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MicroRNA-17~92 Plays a Causative Role in Lymphomagenesis by Coordinating Multiple Oncogenic Pathways

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Thomas Schwarz-Romond

Editor Pre-decision Correspondence 28 June 2013

I received comments from two scientists on your paper. Both appreciate that the paper adds to our understanding of miR17-92 functions in tumor formation.
However, both also emphasize that the major interest would come from the novel targets as identified by the PAR-CLIP approach. Therefore, two major concerns arise for efficient consideration here:

-Ref#1 finds the validations in figure 6/7/8 currently too limited, though offering constructive comments to overcome this.

-More importantly (and emphasized by both refs), significant novelty would arise from data that support the relevance of some of the new targets identified.

Please understand that I am NOT asking for a full, functional characterization at this stage. Rather, possible direct support for the regulation of some of the novel miR17-92 targets with some correlative evidence either by IF/tissue-arrays and/or publically available expression data would be fine and, as far as I am concerned, leave sufficient space for more detailed future characterization/publications.

I would like to give you the opportunity to position yourself to these comments with the hope that some of such work might already be underway in your lab and thus, could be added in a relatively timely manner.

I would be grateful to receive a written response, possibly outlining a few, though crucial experimental expansions BEFORE reaching a final decision how to proceed.

Please be aware that we fully understand the overall value and merit of your work, though it appears in light of the current critiques, a bit borderline for our general and highly selective title.

I look very much forward to your responds in this matter.

1st Editorial Decision 04 July 2013

Thank you very much for submitting your study on the tumorigenic role of the miR17-92 cluster, respective identification of its target spectrum for consideration to The EMBO Journal editorial office.

I also appreciate your recent communication that outlines current experimental limitations in further reaching target validation.

I do note however further experimental results that potentially increase the disease relevance of PI3K-, respective NF-kB signaling downstream of miR17-92.
Based on such amendments, I am happy to expose a suitably revised study once more to at least one of the original referees for final assessment.

Please be reminded that The EMBO Journal considers only one round of revisions and the ultimate decision on publication has to depend on its strength/positive external assessment of your amended manuscript.

REFEREE REPORTS:

Referee #1:

The study by Oda et al investigates the role of mir17-92 in lymphomagenesis. They use PAR-CLIP to identify miR target genes and confirm inhibition of several PI3K pathway regulators and new regulation of NfkB pathway members. Overall, this is a good study that expands on the role of miR17-92 in Myc driven lymphomagenesis. This is a crowded field, the regulation of PI3K genes has been reported previously and several studies have explored the topic especially in the EμMyc lymphoma model. However, the Parclip approach, the variety of lymphomas in the 17-92 transgenics, and the implication of NFkB are new and noteworthy.

Specific comments:
1) The authors should be more specific regarding the term lymphomagenesis esp. in the title. Exactly what human lymphoma is their study relevant to?
2) Figure 1 indicates lymphoma development in the transgenic 17-92 mice. My version appears to lack Panel F (the immune phenotype, which is important to define tumor pathology. Please add back).
3) Exactly how were the tumor pathologies defined in Fig. 2A?
4) Figure 3 shows the partial requirement for 17-92 in Myc driven lymphomas. It would very be interesting to know more about the genetics of the 'escape' tumors, e.g. do they activate PI3K signals through alternate lesions?
5) Figure 4 is largely confirmatory and may be best presented as a supplement.
6) Reports the par-clip data. 868 genes were identified. How were these genes prioritized. In essence, it appears the parclip provide no prioritization over simple target prediction which,

depending on program, seems to identify an equal number of targets. Along those lines, epigenetic regulators are not mentioned, however they are common targets of mutations in lymphoma and given that Parclip identified some 10% of the transcriptome it would be almost noteable if this class of lymphoma relevant genes was unaffected.
7) Figure 6/7/8 provide immunoblot and fucnitonal validation for selected targets. This is overall well done and some additional data could be added: Rb phosphorylation would provide a nice summary of cell cycle activation, similarly Akt phosphorylation would indicate relevance of Pten decrease. Luciferase assays confirming direct interaction of microRNA and UTR/CDS and RT-PCRs would provide additional confirmation. Again, it is not clear how these proteins were chosen from the parclip data.

Overall this is a good study that adds to our understanding of 17-92 and reports some new pieces of information. The parclip data provide a nice list of experimentally identified targets. However, it also leaves the reader to wonder which of these targets are important. The authors have clearly made a reasonable choice to focus on relevant pathways (cell cycle , PI3K,NFkB), however this comes at the cost of not finding new and potentially important interactions.

Referee #2:

In this study, Odo, Lai, and Jin et al. evaluated miR-17~92 as an oncomiR involved in lymphoma. Using a B cell-specific miR-17~92 knockout mouse model, they showed that the miRNA cluster is linked to Myc-mediated lymphomagenesis. They gained some mechanistic insights through the use of PAR-CLIP to identify miR-17~92 target genes in cultured human B cells. The authors then evaluated some of these targets which are germane to cancer and lymphoma by examining their activity/gene expression levels in primary B cells derived from a mir-17~92 overexpression transgenic mouse model.

The major claims are that (1) miR-17~92 is an oncomiR that is specifically involved in Myc-mediated lymphomagenesis, (2) moderate overexpression of mir-17~92 by itself in B cells leads to lymphoma in mice, and (3) miR-17~92 drives lymphomagenesis by suppressing negative regulators of the PI3K and NF B pathways as well as pro-apoptotic and cell cycle regulator proteins.

Comments:

1. As the first known miRNA oncogene, the implications of miR-17~92 as an oncomiR and its involvement with Myc-mediated lymphoma (see PMID: 20008931) are not very novel. However, the authors utilize certain nuances in their in vivo models that increase the novelty of the work; such as the CD19-driven knockout of miR-17~92 in order to avoid developmental issues that confound similar miR-17~92 knockout models (see PMID: 18329372).
2. High throughput target analysis has already been performed in miR-17~92 transgenic models; however, the authors' use of PAR-CLIP to identify miR-17~92 targets is a more robust/relevant method than computational or array-based techniques. The use of PAR-CLIP also provides some insights into miRNA biology that the authors briefly mention in the discussion; perhaps this should be expanded upon.
3. A criticism of the PAR-CLIP work is the use of EBV-transformed human B cells. Instead, PAR-CLIP investigation of the miR-17~92 overexpression/knockout models may yield results that are more relevant to miR-17~92, and aid in the identification of novel targets. This may by particularly pertinent given the cell line-specificity of miR-17~92 targets as mentioned by the authors.
4. The involvement of miR-17~92 in the PI3K pathway is well known and its involvement in the NF B pathway is not surprising. So, the novelty of the work could be strengthened through more thorough functional analysis (e.g. RNAi or overexpression studies) of the identified target genes.
5. Figure 1F appears to be missing.
6. The work should be well-received by a general audience as it involves pertinent miRNAs and disease phenotypes, cutting-edge techniques and mouse models, and the superb writing clarity and data presentation are appreciated.

1st Revision - authors' response 10 July 2013

Summary

We are grateful to the reviewers for their critical reading of our manuscript and very much appreciate their thoughtful and constructive suggestions and comments. Since the original submission, we have been working hard to improve our manuscript. We would like to summarize the progress we have made and setbacks encountered, and present new data that demonstrate the functional importance of the PI3K and NFkB pathways in miR-17~92-driven lymphomagenesis, which we believe significantly enhance our manuscript. We will also explain why some of the experiments proposed by the referees cannot be done or would not generate informative results.

Realizing the limitation of our target gene validation, we took several approaches to assess the impact of transgenic miR-17~92 expression on the mRNA and protein levels of PAR-CLIP-identified target genes. We performed microarray analysis of WT and miR-17~92 transgenic B cells and found that they share almost identical transcriptomes. The effect of transgenic miR-17~92 expression on its target gene mRNA levels is negligible. We also compared WT B cells and B cells deficient of all miR-17~92 family miRNAs and came to the same conclusion (data not shown). Therefore, miR-17~92 miRNAs predominantly act to regulate the translation of their target mRNAs.

We next took a mass spectrometry-based quantitative proteomics approach to assess the impact of transgenic miR-17~92 expression on the target gene protein levels. The currently available genome-wide proteomics technology allows us to quantify 4000-5000 proteins, most of which are cellular proteins of relatively high abundance. Only 200 or so (25%) PAR-CLIP-identified target gene proteins are present among the 5000 proteins we were able to quantify. These 200 or so quantifiable target genes do not show any significant difference in their protein levels between TG and WT B cells, and do not include any of the target genes validated in this study, suggesting that the protein levels of all validated miR-17~92 target genes are below the detection threshold of proteomics. Therefore, technology advancement is essential before proteomics can be used to validate miRNA target genes.

We have taken an in vivo approach to assess the functional relevance of the identified target genes in miR-17~92-driven lymphomagenesis. As shown in the new Figs. 9 and S7, we transplanted miR-17~92-driven lymphomas from TG mice into Rag1-/- mice and treated the recipient mice with chemical inhibitors that specifically block the PI3K pathway (AZD8055) or the NFkB pathway (BMS-345541). Strikingly, inhibition of either pathway significantly controlled tumor growth and prolonged the survival of lymphoma-bearing mice. These data demonstrate that miR-17~92-driven lymphomas are addicted to the PI3K and NFkB pathways, and suggest that there is potentially therapeutic value in treating patients bearing miR-17~92-driven lymphomas with inhibitors targeting these two pathways. These new results establish the functional relevance of the identified target genes, and will significantly enhance the novelty of our manuscript, because this demonstrates, for the first time to our knowledge, that chemical inhibition of miRNA downstream pathways has therapeutic value in treating cancers driven by miRNA dysregulation.

We have performed additional experiments to address referees' concerns and the results are now incorporated in the revised manuscript. Below please find a point-by-point response to the referees' comments. We hope that this revised manuscript is now suitable for publication in *The EMBO Journal*.

Referee #1:

The study by Oda et al investigates the role of mir17-92 in lymphomagenesis. They use PAR-CLIP to identify miR target genes and confirm inhibition of several PI3K pathway regulators and new regulation of NfkB pathway members. Overall, this is a good study that expands on the role of miR17-92 in Myc driven lymphomagenesis. This is a crowded field, the regulation of PI3K genes has been reported previously and several studies have explored the topic especially in the EμMyc lymphoma model. However, the Parclip approach, the variety of lymphomas in the 17-92 transgenics, and the implication of NFkB are new and noteworthy.

Specific comments:

1) The authors should be more specific regarding the term lymphomagenesis esp. in the title. Exactly what human lymphoma is their study relevant to?

Gene amplification and overexpression of miR-17~92 has been found in a broad spectrum of human cancers. As to human lymphomas, miR-17~92 overexpression was found in diffuse large B cell lymphomas, Burkitt lymphomas, mantle cell lymphomas, and other types of lymphomas. The main point of our study is to establish the causative role of miR-17~92 overexpression in the development of lymphoma (i.e. lymphomagenesis) in general. Consistent with the fact that miR-17~92 overexpression was found in many types of human lymphomas, mutant mice with B cell-specific miR-17~92 transgenic expression developed a broad spectrum of lymphomas. Therefore, our mouse model does not mimic any specific type of human lymphoma.

2) Figure 1 indicates lymphoma development in the transgenic 17-92 mice. My version appears to lack Panel F (the immune phenotype, which is important to define tumor pathology. Please add back).

This is our mistake. About half of the lymphoma cases are CD19+B220lowCD5+CD43+ (Fig. S1B), while the others are quite heterogeneous. The immune phenotype of each lymphoma case is presented in column E of Table S1.

3) Exactly how were the tumor pathologies defined in Fig. 2A?

The lymphoma pathological characterization is performed following the guidelines in the Bethesda proposals for classification of lymphoid neoplasms in mice (*Morse et al, Blood, 100:246-58, 2002*), in which an international panel of experts in mouse and human lymphomas constructed a proposed classification system with the goal of categorizing mouse lymphomas in ways that could be consistently recognized by pathologists and related to human disorders where possible. The classification of the spectrum of lymphoid proliferations and lymphomas developing in the miR-17~92 transgenic mice was performed by Dr. Kelly Bethel of Scripps Clinic Medical Group, an experienced subspecialist hematopathologist of human lymphomas, in practice for fifteen years with translational research experience. Cases were classified based on gross necropsy findings, immunophenotypic studies, cytology and histology of H&E fixed sections with selected immunohistochemistry, clonality assessment by Southern blot, and transplantability assessment. As with any series of lymphomas, and especially when the lymphomas are developing within an altered underlying immune system, not all cases will fall neatly into described categories and as the authors of the Bethesda proposals assert, the lymphoma classification system is an evolving entity in mice, as it is in humans. However, certain broad conclusions can be drawn from the spectrum of lymphomas observed in the miR-17~92 transgenic mice. The majority of cases meet the published criteria for classification as Diffuse Large B-cell Lymphoma, with the expected phenotype CD19+, B220+, IgM+ and the following histological and molecular characteristics:

(1) Sheets of large to intermediate sized, transformed-appearing lymphoid cells with numerous mitotic figures (Fig. 2A and Fig. S2A);
(2) A moderate to high proliferative rate as assessed by Ki-67 staining (Fig. 2A);
(3) Positive for Bcl6 (Fig. 2B, Table S1).

The TG mice also developed occasional cases of follicular lymphoma (FL), recognized predominantly by the following characteristics:

(1) Architectural follicularity (i.e. forming a variably nodular pattern) (Fig. S2A);
(2) Admixed centrocytic (cleaved) and centroblastic (non-cleaved) appearing lymphocytes (Fig. 2A);
(3) A low to moderate proliferative rate as assessed by Ki-67 staining (Fig. 2A);
(4) Positive for Bcl6 (Fig. 2B, Table S1).

One TG mouse developed anaplastic plasmacytoma (AP), recognized by the following characteristics:

(1) $CD138^+$ plasmablastic appearing cells (Fig. 2A);
(2) Numerous mitoses and a high Ki-67 positivity rate (Fig. 2A).

The major histological and molecular characteristics of lymphomas developed in miR-17~92 trasngenic mice are also described on pages 7~10 of the revised manuscript, and the detailed information on each lymphoma case is presented in Table S1.

4) Figure 3 shows the partial requirement for 17-92 in Myc driven lymphomas. It would very be interesting to know more about the genetics of the 'escape' tumors, e.g. do they activate PI3K signals through alternate lesions?

Figure 4 shows a detailed analysis of lymphomas developed in Myc;miR-17~92 fl/fl;CD19Cre mice. Strikingly, all lymphomas contained two intact alleles of miR-17~92 and exhibited upregulation of miR-17~92. They "escaped" CD19Cre-mediated miR-17~92 deletion by undergoing malignant transformation either in early B cell precursors (before CD19 promoter is turned on) or in the few mature B cells in which the CD19Cre is not turned on (please note that CD19Cre is turned on in about 93% of splenic B cells). Therefore, the requirement for miR-17~92 in Myc driven lymphomas is very stringent and there was not a single case of "escape" tumor that harbors miR-17~92 deletion in our colony.

5) Figure 4 is largely confirmatory and may be best presented as a supplement.

As discussed above, Figure 4 is important because it shows that all lymphomas developed in Myc;miR-17~92 fl/fl;CD19Cre mice contained two intact alleles of miR-17~92 and exhibited upregulation of miR-17~92.

6) Reports the par-clip data. 868 genes were identified. How were these genes prioritized. In essence, it appears the parclip provide no prioritization over simple target prediction which, depending on program, seems to identify an equal number of targets. Along those lines, epigenetic regulators are not mentioned, however they are common targets of mutations in lymphoma and given that Parclip identified some 10% of the transcriptome it would be almost noteable if this class of lymphoma relevant genes was unaffected.

To prioritize PAR-CLIP identified target genes, we performed pathway analysis of the 868 genes using the Ingenuity software and identified "molecular mechanisms of cancer" as the most enriched pathway (Fig. S4). From this category, we further narrowed down to a list of 18 target genes most likely implicated in lymphomagenesis based on published literatures (Fig. 5C). We then performed Western blot analysis of these 18 targets in TG and WT B cells to identify the ones whose proteins levels are significantly altered by transgenic miR-17~92 expression.

Bioinformatic programs often identify more target genes than PAR-CLIP. For example, TargetScan, one of the most commonly used miRNA target prediction program, identifies 1,700+ target genes for miR-17~92 miRNAs. Among these 1,700+ genes, only 397 overlaps with our PAR-CLIP-identified target genes. This is probably due to the fact that PAR-CLIP only identifies miRNA-mRNA interactions that actually occur in experimental cells, while bioinformatic programs identify miRNA-mRNA interactions that can theoretically happen without considering cellular contexts.

Epigenetic regulators are thought to control gene expression at the transcriptional level by chemically modifying histones or DNA. We performed microarray analysis to examine the impact of transgenic miR-17~92 expression on the transcriptome of B cells. In preliminary results we noticed that TG and WT B cells share almost identical transcriptomes (not shown). Therefore, we thought it was unlikely that transgenic miR-17~92 expression alters the protein levels of important epigenetic regulators, which would lead to significant changes in the transcriptome of TG B cells. Nevertheless, we examined the PAR-CLIP identified target gene list and found nine putative epigenetic regulators (Jarid2, Mbd2, Mbd6, Mbtd1, Med17, Med19, Mll2, Mll4, and Rcor1). Among

these nine, only Mll2 was previously implicated in lymphomagenesis. We are interested in exploring the role of Mll2, as well as other epigenetic regulators, in miR-17~92-driven lymphomagenesis in the future.

7) Figure 6/7/8 provide immunoblot and functional validation for selected targets. This is overall well done and some additional data could be added: Rb phosphorylation would provide a nice summary of cell cycle activation, similarly Akt phosphorylation would indicate relevance of Pten decrease. Luciferase assays confirming direct interaction of microRNA and UTR/CDS and RT-PCRs would provide additional confirmation. Again, it is not clear how these proteins were chosen from the parclip data.

We have now examined Rb phosphorylation. Consistent with our finding that TG B cells exhibit faster cell proliferation than WT B cells, Rb phosphorylation occurred earlier in TG B cells than in WT B cells. This result is now incorporated into Fig. 6D.

We also examined Akt phosphorylation but there was no difference between TG and WT B cells, though TG B cells exhibited significantly increased phospho-S6 levels (Fig. 7A). This suggests additional complexity in the regulation of PI3K pathway by miR-17~92.

As mentioned before, transgenic miR-17~92 expression on its target gene mRNA levels is negligible. Therefore, it is unlikely that RT-PCR analysis of target mRNA levels will reveal any significant difference between TG and WT B cells.

As we have discussed extensively in our manuscript, the consequence of miRNA-target mRNA interaction is dependent on cellular context. It has been shown by other investigators that miR-17~92 repressed the activity of luciferase reporters harboring the 3'UTR of Tgfbr2, a PAR-CLIP-identified miR-17~92 target gene. However, the Tgfbr2 protein levels are the same in TG and WT B cells (Fig. S5). In principle, all PAR-CLIP-identified miR-17~92 target gene mRNAs should interact with miR-17~92 miRNAs directly. Luciferase reporter assay measures the functional consequence of individual interactions in heterologous cell lines (e.g. HeLa, HEK293), and the functional consequence of these interactions may not necessarily be the same in B cells. Luciferase assay in B cells would overcome this problem, but B cells, esp. primary mouse B cells, are notoriously difficult to culture and transfect. Therefore, the value of luciferase reporter assay is questionable in the context of this manuscript.

Overall this is a good study that adds to our understanding of 17-92 and reports some new pieces of information. The parclip data provide a nice list of experimentally identified targets. However, it also leaves the reader to wonder which of these targets are important. The authors have clearly made a reasonable choice to focus on relevant pathways (cell cycle , PI3K,NFkB), however this comes at the cost of not finding new and potentially important interactions.

We are glad that the referee appreciated the value and merit of our work. We have taken an in vivo approach to assess the functional relevance of the identified target genes in miR-17~92-driven lymphomagenesis. As shown in the new Figs. 9 and S7, we transplanted miR-17~92-driven lymphomas from TG mice into Rag1-/- mice and treated the recipient mice with chemical inhibitors that specifically block the PI3K pathway (AZD8055) or the NFkB pathway (BMS-345541). Strikingly, inhibition of either pathway significantly controlled tumor size and prolonged the survival of lymphoma-bearing mice. These data demonstrate that miR-17~92-driven lymphomas are addicted to the PI3K and NFkB pathways, and suggest that there is potentially therapeutic value by treating patients bearing miR-17~92-driven lymphomas with inhibitors targeting these two pathways. These new results establish the functional relevance of the identified target genes, and will significantly enhance the novelty of our manuscript, because this demonstrates, for the first time to our knowledge, that chemical inhibition of miRNA downstream pathways has therapeutic value in treating cancers driven by miRNA dysregulation.

We attempted to use microarray and proteomics to assess the impact of transgenic miR-17~92 expression on the mRNA and protein levels of PAR-CLIP-identified target genes. The microarray approach did not generate any insights because miR-17~92 miRNAs predominantly act to regulate the translation of their target mRNAs, not their stability. The proteomics approach was not

productive either, because proteomics mainly quantifies proteins of high abundance, while all of our validated target genes are expressed at levels below the detection threshold of proteomics. Therefore, we cannot exclude the possibility that new and important miR-17~92 target genes exist and remain to be identified. Our PAR-CLIP-identified target gene list provides a valuable starting point for future investigation.

Referee #2*:*

In this study, Odo, Lai, and Jin et al. evaluated miR-17~92 as an oncomiR involved in lymphoma. Using a B cell-specific miR-17~92 knockout mouse model, they showed that the miRNA cluster is linked to Myc-mediated lymphomagenesis. They gained some mechanistic insights through the use of PAR-CLIP to identify miR-17~92 target genes in cultured human B cells. The authors then evaluated some of these targets which are germane to cancer and lymphoma by examining their activity/gene expression levels in primary B cells derived from a mir-17~92 overexpression transgenic mouse model.

The major claims are that (1) miR-17~92 is an oncomiR that is specifically involved in Myc-mediated lymphomagenesis, (2) moderate overexpression of mir-17~92 by itself in B cells leads to lymphoma in mice, and (3) miR-17~92 drives lymphomagenesis by suppressing negative regulators of the PI3K and NFκB pathways as well as pro-apoptotic and cell cycle regulator proteins.

Comments:
1. As the first known miRNA oncogene, the implications of miR-17~92 as an oncomiR and its involvement with Myc-mediated lymphoma (see PMID: 20008931) are not very novel. However, the authors utilize certain nuances in their in vivo models that increase the novelty of the work; such as the CD19-driven knockout of miR-17~92 in order to avoid developmental issues that confound similar miR-17~92 knockout models (see PMID: 18329372).

We are glad that the referee appreciated the value and merit of our work.

2. High throughput target analysis has already been performed in miR-17~92 transgenic models; however, the authors' use of PAR-CLIP to identify miR-17~92 targets is a more robust/relevant method than computational or array-based techniques. The use of PAR-CLIP also provides some insights into miRNA biology that the authors briefly mention in the discussion; perhaps this should be expanded upon.

We have now added a paragraph discussing the utility of applying the PAR-CLIP method to elucidate the molecular mechanisms of action of miRNAs, and inhibiting miRNA downstream pathways as a therapeutic avenue to treat human cancers driven by miRNA dysregulation.

3. A criticism of the PAR-CLIP work is the use of EBV-transformed human B cells. Instead, PAR-CLIP investigation of the miR-17~92 overexpression/knockout models may yield results that are more relevant to miR-17~92, and aid in the identification of novel targets. This may by particularly pertinent given the cell line-specificity of miR-17~92 targets as mentioned by the authors.

It would be ideal to perform PAR-CLIP analysis of B cells with miR-17~92 overexpression or deletion. However, in vitro culture and metabolically labeling of mRNA with 4SU is a critical step of this method, and primary mouse B cells are notoriously difficult to culture and most of them die after a few days in culture. We used EBV-transformed primary human B cells to overcome this viability problem. We used human B cells because EBV transforms only human B cells, not mouse B cells.

4. The involvement of miR-17~92 in the PI3K pathway is well known and its involvement in the NFκB pathway is not surprising. So, the novelty of the work could be strengthened through more thorough functional analysis (e.g. RNAi or overexpression studies) of the identified target genes.

There are two problems associated with this. First, there are no good in vitro models (i.e. cell-based assays) that mimic lymphomagenesis. Second, primary mouse B cells can only survive 2~3 days when activated and cultured *in vitro*, and they are resistant to transient transfection and infection by retroviruses or lentiviruses. Therefore, there is no good in vitro assays to evaluate the functional contribution of individual target genes to miR-17~92-driven lymphomagenesis. Instead, we have taken an in vivo approach to assess the functional relevance of the identified target pathways. As shown in the new Figs. 9 and S7, we transplanted miR-17~92-driven lymphomas from TG mice into Rag1-/- mice and treated the recipient mice with chemical inhibitors that specifically block the PI3K pathway (AZD8055) or the NFkB pathway (BMS-345541). Strikingly, inhibition of either pathway significantly controlled tumor growth and prolonged the survival of lymphoma-bearing mice. These data demonstrate that miR-17~92-driven lymphomas are addicted to the PI3K and NFkB pathways, and suggest that there is potentially therapeutic value by treating patients bearing miR-17~92-driven lymphomas with inhibitors targeting these two pathways. These new results establish the functional relevance of the identified target genes, and will significantly enhance the novelty of our manuscript, because this demonstrates, for the first time to our knowledge, that chemical inhibition of miRNA downstream pathways has therapeutic value in treating cancers driven by miRNA dysregulation.

5. Figure 1F appears to be missing.

This is our mistake. About half of the lymphoma cases are CD19+B220lowCD5+CD43+ (Fig. S1B), while the others are quite heterogeneous. The immune phenotype of each lymphoma case is presented in column E of Table S1.

6. The work should be well-received by a general audience as it involves pertinent miRNAs and disease phenotypes, cutting-edge techniques and mouse models, and the superb writing clarity and data presentation are appreciated.

Thanks again for the positive assessment of our manuscript and the encouragement.

2nd Editorial Decision 15 July 2013

Thank you very much for the revised study that has been assessed from one of the original referees (comments enclosed below).

Before formal acceptance, please notice that The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels/blots, with the aim to make primary data more accessible and transparent to the reader. This entails presentation of un-cropped/unprocessed scans for KEY data of published work. We would be grateful for one PDF-file per figure with such information. These will be linked online as supplementary "Source Data" files.

Please allow me to congratulate you to the study. I look forward to receiving relevant source data and assure you that the editorial office will be in touch soon with necessary paperwork related to official acceptance.

REFEREE REPORT:

Referee #1

I am satisfied with the revisions. The basic question that remains for me (and is beyond the scope of this MS): if miRs within this cluster interact with ~1000 mRNAs, how does this result in any significant effect on gene expression - there seems to be a stoichiometric problem that the field has not answered.