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Similarity in targets with REST points to neural and glioblastoma related miRNAs

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ABSTRACT

There are groups of genes that need coordinated repression in multiple contexts, for example if they code for proteins that work together in a pathway or in a protein complex. Redundancy of biological regulatory networks implies that such coordinated repression might occur at both the pre- and post-transcriptional level, though not necessarily simultaneously or under the same conditions. Here, we propose that such redundancy in the global regulatory network can be detected by the overlap between the putative targets of a transcriptional repressor, as identified by a ChIP-seq experiment, and predicted targets of a microRNA (miRNA). To test this hypothesis, we used publicly available ChIP-seq data of the neural transcriptional repressor RE1 silencing transcription factor (REST) from 15 different cell samples. We found 20 miRNAs, each of which shares a significant amount of predicted targets with REST. The set of predicted associations between these 20 miRNAs and the overlapping REST targets is enriched in known miRNA targets. Many of the detected miRNAs have functions related to neural identity and glioblastoma, which could be expected from their overlap in targets with REST. We propose that the integration of experimentally determined transcription factor binding sites with miRNA-target predictions provides functional information on miRNAs.

INTRODUCTION

Cell control requires the interplay of a molecular network acting at multiple biological levels, of which transcriptional regulation of gene expression is possibly the most important one. Accordingly, most efforts to understand cell control have been focused on transcription factors (TFs).

The discovery of microRNAs (miRNAs), however, brought the post-transcriptional regulation of gene expression to the limelight. miRNAs are an RNA species of about 23 nt that bind to *cis*-regulatory elements in target mRNAs and help to tune their expression pattern by repressing translation or destabilizing the mRNA ((1), see (2) and references therein). miRNAs seem to be very abundant (3) and the number of experimentally known ones is increasing steadily (there are currently 2577 mature human miRNAs in miRBase release 20 (4)).

However, the small molecular size and mode of action of miRNAs make the study of their function more difficult than that of TFs. miRNA regulatory activity seems to be more subtle than that of TFs: while knockdowns of TFs often cause severe and detectable phenotypes (5), this is rarely the case for miRNAs (6). There may be multiple reasons for this: miRNAs only have an impact on the regulation of genes that have already been transcribed, therefore acting further downstream than TFs; co-regulation of the same transcript by multiple miRNAs is common and therefore the effect of loss of one miRNA might not have a big impact (6,7); the same miRNA usually regulates genes involved in a wide range of processes (1). As a result, over-expression or knockout of miRNAs is not as helpful for the verification of their function as for protein coding genes. For this reason, miRNA functional characterization is highly dependent on the identification and analysis of their target genes (8) and this has driven the development of computational methods for the prediction of miRNAs and their transcript targets. However, the accuracy of such methods is still low and they result in too many predictions (false positive rates from 24 to 70% (9)). Strategies to filter large lists of candidate targets should help to direct experimental efforts to the most likely and relevant miRNA-target interactions.

Here, we propose to use the increasingly large body of experimental knowledge on transcriptional regulation to improve the precision of miRNA target predictions. Our hypothesis is that there are groups of genes whose expression needs to be coordinately repressed in multiple contexts because they are functionally related (for example, if they code for the members of a pathway). Such repression might occur at different regulatory levels, for example via a transcriptional repressor or by a miRNA. We note that these multiple mechanisms of repression may generally occur in unrelated tissues, cell types or developmental stages: a given group of

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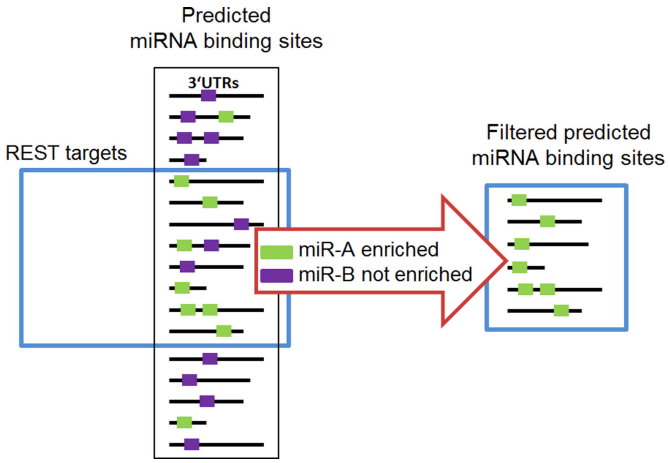

Figure 1. Description of our approach. We collected predictions of miRNA binding sites (green and purple boxes, for example for miR-A and miR-B, respectively) in 3'UTRs of all human genes (represented in the black box on the left). The subset of those belonging to genes identified as bound by a given transcriptional repressor (e.g. REST) in a ChIP-seq experiment (blue box on the left) are selected for analysis. Enrichment analysis (large arrow; see the Materials and Methods section) identifies that miR-A binding sites are enriched in the subset whereas miR-B binding sites are not. As a result we filter the set of predicted miR-A binding sites on the REST targets (box on the right).

genes could be repressed by a transcriptional repressor in one cell type and by a miRNA in another.

Therefore, given a list of genes targeted by a transcriptional repressor in one cell sample, this list can be examined to find out whether a specific miRNA is predicted to target a significant fraction of these genes. This finding could be taken as an indication that the corresponding miRNA-target predictions are biologically relevant, although it will not inform us of where their regulatory effect will take place. A graphical explanation of our approach is depicted in Figure 1.

Technical developments in high-throughput measurement of TF binding support our approach. The ChIP-seq technique, a combination of chromatin immunoprecipitation (ChIP) and massively parallel DNA sequencing, allows the identification of interactions between proteins and DNA (10). Due to fast progress in this field, nowadays an increasing number of ChIP-seq datasets are available on public platforms. Lists of potential target genes of certain TFs can be generated by identifying and annotating the genes close to the binding sites found.

Here, we present the application of our approach using 15 datasets measuring the binding sites of the repressor RE1 silencing transcription factor (REST; also known as neuron-restrictive silencer factor, NRSF) in 15 different cell types (Table 1). REST has a wide spectrum of activities related to the fine-tuning of neuronal gene expression both in neural tissue (e.g. neuronal progenitor cells (11) and adult brain (12)) and in non-neural cell types like Jurkat T cells where REST down-regulates neural genes (13). But it has also been found to regulate a wide range of non-neural targets (10,14).

MATERIALS AND METHODS

Datasets

The high-throughput datasets reporting genomic binding sites for the transcriptional repressor REST and for other factors used in this study were generated and published by the ENCODE project and can be accessed from the link http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeHaibTfbs/ (15).

Assigning binding site locations to genes and miRNAs from ChIP-seq data

Peak locations from ChIP-seq data were assigned to RefSeq genes of the reference genome hg19 according to prioritized criteria: if the peaks were situated (i) in their known or predicted promoter region (according to a database on human and murine promoters: MPromDb (16)); (ii) up to 1000 bp upstream of their transcription start site (TSS); (iii-a) for a single exon gene, up to 1000 bp downstream of the TSS; (iii-b) for a multi-exon gene, anywhere between the TSS to coding start plus first intron of a gene; (iv) up to 5000 bp upstream of their TSS; and (v) up to 5000 bp downstream of their transcription end site. This method stems from the observation that the highest frequency of binding sites for certain TFs can be found mainly in the first intron or in the core promoter region (17). If multiple replicates of the ChIP-seq experiments were available, a gene or miRNA was considered to be potentially regulated by the factor if it was identified in at least two replicates. REST was considered to bind to a miRNA-gene if a peak was found up to 10 000 bp up- or downstream of the miRNA sequence (miRNA positions from miRBase, release 16).

miRNA binding site predictions

Predictions of miRNA binding sites for all annotated human 3'-UTRs, according to the UCSC genome database, were obtained from TargetScanHuman 6.2 (http://www.TargetScan.org/ (18)). The dataset comprised miRNA binding site predictions in the 3'UTRs of human genes after pooling the predictions for all variants associated with each gene. The predictions corresponded to 4 492 024 unique miRNA-gene pairs. To ensure higher accuracy (19), only conserved miRNA binding sites across vertebrates and broadly conserved miRNA families were used for the analysis, resulting in 72 770 unique miRNA-gene pairs for 11 161 genes.

Calculating over-representation of miRNA binding sites in 3′UTRs of factor-bound genes

miRNA binding site over-representation for targets of a miRNA in sets of factor-bound genes (e.g. REST-bound genes) was computed in the following way (see Figure 2). Genes with miRNA target predictions in TargetScanHuman were taken. Given n number of factor-bound genes, and given that m_A of them are predicted to be target of a given miRNA family miR-A, we took n genes at random from the background of all TargetScanHuman genes with predicted miRNA targets 10 000 times and counted

Table 1. miRNA families with predicted binding sites significantly enriched in the 3'UTRs of REST target genes (FDRs < 0.1)

miRNA family	Times found	ECC1	GM12878	H1-hESC	H1-neurons	HCT-116	HeLa-S3	HepG2	HL-60	K562	MCF-7	PANC-1	PFSK-1	SK-N-SH	U87
miR-101/101ab	1	–	–	–	0.058	–	–	–	–	–	–	–	–	–	–
miR-129-5p/129ab-5p	8	–	0.008	0.054	0.015	–	0.031	0.031	–	0.010	–	0.092	–	–	0.015
miR-132/212/212-3p	1	–	–	–	0.078	–	–	–	–	–	–	–	–	–	–
miR-138/138ab	6	–	–	0.070	-	0.096	–	0.097	–	0.010	–	0.097	–	–	0.015
miR-139-5p	1	–	–	–	0.022	–	–	–	–	–	–	–	–	–	–
miR-153*	12	0.077	0.008	0.005	0.010	0.008	0.094	0.036	–	0.015	0.008	0.005	–	0.015	0.015
miR-185 family	8	0.061	0.058	0.096	-	0.005	0.008	–	–	–	0.004	0.005	–	–	0.048
miR-190/190ab	1	–	–	0.079	-	–	–	–	–	–	–	–	–	–	–
miR-208 family**	1	–	–	–	0.023	–	–	–	–	–	–	–	–	–	–
miR-217	1	–	–	–	0.010	–	–	–	–	–	–	–	–	–	–
miR-218/218a	10	0.054	0.005	0.005	-	0.005	0.073	–	0.069	–	0.004	0.086	0.065	–	0.008
miR-300/381/539-3p	2	–	–	–	0.019	–	–	–	–	–	–	–	–	0.082	–
miR-326/330/330-5p	1	–	–	–	-	–	–	–	–	–	–	–	–	–	0.048
miR-329 family	4	–	–	0.079	-	0.052	–	–	–	–	0.021	0.092	–	–	–
miR-34 family	1	–	–	–	-	–	–	–	–	–	–	–	–	–	0.086
miR-374ab	1	–	–	–	-	–	–	0.061	–	–	–	–	–	–	–
miR-421	2	–	0.071	–	-	0.064	–	–	–	–	–	–	–	–	–
miR-448/448-3p*	13	0.015	0.008	0.005	0.022	0.005	0.008	0.015	0.015	0.019	0.008	0.005	–	0.015	0.015
miR-499-5p**	1	–	–	–	0.018	–	–	–	–	–	–	–	–	–	–
miR-543	2	–	–	–	0.064	–	–	–	–	–	–	–	0.082	–	–

Neural cell lines are highlighted in gray. No significant results were found for cell line A549 (not included).
*These miRNA families have overlapping seeds (non independent results, see text for details).
**These miRNA families have overlapping seeds (non independent results, see text for details).

the targets of this miRNA family (z_A). To account for the fact that factor-bound genes might have a higher tendency to bear predicted miRNA targets (e.g. because they would have longer 3′UTRs), we computed a factor (r) to correct the values of z_A. Given the set of n factor-bound genes, we counted the total number of miRNA to gene associations m_t in the set for all 153 miRNA families considered in TargetScanHuman. For every random set the same was done to obtain z_t. We multiplied the number of targets of the respective miRNA family, z_A, by the ratio r between m_t and z_t, resulting in a corrected value z_A*. Then, we counted how many times z_A* was smaller than m_A. The sum of successful tests divided by the number of randomizations (10 000) was taken as a p-value of miR-A enrichment in the target genes. The p-value was corrected for multiple testing using the Benjamini and Hochberg method to yield a false discovery rate (FDR).

Factor r is larger than 1 if the factor-bound genes have a tendency to have more predicted miRNA targets than the background. In general, we observed values of r between 1 and 1.47 for all REST ChIP-seq datasets.

Calculating over-representation of REST-bound genes in miRNA family targets

For each miRNA family with n target genes (m of them being REST targets) we picked at random n genes from the TargetScanHuman list 10 000 times and counted how many times we observed $\geq m$ REST targets. The counts were taken as p-values of over-representation of REST-bound genes in the miRNA family targets, which were then Bonferroni corrected for multiple testing. We considered as REST targets the sum of genes bound by REST in our ChIP-seq datasets.

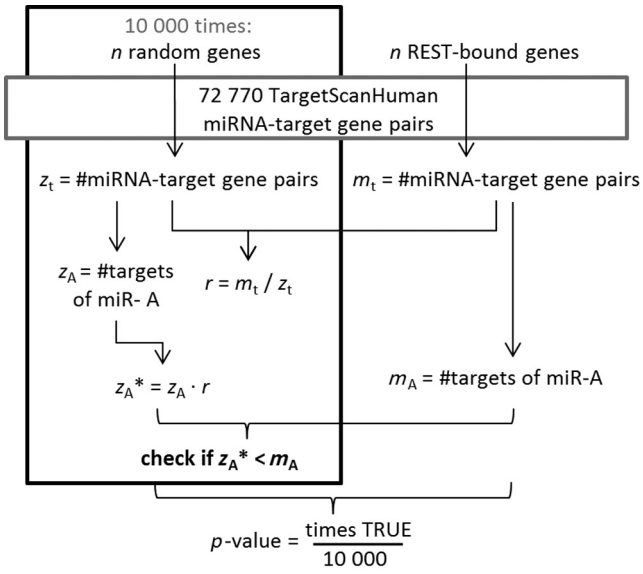

Figure 2. Illustration of the calculation of over-representation of a miRNA family: *p*-value calculation by 10 000 random tests for REST target genes.

miRNA expression in tissues

We used the atlas on mammalian miRNA expression (20) to define miRNA tissue expression. This atlas contains clone counts obtained by small RNA library sequencing. We focused on miRNAs that had a total of at least 10 copies detected over all tissues in the atlas and were present in TargetScanHuman. A miRNA was considered to be expressed in a cell type when the relative cloning frequency exceeded 3% of the total clone counts over all tissues. Results were pooled for miRNA families.

Significance of target gene enrichment

At the time of our analysis the largest collection of experimentally validated target genes of miRNAs was found in the TarBase 6.0 database containing 38 384 miRNA target-gene pairs for human, of which 3077 were predicted by TargetScanHuman 6.2 (21). The proportion of validated miRNA-target pairs in all 72 770 TargetScan predictions was calculated for over-represented miRNA families, if available, and contrasted with the proportion of valid pairs in the filtered subset (Supplementary Table S3) for each family separately and for all miRNA families with more than 10 validated interactions in TargetScanHuman 6.2.

Presence of paired miRNA binding sites

miRNA binding site positions were extracted from the TargetScanHuman 6.2 dataset. We compared the amount of miRNA binding sites that can be found in close proximity to another binding site (dual sites, 8–40 nucleotides), from the filtered set of genes bound by REST in the ChIP-seq experiments, to all remaining genes from the dataset as background. On average the filtered set had a higher number of miRNA binding sites than the background; therefore we classified all genes according to their number of binding sites in the 3′UTR. All classes with 2–26 binding sites were included in the analysis. Then, a set of n genes composed as a sum of m_i genes for each class $i = 2, \ldots, 26$ was obtained both for the filtered set and for the background. The number of dual sites and the number of non-dual sites in each set of genes were compared by Fisher's exact test. We repeated the test 1000 times. The resulting *p*-values were corrected for multiple testing using the Benjamini and Hochberg method.

Statistics

The luciferase data were analyzed using a t-test. All further *p*-values except for those generated by simulation were computed using Fisher's exact test.

Cloning

We aimed to study the impact of miRNA-448 on the UTR-dependent regulation of the *PIK3R1* gene. To this end, we cloned regions of the human *PIK3R1* 3′UTR using the following PCR primers with restriction site overhangs for Region A (chr5: 67593865–67593884: Region-A-Fw: AGTActcgagGCCTGGTTTAGCCTGGATGT, Region-A-Rv GATgcggccgcCCCACCACCCCACTTGATAC) and for Region B (chr5: 67595300–67595319: Region-B-Fw: GTCTctcgagTAGGGCAGGAGTGAGAGGTC, Region-B-Rv: TGAgcggccgcAAAACGACAAATGCGGTGGG). Regions A and B were inserted in the multiple cloning site (MCS) of a modified version of the psiCHECK2 vector (Promega) containing two separate luciferase genes (renilla and firefly) under control of constitutive promoters. The MCS was located at the 3′ end of the renilla luciferase gene, i.e. changes in the renilla luciferase activity are controlled by the inserted UTR. The firefly luciferase was used to normalize the measurement. Region B contains a putative binding site for the miRNA-448 at its 5′ end (GATTTAGATATGCAAAAGCTGG). This region was deleted by shortening the Region B using a PflMI/XhoI digest followed by Klenow blunt end filling and re-ligation yielding a vector containing a modified Region B, namely B-mut. All positions refer to human assembly NCBI37/hg19 (February 2009).

Transfection and stimulation

HEK293 cells were seeded in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) medium (10^6 cells per well) and transfected with the reporter plasmids (1 μg DNA/per well) using 3 μl Roti®-Fect (Carl Roth, P001.4) per well at 80% confluence. Twenty-four hours post transfection, the cells were washed with equilibrated PBS and trypsinized for 4 min at 37°C. Cells were removed, washed and centrifuged and re-suspended in DMEM medium and seeded in 96-well plates that were previously prepared for reverse transfection according to manufacturer's recommendation. The plates contained 10 pmol miRNA-448 per well (Invitrogen, hsa-miR-448, Assay-ID: MC10520), 0.3 μl Lipofectamine® RNAiMAX per well (Life Technologies, Cat. 13778030) and 18 μl Opti-MEM® Medi um

(Life Technologies, Cat. 11058021) per well. Controls contained the same amount of Lipofectamine® RNAiMAX and Opti-MEM® Medium accordingly.

Dual reporter assay

The reporter assay was performed 24 h after miRNA transfection ($n = 6$). The supernatant was removed from the cells and 20 μl passive lysis buffer (Promega, Cat. E1941) was added. After 20 min incubation at 20°C, the dual assay was performed according to the Hampf and Gossen Protocol (22) using 100 μl of the Firefly buffer (Tricine 20 mM, $MgSO_4$ 2.67 mM, EDTA 100 μM, ATP 530 μM, DTT 33.3 mM, Coenzyme A 270 μM, D-Luciferin 470 μM, pH 7.8) and 100 μl of the Renilla buffer (NaCl 1.1 M, $K2HPO_4$ 220 mM, Na-EDTA 2.2 mM, BSA 6.58 mM, coelenterazine 1.43 μM, pH 5.1). The 96-well plates were measured using the Luminoskan luminometer (LabSystems). We used automated injection of the buffers and light measurement using an in-house developed remote control for the Luminoskan luminometer. All data are reported as the ratio of the relative light units of the Renilla/Firefly measurements rescaled in a way that the mean of control of Region B is equal to 1.0 in order to improve readability.

RESULTS

To study the overlap between experimentally verified targets of the transcriptional repressor REST and predicted miRNA targets we used genome-wide experimental datasets of DNA binding sites for REST from ChIP-seq experiments on 15 human cell lines. From each of these datasets we obtained a list of genes likely to be regulated by REST according to their close proximity to REST binding sites (see the Materials and Methods section for details). The size of each gene list as well as a short description of the cell lines can be found in Supplementary Table S1.

Several miRNAs have over-represented targets in sets of REST-bound genes

For each of the sets of potential REST targets we computed the significance of over-representation of targets of particular miRNAs (defined by the TargetScanHuman database, version 6.2 (18)) as compared to random sets of genes by means of p-values (or FDRs) (Supplementary Table S2; see the Materials and Methods section). We could do this for a total of 153 miRNA families (broadly conserved or conserved according to TargetScanHuman definitions) for which targets were identified in the 3′-UTRs of the REST-bound genes (see the Materials and Methods section for details). An arbitrary level of significance (FDR < 0.1) was chosen to select results for further analysis. A number of results still remained significant even after a stricter cutoff of 0.05.

A total of 20 miRNA families were found to be significantly over-represented at that FDR cutoff of 0.1 in one or more of the REST-target gene datasets (Table 1). In 14 of 15 cell lines, enrichment was found (no significant results in cell line A549) and 50% of the miRNA families were detected more than once. miR-138/138ab, miR-129-5p/129ab-5p, the miR-185 family, miR-218/218a, miR-153 and miR-448/448-3p were found significantly over-represented in 6, 8, 8, 10, 12 and 13 of the 15 samples, respectively.

To assess whether miRNA families could have been found to be enriched in many of the samples simply due to the number of common genes in the tested REST-bound gene lists of each cell type, similarity between all pairs of samples according to the Jaccard-index was calculated based on: (i) genes present in the gene list (and TargetScanHuman) or (ii) miRNAs found enriched for each cell type (Figure 3).

Clustering for Jaccard-indices generated from gene counts resulted in separation of the cell lines mostly into neural and non-neural (Figure 3a, Supplementary Table S6). Cluster I contains non-neural cell lines with the exception of cell line U87, which is a glioblastoma cell line. Cluster II includes H1-neurons, SK-N-SH neuroblastoma and PFSK-1 cerebral brain tumor.

In contrast, the clustering for Jaccard-indices generated from miRNA counts reveals a cluster of eight highly similar cell lines (cluster III), which includes both neural and non-neural cell lines, with other two clusters (IV and V) that clearly separate away (Figure 3b). This result is very different from the clustering obtained using the gene counts. For example, the similarity between cell lines HepG2 and K562 is rather low according to the Jaccard-index by genes (0.33) but is very high by miRNAs (0.80). Thus, our finding that some miRNAs are found enriched in many cell types cannot only be explained by common genes in the test sets. This result indicates that our analysis uncovers miRNAs that show a remarkable overlap of targets with REST in different conditions.

We furthermore tested whether each of the 20 miRNAs has an enrichment of REST-bound targets (according to ChIP-seq) in their set of targets (see the Materials and Methods section). A total of 11 miRNA families out of the 20 have a significant enrichment (Bonferroni adjusted p-value < 0.05; see the Materials and Methods section). The complete set of miRNAs was significantly less associated with REST (43 of 153 miRNAs; p-value $= 0.006$; see Supplementary Table S8).

Filtering associations between over-represented miRNAs and REST-bound genes

Our results are based on 8438 associations of the 20 significantly enriched miRNA families with 3814 predicted target genes that are also predicted REST targets (Supplementary Table S3, Figure 4). We hypothesize that such results reflect the existence of groups of genes that require coordinated repression at pre- and post-transcriptional regulatory levels and therefore point to high-confidence miRNA-target predictions. To demonstrate this point we tested whether these miRNA-target associations were significantly enriched in experimentally proven interactions. Such a test is challenging for several reasons: (i) the largest repository of experimentally validated miRNA targets, TarBase 6.0 (21), contains information about just 4.2% of the TargetScanHuman 6.2 miRNA-gene pairs; (ii) twelve of the 20 over-represented miRNA families have no validated target genes and a further three have less than 10.

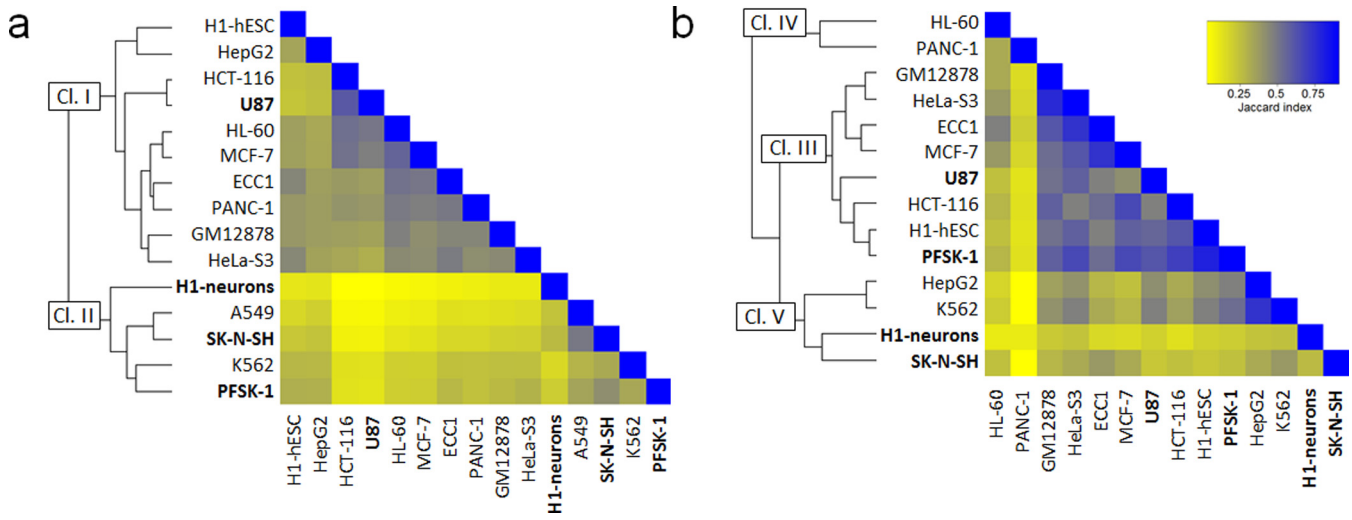

Figure 3. Heatmaps of Jaccard-indices comparing the similarity between 15 cell types. (**a**) Jaccard-indices comparing the similarity in the sets of genes potentially regulated by REST in each of 15 samples. (**b**) Jaccard-indices comparing the similarity in the sets of detected over-represented miRNA families in each sample (A549 not included as there was no over-represented miRNA in this sample). Cell types of neural origin are represented using boldface font.

In Table 2, we present the proportions of experimentally validated targets of miRNA families in the total TargetScanHuman dataset and in the subset of REST-bound genes for the miRNA families with more than 10 validated interactions. Enrichment, if modest, was observed in four out of five cases, and was mildly significant when considering the data collectively (p-value = 0.07). miR-218/218ab, the only miRNA family that was over-represented in many cell types (10 of 15), yielded a 1.25-fold significant enrichment (p-value = 0.028). It is likely that the selection of miRNA-target predictions works better with miRNAs over-represented in more than one tissue, but this cannot be stated with certainty until more experimentally validated targets become available.

Over-represented miRNAs and REST regulation

To further investigate the possible functional relation of the set of 20 enriched miRNAs with REST, we contrasted them with a list of 40 REST-regulated miRNAs (23), of which 22 were present in our set of TargetScanHuman miRNAs. Six of the 20 miRNAs, e.g. miR-129-2, miR-330 and miR-153, are known or predicted to be REST-regulated miRNAs (Table 3). This enrichment was significant (p-value = 0.044; Fisher's exact test). Furthermore, we examined whether REST was binding nearby the regions coding the 20 miRNAs (10 kb up- and downstream; see the Materials and Methods section for details). REST binding (suggesting possible regulation) was found near 16 of the 20 enriched miRNA families (Table 3), though this association was not significant in comparison to other miRNAs (p-value = 1.0). In summary, we found evidence that REST could be repressing a significant amount of the over-represented miRNAs, suggesting that REST turns off these miRNAs whenever it is present.

According to Mangan and Alon (24), the three node network formed by REST, a REST regulated miRNA, and their common target is described as an incoherent feed-forward loop of type 2 (containing only repressive relations). This network motif is very rare in comparison to other motifs. Its biological function is not well understood and differs depending on system and input signals (see (25) for details).

Over-represented miRNAs and neural function

Since REST has a role as neural repressor, we wondered to what extent the 20 enriched miRNAs targeting REST-regulated genes were related to a neural function. The atlas on mammalian miRNA expression (20) contains tissue expression data for 16 of the 20 over-represented miRNAs. According to the atlas, 9 of the 16 tested miRNA families are expressed in neural tissue in human (Table 3; enrichment p-value = 0.05). More specifically, we observed an enrichment of the over-represented miRNA families in adult hippocampus (p-value = 0.025, see the Materials and Methods section for details).

Some of these miRNAs have well-known functions in neural tissue. For example, miR-153 is specific to human brain ((26), Table 3). It plays a role in the neuro-pathological conditions of Parkinson's and Alzheimer's disease (27,28). miRNA-138 is expressed in parts of the brain, and in a feedback loop with SIRT1 it controls axon regeneration (29). Erroneous expression is implicated in panic disorder (30) and various cancer types (31,32).

These evidence collectively suggest a mechanism by which REST represses a broad set of genes in non-neural tissues and miRNAs fine-tune their expression in tissues where REST is absent (e.g. in neural tissues).

The role of over-represented miRNAs in glioblastoma

Expression profiles of miRNAs in glioblastoma have been studied extensively throughout the past two years. More than 28 miRNAs were suggested to have tumor suppressing properties in glioma or glioblastoma (see Supplemen-

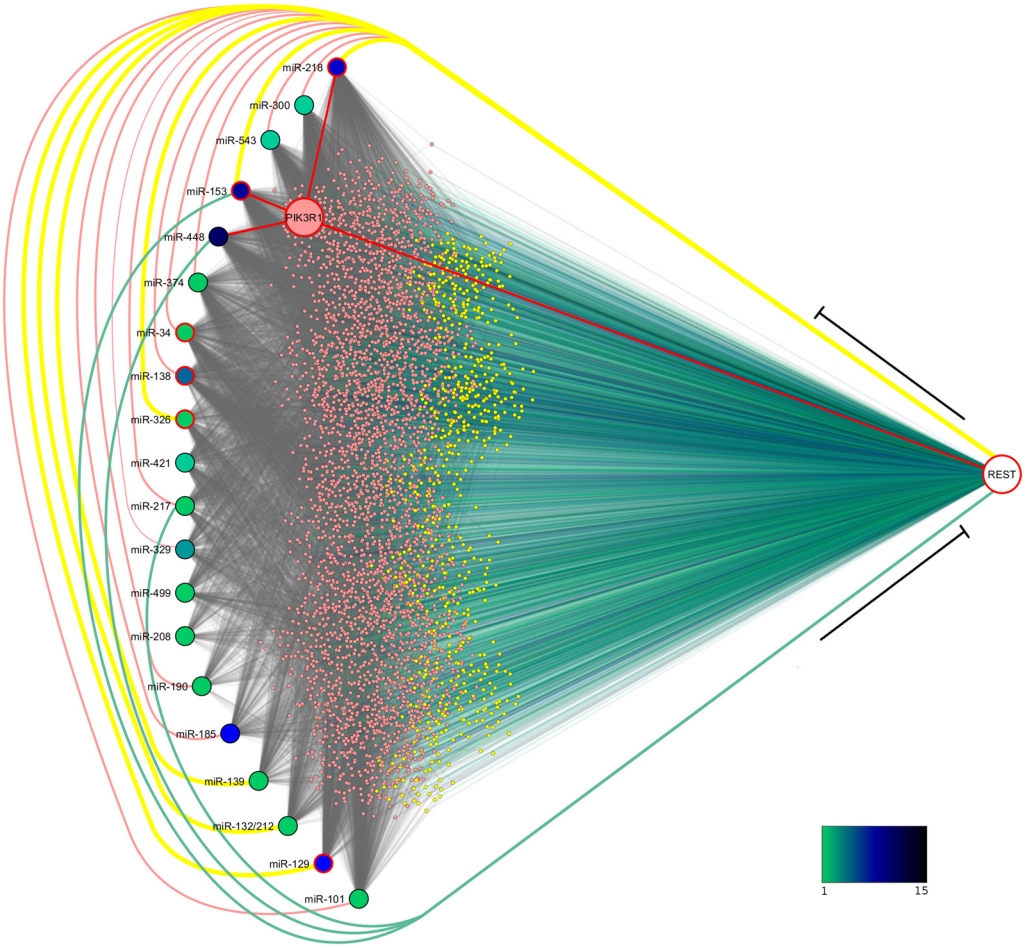

Figure 4. Graph depicting the network of genes redundantly regulated both by REST and by the 20 over-represented miRNA families. Small circles in the center represent genes (yellow: neural function, pink: any other function). Lines between genes and REST (right) indicate the number of tissues in which REST was found close to the particular gene by ChIP-seq (color hue from light green to black). Circles on the left represent the 20 miRNAs (red border: involvement in glioblastoma) and are colored in hue from light green to black according to the number of tissues where they were found to be over-represented. Connections between miRNAs and their predicted targets are shown in gray color. Curves between REST and the miRNAs going around the top of the figure indicate regulation of the miRNAs by REST (known, yellow, or deduced from ChIP-seq data, pink). Possible regulation of REST by miR-153, miR-217 and miR-448 predicted by TargetScanHuman 6.2 is indicated by green edges. Direct relations between REST, the miRNAs and PIK3R1 are shown with red edges. miRNAs were sorted according to hierarchical clustering with respect to their connections to genes. miRNA family names are shortened to the first member of each family.

Table 2. Significance of enrichment in miRNA-target interactions in filtered subset

miRNA family	All pairs	Validated pairs[a]	Filtered pairs	Filtered validated pairs	Proportion valid all (%)	Proportion valid filtered (%)	Fold enrichment	*p*-value	Times observed[b]
miR-101/101ab	804	65	635	50	8.08	7.87	0.97	0.726	1
miR-132/212/212-3p	407	25	332	21	6.14	6.33	1.03	0.498	1
miR-218/218a	931	16	746	16	1.72	2.14	1.25	0.028	10
miR-34 family	680	43	500	36	6.32	7.20	1.14	0.078	1
miR-374ab	656	11	530	11	1.68	2.08	1.24	0.094	1
merged data	3478	160	2743	134	4.60	4.89	1.06	0.071	

[a]Pairs of associations between miRNAs and genes experimentally validated according to Tarbase 6.0.
[b]Number of tissues where the miRNA was found to be enriched (Table 1).

Table 3. Regulation of filtered miRNA families (Table 1) by REST

miRNA family	REST targets		Expressed in neural tissue[b]	Glioma/glioblastoma suppressor (gs)[c]
	Johnson and Buckley (23)	Samples with ChIP signal[a]		
miR-101/101ab		5		
miR-129-5p/129ab-5p	Known[d]	15	d	gs
miR-132/212/212-3p	Known	15	e,d	
miR-138/138ab		6	d	gs
miR-139-5p	Known	14	e,s,d	
miR-153	Likely[e]	8	s,d	gs
miR-185 family		1		
miR-190/190ab		3		
miR-208 family		0	n.a.	
miR-217		1		
miR-218/218a	Likely[e]	1	d	gs
miR-300/381/539-3p		1	n.a.	
miR-326/330/330-5p	miR-330 known	13		gs
miR-329 family		1		
miR-34 family		2	d	gs
miR-374ab		7		
miR-421		0	d	
miR-448/448-3p		0	n.a.	
miR-499-5p		0	d	
miR-543		2	n.a.	

[a]Supplementary Table S4 contains a detailed list of the miRNAs that were bound by REST in a certain cell type according to the ChIP-seq data.
[b]miRNAs are defined as detected (d) if non-cancerous neural tissue copy count was more than 3% of total counts for all tissues in (20), and specific (s) or enriched (e) in brain according to (26).
[c]Details are available in Supplementary Table S5.
[d]According to (23) and (45).
[e]miR-153 and miR-218 are in the introns of a REST regulated gene. Liang *et al.* found that 77% of tested intronic miRNAs are co-expressed with their host genes (46).

tary Table S5). Although these studies cannot be compared easily due to usage of different non-neoplastic references and experimental setups (33), we found that four (miR-129-5p/129ab-5p, miR-138, miR-153 and miR-218/218a) out of six miRNAs over-represented in many tissues (>5, Table 1) seem to function as tumor suppressors in glioblastoma (Table 3; Supplementary Table S5), corresponding to an enrichment with a *p*-value of 0.011.

These results led us to wonder whether the remaining miRNAs (miR-185 family and miR-448/448-3p) could also be involved in glioblastoma. Notably, miR-448, our most over-represented miRNA (in 13 of 15 cell lines), is not known to be expressed in neural tissue. Instead, it has been reported to be involved in differentiation of adipocytes by targeting KLF5 (34) and to be part of an inhibitory feedback loop with NF-κB in breast cancer cells (35). Its function remains to be clarified.

Verification of the effect of miR-448 on the 3′UTR of *PIK3R1*

Looking for a way to assess whether the miR-185 family or miR-448/448-3p could be involved in glioblastoma and how they could have an associated phenotypic impact, we searched our list of filtered predictions (Supplementary Table S3) for genes related to glioblastoma with binding sites for these two miRNAs.

PIK3R1 is an oncogene relevant to proliferation and invasiveness of glioblastoma multiform cells (36) and was predicted to be targeted by miR-448 as well as by the other two most enriched miRNAs, miR-153 and miR-218/218ab (Table 1), which are already known to be glioblastoma suppressors. Therefore, we decided to test the effect of miR-448 on the 3′UTR of this oncogene.

Two approximately 850 bp long regions of the 3′UTR of the *PI3KR1* (Figure 5a) were cloned into a dual reporter plasmid. The action of the miRNA-448 was tested on both regions. Region B shows a clear impact upon UTR dependent gene regulation of the reporter when exposed to miR-448 (Figure 5b). If we perform the same experiment with a version of the construct with a deleted putative binding site for the miR-448, the effect is completely abolished (del-B). Region A does not respond to the miRNA-448 in our assay. In summary, *PIK3R1* expression can be down-regulated by miR-448 *in vitro* by targeting Region B.

Therefore, the enrichment analysis identified parts of a regulatory network with both REST and miR-448 as possible alternative regulators of *PIK3R1*. Since *PIK3R1* is predicted to be the target of another two miRNAs that are tumor suppressors, we suggest that miR-448 could also be a tumor suppressor. Whether miR-448 has a true regulatory function *in vivo* and particularly in glioblastoma remains to be clarified.

In summary, studying the overlap between miRNA targets and genes potentially regulated by REST, we obtained a network of genes (many of them with neural functions) targeted both by REST and by 20 miRNAs (Figure 4). This network is enriched in known miRNA targets and in miRNAs with neural related functions. We hypothesize that the study of this network provides information on miRNA targets and function.

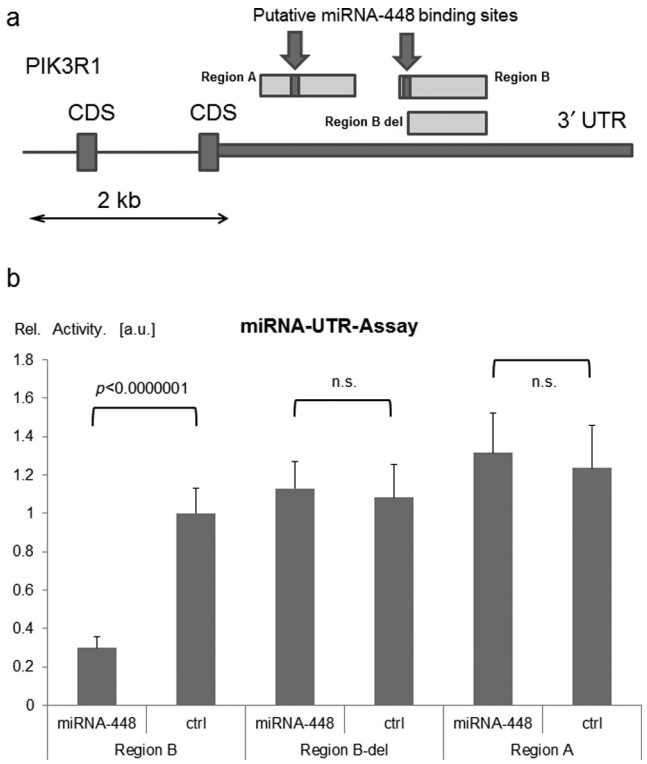

Figure 5. miRNA-UTR-Assay. The action of the miRNA-448 was tested on two approximately 850 base long regions of the 3′UTR of the *PIK3R1* gene (Region A and Region B). (**a**) Genomic location of the experimentally investigated part A and B and the mutated form of part B in relation to the 3′ UTR of the *PIK3R1* gene (thin) and the coding region (CDS) of the two downstream exons (thick) on chromosome 5. The putative binding sites of the miRNA-448 are indicated by the arrows and small dark boxes. Intronic region is given by the thin line. (**b**) Relative luciferase activity (Renilla/Firefly) in a *PIK3R1* 3′UTR dependent reporter assay, with mean control of Region B set to 1.0. The results indicate a clear action of the miRNA-448 on Region B when compared to controls. The effect of the miRNA-448 is completely abolished after deletion of the putative binding site in Region B (Region B-del). Region A does not show a miRNA-448 dependent regulation in this assay. Data are presented as mean. The error bars indicate standard deviation (SD).

DISCUSSION

We have presented here a protocol to study predictions of miRNA targets by integration with data on transcription repressor binding. The observation that the transcripts of genes targeted by a regulatory protein are enriched in targets for particular miRNAs can be assessed for significance. One possible reason for such enrichment may stem from the existence of gene groups that are co-repressed at the pre-transcriptional level in one cell type and at the post-transcriptional level in another cell type. While these effects might not occur simultaneously, the underlying global regulatory network may reflect the overlap between levels.

We applied our approach to 15 datasets indicating binding sites for the transcriptional repressor REST. Previous observations suggested the existence of multiple miRNAs cooperating with TFs in gene regulation (7). Here we are rather studying the overlap between the sets of targets of a transcriptional repressor, REST, and of particular miRNAs. We assessed the existence of such significant overlaps separately for each sample and miRNA. Some miRNA families were detected in many of the analyzed cell types, indicating reproducibility in different contexts, and we showed that such result did not solely depend on regulated genes that are common between the cell types. Due to the strong repressive effect of REST, we do not expect REST and these miRNAs to act on common targets in the same condition. It is more likely that REST will be active in non-neural tissue and the miRNAs will be active in tissues where REST is absent to fine-tune the expression of REST targets.

We collected a set of 20 miRNAs whose targets overlapped significantly with REST targets. The set of predicted associations between these 20 miRNAs and REST targets was significantly enriched in experimentally proven miRNA-target associations and therefore we propose that it can be used to expand our knowledge about miRNA targets. Additionally, we found that this set of selected targets is globally enriched in closely spaced sites (dual sites; 80% of randomization tests with p-value < 0.05, see the Materials and Methods section). This hints at a mechanism of miRNA action by which multiple miRNAs can act together with a synergistic effect (37).

Many of the detected miRNAs have functions related to neural identity, which could be expected from their overlap in targets with REST. Four of the miRNAs found more often in our study have known tumor suppressing effects in glioblastoma, a brain tumor originating from glial cells: miR-129-5p/129ab-5p, miR-138, miR-153 and miR-218/218a. Interestingly, two independent studies reported that elevated expression of REST promotes maintenance of self-renewal and oncogenic properties of glioblastoma cells (38,39).

We then used our results to study the function of miR-448, which is the miRNA that we found in most samples and is currently poorly characterized. We could experimentally verify its potential effect on the 3′UTR of the glioblastoma oncogene *PIK3R1*. Since TargetScanHuman 6.2 predicts its effect on REST, it becomes an interesting candidate for the study of the networks associated with REST and glioblastoma. Collectively, our results suggest that our method can be used both to uncover functional fractions of the miRNA regulatory network without the need of extensive miRNA profiling and to assign putative function to miRNAs.

We note that our approach provides hints about the global underlying regulatory network in a static manner, i.e. we provide evidence for high-confidence miRNA-target associations, but we cannot indicate whether the predicted repression of these targets by the corresponding miRNAs will happen in one or another tissue.

Our approach relies on the quality of both the gene binding sites and the miRNA binding sites. For example, the experimental setup used here for the detection of genes regulated by REST does not distinguish different isoforms of REST that have tissue-specific patterns of expression, likely having different regulatory properties (40). The predictions of TargetScanHuman 6.2 correspond to associations of just about 11 000 genes and 153 broadly conserved miRNAs. These are relatively small numbers, for example in comparison to the number of known transcripts and of known human genes. We expect that the number of known miR-

NAs and targets will grow with the development of high-throughput techniques to measure experimentally RNA-protein interactions such as HITS- and PAR-CLIP (41,42). This will increasingly allow using experimentally validated miRNA binding sites, which will improve the outcome of our method.

TargetScanHuman 6.2 uses 7-nt-long seed sequences to scan for miRNA targets and then predicts a target site if the seed has a perfect match, or a match of the last six nucleotides followed by an (anchoring) adenine. Therefore, miRNAs with seeds sharing the last six nucleotides can have overlapping pattern matches (Supplementary Figure S1) and, as a result, will have much more similar target gene lists than by random expectation. The effect cannot be erased during the over-representation analysis but should be kept in mind when looking at the output. For example, the results in Table 1 are not independent for two of the pairs of miRNAs shown in Figure 4. These pairs and miR-101/101ab were tested for over-representation again, and this time only non-overlapping genes were used. miR-101/101ab and miR-448/448-3p remain enriched with FDR < 0.2. miR-153 was not over-represented in this analysis in any of the tissues. Also for the miR-208 family and miR-499-5p, no significant result could be obtained (data not shown). This means that we cannot tell if one, both or none are truly over-represented.

Here, we have applied our procedure to filter predictions of miRNA targets using information on targets of REST. It can in principle be performed on any dataset mapping the genomic binding sites of a certain transcriptional regulator, provided that the number of genes guarantees reliable statistical results and that a certain amount of common targets exist between the regulatory protein and miRNAs. We tested the procedure on ChIP-seq datasets of binding sites for other regulators than REST in HepG2 cells, including transcriptional activators, and miRNA target enrichment was often found (Supplementary Table S7).

Of note, we detected over-representation of miRNA families for Pol2 bound genes. Pol2 binds DNA but it is expected to act neither as a transcriptional repressor nor as an activator. This highlights the fact that miRNA enrichment in 3′UTRs can be due to reasons not directly associated with similarity of regulatory effects between miRNAs and transcriptional factors. However, we believe that our approach will be useful to point to biologically significant miRNA targets if applied to gene datasets expected to be co-regulated.

Our approach provides a novel methodology that can take advantage of one or more sets of targets of any given gene to filter miRNA target predictions on wide gene sets. Previous work integrated miRNA and transcriptional regulation data but following different objectives. For example, Tsang *et al.* searched for co-regulated miRNAs and genes using microarray measurements of transcript expression (43). Hackl *et al.* looked for over-representation of miRNA binding sites in co-expressed genes and found promising results (44). Shalgi *et al.* did enrichment analysis of miRNA binding sites on genes with computationally predicted TF binding sites (7). No study has been performed on enrichment of miRNA binding sites in experimentally determined targets (by ChIP-seq or other techniques) of a given regulatory protein.

In summary, we found a significant overlap between targets of REST and the targets of a set of miRNA families. The sets of predicted target genes that these miRNAs have in common with REST tend to contain a proportion of experimentally known miRNA targets higher than in predictions without detected REST binding. Furthermore, we have shown that our approach can be used to propose novel functions for miRNAs from the context of the network spanned by REST and the over-represented miRNAs. The method can be applied to other transcriptional repressors and is expected to improve as miRNA-target predictions improve and with the publication of further datasets profiling genome-wide binding sites of transcriptional regulators in multiple cell types.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Bartel,D.P. (2009) MicroRNAs: target recognition and regulatory functions. *Cell*, **136**, 215–233.
2. Huntzinger,E. and Izaurralde,E. (2011) Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat. Rev. Genet.*, **12**, 99–110.
3. Lim,L.P., Lau,N.C., Weinstein,E.G., Abdelhakim,A., Yekta,S., Rhoades,M.W., Burge,C.B. and Bartel,D.P. (2003) The microRNAs of *Caenorhabditis elegans*. *Genes Dev.*, **17**, 991–1008.
4. Kozomara,A. and Griffiths-Jones,S. (2011) miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.*, **39**, D152–D157.
5. Kamath,R.S., Fraser,A.G., Dong,Y., Poulin,G., Durbin,R., Gotta,M., Kanapin,A., Le Bot,N., Moreno,S., Sohrmann,M. *et al.* (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature*, **421**, 231–237.
6. Miska,E.A., Alvarez-Saavedra,E., Abbott,A.L., Lau,N.C., Hellman,A.B., McGonagle,S.M., Bartel,D.P., Ambros,V.R. and Horvitz,H.R. (2007) Most *Caenorhabditis elegans* microRNAs are individually not essential for development or viability. *PLoS Genet.*, **3**, e215.
7. Shalgi,R., Lieber,D., Oren,M. and Pilpel,Y. (2007) Global and local architecture of the mammalian microRNA-transcription factor regulatory network. *PLoS Comput. Biol.*, **3**, e131.
8. Elefant,N., Altuvia,Y. and Margalit,H. (2011) A wide repertoire of miRNA binding sites: prediction and functional implications. Bioinformatics, **27**, 3093–3101.

9. Thomson,D.W., Bracken,C.P. and Goodall,G.J. (2011) Experimental strategies for microRNA target identification. Nucleic Acids Res., **39**, 6845–6853,
10. Johnson,D.S., Mortazavi,A., Myers,R.M. and Wold,B. (2007) Genome-wide mapping of *in vivo* protein-DNA interactions. *Science*, **316**, 1497–1502.
11. Chen,Z.-F., Paquette,A.J. and Anderson,D.J. (1998) NRSF/REST is required *in vivo* for repression of multiple neuronal target genes during embryogenesis. *Nat. Genet.*, **20**, 136–142.
12. Palm,K., Belluardo,N., Metsis,M. and Timmusk,T. (1998) Neuronal expression of zinc finger transcription factor REST/NRSF/XBR gene. *J. Neurosci.*, **18**, 1280–1296.
13. Ballas,N. and Mandel,G. (2005) The many faces of REST oversee epigenetic programming of neuronal genes. *Curr. Opin. Neurobiol.*, **15**, 500–506.
14. Ooi,L. and Wood,I.C. (2007) Chromatin crosstalk in development and disease: lessons from REST. *Nat. Rev. Genet.*, **8**, 544–554.
15. Consortium,T.E.P. (2011) A user's guide to the encyclopedia of DNA elements (ENCODE). *PLoS Biol.*, **9**, e1001046.
16. Gupta,R., Bhattacharyya,A., Agosto-Perez,F.J., Wickramasinghe,P. and Davuluri,R.V. (2011) MPromDb update 2010: an integrated resource for annotation and visualization of mammalian gene promoters and ChIP-seq experimental data. *Nucleic Acids Res.*, **39**, D92–D97.
17. Soler,E., Andrieu-Soler,C., de Boer,E., Bryne,J.C., Thongjuea,S., Stadhouders,R., Palstra,R.J., Stevens,M., Kockx,C., van Ijcken,W. *et al.* (2010) The genome-wide dynamics of the binding of Ldb1 complexes during erythroid differentiation. *Genes Dev.*, **24**, 277–289.
18. Lewis,B.P., Shih,I.H., Jones-Rhoades,M.W., Bartel,D.P. and Burge,C.B. (2003) Prediction of mammalian microRNA targets. *Cell*, **115**, 787–798.
19. Beak,D., Villén,J., Shin,C., Camargo,F.D., Gygi,S.P. and Bartel,D.P. (2008) The impact of microRNAs on protein output. *Nature*, **455**, 64–71.
20. Landgraf,P., Rusu,M., Sheridan,R., Sewer,A., Iovino,N., Aravin,A., Pfeffer,S., Rice,A., Kamphorst,A.O., Landthaler,M. *et al.* (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*, **129**, 1401–1414.
21. Vergoulis,T., Vlachos,I.S., Alexiou,P., Georgakilas,G., Maragkakis,M., Reczko,M., Gerangelos,S., Koziris,N., Dalamagas,T. and Hatzigeorgiou,A.G. (2012) TarBase 6.0: capturing the exponential growth of miRNA targets with experimental support. Nucleic Acids Res., **40**, D222–D229.
22. Hampf,M. and Gossen,M. (2006) A protocol for combined Photinus and Renilla luciferase quantification compatible with protein assays. *Anal. Biochem.*, **356**, 94–99.
23. Johnson,R. and Buckley,N.J. (2009) Gene dysregulation in Huntington's disease: REST, microRNAs and beyond. *Neuromolecular Med.*, **11**, 183–199.
24. Mangan,S. and Alon,U. (2003) Structure and function of the feed-forward loop network motif. *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 11980–11985.
25. Wall,M.E., Dunlop,M.J. and Hlavacek,W.S. (2005) Multiple functions of a feed-forward-loop gene circuit. *J. Mol. Biol.*, **349**, 501–514.
26. Sempere,L.F., Freemantle,S., Pitha-Rowe,I., Moss,E., Dmitrovsky,E. and Ambros,V. (2004) Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.*, **5**, R13.
27. Doxakis,E. (2010) Post-transcriptional regulation of alpha-synuclein expression by mir-7 and mir-153. *J. Biol. Chem.*, **285**, 12726–12734.
28. Liang,C., Zhu,H., Xu,Y., Huang,L., Ma,C., Deng,W., Liu,Y. and Qin,C. (2012) MicroRNA-153 negatively regulates the expression of amyloid precursor protein and amyloid precursor-like protein 2. *Brain Res.*, **1455**, 103–113.
29. Liu,C.M., Wang,R.Y., Saijilafu,, Jiao,Z.X., Zhang,B.Y. and Zhou,F.Q. (2013) MicroRNA-138 and SIRT1 form a mutual negative feedback loop to regulate mammalian axon regeneration. *Genes Dev.*, **27**, 1473–1483.
30. Muinos-Gimeno,M., Espinosa-Parrilla,Y., Guidi,M., Kagerbauer,B., Sipila,T., Maron,E., Pettai,K., Kananen,L., Navines,R., Martin-Santos,R. *et al.* (2011) Human microRNAs miR-22, miR-138–2, miR-148a, and miR-488 are associated with panic disorder and regulate several anxiety candidate genes and related pathways. *Biol. Psychiatry*, **69**, 526–533.
31. Nicoloso,M.S., Sun,H., Spizzo,R., Kim,H., Wickramasinghe,P., Shimizu,M., Wojcik,S.E., Ferdin,J., Kunej,T., Xiao,L. *et al.* (2010) Single-nucleotide polymorphisms inside microRNA target sites influence tumor susceptibility. *Cancer Res.*, **70**, 2789–2798.
32. Wang,Y., Huang,J.W., Li,M., Cavenee,W.K., Mitchell,P.S., Zhou,X., Tewari,M., Furnari,F.B. and Taniguchi,T. (2011) MicroRNA-138 modulates DNA damage response by repressing histone H2AX expression. *Mol. Cancer Res.*, **9**, 1100–1111.
33. Visani,M., de Biase,D., Marucci,G., Taccioli,C., Baruzzi,A., Pession,A. and Group,P.S. (2013) Definition of miRNAs expression profile in glioblastoma samples: the relevance of non-neoplastic brain reference. *PLoS One*, **8**, e55314.
34. Kinoshita,M., Ono,K., Horie,T., Nagao,K., Nishi,H., Kuwabara,Y., Takanabe-Mori,R., Hasegawa,K., Kita,T. and Kimura,T. (2010) Regulation of adipocyte differentiation by activation of serotonin (5-HT) receptors 5-HT2AR and 5-HT2CR and involvement of microRNA-448-mediated repression of KLF5. *Mol. Endocrinol.*, **24**, 1978–1987.
35. Li,Q.Q., Chen,Z.Q., Cao,X.X., Xu,J.D., Xu,J.W., Chen,Y.Y., Wang,W.J., Chen,Q., Tang,F., Liu,X.P. *et al.* (2010) Involvement of NF-kappaB/miR-448 regulatory feedback loop in chemotherapy-induced epithelial-mesenchymal transition of breast cancer cells. Cell Death Differ. , **18**, 16–25.
36. Weber,G.L., Parat,M.O., Binder,Z.A., Gallia,G.L. and Riggins,G.J. (2011) Abrogation of PIK3CA or PIK3R1 reduces proliferation, migration, and invasion in glioblastoma multiforme cells. *Oncotarget*, **2**, 833–849.
37. Grimson,A., Farh,K.K., Johnston,W.K., Garrett-Engele,P., Lim,L.P. and Bartel,D.P. (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol. Cell*, **27**, 91–105.
38. Conti,L., Crisafulli,L., Caldera,V., Tortoreto,M., Brilli,E., Conforti,P., Zunino,F., Magrassi,L., Schiffer,D. and Cattaneo,E. (2012) REST controls self-renewal and tumorigenic competence of human glioblastoma cells. *PLoS One*, **7**, e38486.
39. Kamal,M.M., Sathyan,P., Singh,S.K., Zinn,P.O., Marisetty,A.L., Liang,S., Gumin,J., El-Mesallamy,H.O., Suki,D., Colman,H. *et al.* (2012) REST regulates oncogenic properties of glioblastoma stem cells. *Stem Cells*, **30**, 405–414.
40. Palm,K., Metsis,M. and Timmusk,T. (1999) Neuron-specific splicing of zinc finger transcription factor REST/NRSF/XBR is frequent in neuroblastomas and conserved in human, mouse and rat. *Brain Res. Mol. Brain Res.*, **72**, 30–39.
41. Hafner,M., Landthaler,M., Burger,L., Khorshid,M., Hausser,J., Berninger,P., Rothballer,A., Ascano,M. Jr, Jungkamp,A.C., Munschauer,M. *et al.* (2010) Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell*, **141**, 129–141.
42. Licatalosi,D.D., Mele,A., Fak,J.J., Ule,J., Kayikci,M., Chi,S.W., Clark,T.A., Schweitzer,A.C., Blume,J.E., Wang,X. *et al.* (2008) HITS-CLIP yields genome-wide insights into brain alternative RNA processing. *Nature*, **456**, 464–469.
43. Tsang,J., Zhu,J. and van Oudenaarden,A. (2007) MicroRNA-mediated feedback and feedforward loops are recurrent network motifs in mammals. *Mol. Cell*, **26**, 753–767.
44. Hackl,H., Burkard,T.R., Sturn,A., Rubio,R., Schleiffer,A., Tian,S., Quackenbush,J., Eisenhaber,F. and Trajanoski,Z. (2005) Molecular processes during fat cell development revealed by gene expression profiling and functional annotation. *Genome Biol.*, **6**, R108.
45. Gao,Z., Ding,P. and Hsieh,J. (2012) Profiling of REST-dependent microRNAs reveals dynamic modes of expression. *Front Neurosci.*, **6**, Article 67.
46. Liang,Y., Ridzon,D., Wong,L. and Chen,C. (2007) Characterization of microRNA expression profiles in normal human tissues. *BMC Genomics*, **8**, 166.