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MicroRNA-455 regulates brown adipogenesis via a novel HIF1an-AMPK-PGC1 signaling network

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Transfer Note:

Please note that this manuscript was originally submitted to The EMBO Journal where it was peer-reviewed. It was then transferred to EMBO reports with the original referees' comments and the authors' response, attached. (Please see below)

Original referees' comments and authors' response – The EMBO Journal

Response to Referees

We thank the referees for their insightful and constructive comments. We have taken significant efforts to incorporate the comments to considerably improve the manuscript. The point-by-point responses to the referees' comments are shown in black below.

Referee #1:

Zhang et al performed a microarray analysis and identified microRNAs differentially regulated in C3H10T/2 during differentiation to brown adipocyte lineage. For their further analysis they focused on miR-455, which exhibited a high level of expression in brown adipose tissue (BAT). miR-455 promotes brown adipocyte differentiation and miR-455 transgenic mice exhibited marked browning of subcutaneous white fat. As potential targets for miR-455, Zhan et al identify HIF1 α , Runx1t1 and Necdin.

A previous study had already shown that miR-455 is specifically upregulated during brown adipogenesis and that its expression correlates with UCP-1 (Walden et al. 2009).

Nevertheless, this is an interesting ms. However, several points need to be addressed.

Major points:

1. The authors need to mention the previous study that already showed regulation of miR-455 in brown adipocytes and the correlation with UCP-1 levels in the text. It is disturbing that the authors did not cite this paper by Walden et al.

Response: Thank you for the reminder. We have added Walden et al paper in the reference.

2. The authors need to provide the biostatistical analysis of the microRNA profiling (e.g. how many and which microRNAs were up- or down regulated) and deposit the microRNA profiling data so that they are accessible to the Reviewers as well as to the scientific community. The same is true for the mRNA screen.

Response: In this revised manuscript, we have included descriptions for biostatistical analysis (page 22: Materials and Methods, Identification of microRNAs). We will deposit the data of microRNA and mRNA arrays to the Gene Expression Omnibus (GEO) repository as soon as the paper is accepted for publication and include the accession number in the paper.

3. In primary white adipocytes, expression of miR-455 induces a large increase in the levels of PPAR γ (Fig. E3). This is in contrast, to the F442A cell line, which exhibited only modestly enhanced adipogenic differentiation (Fig.E2). The important question is, whether miR-455 enhances both the adipogenic AND the thermogenic program. Unfortunately, the authors used 2 different approaches to express miR-455 in the primary cells and the F442A cell line. To address this problem, the authors should express a similar level of miR-455 in primary SVF cells isolated from subcutaneous WAT as in F442A cells using lentiviral vectors.

Response: Given the fact that primary SVF cells and immortalized cell line F442A cells are two distinct types of cells, it is technically difficult to express miR455 to exact the same level in primary SVFs and in the 3T3-F442A cells. To address the question whether miR-455 can induce both adipogenic and thermogenic program, we, overexpressed two different levels of miR-455 in SVFs isolated from sWAT by lentiviral transduction (Supplementary Figure S4). The cells were then induced to adipogenic differentiation. Interestingly, expression of adipogenic genes PPAR γ and aP2 were significantly induced by miR-455 in a dose-dependent manner, suggesting that miR455 can enhance adipogenic program. To determine the effect of miR455 on thermogenic program, we treated the abovementioned cells with or without norepinephrine (NE) for 4 hrs on Day 10. While expression of thermogenic genes PGC1 α , UCP1 COXII was not significantly altered with miR455 overexpression, their expression was significantly increased in a miR-455 dose-dependent fashion upon NE treatment, suggesting that miR-455 increases the propensity of these primary cells derived from sWAT to NE by enhancing the thermogenic program. Notably, in this setting, the magnitude of induction of PPAR γ by miR-455 in sWAT-SVF cells are comparable to the level achieved in 3T3-F442A cell (Supplementary Figure S3D,E). Taken together, these data suggest that miR-455 plays significant and specific role in modulating both adipogenesis and thermogenic function.

4. What is the upstream regulator of miR-455? Bmp7 and miR-455 are not additive: of all the markers analysed, only aP2 levels are increased significantly in Bmp7 and miR-455 treated cells. This point needs to be addressed.

Response: Our data demonstrated that miR-455 is a downstream effector of BMP7 (Figure 1A and C,D) as well as cold exposure (Figure 1E). BMP7 induce Smads and p38MAPK/ATF2 signaling

pathways, and cold activates cAMP signaling via beta3-adrenergic receptor. Indeed, we performed a bioinformatics analysis and found that a number of Smad-binding elements (SBE) and cAMP-responsive elements (CRE) elements present in the promoter region of miR-455 (Supplementary Fig S2). SBEs mediate Smads-induced transcription and CREs are responsible for mediating p38MAPK/ATF2 and cAMP-induced transcription. We have added new statements to clarify the upstream regulators of miR-455 in the revised manuscript (page 6). Because BMP7 and miR-455 appear to function sequentially within the same signaling cascade and thus they only have minor, if any, additive effect as observed in our study (Figure 2G-I).

5. Cold/NE significantly suppresses Necdin. How much of this effect is mediated by miR-455? The authors should test this question by treating cells exposed to NE with and without the LNA anti-miR-455 inhibitor.

Response: In response to this comment, we transfected brown preadipocytes with LNA-Scramble control or LNA-anti-miR455, and treated the cells with or without NE, and then extracted protein for western blotting analysis (Data for reviewer Figure 1). NE treatment significantly suppressed Necdin protein level compared to vehicle treatment, and LNA-anti-miR455 transfection markedly upregulated the protein level of Necdin compared to Scramble transfection. Since miR-455 expression was also induced by NE treatment, in cells transfected with LNA-anti-miR455 and treated with NE, miR-455 expression was compromised. Consequently, cells receiving LNA-anti-miR455 and NE treatment displayed an intermediate level of Necdin protein expression between NE treatment alone and LNA-anti-miR455 transfection alone. These data suggest that miR-455 mediates at least part of NE's effect on Necdin protein expression. Since it is impossible to knockdown miR-455 expression completely using LNA transfection technique, a knockout strategy might warrant better addressing this question, but is beyond the scope of current study.

Minor points

- Runx1t1 inhibits cebpbeta. The authors need to measure the levels of cebpbeta and the activity of cebpbeta in miR-455 expressing cells.

Response: We examined C/EBPb gene expression by Q-RT-PCR in miR-455-overexpressing brown preadipocytes (Data for reviewer Figure 2A). While miR-455 significantly induced the expression of both general adipogenic genes (such as aP2) and brown fat-specific gene (such as UCP1), it did not significantly increase the expression of C/EBPbeta gene.

C/EBPb has been shown to transactivate C/EBPa, PPARG and aP2 by binding to the CCAAT-binding motifs of their promoters [1], and transactivate PGC1a by binding to cAMP-responsive element (CRE) of PGC1a promoter [2]. To address the regulation of C/EBPb transactivity by miR-455, we performed ChIP assay in vector control- and miR-455-overexpressing brown preadipocytes (Data for reviewer Figure 2B). The cells were first induced to adipogenic differentiation by standard differentiation protocol. 5 hours after induction of differentiation, cells were incubated in formaldehyde for crosslinking, and then harvested for total lysates (protein and genomic DNA) for ChIP assay. ChIP assay was performed according to standard ChIP protocol using anti-C/EBPb or control Normal antibody. The co-precipitated promoter DNAs were detected by PCR using primer pairs flanking the CCAAT binding motif on the promoters of C/EBPa, PPARG, aP2, and flanking the cAMP binding element (CRE) on PGC1a. Anti-C/EBPb antibody significantly enriched the promoter DNAs of C/EBPa, PPARG, aP2 and PGC1a, showing that C/EBPb efficiently bound to the promoter regions of these genes. Importantly, miR-455-overexpressing cells had significantly increased enrichment of these promoter DNAs, demonstrating that miR-455 overexpression significantly enhanced the binding of C/EBPb to the CAAT element of these gene promoters (Data for reviewer Figure 2B).

In addition, we also performed luciferase assay to examine the effect of miR-455 on C/EBPb transactivity (Data for reviewer Figure 2C). PGC1a promoter-luciferase construct was cotransfected with C/EBPb and/or miR-455 into brown preadipocytes to assay the transactivation of PGC1a promoter by C/EBPb. Cotransfection of C/EBPb significantly activated PGC1a promoter, consistent with previous report [2]. Importantly, cotransfection of miR-455 further increased PGC1a promoter activity in a dose-dependent manner. In the absence of C/EBPb, miR-455 had minimal effect on

PGC1a promoter activity. This data demonstrated that miR-455 potentiated C/EBP β on the transactivation of PGC1a promoter presumably through suppression of Runx1 β .

- In Fig.4c, the differences between in sWAT of wt and transgenic mice are not clearly visible. Please provide new histology data.

Response: We have added new clear figures of the histology data.

Referee #2:

Zhang et al. examined the role of Mir-455 in regulating brown adipogenesis in culture and in mice. The authors showed that Mir-455 enhanced brown adipogenesis, while inhibition of Mir-455 reduced adipogenesis in brown pre-adipocytes. In vivo studies demonstrated that Mir-455 overexpression promoted 10T1/2 cells to differentiate into brown adipocytes in implantation experiments, and transgenic animals overexpressing Mir-455 under the aP2 promoter exhibited greater energy expenditure, heat production and an improved metabolic phenotype. The authors suggest that Mir-455 regulates commitment of brown adipocyte lineage via inhibition of its targets Necdin, HIF1 α and modulation of the AMPK-PGC1 α axis. The authors provided compelling evidence supporting the conclusion and the data are interesting. On the other hand, there are several limitations that compromise the conclusions.

1. It remains unclear if Mir-455 really triggers "commitment" of brown fat lineage or simply activates adipogenesis. The authors should test if loss of Mir-455 in brown pre-adipocytes leads to white adipocyte or skeletal muscle lineage.

Response: We performed miR-455 lose-of-function study in brown preadipocytes by transfecting the cells with LNA-antimiR455 (main text Fig 3). To determine whether knocking down miR-455 renders these cells toward other cellular lineage, we examined the expression of Leptin (a white adipocyte marker) and MyoD (a myocyte marker). As shown in Data for reviewer Figure 3, the expression of neither Leptin nor MyoD was affected by loss of miR-455. These data shows that the expression of leptin and MyoD are not controlled by miR-455, and suggests that loss of miR-455 itself in committed brown adipocytes could not lead to white adipogenic or myogenic lineage.

2. The data in Figure 2 and 3 indicate that Mir-455 simply promotes adipogenesis and the PPAR γ pathway. C3H10T1/2 cells and brown preadipocytes were used for the cell culture experiments and implantation studies, however, the authors need to examine the Mir-455 effects in ScaPCs or in PDGFR α positive precursors.

Response: In response to this comment, we have performed new experiments in ScaPCs (Supplementary Fig S5). ScaPC cells established from sWAT were transduced with miR-455 or control (vector) lentiviruses, and were induced to differentiation by standard differentiation protocol (see Materials and Methods). On Day 8, the differentiated cells were treated with vehicle or 100 μ M norepinephrine (NE) for 4 hours. Consistent with other data shown in this manuscript (Figure 2, S3, S4), miR-455 overexpression significantly increased adipocyte differentiation of ScaPCs, as evidenced by increased expression of general adipogenic markers such as PPAR γ and aP2 and enhanced lipid accumulation. Expression of brown fat-specific genes (UCP1, PGC1 α , and PRDM16) and mitochondria gene (COXII) only showed trends towards increased expression without NE treatment. NE treatment significantly potentiated miR-455-induced expression of brown fat-specific genes. These data demonstrated that miR-455 was able to commit ScaPC cells to the brown adipogenic lineage, providing one possible mechanism for the browning of sWAT in aP2-miR455 transgenic mice.

3. Phosphorylation of AMPK and PGC1 α by Mir-455 is interesting. The authors should validate this finding in vivo (for example, transgenic mice).

Response: In response to this comment, we isolated both BAT and sWAT from WT and FAT455 transgenic mice, and analyzed AMPK α 1 phosphorylation by western blotting. FAT455 mice showed significantly higher level of AMPK α 1 phosphorylation compared to WT mice (Supplementary Fig S12B), consistent with the in vitro observation (Fig 6A). Serine and Threonine phosphorylation of

PGC1a was accessed by western blotting after immunoprecipitation using anti-PGC1a antibody. FAT455 mice showed significant increases in Ser- and Thr-phosphorylation of PGC1a compared to WT littermates in both sWAT and BAT (Supplementary Fig S12C), consistent with in vitro result (main text Fig 6F). We have added these new data in this revised manuscript (Page16).

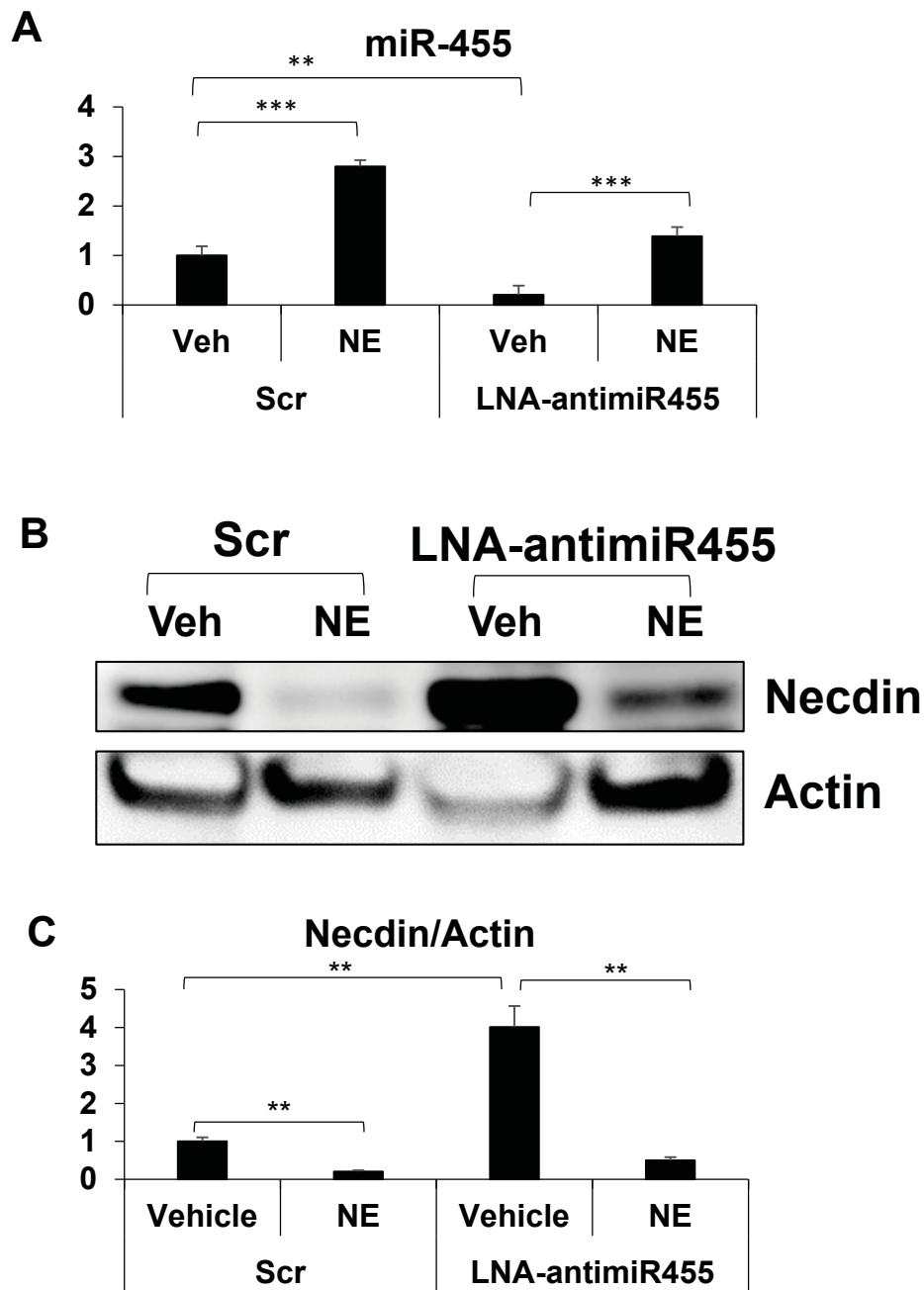
4. Mir-455 promoted brown adipogenesis in fat implantation studies without any stimuli, however, transgenic Mir-455 expression induced UCP1 expression in the transgenic mice upon cold exposure or norepinephrine stimulation. This is puzzling and needs to be reconciled. Is it because expression of Mir-455 in adipose implantation was much higher than the "physiological" level?

Response: The regular room temperature condition (23 °C) can activate brown adipogenic activity of a mouse. This phenomenon is more pronounced for the nude mice as they do not have furs. Indeed, transplanted brown adipocyte precursor cells into nude mice have been reported to activate brown adipogenic program without further cold exposure [3-5]. Thus, we were able to observe brown/beige fat activation in nude mice housed in the room temperature environment and in the aP2-miR455 transgenic mice exposed to cold or upon beta3 adrenergic stimulation.

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3. Schulz TJ, Huang TL, Tran TT, Zhang H, Townsend KL, Shadrach JL, Cerletti M, McDougall LE, Giorgadze N, Tchkonina T, Schrier D, Falb D, Kirkland JL, Wagers AJ, and Tseng YH (2011) Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. *Proc. Natl. Acad. Sci. U. S. A.*, **108**: 143-148.
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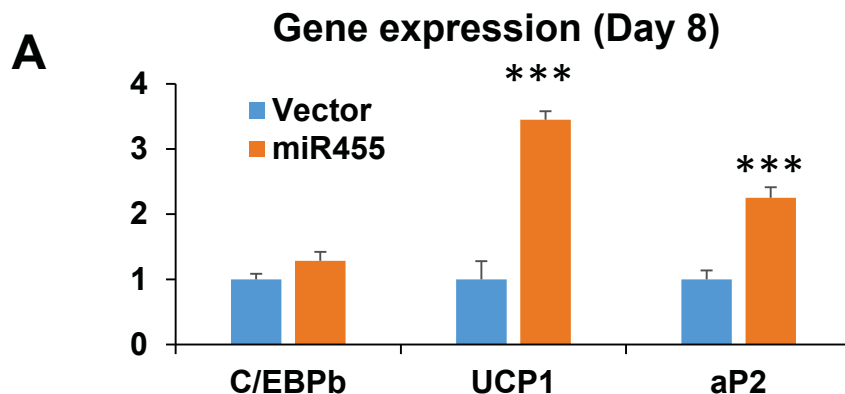
Data for Reviewer Figure 1



Brown preadipocytes were transfected with LNA-antimiR455 or Scramble control at 85% confluence. 24 hours after transfection, the cells were treated with norepinephrine (NE) for 48 hours. Then RNA and protein were isolated for Q-RT-PCR and western blot analysis. Expression of miR-455 (A) and one representative blot (B) was shown. Densitometry (C) was performed from three blots.

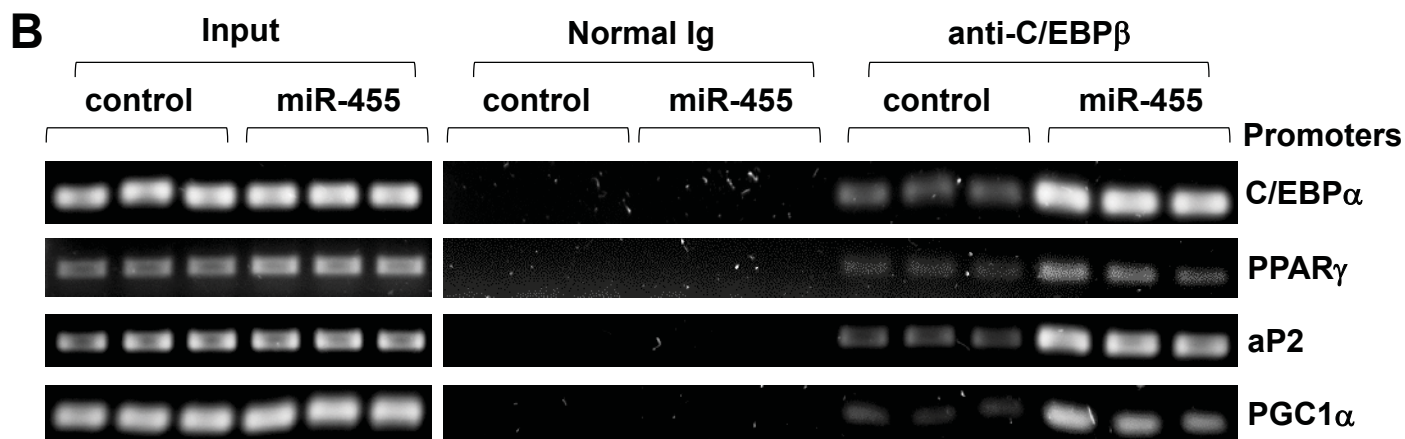
Discussion: NE treatment significantly suppressed Necdin protein level compared to vehicle treatment, while LNA-antimiR455 transfection significantly upregulated the protein level of Necdin compared to Scramble transfection. Importantly, further treatment of LNA-antimiR455-transfected cells with NE brought down Necdin protein to the intermediate level between NE treatment alone and LNA-antimiR455 transfection alone. All these data strongly support the notion that miR-455 mediates most of NE's effect on Necdin protein expression.

Data for Reviewer Figure 2



Brown adipocytes overexpressing miR-455 or control (vector) were differentiated by standard differentiation protocol. On Day 8, RNA were harvested for gene expression analysis by Q-RT-PCR.

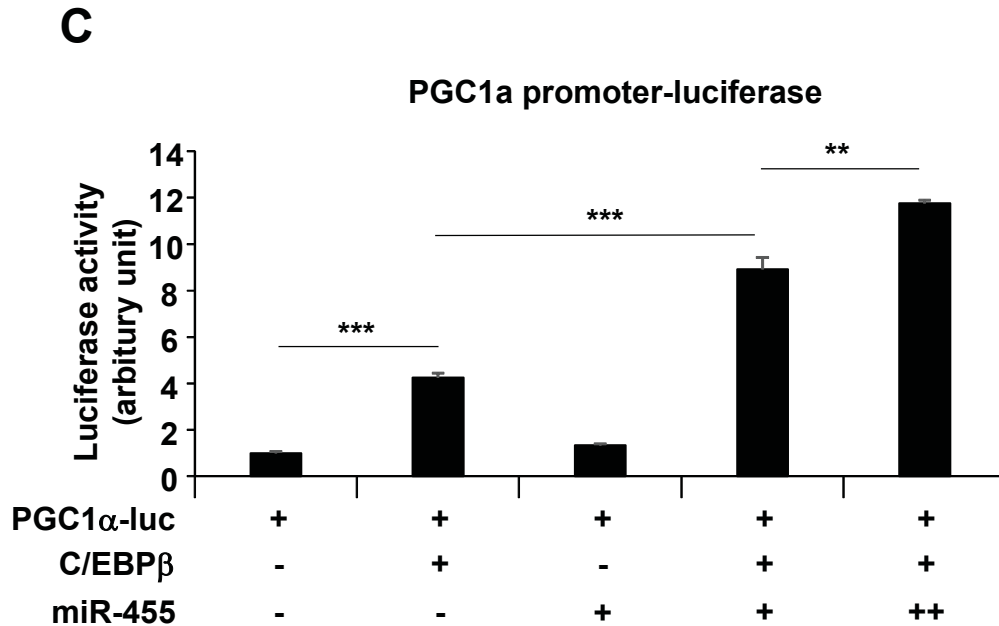
Discussion: While miR-455 overexpression significantly induced expression of brown fat-specific marker (like UCP1) and general adipogenic marker (like aP2), it did not significantly affect C/EBP β expression.



Brown preadipocytes overexpressing miR-455 or control (vector) were differentiated by standard differentiation protocol. 5 hours after differentiation, cells were crosslinked by formaldehyde and harvested for total protein and genomic DNA for ChIP assay. At the time point of 5 hours after differentiation, C/EBP β has expressed to 100% level (Lane (1999) 266, 677–683), but Runx1t1 has not been suppressed significantly (Rochford (2004) MCB 24: 9863–9872). Therefore, this time point choice allows us to catch the effect of miR-455 on Runx1t1 suppression and hence its effect on C/EBP β transactivity. ChIP assay was performed according to standard ChIP protocol using anti-C/EBP β antibody and primer pairs flanking the CCAAT binding motif on the promoters of C/EBP α , PPAR γ , aP2, and flanking the cAMP binding element (CRE) on PGC1 α . 20% total lysates were used as input loading control.

Discussion: Anti-C/EBP β antibody significantly enriched the promoter DNAs of C/EBP α , PPAR γ , aP2 and PGC1 α , showing that C/EBP β efficiently bound to the promoter regions of these genes. Importantly, miR-455-overexpressing cells had significantly increased enrichment of these promoter DNA, demonstrating that miR-455 overexpression significantly enhanced the binding of C/EBP β and hence its transactivity to the CAAT element of these gene promoters. This is achieved through miR-455-mediated suppression of Runx1t1.

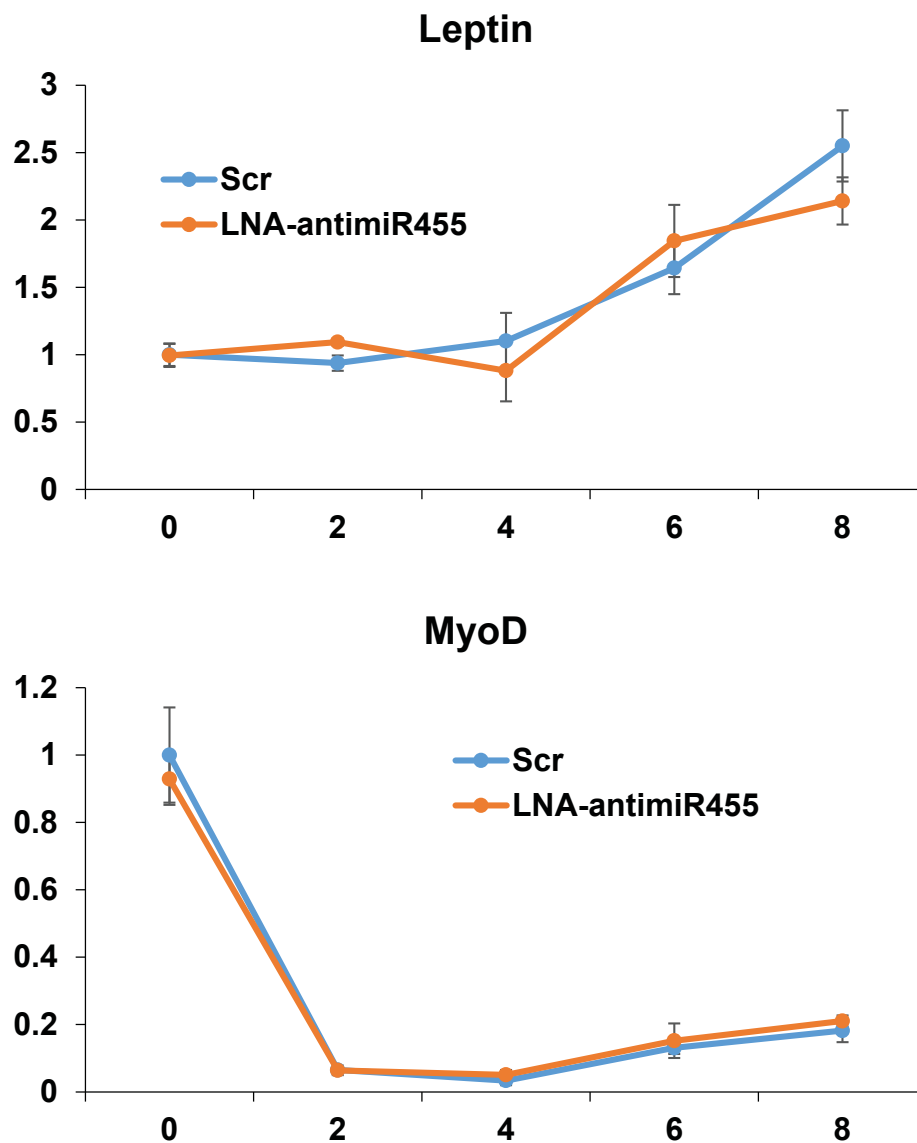
Data for Reviewer Figure 2



PGC1a promoter(2kb)-luciferase (firefly) construct was cotransfected with C/EBPb, miR-455 or control expression vectors and TK-Rluciferase (renilla) into brown preadipocytes (TK-Rluc is presented in all transfection as internal control) 24 hours after transfection, the cells were treated with 10uM forskolin in serum-free condition for 12 hours. The cell lysates were then harvested for luciferase activity assay. The firefly luciferase activity was normalized to renilla luciferase activity. Student's t-test was used for statistical analysis, and mean \pm SEM from quadruplicates are shown.

Discussion: Cotransfection of C/EBPb significantly activated PGC1a promoter, consistent with previous report (Karamanlidis et al (2007) 282: 24660–24669). Importantly, cotransfection of miR-455 further increased PGC1a promoter activity in a dose-dependent manner. However, in the absence of C/EBPa, miR-455 had only minimal effect on PGC1a promoter activity. This data demonstrated that miR-455 potentiated C/EBPb on the transactivation of PGC1a promoter.

Data for Reviewer Figure 3



Brown preadipocytes were transfected with scramble or LNA-antimiR455 at 85% confluency. Two days after transfection, the cells were induced to differentiate by standard differentiation protocol (see Experimental Procedures Materials and Methods). Cells were harvested at the indicated time points, and RNAs were harvested and quantified by Q-RT-PCR for gene expressions.

Discussion: LNA-antimiR455 knockdown in brown preadipocytes did not suppress Leptin (white fat marker) expression or induce MyoD (myogenic marker) expression, showing that knockdown of miR-455 itself did not lead to white adipogenic or myogenic lineage.

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed comments from the referee that was asked to assess it, and I am happy to tell you that we can in principle accept the manuscript now.

The referee notes that the "data for reviewers" should be included in the manuscript, and that the miRNA and mRNA data must be submitted and an accession numbers for these data included in the manuscript.

Regarding statistics, please define "n", the error bars (SEM, SD, etc), tests used to calculate p-values and "*" in each figure legend. This also applies to all supplementary figures. The figures and their legends need to be self-explanatory. Please note that if n=2 no error bars can be shown and no statistics calculated. Please either repeat the experiments at least one more time, or remove the error bars and show the individual data points instead along with their mean.

EMBO press has changed the presentation of supplementary data to expanded view now, please see our guide for authors online for more details. Basically, we can embed expanded view files in the manuscript text now as expandable items. Unfortunately, this cannot be done for more than 5 expanded view figures at the moment, as we are in a transition phase. Can you therefore please choose the 5 most important supplementary data, label them EV1-5, and include the legends for these figures in the main manuscript file, after the main figure legends. The remaining supplementary data and tables need to be combined into the Appendix file.

I am looking forward to receiving the final files as soon as possible.

REFeree REPORTS:

Referee #1:

The authors have addressed the questions of the Reviewer.

However, there are two things that have to be addressed:

The figures shown in "data for reviewer" should be included in the ms (e.g. supplement).

The authors stated they will submit the profiling data "as soon as the paper is accepted". This should have been done before, so that the Reviewers would have been able to review also these data.

Please make sure that these data will be submitted.

1st Revision - authors' response

19 July 2015

Comments of Referee #1:

The authors have addressed the questions of the Reviewer.

However, there are two things that have to be addressed:

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Please make sure that these data will be submitted.

Responses to Referee 1's comments:

1. We have incorporated the original "data for reviewer" to the manuscript. Specific changes of figures are listed below

Data for Reviewer Figure 1 → Appendix Figure S7

Data for Reviewer Figure 2 → Appendix Figure S9

Data for Reviewer Figure 3 → Appendix Figure S4D

Other figures with modifications are Figure EV1, 2 and 5

2. We have deposited the expression data of C3H10T1/2 cells into the Gene Expression Omnibus, and have included the GEO accession number (GSE71157) in the revised text (page 21 and 22 of revised text).

2nd Editorial Decision

24 July 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.