Rbfox3 controls the biogenesis of a subset of microRNAs

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RNA-binding proteins (RBPs) regulate numerous aspects of gene expression; thus, identification of their endogenous targets is important for understanding their cellular functions. Here we identify transcriptome-wide targets of Rbfox3 in neuronally differentiated P19 cells and mouse brain by using photoactivatable ribonucleoside–enhanced cross-linking and immunoprecipitation (PAR-CLIP). Although Rbfox3 is known to regulate pre-mRNA splicing through binding the UGCAUG motif, PAR-CLIP analysis revealed diverse Rbfox3 targets including primary microRNAs (pri-miRNAs) that lack the UGCAUG motif. Induced expression and depletion of Rbfox3 led to changes in the expression levels of a subset of PAR-CLIP-detected miRNAs. In vitro analyses revealed that Rbfox3 functions as a positive and a negative regulator at the stage of pri-miRNA processing to precursor miRNA (pre-miRNA). Rbfox3 binds directly to pri-miRNAs and regulates the recruitment of the microprocessor complex to pri-miRNAs. Our study proposes a new function for Rbfox3 in miRNA biogenesis.

Although the Rbfox proteins and their splice isoforms can regulate alternative splicing of the same exons to some extent when exogenously expressed, their in vivo targets may differ, owing to the differences in expression profile and interaction with other proteins. Recent studies involving depletion of Rbfox in animals and cultured cells have shown that Rbfox has important roles in a number of biological processes17–22. However, the exact function of Rbfox in these biological processes is largely unknown.

To understand the biological function of RBPs, it is necessary to determine their binding targets in a specific cellular context. Cross-linking and immunoprecipitation of RNA–RBP complexes followed by high-throughput sequencing (CLIP-seq or HITS-CLIP) has been widely used to obtain a snapshot of where an RBP binds in intact cells23–26. A modified version, PAR-CLIP, uses photoreactive ribonucleoside analogs such as 4-thiouridine (4SU) to obtain high-resolution data. Irradiation of cells by low-energy 365-nm UV light to cross-link RBPs with photoreactive 4SU incorporated into nascent RNAs leads to more efficient and specific cross-linking27. In vivo PAR-CLIP has also been successfully used in C. elegans28 in addition to cultured cells. Comprehensive analyses of RBP targets by CLIP-seq have provided transcriptome-wide targets related to a known function of the RBP and have yielded mechanistic insights into RBP function23–25. They also have yielded unexpected targets implicating previously unrecognized functions of the RBP23,26. Although to date most of the studies on Rbfox have focused on splicing regulation, the roles of Rbfox may not be limited to splicing. Rbfox3 studies thus far have been based on selected individual genes; thus, a genome-wide analysis is needed to better understand the Rbfox3 function.

miRNAs are short single-stranded RNA molecules that post-transcriptionally regulate gene expression by inhibiting translation and triggering degradation of target mRNAs and are essential for...
normal development and cellular homeostasis. Dysregulation of miRNA expression is linked to numerous human diseases including cancer and heart disease. Thus, studying miRNA processing is important for understanding the regulatory architecture and the biological function of miRNAs. The long pri-miRNAs are transcribed by RNA polymerase II from the genome and then are cleaved into hairpin-structured pre-miRNAs by the microprocessor complex composed of the RNase III enzyme Drosha and the double-stranded RNA-binding protein Dgcr8 in the nucleus. Pre-miRNAs are then exported by Xpo5 to the cytoplasm, where they are processed by the Dicer (RNase III)-containing complex to produce mature miRNAs. Interestingly, expression levels of pri-miRNAs are not always correlated with those of their pre-miRNAs, thus suggesting that each pri-miRNA is differentially processed. Although recent studies have identified several proteins including RBPs that regulate pri-miRNA processing by interacting with the microprocessors or by binding to pri-miRNAs, detailed mechanisms of how the associated RBPs regulate pri-miRNA processing are largely unknown.

In this study, we set out to identify the endogenous targets of Rbfox3 in neuronal cells at the genome scale, using PAR-CLIP. Unexpectedly, we found that Rbfox3 interacts with a wide range of RNA sequences other than UGCAUG and that pri-miRNAs are overrepresented in Rbfox3 targets. Rbfox3 binds specifically to a subset of pri-miRNAs and functions as either a positive or negative regulator in pri-miRNA processing.

RESULTS

PAR-CLIP identifies RNA-binding sites of endogenous Rbfox3

To identify endogenous binding sites of Rbfox3, we carried out PAR-CLIP experiments, using neuronally differentiated P19 embryonal carcinoma cells and mouse neural tissues. We previously showed that P19 cells express Rbfox3 only during neuronal differentiation triggered by retinoic acid (RA). We reconfirmed that observation here (Fig. 1a): Rbfox3 was expressed in RA-treated and neuronally differentiated P19 cells expressing the control short hairpin RNA (shRNA) against GFP (P19-GFP) but not in undifferentiated P19-GFP cells. RA treatment did not increase Rbfox3 protein levels in P19 cells expressing T2 shRNA against Rbfox3 (P19-T2). We incubated P19-GFP and P19-T2 cells with photoactivatable 4SU and irradiated them with UV. We then immunoprecipitated cross-linked endogenous Rbfox3–RNA complexes with mouse anti-Rbfox3 and radiolabeled them in vitro with T4 polynucleotide kinase. Immunoprecipitated Rbfox3–RNA complexes were specifically detected with rabbit anti-Rbfox3 at the 50–60-kDa region after lithium dodecyl sulfate (LDS)-PAGE in the RA-treated P19-GFP cell sample but not in the untreated P19-GFP or in the RA-treated P19-T2 cell samples (Fig. 1b). An autoradiogram of the equivalent gel showed the radiolabeled complexes at the 50–60-kDa region, and we detected these complexes only in the RA-treated P19-GFP cell sample (Fig. 1c). These data unambiguously demonstrate that the complexes at 50–60 kDa are specific for Rbfox3. These complexes have a molecular mass approximately 10–20 kDa larger than that of Rbfox3 itself (40 kDa), consistently with cross-linking with 30–60 nt RNAs. We converted the cross-linked RNAs into cDNA and sequenced them. Two cell cultures generated 3.2 million reads (Supplementary Data Set 1), and 1 million reads uniquely mapped to the mouse genome. We analyzed the mapped reads with PARalyzer, in which the criteria call for at least 10 reads appearing in one or more events of T-to-C conversion, characteristic for cross-linked 4SU, in each cluster. Overall, we found 4,124 clusters (binding sites) from the 3.2 million reads, which were distributed...
Figure 2 Rbfox3-regulated miRNAs in P19 cells. (a) Heat map showing comparative miRNA microarray analysis among three different conditions of P19 cell cultures. Samples are miRNAs isolated from Rbfox3-specific T2 shRNA–expressing P19 cells (T2) or control GFP shRNA–expressing P19 cells (GFP), with RA treatment (+) or no treatment (−) as indicated. Color intensities are shown on a log2 scale. The leftmost column shows positive (+) and negative (−) detection in PAR-CLIP. Relative Rbfox3 expression levels in these cultures are shown in f. (b) Splinted ligation-mediated miRNA detection analysis. Autoradiograms after urea-PAGE of radiolabeled detection oligonucleotides ligated specifically to the indicated mature miRNAs from untreated (−) P19-GFP cells and RA-treated P19-GFP and P19-T2 cells are shown. (c) Agarose gel electrophoresis analysis of total RNAs. Ethidium staining of 18S RNA from the indicated three P19 cell cultures is shown. (d) RT-PCR analysis of pri-miRNAs. PCR products for the indicated pri-miRNAs from the indicated P19 cell cultures after reverse transcription (RT, +) or without RT (−) are shown after agarose gel electrophoresis. RT (−) serves as a negative control for genomic DNA contamination. M, marker. (e) qRT-PCR of pri-miRNAs. No statistically significant difference in pri-miRNA abundance among the indicated three different P19 cell cultures was observed. Average ± s.e.m.; n = 3 cell cultures; P > 0.05 (two-sided t test). (f) Immunoblot analysis of Rbfox3 and the microprocessor complex.

as follows: 41% mapped to intergenic regions (including intergenic miRNA), 38% to intronic regions and 21% to exonic regions (Fig. 1d and Supplementary Data Set 1). Strikingly, 9% (399 clusters) of Rbfox3-bound RNA clusters mapped to miRNA hairpin loci. (The term ‘miRNA hairpin loci’ denotes RNA clusters mapped to miRNA hairpins, and it does not distinguish pri-miRNA, pre-miRNA and mature miRNA. miRNA hairpins are annotated in the miRBase (http://www.mirbase.org/)). Among the 1,977,090 reads that mapped to miRNA hairpin loci, 37,057 reads (1.9%) contained the T-to-C conversion. One of the reasons for the apparently low conversion ratio, which is still considered to be above the background, might be the low 4SU incorporation in neuronally differentiated P19 cells (0.208 ± 0.088%, mean ± s.d.; n = 3 cell cultures), which is less than one-tenth of that reported for actively proliferating cells27. The target distributions of the data set from the 1 million reads were similar to those from the 3.2 million reads. Moreover, 72% unique clusters (75% intergenic, 72% intronic, 66% exonic and 84% miRNA hairpin loci) were also in the list of clusters from the 3.2 million reads with a minimal 5-n t overlap, thus demonstrating the high reproducibility of the PAR-CLIP experiments.

To further investigate Rbfox3 targets in the mouse central nervous system (CNS), we developed an in vivo mouse PAR-CLIP protocol (Supplementary Fig. 1a–c). Although 4SU incorporation in total RNA in the brain (0.026 ± 0.012%, mean ± s.d.; n = 3 mice) was approximately one-tenth of that for RA-treated P19 cells, we identified 803 clusters. Of these clusters, 41% mapped to intergenic regions, 36% to intronic regions and 23% to exonic regions (Supplementary Fig. 1d and Supplementary Data Set 2), results similar to those from P19 cells. Moreover, 80% of the clusters (80% intergenic, 79% intronic, 80% exonic and 90% miRNA hairpin loci) in the in vivo PAR-CLIP analysis were also in the list of clusters from P19 cells. Notably, the binding of Rbfox3 to miRNA hairpin loci (157 clusters) was also present in the in vivo PAR-CLIP experiment (Supplementary Fig. 1d; number of clusters overlapping among three experiments in Fig. 1e).

To our surprise, only a few clusters included the (U)GCGAUA motif, and our attempts at using several motif-discovery algorithms failed to find any motif including (U)GCGAU with statistically significant enrichment in the clusters. Despite high reproducibility of the detected target sites of Rbfox3 in intact cells, their primary sequences showed broad sequence specificity. The following study focuses on the potential role of Rbfox3 through its binding to noncanonical RNA targets.

Rbfox3 affects miRNA biogenesis in cells

The observation that Rbfox3 binds to miRNA hairpin loci prompted us to investigate whether Rbfox3 could alter the expression level of miRNAs during neuronal differentiation of P19 cells. We performed miRNA microarray analyses, using the RNAs extracted from untreated P19-GFP, RA-treated P19-GFP or RA-treated P19-T2 cells. In total, 97 miRNAs were differentially expressed between any two different culture conditions (Supplementary Data Set 3). Among them, the expression levels of 34 miRNAs decreased during differentiation, and Rbfox3 knockdown prevented these changes. The expression levels of ten miRNAs increased during differentiation, and Rbfox3 knockdown inhibited these increases (Fig. 2a and Supplementary Data Set 3; n = 3 cell cultures; P < 0.01; RNA quality in Fig. 2c). Therefore expression of these 44 miRNAs appeared to correlate with Rbfox3 expression. Thirty miRNAs out of the 44 miRNAs (68%) were also identified as Rbfox3 targets by PAR-CLIP,
results suggesting that the Rbfox3 binding to miRNA hairpin loci correlates with the expression level of a subset of miRNAs.

In order to confirm the results obtained by microarray data, we analyzed expression of candidate miRNAs by using a splinted ligation-mediated miRNA-detection method\(^1\). The expression level of miR-214, which was not detected by PAR-CLIP analysis, was unchanged under all three conditions (Fig. 2b, lanes 4–6). Although let-7i was induced by RA, Rbfox3 knockdown did not affect let-7i expression (Fig. 2b, lanes 1–3). In agreement with the microarray data, induction of Rbfox3 was accompanied by increased expression of miR-15a and miR-30c (Fig. 2b, lanes 8 and 11), whereas Rbfox3 knockdown (T2) prevented these increases (Fig. 2b, lanes 9 and 12). In contrast, levels of miR-485 and miR-666 were decreased by Rbfox3 induction (Fig. 2b, lanes 14 and 17), whereas Rbfox3 knockdown restored their expression levels (Fig. 2b, lanes 15 and 18). We next asked whether the Rbfox3-associated changes in miRNA expression originated from the changes in the pri-miRNA expression level. Quantitative reverse-transcription PCR (qRT-PCR) showed that pri-miR-15a and pri-miR-485 levels were not significantly altered in the various states of P19 cells (Fig. 2d,e). There was no obvious change in the protein levels of Drosha and Dgcr8 (Fig. 2f), proteins essential for pri-miRNA processing to pre-miRNA.\(^44,45\)

To further investigate the effect of Rbfox3 on miRNA expression, we measured miRNA expression levels after exogenous Rbfox3 expression in P19 cells. In agreement with the knockdown experiments, exogenous Rbfox3 expression increased and decreased miR-15a and miR-485 levels, respectively (Fig. 3a; RNA quality in Fig. 3b). There was no significant change in the levels of the pri-miRNAs, Drosha and Dgcr8 (Fig. 3c–e). However, the possibility remains that Rbfox3 might affect steps before pri-miRNA processing. Therefore, we analyzed the effect of Rbfox3 on pri-miRNA levels in Drosha-knockdown P19 cells, in which conversion of pri-miRNA to pre-miRNA is prohibited. The pri-miR-15a and pri-miR-214 levels increased moderately, but the pri-miR-485 level was unchanged by Drosha knockdown (Supplementary Fig. 2). These observations reflect that only a small fraction of the pri-miRNAs (host transcripts) is processed by the Drosha complex. Exogenous Rbfox3 expression did not affect the levels of all three pri-miRNAs in either control or Drosha-knockdown cells (Supplementary Fig. 2). Therefore the Rbfox3-mediated change in the abundance of mature miRNA most probably occurs during post-transcriptional steps. Because Rbfox3 is predominantly localized in the nucleus with nucleolar exclusion (Fig. 3f), Rbfox3 might have a role in regulating miRNA biogenesis at the stage of pri-miRNA processing into pre-miRNA. To examine this possibility, we measured the endogenous pre-miRNA levels after exogenous Rbfox3 expression, using RNA blot analysis. Consistently with our hypothesis, pre-miR-15a and pre-miR-485 levels were increased and decreased by exogenous Rbfox3 expression, respectively, whereas the pre-miR-214 level was unchanged (Fig. 3g). These results suggest that Rbfox3 has an effect on the processing of pri-miRNA into pre-miRNA for a subset of miRNAs in the nucleus.

Rbfox3 binds to pri-miRNAs lacking the UGCAUG motif

Although PAR-CLIP analysis indicated that Rbfox3 binds a subset of miRNA hairpin loci in P19 cells and in mouse brain tissue, the cluster sequences determined by PAR-CLIP overlap with the mature and/or pre-miRNA sequences. Therefore we analyzed interactions of Rbfox3 with pri-miRNAs in neurally differentiated P19 cells by RNA–protein–complex immunoprecipitation (RIP) followed by RT-PCR with primers that detect only pri-miRNAs but not mature or pre-miRNAs. Rbfox3 interacted with pri-miR-15a and pri-miR-485 but not pri-miR-214 (Fig. 4a). These results confirm the interaction of Rbfox3 with specific pri-miRNAs in the cells, and the specificity is consistent with the PAR-CLIP data.

To determine whether Rbfox3 itself binds directly to pri-miRNA, we performed in vitro UV cross-linking experiments with radiola-beled pri-miRNA and in vitro–synthesized Rbfox3 protein. Rbfox3 directly interacted with pri-miR-15a, pri-miR-485 and pri-miR-150 but not with pri-miR-214 (Fig. 4b), in agreement with the PAR-CLIP and RIP–RT-PCR results. Next, we addressed the mechanism of Rbfox3 binding to the pri-miRNA compared with its binding to the
UGCAUG motif. On the basis of the solution structure of Rbfox1 and the UGCAUG oligonucleotide, two phenylalanine residues, F108 and F142 within the Rbfox3 RRM, are critical for the recognition of the 5′-UGCAUG nucleotides and the second 5′-UG nucleotides, respectively. We postulated that the critical amino acid residues for Rbfox3 binding to the pri-miRNA and to the UGCAUG motif might be different. We examined this possibility by using mutant Rbfox3 in which F108 or F142 was changed to alanine. The two mutant proteins with F108A and F142A completely lost their binding activities to the wild-type intronic distal downstream enhancer (WT IDDE), which contains two copies of the UGCAUG element. However, whereas the F142A mutant essentially did not bind at all to pri-miR-15a, 40% of the binding of the F108A mutant to pri-miR-15a was retained. These results suggest that Rbfox3 may use different binding mechanisms in a target-specific manner.

Rbfox isoforms have different binding activities to RNAs

Extensive alternative splicing of all three Rbfox-encoding genes generates C-terminal variants. Interestingly, although all Rbfox family proteins contain an almost identical RRM, individual isoforms of the Rbfox family showed different binding activities to pri-miR-15a and pri-miR-485 as well as to WT IDDE. All four isoforms of Rbfox3 efficiently bound to pri-miR-15a and pri-miR-485, and two Rbfox2 isoforms (C72 and C0) also bound strongly to pri-miR-485 but not to pri-miR-15a. Two isoforms of Rbfox1 (A16 and A30) and the C40 isoform of Rbfox2 showed weaker binding to pri-miR-485 than did the C72 and C0 isoforms. Each isoform of Rbfox also had different binding activity to the WT IDDE. Therefore the region outside of the RRM, especially the C-terminal domain, appeared to affect the binding activities of Rbfox proteins to the target RNAs, regardless of the presence or absence of the UGCAUG motif. We next examined whether Rbfox1 and Rbfox2 also have an effect on pri-miRNA processing. RNA blot analysis showed that exogenous expression of Rbfox1 or Rbfox2 increased or decreased the expression of pre-miR-15a or pre-miR-485, respectively, although the extents of the changes were smaller than for Rbfox3. The pre-miR-214 level was unchanged by exogenous Rbfox expression. There were no significant changes observed in the expression of Rbfox isoforms in P19 cells. No statistical difference was observed.

Figure 5 Differential RNA binding activities and pre-miRNA generating activities among Rbfox isoforms. (a) In vitro UV-cross-linking of various Rbfox isoforms with RNAs. Top three gels, autoradiograms showing the indicated myc-tagged Rbfox isoforms cross-linked with the indicated radiolabeled RNAs after anti-myc immunoprecipitation, RNase treatment, and LDS-PAGE. Bottom gel, anti-myc immunoblot (IB). (b) Diagram of Rbfox isoforms. The amino acids in the same colors are the same within each member of the Rbfox family but not among the three different Rbfox-encoding genes. (c) RNA blot analysis of pre-miRNAs after exogenous expression of various Rbfox isoforms in P19 cells. Samples for the top five gels are total RNA from P19 cells transfected with expression constructs for the indicated proteins or with the empty vector (control). Numbers under the top two gels indicate relative intensities of radioactive bands. The RNA blot for U6 and ethidium staining of 18S rRNA serves as loading and quality controls. Bottom gel, anti-myc immunoblot (IB). (d,e) RT-PCR gel (d) and quantification (e) detecting pri-miRNAs after exogenous expression of Rbfox isoforms in P19 cells. No statistical difference was observed. Average ± s.e.m.; n = 3 cell cultures; P > 0.05 (two-sided t test). Isoforms A16, C40 and H1S were used (as in b). Uncropped images of a and c are provided in Supplementary Figure 5.
changes in the pri-miRNA levels by exogenous Rbfox expression (Fig. 5d,e). These results suggest that the C-terminal domain of the Rbfox family protein influences the strength of its RNA-binding activity and thus its ability to promote or inhibit pri-miRNA processing.

Rbfox3 can bind to various regions of pri-miRNA structure
To determine the precise sequence on pri-miRNA bound by Rbfox3, we introduced nucleotide mutations in the stem-loop regions of pri-miR-15a and pri-miR-485. We used mutant pri-miRNAs in \textit{in vitro} UV-cross-linking experiments. Mutation in the stem region of pri-miR-15a did not decrease Rbfox3 binding (Fig. 6a, mutants (mt) mt-1–mt-4, and \textit{Supplementary Fig. 3a}). Mutation of the terminal loop in pri-miR-15a—to extend the stem base-pairing (mt-5), change nucleotides while maintaining the loop structure (mt-6 and mt-7) or delete nucleotides (mt-8)—made the binding to Rbfox3 less efficient (Fig. 6a). These results suggest that Rbfox3 binds the terminal loop of pri-miR-15a, and both the sequence and the loop structure...
Figure 8  Regulation of pri-miRNA processing by Rbfox3. (a) Immunoblots detecting pri-miRNA–recruited microprocessor complex in the presence or absence of Rbfox3. Samples are proteins associated with indicated pri-miRNAs immobilized on beads or beads alone after incubation with NE from untreated P19-GFP cells in the presence (+) or absence (−) of in vitro–synthesized myc-Rbfox3. 5% of input was loaded in lanes 1 and 2. The band indicated with an asterisk in lane 2 is most probably a truncated fragment of myc-Rbfox3. Uncropped images are provided in Supplementary Figure 5. (b) A model for Rbfox3-mediated regulation of pri-miR-15a and pri-miR-485 processing. In the case of pri-miR-15a, Rbfox3 binding to the terminal loop causes enhancement of the microprocessor (Drosha and Dgcr8) recruitment, thereby increasing pre-miR-15a cropping. In the case of pri-miR-485, Rbfox3 binding to the stem region causes inhibition of the microprocessor recruitment, thereby decreasing pre-miR-485 cropping.

are important for its binding. In the case of pri-miR-485, we detected an overall decrease of Rbfox3 binding by mutation in the stem region (Fig. 6b, mt-1–mt-7 and mt-9, and Supplementary Fig. 3b). Specifically, disruption of base-pairing in the stem region proximal to the terminal loop decreased Rbfox3 binding (mt-7 and mt-9). Recovering base-pairing with a compensatory mutation restored the binding to Rbfox3 (mt-8 and mt-10), thus suggesting that the stem structure is more important than the primary sequence of this region. However, various mutations in the terminal loop of pri-miR-485 did not affect Rbfox3 binding (Fig. 6b, mt-11–mt-13). These results suggest a possible mechanism for the effect of Rbfox3 on miRNA processing: Rbfox3 enhances pri-miRNA processing by binding to the terminal loop region, whereas it represses pri-miRNA processing by binding to the stem region. To obtain further support for this idea, we determined nucleotides cross-linked with Rbfox3 for additional pri-miRNAs by in vitro PAR-CLIP with in vitro–transcribed RNA with 4S-UTP. In this analysis, the conversion of U to C due to cross-linking to Rbfox3 was not necessarily detected in the exact binding sites as observed in the IDDE, in which the conversion occurred 3 nt away from the UGCAUG element (Supplementary Fig. 4c). In the case of pri-miR-328, in which mature miRNA increased in the presence of Rbfox3 (Fig. 2a), the cross-linked U was very close to the terminal loop, in a position similar to that in pri-miR-15a (Supplementary Fig. 4a). In contrast, in the case of pri-miR-300 and pri-miR-485, whose mature miRNAs decreased in the presence of Rbfox3 (Fig. 2a), the cross-linked U residues were located in the stem region far away from the terminal loop (Supplementary Fig. 4b). These results support the model that positive and negative effects of Rbfox3 on pri-miRNA processing depend on the binding location of Rbfox3 on the stem-loop structure of pri-miRNAs.

Rbfox3 regulates pri-miRNA processing to pre-miRNA in vitro

To test the direct involvement of Rbfox3 in the pri-miRNA processing into pre-miRNA, we performed in vitro pri-miRNA processing assays by incubating radiolabeled pri-miRNA substrates with nuclear extracts (NE) from undifferentiated P19-GFP, neuronally differentiated (RA-treated) P19-GFP, or neuronally differentiated (RA-treated) P19-T2 cells. We readily detected production of pre-miR-15a in NE from neuronally differentiated P19-GFP cells but not from undifferentiated P19-GFP cells (Fig. 7a, lanes 2 and 3). Moreover, pre-miR-15a generation was remarkably reduced in NE from Rbfox3-knockdown P19-T2 cells (Fig. 7a, lane 4). In contrast, pre-miR-485 production was reduced in NE from neuronally differentiated P19-GFP cells compared to undifferentiated P19-GFP cells (Fig. 7b, lanes 2 and 3). Rbfox3 knockdown restored the pre-miR-485 production (Fig. 7b, lane 4). Because there is a possibility that compositions of NE from three different culture conditions might be different in more ways than only the Rbfox3 level, we repeated the in vitro processing reactions, using the same NE from undifferentiated P19-GFP cells with different amounts of in vitro–synthesized Rbfox3. Increasing the amounts of Rbfox3 increased or decreased the production of pre-miR-15a or pre-miR-485, respectively, in a dose-dependent manner (Fig. 7c,d). RNA blot analysis after pri-miRNA processing assays with the complementary sequence to the mature miRNA used as a probe detected pre-miR-15a and pre-miR-485 with similar sizes to those detected in intact cells (Figs. 3g and 5b) and thus verified the pre-miRNA products (Figs. 7g,h). These results demonstrate that Rbfox3 functions as a positive or negative regulator at the stage of pri-miRNA processing to pre-miRNA. Next we tested processing of mutant pri-miRNAs that bind poorly to Rbfox3. Mt-6 of pri-miR15a, in which the terminal loop is mutated, was not processed into pre-miRNA even in the presence of Rbfox3 (Fig. 7e, lane 6), consistently with the idea that Rbfox3 binding to the terminal loop is required to process pri-miR-15a into pre-miR-15a. Mt-9 of pri-miR-485, in which stem base pairs are disrupted, is not efficiently processed into pre-miRNA regardless of the presence or absence of Rbfox3 (Fig. 7f, lanes 5 and 6). This suggests that mt-9 is neither a substrate for the microprocessor nor a target for Rbfox3 binding. To test whether the terminal loop of pri-miR-15a can function as an Rbfox3-dependent enhancer element for pri-miRNA cleavage in other pri-miRNA contexts, we generated a chimeric pri-miRNA. We replaced the terminal loop of pri-miRNA-214 with the pri-miR-214(ST)-15a(TL) to generate a chimeric pri-miRNA using as a probe detected pre-miR-15a and pre-miR-485 with similar sizes to those detected in intact cells (Figs. 3g and 5b) and thus verified the pre-miRNA products (Figs. 7g,h). These results demonstrate that Rbfox3 functions as a positive or negative regulator at the stage of pri-miRNA processing to pre-miRNA. Next we tested processing of mutant pri-miRNAs that bind poorly to Rbfox3. Mt-6 of pri-miR15a, in which the terminal loop is mutated, was not processed into pre-miRNA even in the presence of Rbfox3 (Fig. 7e, lane 6), consistently with the idea that Rbfox3 binding to the terminal loop is required to process pri-miR-15a into pre-miR-15a. Mt-9 of pri-miR-485, in which stem base pairs are disrupted, is not efficiently processed into pre-miRNA regardless of the presence or absence of Rbfox3 (Fig. 7f, lanes 5 and 6). This suggests that mt-9 is neither a substrate for the microprocessor nor a target for Rbfox3 binding. To test whether the terminal loop of pri-miR-15a can function as an Rbfox3-dependent enhancer element for pri-miRNA cleavage in other pri-miRNA contexts, we generated a chimeric pri-miRNA. We replaced the terminal loop of pri-miRNA-214 with the pri-miR-15a terminal loop, and the stem and flanking regions remained as pri-miR-214 (pri-miR-214(ST)-15a(TL)). Both wild-type pri-miR-214 and the chimeric pri-miR-214(ST)-15a(TL) were scarcely processed into pre-miRNAs in the NE in the absence of Rbfox3 (Fig. 7j, lanes 5 and 8). Remarkably, addition of Rbfox3 to the NE resulted in generation of pre-miRNA from the chimeric pri-miRNA (Fig. 7j, lane 9), whereas Rbfox3 did not show any effect on the pri-miR-214 cleavage (Fig. 7j, lane 6). We also confirmed Rbfox3 binding to the chimeric pri-miRNA but not to pri-miR-214 (Fig. 7i). Thus, the terminal loop of pri-miR-15a enables Rbfox3 regulation of unregulated pri-miRNA processing.
Rbfox3 modulates Drosha–Dgcr8 binding to pri-miRNAs

Next, we investigated how Rbfox3 exerts its activity as a negative or positive regulator in pri-miRNA processing. Because the Drosha–Dgcr8 complex is critical for pri-miRNA processing in the nucleus, we explored the possibility that Rbfox3 strengthens or weakens the association of the Drosha–Dgcr8 complex with pri-miRNA. Pulldown experiments with immobilized pri-miRNA on agarose beads, the NE from undifferentiated P19 cells and in vitro–synthesized Rbfox3 confirmed that Rbfox3 binds to pri-miR-15a and pri-miR-485 but not to pri-miR-214 (Fig. 8a, lanes 6, 8 and 10). In the absence of Rbfox3, pri-miR-15a recruited Drosha and Dgcr8 to a small extent, whereas pri-miR-485 and pri-miR-214 did so to a larger extent (Fig. 8a, lanes 5, 7 and 9). Strikingly, Rbfox3 addition enhanced the recruitment of the Drosha–Dgcr8 complex to pri-miR-15a, whereas it repressed Drosha–Dgcr8 recruitment to pri-miR-485 (Fig. 8a, lanes 6 and 8). There was no change with pri-miR-214 (Fig. 8a, lane 10). These results suggest that Rbfox3 modulates pri-miRNA processing through the recruitment of the Drosha–Dgcr8 complex to pri-miRNA.

DISCUSSION

We used PAR-CLIP analysis to identify more than 4,000 binding sites for endogenous Rbfox3 in neurally differentiated P19 cells and found that these sites were reproducible in mouse CNS tissues. This study underscores the unexpected finding that Rbfox3 binds the Rbfox2-binding sequence in embryonic stem cells by CLIP-seq and RNA-binding sites, Rbfox proteins have been thought to bind the extensive evidence that supports the direct involvement of Rbfox3 in the microprocessor complex to the stem-loop structure of pri-miRNA, consequently changing the regulation of pri-miRNA. Our results revealed that Rbfox3 binds to the terminal loop of pri-miR-15a and promotes the recruitment of Drosha and Dgcr8, thus resulting in enhanced pri-miRNA cleavage. However, Rbfox3 binding to the stem region of pre-miR-485 and inhibits the recruitment of Drosha and Dgcr8, to result in reduced pri-miRNA cleavage (Fig. 8b). Moreover, there are similar correlations between the Rbfox3-binding location on pri-miRNA and the Rbfox3 effect on pri-miRNA biogenesis for pri-miR-328 and pri-miR-300. We speculate, on the basis of these data, that regulation of pri-miRNA processing by Rbfox3 may depend on its binding position within the stem-loop structure. In addition, Rbfox3 may affect the efficiency of stem-loop formation of pri-miRNA, consequently changing the recruitment of the microprocessor complex to the stem-loop structure. To understand how Rbfox3 binding to the terminal loop of the pri-miRNA depends on the involvement of the microprocessor complex in the stem-loop structure, we performed structure probing of pri-miRNA before and after Rbfox3 binding to PRI-miRNA. Although previous studies have demonstrated that Hnrnpa1 and Khsrp 37,50. A more recent study further identified a conserved primary sequence motif in terminal loops critical for cleavage of a set of pri-miRNAs51. Notably, pri-miR-15a and two more pri-miRNAs (10a and 30c-2) out of four pri-miRNAs whose processing is enhanced by Rbfox3 have phylogenetically conserved terminal loops. Previous studies have demonstrated that Hnrnpa1 and Khsrp bind to the terminal loops of pri-miRNAs. Hnrnpa1 can promote and inhibit pri-miRNA processing depending on the pri-miRNA
context, whereas Khsrp promotes pri-miRNA processing\textsuperscript{35,37,50}. Srsf1 and Smad proteins have been reported to bind the stem region of pri-miRNAs and activate Drosha cleavage\textsuperscript{36,38}. Although these examples focused on regulation of individual miRNAs, a recent study has demonstrated that Srp20 (Srsf3) has an important role in Drosha cleavage of a large number of pri-miRNAs through its binding to a phylogenetically conserved CNNC motif in the 3' single-stranded flanking region of pri-miRNAs\textsuperscript{51}. In the case of Rbfox3, pri-miRNA cleavage is regulated positively and negatively depending on the pri-miRNA context.

Rbfox3 is expressed in a wide range of postmitotic neurons from early development to adulthood. Although Rbfox3 is expected to play a part in the differentiation of neuronal cells and to function in mature neurons, its biological function has just begun to be addressed in cultured cells and in animals\textsuperscript{21}. To understand the biological function of Rbfox3, it is essential to study unbiased transcriptome-wide Rbfox3-RNA interactions, which were previously limited to the bioinformatics analysis of the UGCAUG motif\textsuperscript{12}. Our study does not deny the previous notion that Rbfox proteins regulate alternative splicing through their binding to the UGCAUG motif, but it adds the idea that Rbfox3 can bind to non-UGCAUG RNAs and can regulate other RNA processing events such as miRNA biogenesis. In regard to other members of the Rbfox family, we have shown that some isoforms of Rbfox1 and Rbfox2 are also capable of binding to pri-miRNAs and regulating their biogenesis. In addition, the ability of Rbfox family proteins to form homo- or heterodimers suggests that Rbfox1 and Rbfox2 might also participate in miRNA processing with Rbfox3. Future studies will elucidate the biological relevance of Rbfox-regulated miRNA biogenesis.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The PAR-CLIP sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number SRP032810. The microarray data have been deposited in the NCBI Gene Expression Omnibus repository under accession number GSE52377.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.K.K. and S.K. designed the study. K.K.K. performed the biological experiments, and K.K.K., R.S.A. and S.K. interpreted the data. Y.Y. and J.Z. performed bioinformatics analyses. K.K.K., R.S.A. and S.K. wrote the manuscript with input from Y.Y. and J.Z.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Online Methods

Cell culture, transfection, shRNAs, and siRNA. P19 mouse embryonic carionoma cells were purchased from ATCC and maintained in MEMα containing 7.5% calf serum and 2.5% FBS. Establishment of the clonal P19 cell lines expressing T2 siRNA for Rbfox3 and control shRNA for GFP was described previously.15 Neuronal differentiation of P19 cells was induced by retinoic acid as described previously.13 In brief, cells were cultured in a bacterial-grade Petri dish in a medium containing 5 × 10^−7 M all-trans retinoic acid for 4 d. The cell aggregates were resuspended by mild pipetting and trypsin/EDTA treatment. The resuspended cells were then transferred to a poly-D-lysine–coated tissue-culture dish and cultured for an additional 4 d. P19 cells were transfected with plasmids alone or together with siRNA by electroporation (Amaxa Nucleofector, Lonza). siRNA against Drosha was purchased from Santa Cruz (sc-44812).

Preparation of cell extracts, immunoblot analysis and antibodies (Abs). Cell extracts were prepared with radioimmunoprecipitation assay (RIPA) buffer (Sigma) supplemented with a protease-inhibitor cocktail (Roche) as described previously.13 For immunoblot analysis, the lithium dodecyl sulfate (LDS)-denatured and reduced protein samples were resolved on a 4–12% polyacrylamide NuPAGE Bis-Tris gel (Invitrogen) and transferred onto a nitrocellulose membrane. Binding of antibodies was detected by the SuperSignal system (Pierce). The primary Abs used in this study were rabbit polyclonal anti-Rbfox3 (1:500, Millipore, MAB377), mouse monoclonal anti-myc (1:5,000, Invitrogen, 46-0603), mouse monoclonal anti-Gapdh (1:5,000, Biodesign, HS6504M), rabbit polyclonal anti-Drosha (1:1,000, Abcam, ab12286), and goat polyclonal anti-Dgcr8 (1:1,000, Abcam, ab109098). The antibodies have been validated for immunoblot analysis of mouse samples on the manufacturers’ websites.

Immunofluorescence microscopy. Cell staining and immunofluorescence microscopy were carried out as described previously.13 In brief, P19 cells transfected with an Rbfox3 expression plasmid were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline. Fixed cells were incubated with primary mouse monoclonal anti-Rbfox3 (1:500, Millipore, MAB377) and rabbit polyclonal anti-Drosha (1:1,000, Abcam, ab12286). Alexa–488– and Alexa–594–conjugated goat IgG Abs against mouse IgG and rabbit IgG (1:500, Molecular Probes, A11001, A11012) were used as secondary Abs. The antibodies have been validated for immunocytochemical and immunofluorescence analysis of mouse samples on the manufacturers’ websites. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 5 μg/ml).

Plasmids and DNA templates. The expression plasmids for Rbfox3 in the pCS3+–MT vector, which contains the cytomegalovirus promoter, the SP6 promoter and a myc epitope, have been described previously. Mouse Rbfox3 isoforms H1L, H1S, H2L, and H2S correspond to mRbfox3-L (374), mRbfox3-S (327), mRbfox3-L (361), and mRbfox3-S (314), respectively, in a previous publication.13 Rbfox3 A16 and A30 and Rbfox2 C40 (same as F11) were described previously. The cDNAs for Rbfox2 C72 and C0 were obtained from mouse brain total RNA by RT–PCR with the primers 5′-ctc agg cct cta gtt ATG GAG AAA AAG AAA ATG GTA ACAA CTC-3′ and 5′-ctc agg cct cta gta GGA GGC AAA TCG GTG GTA-3′. Lowercase letters represent adapter sequences including restriction-enzyme sites. The F108A and F142A mutant clones of Rbfox3 were generated by GenScript. The template DNA containing the T7 promoter upstream of the IDDE, which was used for in vitro transcription, was described previously. DNA templates containing the T7 promoter and encoding an approximately 200 nt region of pri-miRNAs were generated by PCR from MicroRNA Expression Plasmids (OnGene) with the following primer sets: 5′-TTG TTA TAC GAC TCA CTA TAG GGA AAG ATG CGA TGT GCT TGT C-3′ and 5′-GCT ATC ATG AGT AAT AAA AAG-3′ for pri-miR-15a; 5′-TTG TTA TAC GAC TCA CTA TAG GGA AGG AAC ATG CCC-3′ and 5′-AAG-3′ for pri-miR-150; 5′-TTG TTA TAC GAC TCA CTA TAG GGA AGG AAC ATG CCC-3′ and 5′-AAG-3′ for pri-miR-666; 5′-TTG TTA TAC GAC TCA CTA TAG GGA AGG AAC ATG CCC-3′ and 5′-AAG-3′ for pri-miR-214; and 5′-TTG TTA TAC GAC TCA CTA TAG GGA AGG AAC ATG CCC-3′ and 5′-AAG-3′ for pri-miR-485.

RNA-protein UV-cross-linking. Preparation of RNA substrates and myc-tagged proteins was carried out as described previously. In brief, the RNA substrates were synthesized from PCR-generated DNA templates by T7 RNA polymerase in the presence of [α-32P]UTP with a MaxiScript kit (Ambion). The myc-tagged proteins were synthesized in vitro from pCS3+–MT constructs with the TNT Coupled Reticulocyte Lysate System with SP6 RNA polymerase (Promega). Binding reactions were carried out in a 25-μl mixture that contained 10 mM HEPES, pH 7.9, 2 mM MgCl2, 1 mM ATP, 20 mM creatine phosphate, 50 ng yeast tRNA, 2 mM DTT, 2% polyethylene glycol (MW 3,550), the reticulocyte lysate reaction mixture and 1 × 106 c.p.m. RNA probe for 20 min at 30 °C. Reaction mixtures were irradiated with 254 nm UV in a UV Stratalinker 2400 (Stratagene) for 20 min on ice, digested with 2 μl RNase/A–T1 (Ambion) and immunoprecipitated with anti-Myc agarose.
with 2 μg of anti-myc. Samples were subject to LDS-PAGE and subsequent autoradiography. The radioactive bands were quantified with NIH Image.

RNA affinity purification. Pri-miRNAs were generated by in vitro transcription from PCR-generated DNA templates containing the T7 promoter with a MAXIscript kit (Ambion). The RNAs were treated with sodium m-periodate (Sigma) and coupled to adipic acid dihydrazide agarose beads (Sigma) as described previously. By this procedure, the 3′ end of the RNA molecule attaches to agarose. Affinity purification of pri-miRNA–binding proteins from nuclear extract of P19 cells was performed as described. Proteins associated with the immobilized pri-miRNAs were analyzed by immunoblotting.

In vitro pri-miRNA processing assays. Pri-miRNA substrates were synthesized from PCR-generated DNA templates by T7 RNA polymerase in the presence of [α-32P]UTP with a MAXIscript kit (Ambion). Nuclear extracts of P19 cells were prepared by Nuclear Extract Kit (Active Motif) and dialyzed in a buffer containing 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.4 mM PMSF, 0.5 mM DTT and 20% (v/v) reticulocyte lysate extract, 10 mM HEPES, pH 7.9, 2 mM MgCl2, 1 mM ATP, 20 mM creatine phosphate, 50 ng yeast tRNA, 2 mM DTT, 2% polyethylene glycol (MW 3,550), and 100,000 c.p.m. of each pri-miRNA probe for 30 min at 30 °C. The reaction mixtures were then extracted with phenol-chloroform, and the RNAs were precipitated with ethanol. The RNA samples were resolved on a urea-6% polyacrylamide TBE gel (Invitrogen) with Dynalmarker Small RNA plus as a size marker and exposed to X-ray film (Kodak) at −80 °C.

RNA–protein–complex immunoprecipitation (RIP). Whole cell extracts were prepared from RA-treated neurally differentiated P19 cells. Approximately 300 μl of cell pellets was lysed in 1 ml of the RIPA buffer, and 500 μl of cell extracts was subjected to RIP with 10 μg mouse anti-Rbfox3 (Millipore) or control IgG with Magna RIP kit (Millipore). RNA recovered from immunoprecipitates was subjected to RT-PCR.

RNA blot analysis. RNAs were resolved on a urea-10% polyacrylamide TBE gel (Invitrogen) under denaturing conditions with Dynalmarker Small RNA Plus as a size marker. RNAs in the gel were transferred electronically onto a Zeta-probe GT membrane (Bio-Rad) and then cross-linked to the membrane by UV-light at 1,200 mJ in a UV Stratalinker 2400 (Stratagene). The membrane was exposed to X-ray film (Kodak) and then subjected to RT-PCR.

Determination of 4SU incorporation rate into total RNA. After incubation with 4SU, total RNA was isolated from neurally differentiated P19 cells and mouse brain tissue and was subjected to enzymatic digestion with or without dephosphorylation to generate single nucleosides or nucleotides, respectively, for HPLC analysis. Briefly, 40 μg of purified total RNA was incubated in a 28-μl volume overnight with 0.09 U snake venom phosphodiesterase (Worthington Biochemical) alone or together with 0.4 U bacterial alkaline phosphatase (Worthington Biochemical). The resulting single nucleosides or nucleotides were analyzed by a modified ion-pairing high-performance liquid chromatography (HPLC) with the Agilent 1100 HPLC equipped with a reverse-phase column, Supelco LC-18-T (150 × 4.6 mm, 3 μm particle size). Chromatography was performed at the flow rate of 0.7 ml/min, and nucleoside or nucleotide detection was performed at 260 nm and 333 nm. The HPLC reverse-phase column was calibrated with AMP, CMP, GMP, UMP, U and 4SU obtained from Sigma–Aldrich.

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