Paraspeckles modulate the intranuclear distribution of paraspeckle-associated Ctn RNA

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Paraspeckles are sub-nuclear domains that are nucleated by long noncoding RNA Neat1. While interaction of protein components of paraspeckles and Neat1 is understood, there is limited information on the interaction of non-structural RNA components with paraspeckles. Here, by varying paraspeckle number and size, we investigate how paraspeckles influence the nuclear organization of their non-structural RNA component Ctn RNA. Our results show that Ctn RNA remains nuclear-retained in the absence of intact paraspeckles, suggesting that they do not regulate nuclear retention of Ctn RNA. In the absence of Neat1, Ctn RNA continues to interact with paraspeckle protein NonO to form residual nuclear foci. In addition, in the absence of Neat1-nucleated paraspeckles, a subset of Ctn RNA localizes to the perinucleolar regions. Concomitant with increase in number of paraspeckles, transcriptional reactivation resulted in increased number of paraspeckle-localized Ctn RNA foci. Similar to Neat1, proteasome inhibition altered the localization of Ctn RNA, where it formed enlarged paraspeckle-like foci. Super-resolution structured illumination microscopic analyses revealed that in paraspeckles, Ctn RNA partially co-localized with Neat1, and displayed a more heterogeneous intra-paraspeckle localization. Collectively, these results show that while paraspeckles do not influence nuclear retention of Ctn RNA, they modulate its intranuclear compartmentalization.

The eukaryotic nucleus is the site where crucial cellular events such as DNA replication, RNA synthesis and processing take place¹. To facilitate the efficient co-ordination of these pathways, the nucleus is further compartmentalized into sub-nuclear domains such as nuclear speckles, Cajal bodies and nucleoli²⁻⁴. These subnuclear domains are known to regulate several important cellular processes such as ribosomal RNA transcription and pre-mRNA splicing⁵⁻¹¹. Recent studies have shown that nuclear bodies are not random aggregates of proteins or RNAs, but are steady-state structures that are formed by dynamic interactions of protein-protein and/or protein-RNA components¹²⁻¹⁴. The interactions between protein and RNA components of nuclear domains have been investigated in-depth for some nuclear domains, but remain uncharacterized for others. A comprehensive understanding of these interactions will provide insights into the biogenesis, maintenance and function of sub-nuclear domains.

Paraspeckles are sub-nuclear bodies that are detected as variable number of discrete dots, and are preferentially located adjacent to nuclear speckles⁷⁻⁹. Paraspeckles contain ribonucleoprotein complexes that are formed around NEAT1 (Nuclear Enriched Abundant Transcriptor 1/MENε/β long noncoding RNA)⁴⁻¹⁴. Two non-coding Neat1 RNA isoforms are transcribed from the same promoter through alternative 3' end processing⁶⁻¹⁰,¹²,¹⁴. The mouse Men ε (Neat1_v1) is a 3.17-kb long poly(A) RNA, while Men β (Neat1_v2) is a 20.7-kb long transcript that harbors a triple helical structure in its 3’end¹²,¹⁴,¹⁶. Apart from Neat1, paraspeckles contain several paraspeckle-localized RNA-binding proteins, collectively termed as PSPs (paraspeckle-associated proteins). In addition to the three core PSPs that are members of the Drosophila Behavior Human Splicing (DBHS) family - NonO, SFPQ and PSP1, paraspeckles also contain an additional ~40 or more PSPs²⁻⁷,¹²,¹³. The protein components

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of paraspeckles are known to participate in several RNA metabolic pathways, including pre-mRNA processing and RNA stability.

Previous studies demonstrated Neat1 as the organizational component of paraspeckles.\(^\text{8,10,12–14}\). NEAT1_v2 forms the paraspeckle core, whereas NEAT1_v1 is recruited as a subsidiary factor.\(^\text{12,18}\) The depletion of Neat1 has been shown to disrupt paraspeckle structure.\(^\text{10,12,14}\). Studies using a LacI reporter live cell imaging system to visualize the inducible transcription of Neat1 and paraspeckle proteins demonstrate that active transcription of Neat1 regulates paraspeckle maintenance\(^\text{13}\). This is supported by other studies showing the disruption of intact paraspeckles upon transcription inhibition, and reformation upon transcription reactivation\(^\text{13}\).

Neat1 plays an important role in basic physiological functions and diseases\(^\text{20,21}\). Upon immune stimuli, Neat1 facilitates the relocation of splicing factor proline/glutamine-rich (SFPQ), a Neat1-associated PSP, from the IL8 (Interleukine 8) promoter to paraspeckles, leading to transcriptional activation of IL8\(^\text{22}\). Neat1 is also known to repress transcription of several genes, including ADARB2 (Adenosine deaminase that Acts on RNA 3), by sequestering the transcription repressor SFPQ from the promoters of these protein-coding genes\(^\text{22}\). Finally, NEAT1-enriched paraspeckles have been suggested to be involved in the nuclear retention of A-to-I edited transcripts.\(^\text{8}\) Neat1-knockout mice are viable under laboratory growth conditions, and thus, paraspeckles are considered to be nonessential nuclear bodies that are formed upon certain environmental triggers such as viral infection, proteasome inhibition and differentiation\(^\text{14,22–24}\).

Members of the paraspeckle-resident PSPs interact with Neat1 and influence the spatial arrangement of Neat1 within paraspeckles. For example, NonO and SFPQ selectively associate with, and stabilize NEAT1_v2, thus contributing to the organization of the paraspeckle structure\(^\text{12}\). Although the interaction of PSPs and Neat1 has been studied in detail, information regarding the organization and behavior of non-structural RNA components of paraspeckles is scant\(^\text{25,26}\). Ctn RNA is an 8 kb long, mouse-specific, nuclear-retained RNA that is induced as part of the antiviral response\(^\text{26}\). Apart from its homogenous distribution in the nucleoplasm, it also localizes to paraspeckles. Ctn RNA regulates the expression of its protein-coding partner, Cat2 (mouse cationic amino acid transporter 2)\(^\text{26}\). mCat2 facilitates the cellular uptake of L-arginine, which is utilized as a substrate for the synthesis of nitric oxide (NO) in the cell. Both Slc7a2 gene, mRNA are encoded by the transcripts\(^\text{9}\).

NEAT1-associated PSP , from the IL8 promoter to paraspeckles, leading to transcriptional activation of IL8\(^\text{22}\). NEAT1_v2 is an 8 kb long, mouse-specific, nuclear-retained RNA that is induced as part of the antiviral response\(^\text{26}\). Apart from its homogenous distribution in the nucleoplasm, it also localizes to paraspeckles. Ctn RNA regulates the expression of its protein-coding partner, Cat2 (mouse cationic amino acid transporter 2)\(^\text{26}\). mCat2 facilitates the cellular uptake of L-arginine, which is utilized as a substrate for the synthesis of nitric oxide (NO) in the cell. Both Slc7a2 gene, mRNA are encoded by the transcripts\(^\text{9}\).

To investigate if paraspeckles regulate the nuclear retention of Ctn RNA, we determined the cellular localization of Ctn RNA in WT-MEFs (Mouse embryonic fibroblasts) and Neat1-KO (knockout) MEFs by RNA-FISH (RNA-Fluorescent in situ hybridization) analysis\(^\text{24}\). Neat1 lncRNA has been shown to nucleate paraspeckles and thus, in the absence of Neat1, the paraspeckle core is disrupted\(^\text{9,10,12,14,19}\). We observed that in WT-MEFs, Ctn RNA co-localized with Neat1 with the intact paraspeckles. In addition, Ctn RNA also displayed homogenous nuclear distribution (Fig. 1Aa–c). In Neat1-KO MEFs too, where intact paraspeckles were absent, Ctn RNA continued to localize in the nucleus (Figs 1Ad–f and S1A–C). Since paraspeckle protein NonO has been shown to interact with, and influence the nuclear localization of hyper-edited RNAs, we ascertained if NonO regulates nuclear retention of A-to-I edited transcripts in the absence of intact paraspeckles\(^\text{8,10,26–28}\). Ctn RNA is a paraspeckle-associated transcript that is A-to-I edited within its long 3′UTR\(^\text{26}\). The long 3′UTR of Ctn RNA contains several inverted repeats of SINE origin, and several of the adenosines within these repeats undergo Adenosine-to-Inosine (A-to-I) editing by ADAR family of cellular enzymes\(^\text{26}\). Upon cellular stress, Ctn RNA is cleaved at the 3′UTR and is exported into the cytoplasm where it is translated to form mCAT2 protein\(^\text{26}\). Knockdown of Ctn RNA does not affect paraspeckle integrity, suggesting that it is a non-structural RNA component of paraspeckles\(^\text{10,26}\). Ctn RNA has been shown to interact with PSPs ~ NonO and PSPI\(^\text{26}\). However, apart from this limited information, not much is known about the interaction of Ctn RNA with paraspeckles. In this study, we investigated how alteration in paraspeckle number and size affects the association of Ctn RNA with paraspeckles. In addition, by utilizing Ctn RNA as a model system, we determined the potential involvement of A-to-I editing in the nuclear retention and paraspeckle association of RNA.

**Results**

**Ctn RNA is nuclear-retained in the absence of intact paraspeckles, and forms ‘residual’ paraspeckle foci.** Previous studies have speculated that paraspeckles are involved in the nuclear retention of A-to-I edited transcripts\(^\text{8,10,26–28}\). Ctn RNA is a paraspeckle-associated transcript that is A-to-I edited within its long 3′UTR\(^\text{26}\). To investigate if paraspeckles regulate the nuclear retention of Ctn RNA, we determined the cellular localization of Ctn RNA in WT-MEFs (Mouse embryonic fibroblasts) and Neat1-KO (knockout) MEFs by RNA-FISH (RNA-Fluorescent in situ hybridization) analysis\(^\text{24}\). Neat1 lncRNA has been shown to nucleate paraspeckles and thus, in the absence of Neat1, the paraspeckle core is disrupted\(^\text{9,10,12,14,19}\). We observed that in WT-MEFs, Ctn RNA co-localized with Neat1 with the intact paraspeckles. In addition, Ctn RNA also displayed homogenous nuclear distribution (Fig. 1Aa–c). In Neat1-KO MEFs too, where intact paraspeckles were absent, Ctn RNA continued to localize in the nucleus (Figs 1Ad–f and S1A–C). Since paraspeckle protein NonO has been shown to interact with, and influence the nuclear localization of hyper-edited RNAs, we ascertained if NonO regulates nuclear retention of A-to-I edited Ctn RNA\(^\text{9}\). We performed RNA-FISH to determine Ctn RNA and Neat1 co-localization in control and NonO-depleted WT-MEFs (Figs 1Ca–f, and S1D–E). Paraspeckle proteins NonO and SFPQ associate with, and stabilize the longer isoform of Neat1, thus, stabilizing paraspeckle structure\(^\text{12}\). Neat1 RNA-FISH analysis confirmed the reduction in paraspeckle number in NonO-depleted cells (Figs 1C and S1D,E). However, NonO-depleted cells continued to show nuclear and paraspeckle association of Ctn RNA, suggesting that NonO does not influence the nuclear retention of Ctn RNA (Fig. 1C).

Next, we determined the total levels of Ctn RNA in nuclear cytoplasmic fractions by RT-qPCR (Reverse transcription quantitative PCR) analysis. In order to measure Ctn RNA levels specifically, and not mCat2, we used a primer pair that is unique to the long 3′UTR of Ctn RNA (Fig. S2A). In agreement with RNA-FISH analysis, we observed predominantly nuclear enrichment of Ctn RNA in the presence or absence of Neat1 or NonO (Figs 1E,F and S2B–E). We also investigated if the disruption of paraspeckle resulted in any changes in the relative abundance of Ctn RNA. To this end, we measured total Ctn RNA levels in WT and Neat1-KO MEFs or control and NonO-depleted MEFs. We did not observe any change in the total levels of Ctn RNA in the absence of Neat1 or NonO (Fig. 1G,H). Together, these results show that paraspeckles do not affect the nuclear retention or cellular abundance of Ctn RNA.

In WT-MEFs, Ctn RNA is localized to paraspeckles, as confirmed by co-staining of cells with Neat1 (Fig. 1Aa–c). However, there was a dramatic reduction in the number of intranuclear Ctn RNA foci in the Neat1-KO cells (Fig. 1Ad–f,B). We observed a few, but prominent Ctn RNA positive intranuclear ‘residual’ foci in Neat1-KO MEFs (3–4/nucleus in KO cells instead of 15–20 paraspeckles/nucleus in WT cells) (Fig. 1A,B). In the absence of Neat1, a subset of Ctn RNA localized to the perinucleolar space (Fig. 1A). On the other hand, in NonO-depleted
cells, we observed that a few less prominent residual Ctn RNA foci continued to persist, some of which did not co-localize with Neat1 (Fig. 1Cd–f, see arrowhead). Collectively, these results suggest that in absence of intact paraspeckles, Ctn RNA is nuclear-retained.
paraspeckle structure, Ctn RNA foci form fewer residual nuclear foci, and also localize to the perinucleolar compartment.

**Ctn RNA associates with other paraspeckle components in absence of Neat1.** Next, we wanted to determine if disruption of paraspeckle structure affects the interaction of Ctn RNA with other paraspeckle components. Ctn RNA has been shown to interact with paraspeckle proteins such as NonO and PSP126. In the current study, we ascertained if the Ctn RNA continues to associate with paraspeckle proteins in the absence of Neat1. To this end, we determined the localization of Ctn RNA and paraspeckle protein NonO by performing RNA-FISH followed by immunostaining for NonO in WT and Neat1-KO MEFs. We observed that as shown in previous studies, Ctn RNA and NonO co-localized in WT-MEFs (Fig. 2Aa–d)26. Surprisingly, in the Neat1 KO cells, a few but not all of the bright Ctn RNA foci co-localized with NonO-stained foci (Fig. 2Ae–h,B; please see the arrows). However, due to the reduction in the number of Ctn RNA and NonO foci in the Neat1 KO cells, it was not possible to determine if the efficiency of this interaction was same as seen in case of WT-MEFs. Therefore, to quantitate the interaction between Ctn RNA and NonO, we performed ribonucleoprotein immunoprecipitation (RIP) studies using the NonO antibody followed by RT-qPCR. We observed that NonO displayed increased interaction with Ctn RNA in Neat1-KO cells but not with Malat1, another highly abundant nuclear-retained long noncoding RNA (Fig. 2C). However, we also observed that the total levels of NonO were marginally increased in Neat1-KO MEFs (Fig. 2D). Thus, it is possible that the increased interaction between Ctn RNA and NonO in Neat1-KO MEFs, is due to the higher levels of NonO in the Neat1-KO MEFs. Collectively, these studies conclude that Ctn RNA is able to interact with other paraspeckle components in the absence of Neat1 or an intact paraspeckle structure.

**ADARs do not regulate the association of Ctn RNA to paraspeckles.** There has been an ongoing debate about the potential involvement of A-to-I editing in the retention of hyper-edited transcripts to nucleus and also to paraspeckles9,26,29–32. While some earlier studies have shown that inverted Alu (IRAlu) containing RNA is retained in the nucleus by paraspeckles, other studies show that IRAIu containing RNA are efficiently exported into the cytoplasm9,26,29–32. A very recent study reported that in LPS-induced immune cells, a significant

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Figure 2. Ctn RNA interacts with paraspeckle component NonO in absence of intact paraspeckles.
(A) RNA-FISH to detect Ctn RNA (green) and NonO (red) in DRB-recovered WT and Neat1-KO MEFs. Scale bar indicates 10 μm. Arrow (a–h) indicates Ctn RNA and NonO positive nuclear foci. DNA is counterstained with DAPI (blue). (B) Graph showing percentage co-localization in WT and Neat1-KO MEFs. (C) NonO-RIP (RNA immunoprecipitation) followed by RT-qPCR analysis of Ctn RNA to determine interaction of NonO and Ctn RNA in WT and Neat1-KO MEFs. (D) Western blot showing NonO levels in WT and Neat1-KO MEFs. Tubulin was used as a loading control. Gapdh was used as the normalization control in RT-qPCR experiments. Error bars in (B,C) represent mean ± SD of three independent experiments. **P < 0.01 using Student’s t test.
fraction of transcripts with hyper-edited regions tends to be retained in the nucleus. In its 3′UTR, Ctn RNA harbors three inverted repeats (IR) of SINE origin that are inverted with respect to the forward repeat (FwR) (Fig. S2A). Double stranded regions formed by pairing of FwR and IR are frequently A-to-I edited by RNA editing enzyme Adenosine deaminases acting on RNA (ADARs). While three forms of this enzyme exist in human cells—ADAR1, 2 and 3, only ADAR1 and ADAR2 display editing activity. In case of Ctn RNA, we have previously reported that the several adenosines within the FwR and IR2 repeats undergo A-to-I editing. Taken together with the fact that the 3′UTR was involved in the nuclear localization of Ctn RNA, it was hypothesized that editing of Ctn RNA and its association with paraspeckle components such as NonO could influence it nuclear retention. Thus, we ascertained if A-to-I editing regulates the paraspeckle association and nuclear retention of Ctn RNA.

Figure 3. ADARs do not influence the association of A-to-I edited Ctn RNA to paraspeckles. (A) RNA-FISH to detect Ctn RNA (green) and Neat1 (red) in DRB-recovered WT and Adar1/Adar2 double knockout-KO (DKO) MEFs. DNA is counterstained with DAPI (blue). Scale bar indicates 10 μm. (B) % co-localization of Ctn RNA and Neat1 in the paraspeckles of DRB-recovered WT and Adar1/Adar2 double knockout-KO (DKO) MEFs.
**Ctn RNA foci increase in number upon transcription reactivation.** The adenosine analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) inhibits RNA polymerase II-mediated transcriptional elongation by inactivating the activity of PETFβ kinase. Previous studies have shown that paraspeckle protein PSP1 re-localized to perinucleolar caps after 5 min of DRB treatment and remarkably, paraspeckles structure was disrupted ~40 min after DRB treatment. Interestingly, paraspeckles reformed upon removal of DRB from the culture medium indicating that paraspeckle maintenance is coupled with Neat1 transcription. We have previously reported that Ctn RNA foci were also disrupted upon RNA pol II transcription inhibition, including treatment of cells with DRB. Therefore, we investigated the reassembly of Ctn RNA foci upon transcription reactivation. To this end, we treated transformed WT-MEFs with DRB for three hours. Next, we removed DRB by washing cells with medium, cultured the cells in fresh media for another 3 hours, performed co-RNA-FISH and counted the number of Ctn RNA foci in both untreated and DRB recovered cells (Fig. 4A). In agreement with previous studies that demonstrated increased paraspeckle number upon DRB recovery, we observed a two to three-fold increase in the number of paraspeckles/cell as observed by Neat1 staining (~4/cell in control to ~8/cell in treated) (Fig. 4B–D). In addition, we also observed a concomitant two-fold increase in number of cells with Ctn RNA decorating Neat1 positive paraspeckles (Fig. 4B,C,E) (~30% of cells showed Ctn RNA foci in control cells whereas ~70% of transcription-reactivated cells showed Ctn RNA positive paraspeckles). Furthermore, Ctn RNA staining within the paraspeckles appeared more prominent in transcription-reactivated cells, indicating a possible increase in the number of Ctn RNA molecules per paraspeckle during DRB-recovery.

Previous studies have shown that while Neat1 is a highly unstable RNA (half-life ~2 h), Ctn RNA constitutes a stable pool of RNA (half-life ~8 h). We wanted to determine if the increased paraspeckle number and Ctn RNA association with paraspeckles during transcription reactivation was due to changes in the total levels of Neat1 and Ctn RNA. To this end, we measured the total levels of Neat1 and Ctn RNA in control and DRB-recovered transformed WT-MEFs by RT-qPCR (Fig. 4F). The results showed that neither Neat1 nor Ctn RNA displayed any significant changes in abundance upon DRB recovery (Fig. 4F). Together, these results demonstrate that Ctn RNA shows increased localization to paraspeckles upon transcriptional reactivation without a corresponding increase in total levels of Neat1 or Ctn RNA.

**Ctn RNA forms enlarged foci upon proteasome inhibition.** Recently, it was demonstrated that paraspeckles become dramatically enlarged upon proteasome inhibition. Surprisingly, this enlargement in paraspeckle size was shown to be a result of Neat1 transcription activation, and not because of the accumulation of undegraded PSPs. In fact, upon proteasome inhibition, PSPs were sequestered into paraspeckles as evidenced by 50% depletion of these proteins from the nucleoplasm. To determine if paraspeckle RNA component, Ctn RNA showed any changes upon proteasome inhibition, we treated transformed WT-MEFs with the proteasome inhibitor MG132 for 17 h and compared the Ctn RNA foci with control (DMSO-treated) cells (Fig. 5A). We observed that Ctn RNA also formed enlarged nuclear foci upon MG132-treatment (Figs 5B and S4).

We wondered whether proteasome-inhibition also altered transcription from Ctn RNA locus. To test this aspect, we measured Ctn RNA levels in DMSO and MG132-treated transformed WT-MEFs by RT-qPCR analysis (Fig. 5C,D). Consistent with a previous report, we observed significant increase in the levels of Neat1 in proteasome-inhibited cells (Fig. 5C). On the other hand, Ctn RNA showed only a small but significant increase in total RNA levels (Fig. 5D; ~2 fold of increase of Ctn RNA compared to ~16 fold of Neat1). Collectively, these results showed that upon proteasome inhibition, Ctn RNA formed enlarged foci. In addition, the Ctn RNA levels were only moderately increased in comparison to the marked increase in Neat1 levels.

Ctn RNA and Neat1 localization studies, especially in the proteasome-inhibited cells, using conventional fluorescent microscopy indicated that only a fraction of the paraspeckle-associated Neat1 and Ctn RNA displayed complete co-localization (Fig. 5B–h). To achieve a better understanding of the localization of these RNA molecules in paraspeckles, we used Super-resolution structured illumination (SR-SIM) microscopy to determine the molecular organization of Ctn RNA and Neat1 in MG132-treated and DRB-recovered transformed WT-MEFs (Fig. 6). We observed that under both conditions, Ctn RNA did not completely overlap with Neat1 positive paraspeckles (Fig. 6B–E). This suggests that Ctn RNA decorated only a part of Neat1-nucleated paraspeckles (Fig. 6A–C). Furthermore, we observed that not all Neat1-nucleated paraspeckles contained Ctn RNA (Fig. 6A; see arrow). We further quantitated the ratio and degree of overlap of Neat1 and Ctn RNA in paraspeckles (Fig. 6D–c,Ea–c). The results showed that in DRB recovered cells, both Neat1 and Ctn RNA foci display a more homogenous intraparaspeckle distribution and largely overlap with each other (Fig. 6Da–c). However, upon MG132 treatment, Ctn RNA and Neat1 showed altered peak ratios and degree of overlap suggestive of a more heterogeneous intraparaspeckle distribution (Fig. 6Ea–c).

**Discussion**

Previous studies suggest that sub-nuclear domains could be formed in two ways: (1) random self-organization or (2) ordered assembly. Studies using a LacI reporter live cell imaging system have shown that paraspeckles do not organize by random self-organization. The study by Mao et al. suggested that while PSPs within paraspeckles could facilitate the recruitment of other PSPs, they are unable to recruit RNA components to form bona fide paraspeckles. Instead, Neat1 serves as the seeding molecule that recruits other components during the paraspeckle assembly. Since paraspeckle components do not interact in a random manner, the interactions between these components merit investigation in order to understand how this sub-nuclear body assembles. While a number of studies have investigated the interaction of paraspeckles, information on the intranuclear organization of Ctn RNA is largely unavailable.

**Paraspeckles regulate the intranuclear organization of Ctn RNA.** In ~30% of MEFs, a significant fraction of Ctn RNA forms discrete foci that localize to paraspeckles. In contrast, upon disruption of paraspeckle
Figure 4. Number of Ctn RNA positive foci and their association with paraspeckles is increased upon transcriptional reactivation. (A) Schematic showing the experimental design. (B) RNA-FISH to detect Ctn RNA (green) and Neat1 (red) in control (ethanol) and DRB-recovered transformed WT-MEFs. (C) RNA-FISH analysis of Ctn RNA and Neat1 localization in a single cell of control (ethanol-treated) and DRB-recovered transformed WT-MEF. (D) Graph showing average number of paraspeckles per cell in control (ethanol-treated) and DRB-recovered transformed WT-MEFs. (E) Graph showing percentage of cells positive for Ctn RNA foci in control (ethanol-treated) and DRB-recovered transformed WT-MEFs. (F) RT-qPCR analysis of Ctn RNA levels in control (ethanol-treated) and DRB-recovered transformed WT-MEFs. Gapdh was used as the normalization control in RT-qPCR experiments. Scale bar indicates 10 μm. DNA is counterstained with DAPI (blue). Error bars in (D–F) represent mean ± SD of three independent experiments. *P < 0.05, ns: not significant using Student’s t test.
structure, while Ctn RNA continues to form residual foci, a subset of Ctn RNA localizes to the perinucleolar space. This suggests that paraspeckles mediate the efficient compartmentalization of Ctn RNA, and in their absence Ctn RNA appears to acquire a more stochastic distribution in the nucleoplasm. Furthermore, in the absence of Neat1, Ctn RNA continues to associate with other PSPs, as indicated by RNA-FISH and RNA immunoprecipitation studies. This is in contrast to a previous study where the authors showed that structures induced by tethering of individual PSPs (instead of Neat1) to a LacI reporter construct did not retain Ctn RNA. It is possible that artificial tethering of proteins may prevent interaction of these proteins with Ctn RNA, which might otherwise occur under physiological conditions.

Figure 5. Ctn RNA forms enlarged foci in proteasome-inhibited cells. (A) Schematic showing the experimental design. (B) RNA-FISH analysis of Ctn RNA (green) and Neat1 (red) localization in control (DMSO-treated) and MG132-treated transformed WT-MEFs. Scale bar indicates 10 μm. DNA is counterstained with DAPI (blue). (C) RT-qPCR analysis of Neat1 RNA levels in control (DMSO-treated) and MG132-treated transformed WT-MEFs. (D) RT-qPCR analysis of Ctn RNA levels in control (DMSO-treated) and MG132-treated transformed WT-MEFs. Gapdh was used as the normalization control in RT-qPCR experiments. Error bars in (C,D) represent mean ± SD of three independent experiments. ***P < 0.001, *P < 0.05 using Student’s t test.
Figure 6. Intra-paraspeckle localization of Ctn RNA. (A) Super-resolution structured illumination microscopy (SR-SIM) of Ctn RNA (green) and Neat1 (red) localization in DRB recovered and MG132-treated transformed WT-MEFs. Scale bar indicates 10 μm. Arrow (a–h) indicates paraspeckle where Ctn RNA does not show co-localization with Neat1. (B,C) Co-localization of a single paraspeckle in (B) DRB recovered and (C) MG132-treated transformed WT-MEF. (D,E) Quantitation of Ctn RNA and Neat1 co-localization in (Da–c) DRB recovered and (Ea–c) MG132 treated transformed WT-MEFs (performed using ZEN 2012). Numbers in image indicate the specific paraspeckle analyzed and corresponds to the number mentioned in the graph. For example, “1” in image refers to “paraspeckle 1” in graph.
Paraspeckles do not impact nuclear retention of A-to-I edited Ctn RNA. Previous studies have suggested that paraspeckles are potentially involved in the nuclear retention of A-to-I edited transcripts in human cells. For example, in undifferentiated human embryonic stem cells where intact paraspeckles are absent, in spite of robust A-to-I editing activity, the edited transcripts were not retained in the nucleus. Furthermore, knockdown of Neat1 and the consequent disruption of paraspeckle structure in HeLa cells resulted in the nucleocytoplasmic export of inverted Alu (IRAlu) containing mRNA. In another study, it was documented that mRNAs with structured or edited 3′UTRs can be bound by a nuclear complex containing NonO, and such interaction prevents their export to the cytoplasm. We observed that in the absence of intact paraspeckles (due to deletion of Neat1) or A-to-I editing (due to deletion of both ADAR1 & 2), Ctn RNA continued to localize in the nucleoplasm. Thus, in the case of Ctn RNA, Neat1 or intact paraspeckles do not influence its nuclear retention in MEFs. We observed Ctn RNA positive residual nuclear foci in Neat1 KO cells, some of which co-localized with other PSPs. At present, we cannot exclude the potential involvement of these residual nuclear foci in the nuclear retention of Ctn RNA. It is possible that association of Ctn RNA with other PSPs (such as SFPQ) in Neat1 or NonO-depleted cells could facilitate the nuclear retention of Ctn RNA. Our results also demonstrate that A-to-I editing of Ctn RNA is not essential for its association with paraspeckles since Adar1/Adar2 double KO MEFs showed paraspeckle localization of Ctn RNA.

Ctn RNA foci are responsive to environmental triggers – transcriptional reactivation and proteasome inhibition. Previous studies have shown that transcriptional reactivation results in the reassembly of paraspeckles due to initiation of Neat1 transcription. In our study, we observe that Ctn RNA showed increased localization to paraspeckles upon transcriptional reactivation without a concomitant increase in Ctn RNA or Neat1 levels. Earlier studies have demonstrated that paraspeckles tend to assemble in close proximity to the site of transcription of Neat1. In contrast, Ctn RNA gene loci are located further away from paraspeckles as compared to Neat1 transcription site. Viewed in conjunction with our results, this suggests that after transcription, Ctn RNA localizes to paraspeckles possibly for its further processing or A-to-I editing. Future studies will investigate the potential involvement of paraspeckle in the processing of Ctn RNA.

In addition to being responsive to transcriptional reactivation, paraspeckles also show enlargement upon proteasome inhibition. This enlargement is mainly due to the transcriptional upregulation of Neat1. The positive effect of proteasome inhibition on transcription has also been shown to occur in case of cyclooxygenase-2 (Cox-2) where increased level of this protein in response to proteasome inhibition has been attributed to its increased transcription. Interestingly, in case of Ctn RNA, we see only a marginal increase in total Ctn RNA levels upon proteasome treatment, we observe a significant increase in the size of Ctn RNA foci. At the ultrastructural level, as observed by SR-SIM, Ctn RNA adopts a more heterogeneous intraparaspeckle distribution in MG132-treated cells – with altered Neat1: Ctn RNA ratios and foci overlap. Therefore, Ctn RNA shows altered intra-paraspeckle organization upon proteasome inhibition.

In summary, the results from this study further our knowledge about the organization and behavior of RNA components within the paraspeckle at several levels. First, non-structural RNA components of paraspeckles, namely, Ctn RNA forms residual foci in the absence of Neat1, though at a significantly lower level. Therefore, while Ctn RNA by itself forms ‘paraspeckle-like’ foci, Neat1 improves the efficiency of foci formation. Second, Ctn RNA can associate with other paraspeckle components (PSPs) in the absence of Neat1. The efficiency of such interactions remains largely unaffected even in the presence or absence of intact paraspeckles. From this, we infer that the non-structural RNA components do not require an intact paraspeckle structure to associate with other PSPs. Lastly, RNA components of paraspeckles – both Neat1 and Ctn RNA are responsive to environmental triggers, strengthening the view that paraspeckles function in response to certain stimuli.

Materials and Methods

Cell culture. Adar1/Adar2-KO MEFs were obtained from the Jantsch lab. Transformed WT-MEFs, Neat1-KO MEFs and Adar1/Adar2-KO MEFs were grown in DMEM containing high glucose, supplemented with penicillin-streptomycin and 10% foetal bovine serum (FBS) (HyClone, Logan, UT). For the 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) recovery experiments, cells were treated with Ethanol or 25μg/ml DRB (SIGMA, USA). For proteasome inhibition experiments, cells were treated with DMSO or 5μM MG132 (SIGMA, USA).

Reverse Transcription (RT), quantitative PCR, PCR. Total cellular RNA was isolated using Trizol (15996-018, Invitrogen, USA) according to the manufacturer’s instructions and reverse transcribed into cDNA using Superscript III First-Strand Synthesis System for RT-PCR (ThermoFisher Scientific, USA). qPCRs were performed using the Applied Biosystems StepOne Plus Real-Time PCR Systems (Applied Biosystems, USA). Transcript levels were quantitated against a standard curve by Real-Time RT-PCR using the SYBR Green fluorescent dye and data analysed using the Applied Biosystems StepOne Plus Real-Time PCR Systems (Applied Biosystems, USA). Primer sets showing comparable high efficiencies were used for the analyses. The qPCR results were analysed using the comparative Ct method.

Transfection and siRNA/sh-RNA-mediated knockdown. NonO (L-048587-00-0005, 40 nM) (ON-TARGETplus smartpool siRNA, GE Dharmaco, USA) and Adar1 siRNA (L-048587-00-0005, 150 nM) (ON-TARGETplus smartpool siRNA, GE Dharmaco, USA) were used to deplete NonO and Adar1, respectively. The siRNAs were transfected to cells using Lipofectamine RNAiMAX reagent as per the manufacturer’s instructions (Invitrogen, USA) and incubated for 48 hrs. Knockdown was confirmed using NonO antibody (gift from Dr Yasuyuki Kurihara, Yokohama National University, Yokohama, Japan) and ADAR1 antibody (sc-73408; Santa Cruz Biotechnology, USA). Loading controls used were α-tubulin (TS168, SIGMA, USA) and B′-U2snRNP.
Ribonucleoprotein Immunoprecipitation (RIP). RIP was performed using an established protocol\(^\text{45}\). WT and Neat1-KO cells (1 × 10^6) were used for RNA immunoprecipitation utilizing reversible chemical crosslinking of RNA-protein interactions by formaldehyde followed by immunoprecipitation using Anti-NonO antibody (9–99, gift from Dr. David Spector, CSHL, USA). Following IP, extracts were reverse cross-linked and total RNA was extracted using Trizol LS (Invitrogen, USA). Extracted RNA was treated with RNase-free DNase I (SIGMA, USA), and RT was conducted using random-hexamer primers as per the manufacturer’s instructions (Applied Biosystems, USA). qPCR was performed using gene-specific primers.

Nuclear and cytoplasmic fractionation. Transformed WT-MEFs, Neat1-KO MEFs, control and NonO siRNA treated transformed WT-MEFs (1 × 10^6) cells were used for fractionation. Cells were washed with PBS and re-suspended in RSB buffer (10 mM Tris–HCl pH 7.4, 100 mM NaCl, 2.5 mM MgCl\(_2\), RNAse Inhibitor) and lysed in RSB buffer containing Digitonin (8 μg/ml) (SIGMA-ALDRICH, USA) for 10 min on ice. Cells were centrifuged (2000 rpm, 4°C, 10 min) and the supernatant (cytoplasmic fraction) collected. The pellet (nuclear fraction) was washed with RSB and digitonin by the procedure described above. Trizol LS (10296-028, Invitrogen, USA) was added to the cytoplasmic fraction while Trizol was added to the nuclear fraction. Ct values of nuclear or cytoplasmic fractions were normalized to total RNA.

RNA-FISH. To detect Ctn RNA and Neat1, RNA-FISH analysis was performed as previously described\(^\text{45}\). Ctn RNA localization to paraspeckles was increased during transcriptional reactivation. Therefore, for Ctn RNA FISH, cells were treated with the transcriptional inhibitor DRB followed by reactivation of transcription by removal of inhibitor with medium for 3 hrs. After RNA-FISH, immunofluorescence staining of NonO was performed using NonO antibody (1:100 for 2 hr at room temperature; sc-376865, Santa Cruz Biotechnology, USA) as previously described\(^\text{45}\). The paraspeckle number and percent co-localization was counted or measured respectively, by eye as the Ctn RNA/Neat1/NonO foci are very distinct and prominent. Unless indicated, hundred cells were counted in each experiment and the experiments were performed in biological replicate.

Super-resolution structured illumination microscopy (SR-SIM) image acquisition and image processing. Images were acquired by SR-SIM ELYRA system with Axio Observer Z1 microscope from ZEISS. 3-color imaging was performed using 488 nm, 561 nm and 632 nm lasers. Exposure time was 100 ms or less for re-suspended in RSB buffer (10 mM Tris–HCl pH 7.4, 100 mM NaCl, 2.5 mM MgCl\(_2\), RNAse Inhibitor) and lysed in RSB buffer containing Digitonin (8 μg/ml) (SIGMA-ALDRICH, USA) for 10 min on ice. Cells were centrifuged (2000 rpm, 4°C, 10 min) and the supernatant (cytoplasmic fraction) collected. The pellet (nuclear fraction) was washed with RSB and digitonin by the procedure described above. Trizol LS (10296-028, Invitrogen, USA) was added to the cytoplasmic fraction while Trizol was added to the nuclear fraction. Ct values of nuclear or cytoplasmic fractions were normalized to total RNA.

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**Author Contributions**

The study was designed by A.A. and K.V.P. A.A., S.G.P. and K.V.P wrote the main manuscript. A.A., M.J. and V.T. performed the experiments. M.F.J., S.N. and T.H. provided essential reagents. All the authors have contributed in reviewing the manuscript.

**Additional Information**

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