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Reviewer A

The authors claimed that TKI induced EMT in HCC827 NSCLC cells involved in miR-200/miR-141/ZEB1 or FGFR1 signaling which was validated by CRISPR-Cas9 mediated gene knockout technology. However, there were some concerns existed that make the manuscript is not acceptable at the present form. The major concerns are listed below:

Comment 1A. The manuscript raised a badly confusion for the use between HCC827EMT and MET TKI-R. For example, the authors claimed to use HCC727MET cells for Figure 6C but the figure displayed MET clone. The authors should clearly present the rationale for using these two HCC827 TKI-R clones and the differences between HCC827EMT and MET TKI-R cells.

Reply: We apologize for the confusion. We had in the original submission mislabeled several panels in figure 6 (sections C and D) "MET" instead of the correct "EMT" ! We are embarrassed this type of typo could be unseen in our internal revisions beforehand submission and understand this made the interpretation of the results impossible from the original figure, and accordingly seriously questioning the whole argumentation. We have of course corrected this in the revised version, and at the same time very carefully revised for possible existence of similar typos.

With this said, we have from the basis of the concern from the reviewer in the revised version of the manuscript introduced a now stringent and much more logical naming of TKI-resistance mechanisms. We now systematically name parental HCC827 cells for HCC827PAR and cells that are characterized to have EMT-associated EGFR TKI-resistance (EMT-E-TKI-R) for HCC827EMT. The abbreviation EMT-E-TKI-R is restricted to describe the process of EMT-associated EGFR TKI-resistance. The process of EGFR TKI-resistance in consequence of MET-amplification is abbreviated MET-E-TKI-R. The HCC827 cells we have generated possessing MET-E-TKI-R are named HCC827MET but these have not been experimentally addressed in the manuscript but solely mentioned in the introduction (Jakobsen KR et al. Oncogenesis doi:10.1038/oncsis.2017.17). HCC827GR5 cells have a MET-E-TKI-R phenotype and we have selected to maintain the name HCC827GR5 as it is widely used in the literature and databases from where we have extracted expression data for this particular cell type. The times HCC827GR5 cells are mentioned in the manuscript we have also specified this represents a MET-E-TKI-R cell model. Furthermore, we have throughout the entire manuscript clearly presented rationales for usage of the different cell line models. Finally, a new supplemental figure S1 is included to give an overview of the used HCC827 derived cells in the study.

Changes in the text: Figure 6 has been extensive revised to use the correct naming of HCC827EMT instead of the erroneous MET. Throughout the entire manuscript, we have shifted to use of the nomenclature EMT-E-TKI-R and MET-E-TKI-R where relevant. New supplemental figure S1.

Comment 1B. In addition, the authors described that there were two clones of HCC827EMT (clone 4 and 10). Did they mixed these two clones for whole experiments of this study? The authors should clearly describe it.

Reply to comment 1B. All omics experiments (RNA-seq, DNA methylation array, and H3K36me3-Chip) have addressed HCC827EMT clone 4 and clone 10 as well as two independent biological samples of HCC827PAR, as separate samples to account for biological variance. However, the subsequent pipelines for data visualization and data interpretation were based on the comparison of the collective results from HCC827 clones 4 and 10 relative to the collective results from two HCC827PAR samples. This procedure has now been specified in the revised methods section as well as revised result sections for the various omics approaches. For experiments related to Figure 6 and MIR expression and DNA methylation, HCC827EMT subclone 10 was analyzed. Similar results were obtained with HCC827EMT subclone 4. This information is given in the revised figure legend.

Changes in the text: Revised methods section for RNA-seq (P8 Line 3, P 8 Lines 7-9), DNA-methylation array (P10 line 17-18 and P 11 Lines 1-3), and H3K36me3-ChIP (P 9 line 3 and 21-23 and P10 lines 4-5). Revised results section for RNA-seq (P14 lines 5-12), DNA-methylation array (P15 lines 3-5), and H3K36me3-ChIP (P16 lines 15-16). Revision of figure 6 legend.

Comment 2. For FGFR1 knockout cells, only sgRNA 3 clone displayed a reverse EMT which raised a concern of the off-target effect. Furthermore, knockout of FGFR1 seems not sensitize HCC827EMT cells to erlotinib treatment due to the only significant decreased viability observed in 0.01 microM but not for other concentrations.

Reply: (Please also notice reply to comment 14 from reviewer C.) We agree the presentation of FGFR1 depletion data were very confusion. We have revised to presentation of data to be more clear and stringent relative to what results actually show:

1) given FGFR1 sgRNA 2 only had minimal, if any effect, for FGFR1 protein levels, sgRNA 2 had a resulting gene expression profile differing from FGFR1 sgRNAs 1 and 3, and sgRNA 2 MTS results differing from FGFR1 sgRNAs 1 and 3, we have removed FGFR1 sgRNA 2 from the revised data presentation.

2) We agree the results with sgRNA 3 and sgRNA1 are not exact similar but at least a tendency of reverse EMT is also observed with sgRNA1. The exact observations, in terms of differences and similarities, of results obtained with FGFR1 sgRNAs 1 and 3 are in the revised manuscript much more clearly described and discussed.
3) For the MTS assays we have separated results into two panels (following a request from reviewer C) and as said removed data for sgRNA2. This has clarified the results. In this line, we have accepted the notion from the reviewer concerning erlotinib sensitization and reformatted the text in the results accordingly. Moreover, the revised discussion includes a discussion of these issues.
4) We have in a new supplemental figure S5 included relevant data for FGFR1 sgRNA targeting/off targeting as well as uncropped western blots visualizing the FGFR1 depletion.

Changes in the text: Results section (p24 lines 13-25 and P25lines 1-5), Figure 7 removed data for FGFR1 sgRNA 2. Figure 7 with split of panel D into two graphs. Revised discussion section (P28 lines 15-23). New supplementary figure S5.

Comment 3. It is not clear about the link between the methylation status of miR-200c/miR-141 and the disruption of ZEB1 expression in HCC827EMT TKI-R cells. ZEB1 is a transcriptional factor not a DNA methyltransferase. The authors should clearly describe the rationale for analyzing the changes of DNA methylation status of miR-200c/miR-141 gene locus.

Reply: We agree it was unclear from the original manuscript why this experiment was included in Figure 6E. We have revised the corresponding results section to specify why. Summarized, literature states that *MIR200C-MIR141* precursor locus DNA methylation inversely correlates with expression of the genuine *MIR200C* and *MIR141*, and DNA methylation at the *MIR200C-MIR141* precursor locus is acquired in result of EMT. We also find increased DNA methylation of the *MIR200C-MIR141* precursor locus and decreased *MIR200C* and *MIR141* expression in the HCC827 EMT-E-TKI-R. At the same time, we show that ZEB1 depletion delays progression of EMT following erlotinib treatment. Thus, we addressed the logical question if ZEB1 depletion also impacted EMT-mediated DNA methylation of the *MIR200C-MIR141* precursor locus and therefor the expression of *MIR200C* and *MIR141*. This was indeed observed supporting the link between DNA methylation of the *MIR200C-MIR141* precursor locus, ZEB1 expression status, and EMT.

Changes in the text: Results section is revised (P21 lines 14-18 and P22 lines 1-13).
Introduction P5, line 11.

Reviewer B

This study was aimed to evaluate genome-wide changes in mRNA-expression, DNA-methylation and the histone-modification H3K36me3 in the EGFR-mutated HCC827 NSCLC cell and to examine their function by CRISPR/Cas9-mediated gene depletions. Authors reported that the involvement of the MIR141/MIR200C-ZEB1/ZEB2-FGFR1 signaling axis in EMT TKI-R was functionally verified by CRISPR/Cas9-mediated depletion of the central axis components MIR200C-MIR141, ZEB1, and FGFR1. Multiple studies have reported related information. Therefore, this research does not have impactful findings regarding this topic.

Reply to this notion: Despite the above is not stated as a specific comment from the reviewer we will allow us to reply this point. We can understand the criticism of the reviewer, and agree what some of the described findings, overall speaking, in the original version of the manuscript could appear redundant with existing literature. With this said, we are confident that the presented study in the major revised manuscript version in its majority presents novelty and important extensions to the current knowledge concerning EMT in NSCLC EGFR TKI resistance. We have in the revised manuscript addressed the concerns from the reviewer (see below) and inserted descriptions, which now much better clarifies what, at least to some extent, is already known and what is novel.

We find it important to specify that the existing literature for various aspects of EMT in NSCLC not can be directly translated to the specific type of EMT hereby addressed, namely EMT in NSCLC EGFR TKI-resistance. In the revised manuscript, we exemplify this at several places. Moreover, to the best of our knowledge is the combination of genome wide DNA methylation, H3K36me3, and RNA-seq data for EMT-E-TKI-R not yet existing in the literature (and for H3K36me3 for no EMT models in NSCLC) and accordingly not beforehand interpreted in a systematic comparison of results as here given in the revised manuscript. On top of this we agree that CRISPR/Cas9 have been used to address FGFR1 related to EMT, but again we find that the CRISPR/Cas9 data we here present, including the depletion of *MIR200C* and *MIR141* function by disrupting the seed-sequences, as well as ZEB1 by CRISPR/Cas9, is presented in a novel EMT context going beyond what is already existing in the literature. We are confident this altogether in the revised manuscript will present an important extension as well as substantiation of current knowledge concerning EMT in NSCLC EGFR TKI-resistance

Comment 4. In the abstract, the conclusions do not clearly emphasize the important findings in this study.

Reply: We agree that several parts of the abstract was unclear and vague. We have accordingly extensively revised the abstract.

Changes in the text: entire abstract revised and with in particular focus on the sections

"results" and "conclusions" in the abstract.

Comment 5. The authors describe that depletion of functional MIR200C and MIR141 is sufficient to drive increased expression of mesenchymal markers, and that an EMT marker expression profile as observed in HCC827EMT cells is not mimicked. However, microRNA-200c has already been reported to inhibit epithelial-mesenchymal transition, invasion, and migration of lung cancer by targeting HMGB1 in the previous study. (PLoS One. 2017 Jul 20;12(7):e0180844.) Another study also reported that the miR-200c/LIN28B axis plays an important role in cells with acquired resistance to EGFR-TKI that harbor EMT features and might be a useful therapeutic target for overcoming resistance.(Sci Rep. 2017 Jan 13;7:40847.)
Reply: We acknowledge that both manuscripts are of high importance and have included the references and their most important content in the revised manuscript. We have detailed included the information contributed by these two references for NSCLC EMT.

Changes in the text: P5 lines 24-25 and P6 line 1; P22 lines 5-7: P21 lines 16-18, P22 lines 24-25 and P23 line 1.

Comment 6. The authors describe that Genome-wide H3K36me3-modification changes in EMT TKI-R. However, a previous study has already reported that the suppressive role of SETD2/H3K36me3 in cell proliferation, migration, invasion, and EMT during lung adenocarcinoma carcinogenesis, via regulation of the STAT1– IL- 8 signaling pathway. (Cancer Sci. 2022 Apr;113(4):1195-1207)
Reply 6: We have included the reference in the revised manuscript and addressed the content of the reference in details in the revised manuscript. The reference contributes important information concerning SETD2 and H3K36me3 in NSCLC EMT. However, the reference does not directly address EMT in EGFR TKI-resistance, and the reference does not address genome wide changes in H3K36me3 by ChIP-seq as we present in our manuscript. Finally, in the reference presented observation that H3K36me3 acts to suppress CXCL8 (IL8) in relation to development of EMT could not be re-identified in EMT-E-TKI-R (as also noted in the revised manuscript). Thus, we agree the reference is important by also highlights the importance of public availability of conflicting/supportive/additional data like we present with the revised manuscript.

Changes in the text: P5 lines 15-17, p16 lines 10-12, P17 lines 6-9. P31 lines 16-18.

Comment 7.The authors reported that ZEB1 depletion delays both progression of EMT and MET-activation during TKI-R development. However, a previous study has already reported that ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. (J Clin Invest. 2016 Sep

1;126(9):3219-35)

Reply 7: We have included this very important reference in the revised manuscript and addressed the content of the reference in details in the revised introduction, result section, and discussion. Whereas the reference clearly specifies the importance of ZEB1 for NSCLC EMT, generally speaking, the reference does not specifically address ZEB1 in EMT related to EGFR-TKI-resistance. We find our presented results a natural extension of the results presented in the reference and with importance to further substantiate the function of ZEB1 in NSCLC EMT related to TKI-resistance. Moreover whereas ZEB1 depletion previous was analyzed with siRNS/shRNA the hereby usage of CRISPR/Cas9 methodology represents a logical pathway for extension of current knowledge. This in particular given this allows performing long-term TKI-treatment experiments at ZEB1-depleted conditions as we present in the manuscript.

Changes in the text: P5 lines 24-25, P 6 line 1, P19 lines 22-23, P29 4-7, P31 lines 13-18.

Comment 8. In the page 1 line 33, the authors described that "before and after acquisition of EMT EMT-mediated TKI-R" Is EMT EMT-mediated a typo?

Reply 8: We thanks for the observation and have corrected this type in the revised manuscript.

Changes in the text: abstract P2 line 8.

Reviewer C

The manuscript describes a genomic analysis at mRNA and epigenetic levels of lung cancer cells resistant to a tyrosine kinase inhibitor, which identified the miR141/miR200C-ZEB1/ZEB2-FGFR1 axis as a possible player in the EMT process. Although of importance in the field, the manuscript is not strong when presenting the functional modulation of the targets at the molecular levels, lacking better characterization of the CRISPR KO models and the EMT phenotype per se as the authors remain mostly in mRNA analysis of markers.

Specific comments:

Comment 9. - explain why clone selection wasn't performed after generating ZEB1 CRISPR KO cells.

Reply 9: Our previous CRISPR/Cas9 experience have revealed that among cell clones isolated after gene depletion, phenotypic variation often is present which is not specifically related to the introduced depletion. Since this could reflect "random" phenotypic drift arising from cell outgrowth in the colonies starting from single cells, we have found that usage of cell populations more reproducible will deduce the effect of specific gene depletions. This is of course given that a

high indel/knock-out efficiency is obtained. Given that we observed high indel and knock-out percentages we subsequently analysed cell populations instead of cell clones to minimize phenotype effects arising from outgrowth of colonies from single cells.

Changes in the text: This information is in a summarized format inserted in methods section "CRISPR/Cas9 procedures" (P11 lines 18-24 and P12 lines 1-2)

Comment 10. - sgRNA #3 has some variations compared to the other two. Please explain why there is increased expression of ZEB1 mRNA in P2 (Fig S2C)

Reply 10: We agree on the presence of gene-expression differences between the HCC827Cas9 cells harboring different *ZEB1* sgRNAs. Whereas sgRNA 1 and sgRNA 2 in general have similar effects, the sgRNA 3 effects were for some genes similar to sgRNAs 1 and 2, e.g. *ZEB2* and *VIM*, whereas for other genes resembled what was observed for sgRNA C, e.g. *ZEB1* and *MSRB3*. This eventual could be a consequence of specifically sgRNA 3 generating a ZEB1-derived 100kD protein visible after cell passages with erlotinib (Figure S3). For ZEB1 expression, the difference was even more striking (exemplified with P2). Whereas sgRNAs 1 and 2 resulted in a decrease, sgRNA 3 was resulting in an increase, in ZEB1 mRNA expression. Our best explanations could be:

1) That indels from sgRNAs 1 and 2 results in nonsense mediated decay of the mRNA whereas this was not a result of the sgRNA 3 indels (note to this the presence of a ZEB1 band in western blot after sgRNA 3 processing which represents a shorter variant of ZEB1).

2) The ZEB1 protein variants generated from sgRNA 1 and 2 processing relative to sgRNA 3 processing somehow influences an EMT auto-regulatory loop differently with resulting different impacts on ZEB1 mRNA expression levels.

We find both these explanations speculative and the entire set of results from usage of ZEB1 sgRNA 3 with potential of confusing the data interpretation. We have in accordance with this, selected to remove data related to ZEB1 sgRNA 3 from the revised manuscript and instead focused on the results obtained with ZEB1 sgRNAs 1 and 2. In consequence, the presented results are much more coherent and understandable since ZEB1 sgRNAs 1 and 2 give very similar results in most experiments.

Moreover, for nomenclature reasons we have renamed ZEB1 sgRNAs 1 and 2 with prefix Z to: Z1 and Z2. This accordingly improves readability and separation to the naming relative to FGFR1 sgRNAs (renamed from 1 and 3 to F1 and F3) and *MIR200C* and *MIR141* sgRNAs (renamed from 2 and 1 to M2 and M1).

Changes in the text: In figure 5 and supplement figure S3 panel D results from ZEB1 sgRNA 3 are removed. Also in the results section description of ZEB1 sgRNA 3 is removed (P20 lines 21-25 and P21 lines 1-3). Notice that in revised figure S3 panel C ZEB1 sgRNA 3 still appears

given uncropped western blots now are shown (see also next comment).

Comment 11. - western blotting is not convincing regarding the KO of ZEB1, since unspecific bands are seen, including smaller bands in the KO cells at P6. Please present the uncropped images and size markers.

Reply 11: We have in a modified figure S3 section C now included uncropped images as well as size markers. Expected positions of relavant proteins are also indicated. We note that ZEB1 appears visible only at P3 and P6 after erlotinib treatment with sgRNA C in accordance with increased expression following EMT. A band with expected size of ZEB1 is less pronounced in presence of ZEB1 sgRNAs Z1 and Z2. Related to the comment above we note appearance of a minor sized ZEB1 protein product with sgRNA Z3. This in alignment of sgRNA Z3 removed from the analysis in the revised manuscript as described in details above.

Note that uncropped FGFR1 westerns also have been included in the revised manuscript (new supplemental figure S5)

Changes in the text: New panel C added to figure S3.

Comment 12. - EMT markers should be tested at the protein level, either by western blot or immunofluorescence. Does cell shape reflect the changes in markers in the different KO cells generated?

Reply 12: Both of the in this study examined HCC827EMT cell clones 4 and 10 were previously established and both well characterized to have mesenchymal morphology relative to the epithelial morphology of HCC827PAR cells (Jakobsen KR et al. Oncogenesis doi:10.1038/oncsis.2017.17). Summarized, mRNA-expression analyses, western blot analyses, and immunological staining's for E-cadherin and Vimentin verified decreased and increased expression, respectively, in both HCC827EMT cell clones 4 and 10 relative to HCC827PAR cells (Jakobsen KR et al. Oncogenesis doi:10.1038/oncsis.2017.17). We are accordingly confident concerning the EMT-status. This information is included in the revised manuscript.

For the different KO cells generated, we have not examined the association between cell shape and EMT marker expression. We note that in the FGFR1 depletion model, cells have a minor shift towards a more epithelial EMT-marker expression profile than control cells, and we envisage a significant morphological change will be difficult to determine. For the *MIR200C* and *MIR141* seed-sequence depletion, partial EMT characteristics are only evident with increased expression of mesenchymal markers but not concomitant decrease in epithelial markers. Again, a significant morphological change is envisaged difficult to determine. Finally, for the ZEB1 depletion model, at P15 the ZEB1 depleted cells have an EMT-marker expression not aligned with the other vice observed EMT, namely concomitant FGFR1 expression without VIM and ZEB2, but with TWIST1 (figures 5 and S2). The detailed characterization of these

cells in terms of their EMT-status, morphological speaking, could be interesting but could be worth a separate study. We have noted this in the revised discussion section.

Changes in the text: In the modified methods section "Cell lines" this has now been described (P7 lines 4-10). Discussion section revised (P29lines 20-23).

Comment 13. - Depletion of MIR200C and MIR141 by CRISPR should be tested somehow. What kind of indels were generated? Do the indels reflect the miRNAs functionality?

Reply 13: We have in the study used CRISPR/Cas9 to create indels towards the seed-sequences of *MIR200C* and *MIR141*. By this approach, which we find very elegant, we are confident the functionality of the MIRs will be depleted as described below in more details. We agree that using this was approach was not described in the original submission. Accordingly, this concern from the reviewer is now addressed in a new figure S4 included in the revised manuscript as well as a modification of the methods section describing CRISPR/Cas9. Finally, the results section now describes the approach targeting indels to the seed-sequences of *MIR200C* and *MIR141* by CRISPR/Cas9. Summarized, sgRNAs targeting *MIR200C* and *MIR141* were designed in the UCSC Genome Browser (https://genome.ucsc.edu/) on human Feb. 2009 (GRCh37/hg19) using the CRISPR Targets track to target the seed sequences of the miRNAs, as perfect complementarity of the seed sequence to the target site is important for miRNA function (Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215-33). A double miRNA KO HCC827 cell line was produced by first transducing HCC827Cas9 cells with MIR141 targeting sgRNA (GTGAAGCTCCTAACACTGTC, MIT Specificity Score = 79 and Doench/Fusi 2016 score = 46%) and 200 µg/ml hygromycin selection (Invitrogen, 10687010) followed by transduction with MIR200C targeting sgRNA (TTGGGAGTCTCTAATACTGC, MIT Specificity Score = 81 and Doench/Fusi 2016 score = 50%) and 0.5 µg/ml puromycin selection (Sigma-Aldrich, P8833). Each selection was conducted for 14 to 18 days. The subsequent Synthego analyses revealed seed-sequence indels as shown in the new figure S4.

Changes in the text: New figure S4, modification of the Methods section related to CRISPR/Cas9, and modification of the entire results section " Interaction between *MIR200*-family, ZEB1 and ZEB2 expression in EMT-E-TKI-R." to now describe the approach generating MIR seed-sequence indels by CRISPR/Cas9 (P22 lines 14-19).

Comment 14. - For FGR1 KO, Synthego analysis of the KO efficiency seems to not reflect protein levels by Western blot, which shows a much lower efficiency. Please explain.

Reply 14: We completely agree what the original included FGFR1 sgRNA 2 whereas having a

high indel/knock-out percentage still presented a FGFR1 band in western blot comparable with the FGFR1 band observed with control sgRNA C. We have no stringent explanation for this. But on top of this FGFR1 sgRNA 2 also displayed a gene-expression phenotype in HCC827 cells different than observed for sgRNAs 1 and 3, and in MTS-assay the results for FGFR1 sgRNA 2 were also different than for FGFR1 sgRNAs 1 and 3. Thus, since we cannot argument that FGFR1 depletion really succeeded with FGFR1 sgRNA 2 and accordingly after reviewing data have concerns for the resulting result interpretation, we have selected to remove the FGFR1 sgRNA 2 data from the revised manuscript. This makes the interpretation of results concerning FGFR1 depletion much more coherent and confident. Please note that the western blot presented in the new figure S5 still includes FGFR1 F2 as blots are presented uncropped. Furthermore, for FGFR1 sgRNA 1 and sgRNA 3, FGFR1 protein depletion relative to sgRNA C is clear but still not reaching a proportional effect scheduled from the Synthego analysis. This is described in the revised results section. Finally, please note we have renamed FGFR1 sgRNAs 1 and 3 with prefix to F1 and F3 (see also reply to comment 10).

Changes in the text: Data related to FGFR1 sgRNA 2 is removed from the text (entire results and discussion secton) as well as from the revised figure 7 panels C and D. In results section "Receptor kinase expression in EMT-E-TKI-R" is given information concerning lack of absolute coherence between FGFR1 protein expression and FGFR1 depletion deduced by Synthego (P24 lines 16-22).

Comment 15. - No testing of the sgRNAs at the DNA level is described, like T7E1 assay.

Reply 15: With our previous experience with CRISPR/Cas9 we have a pipeline which in most cases secure that utilized sgRNAs will have high efficiency and specificity without *in prior in vitro* testing. Former, we performed a lot of sgRNA pre-screening but have evaluated this effort can be excluded and the time instead used on testing more sgRNAs and target specificity analyses.

In the previous version of the manuscript, design and testing of sgRNAs were sparsely described. This is now modified in the revised manuscript. The method section includes a novel section concerning sgRNA design and testing and assumptions to decrease potential off-targeting. Moreover, we have in supplemental figures S3, S4 and S5 now included information related to sgRNA efficiency and specificity.

For all used sgRNAs actual genomic targeting was inspected by PCR, gel-extraction, sequencing, and Synthego ICE analysis. For sgRNAs towards *MIR200C* and *MIR141* seed-sequences we in addition confirmed that no off-targeting was present across the MIR200 family as well as to the potential off-targets for *MIR141* sgRNAs in genes *WT1* and *CDHR1*.

Changes in the text: Modification of the methods section "CRISPR/Cas9 procedures (P11 lines 21-24 and P12 lines 1-2, P11 lines 18-20). New panels in Figure S3 (A, B, and C). New figure S4. New figure S5.

Comment 16. - No test of off-target effects of the sgRNAs used is described.

Reply 16: (See also reply to comment 15). As described in reply to comment 15 we have in the revised manuscript included a much more in depth theoretical analyses of sgRNA targeting and potential off-targeting. This was done for sgRNAs targeting *ZEB1*, *FGFR1*, *MIR200C* and *MIR141*. We acknowledge that eventual off-targeting could be present and for example for *FGFR1* sgRNAs where the functional outputs for two different sgRNAs showed some differences this eventuality is discussed in the revised manuscript. With the *MIR200C* and *MIR141* sgRNAs targeting and off-targeting was in addition to the common described protocols also involving sequence alignment analyses as discussed below. Note this is now summarized in the novel figure S4.

To examine if the *MIR200C* and *MIR141* sgRNAs could target the seed sequences of the other *MIR200* family members, sequence alignments were performed. Sequence alignment of the *MIR200C* sgRNA to the other *MIR200* family members all displayed more than six mismatches and no SpCas9 PAMs were flanking the potential target sites. Although the potential target sites of *MIR141* sgRNA at the other *MIR200* family members were flanked by SpCas9 PAMs, all family members contained seven or more mismatches to the *MIR141* sgRNA.

To further examine the possibility of *MIR200C* off-target effects in the genomic location of *MIR141* and *vice versa*, Sanger sequencing of *MIR141* genomic sequence was performed on DNA from *MIR200C* single KO cells and of *MIR200C* genomic sequence on DNA from *MIR141* single KO cells. No indels was observed. Furthermore, genomic off-target effects were predicted through the tool CRISPOR. The total number of potential off-targets with up to four mismatches and a CFD score > 0.02 are shown in the table below.

Number of potential off-targets per sgRNA with CFD score > 0.02 grouped by mismatches

sgRNA	# off-targets 0 mismatches	# off-targets 1 mismatch	# off-targets 2 mismatches	# off-targets 3 mismatches	# off-targets 4 mismatches	Total
MIR200C	0	0	1	4	76	81
MIR141	0	0	2	11	63	76

MIR200C sgRNA full table of potential off-targets: https://genome.ucsc.edu/cgi-bin/hgc?c=chr12&l=7072887&r=7072922&o=7072893&t=7072916&g=crisprAllTargets&i=&db=hg19

MIR141 sgRNA full table of potential off-targets: https://genome.ucsc.edu/cgi-bin/hgc?c=chr12&l=7073307&r=7073338&o=7073307&t=7073330&g=crisprAllTargets&i=&db=hg19

Two potential *MIR141* sgRNA off-targets located in *WT1* (CFD score: 0.419 and 4 mismatched nucleotides) and *CDHR1* (CFD score 0.352 and 3 mismatched nucleotides) were chosen for

further examination, as they were located within exons, and the genes have been associated with EMT and cell adhesion, respectively (Park J, Kim D-H, Shah SR, Kim H-N, Kshitiz, Kim P, et al. Switch-like enhancement of epithelial-mesenchymal transition by YAP through feedback regulation of WT1 and Rho-family GTPases. Nature Communications. 2019;10(1):2797 and *CDHR1* Gene: genecards.org). Synthego Ice analysis did not detect any indels in the potential off-target sites.

Changes in the text: New sections A, B, and C in supplemental figure S3, New figure S4, new figure S5. Modification of Methods section to include relevant information concerning this (P11 lines 21-24 and P12 lines 1-2)

Comment 17. - groups are hard to distinguish in Fig 7E

Reply 17: We completely acknowledge this and accordingly instead present two graphs – one for *FGFR1* sgRNA F1 and one for sgRNA F3. By this, we eliminates the illustrative problem that the curves obtained for *FGFR1* sgRNAs F1 and F3 are were similar and accordingly overlapping in illustrations. See also response to reviewer 1 concerning the removal of *FGFR1* sgRNA2 from the illustrations. The revised figure panel now more clearly illustrates that the curves of the two *FGFR1* sgRNAs F1 and F3 relative to the curve of control sgRNA C.

Changes in the text: Two new figure panels in figure 7 section D, which replaces the original single panel.

Comment 18. - units should be standardized to µL

Reply 18: This is now corrected throughout the revised Methods section

Changes in the text: At several positions in the Methods section, this has been corrected.

Additional note from the authors.

The original submission included by mistake a panel D in figure S3 (previously abbreviated figure S2). The shown data were not assigned a labeling number in the results or discussion. This panel has been removed in the revised version of the manuscript.