miR-301a as a NF-κB Activator in Pancreatic Cancer Cells

Zhongxin Lu, Yan Li, Apana Takwi, Benhui Li, Jingwen Zhang, Daniel J. Conklin, Ken H. Young, Robert Martin and Yong Li

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Transaction Report:

(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 12 July 2010

Thank you very much for submitting your research paper for consideration to The EMBO Journal editorial office. The work has in the meantime being assessed by three expert scientists with all of them indicating some interest in the study. However, all three also agree that significant additional work would be needed to reach a level of clarity and consistency that would make the paper suitable for eventual publication. As no general conceptual concerns exist and the comments are very explicit, there seems not much sense to repeat every criticism here in full. Overall, ref#1 is curious what indeed would trigger the reported feedback loop and whether induced signals are found within the whole tumor. Ref#2 would like to see an extension of the tumor samples. Given the overall positive responses from our referees, I am happy to offer you the chance to amend the current version of the study by major revisions. Thus, the final decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript that will be assessed involving some of the original expert scientists!

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

Yours sincerely,

Editor
The EMBO Journal
REFEREE REVIEWS

Referee #1 (Remarks to the Author):

Pancreatic cancer (PC) is the most deadly human cancer. NFκB-signaling is constitutively activated in most cases of this cancer, although mutations in components of this pathway were not detected. The authors investigated how NFκB signaling is activated in PC.

They identified a microRNA (miR-301) which is strongly upregulated in PC, but not in other cancer types. Overexpression of miR-301 increased NFκB pathway activity by increasing binding of NFκB to target promoters. They identified the NFκB repressing factor Nkrf as target of miR-301 and could further demonstrate that NFκB activates transcription of miR-301, thus showing that miR-301 and NFκB are linked in a positive feedback loop. In additional work it was demonstrated that this feedback loop is active and crucial for pancreatic cancer cells, also in xenograft models. Moreover a correlated overexpression of miR-301 and NFκB was detected in human PCs.

This work is original and well performed. The description of this positive feedback loop might have high relevance for understanding the biology of PC and for future translational aspects of this disease.

Some open questions should be addressed before publication:
1. Fig. 6b: The in situ hybridization for miR-301 is not fully convincing and quantification is difficult to perform. What is the control probe? Control staining should be shown. A quantitative PCR for miR-301 should be performed in addition.
2. Still it is not known what is responsible for activating the whole cascade (positive feedback loop) without an initial genetic alteration. Alternatively environmental triggers could locally start the cascade. Is the activation of NFκB signaling homogenous within the whole tumor or locally increased? All these aspects should at least be better discussed.

Minor point:
Page 9, mistake in second chapter: shouldn't it read ".... The native Cox2 promoter.... was inhibited...."?

Referee #2 (Remarks to the Author):

In this manuscript, the authors identified miR-301a as a NF-κB activator in pancreatic cancer cells by miRNA library screening using a cell line stably expressing NF-κB responsive luciferase construct. Through computational analysis, the authors demonstrated that a NF-κB activity repressor, NKRF is a miR-301a target that mediates the activation of NF-κB by miR-301a and miR-301a is also induced by NF-κB. Therefore, the authors identified a novel positive feedback mechanism in pancreatic cancer between NF-κB and miR-301a, which they further examined in the pancreatic cancer cell lines and several tumor tissue samples. Finally, the authors showed that miR-301a inhibition or NKRF upregulation in pancreatic cancer cells could suppress tumor xenograft growth and reduce NF-κB target gene expression.

This manuscript is highly interesting and provided mechanistic insight regarding to the link between elevated expression of miR-301a and activation of NF-κB in pancreatic cancer. However, the manuscript is not very well written and many figure legends are not self-explanatory to the corresponding figures, which made this manuscript somewhat hard to read and understand.

Major Points:
1. In the Fig. 5A, the presentation of miR-301a expression data is confusing. The authors should present the data consistent with other gene expression data in the manuscript. This is also the case for Fig. S3. The author should also state how many pancreatic cancer tissue and NAT samples were profiled for miR-301a expression. I believe the data were presented in average. If so, the authors should present miR-301a expression data in each tumor and NAT samples individually to eliminate ambiguity and confusion. It would also be helpful to include more pancreatic cancer samples to examine miR-301a expression level (five were presented in Fig. S3).
2. I am curious why the authors used 293T & Hela cells to examine the positive feedback loop of miR-301a & NF-κB instead of pancreatic cancer cell lines at the first place. If they had done so, they may be able to merge Fig. 4 & 5 into one figure, which could make the manuscript easier to read and follow.

3. In Fig. 7B, G & J, the tumor volume should be presented as growth curve from individual mouse with error bars. In Fig. 7G & J, parental cell line growth curve should be included.

Minor Points:

1. In the Fig. 1D, how did the authors measure the 2.8-fold increase of DNA binding in miR-301a expressing cells?
2. In Fig. 6, the review disagree that there is "strong staining" for RelA in nucleus in pancreatic cancer as the picture presented. The reviewer believes that this should be read as "repressed" but not "activated".
3. In this sentence, "when either anti-miR-301a (SI Fig. S4A) or Nkrf (Fig. S4B) was introduced, the native Cox2 promoter, but not the mutant with an altered NF-κB binding site, was activated". The reviewer believes that this should be read as "repressed" but not "activated".

4. In Fig. 7B & C, the VEGF-C and CD31 IHC data should be quantified and presented.
5. In Fig. 4E, F & G, although expressing miR-301a resulted ~50% down regulation of NKRF, which is similar to the level achieved by using TNF-a or siNKRF, the expression of FAM33A & MMP2 were increased significantly higher by miR-301a than that of TNF-a or siNKRF, suggesting NKF may not be the only factor targeted by miR-301a in regulating NF-κB pathway. The authors should discuss this.
6. It would be interesting for the authors to speculate how RelA is regulated by miR-301a on protein level but not on RNA level (Fig. 6E).
7. It would be nice to show knocking down of VEGF-C could abolish the miR-301a phenotype in tumor xenografts.

Referee #3 (Remarks to the Author):

Summary

The authors describe a screen that identified miR-301a as regulating NFκB activity. miR-301a represses NKRF and this appears to be the mechanism whereby NFκB is regulated. It is also induced by NFκB in some cells but not others, indicating an interesting positive feedback loop. Lastly, it is deregulated in pancreatic adenocarcinomas and miR-301 overexpression can knockdown Nkrf and can inhibit tumor formation in nude mice.

Overall assessment:

Strengths:

Nice screening strategy that resulted in the identification of an interesting miRNA species.

Extensive work to characterize a target

Weaknesses:

Figure labeling is confusing

There is a plethora of data that show similar things and distract from the logical progression of the paper- the paper could be streamlined to enhance the main points, and some of the data could be made supplementary.

Specific criticisms (tied to a figure number, these comments apply to the corresponding results section and figure legend as well):

Figure 1/legend/result:

The Tier-1/Tier-2 assay is not well-described in the text or figure legends. Which is the inducible promoter in (A) and what type of cells are these transfections being performed in? In (C) why are you normalizing luc by Rluc- is the data based on the same system shown in (B)? Shouldn't it be the other way around? Please state explicitly in text and not just in the methods section. Also what is NFKBIA in figure legends?

Figure 2A, why are the two negative controls different from each other? (2.4 versus 0.52). Are these
two different blots? If so, do not show them together like this. It would be better to normalize to the negative control.

Figure 3
Please state what PANC cells are in the results section. For D,E,F, and G you need to label the y-axis with more than just abbreviations (Relative Quantity). The results are not readily understandable from the figures.

Figure 4
Please re-label your y-axes

Figure 5
Please re-label your y-axes

Figure 6
There appears to be nuclear vs cytoplasmic localization of Nkrf by immunohistology, does this have any relevance?

Referee #1 (Remarks to the Author):

1. **Fig. 6b:** The in situ hybridization for miR-301 is not fully convincing and quantification is difficult to perform. What is the control probe? Control staining should be shown. A quantitative PCR for miR-301 should be performed in addition.

   We updated the in situ images that showed the cellular structure with miR-301a localized in perinuclear regions. We use a scrambled RNA from the manufacture (Exiqon) as a negative control, which did not show any positive staining in all tissues (one staining image is shown in Fig. 5B). We used qPCR to show miR-301a overexpression in more tumor specimens (Fig. S3).

2. Still it is not known what is responsible for activating the whole cascade (positive feedback loop) without an initial genetic alteration. Alternatively environmental triggers could locally start the cascade. Is the activation of NFkB signaling homogenous within the whole tumor or locally increased? All these aspects should at least be better discussed.

   We can think of a few possibilities regarding what triggers miR-301a overexpression and NF-κB activation. First, KRas mutations are frequent in pancreatic adenocarcinomas and are detected in 20-30% of early neoplasms with the frequency rising to nearly 100% at advanced stages, higher than any other types of cancer. One of the downstream effectors of Ras is Akt, which can activate IKK that subsequently activates NF-κB. Activated NF-κB, in turn, promotes the transcription of miR-301a. To test this possibility, we introduce HRas V12 (activated Ras) into 293T cells and determine the expression levels of miR-301a. miR-301a levels were not significantly changed with Ras activation, indicating that Ras activation alone cannot trigger miR-301a overexpression. We then down-regulated KRas using siRNAs in PANC-1 and Mia-PaCa-2 cells and found that the expression of miR-301a was not inhibited by KRas down-regulation. Collectively, these results suggest that Ras activation is unlikely responsible for miR-301a overexpression. Second, miR-301a is located in the human Chromosome 17q22-17q23.1, a region that has been reported to be amplified in 46% of 13 primary tissues and 24 cell lines of pancreatic adenocarcinomas. Other possibilities include epigenetic regulation of miR-301a expression and/or other trans or cis regulatory factors. The scope of these studies is large, so we intend to examine the molecular mechanisms of miR-301a overexpression in future investigations.

   We did not observe hot spots for RelA or miR-301a staining, indicating that the NF-κB activation is unlikely locally initiated.

*Minor point:*
Page 9, mistake in second chapter: shouldn't it read ".... The native Cox2 promoter.... was inhibited...."?

We have corrected the sentence. “the activities of the native Cox2 promoter … were inhibited”

Referee #2 (Remarks to the Author):

Major Points:

1. In the Fig. 5A, the presentation of miR-301a expression data is confusing. The authors should present the data consistent with other gene expression data in the manuscript. This is also the case for Fig. S3. The author should also state how many pancreatic cancer tissue and NAT samples were profiled for miR-301a expression. I believe the data were presented in average. If so, the authors should present miR-301a expression data in each tumor and NAT samples individually to eliminate ambiguity and confusion. It would also be helpful to include more pancreatic cancer samples to examine miR-301a expression level (five were presented in Fig. S3).

We changed Fig. 5A to present the expression levels of miR-301a using the relative quantities compared to that of normal pancreas. Fig. S3A was used to demonstrate why U6 was used as a reference across cell lines, normal pancreas, tumor specimens, and normal adjacent tissues. Fig. S3B presents miR-301a expression data in each tumor and NAT samples individually. We increased the tumor sample number from 5 to 24 in Fig. 5 (Fig. S3).

2. I am curious why the authors used 293T & Hela cells to examine the positive feedback loop of miR-301a & NF-κB instead of pancreatic cancer cell lines at the first place. If they had done so, they may be able to merge Fig. 4 & 5 into one figure, which could make the manuscript easier to read and follow.

HeLa cells were used to demonstrate that the regulation of Nkrf and NF-κB activation by miR-301a is not cell line-specific to 293T and pancreatic cancer cells. Pancreatic cancer cells are unique, as they express high levels of miR-301a endogenously. We merged Fig. 4 & 5 into one figure to shorten the manuscript.

3. In Fig. 7B, G & J, the tumor volume should be presented as growth curve from individual mouse with error bars. In Fig. 7G & J, parental cell line growth curve should be included.

We included these data.

Minor Points:

1. In the Fig. 1D, how did the authors measure the 2.8-fold increase of DNA binding in miR-301a expressing cells?

We quantified the Signal Densities (SD) using ImageQuant 5.1 (Molecular Dynamics, Inc.). The densitometry of band intensity revealed that the SD (calculated as shift/(shift+free probe)) from Lane 5 is 2.8 relative to that from Lane 1.

2. In Fig. 6, the reviewer disagree that there is "strong staining" for RelA in nucleus in pancreatic cancer as the picture presented. The miR-301a in situ signal is not impressive and it is hard to see the tissue morphology.

We rephrased the statement: “There is sporadic nuclear RelA staining in pancreatic tumor sections, but not in sections from normal adjacent tissue or normal pancreas, consistent with previous reports”. We updated the miR-301a in situ data to demonstrate the tumor cell morphology.

3. In this sentence, "when either anti-miR-301a (SI Fig. S4A) or Nkrf (Fig. S4B) was introduced, the native Cox2 promoter, but not the mutant with an altered NF-κB binding site, was activated". The reviewer believes that this should be read as "repressed" but not "activated".

We changed it to: "when either anti-miR-301a (SI Fig. S4A) or Nkrf (Fig. S4B) was introduced, the native Cox2 promoter, but not the mutant with an altered NF-κB binding site, was repressed".
We have corrected this sentence. “the activities of the native Cox2 promoter … were inhibited”

4. In Fig. S7B & C, the VEGF-C and CD31 IHC data should be quantified and presented.

We thank the reviewer for this suggestion. We included the IHC scores in Fig. S7D. Indeed, after careful examination and blind-scoring our sections, we found there was a lack of statistical significance in CD31 staining in xenografts with PANC-1 cells treated with either siNkrf or a control (Fig. S7D), suggesting that regulation of miR-301a:Nkrf expression may attenuate tumor growth through mechanisms other than angiogenesis, or in addition to angiogenesis. However, VEGF-C staining did follow NF-κB activation as we modulated miR-301a or Nkrf; fully supporting the main conclusion that miR-301a is a NF-κB activator.

5. In Fig. 4E, F & G, although expressing miR-301a resulted ~50% downregulation of NKRF, which is similar to the level achieved by using TNF-α or siNKRF, the expression of FAM33A & MMP2 were increased significantly higher by miR-301a than that of TNF-α or siNKRF, suggesting NKRF may not be the only factor targeted by miR-301a in regulating NF-κB pathway. The authors should discuss this.

We thank the reviewer pointing this out. We amended a short discussion in the Results Section. “It is noteworthy that miR-301a overexpression led to ~50% down-regulation of Nkrf, which is similar to the level achieved by TNF-α or siNkrf treatment, yet the expression of FAM33A and MMP2 were increased significantly higher by miR-301a than that of TNF-α or siNkrf in HeLa cells (Fig. 4E, 4F & 4G). This result indicates suggest that miR-301a may target other genes beyond Nkrf to regulate NF-κB activation. Given that recent proteomic studies have revealed that one miRNA is expected to repress the expression of a large number of genes (Baek et al, 2008; Selbach et al, 2008), Nkrf is likely one of the targets rather than the target of miR-301a in NF-κB signaling.”

6. It would be interesting for the authors to speculate how RelA is regulated by miR-301a on protein level but not on RNA level (Fig. 7E).

It suggests that inhibition of NF-κB by Nkrf (upregulated by anti-miR-301a) could be through Nkrf:RelA binding (supported by a previous article, Nourbakhsh & Hauser, 1999), which might promote RelA degradation (a pure speculation). This does, however, suggest that Nkrf may be involved in the regulation of RelA at the posttranslational level.

7. It would be nice to show knocking down of VEGF-C could abolish the miR-301a phenotype in tumor xenografts.

We did not perform the VEGF-C knockdown assay, but we noted that “VEGF inhibition using monoclonal antibodies such as Bevacizumab (a.k.a. A4.6.1, RhuMAb, or Avastin® targeting all VEGF isoforms, including VEGF-C) has been reported to reduce xenograft pancreatic tumor growth and angiogenesis (Hotz et al, 2003).”

Referee #3 (Remarks to the Author):

“There is a plethora of data that show similar things and distract from the logical progression of the paper- the paper could be streamlined to enhance the main points, and some of the data could be made supplementary.”

We have streamlined some sections of the manuscript by merging Fig. 4 and Fig. 5, as well as rewritten some discussions.

Figure 1/legend/result: The Tier-1/Tier-2 assay is not well-described in the text or figure legends. Which is the inducible promoter in (A) and what type of cells are these transfections being performed in? In (C) why are you normalizing luc by Rluc- is the data based on the same system shown in (B)? Shouldn’t it be the other way around? Please state explicitly in text and not just in the methods section. Also what is NFκBβIA in figure legends?
We added the details of the screen assay from Supplemental Text 1 to the main text. Briefly, the inducible promoter in (A) is a minimal CMV (mCMV) promoter, which is downstream of transcription response elements (TREs, which are four tandem repeats of GGGACTTTCC for NF-κB). In (C) luc normalized by Rluc is based on (A). NFκBIA encodes IkBα (we added this information in the figure legend). All these information were added into the main text.

*Figure 2A, why are the two negative controls different from each other? (2.4 versus 0.52). Are these two different blots? If so, do not show them together like this. It would be better to normalize to the negative control.*

We normalized the Nkrf relative quantity (Nkrf) (using actin as a reference) to the negative control in the revision.

*Figure 3*
Please state what PANC cells are in the results section. For D, E, F, and G you need to label the y-axis with more than just abbreviations (Relative Quantity). The results are not readily understandable from the figures.

We added the information in the text and figure legends.

*Figure 4 & Figure 5*
Please re-label your y-axes

We re-labeled the y-axes in both Fig. 4 and Fig. 5.

*Figure 6*
There appears to be nuclear vs cytoplasmic localization of Nkrf by immunohistology, does this have any relevance?

We discuss the subcellular localization of Nkrf and the relevance to its functions. “Nkrf is abundant in many human cell lines and adult tissues (Nourbakhsh & Hauser, 1999) with localization in cytoplasm, nucleoplasm, and dominantly nucleoli (Niedick et al, 2004), supporting its availability to participate in the regulation of its target genes. The DNA-independent function is likely from cytoplasmic Nkrf proteins that inhibit the expression of MMP2, COX2, MYC, and FAM33A-miR-301a by binding NF-κB (Nourbakhsh & Hauser, 1999), i.e., in the cytoplasm, Nkrf acts to hold NF-κB in an inactive state. This is in contrast to the DNA-dependent regulation of NOS2A, IFNB, and IL in nucleoplasm, where Nkrf interacts with NREs to mediate transcriptional repression by inhibition of the NF-κB activity at their promoters. The biological activity of nucleolar Nkrf, however, remains unknown with a speculation that the Nkrf-regulated genes are kept inactive in nucleoli but are released to the nucleoplasm upon transcriptional activation (Niedick et al, 2004).”
REFEREE REVIEWS

Referee #2 (Remarks to the Author):

The revised manuscript has been significantly improved. However, several minor points still remain as following:

1. Although the authors have tried to clarify their screening strategy in the first part of "Results" section, the description is still not straightforward and clear enough. The 2-tier screening is essentially screening a cell line expressing a NF-κB response element using a miRNA expressing library (tier-1) and then, followed by validation of the screening hits with reporter assay (tier-2). So, maybe the authors can improve their language in this section to further clarify this.

2. Figure 4 is too busy. Some of the data should go to supplemental information.

3. On page 10, second line, "One shRNA (short ........ a miRNA)......". This sentence is confusing. shRNA is small hairpin RNA. If this particular shRNA uses miRNA precursor as a backbone (such as mir-30), the authors do not need to specify it in the main text. This information should be included in the "Material and Methods" instead. The authors should also clearly specify where shRNA or siRNA is used in the text and figures instead of using siRNA for both.

4. In the updated Figure 6B, G, and J, the growth rates of parental tumor (PANC-1) are significantly different with same amount of input cells (B&J compared to G at 8 weeks after inoculation). I am wondering what caused this big difference? In Fig. 6B, how many mice were used? In all animal experiments, do the error bars represent SD or SEM? These information should be included in the figure legend.

Referee #3 (Remarks to the Author):

The paper is improved significantly from the prior version. The studies are more clear, but I still feel that the paper suffers from a lack of clarity. Nonetheless, this is an important finding and should be considered for publication if the authors can clarify some of the outstanding issues and have the manuscript carefully proof-read.

1- In the introduction the description of the Nkrf-NRE-NF-κB relationship is extremely confusing. The authors jump around between what was already known and what they are claiming in this study. This needs to be clarified.

2- I appreciate the effort to demystify the "Tier 1" and "Tier 2" assays. Would it not have been simpler to call the first a "NFκB transcription reporter" and the second a "miRNA-suppression luciferase assay"? Also, the screening aspect of this assay is overstated because in the second assay, the authors choose which UTR to screen with. Hence if you use Nkrf's UTR, you will find miRNAs that target Nkrf. It is not an unbiased screen and that needs to be pointed out, OR an explanation of why NKRF's UTR was chosen out of the screen.

3- The data on the 3'-compensatory binding in the lack of the seed match is fascinating. Most studies to date have relied on destroying seed complementarity in efforts to demonstrate specific targeting. The authors should add a line explaining what they mutated to show specific targeting.

4- The section title "mir-301 inhibition or Nkrf upregulation reduces xenograft growth" could be made simpler and clearer. Just state miR-301 inhibition reduces xenograft growth.

5- Despite the reduction in the number of figures, the paper remains enormous and the main message (of a novel miR-regulated NF-κB activating pathway that may cause cancer) seems to be lost. I would recommend working on shortening it a bit further.
We thank both reviewers for their speedy and thoughtful suggestions and comments and have revised the manuscript using reviewers’ critiques as a guide.

Referee #2 (Remarks to the Author):

1. Although the authors have tried to clarify their screening strategy in the first part of "Results" section, the description is still not straightforward and clear enough. The 2-tier screening is essentially screening a cell line expressing a NF-κB response element using a miRNA expressing library (tier-1) and then, followed by validation of the screening hits with reporter assay (tier-2). So, maybe the authors can improve their language in this section to further clarify this.

We shorten the paragraph to two sentences: The first assay (NF-κB-dependent reporter) uses a cell line 293NFκB (SI Text) that expresses a firefly luciferase gene (luc) under the control of a minimal CMV (mCMV) promoter and NF-κB transcription response elements. miRNAs that upregulate the NF-κB-dependent reporter will be subjected to the second assay (UTR-dependent reporter) to test whether they suppress the expression of a Renilla luciferase gene (Rluc) with a chosen 3’ UTR.

2. Figure 4 is too busy. Some of the data should go to supplemental information.

We moved the data on 293T and HeLa cells to Fig. S6.

3. On page 10, second line, "One shRNA (short ...... a miRNA)......". This sentence is confusing. shRNA is small hairpin RNA. If this particular shRNA uses miRNA precursor as a backbone (such as mir-30), the authors do not need to specify it in the main text. This information should be included in the "Material and Methods" instead. The authors should also clearly specify where shRNA or siRNA is used in the text and figures instead of using siRNA for both.

All siRNAs used were based on small hairpin RNAs using pre-miR-30 as a backbone. We have clarified this in the Materials and Methods. The term “siRNA” is used throughout the whole manuscript.

4. In the updated Figure 6B, G, and J, the growth rates of parental tumor (PANC-1) are significantly different with same amount of input cells (B&J compared to G at 8 weeks after inoculation). I am wondering what caused this big difference? In Fig. 6B, how many mice were used? In all animal experiments, do the error bars represent SD or SEM? These information should be included in the figure legend.

We thank the reviewer for pointing this out. We should have clarified the use of “parental” in Fig. 6G and 6J. In the revised version, we use the term “Control”. In Fig. 6B, the “Control” was a group of mice injected with PANC-1 cells carrying the parental vector (that was used to express TuD:anti-miR-301a). In Fig. 6G, the “Control” was mice injected with naïve PANC-1 cells. In Fig. 6J, the “Control” was mice injected with PANC-1 cells carrying the TuD:anti-miR-301a (the parental cells for siControl and siNkrf), so it is expected to generate smaller tumors than PANC1 cells (Control in Fig. 6G) or PANC-1 cells with a empty vector (Control in Fig. 6B). There is no statistical difference in tumor volumes between “Control” in Fig. 6B and “Control” in Fig. 6G, or between “Control” and “siControl” in Fig. 6J. 5 mice per group were used throughout the study. Error bars represent SD.

Referee #3 (Remarks to the Author):

1- In the introduction the description of the Nkrf-NRE-NF-κB relationship is extremely confusing. The authors jump around between what was already known and what they are claiming in this study. This needs to be clarified.

We removed the NRE part in the introduction Section. We discuss the requirement of the NRE sequence to Nkrf function in the discussion Section as it is crucial to explain the modulation of NRE-less genes by miR-301a:Nkrf.
2- I appreciate the effort to demystify the "Tier 1" and "Tier 2" assays. Would it not have been simpler to call the first a "NFκB transcription reporter" and the second a "miRNA-suppression luciferase assay"? Also, the screening aspect of this assay is overstated because in the second assay, the authors choose which UTR to screen with. Hence if you use Nkrf's UTR, you will find miRNAs that target Nkrf. It is not an unbiased screen and that needs to be pointed out, OR an explanation of why NKRF's UTR was chosen out of the screen.

We appreciated the reviewer's point and shortened the description of the assay. We now only use the word “screening” for the first assay. See the response to No. 1 concern from Review #2.

3- The data on the 3’-compensatory binding in the lack of the seed match is fascinating. Most studies to date have relied on destroying seed complementarity in efforts to demonstrate specific targeting. The authors should add a line explaining what they mutated to show specific targeting.

We added that 9 out of the 10 compensatory base pairs are disrupted in miR-130a:Nkrf compared to miR-301a (SI Fig. S2).

4- The section title "mir-301 inhibition or Nkrf upregulation reduces xenograft growth" could be made simpler and clearer. Just state mir-301 inhibition reduces xenograft growth.

We made the change.

5- Despite the reduction in the number of figures, the paper remains enormous and the main message (of a novel miR-regulated NF-κB activating pathway that may cause cancer) seems to be lost. I would recommend working on shortening it a bit further.

Beyond scores of grammatical changes, we modified the manuscript with some major improvements. First, we streamlined the screening assay that identified miR-301a as the most potent NF-κB activator. This condensed three paragraphs into one. Second, we moved part of Figure 4 into a supplemental figure (SI Figure 6). Third, we compressed the description of data from 293T and HeLa cells into a few sentences from three paragraphs. The revised manuscript sticks to the following logic: identification of miR-301a as a potent NF-κB activator; finding of the target of miR-301a that inhibit NF-κB, Nkrf; that miR-301a is regulated by NF-κB; that miR-301a inhibition in pancreatic cancer cells reduces NF-κB activation; that Nkrf is down-regulated in pancreatic cancer (a missing link for the feedback loop); and that miR-301a inactivation attenuates tumor growth, a finding that may have therapeutic values. We believe that these modifications tightened the manuscript with a reduction of >10% of total words.