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SHORT COMMUNICATION

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sta-1 is repressed by *mir-58* family in *Caenorhabditis elegans*

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ABSTRACT

The miR-58 family comprises 6 microRNAs with largely shared functions, and with an overall high expression, because one of its members, miR-58, is the most abundant microRNA in *Caenorhabditis elegans*. We recently found that 2 TGF-β signaling pathways, Sma/Mab and Dauer, responsible for body size and dauer formation respectively, among other phenotypes, are downregulated by the miR-58 family. Here, we further explore this family by showing that it also acts through the *sta-1* 3′UTR. *sta-1* encodes a transcription factor, homologous to mammalian STATs, that inhibits dauer formation in association with the TGF-β Dauer pathway. We also observe that mutants with a constitutively active TGF-β Dauer pathway express higher levels of *sta-1* mRNA. Our results reinforce the view of the miR-58 family and STA-1 as regulators of dauer formation in coordination with the TGF-β Dauer pathway.

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KEYWORDS
Dauer; microRNAs; mir-58; sta-1; TGF-β Dauer

Introduction

Most microRNA-target interactions are merely suggested through genome-wide studies, which involve the analyses of transcriptomes or proteomes physically associated to microRNAs.[1] These studies are essential for outlining networks of putative interactions between miRNAs and their target genes. However, for the confirmation of particular miRNA-target pairings, individual gene-reporter assays are preferred.[2-4] The main reason for this is the greater reliability of such validations, with well-controlled gene-reporter experiments instead of large-scale miRNA/3′UTR "binding" assays. Besides, those discrete experiments are usually interpreted in the context of particular phenotypes and/or cell-signaling pathways, thus implying a functional focus, rather than a more structural one.

We have recently shown through *in vivo* and *in vitro* gene-reporter assays that the miR-58 family regulates 2 TGF-β signaling pathways, Sma/Mab (controlling body size, immune response and other phenotypes) and Dauer (inhibitor of the dauer larval stage).[5] That makes the miR-58 family, miR-58f hereafter, a potential key regulator of growth and stress-related responses, which is consistent with the role of the miR-58f ortholog in *Drosophila*, bantam.[6,7]

In *C. elegans*, miR-58f consists of a set of 6 miRNAs: miR-58.1 (or simply miR-58), miR-58.2 (or miR-1834), miR-80, miR-81, miR-82 and miR-2209.1. Although the expression of these 6 miRNAs is different both quantitatively and spatiotemporally across the various tissues or organs, they present a high degree of functional redundancy.[5,8-10] Thus, while single mutants hardly present any phenotypic abnormality, an available mutant lacking 4 miR-58f members (miR-58.1, miR-80, miR-81 and miR-82), *mir-58f(−)* hereafter, shows remarkable defects in body size, locomotion, and is unable to form dauer larvae.[11] The unaffected *mir-58.2* and *mir-2209.1* in *mir-58f(−)* are unlikely to compensate at all for the 4 missing miRNAs in *mir-58f(−)*, because the expression of *mir-58.2* and *mir-2209.1* is "essentially undetectable."[10] In fact, Warf et al. (2011) were unable to fully validated them as miRNAs.[12] Therefore, the quadruple mutant *mir-58f(−)* appears to have, at most, just a residual miR-58f activity.

Here we further investigate how the miR-58f may regulate dauer formation. In our recent report, we show that miR-58f directly inhibits the expression of *daf-1* and *daf-4*, which code for the heteromeric cell-membrane receptor of the TGF-β Dauer pathway.[5] Additionally, the dauer defective

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phenotype of *mir-58f(−)* was suppressed by the inactivation of *daf-1* in the *mir-58f(−)* background, suggesting that the observed activation of the TGF-β Dauer signaling in *mir-58f(−)* was responsible for its inability to form dauer larvae.[5]

However, microRNAs are thought to regulate the expression of hundred of genes, and therefore, other genes related to the TGF-β Dauer pathway might be controlled by miR-58f. In that regard, our computational predictions of miR-58 targets offered an interesting candidate: *sta-1*. Mammalian STAT (Signal Transducer and Activator of Transcription) transcription factors regulate many aspects of growth, survival and cell differentiation.[13,14] In *C. elegans* two STAT orthologs, *sta-1* and *sta-2*, are present.[15] In spite of their potential relevance, both *sta-1* and *sta-2* have been hardly studied. GFP reporter studies suggest that *sta-1* is expressed in a wide range of tissues including pharynx, intestine, body muscle and neuronal cells.[16] Nevertheless, *sta-1* absence only shows a Hid (high temperature induction of dauer) phenotype.[17] Double mutants of *sta-1* and TGF-β Dauer genes display a synthetic dauer phenotype which suggests that STA-1 regulates dauer formation in cooperation with the TGF-β Dauer pathway.[17]

Here we show that the miR-58 family also represses *sta-1* expression. Using heterologous assays in HeLa cells, we observed that this repression is dependent on 3 nucleotides of the *sta-1* 3′UTR that are complementary to the miR-58f seed region, which strongly suggests a direct interaction. In agreement with that, we also found that *sta-1* is upregulated in a constitutively active TGF-β Dauer background. Our results suggest a new target of miR-58f regulating dauer formation, STA-1, acting in cooperation with the TGF-β Dauer pathway.

Results and discussion

sta-1, *predicted target of* mir-58 *family*

To address the question of whether other genes related to the TGF-β Dauer cell-signaling pathway could also be under the control of miR-58f, we first did a computational search. We used various software programs to predict miRNA/3′UTR interactions, and most of them listed *sta-1* as a putative target of at least one of the 6 members of miR-58f. In particular, 5 programs predicted *sta-1*/miR-58f interactions: TargetScan Worm 6.2,[18,19] miRanda-mirSVR,[20] MicroCosm Targets, PITA[21] and RNAhybrid.[22] TargetScan and RNAhybrid find binding sites for 5 of the 6 miR-58f members, miRanda-mirSVR and PITA for 4 members, and MicroCosm Targets just for miR-80. These 5 bioinformatic resources use different algorithms that take into account features such as the quality of

Table 1. Predicted binding positions of miR-58 family members to *sta-1* 3′UTR.

TargetScan Worm 6.2

sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUCUGAUCUCA.. 3′
cel-miR-80	3′ AGCCGAAAGUUGAUUACUAGAGU 5′
sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUC———UGAUCUCA.. 3′
cel-miR-81	3′ UGAUCGAAAGUGCUACUAGAGU 5′
sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUC———UGAUCUCA.. 3′
cel-miR-82	3′ UGACCGAAAGUGCUACUAGAGU 5′
sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUCUGAUCUCA.. 3′
cel-miR-1834 (mir-58.2)	3′ AACCUAGAGUUACCAACUAGAGA 5′
sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUCUGAUCUCA.. 3′
cel-miR-2209.1	3′ ACAUCACAUUGGCGACUAGAGA 5′

miRanda-mirSVR

sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUCUGAUCUCA.. 3′
cel-miR-58.1	3′ UAACGGCAUGACUUGCUAGAGU 5′
sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUCUGAUCUCA.. 3′
cel-miR-80	3′ AGCCGAAAGUUGAUUACUAGAGU 5′
sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUC———UGAUCUCA.. 3′
cel-miR-81	3′ UGAUCGAAAGUGCUACUAGAGU 5′
sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUC———UGAUCUCA.. 3′
cel-miR-82	3′ UGACCGAAAGUGCUACUAGAGU 5′

Microcosm Targets

sta-1 3′UTR (165–187)	5′..AUCACAAGUGAUUUCUGAUCUCA.. 3′
cel-miR-80	3′ AGCCGAAAGUUGAUUACUAGAGU 5′

RNAhybrid

sta-1 3′UTR (171–187)	5′…———AGUGAUUUCUGAUCUCA.. 3′
cel-miR-2209.1	3′ ACAUCACAUUGGCGACUAGAGA 5′
sta-1 3′UTR (168–187)	5′————ACAAGUGAUUUCUGAUCUCA..3′
cel-miR-80	3′AGCCGAAAGUUGAUU ——ACUAGAGU 5′
sta-1 3′UTR (165–187)	5′….CACAAGUGAUUUC———UGAUCUCA. 3′
cel-miR-81	3′ UGAUCG—AAAGUGCUACUAGAGU 5′
sta-1 3′UTR (165–187)	5′…….AUCACAAGUGAUUUCUGAUCUCA. 3′
cel-miR-82	3′UGACCGAAAGUG———CU———ACUAGAGU 5′
sta-1 3′UTR (171–187)	5′…AGUGAUUUC————UGAUCUCA.. 3′
cel-miR-1834 (mir-58.2)	3′ AAC–CUAGAGUUACCAACUAGAGA 5′

Note. In gray, positions mutated for the luciferase-reporter assays.

match at seed regions, outside of them, conservation across species, thermodynamic parameters, site accessibility, etc. Importantly, all these software programs identified the same region at *sta-1* 3′UTR, regardless of the miR-58f member recognized in the match. Table 1 shows the predicted binding sites in *sta-1* 3′UTR found in all these programs, with the exception of PITA, which does not render such detailed information.

sta-1 *is upregulated in the* mir-58f *mutant*

To test the hypothesis that miR-58f represses *sta-1* transcripts, we began by comparing the mRNA levels of *sta-1* between the quadruple mutant *mir-58f(−)* (missing miR-58.1, miR-80, miR-81 and miR-82) and the wild-type N2. We found that the expression of *sta-1* was significantly higher in *mir-58f(−)* (Fig. 1; $P < 0.001$). In particular, we observed a 4.3-fold increment in mixed-stage populations. In close agreement with our results, Subasic et al.[10] have recently analyzed the transcriptome of *mir-58f(−)* worms at stage L4 and found a 2-fold increase of *sta-1* mRNA compared to N2 ($P = 0.002$).

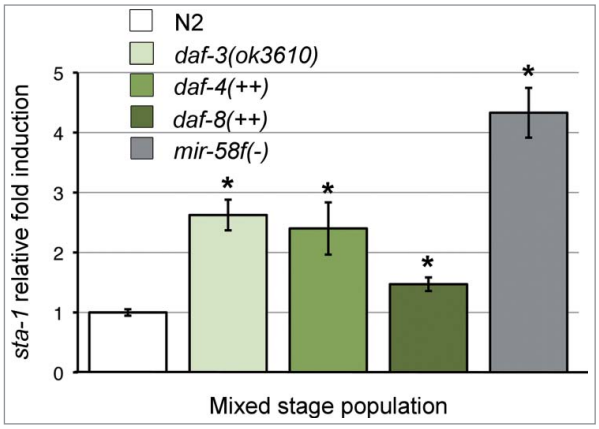

Figure 1. *sta-1* mRNA levels are upregulated in the *mir-58* family mutant and in worms with a constitutively active TGF-β Dauer pathway. mRNA levels of *sta-1* were measured in mixed-staged populations of wild-type strain N2, the quadruple mutant *mir-58f(−)* (MT15563) and 3 additional strains with constitutively active TGF-β Dauer signaling: *daf-3(ok3610)* (RB2589), *daf-4(++)* (*pwIs922*), and *daf-8(++)* (DR2490). Measurements were carried out by quantitative Real-Time PCR with a specific TaqMan probe for *sta-1*. mRNA levels of mutant strains (color bars) were normalized to those of N2 (white bars). Each value represents the average from 4 independent biological replicates. Error bars indicate standard deviations. Significant statistical differences between each mutant and N2 are indicated as $^{*}(P < 0.001)$.

sta-1 *3′UTR mediates repression by* mir-58 *family members*

Our qPCR results (Fig. 1) are consistent with *sta-1* expression being under the control of miR-58f, but qPCR analyses cannot discriminate between direct or indirect interactions between any microRNA and a putative target gene. Therefore, we used a heterologous luciferase-reporter assay in human HeLa cells to investigate whether *sta-1* is a direct target of miR-58f. To that end we cloned the 3′UTR sequence of *sta-1* downstream of the renilla luciferase gene and transfected this construct together with each of the members of miR-58f (except miR-2209.1; and miR-82, because this one only differs from miR-81 in one nucleotide out of the seed region) into HeLa cells.

We observed strong decreases in luciferase activity when comparing co-transfections of wild-type *sta-1* 3′UTRs with each of the miR-58f members, to the co-transfections of the same 3′UTRs but with a standard control miRNA, miR-67 (which was absent from the lists of predicted miRNAs binding *sta-1* 3′UTR). Thus, luciferase activity was between 48% (miR-58.1) and 65% (miR-58.2) lower with miR-58f members than with miR-67 ($P < 0.005$; white columns in Fig. 2). Furthermore, this repression is dependent on the predicted miR-58f binding sites on *sta-1* 3′UTR, because when those sites were mutated luciferase repression was largely abolished (Fig. 2, gray columns; $P < 0.005$). In particular, we show that the miR-58f-mediated luciferase repression was dependent on just 3 nucleotide positions of *sta-1* 3′UTR (CUC mutated to GAG), complementary to sites 2–4 of miR-58f seed-region (Table 1). We conclude that *sta-1* is directly repressed by miR-58f (or at least by the 4 tested miRNAs of the family) through its 3′UTR in a heterologous system, suggesting an equivalent *in vivo* action.

Zisoulis et al.[23] and Grosswendt et al.[24] sequenced thousands of mRNA fragments associated to Argonaute (ALG-1), and therefore likely targets of miRNA regulation. Consistently with our results, both groups found 3 different regions of *sta-1* bound to ALG-1. One of these regions (chromosome IV: 16671301-16671432) contained the 3′UTR fragment that we have shown to be a target for miR-58f (Table 1 and Fig. 2). In summary, *sta-1* appears a *bona fide* miR-58f target gene.

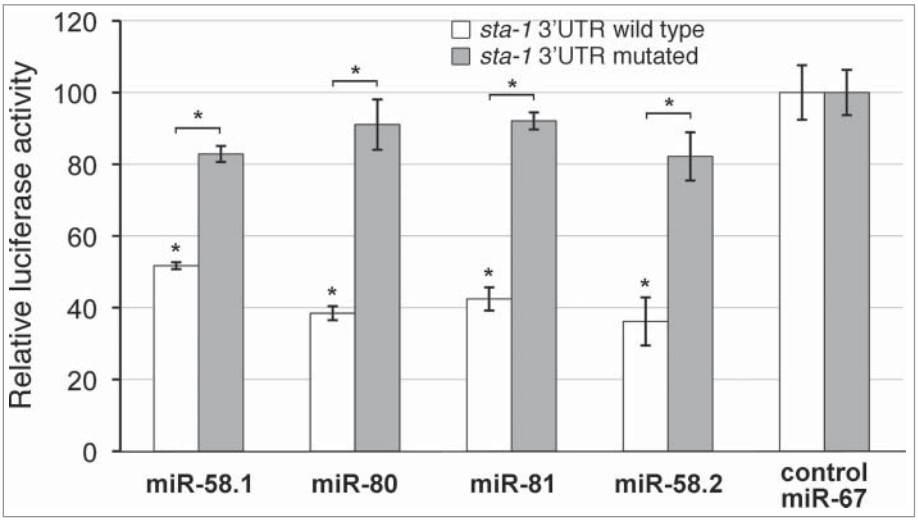

Figure 2. Luciferase-reporter assays show that miR-58 family members repress gene expression through the *sta-1* 3′UTR. Human HeLa cells were transiently transfected with psiCHECK-2 vector containing either the wild-type (white) or mutated (gray) 3′UTR from *sta-1*, along with miR-58 family mimics of miR-58.1, miR-80, miR-81 and miR-58.2 (i.e. miR-1834), or the unrelated miR-67 as negative control. The luciferase activity for each mimic was normalized to the value obtained with miR-67 using the same *sta-1* 3′UTR. Data shown are representative from 2 independent experiments. Error bars indicate standard deviations. *($P < 0.005$), comparing luciferase activities for each inhibitory miRNA to miR-67s (white bars), and between wild-type and mutated *sta-1* 3′UTRs for each microRNA (pairs of white and gray bars).

The activity of the TGF-β Dauer pathway upregulates sta-1 *expression*

As already explained, STA-1 is known to inhibit dauer formation because of the higher percentage of dauers at 27°C of *sta-1(−)*, compared to N2. Moreover, it is thought to interact with at least DAF-1 and DAF-8, 2 proteins of the TGF-β Dauer pathway. However, in apparent contradiction to the above, dauer-inhibitory TGF-β Dauer signaling appears to repress STA-1 activation in a particular set of sensory neurons, some of which are known to be key in the dauer decision.[17] Trying to shed some light on this matter, we then studied whether TGF-β Dauer activation would indeed downregulate *sta-1* expression, or more in accordance with *sta-1* inhibitory role on dauer entry, activation of TGF-β Dauer could instead promote *sta-1* expression.

Accordingly, we compared *sta-1* mRNA levels between N2 and 3 mutants where the TGF-β Dauer pathway is constitutively upregulated.[5,25] We used worms overexpressing *daf-4* (whose protein product is part of the TGF-β Dauer receptor) or *daf-8* (coding for a Smad transcription factor of TGF-β Dauer), as well as a *daf-3* null mutant (DAF-3 is a Co-Smad antagonizing TGF-β Dauer).[26] We found that in all these backgrounds, *sta-1* transcripts were significantly more abundant than in wild-type worms (Fig. 1). We conclude that, in at least mixed-staged populations, TGF-β Dauer signaling appears to stimulate the expression of *sta-1*. This fits well with the role of both, TGF-β Dauer and STA-1, as facilitators of the reproductive developmental program. In our view, however, 2 fundamental questions deserve further work in relation to STA-1/TGF-β Dauer. First, how to reconcile the inhibitory role of STA-1 on dauer formation with its deactivation by TGF-β Dauer in discrete sensory neurons (perhaps its role in those neurons is unrelated to dauer); and second, what is the precise molecular role of STA-1 in relation to the components of TGF-β Dauer.

We summarize our results in Fig. 3 by which the *sta-1* upregulation observed in *mir-58f(−)* (Fig. 1) seems to derive from 2 sources. First, from the impairment of miR-58f to act directly on *sta-1* 3′UTR (Fig. 2), and second, from the activation of TGF-β Dauer caused by the miR-58f null mutations[5] – because that activation, in turn, would upregulate the expression of *sta-1* (Fig. 1). On the other hand, the overexpression of *sta-1* in *daf-3(−), daf-4(++)* or *daf-8 (++)* are only due to the activation of TGF-β Dauer. That may explain why *sta-1* mRNA levels are considerably higher in *mir-58f(−)* compared to *daf-3(−), daf-4 (++)* or *daf-8(++)* mutants (Fig. 1). Finally, we suggest that perhaps the double control of *mir-58f* on dauer, through the TGF-β Dauer pathway and *sta-1*,

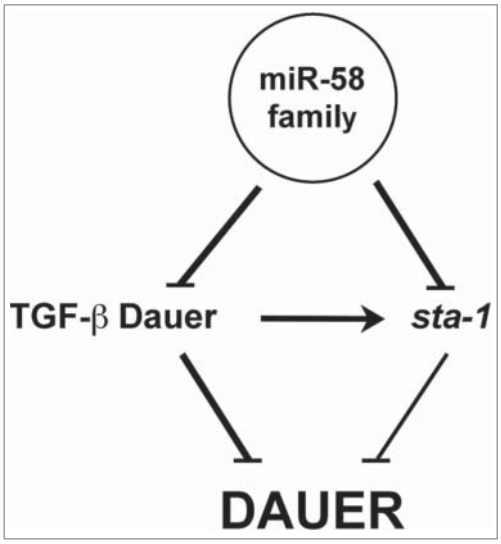

Figure 3. Model of genetic interactions between the family of miR-58, TGF-β Dauer, *sta-1* and the dauer phenotype. Based on our results (this work and reference 5), and on the *sta-1* and TGF-β Dauer literature.[17,26] The thin line between *sta-1* and dauer illustrates the weaker inhibitory role of *sta-1* on dauer compared to the stronger effect from the TGF-β Dauer pathway.[17]

outlined in Fig. 3, may serve as a system to strengthen the critical decision to go either into reproductive development or dauer, and hence contribute to avoid intermediate phenotypes.[5,27]

Materials and methods

C elegans *strains and culture conditions*

The following mutant strains were obtained from *Caenorhabditis* Genetics Center (CGC): Wild-type *Caenorhabditis elegans* N2 strain (Bristol), RB2589 *daf-3(ok3610) X*, and the *mir-58*-family mutant MT15563, which is the available strain with more *mir-58f* members mutated (*mir-80(nDf53) III;mir-58.1(n4640) IV; mir-81 and mir-82(nDf54)X*). DR2490 *mIs27 [$P_{daf\text{-}8}$::daf-8::gfp, rol-6(su1006)]* and *pwIs922 [pvha-6::DAF-4::gfp]* were kindly provided by Drs. D. Riddle and R. Padgett, respectively. We cultured worms on nematode growth medium plates (NGM: 17 g/L of agar, 3 g/L of NaCl, 2.5 g/L of peptone, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KPO_4 buffer pH 6, 5 mg/L of cholesterol) seeded with *E. coli* OP50 at 20°C according to standard procedures.[28] For RNA isolation, we cultured worms in agarose plates (10 g/L of agarose, 3 g/L of NaCl, 10 g/L of peptone, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KPO_4 buffer pH 6, 5 mg/L of cholesterol) instead of NGM agar plates.

Quantitative real-time PCR assays (qPCRs)

We grew worms in agarose plates until we got a mixed stage population. We sampled worms by washing the plates in M9 buffer, allowing them to settle by centrifugation and aspirating off the supernatant. We washed the worm pellet with M9 and repeated the process 2 additional times. Total RNA was extracted with miRNeasy Kit (Qiagen). We collected 4 (mixed stage) biological replicates for each tested strain.

For quantification of mRNAs, we first synthesized cDNAs with SuperScript III First-Strand Synthesis System (Invitrogen). qPCR of cDNAs was run on an Applied Biosystems 7500 Fast thermocycle, using TaqMan Assays (Applied Biosystems). We tested each sample in triplicate. *crn-1* and *cey-1* transcript levels were used as controls, as CRN-1 and CEY-1 protein levels did not change in *mir-58.1(n4640)* mutant strain.[29] To obtain the *sta-1*-fold induction rates in our sample strains versus N2, we analyzed qPCR data with REST-2009 software.[30] For normalization among biological replicates, automatic Ct threshold values in each run (assigned by software 7500 v2.0.6) are referred to the corresponding means across runs, and re-analyzed by program 7500. Recalculated Ct data were then analyzed by REST-2009 that calculate relative changes of transcripts following the 2–ΔΔCT method.[31] *P*-values were also obtained with REST-2009, which performs a pair-wise fixed reallocation randomization test to assess the significance between control strain N2 and samples.

Luciferase assays

Wild-type 3′UTRs of *sta-1* were amplified with forward primer 5′AACACTATTTAAATGTTAAAACAGTC and reverse primer 5′TTTACAGTAAAATAATTTATTGGATGAG. For the mutated 3′UTR, the same forward primer was used together with reverse primer 5′TTTACAGTAAAATAATTTATTGGATCTC, with underlined nucleotides corresponding to the sites mutated (positions 2 to 4 of the predicted miR-58f binding sites). Both amplified fragments were cloned downstream of the renilla-reporter gene in psiCHECK-2 plasmid (Promega).

We grew HeLa cells in DMEM (Lonza) with 10% FBS in 5% CO_2 at 37°C and seeded them at a density of 1×10^4 cells per well into 96-well plates. Cells were transfected in triplicate 24/48 h later with Lipofectamine 2000 (Invitrogen), 150 ng of the

modified psiCHECK-2 plasmid (see above), and 50 nmol of test miRNA mimic (miR-58.1, miR-58.2, miR-80 and miR-81; miRIDIAN, Dharmacon) or the standard control miRNA mimic miR-67 provided by the manufacturer. Dual-luciferase reporter assays were performed 48 h after transfection using Dual-Luciferase Reporter Assay System (Promega), to detect firefly and renilla luciferase activity. Luminescence was measured with an Infinite M200 TECAN luminometer (TECAN Group Ltd, Männedorf, Switzerland). Renilla luciferase activity was first normalized using the firefly luciferase activity as intraplasmid transfection reporter. Resulting values for miRNA-3′UTR co-expression were further normalized to those incubated with control mimic miRNA (miR-67).

Statistical pairwise comparisons were performed with a Welch Two Sample t-test, run on R software version 2.15.1.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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