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 (IRAus) 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 IRAus 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 IRAus. 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 IRAus, 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 IRAus 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...
CARM1 alters mRNA nuclear retention in paraspeckle

Proteins and paraspeckle proteins were recently identified [Naganuma et al. 2012; West et al. 2014].

Recent studies have revealed that both NEAT1 lncRNAs and paraspeckle proteins can mediate the function of paraspeckles in gene regulation, although the detailed mechanisms remain to be fully defined. For example, the abundant NEAT1 RNA was shown to regulate transcription by sequestering the paraspeckle protein PSF [Hirose et al. 2014; Imamura et al. 2014]. Such sequestration of the transcriptional repressor PSF resulted in the activation of IL8 expression upon immune stimuli [Imamura et al. 2014] and ADARB2 transcription [Hirose et al. 2014]. Moreover, genome-wide analyses recently revealed that NEAT1 can associate with hundreds of active chromatin sites [West et al. 2014], consistent with the view that paraspeckles may be involved in transcription regulation.

In addition to NEAT1-mediated regulation, p54\textsuperscript{rb} is involved in the paraspeckle-mediated nuclear retention of mRNAs containing inverted repeats in their 3′ untranslated regions (UTRs) [Prasanth et al. 2005; Chen et al. 2008; Chen and Carmichael 2009; Mao et al. 2011a; Elbarbary et al. 2013]. The mouse CTN-RNA, which contains a dsRNA structure resulting from inverted short interspersed nuclear elements (SINEs) in its 3′ UTR, is retained in the nucleus and at least partially localized to paraspeckles [Prasanth et al. 2005]. In human cells, hundreds of genes contain inverted repeated SINEs [mainly Alu elements] in their 3′ UTRs. Alu elements are conserved from primates and account for almost all of the human SINEs and >10% of the genome. Their abundance leads to the frequent occurrence of inverted repeat sequences [inverted repeated Alu elements] in gene regions [Chen and Carmichael 2008]. We reported previously that mRNAs containing IRA\textsubscript{Alus} in their 3′ UTRs are retained in the nucleus in paraspeckles and in association with p54\textsuperscript{rb} [Chen et al. 2008; Chen and Carmichael 2009]. Such regulation at paraspeckles in human cells was further demonstrated by live-cell imaging [Mao et al. 2011a]. Therefore, this nuclear retention pathway of IRA\textsubscript{Alus} in 3′ UTRs of genes provides an additional layer of gene regulation by sequestering otherwise mature mRNAs within the nucleus.

Interestingly, we and others observed that the nuclear retention of transcripts containing IRA\textsubscript{Alus} is variable, with some such mRNAs located in the nucleus, while others are in the cytoplasm [Chen and Carmichael 2008; Chen et al. 2008; Hundley et al. 2008; Elbarbary et al. 2013]. How is such nuclear retention of mRNAs containing IRA\textsubscript{Alus} at paraspeckles achieved and regulated? Earlier observations suggested that the mouse CTN-RNA is retained in the nucleus until cell stress occurs, resulting in the cleavage and removal of its 3′ UTR nuclear retention signal [inverted repeats of murine SINEs] by an unknown mechanism. The truncated message is then transported efficiently to the cytoplasm for translation [Prasanth et al. 2005]. However, some mRNAs containing IRA\textsubscript{Alus} were seen in the cytoplasm [Chen et al. 2008; Hundley et al. 2008; Elbarbary et al. 2013], implying that other mechanisms may be involved in the release of paraspeckle-mediated nuclear retention of mRNAs containing IRA\textsubscript{Alus}. For instance, it had been shown recently that the dsRNA-binding protein Staufen 1 competed with p54\textsuperscript{rb} for the binding to 3′ UTR IRA\textsubscript{Alus}, resulting in an enhanced nuclear export and translation of these RNAs [Elbarbary et al. 2013]. Another hypothesis is that post-translational modifications on p54\textsuperscript{rb} could alter its binding activity to mRNAs containing IRA\textsubscript{Alus}.

CARM1 (coactivator-associated arginine methyltransferase), also known as PRMT4, was the first identified nuclear receptor coactivator in a family of nine PRMT members [PRMT1–9] [Bedford and Clarke 2009]. CARM1 methylates histone H3 at Arg17, generating a docking site for the recruitment of the methylarginine effector TDRD3 [Yang et al. 2010]. CARM1 also methylates many nonhistone proteins that play important roles in a number of biological processes, including transcriptional regulation [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\textsubscript{Alus}. We demonstrate that CARM1 regulates the nuclear retention of mRNAs containing IRA\textsubscript{Alus} in paraspeckles at two levels. On the one hand, CARM1 methylates p54\textsuperscript{rb}, resulting in the reduced binding capability to mRNAs containing IRA\textsubscript{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\textsubscript{Alus} from paraspeckles under certain cellular stresses, such as poly[C] 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

p54\textsuperscript{rb} 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\textsuperscript{−/−} 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
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, p54 nh, the well-known paraspeckle component, was also identified as a new CARM1 substrate and was chosen for further study [Fig. 1A].

p54 nh as a substrate of CARM1 was further validated by CARM1 in vitro methylation of Escherichia coli-expressed histidine-tagged p54 nh [Fig. 1B]. Furthermore, a pan-screening for the activity of arginine methylase family members on p54 nh revealed that PRMT1 and PRMT6 could also methylate p54 nh, but the reaction of CARM1 on p54 nh was the strongest [Supplemental Fig. S1A]. Moreover, the in vitro methylation of p54 nh truncations revealed that multiple arginine methylation sites on p54 nh 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 p54 nh was expressed in scramble shRNA-treated [scramble] and CARM1 shRNA-treated [CARM1 knockdown] cell lines, and then immunoprecipitation by anti-Flag was performed [Supplemental Fig. S2B] followed by MS analysis. Multiple methylation sites in p54 nh 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 p54 nh. In vitro methylation assays of p54 nh 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.** p54 nh is methylated by CARM1. (A) p54 nh is a CARM1 substrate. Acid-extracted histones from CARM1 wild-type (+/+) 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) p54 nh is methylated in vitro by recombinant CARM1. In vitro methylation reactions were performed using recombinant His-tagged p54 nh 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 p54 nh methylation. Mutants of p54 nh were made as indicated by MS results and expressed as His-tagged fusion proteins. Purified His-p54 nh 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 but was included in the mutation assay. (D) p54 nh interacts with CARM1. HeLa cells expressing Flag-p54 nh or Flag-EGFP [control] were immunoprecipitated with anti-Flag antibody and then immunoblotted with anti-Flag, anti-p54 nh, anti-PSF, anti-CARM1, and anti-PRMT6. Note that p54 nh specifically interacted with CARM1 but not PRMT6 in vivo. [IgGH] IgG heavy chain, [IgGL] IgG light chain. (E) RNA-independent interaction of p54 nh 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-p54 nh antibodies. DAPI was used to indicate DNA. (G) 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 p54 nh are in red. NEAT1 colocalizes with CARM1 [top panels] and p54 nh [bottom panels].
The communoprecipitation (co-IP) assays between p54\textsuperscript{arb} 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-p54\textsuperscript{arb} immunoprecipitation complexes and vice versa (Fig. 1D,E). Importantly, the co-IP was still detectable in the presence of RNase A (Fig. 1E). Colocalization of the endogenous p54\textsuperscript{arb} 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 p54\textsuperscript{arb} is a new substrate for CARM1 and that p54\textsuperscript{arb} methylation by CARM1 may occur within paraspeckles.

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

We next asked whether p54\textsuperscript{arb} methylation by CARM1 could alter paraspeckle-associated p54\textsuperscript{arb} function. Since p54\textsuperscript{arb} is involved in the nuclear retention of mRNAs containing IRAlus in their 3′ UTRs (mRNA-IRA\textit{lus}) (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-IRA\textit{lus} reporter constructs as previously described (Chen et al. 2008). Constructs with a single \textit{Alu} element or IRA\textit{lus} (originally from the 3′ UTR of the \textit{Nicn1} gene) in the 3′ UTR of \textit{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 IRA\textit{lus}, but not by a single \textit{Alu}, in the 3′ UTR of \textit{egfp} (Fig. 2A,B). This repressed EGFP expression was largely due to the nuclear retention of \textit{egfp}-IRA\textit{lus} (Fig. 2A,B).

Strikingly, the EGFP fluorescence in CARM1 knockdown cells transfected with the EGFP-IRA\textit{lus} 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 \textit{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 IRA\textit{lus} in the nucleus. First, nuclear/cytoplasmic (N/C) RNA fractionation analyses in scramble and CARM1 knockdown cells individually transfected with \textit{egfp-Alu} or \textit{egfp-IRA\textit{lus}} constructs clearly showed that \textit{egfp-IRA\textit{lus}} 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 \textit{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 \textit{egfp-IRA\textit{lus}} in scramble and CARM1 knockdown cells by RNA ISH at the single-cell level revealed that \textit{egfp} mRNAs with IRA\textit{lus} 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-IRA\textit{lus} nuclear retention, we would expect to observe that the endogenous mRNA-IRA\textit{lus} would have a different fate in CARM1 depletion cells. There are hundreds of mRNAs containing IRA\textit{lus} in their 3′ UTRs (Chen et al. 2008), and we chose a number of mRNAs that contain IRA\textit{lus} in their 3′ UTRs and are expressed well in HeLa and HEK293 cells (nic\textit{c1}, ic\textit{m}, mrpl\textit{30}, and pcc\textit{b}) (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 \textit{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 IRA\textit{lus} (Supplemental Fig. S5). Note that the longer isoform of each of the mRNAs containing IRA\textit{lus} 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: \textit{nic\textit{c1}} (Fig. 2E) and ic\textit{m} (Supplemental Fig. S6B). The long isoform of \textit{nic\textit{c1}} contains one pair of IRA\textit{lus} in its 3′ UTR, but the short isoform lacks IRA\textit{lus}. Correspondingly, the \textit{nic\textit{c1}} long isoform is preferentially localized to the nucleolus, while the short one is almost exclusively cytoplasmic (Fig. 2E, Chen et al. 2008). Importantly, knockdown of CARM1 increased the amount of \textit{nic\textit{c1}} long isoform retained in the nucleus (Fig. 2E), while the subcellular distribution of the \textit{nic\textit{c1}} short isoform and \textit{actin} mRNA remained unaltered (Fig. 2E). Such altered nuclear retention regulation by CARM1 was also seen in another examined endogenous mRNA, ic\textit{m} (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 IRA\textit{lus}.

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 IRA\textit{lus} in their 3′ UTRs expressed in MCF7 cells (Fig. 2F), implying that these transcribed nascent mRNAs and corresponding mRNAs with 3′ UTR IRA\textit{lus} are located close to paraspeckles and thus are capable of association with paraspeckle protein p54\textsuperscript{arb} 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 sRNA 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 subcellular distribution of RNAs with IR from the same stripped membrane. (B) CARM1 deficiency significantly enhances the nuclear retention of egfp mRNA with IRAlus. (Left) Cytoplasmic and nuclear RNAs were isolated from the same batch of transfected HeLa cells used in A and then resolved on a denaturing agarose gel. Transcripts of egfp-tagged RNAs were probed 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 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-IRAlus in scramble and CARM1 knockdown cells. (C) Enhanced EGFP expression of egfp mRNA with IRAlus in scramble and CARM1 knockdown cells. (D) CARM1 knockdown enhances the nuclear retention of other endogenous mRNAs with IRAlus. RT-qPCR analyzed a number of other endogenous mRNAs with IRAlus using nuclear and cytoplasmic fractionated RNAs. Primers to detect RNAs with IRAlus were designed downstream from IRAlus in 3′ UTRs [Supplemental Fig. S5]. The relative subcellular distribution of each RNA with IRAlus was normalized to the relative amount of actin mRNA. (E) CARM1 knockdown promotes the nuclear retention of endogenous nics1 mRNA in HEK293 cells. (Top) A schematic drawing of transcripts of the Nics1 gene containing two isoforms [Chen et al. 2008]. (Bottom left) Note that the long isoform contains a pair of IRAlus in its 3′ UTR and is preferentially retained in the nucleus. Cytoplasmic and nuclear mRNA fractionation of scramble and CARM1 knockdown cells followed by Northern blotting to detect nics1 mRNAs. tRNA expression and actin revealed a successful cytoplasmic and nuclear fractionation. Equivalent amounts of RNA from different compartments were loaded as indicated by 28S and 18S rRNAs. (Bottom right) The relative subcellular distribution of the long isoform of nics1 mRNA was quantified from the left panels by ImageJ and normalized to the relative amount of nuclear actin mRNA from the same stripped membrane. (F) Genes containing IRAlus in 3′ UTRs are enriched in the NEAT1-bound active chromatin sites. 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\textsuperscript{\textit{nr}} methylation by CARM1 regulates nuclear retention of mRNAs containing IRA\textit{Al}us

The next question is whether this CARM1-regulated nuclear retention is dependent on p54\textsuperscript{\textit{nr}}. To answer this question, we knocked down CARM1 or p54\textsuperscript{\textit{nr}} and then followed the expression and the subcellular distribution of the IRA\textit{Al}us-containing reporter plasmid Flag-mcherry-IRA\textit{Al}us. The pair of IRA\textit{Al}us 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\textit{Al}us mRNAs (Fig. 3B), as other assays revealed (Fig. 2, Supplemental Figs. S3, S4), knockdown of p54\textsuperscript{\textit{nr}} enhanced Flag-mcherry expression (Fig. 3A, lane 4, Supplemental Fig. S7A) with a reduced nuclear retention of Flag-mcherry-IRA\textit{Al}us mRNAs (Fig. 3B). Double knockdown of CARM1 and p54\textsuperscript{\textit{nr}} 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\textit{Al}us is mediated by the paraspeckle-localized protein p54\textsuperscript{\textit{nr}} 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\textit{Al}us, 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\textit{Al}us. (D) Altered Flag-mcherry protein expression in C corresponds to the altered nuclear retention of transcripts of Flag-mcherry-IRA\textit{Al}us. The relative subcellular distribution of transcripts of Flag-mcherry-IRA\textit{Al}us 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 to those in the cytoplasm after normalization to the relative amount of actin mRNA in each fraction. The endogenous mRNA with 3' UTR IRA\textit{Al}us was also assayed and normalized in the same way. (C) IRA\textit{Al}us 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 knockdown cells enhanced expression, while knockdown of p54\textsuperscript{\textit{nr}} increases Flag-mcherry expression. (E) Mutation of CARM1 methylation sites on p54\textsuperscript{\textit{nr}} enhances the p54\textsuperscript{\textit{nr}}-mediated mRNA nuclear retention. Reintroduction of the lysine replacement of Arg357, Arg365, and Arg378 [R357K, R365K, and R378K] of p54\textsuperscript{\textit{nr}} [p54\textsuperscript{\textit{nr}}-MUT; lane 3] into p54\textsuperscript{\textit{nr}} knockdown cells resulted in an enhanced mCherry suppression compared with that of wild-type p54\textsuperscript{\textit{nr}} [lane 2], as revealed by Western blotting. (F) Altered Flag-mcherry expression in E corresponds to the altered nuclear retention of transcripts of Flag-mcherry-IRA\textit{Al}us. The relative subcellular distribution of transcripts of Flag-mcherry-IRA\textit{Al}us and the endogenous mRNA with 3' UTR IRA\textit{Al}us from the same batch of transfected cells as described in E were investigated by RT-qPCR. See for details. In B, D, and F, error bars represent ±SD in triplicate experiments. [*] P < 0.05; [**] P < 0.01; n ≥ 3.
CARM1 stable knockdown cell lines followed by the reporter assays described above (Fig. 3A,B). We found that re-introduction 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 IRA\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 IRA\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. Re-introduction 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 IRA\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 IRA\textsubscript{Alus}

How does p54\textsuperscript{nb} methylation by CARM1 alter mRNA nuclear retention? Since p54\textsuperscript{nb} can bind to IRA\textsubscript{Alus} 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 IRA\textsubscript{Alus} 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 IRA\textsubscript{Alus} (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 ability to bind to mRNAs with IRA\textsubscript{Alus} 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 mRNA-IRA\textsubscript{Alus}, we then performed a reciprocal pull-down assay by tagging an aptamer tRSA (Iioka et al. 2011) to IRA\textsubscript{Alus} RNA [tRSA-IRA\textsubscript{Alus}] (Fig. 4D). Following tRSA immunoprecipitation with nuclear extracts, we found that tRSA-IRA\textsubscript{Alus}, but not the tRSA alone, was preferentially associated with p54\textsuperscript{nb} (Fig. 4D). Overexpression of CARM1 resulted in reduced binding of tRSA-IRA\textsubscript{Alus} with p54\textsuperscript{nb} (Fig. 4D), confirming that methylation by CARM1 on p54\textsuperscript{nb} reduced its ability to bind to RNA-IRA\textsubscript{Alus}. 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 IRA\textsubscript{Alus} 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 IRA\textsubscript{Alus}.

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 IRA\textsubscript{Alus}? 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-IRA\textsubscript{Alus} [Prasanth et al. 2005; Chen et al. 2008]. However, some extensively adenosine [A]-to-inosine [I] edited mRNA-IRA\textsubscript{Alus} were also exported to the cytoplasm [Prasanth et al. 2005; Chen et al. 2008; Hundley 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 IRA\textsubscript{Alus} 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 IRAlus. This is indeed the case. Incubation of ssRNAs or dsRNAs with differentFlag-tagged p54

methylation reduces its

its ability to bind to IRAlus mRNAs. (A) CARM1 deficiency increases the association of p54

mRNAs with IRAlus was assayed by formaldehyde cross-linking RIP from scramble or CARM1 knockdown HeLa cells using anti-p54

followed by RT-qPCR. Bar plots represent the fold enrichments of RNAs immunoprecipitated by anti-p54

tRNA over the same amount of input across different samples, and error bars represent SD in triplicate experiments. Nucleolin mRNA was a control that does not bind to p54

mRNAs, as assayed by UV cross-linking RIP from scramble or CARM1 knockdown HeLa cells using anti-p54

and anti-IgG followed by RT-qPCR. See A for details. (B) CARM1 overexpression attenuates the interaction between p54

and endogenous IRAlus mRNAs. The interaction of p54

and endogenous IRAlus mRNAs was assayed by RIP in HeLa cells expressing Flag-p54

Flag-p54

Flag-EGFP followed by RT-qPCR. See A for details. (C) Lysine replacement of Arg357, Arg365, and Arg378 (R357K, R365K, and R378K) of p54

(p54

increases the association of p54

and endogenous IRAlus mRNAs. The interaction of p54

and endogenous IRAlus mRNAs was assayed by RIP in HeLa cells expressing Flag-p54

Flag-p54

Flag-EGFP followed by RT-qPCR. See A for details. (D) CARM1 expression was induced upon the DOX treatment, as revealed by Western blotting. (E) Partially purified triple mutations of R357K, R365K, and R378K of p54

(p54

exhibit increased ability to bind to IRAlus RNAs. (Left) A schematic drawing of the experimental flow. (Right) Flag-p54

but not Flag-EGFP, exhibited an ability to bind to Dig-labeled IRAlus RNA, Flag-p54

MUT showed an enhanced ability to bind to IRAlus RNA.

Figure 4. p54

methylation reduces its

mRNAs containing IRAlus. This is indeed the case. Incubation of ssRNAs or dsRNAs with differentFlag-tagged p54

truncations individually expressed in HeLa cells revealed that the truncation of p54

containing two RRM do-
mRNAs preferred to bind to ssRNAs, and the truncation of p54

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

carrying only RRM domains showed little association to the in vitro transcribed IRAlus, the inclusion of the coiled-coil domain to this truncation significantly increased its ability to bind to IRAlus (Fig. 5F), confirming that the coiled-coil domain of p54

mediates its binding to dsRNAs, including mRNA-IRAlus. Together, these results strongly suggest that p54

methylation by CARM1 at or near the coiled-coil domain alters the interaction between dsRNAs and p54

via the coiled-coil domain.

CARM1 inhibits NEAT1 transcription and reduces paraspeckle formation

We showed that CARM1 methylated the coiled-coil domain of p54

mRNAs containing IRAlus. 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 NEAT1 gene (Fig. 6D). Further studies by nuclear run-on (NRO) assays showed that the transcription of nascent transcripts of NEAT1 was increased upon CARM1 knockdown, confirming the notion that CARM1 could regulate NEAT1 transcription as a transcriptional cosuppressor.

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

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 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


Figure 5. p54\textsuperscript{mth} methylation by CARM1 alters its binding to dsRNAs. [A] CARM1 knockdown affects p54\textsuperscript{mth} binding to dsRNAs but has little effect on ssRNAs. Biotin-labeled dsRNAs (double-stranded-egfp 76–495) or ssRNAs (single-stranded-egfp 1–798) were made from in vitro transcription (IVT). Nuclear extracts of scramble or CARM1 knockdown (KD) HeLa cells were incubated with biotin-dsRNA or biotin-ssRNA followed by anti-biotin pull-down and Western blotting with antibodies for anti-p54\textsuperscript{mth} and anti-PSF. [Right] The quantitative analyses of the left panels from three independent experiments. [B] Methylated p54\textsuperscript{mth} binds to few dsRNAs. (Left) The 620me antibody was developed to specifically recognize the methylated p54\textsuperscript{mth}. Western blotting analyzed the specificity of 620me antibody purified from rabbit serum immunized with methylated peptides of R357 and R365. p54\textsuperscript{mth} and Actin antibodies were used as controls. [Right] The methylated p54\textsuperscript{mth} (620me) was enriched by biotin-ssRNA but not by biotin-dsRNA pull-downs from wild-type HeLa cell nuclear extracts. See A for details. [C] Lysine replacement of Arg357, Arg365, and Arg378 (R357K, 365K, and R378K) of p54\textsuperscript{mth} [p54\textsuperscript{mth}MUT] increases the association of p54\textsuperscript{mth} and dsRNAs. [Right] The quantitative analyses of the left panels from three independent experiments. See A for details. [D] A schematic drawing of different truncations of p54\textsuperscript{mth} used in E and F. [E] A different domain of p54\textsuperscript{mth} selectively binds to ssRNAs or dsRNAs. (Top) Nuclear extracts of HeLa cells transfected with different p54\textsuperscript{mth} truncations were incubated with biotin-dsRNAs or biotin-ssRNAs followed by anti-biotin pull-down and Western blotting with anti-Flag. (Bottom) The quantitative analyses of the top panels from three independent experiments. [F] Partially purified truncation of p54\textsuperscript{mth} selectively binds to IR\textsubscript{Alu}s RNAs. Different p54\textsuperscript{mth} truncations shown in D were purified and incubated with Dig-labeled IR\textsubscript{Alu}s RNAs. See Figure 4E for details. In A, C, and E, error bars represent ±SD in triplicate experiments. P-values from one-tailed t-test in the pairwise comparison are shown.
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 IRAluS (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 IRAlus 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 repeats 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 IRAlus is variable, with some such mRNAs located in the nucleus, while others are in the cytoplasm [Prasanth et al. 2005; Chen et al. 2008; Hundlely et al. 2008; Chen and Carmichael 2009; Elbarbary et al. 2013]. How the nuclear retention of mRNAs containing IRAlus 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 IRAlus and represses gene expression [Fig. 2, Supplemental Figs. S3, S4, S6].
To achieve this regulation, CARM1 methylates p54nrb (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 p54nrb 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 p54nrb to mRNA-IR\textsubscript{Alu} achieved? Since it is known that p54nrb 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 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}}
mRNAs [Elbarbary et al. 2013]. These results thus suggested that another nuclear retention signal is required for p54\textsuperscript{nrb}-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{nrb} [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{nrb} 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{nrb} 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 lncRNAs [either TUG1 or MALAT1] with an unknown mechanism, resulting in the coordinat ed 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{nrb} occurs at or near the dsRNA-binding coiled-coil domain and that knockdown of CARM1 significantly alters p54\textsuperscript{nrb}-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{nrb} to dsRNAs could result from a direct conformational change of p54\textsuperscript{nrb} 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{nrb} [Yang et al. 2014]. Finally, the dsRNA-binding protein STA\textsubscript{U}1 [Wickham et al. 1999] was recently shown to compete with p54\textsuperscript{nrb} 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 STA\textsubscript{U}1 occurs after the release of mRNAs containing IRA\textsubscript{Alu}s from methylated p54\textsuperscript{nrb}.

Although many lncRNAs have been implicated in gene regulation and mammalian development [Ulitksy and Bartel 2013], how the expression of lncRNAs is regulated has remained poorly understood. Interestingly, we found that paraspeckle-localized CARM1 also plays a role in the transcription regulation of NEAT1 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 corepressor, such as in the cyclic adenosine monophosphate signaling pathway [Xu et al. 2001]. In the case of NEAT1 regulation, we found that CARM1 is enriched at the NEAT1 promoter and acts as a transcriptional repressor [Fig. 6]. Conspicuously, this “negative” regulation of NEAT1 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 NEAT1 promoter and what other factors are involved in this NEAT1 transcription regulation by CARM1. It had been reported recently that NEAT1 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 NEAT1 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{nrb}, resulting in the reduced ability to binding to mRNAs containing IRA\textsubscript{Alu}s [Figs. 3–5]. On the other hand, CARM1 suppresses NEAT1 transcription and paraspeckle formation [Figs. 6, 7I]. 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{nrb} methylation [enhanced mRNA nuclear retention] and the decreased binding of CARM1 to the NEAT1 promoter [enhanced transcription of NEAT1 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−/− 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{nrb}-wt (MUT) or Flag-EGFP stable cell lines, pcDNA3.1 (+)-Flag-p54\textsuperscript{nrb}-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{nrb}, the sequence “GGAG CGAAGTCTTCATTCAT” was inserted into pLVTHM vector with MuI1 and ClaI, 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{nrb} 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-^3^H] methionine (85 Ci/mmol from a 0.5 mCi/mL stock solution, Perkin-Elmer). The reaction was incubated for 1 h at 30°C and then subjected to fluoro- 

ography 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 fluoro- 

ography, 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.

**Immuno precipitation**

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 PMSF, 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 at 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.

**MS for methylation**

pcDNA3.1 (+)-Flag- p54nrb was transfected into scramble and MS for methylation

were subjected to two additional washes with 160 µL of lysis buffer and one additional wash by adding 0.5% deoxycholic acid into the lysis buffer. Finally, the purified nuclei were resuspended in 100 µL of lysis buffer followed by extraction with Trizol. The RNA was extracted per standard protocols. For Northern blotting, equal amounts of RNAs from different fractions were loaded. Northern blotting was performed using the DIG Northern starter kit (Roche). The probes used were produced by digoxigenin (Dig)-labeled in vitro transcription (IVT) of specific PCR products. Primers used for amplifying templates for IVT are listed in the Supplemental Material. For qRT–PCR, after treatment 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 glycine 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 precoted with 2 µg of antibodies for p54nrb (BD) or IgG (Sigma) for 4 h at 4°C. The beads were washed with PBS and then aliquoted into three parts (one each for Glu-C, subtilisin, and trypsin digestion) followed by incubation with DTT and urea for 30 min at 60°C. Next, 15 mM iodoacetamide was added into the beads and incubated for 20 min at room temperature. Digestion was performed using sequencing grade Glu-C, subtilisin, and trypsin for 4 h at 37°C. Liquid chromatography-tandem MS (LC-MS/MS) was carried out on the Thermo Q Exactive Hybrid quadrupole orbitrap mass spectrometer.

**Nuclear and cytoplasmic RNA fractionation, RNA isolation, qRT–PCR, and Northern blotting**

Nuclear and cytoplasmic RNA fractionation was carried out as described (Chen et al. 2008) with slight modifications. Cell pellets were suspended by gentle pipetting in 200 µL of lysis buffer [10 mM Tris at pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% Igepal, 2 mM vanadyl ribonucleoside complex (VRC)] and incubated for 5 min on ice. One-fifth of the lysate was saved as total RNA. The rest of the lysate was centrifuged at 1000g for 3 min at 4°C to pellet the nuclei, and the supernatant was the cytoplasmic fraction. To obtain pure cytoplasmic RNA, the supernatant fraction was further centrifuged at 13,000 rpm for 10 min at 4°C and then collected carefully to a new tube, and RNA was extracted with Trizol. To obtain pure nuclear RNA, the nuclear pellets

were 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

HU ET AL.
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 for 1 h at 4°C. Next, the precleared lysate was incubated with Dynabeads G that were precoated with 2 μg of antibodies for p54<sup>NR</sup>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 100 μL of elution buffer (100 mM Tris-HCl at pH 7.5, 5 mM EDTA, 10 mM DTT, 1% SDS). Five microliters of 10 mg/mL protease K was added into the RNA sample and incubated for 30 min at 55°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.

**ChIP**

ChIP was carried out as described [Zhang et al. 2013b]. HeLa cells (10⁷) were cross-linked in 1% formaldehyde for 5 min at room temperature, quenched by adding 0.25 M glycine, and then collected by cell scraper. After being suspended in 1 mL of ChIP lysis buffer [1% Triton X-100, 0.1% sodium deoxycholate, 50 mM Tris at pH 8.0, 150 mM NaCl, 5 mM EDTA], cells were sonicated until the majority of DNA fragments was 300–500 base pairs (bp). Supernatants were collected and subjected to pre-clearing with Dynabeads G [Invitrogen] with a supplement of 100 μg of ssDNA. Next, the precleared lysates were used for ChIP with 2 μg of CARM1 antibody (Bethyl Laboratories). ChIP was carried out overnight at 4°C. The beads were washed with 600 μL of ChIP lysis buffer, 600 μL of high-salt wash buffer (1% Triton X-100, 0.1% deoxycholate, 50 mM Tris at pH 8.0, 500 mM NaCl, 5 mM EDTA), and 600 μL of LiCl immune wash buffer [0.25 M LiCl, 0.5% Igepal, 0.5% deoxycholate, 10 mM Tris at pH 8.0, 1 mM EDTA] sequentially followed by two washes with 600 μL of 1× TE buffer (10 mM Tris at pH 8.0, 1 mM EDTA) at 4°C. The immunoprecipitated complex was eluted from Dynabeads G by adding 200 μL of fresh-prepared elution buffer [1% SDS, 0.1 M NaHCO₃] with rotation for 15 min at room temperature. Next, the reverse cross-linking was carried out by adding 8 μL of 5 M NaCl and incubation for 4 h at 65°C followed by the addition of 4 μL of 0.5 M EDTA and 10 μL of 10 mg/mL protease K for 2 h at 37°C. DNA was then extracted and analyzed by qPCR.

**NRO assay**

The NRO in HeLa cells was performed as described [Zhang et al. 2013b]. HeLa cells (10⁷) were washed with cold PBS three times and incubated in swelling buffer (10 mM Tris at pH 7.5, 2 mM MgCl₂, 3 mM CaCl₂) for 5 min on ice. Cells were collected, suspended in 1.5 mL of lysis buffer (10 mM Tris at pH 7.5, 2 mM MgCl₂, 3 mM CaCl₂, 0.5% Igepal, 10% glycerol, 2 U/mL RNasin ribonuclease inhibitor [Promega]), gently pipetted, and then centrifuged at 1500g for 10 min at 4°C. The pellets were collected and subjected to another lysis to obtain purer nuclei. The resulting nuclear pellets were resuspended in 100 μL of NRO buffer (50 mM Tris at pH 7.5, 5 mM MgCl₂, 150 mM KCl, 0.1% sarkosyl, 2 U/mL RNase inhibitor, 10 mM DTT) containing 0.1% M ATP, GTP, CTP, and BrUTP [Sigma]. Transcription was performed for 3 min on ice and then 5 min at room temperature. The reaction was stopped by addition of 600 μL of Trizol reagent, and RNA was extracted followed by the DNase I (Ambion) treatment to remove genomic DNA. The purified RNAs were incubated with 2 μg of anti-BrdU antibody [Sigma] or an equal amount of IgG antibody [Sigma] for 2 h at 4°C and then immunoprecipitated for 1 h with Dynabeads Protein G [Invitrogen] precoated with yeast tRNA [Sigma]. Precipitated RNAs were extracted by Trizol reagent and used for cDNA synthesis and qPCR analysis. All measured samples were normalized to β-actin transcription.

**Biotin-labeled RNA pull-down**

Biotinylated RNA pull-down assays were performed as described [Zhang et al. 2013b]. DNA fragments of full-length EGFP with T7 promoter on its 5′ end and fragment EGFP [76–495] with T7 promoters on both ends were in vitro transcribed with the biotin RNA-labeling mix [Roche] and T7 transcription kit [Promega]. HeLa cells (10⁷) were collected and suspended in 1 mL of RIP buffer (25 mM Tris at pH 7.5, 150 mM KCl, 0.5 mM DTT, 0.5% NP40, 1 mM PMSE, 2 mM VRC, protease inhibitor cocktail) followed by sonication. After being centrifuged at 13,000 rpm for 15 min at 4°C, the supernatant was transferred into a new tube and pre-cleared with 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<sub>egfp</sub> (full length of egfp) or ds<sub>egfp</sub> (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-p54<sup>NR</sup>BD, anti-methyl-p54<sup>NR</sup> (620me), and anti-PSF [Sigma].

**tRSA RNA pull-down**

tRSA RNA pull-down assays were carried out as described [Iioka et al. 2011] with modifications. IRA1<sub>his</sub> from the 3′ UTR of Nica1 were cloned into pcDNA3 with the tRSA tag at its 5′ end. tRSA-IR<sub>A</sub> or tRSA was in vitro transcribed using the T7 transcription kit [Promega]. Ten micrograms per reaction of synthetic RNAs was denatured for 5 min at 65°C and cooled to room temperature in the presence of PA buffer (10 mM Tris at pH 7.4, 10 mM MgCl₂, 100 mM NH₄Cl). Next, the RNAs were incubated with 40 μL of streptavidin Dynabeads for 20 min at 4°C in the presence of 2 U/mL RNasin [Promega]. HeLa cells (10⁷) were collected and resuspended in 1 mL of lysis buffer (25 mM Tris at pH 7.4, 150 mM KCl, 0.5 mM DTT, 0.5%, NP40, 1 mM PMSE, 2 mM VRC, protease inhibitor cocktail) followed by sonication. After centrifuging at 13,000 rpm for 15 min 4°C, the supernatant was transferred into a new tube and pre-cleared with 40 μL of streptavidin Dynabeads for 20 min at 4°C followed by the addition of 20 μg/mL yeast tRNA for 20 min at 4°C. Beads prebound with tRSA-IR<sub>A</sub> RNA or tRSA RNA were incubated with pre-cleared lysates for 4 h at 4°C followed by washing with lysis buffer containing 0.5% sodium deoxycholate. To harvest the protein complex, 50 μL of 1× SDS loading buffer was added and incubated for 10 min at 95°C and then analyzed by Western blotting.

**Biotin labeling RNA pull-down**

Biotin-labeled RNA pull-down assays were carried out as described [Arab et al. 2014] with modifications. Two 10-cm dishes of cells expressing Flag-p54<sup>NR</sup>BD (wt) 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 washed for Western blot. The rest were equilibrated in binding buffer (50 mM Tris at pH 8.0, 10% glycerol, 100 mM KCl, 5 mM MgCl₂, 3 mM CaCl₂) for 5 min on ice. Cells were collected, suspended in 1.5 mL of lysis buffer (10 mM Tris at pH 7.5, 2 mM MgCl₂, 3 mM CaCl₂, 0.5% Igepal, 10% glycerol, 2 U/mL RNasin ribonuclease inhibitor [Promega]), gently pipetted, and then centrifuged at 1500g for 10 min at 4°C. The pellets were collected and subjected to another lysis to obtain purer nuclei. The resulting nuclear pellets were resuspended in 100 μL of NRO buffer (50 mM Tris at pH 7.5, 5 mM MgCl₂, 150 mM KCl, 0.1% sarkosyl, 2 U/mL RNase inhibitor, 10 mM DTT) containing 0.1% M ATP, GTP, CTP, and BrUTP [Sigma]. Transcription was performed for 3 min on ice and then 5 min at room temperature. The reaction was stopped after addition of 600 μL of Trizol reagent, and RNA was extracted followed by the DNase I (Ambion) treatment to remove genomic DNA. The purified RNAs were incubated with 2 μg of anti-BrdU antibody [Sigma] or an equal amount of IgG antibody [Sigma] for 2 h at 4°C and then immunoprecipitated for 1 h with Dynabeads Protein G [Invitrogen] precoated with yeast tRNA [Sigma]. Precipitated RNAs were extracted by Trizol reagent and used for cDNA synthesis and qPCR analysis. All measured samples were normalized to β-actin transcription.

**CARM1 alters mRNA nuclear retention in paraspeckle**
A stringent pipeline was developed to identify the room temperature. The Dig-labeled RNA was produced by Dig-labeled IVT of an IRAIus DNA fragment, which was amplified from the 3' UTR of Nec1. After washing with immunoprecipitation buffer containing 500 mM NaCl, the bound RNA was extracted and analyzed by Northern blotting.

Computational pipeline for the analysis of genes containing 3' UTR IRAIus enriched in the NEAT1 DNA CHART

A stringent pipeline was developed to identify 3' UTR IRAIus genes enriched by NEAT1 DNA CHART. To identify 3' UTR IRAIus genes, IRAIus were identified by RepeatMasker [Tarailo-Graovac and Chen 2009]. The 3' UTRs were defined in a RefSeq gene file. Next, the overlap region between IRAIus and the 3' UTR was calculated to ensure that IRAIus were located in the 3' UTRs. In total, 545 genes containing 3' UTR IRAIus were obtained. In MCF7 cells, 388 genes containing 3' UTR IRAIus 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 IRAIus-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 IRAIus enriched by the NEAT1 DNA CHART.

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MgCl2, 10 mM β-mercaptoethanol, 0.1% NP-40 and incubated with 200 ng of Dig-labeled RNA in binding buffer for 1.5 h at room temperature. The Dig-labeled RNA was produced by Dig-labeled IVT of an IRAIus DNA fragment, which was amplified from the 3' UTR of Nec1. After washing with immunoprecipitation buffer containing 500 mM NaCl, the bound RNA was extracted and analyzed by Northern blotting.


Mol Cell 53: 393–406.


Mol Cell 7: 3294–3301.

Mol Cell 35: 467–478.

Mol Cell 10: e1003731.


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

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