Protein arginine methyltransferase CARM1 attenuates the paraspeckle-mediated nuclear retention of mRNAs containing IRAlus

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In many cells, mRNAs containing inverted repeated Alu elements (IRAulus) in their 3′ untranslated regions (UTRs) are inefficiently exported to the cytoplasm. Such nuclear retention correlates with paraspeckle-associated protein complexes containing p54nrb. However, nuclear retention of mRNAs containing IRAulus is variable, and how regulation of retention and export is achieved is poorly understood. Here we show one mechanism of such regulation via the arginine methyltransferase CARM1 (coactivator-associated arginine methyltransferase 1). We demonstrate that disruption of CARM1 enhances the nuclear retention of mRNAs containing IRAulus. CARM1 regulates this nuclear retention pathway at two levels: CARM1 methylates the coiled-coil domain of p54nrb, resulting in reduced binding of p54nrb to mRNAs containing IRAulus, and also acts as a transcription regulator to suppress NEAT1 transcription, leading to reduced paraspeckle formation. These actions of CARM1 work together synergistically to regulate the export of transcripts containing IRAulus from paraspeckles under certain cellular stresses, such as poly(I:C) treatment. This work demonstrates how a post-translational modification of an RNA-binding protein affects protein–RNA interaction and also uncovers a mechanism of transcriptional regulation of the long noncoding RNA NEAT1.

[Keywords: paraspeckles; p54nrb; nuclear retention; CARM1; NEAT1]

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The mammalian nucleus is highly organized into chromosome territories and a number of distinct membrane-less nuclear bodies or subnuclear structures that can affect nuclear neighborhoods and gene regulation (Zhao et al. 2009). Distinct nuclear bodies contain specific protein and RNA components that define particular nuclear processes (Mao et al. 2011b).

Paraspeckles, first identified in 2002 (Fox et al. 2002), are composed of the long noncoding RNA [lncRNA] NEAT1, which confers structural integrity and multiple proteins for its potential functions [Prasanth et al. 2005; Chen et al. 2008; Chen and Carmichael 2009; Clemson et al. 2009; Sasaki et al. 2009; Sunwoo et al. 2009; Naganuma et al. 2012]. There are two isoforms of NEAT1 lncRNAs, NEAT1_v1 and NEAT1_v2, produced by alternative 3′ end processing [Naganuma et al. 2012]. A live-cell imaging system aiming to visualize the inducible transcription of NEAT1 lncRNAs and paraspeckle proteins revealed that both the active transcription of NEAT1 and NEAT1 lncRNAs regulate paraspeckle maintenance and dynamics [Mao et al. 2011a]. In addition to several well-studied Drosophila behavior and human splicing [DBHS] family proteins [including PSP1α, p54nrb, and PSF, which were known to be localized to paraspeckles] [Fox et al. 2002, 2005; Bond and Fox 2009], new paraspeckle proteins [including many RNA-binding proteins...
Coordination of gene regulation by sequestering otherwise mature mRNAs containing IR elements ([IR]s) in gene regions (Chen et al. 1999), mRNA splicing (Cheng et al. 2007), muscle differentiation (Chen et al. 2002), adipocyte differentiation (Yadav et al. 2008), and T-cell development (Li et al. 2013).

Here we describe a new mechanism of gene regulation by CARM1. Disruption of CARM1 significantly enhances the nuclear retention of mRNAs containing IRA[Alus]. We demonstrate that CARM1 regulates the nuclear retention of mRNAs containing IRA[Alus] in paraspeckles at two levels. On the one hand, CARM1 methylates p54 neuropilin-related (p54nrp) for the binding to 3′ UTR IRA[Alus], resulting in the reduced binding capability to mRNAs containing IRA[Alus]; on the other hand, CARM1 suppresses NEAT1 transcription and paraspeckle formation. Actions of CARM1 at these two levels synergistically work together to regulate the export of transcripts containing IRA[Alus] from paraspeckles under certain cellular stresses, such as poly[IC] treatment. This represents one of a few examples where post-translational modification of an RNA-binding protein affects protein–RNA interaction and gene regulation. In addition, it shows how transcriptional regulation of the lncRNA NEAT1 can occur.

Results

p54nrp is methylated by CARM1

Very few substrates for CARM1 have been characterized, which limits the understanding of the roles of this enzyme. The identification of the repertoire of substrates for CARM1 is an essential step toward a complete understanding of its biological functions. CARM1 substrates are methylated in wild-type mouse embryonic fibroblast (MEF) cells but remain unmethylated in CARM1 knockout cells. Thus, the CARM1 knockout nuclear extracts are good substrates for in vitro methylation assays that would allow the identification of additional CARM1 substrates. We performed in vitro methylation assays using acid-extracted histones from the nuclei of wild-type and CARM1−/− MEFs as substrates and GST–CARM1 as an enzyme. The reaction mixtures were then run on an SDS-PAGE gel, and the indicated protein bands corresponding to potential CARM1 substrates were used for trypsin digestion and subsequent mass spectrometry (MS). Of
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the six identified substrates found in duplicated experiments, PABP1 [Lee and Bedford 2002] and SmB [Cheng et al. 2007] were previously described as CARM1 substrates. Interestingly, p54nrb, the well-known paraspeckle component, was also identified as a new CARM1 substrate and was chosen for further study [Fig. 1A].

p54nrb as a substrate of CARM1 was further validated by in vitro methylation of Escherichia coli-expressed histidine-tagged p54nrb [Fig. 1B]. Furthermore, a pan-screening for the activity of arginine methylase family members on p54nrb revealed that PRMT1 and PRMT6 could also methylate p54nrb, but the reaction of CARM1 on p54nrb was the strongest (Supplemental Fig. S1A). Moreover, the in vitro methylation of p54nrb truncations revealed that multiple arginine methylation sites on p54nrb were enriched in the coiled-coil domain and the C-terminal region of the protein (Supplemental Fig. S1B).

To identify the specific methylation sites catalyzed by CARM1, we developed CARM1 knockdown HeLa and HEK293 cell lines [Supplemental Fig. S2A]. Flag-tagged p54nrb was expressed in scramble shRNA-treated [scramble] cell lines, and then immunoprecipitation by anti-Flag was performed [Supplemental Fig. S2B] followed by MS analysis. Multiple methylation sites in p54nrb were identified from MS [Supplemental Fig. S2C]. Among these sites, R357, R365, and R378 were present in the anti-Flag precipitated complex in scramble cell lines but little in CARM1 knockdown cell lines [Supplemental Fig. S2D, data not shown], confirming that they are CARM1-methylated sites on p54nrb. In vitro methylation assays of p54nrb mutants in which these arginines were replaced with lysines further confirmed that R357, R365, and R378 were the major sites methylated by CARM1 [Fig. 1C].

Figure 1. p54nrb is methylated by CARM1. (A) p54nrb is a CARM1 substrate. Acid-extracted histones from CARM1 wild-type (wt; +/+) and knockout (−/−) MEFs were used as substrates, and GST-CARM1 was used as an enzyme to perform standard in vitro methylation assays. Reactions were done in duplicate, separated on SDS-PAGE, and transferred to PVDF membranes for fluorography and Ruby staining. The indicated methylated proteins were processed for protein identification using MS. (B) p54nrb is methylated in vitro by recombinant CARM1. In vitro methylation reactions were performed using recombinant His-tagged p54nrb with recombinant GST-CARM1 in the presence of [3H]AdoMet. Reactions were separated on SDS-PAGE and transferred to PVDF membranes for fluorography, Coomassie blue gel staining, and immunoblotting with anti-His antibody. (C) Lysine replacement of Arg357 and Arg365 (R357K, 365K) and of Arg378 and Arg383 (R378K, 383K) reduces p54nrb methylation. Mutants of p54nrb were made as indicated by MS results and expressed as His-tagged fusion proteins. Purified His-p54nrb mutants were incubated with GST-CARM1 in the presence of [3H]AdoMet. Methylated proteins were separated by SDS-PAGE and visualized by fluorography. The same membrane was subjected to Ponceau staining to verify equal loading. Note that R383 was not identified by MS results and was included in the mutation assay. (D) p54nrb interacts with CARM1. HeLa cells expressing Flag-p54nrb or Flag-EGFP [control] were immunoprecipitated with anti-Flag antibody and then immunoblotted with anti-Flag, anti-p54nrb, anti-PSF, anti-CARM1, and anti-PRMT6. Note that p54nrb specifically interacted with CARM1 but not PRMT6 in vivo. [IgGH] IgG heavy chain, [IgGL] IgG light chain. (E) RNA-independent interaction of p54nrb and CARM1 in HeLa cells. Total extracts of HeLa cells treated with RNase A were immunoprecipitated with CARM1 antibody or mock antibody and then immunoblotted with anti-CARM1 and anti-p54nrb antibodies. DAPI was used to indicate DNA. (F) CARM1 is a new component of paraspeckles. RNA in situ hybridization [ISH] was performed with digoxigenin [Dig]-labeled antisense NEAT1 probe [green] in HeLa cells, and representative images are shown. CARM1 and p54nrb are in red. NEAT1 colocalizes with CARM1 [top panels] and p54nrb [bottom panels].
The communoprecipitation (co-IP) assays between p54nr and CARM1 performed in HeLa [Fig. 1D,E] and HEK293 (data not shown) cells both revealed that they interacted with each other endogenously. CARM1 was detected in the Flag-p54nr immunoprecipitation complexes and vice versa [Fig. 1D,E]. Importantly, the co-IP was still detectable in the presence of RNase A [Fig. 1E]. Localization of the endogenous p54nr and CARM1 further revealed that they colocalized in the nucleus in HeLa cells [Fig. 1F] and largely within paraspeckles, as revealed by NEAT1 RNA in situ hybridization (ISH) and CARM1 immunostaining [Fig. 1G]. Together, these results suggest that p54nr is a new substrate for CARM1 and that p54nr methylation by CARM1 may occur within paraspeckles.

**CARM1 deficiency leads to enhanced nuclear retention of mRNAs containing IRAIus**

We next asked whether p54nr methylation by CARM1 could alter paraspeckle-associated p54nr function. Since p54nr is involved in the nuclear retention of mRNAs containing IRAIus in their 3′ UTRs [mRNA-IRAIus] [Chen et al. 2008; Mao et al. 2011a], we first performed mRNA nuclear retention assays in the scramble and CARM1 knockdown cell lines with mRNA-IRAIus reporter constructs as previously described [Chen et al. 2008]. Constructs with a single Alu element or IRAIus [originally from the 3′ UTR of the Nicn1 gene] in the 3′ UTR of egfp mRNA were individually transfected into the scramble and CARM1 knockdown HeLa cells. Consistent with previous reports [Chen et al. 2008; Mao et al. 2011a], the expression of EGFP protein was repressed by IRAIus, but not by a single Alu, in the 3′ UTR of egfp [Fig. 2A,B]. This repressed EGFP expression was largely due to the nuclear retention of egfp-IRAIus [Fig. 2A,B].

Strikingly, the EGFP fluorescence in CARM1 knockdown cells transfected with the EGFP-IRAIus construct was much more significantly reduced than that in scramble cells [Fig. 2A]. This enhanced repression effect was also confirmed by Western blotting with anti-EGFP antibody [Fig. 2A, top right panels, lanes 2,4]. Meanwhile, Northern blotting of transcripts of egfp from the same batch of transfected HeLa cells revealed that both transfections yielded comparable or higher levels of egfp mRNAs [Fig. 2A, bottom right panels, lanes 2,4], suggesting that the observed enhanced EGFP expression repression in CARM1 knockdown cells is post-transcriptional. We also observed similar phenomena using other constructs containing inserts of a single Alu element or IRAIus derived from the 3′ UTR of the Lin28 gene in the 3′ UTR of egfp mRNA [Supplemental Fig. S3A,B] and in other cell lines, such as in the scramble and CARM1 stable knockdown HEK293 cells [Supplemental Fig. S4A,B].

What mechanism accounts for reduced EGFP expression in CARM1 knockdown cells? Further analyses revealed that CARM1 knockdown strikingly enhanced the sequestration of mRNAs containing 3′ UTR IRAIus in the nucleus. First, nuclear/cytoplasmic [N/C] RNA fractionation analyses in scramble and CARM1 knockdown cells individually transfected with egfp-Alu or egfp-IRAIus constructs clearly showed that egfp-IRAIus mRNA was more efficiently retained in the nuclei of CARM1 knockdown cells than those in the nuclei of scramble cells [Fig. 2B, left panels {lanes 3,4,7,8} and right panel], while the N/C ratio of egfp-Alu mRNA was not altered [Fig. 2B, left panels {lanes 1,2,5,6} and right panel]. Second, visualization of the subcellular distribution of egfp-IRAIus in scramble and CARM1 knockdown cells by RNA ISH at the single-cell level revealed that egfp mRNAs with IRAIus were more highly enriched in the nucleus in CARM1 knockdown cells than those in scramble cells [Fig. 2C]. Third, if CARM1 were essential for the paraspeckle-associated mRNA-IRAIus nuclear retention, we would expect to observe that the endogenous mRNA-IRAIus would have a different fate in CARM1 depletion cells. There are hundreds of mRNAs containing IRAIus in their 3′ UTRs [Chen et al. 2008], and we chose a number of mRNAs that contain IRAIus in their 3′ UTRs and are expressed well in HeLa and HEK293 cells [nicn1, icmt, mpl30, and pccb] [Supplemental Fig. S5]. For each of these mRNAs, CARM1 knockdown resulted in more efficient retention in the nucleus in both cell lines [Fig. 2D,E, Supplemental Figs. S4D, S6], while the N/C distribution of actin mRNA remained unchanged. For experiments shown in these figures, we used PCR probes that specifically recognize mRNAs with the extended 3′ UTRs that contain IRAIus [Supplemental Fig. S5]. Note that the longer isoform of each of the mRNAs containing IRAIus examined increased its N/C ratio after knockdown of CARM1 [Fig. 2D, Supplemental Figs. S4D, S6A]. We further used Northern blotting to confirm the subcellular distribution of two such endogenous mRNAs: nicn1 [Fig. 2E] and icmt [Supplemental Fig. S6B]. The long isoform of nicn1 contains one pair of IRAIus in its 3′ UTR, but the short isoform lacks IRAIus. Correspondingly, the nicn1 long isoform is preferentially localized to the nucleus, while the short one is almost exclusively cytoplasmic [Fig. 2E, Chen et al. 2008]. Importantly, knockdown of CARM1 increased the amount of nicn1 long isoform retained in the nucleus [Fig. 2E], while the subcellular distribution of the nicn1 short isoform and actin mRNA remained unaltered [Fig. 2E]. Such altered nuclear retention regulation by CARM1 was also seen in another examined endogenous mRNA, icmt [Supplemental Fig. S6B], by Northern blotting. Together, these results demonstrate that the absence of CARM1 leads to an enhanced nuclear retention of mRNAs containing 3′ UTR IRAIus.

Recently, NEAT1 was shown to bind to hundreds of active chromatin sites in MCF7 cells [West et al. 2014]. We found that ~40% of such genes contain at least one pair of IRAIus in their 3′ UTRs expressed in MCF7 cells [Fig. 2F], implying that these transcribed nascent RNAs and corresponding mRNAs with 3′ UTR IRAIus are located close to paraspeckles and thus are capable of association with paraspeckle protein p54nr for their nuclear retention.
Figure 2. CARM1 deficiency enhances nuclear retention of mRNAs containing IRAlus. (A) CARM1 knockdown (KD) suppresses EGFP expression of egfp mRNA containing IRAlus in its 3′ UTR. (Left) IRAlus and Alu from the 3′ UTR of Nicn1 were inserted into the 3′ UTR of egfp mRNA [Chen et al. 2008]. Stable HeLa cell lines with the scramble shRNA treatment and CARM1 knockdown were transfected with the indicated plasmids, and representative images are shown. The white dotted line indicates the NEAT1 CHART-seq data in MCF7 cells were retrieved from West et al. (2014). Genes containing IR from different compartments were loaded as indicated by 28S and 18S rRNAs. (Right) The relative translation efficiency (the relative intensity of each corresponding band from Western blotting and Northern blotting shown in the middle panels) of egfp-Alu mRNA and mRNA with egfp-IRA was quantified from the same batch of transfected HeLa cells as described in the left panels was investigated by Western blotting by probing with anti-EGFP antibody and by Northern blotting by probing with a Dig-labeled egfp fragment. Actin was used as loading control for Western blotting. Equivalent amounts of total RNAs were loaded for Northern blotting as indicated by 28S and 18S rRNAs. (Left) The relative subcellular distribution of RNAs with IR from different subcellular compartments was investigated by Western blotting and Northern blotting. Equivalent amounts of total RNAs were loaded as indicated by 28S and 18S rRNAs. Primers to detect RNAs with IR from the same stripped membrane. (F) The expression of EGFP and transcripts of egfp from the same batch of transfected HeLa cells as described in the left panels was investigated by Western blotting by probing with anti-EGFP antibody and by Northern blotting by probing with a Dig-labeled egfp fragment. Actin was used as loading control for Western blotting. Equivalent amounts of total RNAs were loaded for Northern blotting as indicated by 28S and 18S rRNAs. (Right) The relative subcellular distribution of RNAs with IR from different compartments was loaded as indicated by 28S and 18S rRNAs. (F) Genes containing IRAlus in 3′ UTRs were identified by NEAT1 CHART-seq data in MCF7 cells were retrieved from West et al. (2014). Genes containing IRAlus in 3′ UTRs were identified from RefSeq by a home-brewed pipeline. In B, D, and E, error bars represent ±SD in triplicate experiments. (* P < 0.05; (**) P < 0.01; n = 3.
p54<sup>arb</sup> methylation by CARM1 regulates nuclear retention of mRNAs containing IRA<sub>Alu</sub>s

The next question is whether this CARM1-regulated nuclear retention is dependent on p54<sup>arb</sup>. To answer this question, we knocked down CARM1 or p54<sup>arb</sup> and then followed the expression and the subcellular distribution of the IRA<sub>Alu</sub>-containing reporter plasmid Flag-mcherry-IRA<sub>Alu</sub>s. The pair of IRA<sub>Alu</sub>s used in this construct is from the 3′ UTR of the Nicn1 gene (Chen et al. 2008). While knockdown of CARM1 reduced Flag-mcherry expression (Fig. 3A, lane 2; Supplemental Fig. S7A) with an enhanced nuclear retention of Flag-mcherry-IRA<sub>Alu</sub>s mRNAs (Fig. 3B), as other assays revealed (Fig. 2, Supplemental Figs. S3, S4), knockdown of p54<sup>arb</sup> enhanced Flag-mcherry expression (Fig. 3A, lane 4; Supplemental Fig. S7A) with a reduced nuclear retention of Flag-mcherry-IRA<sub>Alu</sub>s mRNAs (Fig. 3B). Double knockdown of CARM1 and p54<sup>arb</sup> showed little change in both protein expression and mRNA nuclear retention (Fig. 3A [lane 3], B). These results confirm that the nuclear retention of mRNAs with 3′ UTR IRA<sub>Alu</sub>s is mediated by the paraspeckle-localized protein p54<sup>arb</sup> and that CARM1 suppresses this nuclear retention pathway (Supplemental Fig. S7C).

We next asked whether the catalytic activity of CARM1 is required for the observed nuclear retention. We expressed the wild-type or the catalytically inactive E266Q mutant of CARM1 (Lee et al. 2002) in scramble or CARM1 knockdown [KD] (lanes 5,6) HeLa cells expressing Flag-mcherry-IRA<sub>Alu</sub>s, followed by Western blotting. Note that expression of wild-type CARM1 but not the E266Q-CARM1 in CARM1 knockdown cells could rescue the suppressed expression of Flag-mcherry-IRA<sub>Alu</sub>s. (D) Altered Flag-mcherry protein expression in C corresponds to the altered nuclear retention of transcripts of Flag-mcherry-IRA<sub>Alu</sub>s. The relative subcellular distribution of transcripts of Flag-mcherry-IRA<sub>Alu</sub>s from the same batch of transfected cells as described in A was investigated by RT-qPCR. See B for details. (E) Mutation of CARM1 methylation sites on p54<sup>arb</sup> enhances the p54<sup>arb</sup>-mediated mRNA nuclear retention. Reintroduction of wild-type (WT) CARM1 into scramble (lanes 2,3) or CARM1 knockdown [KD] (lanes 5,6) HeLa cells expressing Flag-mcherry-IRA<sub>Alu</sub>s, followed by Western blotting. Note that knockdown of CARM1 decreased Flag-mcherry expression, while knockdown of p54<sup>arb</sup> increases Flag-mcherry expression. (B) The altered Flag-mcherry protein expression in A corresponds to altered nuclear retention of transcripts of Flag-mcherry-IRA<sub>Alu</sub>s. The relative subcellular distribution of transcripts of Flag-mcherry-IRA<sub>Alu</sub>s from the same batch of transfected cells as described in A was investigated by RT-qPCR by probing mCherry. The level of nuclear retention was presented as the ratio of nuclear-retained mRNA to the relative amount of actin mRNA in each fraction. The endogenous mRNA with 3′ UTR IRA<sub>Alu</sub>s was also assayed and normalized in the same way. (C) IRA<sub>Alu</sub>s mRNA nuclear retention is dependent on the catalytic activity of CARM1. Reintroduction of wild-type [WT] CARM1 or the catalytic inert E266Q-CARM1 into scramble [lanes 2,3] or CARM1 knockdown [KD] (lanes 5,6) HeLa cells expressing Flag-mcherry-IRA<sub>Alu</sub>s, followed by Western blotting. Note that expression of wild-type CARM1 but not the E266Q-CARM1 in CARM1 knockdown cells could rescue the suppressed expression of Flag-mcherry-IRA<sub>Alu</sub>s. (D) Altered Flag-mcherry protein expression in C corresponds to the altered nuclear retention of transcripts of Flag-mcherry-IRA<sub>Alu</sub>s. The relative subcellular distribution of transcripts of Flag-mcherry-IRA<sub>Alu</sub>s from the same batch of transfected cells as described in C were investigated by RT-qPCR. See B for details. (E) Mutation of CARM1 methylation sites on p54<sup>arb</sup> enhances the p54<sup>arb</sup>-mediated mRNA nuclear retention. Reintroduction of wild-type (WT) CARM1 into scramble (lanes 2,3) or CARM1 knockdown [KD] (lanes 5,6) HeLa cells expressing Flag-mcherry-IRA<sub>Alu</sub>s, followed by Western blotting. Note that knockdown of CARM1 decreased Flag-mcherry expression, while knockdown of p54<sup>arb</sup> increases Flag-mcherry expression. (B) The altered Flag-mcherry protein expression in A corresponds to altered nuclear retention of transcripts of Flag-mcherry-IRA<sub>Alu</sub>s. The relative subcellular distribution of transcripts of Flag-mcherry-IRA<sub>Alu</sub>s from the same batch of transfected cells as described in A was investigated by RT-qPCR by probing mCherry. The level of nuclear retention was presented as the ratio of nuclear-retained mRNA to the relative amount of actin mRNA in each fraction. The endogenous mRNA with 3′ UTR IRA<sub>Alu</sub>s was also assayed and normalized in the same way. (C) IRA<sub>Alu</sub>s mRNA nuclear retention is dependent on the catalytic activity of CARM1. Reintroduction of wild-type [WT] CARM1 or the catalytic inert E266Q-CARM1 into scramble [lanes 2,3] or
CARM1 stable knockdown cell lines followed by the reporter assays described above (Fig. 3A, B). We found that reintroduction of the wild-type CARM1, but not the E266Q-CARM1, into CARM1 knockdown cells in which p54\textsuperscript{nb} is largely unmethylated due to CARM1 knockdown could largely restore the reduced Flag-mcherry expression [Fig. 3C, lanes 4–6; Supplemental Fig. S7B], the enhanced nuclear retention of Flag-mcherry-IR\textsubscript{Alu} mRNAs, and the endogenous mRNA with 3′ UTR IR\textsubscript{Alu} (Fig. 3D). However, reintroduction of either wild-type or E266Q-CARM1 into scramble treated cells in which CARM1 exists and p54\textsuperscript{nb} is methylated had little effect on the reporter assay or the endogenous mRNA with 3′ UTR IR\textsubscript{Alu} [Fig. 3C (lanes 1–3), D].

Moreover, we set up to examine whether the p54\textsuperscript{nb} variant carrying the lysine replacement of Arg357, Arg365, and Arg378 (R357K, 365K, and R378K) that showed a reduced p54\textsuperscript{nb} methylation by CARM1 (Fig. 1C) could enhance the p54\textsuperscript{nb}-mediated mRNA nuclear retention. Reintroduction of the p54\textsuperscript{nb} variant that carries these three point mutants (R357K, 365K, and R378K) into p54\textsuperscript{nb} knockdown cells not only was able to restore the p54\textsuperscript{nb} function but also exhibited a stronger effect on protein expression suppression and the corresponding mRNA nuclear retention in the reporter assay [Fig. 3E, F]. Meanwhile, a similar observation was also seen in the examined endogenous mRNA with 3′ UTR IR\textsubscript{Alu} [Fig. 3F]. Together, these results strongly suggest that CARM1 methylation on p54\textsuperscript{nb} attenuates the p54\textsuperscript{nb}-mediated mRNA nuclear retention.

The absence of CARM1 enhances the association of p54\textsuperscript{nb} and mRNAs with IR\textsubscript{Alu}

How does p54\textsuperscript{nb} methylation by CARM1 alter mRNA nuclear retention? Since p54\textsuperscript{nb} can bind to IR\textsubscript{Alu} mRNAs and subsequently retain them in the nucleus [Prasanth et al. 2005; Chen et al. 2008; Mao et al. 2011a], we performed p54\textsuperscript{nb} RNA immunoprecipitation (RIP) assays to examine whether the methylation of p54\textsuperscript{nb} could alter its binding activity with the IR\textsubscript{Alu} mRNAs. Both formaldehyde cross-linking RIP and UV cross-linking RIP with the anti-p54\textsuperscript{nb} antibody in scramble and CARM1 knockdown cell lines revealed that the absence of CARM1 augmented the association of p54\textsuperscript{nb} and mRNAs with IR\textsubscript{Alu} [Fig. 4A, B]. Importantly, the lysine replacement of Arg357, Arg365, and Arg378 (R357K, 365K, and R378K) of Flag-p54\textsuperscript{nb} that showed a reduced p54\textsuperscript{nb} methylation by CARM1 [Fig. 1C] also increased its binding to mRNAs with IR\textsubscript{Alu} compared with the wild-type Flag-p54\textsuperscript{nb} [Fig. 4C], with an anti-Flag antibody UV cross-linking RIP in HeLa cells.

To further confirm that p54\textsuperscript{nb} unmethylated in its coiled-coil domain has an increased ability to bind to mRNAs-IR\textsubscript{Alu}, we then performed a reciprocal pull-down assay by tagging an aptamer tRSA [Lioka et al. 2011] to IR\textsubscript{Alu} RNA (tRSA-IR\textsubscript{Alu}) [Fig. 4D]. Following tRSA immunoprecipitation with nuclear extracts, we found that tRSA-IR\textsubscript{Alu}, but not the tRSA alone, was preferentially associated with p54\textsuperscript{nb} [Fig. 4D]. Overexpression of CARM1 resulted in reduced binding of tRSA-IR\textsubscript{Alu} with p54\textsuperscript{nb} [Fig. 4D], confirming that methylation by CARM1 on p54\textsuperscript{nb} reduced its ability to bind to RNA-IR\textsubscript{Alu}s. Moreover, the partially purified Flag-p54\textsuperscript{nb}-MUT carrying the lysine replacements of R357K, 365K, and R378K significantly increased its ability to bind to the in vitro transcribed IR\textsubscript{Alu}s compared with the wild-type protein [Fig. 4E]. Since R357 and R365 are located at the coiled-coil domain and R378 is located very close to this domain at the C terminus of p54\textsuperscript{nb} [Supplemental Fig. S2D], these results strongly suggest that methylation on p54\textsuperscript{nb} at or near the coiled-coil domain by CARM1 reduces its capability to associate with RNAs containing IR\textsubscript{Alu}s.

p54\textsuperscript{nb} methylation at or near the coiled-coil domain by CARM1 alters its binding to dsRNAs

How does unmethylated p54\textsuperscript{nb} bind to mRNAs with IR\textsubscript{Alu}? Since p54\textsuperscript{nb} is known to bind to inosine-containing RNAs [Zhang and Carmichael 2001], one possibility is that p54\textsuperscript{nb} binds to edited mRNA-IR\textsubscript{Alu}s [Prasanth et al. 2005; Chen et al. 2008]. However, some extensively adenosine (A)-to-inosine (I) edited mRNA-IR\textsubscript{Alu}s were also exported to the cytoplasm [Prasanth et al. 2005; Chen et al. 2008; Hundleby et al. 2008; Elbarbary et al. 2013], and knockdown of ADAR1 did not alter the nuclear retention of these RNAs [Elbarbary et al. 2013], indicating that p54\textsuperscript{nb} can bind to RNAs by recognizing other RNA structures, such as dsRNAs formed by the IR\textsubscript{Alu}s at the 3′ UTR of mRNAs. We therefore examined whether p54\textsuperscript{nb} could bind to dsRNAs and whether p54\textsuperscript{nb} methylation by CARM1 could selectively alter its binding to these molecules.

The in vitro binding assays revealed that full-length of p54\textsuperscript{nb} bound to both dsRNAs and ssRNAs [Fig. 5A]; importantly, knockdown of CARM1 altered the binding ability of p54\textsuperscript{nb} to only dsRNAs but not to ssRNAs [Fig. 5A]. With a newly developed antibody [620me] that specifically recognizes the methylated R357 and R365 on a p54\textsuperscript{nb} peptide [Fig. 5B, left panels], we found that the methylated p54\textsuperscript{nb} (620me) was enriched in ssRNA but was barely detected in dsRNA pull-downs [Fig. 5B, right panels]. This observation further suggests that CARM1 methylation on p54\textsuperscript{nb} reduces its binding capacity to dsRNAs. Moreover, the partially purified Flag-p54\textsuperscript{nb}-MUT carrying the lysine replacements of R357K, 365K, and R378K promoted its binding capability to the in vitro transcribed dsRNAs but showed little binding alteration to ssRNAs [Fig. 5C]. These results thus support the notion that p54\textsuperscript{nb} methylation by CARM1 alters its binding to dsRNAs and that the unmethylated p54\textsuperscript{nb} binds more strongly to dsRNAs.

As it has been reported that p54\textsuperscript{nb} bound to ssDNAs [and RNAs] through its N terminus and to DNA through its C terminus [Yang et al. 1993], we speculated that the coiled-coil domain at the C terminus of p54\textsuperscript{nb} might mediate its binding to dsRNAs, such as mRNAs.
containing IRAls. This is indeed the case. Incubation of ssRNAs or dsRNAs with different Flag-tagged p54\textsuperscript{nr} truncations individually expressed in HeLa cells revealed that the truncation of p54\textsuperscript{nr} containing two RRM domains preferred to bind to ssRNAs, and the truncation of p54\textsuperscript{nr} with the addition of the coiled-coil domain exhibited a much higher capacity to bind to dsRNAs (Fig. 5D,E). Importantly, while the partially purified Flag-tagged p54\textsuperscript{nr} carrying only RRM domains showed little association to the in vitro transcribed IRA\textsubscript{Alu}s, the inclusion of the coiled-coil domain to this truncation significantly increased its ability to bind to IRA\textsubscript{Alu}s (Fig. 5F), confirming that the coiled-coil domain of p54\textsuperscript{nr} mediates its binding to dsRNAs, including mRNA-IRA\textsubscript{Alu}s. Together, these results strongly suggest that p54\textsuperscript{nr} methylation by CARM1 at or near the coiled-coil domain alters the interaction between dsRNAs and p54\textsuperscript{nr} via the coiled-coil domain.

CARM1 alters mRNA nuclear retention in paraspeckle

We showed that CARM1 methylated the coiled-coil domain of p54\textsuperscript{nr}, resulting in the reduced binding of p54\textsuperscript{nr} to mRNAs containing IRA\textsubscript{Alu}s. CARM1 is also known for its activity in transcription regulation [Chen et al. 1999]. CARM1 largely functions as a transcriptional coactivator [Chen et al. 1999] but also acts as a transcriptional cosuppressor [Xu et al. 2001]. We then asked whether the paraspeckle-localized CARM1 could also affect NEAT1 transcription. Surprisingly, we found that in CARM1 knockdown stable cell lines, the expression of both isoforms of NEAT1 was increased [Fig. 6A]. Correspondingly, the number of paraspeckles was also modestly increased in both CARM1 stable knockdown HeLa cells [Fig. 6B] and HEK293 cells [Fig. 6C]. CARM1 chromatin
immunoprecipitation (ChIP) revealed that CARM1 was enriched at the promoter of the \textit{NEAT1} gene (Fig. 6D). Further studies by nuclear run-on (NRO) assays showed that the transcription of nascent transcripts of \textit{NEAT1} was increased upon CARM1 knockdown, confirming the notion that CARM1 could regulate \textit{NEAT1} transcription as a transcriptional cosuppressor.

Collectively, we propose a model for CARM1-regulated nuclear retention of mRNAs containing IR\textit{Alu}s at paraspeckles. p54$^{\text{mth}}$ methylation by CARM1 at the coiled-coil domain reduces its ability to bind to mRNAs containing IR\textit{Alu}s (Figs. 2–5). This is in coordination with the transcriptional suppression of CARM1 at the \textit{NEAT1} promoter [Fig. 6]. Thus, CARM1 can synergistically act at two levels within paraspeckles to regulate its function for mRNA nuclear retention [Fig. 7I].

\textbf{CARM1 is involved in the poly(I:C)-stimulated enhancement of nuclear retention regulation}

Finally, it will be of interest to identify conditions that affect CARM1-regulated mRNA nuclear retention within paraspeckles. It is known that the transcription of \textit{NEAT1} RNAs and the formation of paraspeckles are induced upon virus infection or poly(I:C) treatment (Saha et al. 2006; Zhang et al. 2013a; Imamura et al. 2014). We therefore
investigated whether CARM1-regulated nuclear retention is affected by poly(I:C) treatment. In our experiments, transfection of poly(I:C) led to increased expression of interferon β and NEAT1 RNAs (Fig. 7A,B). Importantly, we found that the enrichment of CARM1 at the NEAT1 promoter was reduced upon poly(I:C) treatment (Fig. 7C), corresponding to the observed up-regulation of NEAT1 RNAs (Fig. 7B). Furthermore, the expression of NEAT1 RNAs remained at high levels in CARM1 knockdown cells, and NEAT1 expression could not be further induced upon poly(I:C) treatment (Fig. 7D). In addition, we found that the treatment of cells with poly(I:C) led to a strong reduction of p54nrb methylation on these arginine residues (Fig. 7E), while the expression of p54nrb remained unchanged. Finally, poly(I:C) treatment also resulted in an enhanced nuclear retention of a number of examined mRNAs containing IR Alus (Fig. 7F). Interestingly, we found that upon poly(I:C) treatment, the expression of CARM1 protein remained largely unchanged (Fig. 7G), while the paraspeckle-localized CARM1 was significantly reduced (Fig. 7H), indicating that CARM1 may rapidly change its subcellular localization with an unknown mechanism upon the stress treatment. Taken together, these results suggest that actions of CARM1 at p54nrb methylation and NEAT1 transcription synergistically work together to regulate the export of transcripts containing IR Alus from paraspeckles under certain cellular stresses, such as the poly(I:C) treatment (Fig. 7I).

Discussion

Paraspeckles play a role in gene regulation through nuclear retention mediated by the association of their key protein component, p54nrb, with mRNAs containing inverted repeats [Alu repeat in humans] in their 3’ UTRs [Prasanth et al. 2005; Chen et al. 2008; Chen and Carmichael 2009; Mao et al. 2011a]. Such nuclear-retained mRNAs are inefficiently exported to the cytoplasm, resulting in silencing of gene expression. However, the level of nuclear retention of transcripts containing IR Alus is variable, with some such mRNAs located in the nucleus, while others are in the cytoplasm [Prasanth et al. 2005; Chen et al. 2008; Hundleby et al. 2008; Chen and Carmichael 2009; Elbarbary et al. 2013]. How the nuclear retention of mRNAs containing IR Alus at paraspeckles is regulated has remained elusive. Here we demonstrate that CARM1 is a novel component of paraspeckles [Fig. 1]. Disruption of CARM1 significantly enhances the nuclear retention of mRNAs containing 3’ UTR IR Alus and represses gene expression [Fig. 2; Supplemental Figs. S3, S4, S6].
To achieve this regulation, CARM1 methylates p54\textsuperscript{rb} (Fig. 1; Supplemental Figs. S1, S2) and reduces its ability to associate with dsRNAs, such as mRNAs with IR\textsubscript{Alu} (Figs. 4, 5). On the other hand, CARM1 suppresses NEAT1 transcription and inhibits paraspeckle formation (Fig. 6).

While p54\textsuperscript{rb} is required for nuclear retention of mRNAs with 3' UTR IR\textsubscript{Alu}, the catalytic activity of CARM1 is also required for this effect (Fig. 3). How is binding of p54\textsuperscript{rb} to mRNA-IR\textsubscript{Alu} achieved? Since it is known that p54\textsuperscript{rb} binds to inosine-containing RNAs [Zhang and Carmichael 2001] and that a strong correlation between A-to-I RNA editing and retention was seen in mRNAs containing inverted repeats [Prasanth et al. 2005; Chen and Carmichael 2008; Chen et al. 2008], it has been thought that one of the consequences of IR\textsubscript{Alu} RNA editing is to retain edited mRNAs within nuclear paraspeckles. However, some mRNAs with edited IR\textsubscript{Alu} in their 3' UTRs were also observed in the cytoplasm [Chen et al. 2008; Hundley et al. 2008]. Knockdown of ADAR1, which is responsible for A-to-I RNA editing, has little effect on the export of nuclear-retained IR\textsubscript{Alu}.

Figure 7. CARM1 is involved in the poly(I:C)-stimulated enhancement of nuclear retention. (A) Poly(I:C) treatment induces the expression of IFN\textbeta. HeLa cells were transfected with 2 \mu g/mL poly(I:C) for 6 h and then harvested for analysis. (B) Poly(I:C) treatment induces NEAT1 expression. (C) Poly(I:C) treatment reduces the occupancy of CARM1 at the NEAT1 promoter. HeLa cells were transfected with poly(I:C) for 6 h and then harvested for CARM1 ChIP followed by qPCR. Data are presented as the percentage of CARM1 coprecipitating DNAs along the NEAT1 gene versus input under each indicated condition. (D) Poly(I:C) treatment induces NEAT1 expression in scramble-treated HeLa cells but not in CARM1 knockdown (KD) HeLa cells. (E) Poly(I:C) treatment reduces p54\textsuperscript{rb} methylation. HeLa cells were transfected with 2 \mu g/mL poly(I:C) for 6 h and then harvested for Western blotting analyses. (F) Poly(I:C) treatment enhances the nuclear retention of mRNAs containing IR\textsubscript{Alu}. HeLa cells were transfected with poly(I:C) for 6 h and then harvested for the nuclear and cytoplasmic fractionations. The relative subcellular distribution of transcripts of endogenous transcripts with IR\textsubscript{Alu} was investigated by RT-qPCR, normalized to the relative amount of actin mRNA in each fractionation, and compared with control HeLa cells. (G) The expression of CARM1 remains unchanged upon poly(I:C) treatment, as revealed by Western blotting. (H) The poly(I:C) treatment attenuates CARM1 localization to paraspeckles. (Left) Control and poly(I:C) transfected HeLa cells were stained with anti-p54\textsuperscript{rb} and anti-CARM1 antibodies, and representative images are shown. (Right) The statistics of p54\textsuperscript{rb} and CARM1 colocalization. The IODs (integrated optical densities) of punctuated anti-CARM1 and anti-p54\textsuperscript{rb} signals were measured by Image-Pro Plus from images taken with the same parameters (n > 100 double-positive staining cells). The ratio of IOD\textsubscript{CARM1} to IOD\textsubscript{p54\textsuperscript{rb}} was used to evaluate the extent of colocalization. The P-value from a one-tailed t-test in the pairwise comparison is shown. (I) A model of how the nuclear retention of IR\textsubscript{Alu} mRNAs at paraspeckles is regulated. (Left) Under normal conditions, CARM1 suppresses NEAT1 transcription and paraspeckle formation and also methylates p54\textsuperscript{rb}, resulting in the reduced ability to bind to mRNAs containing IR\textsubscript{Alu}. (Right) Upon appropriate stimulation, such as upon poly[I:C] treatment, actions of CARM1 are attenuated, resulting in an increased expression of NEAT1 RNA, unmethylated p54\textsuperscript{rb}, and enhanced nuclear retention of IR\textsubscript{Alu} mRNAs at paraspeckles. See the text for details. In B–D and F, error bars represent ±SD in triplicate experiments. [*] P < 0.01; n = 3.
mRNAs [Elbarbary et al. 2013]. These results thus suggested that another nuclear retention signal is required for p54\textsuperscript{nrp}-mediated mRNA nuclear retention in addition to RNA editing. Another formal possibility is that long imperfect duplexes formed by IRA\textsubscript{Alu}s in the 3’ UTRs of genes might influence gene regulation even in the absence of editing. We showed that such unique hairpin structures can directly bind to unmethylated p54\textsuperscript{nrp} [Figs. 4, 5], which in turn could lead to the nuclear retention of mRNAs containing IRA\textsubscript{Alu}s within paraspeckles. Moreover, it has been reported that p54\textsuperscript{nrp} can bind DNA through its C terminus (Yang et al. 1993). Consistent with this view, we demonstrated that the selective binding of dsRNAs to p54\textsuperscript{nrp} also requires its coiled-coil domain [Fig. 5].

Post-translational modifications of proteins play key roles in the regulation of many cellular processes by altering their associated effectors, including both proteins and a few reported RNAs. For instance, methylation/demethylation of Polycomb 2 protein could modulate its interaction with different IncRNAs [either TUG1 or MALAT1] with an unknown mechanism, resulting in the coordinated gene expression program in distinct subnuclear architectural compartments in response to growth signals (Yang et al. 2011). We observed that CARM1 methylation on p54\textsuperscript{nrp} occurs at or near the dsRNA-binding coiled-coil domain and that knockdown of CARM1 significantly alters p54\textsuperscript{nrp}-binding activity to dsRNAs but not ssRNAs [Fig. 5], resulting in enhanced nucleocytoplasmic export of mRNAs containing inverted Alu repeats. Thus, this regulation pathway represents one of a few examples where post-translational modification of an RNA-binding protein affects protein–RNA interaction and gene expression. Furthermore, although our data support the view that the reduced binding capability of methylated p54\textsuperscript{nrp} to dsRNAs could result from a direct conformational change of p54\textsuperscript{nrp} methylation at the coiled-coil domain, we cannot exclude the possibility that these methyl sites may recruit other effector proteins to facilitate the release of mRNA-IRA\textsubscript{Alu}s from methylated p54\textsuperscript{nrp} [Yang et al. 2014]. Finally, the dsRNA-binding protein STAU1 [Wickham et al. 1999] was recently shown to compete with p54\textsuperscript{nrp} for the binding of 3’ UTR IRA\textsubscript{Alu}s, independent of editing [Elbarbary et al. 2013]. It will be of interest to examine whether the binding of 3’ UTR IRA\textsubscript{Alu}s with STAU1 occurs after the release of mRNAs containing IRA\textsubscript{Alu}s from methylated p54\textsuperscript{nrp}.

Although many IncRNAs have been implicated in gene regulation and mammalian development [Ulitsky and Bartel 2013], how the expression of IncRNAs is regulated has remained poorly understood. Interestingly, we found that paraspeckle-localized CARM1 also plays a role in the transcription regulation of NEAT\textsuperscript{1} RNAs and affects paraspeckle formation [Fig. 6]. CARM1 is recognized as a transcriptional coactivator [Chen et al. 1999; Bedford and Clarke 2009] but also acts as a transcriptional repressor, such as in the cyclic adenosine monophosphate signaling pathway [Xu et al. 2001]. In the case of NEAT\textsuperscript{1} regulation, we found that CARM1 is enriched at the NEAT\textsuperscript{1} promoter and acts as a transcriptional repressor [Fig. 6]. Conspicuously, this “negative” regulation of NEAT\textsuperscript{1} transcription and paraspeckle formation by CARM1 could in fact lead to a “positive” gene expression output by allowing the export of more mRNAs with IRA\textsubscript{Alu}s to the cytoplasm for protein translation. This final output in gene expression is therefore consistent with a general role of CARM1 in promoting gene transcription [Chen et al. 1999; Bedford and Clarke 2009]. However, we do not yet know what directs CARM1 to the NEAT\textsuperscript{1} promoter and what other factors are involved in this NEAT\textsuperscript{1} transcription regulation by CARM1. It had been reported recently that NEAT\textsuperscript{1} RNAs can sequester the transcriptional regulator PSF to regulate gene expression [Hirose et al. 2014; Imamura et al. 2014]. Since PSF is largely localized to paraspeckles, it will be of interest to examine whether PSF can autoregulate NEAT\textsuperscript{1} transcription in coordination with CARM1.

The identification of CARM1 functioning at two levels within paraspeckles is particularly interesting. On the one hand, CARM1 methylates p54\textsuperscript{nrp}, resulting in the reduced ability to binding to mRNAs containing IRA\textsubscript{Alu}s [Figs. 3–5]. On the other hand, CARM1 suppresses NEAT\textsuperscript{1} transcription and paraspeckle formation [Figs. 6, 7]. Actions of CARM1 at these two levels synergistically work together to regulate the export of transcripts containing IRA\textsubscript{Alu}s from paraspeckles. In response to certain cellular stressors, such as poly[IC] treatment, we observed the reduced p54\textsuperscript{nrp} methylation [enhanced mRNA nuclear retention] and the decreased binding of CARM1 to the NEAT\textsuperscript{1} promoter [enhanced transcription of NEAT\textsuperscript{1} and paraspeckle formation] [Fig. 7A–F]. Thus, this paraspeckle-mediated nuclear retention was enhanced upon poly[IC] stimulation, leading to less translation of mRNAs containing IRA\textsubscript{Alu}s [Fig. 7I].

**Materials and methods**

**Cell culture, plasmids, transfection, and knockdown with shRNAs**

HeLa, HEK293, and MEF cell lines were cultured using standard protocols. HEK293 CARM1 Flp-in stable cell line, wide-type, and CARM1 \textsuperscript{−/−} MEFs have been described [Cheng et al. 2007]. Transfection was carried out with either X-tremeGENE 9 (Roche) or Lipofectamine 2000 (Invitrogen) according to the manufacturers’ protocols. To generate Flag-p54\textsuperscript{nrp}-wt (MUT) or Flag-EGFP stable cell lines, pcDNA3.1 (+)-Flag-p54\textsuperscript{nrp}-wt (MUT) or pcDNA3.1 (+)-Flag-EGFP was transfected into HEK293 CARM1 Flp-in cells followed by G418 selection. The plasmids pEGFP-SC-Nicn1-Alu (IRA\textsubscript{Alu}s) and pEGFP-SC-Lin28-Alu (IRA\textsubscript{Alu}s) have been described [Chen et al. 2008]. CARM1 knockdown was carried out as described [Ou et al. 2011], and stable HeLa and HEK293 lines were generated. To knock down p54\textsuperscript{nrp}, the sequence “CGAGGCGAAAGTCCATATTACC” was inserted into pLVTHM vector with MLU1 and Clal, and the construct was packaged into lentivirus to infect HeLa cells. All plasmids used are listed in the Supplemental Material.

**In vitro methylation assay**

In vitro methylation assay was carried out as described [Cheng et al. 2007]. GST-CARM1 and His-p54\textsuperscript{nrp} were overexpressed...
and purified from *E. coli*. In vitro methylation reactions were performed in a final volume of 30 µL of PBS (pH 7.4). The reaction contained 0.5–1.0 µg of substrates and 0.2–0.4 µg of recombinant enzymes. All methylation reactions were carried out in the presence of 0.5 µCi S-adenosyl-L-[methyl-3H] methionine [85 Ci/mmol from a 0.5 mM Ci/mL stock solution, Perkin-Elmer]. The reaction was incubated for 1 h at 30°C and then subjected to fluorography by separation on SDS-PAGE [12% gel], transferred to a PVDF membrane, treated with Enhance (Perkin-Elmer), and exposed to film overnight. After in vitro methylation followed by fluorography, the same membrane was subjected to Coomassie blue staining, immunoblotting with anti-His tag, or Ponceau staining. We overlaid the fluorograph with the stained membrane and signals from immunoblots and verified that the stained protein bands matched with the methylated bands.

**Immunoprecipitation**

HeLa cells (10⁷) were harvested and suspended in immunoprecipitation buffer [50 mM HEPES at pH 7.6, 250 mM NaCl, 5 mM EDTA at pH 8.0, 0.1% NP-40, 1 mM PMSE, protease inhibitor cocktail] followed by sonication. After centrifuging at 13,000 rpm for 15 min at 4°C, the supernatant was transferred into a new tube and precleared with 10 µL of Dynabeads G. Next, the precleared supernatant was incubated with 20 µL of Dynabeads G with antibodies for p54nrb (BD) or IgG (Sigma) for 4 h at 4°C followed by washing with immunoprecipitation buffer. To harvest the protein complex, 50 µL of 1× SDS loading buffer (62.4 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.0012% bromophenol blue) was added, incubated for 10 min at 95°C, and analyzed by Western blotting.

**DNA methylation**

pcDNA3.1 (+)-Flag- p54nrb was transfected into scramble and MS for methylation. HeLa cells (10⁷) were harvested and suspended in immunoprecipitation buffer and purified from Dynabeads G by adding 100 µL of elution buffer [100 mM NaCl, 1% NP40, 1% sodium deoxycholate, 2 mM VRC, protease inhibitor cocktail]. The cells were homogenized by sonication and then centrifuged at 13,000 rpm for 10 min at 4°C to remove the insoluble material. Fifty microliters of supernatant were used as input. The rest of the supernatant was precleared by applying 10 µL of Dynabeads G [Invitrogen] with 20 µg/mL yeast tRNA for 1 h at 4°C. Next, the precleared lysate was incubated with Dynabeads G that were precoated with 2 µg of antibodies for p54nrb (BD) or IgG (Sigma) for 4 h at 4°C. The cells were washed three times for 5 min with washing buffer I [50 mM Tris-HCl at pH 7.5, 1 M NaCl, 1% NP40, 1% sodium deoxycholate, 2 mM VRC] and three times for 5 min with washing buffer II [50 mM Tris-HCl at pH 7.5, 1 M NaCl, 1% NP40, 1% sodium deoxycholate, 2 mM VRC, 1 M urea]. The immunoprecipitated complex was eluted from Dynabeads G by adding 100 µL of elution buffer [100 mM Tris-HCl at pH 8.0, 10 mM EDTA, 1% SDS]. Proteinase K [0.2µg/µL] and 200 mM NaCl were added into the RNA sample and incubated for 1 h at 42°C followed by 1 h at 65°C. RNA was then extracted, digested with DNase I (Ambion), equal amounts of RNAs from different fractions were reverse-transcribed into cDNAs with SuperScript II (Invitrogen). β-Actin was used as an endogenous control.

**RNA ISH and immunofluorescence microscopy**

Simultaneous RNA ISH and immunofluorescence were performed as described [Yin et al. 2012] with slight modifications. Hybridization was performed with in vitro transcribed DIG-labeled probes. For colocalization studies, cells were costained with mouse anti-p54nrb (BD) and/or rabbit anti-CARM1 [Bethyl Laboratories]. The nuclei were counterstained with DAPI. Images were taken with a Zeiss LSM 510 microscope or an Olympus IX70 DeltaVision RT deconvolution system microscope. For statistical analysis, >300 cells from each group were observed and calculated. Image analyses of signal intensity were carried out by Image-Pro Plus according to standard protocols and were described previously [Yin et al. 2015].

**Formaldehyde cross-linking RIP**

HeLa cells (10⁷) were washed twice with 5 mL of PBS and cross-linked with 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped by the addition of glycerine to a final concentration of 0.25 M followed by incubation for 5 min at room temperature. After washing twice with 5 mL of cold PBS, cells were collected and suspended in 1 mL of RIP buffer [50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM PMSE, 2 mM VRC, protease inhibitor cocktail]. The cells were homogenized by sonication and then centrifuged at 13,000 rpm for 10 min at 4°C to remove the insoluble material. Fifty microliters of supernatant was saved as input. The rest of the supernatant was precleared by applying 10 µL of Dynabeads G [Invitrogen] with 20 µg/mL yeast tRNA for 1 h at 4°C. Next, the precleared lysate was incubated with Dynabeads G that were precoated with 2 µg of antibodies for p54nrb (BD) or IgG (Sigma) for 4 h at 4°C. The cells were washed three times for 5 min with washing buffer I [50 mM Tris-HCl at pH 7.5, 1 M NaCl, 1% NP40, 1% sodium deoxycholate, 2 mM VRC] and three times for 5 min with washing buffer II [50 mM Tris-HCl at pH 7.5, 1 M NaCl, 1% NP40, 1% sodium deoxycholate, 2 mM VRC, 1 M urea]. The immunoprecipitated complex was eluted from Dynabeads G by adding 100 µL of elution buffer [100 mM Tris-HCl at pH 8.0, 10 mM EDTA, 1% SDS]. Proteinase K [0.2µg/µL] and 200 mM NaCl were added into the RNA sample and incubated for 1 h at 42°C followed by 1 h at 65°C. RNA was then extracted, digested with DNase I (Ambion), and used to synthesize cDNA using random hexamers (SuperScript III, Invitrogen) followed by qPCR analysis [AceQ, Vazyme].

**UV cross-linking RIP**

UV cross-linking RIP was carried out as described [Zhang et al. 2013b]. HeLa cells (10⁷) were washed twice with 5 mL of cold
PBS and irradiated at 150 mJ/cm² at 254 nm in a Stratalinker. Cells were collected and resuspended in 1 mL of RIP buffer. The cells were homogenized by sonication and then centrifuged at 13,000 rpm for 10 min at 4°C to remove the insoluble material. Fifty microliters of supernatant was saved as input. The rest of the supernatant was precleared by applying 10 μL of Dynabeads G (Invitrogen) with 20 μg/mL yeast tRNA buffer for 1 h at 4°C. Next, the precleared lysate was incubated with Dynabeads G that were precoated with 2 μg of antibodies for p54nrb (BD) or IgG (Sigma) for 4 h at 4°C. The beads were washed three times for 5 min with washing buffer I and three times for 5 min with washing buffer II. The immunoprecipitated complex was eluted from Dynabeads G by adding 200 μL of elution buffer (100 mM Tris-Cl, pH 8.0, 1 mM EDTA) at 4°C. The immunoprecipitated complex was eluted from Dynabeads G and incubated for 40 μL of streptavidin Dynabeads for 20 min at 4°C. Next, 20 μg/mL yeast tRNA was added to block unspecific binding and incubated for 20 min at 4°C. The precleared lysate was divided into two parts, and each was supplemented with 2 μg of biotin-labeled ss_egfp (full length of egfp) or ds_egfp (an inverted repeated fragment of egfp) and incubated for 1.5 h followed by addition of 40 μL of streptavidin Dynabeads and incubation for another 1.5 h at room temperature. Beads were washed four times for 5 min with RIP buffer containing 0.5% sodium deoxycholate and boiled in 1× SDS loading buffer for 10 min at 100°C. The retrieved proteins were analyzed by Western blotting with anti-p54nrb (BD), anti-methyl-p54nrb (620me), and anti-PSF (Sigma).

tRNA pull-down

tRNA pull-down assays were performed as described by Arab et al. (2014) with modifications. Two 10-cm dishes of cells expressing Flag-p54nrb-wt (MUT) or Flag-EGFP were used to immunoprecipitate with anti-Flag M2 (20 μL for each reaction) (Sigma). After immunoprecipitation and washing, one out of five beads was saved for Western blot. The rest were equilibrated in binding buffer (50 mM Tris at pH 8.0, 10% glycerol, 100 mM KCl, 5 mM

CARM1 alters mRNA nuclear retention in paraspeckle
A stringent pipeline was developed to identify 3′ UTRs containing IRAlus DNA CHART.

A stringent pipeline was developed to identify 3′ UTR IRAhs genes enriched by NEAT1 DNA CHART. To identify 3′ UTR IRAhs genes, IRAhs were identified by RepeatMasker (Tarailo-Graovac and Chen 2009). The 3′ UTRs were defined in a ReSeq gene file. Next, the overlap region between IRAhs and the 3′ UTR was calculated to ensure that IRAhs were located in the 3′ UTRs. In total, 545 genes containing 3′ UTR IRAhs were obtained. In MCF7 cells, 388 genes containing 3′ UTR IRAhs were expressed [reads per kilobase per million mapped reads (RPKM) ≥ 2] by analyzing the available RNA sequencing data sets (MCF7 RNA-seq; Gene Expression Omnibus [GEO]: GSE47042) (Janky et al. 2014). To analyze the NEAT1 DNA CHART-enriched IRAhs-containing genes, the NEAT1 DNA CHART-seq data sets carried out in MCF7 cells (GEO: GSE47042) (West et al. 2014) were used to call peaks by MACS (version 1.4.2, 20120305) (Feng et al. 2012). One-hundred-forty-nine MCF7 cells expressed genes that contain 3′ UTR IRAhs enriched by the NEAT1 DNA CHART.

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Protein arginine methyltransferase CARM1 attenuates the paraspeckle-mediated nuclear retention of mRNAs containing IR Alus

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