Author's response to reviews

Title: Dissecting the signaling pathways associated with the oncogenic activity of MLK3 P252H mutation

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Author's response to reviews:

Reviewer: Dr. Tamara Tanos

Reviewer’s comment 1: What type of cells were used to determine the transforming potential, growth and invasive properties of P252H mutation?

Author’s response: The transforming potential, growth and invasive properties of P252H mutation were addressed in a previous paper from our group. The cell type used was NIH/3T3 cells. These cells are routinely used in in vitro assays to determine the transforming potential of mutant genes. NIH/3T3 transfected cells were also used for the in vivo experiments in which we addressed the tumorigenic and invasive properties of the mutant, wild-type and mock cells. Since NIH/3T3 cells are from mouse origin, in the present manuscript we decided to use a human cell line and we opted by HEK293 cells for the reasons already mentioned in the manuscript.

Reviewer’s comment 2: What is the MAPK status in HEK293?

Author’s response: MLK3 mutational status was tested before selecting HEK293 cells for the study and after transfection in order to make sure that no other MLK3 mutations existed besides the one we had transfected. Regarding BRAF and KRAS activation status, we know from the literature (e.g. Al-Jehani RM et al JNCI 1998; An L et al Scientific Reports 2013; Pópulo H et al PeerJ 2013) that these cells are wild-type for both genes and therefore are frequently used to study the effects of MAPK pathway activation.

Reviewer’s comment 3:

a) Evaluation of candidate MLK3 regulated genes in a panel of gastrointestinal
Author’s response: We totally agree that validation of the results obtained with HEK293 cells in a panel of gastric and colorectal cancer cell lines (as well as in tumor tissue from cancer patients as suggested by the other reviewer) is mandatory. However, since our previous experiments were done in NIH3T3 cells, which do not have MAPK activation, the aim of the present work was to determine the signaling pathways associated with P252H mutation in a similar genetic context (without MAPK activation – the reason why we chose HEK293 cells and not gastrointestinal cells available) but in a human context. The results obtained with this unbiased first approach are the foundation of a new project focusing on validating the targets in a panel of gastric and colorectal cancer cells, and human samples, as well as on studying MLK3 P252H- associated signaling in the presence/absence of KRAS and BRAF mutations.

b) to check protein levels

Author’s response: We tried to detect changes at the protein level for two of the 4 targets selected for validation (CCND1 and FZD10). Changes in CCDN1 protein levels were in accordance with what we observed at the RNA level, down-regulation in P252H- expressing cells in comparison with wild-type and mock cells. FZD10 antibody (SC-33510), although described as suitable for western-blot, in our hands, it did not work. Because we only had interpretable data for one of the targets we decided not to include this information in the manuscript.

c) Functional assays (e.g. invasion assays) with cells transfected with MLK3 constructs.

Author’s response: In vitro invasion assays using our cells expressing MLK3 mutant and wild-type forms would be a strong complement to our results, however, we don’t think that HEK 293 would be the right model to do it. HEK293 cells, although suitable to study the transcriptional and proteomic alterations upon exogenous expression of mutant genes, are not a good model to determine the associated cellular effects since cell behavior induced by activation of a certain signaling pathway is highly cell type dependent. It is important to note that our expression results showed that exogenous expression of mutant MLK3 in a non-gastrointestinal cell line (HEK293) lead to the deregulation of signaling pathways highly associated with gastrointestinal epithelial homeostasis and gastrointestinal cancer development. These findings gave us confidence in using HEK293 cells as a test tube to determine the downstream effectors of mutant MLK3. We did some migration assays with these cells and we observed a trend for increase migratory capacity in the mutant cells. For the reasons above mentioned, we do not feel that determining the cellular effects in HEK293 cells would add a lot to the message we want to pass with our manuscript. We do recognize that this is a weakness of our model (even the expression profile needs to be interpreted with caution) but we believe that determining the cellular
effects will be more valuable if done in gastrointestinal cell lines and after validating the signaling pathways (in the scope of our future project).

Reviewer’s comment 4: The reviewer did not agree with the statement “Our results showed that mutant MLK3 exerts its oncogenic effect by deregulating several important colorectal cancer-associated signaling pathways such as WNT, MAPK, NOTCH, TGF-beta and p53.”

Author’s response: We agree with the reviewer point of view and therefore the sentence was rephrased to: “Our microarray results showed that mutant MLK3 deregulates several important colorectal cancer-associated signaling pathways such as WNT, MAPK, NOTCH, TGF-beta and p53, helping to narrow down the number of potential MLK3 targets responsible for its oncogenic effects”. This change is highlighted in blue in the results section of the abstract.