A novel class of microRNA-recognition elements that function only within open reading frames

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MicroRNAs (miRNAs) are well known to target 3′ untranslated regions (3′ UTRs) in mRNAs, thereby silencing gene expression at the post-transcriptional level. Multiple reports have also indicated the ability of miRNAs to target protein-coding sequences (CDS); however, miRNAs have been generally believed to function through similar mechanisms regardless of the locations of their sites of action. Here, we report a class of miRNA-recognition elements (MREs) that function exclusively in CDS regions. Through functional and mechanistic characterization of these ‘unusual’ MREs, we demonstrate that CDS-targeted miRNAs require extensive base-pairing at the 3′ side rather than the 5′ seed; cause gene silencing in an Argonaute-dependent but GW182-independent manner; and repress translation by inducing transient ribosome stalling instead of mRNA destabilization. These findings reveal distinct mechanisms and functional consequences of miRNAs that target CDS versus the 3′ UTR and suggest that CDS-targeted miRNAs may use a translational quality-control-related mechanism to regulate translation in mammalian cells.

miRNAs are small noncoding RNAs (ncRNAs) of 21 or 22 nt in length that function in the post-transcriptional regulation of gene expression1–4. These small ncRNAs bind their target sequences in mRNAs, known as MREs, through imperfect base-pairing interactions. Base-pairing via their 5′ portions of sequences from 2–7 nt, a region termed the ‘seed,’ are particularly important for miRNA recognition of target mRNAs. These base-pairing interactions take place in the RNA-induced silencing complex (RISC), in which the 5′ portion of miRNA is exposed for initial base-pairing and a subsequent conformational change allowing for additional base-pairing in the 3′ portion5–7. Thus, although nonessential, 3′ base-pairing may strengthen miRNA targeting. This so-called seed base-pairing rule has been the basis for the development of various computational algorithms for predicting miRNA-target sites in mRNAs8. However, numerous noncanonical MREs have been described in the literature8,9, thus indicating a degree of flexibility for miRNA–mRNA interactions, which may be influenced by a variety of parameters, including RNA secondary structures and RNA-binding proteins that may enhance or repress miRNA targeting in the cell10.

According to the general consensus in the field, miRNAs selectively target 3′ UTRs of mRNAs, because a functional MRE in the 3′ UTR generally loses its ability to mediate miRNA action when it is moved to the CDS10. This selectivity probably results from the action of elongating ribosomes, which may strip off the RISC in CDS but not in the 3′ UTR. However, various reports have characterized a number of functional MREs in CDS11–13. Because an Argonaute (Ago) protein (e.g., Ago paralogs Ago1–4 in mammalian cells) is a main protein component of the RISC, genome-wide RISC targeting sites have been surveyed by UV cross-linking immunoprecipitation and sequencing (CLIP–seq), which has revealed approximately equal distributions of Ago peaks in CDS and the 3′ UTR in mammalian cells14,15. These findings suggest that the RISC may target CDS regions more extensively than previously thought, but such potential CDS MREs have not been widely appreciated or characterized in detail. It has thus remained largely unclear whether those Ago CLIP peaks represent transient sampling events of the RISC in CDS or whether at least a fraction of those peaks reflect functional miRNA actions.

The functional consequence of miRNA action has been a major mystery in the field16, and little progress has been made since a review 10 years ago. Initially, through reporter-based assays, miRNA was found to induce translational repression as well as mRNA decay17–19. Subsequent genome-scale analysis of mRNA levels (by RNA-seq) and translation efficiency (by ribosome profiling normalized to transcript levels) suggested that miRNA-induced mRNA decay is the dominant functional consequence in mammalian cells20. However, a careful analysis of miRNA-induced responses on endogenous gene products has suggested that translation repression appears to occur before mRNA decay21. The mechanism of miRNA-mediated mRNA decay has been well elucidated: the RISC initially recruits GW182 (three paralogs of which are encoded in mammalian cells), which in turn attracts both decapping and deadenylase complexes; these complexes deprotect mRNA ends, thus leading to mRNA decay by both 5′–3′ and 3′–5′ RNA exonucleases22. In contrast, many different mechanisms have been proposed to explain...
miRNA-mediated translational repression, ranging from interference of translation initiation to blockage of ribosome elongation or to ribosome drop-off accompanied by degradation of the nascent peptide. To date, none of these mechanisms have been substantiated. Although the mechanism is unknown, the RISC is generally accepted to be part of the polysome, thus suggesting that the RISC is associated with translating mRNA and modulates translation and/or degradation.

In the current study, we pursued an MRE initially identified in the CDS region of the p53-activating kinase DAPK3 (ref. 30). Interestingly, we found that this MRE is representative of a class of previously unrecognized MREs that function exclusively in CDS. Extensive mutational analysis revealed that this class of MREs requires extensive 3' base-pairing, and there is a minimal requirement for 5' base-pairing in the seed. These CDS MREs effectively repress translation but do not seem to affect mRNA stability, and they require an Ago, but not GW182. Ribosome profiling experiments have shown that these MREs induce a measurable degree of ribosome stalling, but without evident ribosome queuing, thus suggesting that CDS RISC may have both local and global effects on translation. We provide initial evidence that such regulation may be related to a mechanism involved in translation quality control (QC).

Results

An MRE represses translation exclusively in CDS. We previously identified and characterized a target site for both miR-17 and miR-20a in the second coding exon of DAPK3, a gene encoding a p53-activating kinase. These two miRNAs differ by a single nucleotide in the 5' end, which does not affect their base-pairing interactions. Because most previous studies have used miR-20a, we used miR-20a throughout this report to represent both miR-17 and miR-20a. Several approaches confirmed that this site is an authentic MRE: the site was identified in two separate Ago2 mapping experiments as well as by CLASH mapping of miRNA–mRNA interactions in HEK293T cells (Fig. 1a). Importantly, DAPK3 protein levels were specifically repressed by a miR-20a mimic and elevated by a miR-20a antagonist, findings similar to the expected effects on the E2F1 protein via the characterized miR-20a MRE in its 3' UTR (Fig. 1b,c and Supplementary Fig. 1a–d). However, we detected no change in endogenous DAPK3 mRNA levels (Supplementary Fig. 1e,f), thus suggesting that this CDS MRE mediates translational repression rather than mRNA decay.

This MRE is atypical of 'standard' MREs in that it lacks typical seed base-pairing with its cognate miRNA. To characterize this unusual MRE, we inserted it into a standard dual-luciferase reporter in which the MRE is present in the 3' UTR of Renilla luciferase. Unexpectedly, the MRE was nonfunctional in this context (Fig. 1d,e). To ensure that the loss of function was not caused by sequence effects, we inserted varying amounts of 'flanking' sequence derived from exon 2 of DAPK3, but we still did not observe any effect. In contrast, when we placed the MRE in CDS 6 nt upstream of the stop codon or mutated the stop codon to allow for the use of a downstream stop codon, thereby switching the MRE from the 3' UTR to CDS, both reporters responded to the transfected miR-20a mimic (Fig. 1e,f). Again, we observed no changes at the mRNA level under all of these conditions (Fig. 1e). Therefore, this MRE appears to function only in CDS, unlike all previously reported CDS MREs, which are also functional when placed in 3' UTRs, and in contrast to typical MREs, which lose functionality when moved to CDS.

The CDS MRE requires extensive 3' base-pairing to function. We noted that although this atypical MRE lacked conventional 5' seed base-pairing with target mRNA, it had extensive base-pairing potential at the 3' end, in agreement with the directly mapped miRNA targeting sites in CDS in Caenorhabditis elegans. When mutations were introduced into the MRE to reestablish seed-base-pairing (Fig. 2a), the MRE placed in the 3' UTR became responsive to a transfected miR-20a mimic (Fig. 2b), and 3' base-pairing became nonessential (Fig. 2a,b). To determine whether 3' base-pairing was required for the MRE to function in CDS, we introduced mutations into the MRE and found that 3' base-pairing was indeed critical for the MRE to function, even after seed-base-pairing was restored (Fig. 2c,d). We also tested the MRE in the 5' UTR and found that, similarly to its behavior in the 3' UTR, it largely lacked function; however, after restoration of seed-base-pairing, it regained repression of luciferase reporter activity, and with the restored seed, 3' base-pairing was not critically required (Supplementary Fig. 1g–i).

We next sought to determine whether this miR-20a MRE was unique. By mining publicly available CLASH data, we identified two classes of putative MREs. The first class (i.e., those in PRDM4, SAFB, SNRPA, and NDRG1) showed both 5' and 3' base-pairing potential with Let-7b, and when inserted into the luciferase reporter, all repressed luciferase activity regardless of being located in the 3' UTR or CDS (Fig. 2c). In contrast, the second class (i.e., those in BAT2, BRPF1, CD99, and ERAP2) exhibited pronounced similarity to the miR-20a MRE, with substantial 3' base-pairing potential with Let-7b, but lacking typical base-pairing at the 5' seed; when placed into the luciferase reporter, all MREs in this class functioned exclusively in the CDS context (Fig. 2f). Like the miR-20a MRE in DAPK3, none of these MREs that functioned only in CDS caused mRNA decay, in contrast to the four MREs in the first class, all of which decreased mRNA levels (Supplementary Fig. 1j,k). We further showed that all of the effects on luciferase activity and mRNA levels were Let-7 dependent (Supplementary Fig. 1l,m). Western blotting with available antibodies further confirmed the expected effects of transfected Let-7b mimic or antagonist on endogenous CD99, NDRG1, and SAFB proteins (Supplementary Fig. 1n). As with the miR-20a MRE in DAPK3, extensive mutational analysis on BAT2, BRPF1, CD99, and ERAP2 demonstrated the central requirement of 3' base-pairing for each of these MREs to function in CDS, with or without a restored 5' seed (Supplementary Fig. 2); moreover, with full 3' base-pairing, a minimum of 3 bp in the 5' seed was found to be critical, as shown for the ERAP2 MRE (Supplementary Fig. 3). Collectively, these results define a novel class of MREs for which substantive 3' base-pairing with minimal base-pairing at the 5' seed confers their repressive activities exclusively in CDS.

The function of CDS-targeted miRNA depends on Ago but not GW182. Most, if not all, miRNA-mediated repression by standard MREs in the 3' UTR result from targeted mRNA destabilization, results fully consistent with our current data (Fig. 2b, Supplementary Fig. 1j and Supplementary Fig. 3). In contrast, with all atypical MREs that function in CDS, we were unable to detect mRNA decreases that could account for the lower luciferase activity (Fig. 1e, Supplementary Fig. 1e,f and Supplementary Fig. 1k). Interestingly, even with those MREs that functioned in both CDS and the 3' UTR, we did not detect decreased mRNA in the context of CDS (Supplementary Fig. 1j), thus suggesting that CDS-targeted miRNAs may function via a mechanism different from that used by 3' UTR-targeted miRNAs. This result prompted us to ask whether the same protein cofactors that mediate standard MRE function might also be required for CDS MRE to function. An Ago protein and GW182 (also known as TNR6GA) are both required for standard MRE function in the 3' UTR. To test whether Ago is required, we analyzed multiple atypical MREs in Ago2-null mouse embryonic fibroblasts (MEFs) as well as in HeLa cells treated with a pool of short interfering RNAs (siRNAs) against Ago1–4. The function of these atypical MREs was partially compromised in Ago2-null MEFs and completely abolished in HeLa cells depleted of Ago1–4, thus indicating the requirement of an Ago protein for CDS MRE to function (Fig. 3a,b and Supplementary Fig. 4a). In contrast,
knockdown of all three TNRC6 family members, a treatment that prevented standard MRE function in the 3' UTR, did not affect the activity of CDS MRE, on the basis of reporter assays (Fig. 3c and Supplementary Fig. 4b) or the response of endogenous DAPK3 (Fig. 3d and Supplementary Fig. 4c).

Lack of mRNA decay, owing to inefficient recruitment of GW182. Because Ago-associated TNRC6 proteins are required for bridging mRNA-decay machineries and for subsequent deadenylation and decapping13,17, the TNRC6-independent function of atypical MREs might thus explain the observed unchanged mRNA levels. To provide further evidence for this possibility, we inserted the miR-20a MRE and variants with different base-pairing potentials in CDS or the 3' UTR into an inducible or the 3'-UTR to trigger mRNA decay. In contrast, extensive base-pairing interactions at both the 5' and 3' ends were required for stable binding of Ago2 and GW182 to the reporter mRNA carrying the MRE in CDS, thus implying that under certain conditions, some very stable RISCs in CDS might also cause a measurable degree of mRNA decay. Interestingly, in CDS, the miR-20a MRE in DAPK3 with strong base-pairing interactions at the 3' end, but minimal base-pairing in the 5' end still allowed for a degree of Ago2 recruitment, but not GW182 recruitment. Quantification of three independent capture experiments confirmed these results (Fig. 4b and Supplementary Fig. 4d). These data suggest that translating ribosomes may prevent CDS RISC from stably recruiting additional cofactors to mediate mRNA decay.

Evidence for aborted translation induced by CDS-targeted miRNA. To investigate how miRNA might repress translation when acting in CDS, we took advantage of a report showing that the effect of 3'-UTR-targeted Let-7b can be progressively bypassed...
by virus-derived internal ribosome entry sites (IRESs)\(^4\). Using this set of reporters, we confirmed the inhibitory effect of Let-7b on the cap-dependent reporter as well as on the reporter containing the encephalomyocarditis virus (EMCV) IRES, which bypasses the requirement for eIF4E (Fig. 5a). As demonstrated earlier\(^4\), the effect of Let-7b was greatly diminished on the reporter containing the functional MRE in CDS, although the band intensity was much lower that induced by the stop codon (a minor degree of translational elongation (Fig. 5a). Under the same conditions, the wild-type luciferase gave rise to the expected 34-kDa band, whereas a 28-kDa band was detected for the reporter containing the functional MRE in CDS, although the band intensity was significantly attenuated when the mutant MRE was inserted in CDS or only in the CDS (Fig. 5b). Importantly, the presence of the truncated product depended on a functional MRE, because the band intensity was much lower that induced by the stop codon (a minor degree of translational elongation (Fig. 5a). Under the same conditions, the wild-type luciferase gave rise to the expected 34-kDa band, whereas a 28-kDa band was detected for the reporter containing the functional MRE in CDS, although the band intensity was significantly attenuated when the mutant MRE was inserted in the same location as well as when the cells were cotransfected with a miR-20a antagonist. Quantification of three independent experiments confirmed these results (Fig. 5b and Supplementary Fig. 4e). To further substantiate the aborted translation, we used a ‘2A’ strategy, in which a P2A self-cleavage peptide sequence was inserted downstream of each of the reporters from a single mRNA\(^4\). This design enabled us to detect decreases in mCherry relative to GFP signal in response to a functional MRE in CDS. As expected, we found that the mCherry signal was lower in
Dataset 1. Source data for –d are available online.

Fig. 3 | CDS-targeted miRNAs function in an Ago-dependent but GW182-independent manner. a, Western blot of Ago1–4 and GW182 family members (TNRC6A–C) after siRNA-mediated knockdown. Actin, β-actin loading control. ‘si’ prefix denotes siRNA. b, c, Luciferase reporter assays on five MREs from the indicated genes inserted into the CDS region (black bars) or the MREs with restored 5’ seeds in the 3’ UTRs (gray bars) of the Renilla luciferase reporter in transfected HeLa cells, treated with nonspecific control (NC) or a pool of siRNAs against Ago1–4 (b) or three GW182 family members (c). Data represent 3 independent experiments and are expressed as mean ± s.e.m. Additional data for single knockdown -actin loading control.

We next performed parallel ribosome profiling and RNA-seq under the same conditions and with independent replicates to validate the reproducibility of our libraries (Supplementary Fig. 5e). As expected, we observed a typical length distribution of ribosome-protected fragments (RPFs), and more importantly, the 3-nt periodicity (Supplementary Fig. 5f,g). Although ribosome-profiling data on individual transcripts tend to be noisy, we nevertheless inspected the data on the characterized DAPK3 transcript, and, by using combined mapped reads from repeated experiments, we observed a clear induction of Ago2 and a modest increase in RPF on the characterized miR-20a MRE in response to miR-20a overexpression, whereas the RNA-seq profiles remained largely the same (Fig. 6c).

We then performed metagene analysis on the basis of the Ago2 CLIP-seq data in Fig. 6a, as well as on the miR-20a-induced peaks in CDS. For this metagene analysis, we omitted transcripts with Ago2 peaks too close to the translation start or stop site to avoid response to wild-type miR-20a MRE from DAPK3 than to a mutant MRE. miR-20a overexpression had a further effect (also because of miR-20a overexpression) and the miR-20a antagonir diminished this MRE-dependent effect (Fig. 5c and Supplementary Fig. 4f). Together, these data demonstrated aborted translation by a CDS-targeted miRNA.

**Figure 3**

**CDS-targeted miRNA induces a degree of ribosome stalling.** A RISC in the middle of CDS might cause local ribosome stalling and subsequent drop-off as a mechanism inducing translation abortion. To test this possibility, we performed ribosome profiling to characterize ribosome binding behavior relative to miRNA actions on endogenous targets. For this purpose, we needed to first localize RISC complexes in both CDS and the 3’ UTR for comparison and in response to overexpression of a specific miRNA. From two independent Ago2 enhanced CLIP (eCLIP) experiments that demonstrated the reproducibility of our libraries (Supplementary Fig. 5a), we identified specific Ago2 peaks before and after miR-20a transfection (Fig. 6a). We found enriched seed sequences in the 3’ UTR, on the basis of predicted seeds underlying mapped Ago2 peaks for the top 20 expressed miRNAs in HeLa cells (Supplementary Fig. 5b, left) and highly enriched seed sequences for miR-20a underlying miR-20a-overexpression-induced Ago2 peaks in the 3’ UTR (Supplementary Fig. 5b, right, and Supplementary Fig. 5c). Importantly, relative to unaltered Ago2 peaks or random locations in miRNAs, we found elevated base-pairing potential with miR-20a among the induced Ago2 peaks in both CDS and the 3’ UTR, on the basis of using combined Ago2 eCLIP data (Fig. 6b) or independent repeats of the Ago2 eCLIP experiment (Supplementary Fig. 5d).

Together, these data strongly support that the identified Ago2 peaks reflect miRNA sites of action on endogenous miRNAs.

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natural ribosome-pausing events. By aligning RPFs relative to the centers of miR-20a-induced Ago2 peaks, we detected elevated RPFs on the peak center (Fig. 6d). We also observed increased RPFs on the center of Ago2 peaks not induced by miR-20a, and as expected, these accumulated RPFs were not affected by miR-20a overexpression (Fig. 6d). No RPF accumulation was detected on randomly selected sites (Fig. 6d). We reached the same conclusion on the basis of Ago2 peaks identified from independent Ago2 eCLIP experiments (Supplementary Fig. 5h,i).

Translation repression without an evident decrease in ribosome occupancy. Given the observed ribosome stalling induced by CDS-targeted miRNAs, we next sought evidence for potentially compromised translation efficiency, on the basis of RPFs normalized against transcript levels. To this end, we plotted fold changes in RNA-seq, RPFs, and translation efficiency (RPF density/RNA-seq density), all in cumulative fashion, but separately for transcripts without an Ago2 peak (gray) or with Ago peaks in the 3′ UTR (blue) or CDS (red) (Fig. 6e). We observed RNA decay with transcripts containing miR-20a-induced Ago2 peaks in the 3′ UTR, as previously observed20. These results validated our approach based on mapped, rather than predicted, sites of miRNA action in the 3′ UTR. We observed a minor transcript decrease with Ago2 peaks in CDS, thus indicating that some stable RISC in CDS might also cause a degree of mRNA destabilization. Unexpectedly, however, we saw little difference in translation efficiency between transcripts with or without Ago2 binding in CDS (Fig. 6e).

We further validated this finding by using a panel of HeLa cell lines stably expressing a luciferase reporter without an MRE or with a functional miR-20a MRE from DAPK3 either in CDS or the 3′ UTR. As we observed in transfected cells, the MRE repressed translation without decreasing mRNA decay only in CDS (notably, such atypical MRE is not functional in the 3′ UTR) (Supplementary Fig. 6a,b). We next performed polysome profiling on these cells. From the averages of three independent polysome experiments, we found that all reporter transcripts with or without an MRE showed similar polysome profiles to that of endogenous actin mRNA (Supplementary Fig. 6b–d). These data are consistent with our ribosome-profiling data and together suggest that CDS-targeted miRNAs do not appear to alter the overall ribosome occupancy on their mRNA targets, despite a measurable degree of ribosome stalling that can be correlated to translation repression.

CDS-targeted miRNAs may use a related mechanism for translational QC. We suspected that CDS-targeted miRNAs might repress translation via a related mechanism for translational QC, owing to defective tRNA loading, as previously reported45. In that study, the defect induced much stronger ribosome stalling than we observed with CDS MREs but did not alter overall polysome profiles. Such a translational QC mechanism has been extensively characterized in yeast, and is also known to operate in mammalian cells, and a key functional consequence is the induced degradation of nascent polypeptides by the proteasome system46. To provide initial evidence for this mechanism, we engineered a miR-20a MRE near the 3′ end of the luciferase reporter but still within the CDS region. Placing a stop codon in the same location did not affect the luciferase activity, thus indicating that the very last C-terminal sequence is not required for luciferase activity (Fig. 6f). In contrast, the presence of the miR-20a MRE decreased the luciferase activity by endogenous miR-20a, and importantly, the effect was rescued with the proteasome inhibitor MG132 (Fig. 6f). These observations indicate that aborted translation is coupled with degradation of nascent polypeptides by the proteasome system, thus suggesting that CDS-targeted miRNA may exploit a related translational QC mechanism.

Discussion
In this study, we provide multiple lines of evidence for a novel class of MREs that function exclusively in CDS and repress translation...
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**Fig. 5 | CDS-targeted miRNA induces aborted translation.** a, Luciferase assays of reporters carrying a normal cap or IRES from EMCV, HCV, or CrPV, each also containing the miR-20a MRE in the CDS or the Let-7b MRE in the 3′ UTR. Data are based on 3 independent experiments and are expressed as mean±s.e.m. b, Top, Diagram of FLAG-tagged luciferase constructs containing a stop codon or carrying a wild-type or mutant miR-20a MRE from the DAPK3 in CDS. The expected protein sizes are indicated at right. Bottom, western blot of the luciferase reporter proteins expressed in transfected HeLa cells. The second-to-last lane was from cells cotransfected with reporter containing the miR-20a MRE and the miR-20a antagonist. The data are representative of 3 independent experiments and are quantified at the bottom with the other two experimental repeats shown in Supplementary Fig. 4f. Data are expressed as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001 (two-tailed Student’s t test). Source data for this figure are available online.

Without inducing mRNA decay. We show that the function of these MREs requires minimal 5′ base-pairing in the seed and extensive 3′ base-pairing with target miRNAs. Our results extend the already broad range of miRNA actions in mammalian cells, in agreement with numerous Ago binding events detected in CDS by CLIP14,15,47 and miRNA–mRNA interactions captured by RNA ligation16,35,48. Although several reports have documented such miRNA action in CDS11,12,27, those CDS-targeted miRNAs have mediated RNA decay, probably because those CDS-targeted miRNAs might use a mechanism accounting for their predominant effects on translation but not mRNA decay, results opposite from the functional consequences of miRNAs acting on the 3′ UTR.

Such atypical CDS MREs do not seem to trigger nonsense-mediated RNA decay, probably because those CDS-targeted miRNAs function after ‘pioneer translation’ in the cytoplasm40. A strong RNA secondary structure can induce so-called no-go decay, but such a mechanism has been documented only with a reporter-based assay in yeast40, but not on any endogenous transcript in either yeast or mammalian cells, and the endonuclease involved has remained unidentified. Instead, we provide initial evidence that CDS-targeted miRNAs might use a mechanism related to that used in translational QC, which has been mainly characterized in the non-stop decay (NSD) pathway41. Interestingly, no-go decay and NSD pathways appear to utilize the same machinery consisting of a heterodimer of Hbs1 and Dom34 (Pelota in mammals), which are structurally related to the translation-termination factors eRF1 and eRF3, respectively. The NSD pathway is normally activated on mRNAs that lack a natural stop codon, which catalyzes ribosome splitting, tRNA release, ubiquitination of the nascent peptide, and mRNA decay42. This machinery appears to also function in the middle of CDS in response to a tRNA-loading defect, which causes strong ribosome stalling but not mRNA decay43. However, suboptimal codons are extensively correlated with mRNA instability44, although whether suboptimal codons trigger mRNA decay remains unclear. We provide initial evidence that a CDS-targeted miRNA induces degradation of the nascent peptide, thus suggesting a potential mechanistic link to the NSD pathway and providing a new direction for future studies seeking understanding of how CDS-targeted miRNAs may repress translation.

During this investigation, we initially thought that CDS-targeted miRNA might abort translation by inducing ribosome stalling followed by ribosome drop-off. We provide evidence for such aborted translation on two separate reporters. Interestingly, we note that most miRNAs target mRNAs in CDS regions in plants, as has been demonstrated to induce translation abortion at miRNA-target sites45. Unexpectedly, however, our ribosome-profiling data provide no evidence for the anticipated ribosome queuing (ribosome build-up in front of a stalling site and decreased ribosome presence after the site). In fact, such ribosome queuing is rarely seen on most transcripts exhibiting strong ribosome stalling, even in bacteria depleted of a key elongation factor46. This finding suggests that internal ribosome stalling might ultimately
transmit a signal that affects the overall competence of affected mRNAs in translation. One possibility is the sequestration of such mRNAs along with bound ribosomes in P bodies, although this possibility is a subject of active debate. Another possibility is ribosomes, thereby linking translation elongation to initiation, α kinase (GCN2) in response to stalled translation efficiency; these findings clearly require further investigation in future studies.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41594-018-0136-3.

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Fig. 6 | Effects of CDS-targeted miRNAs on ribosome binding. a, Scatter plot of combined Ago2 eCLIP data before and after miR-20a overexpression. Induced (red) and decreased (blue) Ago2 peaks, separately counted in 5’ UTR, CDS, and 3’ UTR. WT, wild type. b, Box plots of base-pairing potential (based on ΔG) between miR-20a and sequences underlying miR-20a-overexpression-induced Ago2 peaks in CDS (left) or the 3’ UTR (right) relative to uninduced Ago2 peaks or random sites. The median value for each group is shown by a horizontal gray line. Filled boxes extend from the first to the third quartiles. The upper/lower whiskers extend from the hinge to the highest/lowest value within 1.5 times the interquartile range of the hinge. Data beyond the ends of the whiskers are outliers and are plotted as points. *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed Student’s t-test). c, UCSC genome browser view of Ago2 eCLIP-seq, ribo-seq, and RNA-seq tracks on the DAPK3 transcript before and after miR-20a overexpression, all based on combined reads from independent replicates of each experiment. d, Delta-gene analysis of RPFs on miR-20a-overexpression-induced Ago2 peaks (left), all other Ago2 peaks (middle), or random sites (right). Gray and red tracks, RPFs before and after miR-20a overexpression, respectively. e, Cumulative distribution of RNA-seq, ribo-seq, and translational efficiency (TE) fold change (FC) in response to miR-20a overexpression. Red and blue, transcripts containing miR-20a-induced Ago2 peaks in CDS or the 3’ UTR (transcripts containing Ago2 in both CDS and the 3’ UTR were not included in the analysis); gray, transcripts without a significant Ago2 binding peak. Additional Ago2 eCLIP and ribosome profiling data are shown in Supplementary Fig. 5. f, Evidence for CDS-targeted miRNA-induced degradation of nascent polypeptide. Left, luciferase assays of the reporters containing a normal stop codon or the stop codon moved 6 nt upstream. Right, luciferase assays of the reporters carrying the miR-20a-target site in the CDS region near the 3’ end, with or without MG132 treatment. Quantitative results were based on 3 independent experiments and are expressed as mean ± s.e.m. *P < 0.01; ***P < 0.001 (two-tailed Student’s t-test). Source data for f are available online. Uncropped blot images are shown in Supplementary Dataset 1.
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Author contributions
Z. Cai, K.Z., X.Z., Y. Zhou, T.N., and X.-D.F. designed the experiments; Z. Cai, K.Z., X.Z., and W.R. performed most experiments; J.Z., B.Z., Z. Chen, D.W., and Y. Zhou analyzed the data; Y. Zhao and L.W. performed the deadenylation assay; R.C., G.L., Q.Z., Y.X., and Z. Cai, K.Z., X.Z., Y. Zhou, T.N., and X.-D.F. wrote the paper.

Competing interests
The authors declare no competing interests.

Additional information
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Cell culture and antibodies. HEla and 293T cells, purchased from the American Type Culture Collection (ATCC), were cultured in DMEM plus 10% FBS. Both cell lines were maintained in a humidified atmosphere at 37 °C, 5% CO2. The antibodies used were as follows: anti-DAPK1 (Abcam, ab109382), Abclonal, A5741, anti-DAPK2 (Abgent, A7033A), anti-DAPK3 (Abgent, AIJ236B), Thermo scientific, PA5-27700, anti-actin (PTGCN, 60008-1-Ig), anti-EF1 (Abcam, ab179445), anti-GW182 (Bethyl, A303-329A), anti-TNRC6B (Abnova, H00023112-M12), anti-TNRC6C (Bethyl, A303-969A), anti-E2F1 (Abcam, ab150104), anti-Ago2 (Abnova, H000271-161-M01), anti-Ago3 (Proteintech, 19692-1-A), anti-Ago4 (CST, 69135), anti-CD99 (Proteintech, 60354-1-Ig; Abclonal, A0282), anti-NDRG1 (Abcam, ab124698), anti-SAFB (Abnova, H00006294-M04), anti-FLAG (Abclonal, AE005), anti-GFP (Proteintech, 66002-1-Ig), and anti-mCherry (Abclonal, AE002). Western blot results were quantified in ImageJ software.

RT-PCR and real-time PCR. For PCR analysis, total RNA was extracted from cells with TRIzol (Life Technologies), and ~1 μg RNA was treated with RQ DNAse, then subjected to random priming with N9 (Takara) by M-MLV reverse transcriptase. Real-time PCR was performed with a Master SYBR Green kit (Tiangen) on aRotor Gene 6000 Real-time Analyzer. All primers and miRNA sequences used in this study are listed in Supplementary Table 1.

Luciferase reporter constructs and dual luciferase assays. The sICHECK2 reporter vector was digested with XhoI and NotI. The miR-20a MRE from DAPK3 was inserted into the vector after annealing of oligonucleotides consisting of MRE F (5′-TGGAGCATCTGACCAGCTAAAGGCATCGGC-3′) and MRE R (5′-GGCCCGGATCCGGCTTAGTGACGATGCGAGC-3′). Specific mutations in the target site were introduced by PCR-based site-specific mutagenesis.

The luciferase reporters containing EMCV, CrPV, and HCV IRESs cloned into pRL-SV40 were harvested for western blotting 48 h after transfection. Luciferase activity was measured 48 h after transfection, by using a Promega dual luciferase kit.

Detection of translation termination by CDS-targeted miRNA. The Renilla luciferase was FLAG-tagged, and the expression plasmid was further modified by insertion of a TAA stop codon at position 1479 (from ATG) or a WT or mutant miR-20a MRE from DAPK3. HEla cells were seeded at ~45% confluency in 24-well plates and transfected the next day with 10 ng sICHECK2 alone or in combination with 100 ng pGL3 and 15 ng of pGL3 plasmids. Luciferase activity was measured 48 h after transfection, by using a Promega dual luciferase kit.

Statistical analysis. Data shown for each experiment were based on three or four technical replicates, as indicated in the individual figure legends. Data are presented as means ± S.E.M., and P values were determined by two-tailed Student’s t test. All experiments were further confirmed in biological replicates.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All deep-sequencing data generated in this study have been deposited in the Gene Expression Omnibus database under accession number GSE115146. Source data for the graphs in all figures are available online. Other data are available from the corresponding author upon reasonable request.
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Software and code

Policy information about availability of computer code

Data collection

qPCR data were collected from Real-time Analyzer (CFX Connect). Luciferase data were collected from Promega (GLOMAX 20/20 LUMINOMETER). Western blotting results were quantified by the ImageJ (1.46r). Ribo-seq and eCLIP-seq data were from Hi-Seq 2500 and RNA-seq were from Hi-Seq X10.

Data analysis

Open source softwares include cutadapt (1.14), Bowtie2 (2.3.4), STAR (020201), CLIPper (0.2.0), featureCounts (1.6.0), edgeR (3.14), RNAhybrid (2.1.2) and BEDTools (2.27.1).

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Life sciences study design

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| Sample size | Sample sizes were described in the legend of each figure. The sample size (in most cases, at least 3) allows us to achieve at least 80% power (standard power) to detect the difference with 95% confidence. |
|-------------|--------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from the analyses. |
| Replication | All of the experiments were confirmed by both technical and biological repeats as described in Methods. |
| Randomization | n/a |
| Blinding | n/a |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| - | Unique biological materials |
| - | Antibodies |
| - | Eukaryotic cell lines |
| - | Palaeontology |
| - | Animals and other organisms |
| - | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| - | ChIP-seq |
| - | Flow cytometry |
| - | MRI-based neuroimaging |

Antibodies

Antibodies used

The antibodies used were from different vendors: Anti-DAPK1 (Abcam, ab109382), ABclonal, AS741; Anti-DAPK2 (Abgent, AP7033A); Anti-DAPK3 (Abgent, A11236b); Thermal scientific, PAS-27700; Anti-ACTIN (PTGCN, 60008-lg); Anti-E2F1 (Abcam, ab179445); Anti-SW182 (Bethyl, A302-329A); Anti-TNRC6B (Abnovo); Anti-TNRC6C (Bethyl, A303-969A); Anti-Ago1 (Abcam, ab105104); Anti-Ago2 (Abnova, H00027161-M01); Anti-Ago3 (Proteintech, 19692-I-AP); Anti-Ago4 (CST, 69135); Anti-CD99 (Proteintech, 60354-1-lg); ABclonal, A2028; Anti-NDRG1 (Abcam, ab124690); Anti-SAFB (Abnovo, H0006294-M04); Anti-FLAG (ABclonal, AE005); Anti-GFP (Proteintech, 66002-I-lg); anti-Mcherry (ABclonal, AE002).

Validation

All validation information for the antibodies can be searched in antibodypedia.
## Eukaryotic cell lines

### Policy information about cell lines

| Cell line source(s) | Three cell lines were used in this study, HeLa and HEK293T cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The third one MEF Ago2 KO cell line was from Dr. Ligang Wu Lab at Shanghai Institutes for Biological Sciences, CAS. |
|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Authentication      | The cell lines were not further authenticated.                                                                                                                                                        |
| Mycoplasma contamination | The cell lines are not contaminated with mycoplasma. We periodically checked potential contamination with mycoplasma, which causes retarded cell growth and low pH in the media. Mycoplasma was tested by Hoechst staining of the cells according to Young L. et al., Nature Protocols, 2010. |
| Commonly misidentified lines (See CCLAC register) | HeLa and HEK293T cell lines used in this study are not listed in the database.                                                                                                                         |