ARS2 Regulates Nuclear Paraspeckle Formation through 3'-End Processing and Stability of NEAT1 Long Noncoding RNA

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ABSTRACT Nuclear paraspeckle assembly transcript 1 (NEAT1) is a long noncoding RNA that functions as an essential framework of subnuclear paraspeckle bodies. Of the two isoforms (NEAT1_1 and NEAT1_2) produced by alternative 3'-end RNA processing, the longer isoform, NEAT1_2, plays a crucial role in paraspeckle formation. Here, we demonstrate that the 3'-end processing and stability of NEAT1 RNAs are regulated by arsenic resistance protein 2 (ARS2), a factor interacting with the capping complex (CBC) that binds to the m7G cap structure of RNA polymerase II transcripts. The knockdown of ARS2 inhibited the association between NEAT1 and mammalian cleavage factor I (CFIm), which produces the shorter isoform, NEAT1_1. Furthermore, the knockdown of ARS2 led to the preferential stabilization of NEAT1_2. As a result, NEAT1_2 RNA levels were markedly elevated in ARS2 knockdown cells, leading to an increase in the number of paraspeckles. These results reveal a suppressive role for ARS2 in NEAT1_2 expression and the subsequent formation of paraspeckles.

KEYWORDS ARS2, NEAT1, long noncoding RNA, paraspeckle

A large proportion of mammalian genomes are transcribed into noncoding RNAs (ncRNAs) (1, 2). Among them, small species, such as microRNA (miRNA) and PIWI-interacting RNA (piRNA), are relatively well characterized. However, the functions of long species (lncRNAs) have not yet been elucidated in detail (1–4). Some lncRNAs, such as nuclear paraspeckle assembly transcript 1 (NEAT1) (5–7), gomafu (also known as myocardial infarction-associated transcript [MIAT]), metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) (8), and taurine-upregulated gene 1 (TUG1) lncRNAs (9), are localized in specific subnuclear structures collectively called nuclear bodies. Nuclear bodies often contain specific RNA and protein components and play important roles in various nuclear events (10, 11).

NEAT1 lncRNA has an essential function in the formation of paraspeckle nuclear bodies (11, 12). Paraspeckles, localizing adjacent to nuclear speckles, contain NEAT1 lncRNA and more than 40 proteins, most of which are RNA-binding proteins (RBPs) (11, 13). Paraspeckles function as a sponge, trapping specific RBPs and mRNAs, and have been physiologically implicated in antiviral responses, DNA damage responses, and the development of the corpus luteum and mammary glands (14–16). The formation of paraspeckles is initiated by NEAT1 gene transcription by RNA polymerase II (RNAPII) and the subsequent binding of paraspeckle proteins to the transcript. NEAT1 has two isoforms, NEAT1_1 and NEAT1_2, which are generated by alternative 3'-end processing of the NEAT1 primary transcript (17, 18). The shorter isoform, NEAT1_1 (approximately 3.7 kb), is produced by early polyadenylation initiated by the recruitment of the CPSF6-NUDT21 complex (also known as mammalian cleavage factor I [CFIm]) to the UGUA repeat sequence, while the longer isoform, NEAT1_2 (approximately 23 kb), is produced by transcription readthrough of the above-described polyadenylation site,

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mediated by hnRNPK inhibiting CFIm recruitment to the NEAT1 transcript (17). To generate the 3' end of NEAT1_2, RNase P recognizes its tRNA-like structure and digests it (18). The resultant 3' end of NEAT1_2 forms triple-helix (TH) structures, which stabilize its 3' end (19). Since NEAT1_2, but not NEAT1_1, is essential for paraspeckle formation, the alternative 3'-end processing of NEAT1 is a key process for regulating the NEAT1_1-to-NEAT1_2 ratio and, hence, paraspeckle formation *per se* (17).

The cap-binding complex (CBC), which binds to the 5'-end m7G cap structure of nascent RNAPII transcripts, recruits a number of proteins to the cap-proximal RNA region and promotes a plethora of RNA-related processes, including transcription, RNA processing, and nuclear export (20–23). One of the CBC-associating proteins is arsenic resistance protein 2 (ARS2). The CBC-ARS2 complex promotes the 3'-end processing of relatively short RNA transcripts, such as U snRNAs, histone mRNAs, and promoter upstream transcripts (PROMPTs) (24, 25). Furthermore, the CBC-ARS2 complex recruits the nuclear exosome-targeting (NEXT) and poly(A) tail exosome-targeting (PAXT) complexes via the adaptor protein ZC3H18, leading to mRNA degradation by the RNA exosome (26, 27).

In the present study, we demonstrate that ARS2 regulates both the 3'-end processing and stability of NEAT1. The knockdown (KD) of ARS2 (ARS2-KD) markedly increased NEAT1_2 expression levels via the inhibition of CFIm recruitment to NEAT1 and the preferential stabilization of NEAT1_2. As a result, paraspeckle formation was significantly induced in ARS2-KD cells. These results indicate that the ARS2-mediated regulation of NEAT1_2 expression has a strong impact on paraspeckle formation.

RESULTS

ARS2-KD leads to an increase in NEAT1_2 expression. To clarify whether ARS2 influences NEAT1 expression, we investigated the previously reported data from iCLIP (individual-nucleotide-resolution UV cross-linking and immunoprecipitation) with a CBC component (CBP20) and several CBC-binding factors (PHAX, ARS2, and ZC3H18) and found that ARS2 could interact with NEAT1 (18) (Fig. 1A). ARS2, as well as CBP20, PHAX, and ZCH18, showed preferential binding to a cap-proximal region of NEAT1 (Fig. 1A), whereas ARS2 also showed a binding signal in a cap-distal region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 1B).

These results prompted us to examine the effects of the KD of ARS2 on NEAT1 expression levels. We measured steady-state NEAT1 RNA levels in U2OS cells, which had been transfected with small interfering RNAs (siRNAs) against CBP80 (siCBP80), PHAX (siPHAX), or ARS2 (siARS2), by reverse transcription-quantitative PCR (qRT-PCR) analysis. In these experiments, we used three distinct pairs of primers (Fig. 1C). NEAT1 5' primers should detect both NEAT1_1 and NEAT1_2, while NEAT1 mid- and 3' primers should detect only NEAT1_2 (Fig. 1C). The expression of the ARS2 protein was successfully reduced by siARS2 (Fig. 1D). The results of the qRT-PCR analysis using NEAT1 5' primers demonstrated that NEAT1 (NEAT1_1 and NEAT1_2) RNA levels were slightly higher in CBP80- and ARS2-KD cells than in control-KD cells (Fig. 1E, blue bars). In contrast, the expression levels of NEAT1_2 alone were markedly increased by more than 5-fold in ARS2-KD cells but not in CBP80- or PHAX-KD cells (Fig. 1E, orange and gray bars). The increase in NEAT1_2 RNA levels was canceled by the rescue of ARS2 expression (Fig. 1F and G), indicating that ARS2 is crucial for this phenomenon. Similar results were obtained in cells treated with a second siARS2 (siARS2#2), which targets a distinct sequence of ARS2 mRNA (Fig. 1H and I). NEAT1_2 RNA levels were also increased in CPSF6-KD cells (Fig. 1J) because the KD of CPSF6 inhibits the 3'-end processing of NEAT1_1 and induces the production of NEAT1_2 (17). RNase protection assays (RPAs) also demonstrated that NEAT1_2 RNA levels were markedly higher in ARS2-KD cells than in control-KD cells (Fig. 1C, J, and K). RPAs also showed that NEAT1_1 RNA levels were not markedly affected (Fig. 1J and K).

To clarify the mechanisms underlying the increase in NEAT1_2 RNA levels in ARS2-KD cells, we investigated whether ARS2 is involved in NEAT1 expression at the step of transcription. Nascent RNAs were pulse-labeled by 5-ethynyl uridine (5EU) for

FIG 1 Increase in NEAT1_2 in ARS2 knockdown cells. (A and B) iCLIP-seq data for the indicated proteins. Reads were mapped to the NEAT1 (A) and GAPDH (B) genes. (C) Schematic diagram of NEAT1_1 and NEAT1_2. The primers and probe used for the qRT-PCR analysis and RNase protection assay (RPA), respectively, are shown. (D and E) U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, the indicated protein and RNA levels in cells were assessed by Western blotting (D) and qRT-PCR analysis (E), respectively. (F and G) U2OS cells were cotransfected with siARS2 and a control plasmid (pcDNA3.1) or an ARS2-expressing plasmid (pcDNA3.1-ARS2). After a 48-h incubation, the indicated protein (F) and RNA (G) levels in cells were similarly assessed. (H and I) U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, the indicated protein (H) and RNA (I) levels in the cells were similarly assessed. (J and K) U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, total RNA was extracted and subjected to an RPA. The intensities of RNase-protected bands are shown below the panels. Data are the means $\pm$ SD ($n = 4$). *, $P < 0.05$; **, $P < 0.01$.

FIG 2 Involvement of ARS2 in the 3'-end processing of NEAT1. (A) U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, nascent RNAs were labeled with 5EU for 30 min. 5EU-labeled nascent RNA levels were assessed by qRT-PCR analysis. (B to E) U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, RNAPII (PolII) (B and C)- or CPSF6 (D and E)-binding DNA levels on the indicated gene loci were assessed by a ChIP assay. (F) U2OS cells were transfected with a FLAG-tagged ARS2-expressing plasmid. After a 48-h incubation, whole-cell lysates were prepared, and immunoprecipitation was performed with an anti-FLAG antibody. A total of 0.5% of the lysate used for immunoprecipitation was loaded into the input lane. The precipitated protein was analyzed by Western blotting. Data are the means $\pm$ SD ($n = 4$). n.s., not significant; *, $P < 0.05$.

30 min and purified by immunoprecipitation with an anti-5EU antibody. Subsequent qRT-PCR analyses demonstrated that nascent NEAT1 RNA levels were not significantly different between ARS2-KD cells and control-KD cells (Fig. 2A). Furthermore, chromatin immunoprecipitation (ChIP) analyses revealed that RNAPII recruitment to the NEAT1 gene locus was not significantly altered in ARS2-KD cells (Fig. 2B and C). These results suggest that ARS2 regulates NEAT1 RNA levels at steps other than transcription.

ARS2-KD leads to a reduction in the recruitment of CFIm to NEAT1. Since ARS2 plays an important role in the 3'-end processing of RNAPII transcripts, such as U snRNAs, histone mRNAs, and PROMPTs (24), we examined whether ARS2 is also involved in the 3'-end processing of NEAT1. To investigate the recruitment of CFIm,

which induces the 3'-end processing of NEAT1_1 (17), we performed ChIP analyses with an antibody against CPSF6, a component of CFIm. The recruitment of CPSF6 to the repeated consensus sequence for CFIm binding (NEAT1 + 3506) was inhibited significantly more in ARS2-KD cells than in control-KD cells (Fig. 2D and E). To gain further mechanistic insights into this recruitment, we examined the interaction between ARS2 and CFIm using immunoprecipitation analyses. Both endogenous CFIm components, CPSF6 and NUDT21, were coimmunoprecipitated with FLAG-tagged ARS2 (Fig. 2F), indicating that ARS2 interacts with CFIm. RNase A treatment reduced the interaction between CFIm and ARS2; however, this interaction was still detected after the RNase A treatment (Fig. 2F). Some ARS2 still interacted with CFIm independently of RNA. Collectively, these results suggest that ARS2 regulates the 3'-end processing of NEAT1_1 through the recruitment of CFIm to NEAT1.

ARS2-KD leads to the stabilization of NEAT1. When we compared NEAT1 RNA levels in ARS2-KD cells and CPSF6-KD cells (Fig. 1I to K), we noted that NEAT1 RNAs appeared to be stabilized in ARS2-KD cells. Previous studies showed that the CBC-ARS2 complex recruited the NEXT or PAXT complexes via binding to ZC3H18, leading to mRNA degradation by the RNA exosome (26, 27). This prompted us to examine whether the same scenario is applicable to NEAT1. The KD of ZC3H18 and RRP40, components of the RNA exosome, led to a significant increase in NEAT1 expression levels (Fig. 3A to C), suggesting that ARS2 and ZC3H18 regulate NEAT1 stability. Therefore, we investigated the stability *per se* of NEAT1_1 and NEAT1_2 by measuring the half-lives of 5EU-pulse-labeled RNAs. Both NEAT1_1 and NEAT1_2 were more stable in ARS2- and ZC3H18-KD cells than in control-KD cells (Fig. 3D). Notably, the half-life of NEAT1_2 was prolonged by more than 7-fold (1.61 to 12.05 h) in ARS2-KD cells (Table 1). The effects of ZC3H18-KD were more modest but still detectable (Fig. 3D and Table 1). The recruitment of CPSF6 to the repeated consensus sequence for CFIm binding (NEAT1 + 3506) was not significantly different between ZC3H18-KD cells and control-KD cells (Fig. 3E and F). These results indicate that ARS2 and ZC3H18 regulate the stability of NEAT1, while ZC3H18 does not regulate the 3'-end processing of NEAT1_1.

ARS2-KD leads to an increase in MALAT1 expression. To examine whether ARS2 regulates the expression of other lncRNAs, we investigated the expression levels of several lncRNAs whose functions have already been clarified. ARS2-KD led to a significant increase in the expression levels of MALAT1, which is localized in nuclear speckles and regulates alternative splicing (8), whereas MIAT and TUG1 RNA levels were not altered (Fig. 4A). HOTAIR RNA levels were conversely lower in ARS2-KD cells than in control-KD cells for an unknown reason (Fig. 4A). Analyses of previously reported data from iCLIP with several CBC-binding factors (23) suggested that ARS2 could bind to MALAT1 (Fig. 4B). These results imply that ARS2 regulates the expression of not only NEAT1 but also other lncRNAs, such as MALAT1.

Both NEAT1_2 and MALAT1 lncRNAs carry TH structures at their 3' ends (19). ARS2 may negatively regulate the stability of NEAT1_2 and MALAT1 lncRNAs by binding to their TH structures. In order to examine the involvement of ARS2 and ZC3H18 in the stability of the TH structure, each 3' end of NEAT1_2 and MALAT1, including the TH structure, was inserted downstream of the renilla luciferase (Rluc) reporter gene. The mRNA levels of Rluc with a poly(A) sequence at the 3' end [Luc-poly(A)] were slightly lower in ARS2- and ZC3H18-KD cells than in control-KD cells (Fig. 4C). In contrast, Rluc mRNA levels with the TH structure (Luc-NEAT1 or MALAT1 3' end) were significantly higher in ARS2- and ZC3H18-KD cells than in control-KD cells (Fig. 4C). These results suggest that ARS2 and ZC3H18 regulate the stability of NEAT1 and MALAT1 lncRNAs by recognizing their TH structures.

ARS2 suppresses the formation of nuclear paraspeckles. Since ARS2-KD led to a marked increase in NEAT1_2 expression (Fig. 1), which is crucial for the formation of nuclear paraspeckles (17), attempts were made to detect nuclear paraspeckles in ARS2-KD cells by RNA-fluorescence *in situ* hybridization (FISH) with a probe against

FIG 3 Involvement of ARS2 in the stability of NEAT1. (A to C) U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, the indicated protein and RNA levels in the cells were assessed by Western blotting (A) and qRT-PCR analysis (B and C), respectively. (D) U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, RNAs were labeled with 5EU for 1.5 h, and cells were then cultured in fresh medium. At the indicated time points, 5EU-labeled RNA levels in cells were assessed by qRT-PCR analysis. (E and F) U2OS cells were transfected with siZC3H18. After a 48-h incubation, CPSF6-binding DNA levels on the indicated gene loci were assessed by a ChIP assay. Data are the means $\pm$ SD ($n = 4$). *, $P < 0.05$.

NEAT1_2. Nuclear paraspeckles were clearly detected in ARS2-KD cells as well as in control-KD cells and colocalized with SFPQ (splicing factor proline and glutamine rich), a marker of paraspeckles (Fig. 5A). The number of paraspeckles was approximately 2-fold higher in ARS2-KD cells than in control-KD cells (Fig. 5B), suggesting that

TABLE 1 Half-lives of NEAT1_1 and NEAT1_2

siRNA ^a	Mean half-life (h) $\pm$ SD	
	NEAT1_1	NEAT1_2
siCtrl	1.39 $\pm$ 0.19	1.61 $\pm$ 0.20
siARS2	6.46 $\pm$ 0.28	12.05 $\pm$ 2.70
siZC3H18	2.76 $\pm$ 0.14	4.43 $\pm$ 0.88

^asiCtrl, control siRNA.

FIG 4 Increase in MALAT1 in ARS2 knockdown cells. (A) U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, the indicated RNA levels in cells were assessed by qRT-PCR analysis. (B) iCLIP-seq data for the indicated proteins. Reads were mapped to the MALAT1 gene. (C) U2OS cells were cotransfected with the indicated siRNAs and the reporter plasmid expressing Rluc with the poly(A) sequence (Luc-polyA) or the 3'-end sequence of the NEAT1_2 gene (Luc-NEAT1-3'end) or the MALAT1 gene (Luc-MALAT1-3'end) downstream of each Rluc gene. After a 48-h incubation, Rluc mRNA levels were assessed by qRT-PCR analysis. Rluc mRNA levels were normalized by coexpressed firefly luciferase (Fluc) mRNA levels. Data are the means $\pm$ SD ($n = 4$). *, $P < 0.05$.

ARS2-KD led to increases in NEAT1_2 expression levels and, hence, the number of paraspeckles.

DISCUSSION

In the present study, we clarified the roles of ARS2 in the 3'-end processing and stability of NEAT1 (Fig. 6). We provide evidence to show that ARS2 recruits CFIm to the NEAT1_1-processing site (Fig. 2) and preferentially destabilizes NEAT1_2 (Fig. 3). Since ARS2 suppresses efficient NEAT1_2 expression, ARS2-KD cells induce the efficient formation of nuclear paraspeckles (Fig. 5) due to the upregulated expression of NEAT1_2 (Fig. 1). Although CBC and ARS2 have been reported to regulate the 3'-end processing and stability of RNAPII RNA transcripts (24, 26), the expression levels of NEAT1_2 were not enhanced by CBP80-KD (Fig. 1E). This may be because the CBC is also required for efficient transcriptional elongation by RNAPII (28). In contrast, ARS2 does not appear to affect the transcriptional elongation of NEAT1 because the recruitment of RNAPII to the NEAT1 gene locus was not altered in ARS2-KD cells (Fig. 2B).

Previous studies demonstrated that ARS2 was recruited to the CBC, bound to the cap structure, and that recruited ARS2 in turn recruited the primary miRNA (pri-miRNA)-processing complex, leading to the efficient processing of pri-miRNAs (29). CBC-ARS2 was also shown to regulate the 3'-end processing of replication-dependent histone mRNAs (RDHs) by binding to FLASH (30–32). Although the RNA processing regulation of a limited number of RNAPII transcripts, such as pri-miRNAs and RDHs, has been mechanistically elucidated in detail, the mechanisms by which ARS2 regulates the 3'-end polyadenylation of mRNAs remain unclear. Hallais et al. recently demonstrated

FIG 5 Increase in paraspeckle numbers in ARS2 knockdown cells. U2OS cells were transfected with siARS2. (A) After a 48-h incubation, cells were subjected to RNA-FISH with a NEAT1 probe and coimmunostaining with an anti-SFPQ antibody. (B) The numbers of paraspeckles per cell were counted. Data are the means $\pm$ SD. ***, $P < 0.0001$.

that ARS2 interacted with CLP1, a component of cleavage factor IIm (CFIm), which is involved in the 3'-end polyadenylation of mRNAs (24). However, in the present study, CLP1-KD had only a minor effect on the NEAT1 expression pattern (data not shown), whereas ARS2- and CPSF6-KD had more prominent effects (Fig. 1). The present results also demonstrate, for the first time, that ARS2 interacts with CFIm and regulates the 3'-end processing of NEAT1 through the recruitment of CFIm to NEAT1 (Fig. 2). Since CFIm regulates the 3'-end polyadenylation of numerous mRNAs (33), it would be interesting to investigate whether CFIm cooperates with ARS2 to induce the 3'-end polyadenylation of mRNAs.

In CBC-mediated RNA decay, CBC-ARS2 recruits the adaptor protein ZC3H18, which in turn recruits the NEXT or PAXT complexes, leading to RNA degradation by the RNA

FIG 6 Model of roles of ARS2 in the 3'-end processing and stability of NEAT1. See Discussion for details.

exosome (26, 27). The NEXT complex, comprising hMTR4, RBM7, and ZCCHC8, mainly targets unprocessed nascent RNAs (26), whereas the poly(A) tails of polyadenylated nuclear RNAs were targeted by the PAXT complex, comprising hMTR4, PABPN1, and ZFC3H1 (27). Since the degradation of NEAT1_1 has been reported to be mediated by PABPN1 (34), polyadenylated NEAT1_1 would be targeted by the PAXT complex containing PABPN1. The mechanisms underlying NEAT1_2 degradation currently remain unknown; however, ARS2-KD strongly stabilizes NEAT1_2 (Fig. 3D). The 3' end of NEAT1_2 is processed by RNase P, which recognizes a tRNA-like structure immediately downstream of the NEAT1_2 3' end (18). The resultant 3' end of NEAT1_2 is not polyadenylated but forms a TH structure (35). MALAT1 lncRNA also has the TH structure at its 3' terminus, and the TH structure has been reported to inhibit nuclear RNA decay (19, 35, 36). As shown in Fig. 4A, ARS2-KD significantly increased the expression levels of MALAT1 lncRNA but not other lncRNAs. Furthermore, Fig. 4C shows that the expression levels of luciferase mRNA with the TH structure were increased in ARS2- and ZC3H18-KD cells. ARS2 may negatively regulate the stability of NEAT1_2 and MALAT1 lncRNAs by recognizing their TH structures. Further studies are needed to clarify the mechanisms underlying the ARS2-mediated regulation of NEAT1_2 stability.

Although our results clearly indicate that ARS2 affects long RNA (NEAT1_2 [22 kb]), previous findings suggested that ARS2 affected relatively short RNA transcripts, such as U snRNAs, RDHs, and PROMPTs (24, 25). Since ARS2 associates with the CBC at the very 5' end of RNAs, it may be difficult for ARS2 to gain access to the 3' end of long RNAs. The structure of paraspeckles may explain how ARS2 affects the expression of such a long RNA (i.e., NEAT1). High-resolution *in situ* hybridization analysis using electron microscopy revealed the structural arrangement of NEAT1 in paraspeckles (6). The 5' end of NEAT1_1/NEAT1_2 and the 3' end of NEAT1_2 are located in the lateral area of paraspeckle bodies. The middle region of NEAT1_2 is located in the medial area of paraspeckle bodies (6). Since the 5' and 3' ends of NEAT1_2 are spatially adjacent, the 3' end of NEAT1_2 may be susceptible to the RNA exosome recruited to the NEAT1_2 5' end by ARS2 and ZC3H18.

NEAT1 is reportedly more unstable than other lncRNAs (12, 37), and transcription inhibition by an RNAPII inhibitor leads to a rapid reduction in NEAT1_2 and the subsequent disruption of paraspeckles (12, 37). The present results reveal the ARS2-mediated suppression of NEAT1_2 expression and inhibition of subsequent paraspeckle formation, indicating that ARS2 has a critical influence on paraspeckle dynamics.

MATERIALS AND METHODS

Cells. U2OS cells (a human osteosarcoma cell line) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μ g/ml), and penicillin (100 U/ml).

Plasmids. Human ARS2 cDNA was amplified by PCR from a HeLa cDNA library and cloned into pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA), resulting in pcDNA3.1-ARS2. The siARS2-targeted sequence of cloned ARS2 was mutated by site-directed mutagenesis of pcDNA3.1-ARS2 using appropriate primers. To insert a FLAG tag sequence, site-directed mutagenesis of pcDNA3.1-ARS2 was performed using appropriate primers, resulting in pcDNA3.1-FLAG-ARS2. The sequences of the primers used in the present study are shown in Table 2.

The reporter plasmids psiCHECK-2-NEAT1-3'end and psiCHECK-MALAT1-3'end, which have the 3'-end sequences of the NEAT1_2 (nucleotides [nt] +22624 to +23003) and MALAT1 (nt +8238 to +8617) genes, respectively, downstream of the Rluc gene, were constructed as follows. The fragments containing the 3'-end sequences of the NEAT1_2 and MALAT1 genes were synthesized by Eurofins Genomics (Tokyo, Japan). These fragments were ligated with an XhoI/NotI fragment of psiCHECK-2 (Promega, Madison, WI), resulting in psiCHECK-2-NEAT1-3'end and -MALAT1-3'end.

Transfection with siRNA. All siRNAs (Stealth siRNA or Silencer select siRNA) used in the present study were obtained from Thermo Fisher Scientific (Waltham, MA). Cells were transfected with siRNAs using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions.

qRT-PCR analysis. Total RNA was isolated from cells using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). cDNA was synthesized using 500 ng of total RNA with a Superscript IV first-strand synthesis system (Thermo Fisher Scientific). Unless otherwise noted, the reverse transcription reaction was performed using both random hexamers and oligo(dT) primers. qRT-PCR analysis was performed using Fast SYBR green master mix (Thermo Fisher Scientific) and StepOnePlus real-time PCR systems (Thermo Fisher Scientific). The sequences of the primers used in this study are listed in Table 2.

TABLE 2 Primers and probes used in this study

Purpose and primer or probe	Sequence
qRT-PCR	
NEAT1-5'-F	CAATTACTGTCGTTGGGATTTAGAGTG
NEAT1-5'-R	TTCTTACCATAACAGAGCAACATACCAG
NEAT1-mid'-F	CAGTTAGTTTATCAGTTCTCCCATCCA
NEAT1-mid'-R	GTTGTTGTCGTCACCTTTCAACTCT
NEAT1-3'-F	TGTGTGTGTAAGAGAGAGAAGTTGTGG
NEAT1-3'-R	AGAGGCTCAGAGAGGACTGTAACCTG
GAPDH-F	ATGAGAAGTATGACAACAGCCTCAA
GAPDH-R	AGTCCTTCCACGATACCAAGTT
MALAT1-F	GAATTGCGTCATTTAAAGCCTAGTT
MALAT1-R	GTTTCATCCTACCACTCCCAATTAAT
MIAT-F	GTGTGTGTCGTCGAGGTG
MIAT-R	CTGGGGTTAGTAAGAAGAGAA
TUG1-F	TAGCAGTTCCCAATCCTTG
TUG1-R	CACAAATCCCATCATTCCC
HOTAIR-F	CAGTGGGGAAGTCTGACTCG
HOTAIR-R	GTGCCTGGTGCTCTCTTACC
Fluc-F	TTCACCGATGCCACATTGA
Fluc-R	GTTCTCAGAGCACACCACGA
Rluc-F	CAAGGAGAAGGGCGAGGTTA
Rluc-R	TGTAGTTGCGGACAATCTGGA
ChIP	
NEAT1 + 3506-F	GCAAACAATTACTGTCGTTGGG
NEAT1 + 3506-R	ACAACAGCATACCCGAGACTAC
NEAT1 + 21517-F	TGTGTGTGTAAGAGAGAGAAGTTGTGG
NEAT1 + 21517-R	AGAGGCTCAGAGAGGACTGTAACCTG
NEAT1-294-F	GACCTCAACAACATCCGGGA
NEAT1-294-R	CTTTTTGGGATCGCGGACAG
NEAT1 + 3511-F	CAATTACTGTCGTTGGGATTTAGAGTG
NEAT1 + 3511-R	TTCTTACCATAACAGCAACATACCAG
NEAT1 + 11353-F	CAGTTAGTTTATCAGTTCTCCCATCCA
NEAT1 + 11353-R	GTTGTTGTCGTCACCTTTCAACTCT
GAPDH+1662-F	ATTTCCACCGCAAAATGGCC
GAPDH+1662-R	CCCGGTGACATTTACAGCCT
RPA (probe)	
NEAT1-RPA-F	TTGTCGACTTGGGGATGATGCAAACA
NEAT1-RPA-R	TAGAATTCTAATACGACTCACTATAGGGCAAAGTACTCCCCACCTAC
GAPDH-RPA-F	TTGTCGACCACAGTCCATGCCATCACT
GAPDH-RPA-F	TAGAATTCTAATACGACTCACTATAGGGTCAAAGGTGGAGGAGTGGG
FISH (probe)	
NEAT1_2-FISH-F	GTCTTTCCATCCACTCACGTCTATT
NEAT1_2-FISH-R	ATGACTAATACGACTCACTATAGGGCACCTAACTCATCTTACAGAC CACCAG
pcDNA3.1-ARS2	
ARS2-CDS-F	GTGCCATGGGTGACAGTGAT
ARS2-CDS-R	AACGGGGGACGGCTCAAAAGA
pcDNA3.1-FLAG-ARS2	
FLAG-ARS2-F	ACGATGACGATAAAATGGGTGACAGTGATGACGAG
FLAG-ARS2-R	CTTTGTAGTCCATGGGTCTCCCTATAGTGAGTCGT

Western blotting. A Western blot assay was performed as previously described (38). Briefly, whole-cell extracts were prepared, and 10 μ g of total protein per lane was loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. After electrophoresis under reducing conditions, bands of protein were transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Darmstadt, Germany). After blocking with 5% skim milk prepared in Tris-buffered saline-Tween 20 (0.1%) (TBS-T), the membrane was incubated with the primary antibodies, followed by incubation in the presence of horseradish peroxidase (HRP)-labeled anti-mouse or -rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA). The antibodies used in this study are shown in Table 3.

RPA. An RPA was performed as previously described (17). The 32 P-labeled antisense RNA probe against NEAT1 (Fig. 1C) was synthesized using T7 RNA polymerase, a linearized plasmid containing a

TABLE 3 Antibodies used in this study

Antigen	Type	Source or reference
ARS2	Rabbit	Bethyl, Montgomery, TX
CPSF6	Rabbit	Bethyl, Montgomery, TX
NUDT21	Rabbit	Proteintech, Rosemont, IL
FLAG	Mouse	Sigma-Aldrich, St. Louis, MO
GAPDH	Rabbit	Abcam, Cambridge, UK
α -Tubulin	Rabbit	Abcam, Cambridge, UK
ZC3H18	Rabbit	Sigma-Aldrich, St. Louis, MO
DIG	Mouse	Abcam, Cambridge, UK
SFPQ	Mouse	MBL, Aichi, Japan
PHAX	Mouse	21
CBP80	Mouse	21

NEAT1 fragment, and appropriate primers. The sequences of the primers used are shown in Table 2. Total RNA was isolated from the cells described above, and 20 μ g of total RNA was hybridized with the 32 P-labeled antisense RNA probe. After hybridization, the unhybridized single-stranded sequences of the probes were digested with an RNase A and RNase T1 cocktail (Thermo Fisher Scientific). The protected probes were analyzed by 8% denaturing PAGE followed by autoradiography.

Measurement of nascent RNA levels. U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, nascent RNAs were labeled with 5EU at 0.5 mM for 1.5 h. 5EU-labeled RNAs were biotinylated and immunoprecipitated using the Click-it nascent RNA capture kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Nascent RNA levels were measured using qRT-PCR.

Immunoprecipitation assay. Immunoprecipitation assays were performed as previously described (39). Briefly, U2OS cells were transfected with a FLAG-tagged ARS2-expressing plasmid (pcDNA3.1-FLAG-ARS2). After a 48-h incubation, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer without SDS and treated with RNase A (200 μ g/ml). FLAG-ARS2-binding proteins were coimmunoprecipitated using a mouse anti-FLAG antibody (M2) (Merck) from the lysate, and precipitated proteins were detected by Western blotting.

ChIP assay. U2OS cells were transfected with siRNAs. After a 48-h incubation, cells were treated with formaldehyde at a final concentration of 1% for cross-linking, and genomic DNA was then fragmented by sonication. DNA fragment-protein complexes were immunoprecipitated using a mouse anti-RNAPII C-terminal domain (CTD) antibody (Abcam, Cambridge, UK) or rabbit anti-CPSF6 antibody (Bethyl, Montgomery, TX). The ChIP assay kit was purchased from Merck. Precipitated DNA copy numbers were measured by quantitative PCR using the primers shown in Table 2.

Evaluation of RNA stabilities. U2OS cells were transfected with the indicated siRNAs. After a 48-h incubation, RNAs were pulse-labeled with 5EU at 0.5 mM for 1.5 h. 5EU-containing medium was washed out, and fresh culture medium was added. Total RNA was isolated from cells at the indicated time points. 5EU-labeled RNAs were then biotinylated and immunoprecipitated using the Click-it nascent RNA capture kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Immunoprecipitated RNA levels were measured by qRT-PCR, and the half-life of each RNA was assessed.

To distinguish expression levels between polyadenylated NEAT1_1 and nonpolyadenylated NEAT1_2, the reverse transcription reaction was performed using oligo(dT) primers for the determination of NEAT1_1 expression levels (Fig. 3D). We confirmed that the qRT-PCR analysis with oligo(dT) primers detected primarily NEAT1_1 (data not shown).

RNA-FISH. RNA-FISH was performed as previously described (17). The RNA probe against NEAT1_2 was synthesized using T7 RNA polymerase and the digoxigenin (DIG) RNA-labeling kit (Merck). A NEAT1_2 fragment was amplified by PCR from a U2OS cDNA library and used as the template. Cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.5% Triton X-100 in PBS, and then incubated with a hybridization solution containing the DIG-labeled RNA probe. After incubation, excess RNA probes were removed by treatment with 10 μ g/ml RNase A. Cells were blocked with a blocking reagent (Merck) and incubated with the primary antibodies, followed by incubation in the presence of an Alexa 488- or Alexa 568-labeled secondary antibody (Thermo Fisher Scientific). The antibodies used in the present study are listed in Table 3.

Analysis of iCLIP-seq data. iCLIP sequencing (iCLIP-seq) data for the indicated proteins in HeLa Kyoto cells were reanalyzed from deposited data sets (GEO accession number [GSE94427](#)), and the reads were mapped to the hg19 human genome assembly according to a method described previously (23).

Statistical analysis. The significance of differences was assessed using unpaired two-tailed Student's *t* test or one- or two-way analysis of variance (ANOVA) with Bonferroni's test. Data are presented as the means $\pm$ standard deviations (SD).

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M.M. designed and performed the experiments, analyzed data, and wrote the manuscript; I.T. performed the experiments; and M.O. obtained funding, designed some experiments, supervised the project, and wrote the manuscript.

We have no potential conflicts of interest.

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