Author's response to reviews

Title: TrkA is amplified in malignant melanoma patients and induces an anti-proliferative response in cell lines

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Author's response to reviews: see over
Dear Dr. Krisha Mae Natan,

Thank you very much for your kindness and the reviewers for their instructive advices and very detailed revision of the manuscript. We have responded point by point to the reviewers’ comments and revised the manuscript, as evidenced by a red track in Microsoft Word. Where possible, we have tried to satisfy all the requirements. We have modified the figures, as suggested, and repeated/ performed the experiments solicited by the reviewers. The results of the new experiments requested are enclosed either as main figures or as supplementary information in the “Additional file 2”; all the corrections we have added to the text are marked in red.

Unfortunately, the amount of clinical samples was not sufficient to attain further analysis of immunohistochemistry or RT-PCR to verify the expression of our gene. Hence, it is not possible to fulfill this specific request. We have then performed an additional bioinformatic analysis of public datasets, as suggested by the reviewers, to substantiate our hypothesis (included as supplementary information), although we only found partial confirmation, possibly because of the general low expression level of this gene across melanoma cell lines and tumor samples.

We believe we have answered to the great majority of the concerns raised by the reviewers and we hope that the revised manuscript would be acceptable for publication.

Yours sincerely,

Luigi Pasini and Alessandro Quattrone
Reviewers’ Comments to Authors:

Reviewer: Dr. Ken Dutton-Regester

Reviewer’s report:

Pasini and colleagues present a report on the amplification of 1q23.1 in melanoma through the use of array CGH and find an association with its amplification and an increase in primary tumor thickness. Within this region lies a candidate hotspot gene of NTRK1 (TrkA) which with following experimental analysis, was found to be mutated in 50% of primary melanomas. Following this, a series of experiments are presented to elucidate a role of TrkA in melanoma via the MAPK and AKT pathways and the authors suggest the data indicates a putative role in both neoplastic transformation and oncogene-induced senescence. This study has decent body of work and raises some interesting future experiments to further elucidate the role of TrkA in transformation and different genetic backgrounds, however, there are a couple of issues that need addressing and further clarification.

Minor Essential Revisions:

Remark:
Line 108-110 Introduction: The authors state the “low resolution of the methodologies employed” in previous studies is a contributor to the lack of detection of novel candidate alterations. I would be cautious in using this statement as multiple previous studies have used higher resolution arrays in melanoma that what was used in this study. For example, Gast 2010 GCC; 49(8):733-45 and Dutton-Regester 2012 GCC; 51(5):452-61. I would suggest revising this statement to reflect more accurately the literature.

Reply:

We thank the reviewer for this remark. Indeed we agree that the statement could sound quite misleading. We were in fact referring to the notion that the majority of high-resolution studies, as the two mentioned by the reviewer, are based on melanoma cells lines (mostly derived from metastatic tumors) or include only a small cohort of primary clinical samples. On the contrary, our study is mainly intended to emphasize the alterations occurring specifically in primary melanomas.

We have refined the statement in the introduction by including the two important studies underlined by the reviewer and marked the sentence red as follows, Line 108-112:

However, the discovery of specific driver genes and the accurate profiling of genomic mutations and CNAs in MM have been mainly based on MM cell lines derived from metastatic samples [16,17] or have included a restricted cohort of clinical primary tumors [18], limiting the detection of novel candidate alterations that may originate in the primary MM.

Remark:
Methods, line 154 “Genome profiling of clinical samples”. Although the details of the genome profiling method is referenced, it would be useful to know what the exact array was used without having to look it up in the original reference. Suggest including a quick sentence in the methods to include this info.

Reply:

The array information has been added in the Methods section, Line 158-167:
In brief, the array CGH was performed using the Agilent 8x60K human CGH oligo microarray chip (Agilent Technologies, Santa Clara, CA; 021924 SurePrint G3 Human CGH 8x60K Microarray, cat. G4450A), mapped to the human genome (USCS genome browser Human, Feb. 2009, GRCh37/hg19). The scanned microarray TIFF images were acquired with the Agilent DNA Microarray Scanner G2505C, by the manufacturer’s software (Agilent ScanControl 8.1.3), and analyzed using the Agilent Feature Extraction Software version 10.7.7.1. The analysis of raw aCGH data was then conducted via the R environment for statistical computing (http://www.r-project.org/) using packages provided by the Bioconductor library (http://www.bioconductor.org/).

Remark:
Table S1, Age at diagnosis and Breslow thickness headings are accidently reversed and needs to be fixed.

Reply:
Thanks for the accurate notification. The mistake has been corrected.

Remark:
Some of the cell morphology figures are not clear (at least in this version under review). In particular, Figure 3a/b and 4a/b is not high enough resolution to clearly see the morphology changes. This could be just a problem in the review article, but the authors should make sure it’s clear in the final version.

Reply:
We apologize for that. We presume that the problem has been caused by an incorrect uploading of the figures in the PDF version of the review article. We have improved the quality of the images and uploaded the figures with the maximal resolution possible.

Remark:
Figure S5 and Figure 4, NFG is labeled wrong (should be NGF) in NFG only treated boxes.

Reply:
Thanks again for the very detailed revision. We have corrected the mistake in the figures.

Remark:
Line 519 correction: Gens > genes.

Reply:
We have corrected the mistake, marked in red in the text, Line 578.

Remark:
Line 627 correction, “might have be adopted” to “might have been adopted”

Reply:
The entire sentence has been reformulated to simplify the discussion.

Remark:
In the discussion there should be quick mention that there is the possibility that another gene within the minimal common region could be responsible for melanoma oncogenesis.

Reply:
We have included in the discussion a brief mention (marked in red as follows) about the importance of considering other candidate oncogenes encompassed by the minimal amplification, emphasizing however the relevance of TrkA within this genomic region based on its cellular function and previous literature, Line 638-646: In accordance with these previous histological data, the importance of NGF signaling in melanocyte biology [7], and its proved involvement in oncogenic pathways [4,5], TrkA gene seems the most promising candidate for driving segmental amplification of the 1q23.1 region in MM, although we
cannot exclude the possibility that the other genes (INSRR, PEAR1, LRRC71, MIR765, ARHGEF11, ETV3L, ETV3) within the 1q23.1 minimal common amplification could also participate in melanomagenesis. However, we did not find any relevant associations between the expression of these genes and MM clinical attributes when querying the public resource cBioPortal (data not reported).

**Major Compulsory Revisions:**

**Remark:**
I am curious to whether there was enough material on the samples used on the arrays to perform expression analysis (either using RT-PCR with RNA or Immunohistochemistry using the FFPE blocks) to see if there is a correlation between amplification and expression of the gene?

**Reply:**
We agree with the reviewer on this very important point. Unfortunately, it is not achievable for us to satisfy this requirement because when we tried to analyze the mRNA levels of TrkA it was not possible to extract an adequate amount of RNA to perform any qPCR analysis on the material left. As well, there is no possibility to assess the expression of TrkA by immunohistochemistry on the tissue samples.

**Remark:**
It’s clear from the small number of cell lines used in this study that expression is not highly expressed. Have the authors considered using existing resources to look into this question in more detail, specifically the CCLE database for melanoma cell lines and probably more important, accessing the TCGA data? The CBioPortal can quickly present some interesting analyses with the TCGA data in respect to correlation of the levels of mRNA and copy number status of TrkA …

**Reply:**
We thank the reviewer for these useful suggestions. We took advantage of the information available in CCLE and TCGA to integrate our results. The additional observations derived from these external resources were integrated in the body of the results of the revised article (red labeled) and in the revised Supplementary Information (Additional file 2: Figure S2).

Mining the compilation of chromosomal copy number and mRNA data for the TrkA gene, mRNA levels were confirmed to be quite low, while genomic amplification resulted to be a relatively frequent event, both across CCLE cell lines and across TCGA tumor samples (Figure S2A and Tables S5 and S6). These observations were consistent independently of the different experimental platforms used (Affymetrix SNP6.0 arrays and Affymetrix U133 plus 2.0 array in CCLE, DNA-seq and RNA-seq in TCGA).

We also explored the correlation between the log2 copy numbers and the mRNA levels of the TrkA gene. On the basis of the data in the CCLE database, TrkA mRNA levels do not increase in association to genomic gain and amplification in a statistically significant way, indicating that TrkA expression remains low regardless of the genomic status (Figure S2C). Similarly, the correlation between TrkA RNA-seq data and the corresponding copy number values did not have statistical significance (Figure S2D).

**Remark:**
… and also in regards to clinical attributes including staging of the tumor. For example, total copy number (or mRNA expression) can be correlated with the staging of the primary tumor in the melanoma TCGA - one might even hypothesize based on the authors conclusions in this paper that expression of TrkA might
be higher in earlier staged primary melanomas with TrkA amplification (as opposed to later stage primary melanomas with TrkA amplification).

**Reply:**

*We tested, as suggested, the association of TrkA DNA copy numbers and mRNA levels with the clinical attributes of melanoma tumor samples. To this aim, we mined the TCGA report for the pre-determined analysis on the association between copy number variation events in region 1q23.1, which hosts the TrkA gene, and clinical attributes. We did not find a statistically significant correlation with any clinical feature (Q-value < 0.30). When mining the association between TrkA log2 mRNA levels with clinical attribute, again no association was statistically significant (Q-value < 0.30). Nevertheless, the set of cases with CNA, point mutations or mRNA level alterations tend to have the worse prognosis (median month survival of 35.91) compared to cases without TrkA alterations (median month survival of 65.87), although the difference is not statistically significant (Additional file 2: Figure S2B). As suggested by the reviewer, visualization of TrkA mRNA levels and DNA copy number by tumor stage was extracted from the cBioPortal and reported in “Additional file 2: Figure S2E”. As already mentioned before, TrkA mRNA levels are overall very low, close to the detection limit, therefore testing the association between expression levels and tumor stage is challenging. For this reason, we cannot exclude nor support the hypothesis that TrkA mRNA levels are higher in the initial tumor stages.**

*These supplementary analysis have been indicated in the revised manuscript results, Line 430-438:*

We tried to substantiate our hypothesis through the analysis of public resources. By looking at The Cancer Genome Atlas (TCGA) data available through the cBioPortal (http://www.cbioportal.org/index.do; ref. [30,31]), the TrkA gene is recurrently altered (14% of 278 reported tumor samples with RNA-seq and CNA data) in MM, via amplification, mRNA level upregulation, and missense mutations (Additional file 2: Figure S2A). Cases with alterations tend to have the worse prognosis (median month survival of 35.91) compared to cases without TrkA alterations (median month survival of 65.87), although the difference is not statistically significant (Additional file 2: Figure S2B).

**And Line 449-462:**

To confirm this finding we surveyed the data available at the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE, http://www.broadinstitute.org/ccle/home) and found that the log2 mRNA levels of TrkA are indeed quite low (median log2 = 3.8; CI: 3.8 - 4.0), although a fraction of these cells lines show gain or amplification of the TrkA locus (Additional file 1: Table S5). This observation brought to the hypothesis that the contribution of TrkA overexpression (acquired through genomic gain) to the initial progression of the primary tumor might be negatively selected afterwards (by down-regulating gene expression), as it is reflected in our cell lines derived from advanced MMs. Analysis of CCLE cell line data revealed that TrkA mRNA levels and genomic amplification are indeed not correlating (Additional file 2: Figure S2C; Spearman r = 0.080). As well, we were not able to detect any significant correlation between TrkA mRNA and copy number levels in tumor samples collected by TCGA at the cBioPortal (Additional file 2: Figure S2D; Spearman r = 0.086) and listed in the Additional file 1: Table S6.

**Legend of Figure S2 was also modified, Line 1144-1164:**

*Additional file 2: Figure S2. Bioinformatic analysis of TrkA mRNA expression and copy number in public database of MM cell lines and tumor samples. Graphical depiction of mRNA expression and copy number data for NTRK1 gene, as downloaded from public available resources of CCLE cell lines (http://www.broadinstitute.org/ccle/home) and TCGA tumor samples (http://www.cbioportal.org/index.do). A, Frequency of TrkA alterations in MM across tumor samples (n = 278) collected at the cBioPortal, Skin Cutaneous Melanoma (TCGA, Provisional); TrkA is*
altered in 39 (14%) of 278 patients/cases (Case Set: All Complete Tumors: All tumor MM samples that have mRNA, CNA and sequencing data of 278 patients / 278 samples). B, Kaplan–Meier curves of overall survival for patients with TrkA alterations (n = 30) and without TrkA alterations (n = 166), as reported at the cBioPortal (log-rank test: p = 0.225). C, genomic copy number and mRNA expression data for NTRK1 in human MM cell lines (n = 61) were extracted from the CCLE_Expression_Entrez_2012-10-18.res : Gene-centric RMA-normalized mRNA expression data and the CCLE_copynumber_2013-12-03.seg.txt :Segmented copy-number profiles in the .seg file format. (hg19), respectively, and used to evaluate the correlation with TrkA amplification (Spearman r = 0.080). D, correlation analysis of genomic copy number and mRNA expression data for NTRK1 in human MM tumor samples (n = 278) from cBioPortal (Spearman r = 0.086). E, Association of TrkA mRNA expression and copy number status with tumor stages, as classified at the cBioPortal (Clinical Attributes) for MM samples (n = 278), based on AJCC staging criteria.

Furthermore, as suggested by the other reviewer, we have slightly modified the discussion section in the revised manuscript to prevent confusion in the interpretation of our study as well as to dampen excessive speculation, Line 695-700:

Further experiments would be required to substantiate this hypothesis. However, when we investigated public datasets we did not find any significant difference between TrkA mRNA expression in primary MMs versus metastatic MMs (data not shown). As well, expression of TrkA does not substantially differ in earlier compared to later MM stages (Additional file 2: Figure S2E).

Reviewer: Gary Edward Gallick

Reviewer’s report:

The manuscript of Pasini et al. demonstrates complex roles of TrkA in melanoma. In an extension of the group’s previous CGH work, they demonstrate amplification of 1Q23.1 as a common locus in malignant melanoma from patients, corresponding with TrkA amplification. The frequency of this event (~50%) is a novel observation and corresponds with tumor thickness and earlier onset of metastasis. Surprisingly, however, no statistical significance in survival was observed, a concern given the cell line work that follows. Although TrkA was found to be amplified in some cell lines, it was not expressed. An inducible TrkA was a sound strategy to determine the effects of its activity in vitro, but the growth inhibitory effect of NGF is in seeming contrast to the apparently metastasis-promoting affect of gene amplification in human specimens. The different relationship of Erk and Akt to growth regulation adds to the manuscript. Thus, there are several interesting, though difficult to explain, findings in the manuscript. There are some concerns, including:

Major compulsory revisions:

Remark:

The survival data are very important and need to be shown in the “main” figures.

Reply:

According to the suggestion, we moved the survival data from Additional file 2: Figure S2 to the main body of the paper and renamed it Figure 2E; we also modified the legend of Figure 2 accordingly by marking the sentence in red as follows, Line 1012-1015:

E, Kaplan–Meier curves of overall survival for patients with TrkA amplification (n = 32) or diploid TrkA (n = 12), as detected by qPCR on primary MM genome (n.s., not statistically significant by log-rank test). Dipl, diploid copy number; Amp, amplification.
Remark:
Similarly, the activation of Erk and Akt pathways should be in the primary figures.

Reply:
We moved the data, showing the activation of ERK and AKT upon 15 min with NGF, from “Additional file 2: Figure S4” to the main body of the paper and renamed it “Figure 4”; we indicated in red text this modification in the Results section and in the Figure legend. Consequently, we modified the order of the main figures in the text (marked in red) and figure legend as follows, Line 1033-1041:

Figure 4. Activation of MAPK and AKT downstream to NGF-TrkA signaling in MM cells. SK-MEL-28 and G-361 cells, stably transduced with doxycycline-inducible TrkA-vector or empty vector were incubated for 48 h with vehicle (DMSO) or doxycycline (500 ng/ml) in FBS-free medium. Cell extracts were collected at 15 min post-stimulation with vehicle or NGF (100 ng/ml) and subjected to Western blotting using the indicated antibodies. Anti-β-tubulin was used as loading control for TrkA and ERK; anti-GAPDH was used as loading control for AKT. The protein markers in kDa are estimated from the molecular weight standard. Images are representative of \( n = 3 \) experiments. Dox, doxycycline; E, empty vector.

Remark:
The dose-dependence of NGF in eliciting Erk and Akt activation should be assessed.

Reply:
We evaluated the NGF dose-response on ERK and AKT activation by Western blot in melanoma cells expressing TrkA, upon pretreatment with or without doxycycline, and quantified the effect as the average of two separate experiments. The result is now reported in the “Additional file 2: Figure S4” along with the respective figure legend (highlighted in red text). Also, the associated experimental details were introduced in the Methods (marked in red), Line 246-249:

To test the activation of NGF-TrkA downstream pathways, cells were treated with 100 ng/ml β-NGF (#PHG0126; Life Technology) for 15 min in FBS-free medium. A dose-response curve was measured by incubating the cells for 15 min in FBS-free medium with 6.25, 12.5, 25, 50, and 100 ng/ml β-NGF. We revised the main text and marked the sentence red as follows, Line 491-495:

This observation indicated that both ERK and AKT were downstream kinases to NGF-TrkA signaling in MM cells, although ERK showed a sustained basal state of endogenous phosphorylation, while in a dose-response assay (Additional file 2: Figure S4) AKT phosphorylation seemed to be more dependent on NGF-TrkA activation.

Legend of Figure S4 was also modified, Line 1185-1195:

Additional file 2: Figure S4. Dose-response activation of AKT and MAPK following stimulation of NGF-TrkA signaling in MM cell lines. SK-MEL-28 and G-361 cells, stably transduced with doxycycline-inducible TrkA-vector were incubated for 48 h with vehicle (DMSO) or doxycycline (500 ng/ml) in 2% FBS medium. Cell extracts were collected at 15 min post-stimulation with vehicle or serial doses of NGF (dose-response curve was created using a 1:2 dilution scheme, starting at 100 ng/ml) and subjected to Western blotting using the indicated antibodies. The protein markers in kDa are estimated from the molecular weight standard. The quantification of signal intensity of phospho-TrkA, phospho-AKT, and phospho-ERK was normalized over vehicle control cells in the absence of doxycycline (set to 1). Data are expressed as mean \( \pm \) SD of \( n = 2 \) independent experiments. Dox, doxycycline.

Remark:
The cell line data are confusing, with TrkA amplified but not expressed. With this concern in mind, it would be very important to show expression (e.g. by IHC) in at least some of the human samples in which TrkA is amplified.
Reply:
We agree with the reviewer on the importance of this point, and on the necessity of evaluating if there is a correlation between amplification and expression of TrkA. Unfortunately, it was not possible for us to perform any further analysis of expression on TrkA (like IHC on tissue or qPCR on the RNA), since there was not sufficient sample left after the array experiment.
Our hypothesis is that TrkA may exert a double role in melanoma progression: the correlation between amplification of the TrkA locus and primary tumor thickness could indicate a potential pro-oncogenic function in melanoma initiation; while the anti-proliferative mechanism we identified in cell lines suggests that TrkA signaling may be activated as part of a physiological protective feed-back circuit. This may be the reason why advanced-melanoma cell lines suppress the expression of this gene. We tried to make this statement clearer through the body of the revised manuscript (changes are highlighted in red text).

Remark:
The quality of the blots for p21 in Figure 5 is inadequate.  
Reply:
We apologize for the paucity of the staining, due to the poor quality of the antibody that we used. We repeated the Western blots on the same protein lysates by using a new batch of p21 antibody. The experiment was repeated for melanoma cells expressing TrkA and empty-vector controls, in the presence or absence of doxycycline, as previously done. Consequently, we adjusted the corresponding figures (Figure 6 and Figure S9).

Minor Essential Revisions:
Remark:
As the in vitro work is done with NGF, it would be important to assess NGF expression in the clinical samples.
Reply:
As mentioned above, unfortunately it is not possible to retrieve sufficient material from what is left after the aCGH experiment to perform any further expression analysis on the samples.

Remark:
The quality of Figures 3A and 3B are insufficient to assess the morphologic changes the authors describe.
Reply:
We apologize for the low quality of the figure in the review version. We presume that the problem has been caused by an incorrect upload of the figures during the PDF conversion of the manuscript. We improved the images and tried to upload the figures with increased resolution.
Remark:
It is important to quantify the effects of Erk and Akt inhibition in response to NGF, given the complex differential response of inhibiting these signaling enzymes.

Reply:
We included quantitative measurements of cell number along with statistical comparison upon treatment with ERK and AKT inhibitors in response to activated NGF-TrkA signaling or empty-vector control. The quantitation refers to the cell morphology images reported in the previous Figure 4A and 4B (now renamed Figure 5A and 5B) and Figure S5: the same cells have been previously stained with Hoechst 33342 and a portion of labeled nuclei measured by the automated Operetta system, as detailed in the revised “Methods” section (red-traced text), as follow Line 324-331:
The total DNA was stained with Hoechst 33342 (Life Technologies) and used for quantifying the absolute number of cells present in the plate. Quantification of fluorescent cells that incorporated Hoechst 33342, EdU or were stained for caspase-3 was carried out by using the Operetta® High Content Imaging System equipped with the Harmony software (PerkinElmer Inc.). Fractions of EdU labeled cells were calculated based on Hoechst signal. Three independent experiments, with two internal replicates, were performed for each condition.

This additional information is now enclosed as “Additional file 2: Figure S5” and the corresponding results and figure legend have been modified (red-traced text).
We also tried to explain more clearly the concept, as the reviewer can see by red-labeled text corrections, and to summarize the significance of our findings at the conclusion of the corresponding result section, Line 558-565:
When NGF-TrkA signaling is activated, MM cells enter a rapid proliferative arrest (without induction of apoptosis). However, the downstream AKT pathway is mainly required to sustain proliferation and survival (inhibiting AKT following NGF-TrkA stimulus accentuates cell loss and growth arrest), while the MAPK pathway may also have divergent functions and mediate an anti-proliferative signaling in response to NFG-TrkA activation (cell viability is improved when MAPK is inhibited following the NGF-TrkA stimulus).

Legend of Figure S5 was also modified, Line 1197-1211:
Additional file 2: Figure S5. Morphological and quantitative analysis of MM cells in response to NGF-TrkA signaling. A, stably transduced MM cells with doxycycline-inducible TrkA-vector (SK-MEL-28-TrkA and G-361-TrkA) were incubated for 48 h in 2% FBS medium with doxycycline (500 ng/ml) and next treated with vehicle (DMSO), MAPK pathway inhibitor U0126 (5 μM), AKT pathway inhibitor LY294002 (25 μM), or the broad range receptor kinase inhibitor CEP-701 (10 μM), in the presence or absence of NGF (100 ng/ml) for 24 h in 2% FBS medium. B, stably transduced MM cells with doxycycline-responsive empty control vector (SK-MEL-28-E and G-361-E) were incubated with doxycycline (500 ng/ml) for 48 h in 2% FBS medium and treated as in A. Images are representative of three independent experiments and were obtained using phase-contrast microscopy from cells growth in 96-well plate at 24 h after treatment. The total cell number was quantified by nuclear staining as described in the methods and quantified by the Operetta High Content Imaging System (mean ± SD of n = 3 independent replicates; Student’s t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001; n.s., not statistically significant). Dox, doxycycline.

Remark:
To this reviewer, the data do not support the possibility that TrkA signaling may be required for the onset of malignant melanoma, but not later, as it does not explain why humans with TrkA amplification develop metastases sooner. One would need to assess expression of TrkA earlier and later in the primary tumor to draw such a conclusion.

Reply:
We understand the reviewer’s concern. We assessed the mRNA levels of TrkA across skin cutaneous melanoma samples within The Cancer Genome Atlas (TCGA), as they
are available through cBioPortal (http://www.cbioportal.org/index.do). Mining TCGA data did not support the hypothesis of a higher TrkA mRNA in earlier melanoma tumors compared to later stages. However, testing the association between quantitative mRNA levels and tumor stage is challenging, because of TrkA barely detectable mRNA levels (Additional file 2: Figure S2E) and the small number of available primary tumor samples in TCGA. For this reason, we cannot exclude nor support the hypothesis that TrkA mRNA levels are higher in the initial tumor stages. The reviewer can follow the modifications we have added to the manuscript by red-tacked text. The results are shown in “Additional file 2: Figure S2” and mentioned in the revised article discussion.

Line 430-438:
We tried to substantiate our hypothesis through the analysis of public resources. By looking at The Cancer Genome Atlas (TCGA) data available through the cBioPortal (http://www.cbioportal.org/index.do; ref. [30,31]), the TrkA gene is recurrently altered (14% of 278 reported tumor samples with RNA-seq and CNA data) in MM, via amplification, mRNA level upregulation, and missense mutations (Additional file 2: Figure S2A). Cases with alterations tend to have the worse prognosis (median month survival of 35.91) compared to cases without TrkA alterations (median month survival of 65.87), although the difference is not statistically significant (Additional file 2: Figure S2B).

Line 449-462:
To confirm this finding we surveyed the data available at the Broad-Novartis Cancer Cell Line Encyclopedia (CCLE, http://www.broadinstitute.org/ccle/home) and found that the log2 mRNA levels of TrkA are indeed quite low (median log2 = 3.8; CI: 3.8 - 4.0), although a fraction of these cell lines show gain or amplification of the TrkA locus (Additional file 1: Table S5). This observation brought to the hypothesis that the contribution of TrkA overexpression (acquired through genomic gain) to the initial progression of the primary tumor might be negatively selected afterwards (by down-regulating gene expression), as it is reflected in our cell lines derived from advanced MMs. Analysis of CCLE cell line data revealed that TrkA mRNA levels and genomic amplification are indeed not correlating (Additional file 2: Figure S2C; Spearman r = 0.080). As well, we were not able to detect any significant correlation between TrkA mRNA and copy number levels in tumor samples collected by TCGA at the cBioPortal (Additional file 2: Figure S2D; Spearman r = 0.086) and listed in the Additional file 1: Table S6.

Legend of Figure S2 was also modified, Line 1144-1164:

Additional file 2: Figure S2. Bioinformatic analysis of TrkA mRNA expression and copy number in public database of MM cell lines and tumor samples. Graphical depiction of mRNA expression and copy number data for NTRK1 gene, as downloaded from public available resources of CCLE cell lines (http://www.broadinstitute.org/ccle/home) and TCGA tumor samples (http://www.cbioportal.org/index.do). A, Frequency of TrkA alterations in MM across tumor samples (n = 278) collected at the cBioPortal, Skin Cutaneous Melanoma (TCGA, Provisional); TrkA is altered in 39 (14%) of 278 patients/cases (Case Set: All Complete Tumors: All tumor MM samples that have mRNA, CNA and sequencing data of 278 patients / 278 samples). B, Kaplan-Meier curves of overall survival for patients with TrkA alterations (n = 30) and without TrkA alterations (n = 166), as reported at the cBioPortal (log-rank test: p = 0.225). C, genomic copy number and mRNA expression data for NTRK1 in human MM cell lines (n = 61) were extracted from the CCLE_Expressio_nEntrez_2012-10-18.res: Gene-centric RMA-normalized mRNA expression data and the CCEL_copynumber_2013-12-03.seg.txt:Segmented copy-number profiles in the .seg file format (hg19), respectively, and used to evaluate the correlation with TrkA amplification (Spearman r = 0.080). D, correlation analysis of genomic copy number and mRNA expression data for NTRK1 in human MM tumor samples (n = 278) from cBioPortal (Spearman r = 0.086). E, Association of TrkA mRNA expression and copy number status with tumor stages, as classified at the cBioPortal (Clinical Attributes) for MM samples (n = 278), based on AJCC staging criteria.
We have also slightly modified the discussion section in the revised manuscript to prevent confusion in the interpretation of our study as well as to dampen excessive speculation, Line 695-700:

Further experiments would be required to substantiate this hypothesis. However, when we investigated public datasets we did not find any significant difference between TrkA mRNA expression in primary MMs versus metastatic MMs (data not shown). As well, expression of TrkA does not substantially differ in earlier compared to later MM stages (Additional file 2: Figure S2E).

Remark:
The discussion is long and overly speculative.

Reply:
We agree with the reviewer. We tried to make it simpler and avoid excessive speculation in the revised version we are proposing. Again, we are aware that the concept of a double oncogenic/antiproliferative mechanism and the hypothesis that TrkA is required for the onset of melanoma while being deleterious once the tumor has become metastatic should be substantiated with solid experimental evidences. However, we think we collected data that may, at least in part, give support to this hypothesis. We exhaustively discussed our results and compared them with previous findings from the literature. We tried to make our statements clearer in the revised manuscript by dampening down speculations. Again, any correction included in the revised discussion is labeled in red.

Remark:
Some attention to grammar is required.

Reply:
We have carefully revised the manuscript and corrected all possible grammatical errors.