphology details cannot be appreciated in these images, because the immunostaining was done for VSV epitope tag, to highlight the cells actually transfected with PlexinD1. However, since we now provide in revised Fig 3A better documentation of the collapsing response induced by uncl-Sema3E in HUVEC (expressing endogenous PlexinD1), and demonstrate in detail the morphological and molecular changes of integrin-based adhesion in the same cells (in main Fig. 3B and Suppl. Figures 4 and 5), we propone to remove this old figure depicting data obtained in transfected COS cells, which is actually pleonastic for the present study.

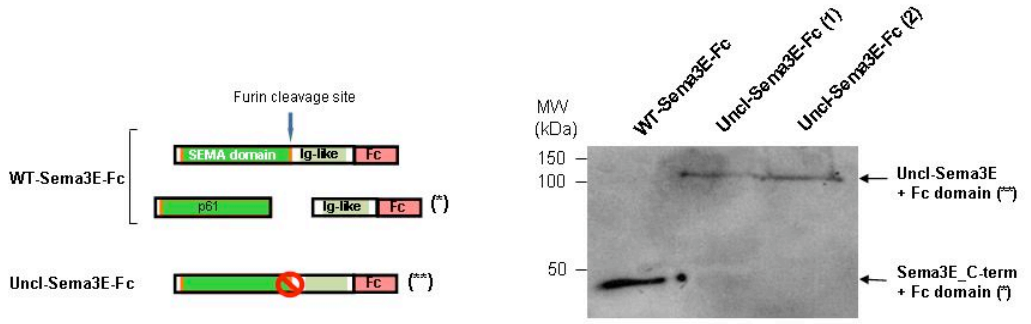
5. The reviewer asked for providing further documentation of the morphological changes induced by uncl-Sema3E in endothelial cells. In the revised manuscript, now we show several additional data addressing this issue. In Figure 3A, we show a better imaging of the collapsed phenotype in HUVEC. We also provide two time-lapse movies illustrating the collapsing process in endothelial cells (Suppl. Movie 1A-C). Moreover, new Figure 3B-C and Suppl. Figures 4 and 5 provide details on the mechanisms responsible for this process, impinging on integrin function regulation.

6. The reviewer asked for testing whether uncl-Sema3E inhibits sprouting angiogenesis induced by known pro-angiogenic factors. We thus performed endothelial cell sprouting assays in 3D in the presence of bFGF (Laib et al. 2009) and found that both uncl-Sema3E and p61-Sema3E can block this process (Fig. 4C). Sema3E-receptor PlexinD1 is not known to directly cross-talk with FGF-Receptors; therefore we assume that this effect may be explained by interference between downstream signaling cascades triggered in endothelial cells. Moreover, our in vivo experiments on LLc tumors that are refractory to VEGF-blockade, indicate that the anti-angiogenic activity of uncl-Sema3E is not due to interference with a specific pro-angiogenic pathway, but rather is explained by a direct inhibitor activity on endothelial cells, impinging on substrate adhesion, migration and survival.

7. We corrected the spelling mistake in Fig. 9A

8. We have modified Fig. 6 in the revised manuscript and shown new data and histological images concerning metastatic foci in RT2 mice in Suppl. Fig. 10.

Figure R2 *(Figure for Reviewer 2)*



A recombinant cDNA encoding wild type (processable) Sema3E was fused to Fc domain at C-terminal (WT-Sema3E-Fc). A point-mutated Sema3E lacking the specific proteolytic site targeted by furins was analogously fused with Fc (Uncl-Sema3E-Fc). A schematic drawing (on the right) depicts the structure of these constructs.
The two expression constructs were transfected in HEK-293 cells and conditioned media were immunoblotted with an anti-Fc antibody, capable to detect the C-terminal tails of the endoded molecules (image on the left). Our results indicate that wild type Sema3E-Fc (first lane on the left) is entirely cleaved by convertases; thus, we could not detect residual full length molecules, but only a small band corresponding to C-term Ig-like domain of Sema3E + Fc domain (indicated by *). Uncl-Sema3E-Fc instead (note that two PAGE lanes were loaded) remains uncleaved, with an apparent molecular weight summing up the size of full-lenhgt Sema3E precursor (87 kDa) and Fc domain (band indicated by **).

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REVIEWER #3

We are grateful to the reviewer for the very positive appreciation of our work and of its perspective implications for cancer therapy, stating that *'the experimental approaches are state-of-the-art and have been thoughtfully designed'*. Our response to the specific requests for clarification follows below.

** Reviewer:* On *Page 5, the last sentence of the page seems misleading*.

Thanks for pinpointing this typo; in fact we should have referred to Figure 1C and not 1D. We corrected the sentence.

** Reviewer: Figure 4E is not showing reduced micro vessel density at early stages of tumor development, as stated in the text. Rather it shows blood vessel coverage with smooth muscle cells (rather than "pericytes" which are in most cases SMA-negative. NG2 would be an appropriate marker for pericytes).*

We thank the reviewer for this comment and for suggestions to address experimentally this issue. In our revised paper, we have stained with anti-NG2 antibodies tumor sections derived from both experiments with MDA-MB435 cells (shown in new Fig. 5E) and RT2 mice (see Fig. 7C). Consistent with our previous data concerning αSMA marker of smooth muscle cells (now shown in Supplemental Figures 6B and 9B), these experiments confirmed the reduced coverage of tumor vessels with pericytes in presence of Uncl-Sema3E. In Suppl. Fig. 6, we actually show two distinct graphs with the quantification of vessel density (panel A) and αSMA^{+} vessel coverage (panel B) assessed in the same tumor samples excised at early stage of development.

During this revision, we furthermore investigated a potential direct role of Uncl-Sema3E in the regulation of pericytes recruitment to vessels. Elucidating this issue is actually complicated by the lack of suitable pericyte cell lines to perform mechanistic experiments in vitro. However, by studying primary human perictyes derived from retina, we found that they express PlexinD1 (although at lower levels compared to endothelial cells) and observed that uncl-Sema3E can act as a repelling factor for these cells in vitro in PlexinD1-dependent manner (Suppl. Fig. 7B), potentially consistent with a direct effect on pericytes in tumor vessels in vivo. Moreover, we found that endothelial cells exposed to Uncl-Sema3E downregulated their production of PDGF-B (Suppl. Fig. 7A), which is a major factor recruiting mural cells to vessels (see Jain and Booth, 2003; von Tell et al, 2006). Thus, in addition to its inhibitor effect on endothelial cell adhesion, migration and survival, Uncl-Sema3E might interfere with pericyte recruitment to vessels resulting in vessel destabilization.

** Reviewer: Figure 5C: It is unfortunate that p61 has not been included in the experiment shown here.*

As suggested by the reviewer, now we show in new revised Figures 5 and 6 several experiments in mice comparing the functional impact of Uncl-3E and p61-Sema3E in tumor growth, tumor angiogenesis, tumor hypoxia, lung metastasis, etc. Our data indicate that both uncleaved Sema3E precursor and p61 fragment are similarly endowed of anti-angiogenic and tumor suppressing activity in vivo. These results are consistent with our analysis of endothelial cells in

vitro (shown in new revised Figure 4), demonstrating that both Uncl-Sema3E and p61 are similarly endowed of endothelial repelling activity in vitro. On the contrary, the effect of the two Sema3E isoforms on metastatic spreading is totally different. This can be appreciated in weakly metastatic MDA-MB435 cells (shown in new Fig. 6C), which express low endogenous levels of Sema3E. Furthermore, since p61 induces metastasis in multiple tumor models (Casazza et al., 2010), we do not think that it should be considered a relevant tool for cancer therapy. This conclusion is further supported by experiments shown in new Fig. 8, which comparatively analyze in vivo the tumor suppressing activity of systemic Uncl-Sema3E or wild type furin-processable Sema3E (known to be readily converted into p61).

** Reviewer: Suppl. Figure 4B and Figure 6E need clarification: Uncl-Sema3E is compared to sunitinib in affecting liver metastasis, yet the mock-treated (control) animals have not been analyzed. They should be included.*

These data are now shown in Suppl. Fig. 10B. See the point below for additional comments on these results.

** Reviewer: an appropriate quantification of liver metastasis incidence vs. size vs. metastatic index (metastatic size per primary tumor volume) is required. The difference in the size of metastatic nodules in uncl-Sema3E-treated animals could be due to a repression of tumor angiogenesis and not so much to a repression of tumor cell dissemination. Along these lines, staging and grading of the primary tumors should be assessed to show the effects of uncl-Sema3E (and sunitinib) on tumor cell invasion.*

The issue raised by the Reviewer is quite important, since our claim is that Uncl-Sema3E not only inhibits tumor angiogenesis and tumor growth, but also restricts cancer cell migration and metastatic spreading. Notably, the RipTag2 transgenic pancreatic tumor model was selected for its particular relevance in the study of tumor angiogenesis; however, it is not a good model to study metastatic dissemination, as a small number of metastases develop to distant organs before the mice die, due to hyperinsulinemia and primary tumor burden. For instance, over a large number of previous experiments, we have observed an incidence of approx. 2 cases of liver metastasis for every 10 control untreated mice (analyzed at the 14^{th} week, shortly before death). Upon treatment with anti-angiogenic molecules blocking VEGF signaling, liver metastases have been found to increase in this model, consistent with what we observed in Sunitinib-treated mice in our study. In contrast, mouse treatment with Uncl-Sema3E produced a strong anti-angiogenic and tumor suppressing effect that was not associated with a significant increase in the occurrence of liver metastasis. Prompted by the reviewer, we decided to focus on this particular aspect by revising the analysis of liver metastasis in our experiments. First of all, by extending the number of analyzed cases, we confirmed that the incidence of liver metastasis in untreated control RipTag2 mice is only 10-20%, consistent with previous literature (Paez-Ribes et al., 2009). By analyzing the livers of Uncl-Sema3E treated mice, we found that 2 out of 14 (approx. 14%) and contained small and isolated metastatic foci (see Suppl. Fig. 10B). Considering the anecdotal finding of metastasis in these mice, in order to assess whether there is any statistically significant difference from untreated controls, we should have performed many more experiments treating RT2 mice with an amount of purified Uncl-Sema3E far beyond our reach. On the contrary, the scenario was dramatically different in Sunitinib-treated mice, which were frequently carrying numerous liver metastasis (5 out of 8 cases, approx. 63%), consistent with previous literature (Paez-Ribes et al., 2009). Notably, the occurrence of distant metastasis by hematogenous dissemination is the main cause of death for cancer patients, therefore avoiding increased metastatic spreading is a major issue in cancer therapy and a potential caveat when treating non-metastatic patients with anti-angiogenic drugs.

Although the RipTag2 mice could not be used as a relevant model to demonstrate the anti-metastatic activity of Uncl-Sema3E in vivo, we however further analyzed RT2 primary tumors by applying recognized histo-pathological criteria to classify them into “encapsulated” non-invasive (IT), microinvasive (IC1) and highly invasive (IC2)(Lopez and Hanahan, 2002; Paez-Ribes et al., 2009) On these bases, we found that, while Sunitinib-treatment strongly induced the invasive phenotype (consistent with previous reports), Uncl-Sema3E significantly reduced tumor invasiveness (Fig. 7E).

When comparing the number of metastatic foci with the size of the primary tumor, we observed that both are reduced upon treatment with Uncl-Sema3E in multiple experimental tumor models in vivo. Although this may suggest that fewer metastasis are simply explained by a smaller primary tumor, as discussed above, several studies have demonstrated that tumor shrinkage achieved by anti-angiogenic drugs may lead to increased invasion and metastatic spreading. This adverse effect is commonly explained by hypoxia-driven changes in cancer cell behavior or defective vessel structure facilitating tumor spreading. In our study, we demonstrated that Uncl-Sema3E does affect tumor vessel structure and causes tissue hypoxia, which is likely responsible for cellular apoptosis and tumor shrinkage. However, hypoxic and apoptotic tumors do not become more invasive and metastatic in the presence of Uncl-Sema3E, consistent with the fact that this molecule can independently inhibit both cancer cells and endothelial cells. A further element against the idea that the anti-metastatic effect displayed by Uncl-Sema3E may be simply explained by its anti-angiogenic activity is provided by our functional analysis in vivo in comparison with p61-Sema3E. In fact, our data indicate that the consistent anti-angiogenic activity observed for both isoforms is independent from their antagonistic regulation of cancer cell invasiveness and metastasis.

Importantly, in order to further address the question raised by the Reviewer, we have also performed new experiments to assess the inhibitory effect of Uncl-Sema3E during the vessel extravasation step in the metastatic process, which we have previously shown to be crucially controlled by p61-Sema3E (Casazza et al, 2010). In new Fig. 2E, we show that Uncl-Sema3E strongly reduces the ability of highly aggressive carcinoma cells to extravasate from the circulation and form initial metastatic seeds in the lungs, displaying a symmetrical opposite effect to p61. Notably, we show that Uncl-Sema3E overexpression has no effect on cancer cell viability (Suppl. Fig. 3A-B); moreover the assay above focuses on an early event (48 hours after tumor cell dissemination) which precedes any angiogenic switch associated with the growth of metastatic foci. Thus, these data further indicate the ability of Uncl-Sema3E to directly interfere with Sema3E-driven cancer cell migration, extravasation and metastatic spreading in mice.

Reviewer: Since the Rip1Tag2 mice have been treated at the end of their life expectancy, it is also important to mention how many mice have died during the treatment, and whether the analyzed mice have been actually selected for surviving mice which in Rip1Tag2 mice correlates with reduced insulin expression = less differentiation = increased tumor cell invasion and metastasis.

We performed our trials in RIP-Tag2 mice between 12-14 weeks of age, aimed to assess the effect of Uncl-Sema3E on regressing or blocking cancer progression in tumor-bearing mice, and in impairing angiogenesis. In this time window, RipTag2 mice are not yet at the very end of their life, as they start to die (mainly due to hypoglycemia) abruptly during week 15 (Bergers et al., 2003; Pietras and Hahahan, 2005). Consistently, in our trials, all the mice lived till 14 weeks of age (including untreated ones). This allowed an accurate measurement of the effect of specific molecules on tumor burden, tumor vasculature and tumor invasiveness after two weeks of continuous treatment. Of course, survival is not a measurable outcome in the same kind of trials. Therefore in our treatment groups, while we detected significant differences in tumor progression and angiogenesis, we did not observe any differences in mortality, and none of the treated or untreated RipTag2 mice died before sacrifice at the end of the trial.

Reviewer: Figure 8: The reference to the panels is wrong in the text (LLC and 4T1 are exchanged). How is sFlk1 increasing metastasis in LLC, when there is no anti-angiogenic effect as determined by a lack of changes in micro vessel density and tumor growth. Is tumor hypoxia still increased in the LLC transplanted, sFlk1-treated mice? Based on the recent findings that anti-angiogenic therapy can increase tumor malignancy, this is an interesting observation that actually could be relevant for the interpretation of the results shown here.

We thank the reviewer for highlighting this mistake in the main text referring to figure panels. This is now corrected.

As previously shown by Shojaei and Ferrara (2007), anti-VEGF therapy does not affect vessel number in LLC tumors and thereby does not result in tumor shrinkage, a condition indicated as intrinsic “resistance” by Bergers and Hanahan (2008). On the other hand, consistent with our data, the treatment with sFlk1 was reported to induce more metastasis in other tumor models in mice (Ebos et al., 2009; Pàez-Ribes et al, 2009). In order to address this issue, we decided to investigate

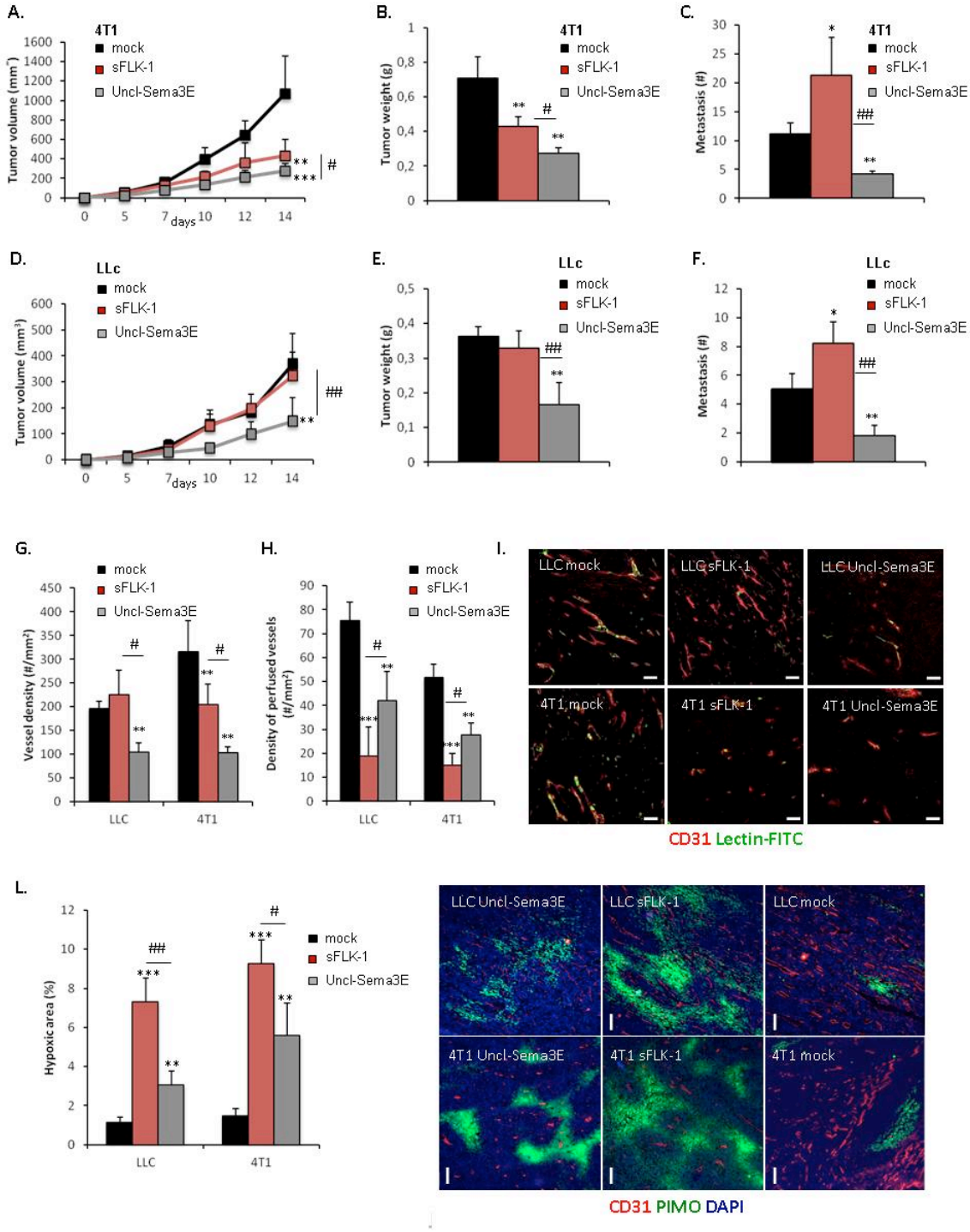
tumor vessel perfusion and tissue hypoxia in tumors treated with sFlk1 and Uncl-Sema3E by performing a new experiment in vivo and inject the mice with Lectin-FITC and Pimonidazole hypoxiprobe before sacrifice. The results of this new experiment are shown in Figure R3 for the Reviewer. Notably, primary tumor growth and metastatic dissemination in the different conditions replicated the results of our previous similar experiments (now shown in main Fig. 9). However, we found that while tumor vessel density was not decreased in sFlk1-refractory LLC tumors, their perfusion was greatly reduced, resulting in a strong increase of tissue hypoxia. This reduced perfusion is probably the consequence of altered vessel architecture, since several vessel-containing areas are in fact hypoxic. The increased hypoxic state is compatible with the observed induction of cancer cell invasion and metastasis reported in previous studies. Moreover, it was shown that a qualitative difference in vessel structure and functionality, affecting tissue oxygenation even in presence of normal vessel density, may influence the metastatic behavior without affecting primary tumor growth (Mazzone et al, 2009; Hamzah et al, 2008). Notably, tumors treated with Uncl-Sema3E also displayed deficient tissue oxygenation (consistent with our results in other tumor models, see Fig. 6A and 7D), and yet the metastatic spreading was not increased, consistent with the described inhibitory effect of Uncl-Sema3E on cancer cell migration. Since the topic of our study was the activity of Uncl-Sema3E, while sFlk1 was included in these experiments merely as reference to a validated anti-angiogenic factor, we feel that showing a detailed analysis of the sFlk1 induced vascular phenotype is out of the scope of the present manuscript. However, we are open to include this figure in the supplemental data, if required.

** Reviewer: Since the manuscripts touches upon initial toxicity issues of treatment with uncl-Sema3E and various applications in preclinical models, together arguing for a development of this therapy approach for clinical use, it seems appropriate to provide some basic data on tissue distribution and long term toxicity (as opposed to the acute toxicity shown here), for example in the heart, liver, kidney and endocrine organs.*

The goal of our study was to provide proof of principle that a modified semaphorin can be the prototype of new molecular tools concomitantly interfering with tumor growth and metastatic progression. In order to support the relevance of our findings in perspective to a potential clinical application, we have ruled out major adverse effect in uncl-Sema3E treated mice by analyzing blood counts, liver and kidney histology and functional parameters (Suppl. Fig. 12 and 13A). Moreover we found that uncl-Sema3E does not interferes with the growth of newborn mice (Suppl. Fig. 13B). These experiments reflected the effect of uncl-Sema3E delivery for up to 25 days. We feel that the request for pharmacokinetic studies and additional "long-term" cytotoxicity experiments is out of the scope of the present study, and more suited for the preclinical validation of a new drug developed by a pharmaceutical company. Moreover, even to provide basic data on these issues, experiments would necessarily require large amounts of purified protein, and we do not have the manpower and resources for large scale production and purification of hundreds of milligrams of Uncl-Sema3E that would be needed to perform long-term experiments with a sufficient number of mice. For these reasons, we are presently unable to fully address reviewer's requests on this specific issue.

In response to the side comment concerning the use of hydrodynamic gene transfer in mice, we would like to underline that this experimental approach is widely used in current gene therapy studies aimed at testing the efficacy of new therapeutic molecules in vivo. About the relevance of data accomplished with this method, we therefore relied on previous literature (e.g. Sawyer et al. 2009; Chen et al. 2009; Hattori et al. 2010; Blain et al. 2010; Hibbitt et al. 2010; Keravala et al. 2011). Notably, in our experiments, we have not observed relevant signs of disease (besides transplanted tumor growth) or tissue toxicity in hydroporated mice (Suppl. Fig. 12).

Figure R3 *(Figure for Reviewer 3)*



The expression of either the validated VEGF-trap molecule sFlk1 or Uncl- Sema3E (or mock control) was achieved in immunodeficient mice by naked cDNA hydroporation (see Methods). Mice treated with either of the two anti-angiogenic molecules were randomized into two experimental groups and transplanted with either 4T1 (VEGF-inhibitor responsive) or LLc (VEGF-inhibitor non-responsive) cancer cells, in analogy to experiments shown in main Fig. 9. Panels A-B and D-E depict tumor growth and endpoint tumor burden in 4T1 and LLc models, respectively (note that the experiment was limited to 14 days). The load of spontaneous lung metastasis is shown in panel C and F, for 4T1 and LLc tumors, respectively. Total vessel density (for both tumor models) is shown in panel G, while density of perfused vessels is shown in panel H (based CD31 staining and

injection of Lectin-FITC in the circulation, see supplemental methods; microscopic images are shown in panel I). Panel L shows detection of hypoxic tumor areas (as revealed by pimonidazole injection). Asterisks indicate statistical significance versus respective mock controls; *p< 0.05; **p< 0.005; ***p< 0.0005.

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2nd Editorial Decision 30 December 2011

Thank you for the submission of your revised manuscript "Tumor growth inhibition and anti-metastatic activity of a mutated furin-resistant Semaphorin 3E isoform" to EMBO Molecular Medicine. We have now received the report from the reviewer who was asked to re-review your manuscript.

You will be glad to see that the reviewer is globally supportive and we can proceed with official acceptance of your manuscript pending the minor editorial changes detailed below.

Please see below for information regarding EMBO Molecular Medicine guidelines for statistical analysis of data. Please mention the actual p value in each case.

In addition, we noted that your point-by-point response contains a figure. Since this would be published in the Review Process File, please let us know whether you agree with its publication, if you would like to delete the figure or whether you would like to opt out (more information below).

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

Statistical analysis
The description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05').

Yours sincerely,

Editor
EMBO Molecular Medicine

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

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

Many independent lines of evidence from different cellular models and mouse models convincingly support the conclusions drawn by the authors.

Referee #3 (Other Remarks):

This reviewer congratulates the authors on this nice piece of work, in particular also on the detailed revision of the manuscript. Substantial experimental data has been added and the manuscript has been revised adequately to respond to all of the reviewers' comments.

2nd Revision - Authors' Response 12 December 2011

We are pleased to resubmit a further revised and edited version of our manuscript entitled “Tumor growth inhibition and anti-metastatic activity of a mutated furin-resistant Semaphorin 3E isoform”, according to editorial recommendations for publication in EMBO Molecular Medicine journal.

We have indicated within figures the actual P values calculated by unpaired Studentís t-test (as now specified in Methods section). In a few cases, following your suggestion, in order to avoid producing too crammed figures, we used symbols explained in figure legend. We have moreover indicated in figure legends the number of independent samples analyzed per each experimental condition (not replicate measures of the same sample). As indicated in Methods, all the graphs display mean values ±SD (represented by error bars).

Our paper should now fulfill all the requirements for publication in EMBO Molecular Medicine; we thus look forward to your reply, and thank you in advance for your consideration.