Published in final edited form as:
Mol Cell. 2009 March 27; 33(6): 717–726. doi:10.1016/j.molcel.2009.01.026.

An Architectural Role for a Nuclear Non-coding RNA: NEAT1 RNA is Essential for the Structure of Paraspeckles

Christine M. Clemson[1,*], **John N. Hutchinson**[2], **Sergio A. Sara**[4], **Alexander W. Ensminger**[2,3], **Archa H. Fox**[4], **Andrew Chess**[2], and **Jeanne B. Lawrence**[1,*]

1 *University of Massachusetts Medical Center, 55 Lake Ave. N. Worcester, Ma. 01655*

2 *Harvard Medical School, 185 Cambridge St., Boston, MA-02114*

3 *Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge MA 02139*

4 *Western Australian Institute for Medical Research, Centre For Medical Research, University of Western Australia, Crawley WA Australia*

Summary

NEAT1 RNA, a highly abundant 4 kb ncRNA, is retained in nuclei in ~10–20 large foci that we show is completely coincident with paraspeckles, nuclear domains implicated in mRNA nuclear retention. Depletion of NEAT1 RNA via RNAi eradicates paraspeckles, suggesting it controls sequestration of the paraspeckle proteins, PSP1 and p54, factors linked to A-I editing. Unlike over-expression of PSP1, NEAT1 over-expression increases paraspeckle number, and paraspeckles emanate exclusively from the NEAT1 transcription site. The PSP-1 RNA binding domain is required for its co-localization with NEAT1 RNA in paraspeckles, and biochemical analyses supports that NEAT1 RNA binds with paraspeckle proteins. Unlike other nuclear retained RNAs, NEAT1 RNA is not A-I edited, consistent with a structural role in paraspeckles. Collectively results demonstrate that NEAT1 functions as an essential structural determinant of paraspeckles, providing a precedent for a ncRNA as the foundation of a nuclear domain.

Introduction

Large (several kb) non-coding RNA (ncRNAs) are increasingly being identified and we have just begun to glimpse the diversity of their potential functions. For example, XIST RNA is involved in silencing the inactive X chromosome (Brockdorff and Duthie, 1998; Brown, 1991; Hall and Lawrence, 2003); NRON RNA regulates the nuclear trafficking of the NFAT protein (Willingham et al., 2005) and HOTAIR RNA represses HOX loci in *trans* (Rinn et al., 2007).

*Correspondence: E-mail: christine.clemson@umassmed.edu; E-mail: jeanne.lawrence@umassmed.edu, (508) 856-6016, fax: (508) 856-5178.

Nitrocellulose Filter Binding Assay
YFP-PSP1 mutants
Image Analysis and Morphometrics
A detailed account of these methods can be found in the supplement..

We previously identified two Nuclear Enriched Autosomal Transcript (NEAT) ncRNAs using nuclear and cytoplasmic RNA fractions from human fibroblasts and lymphoblasts to screen the Affymetrix human U133 chipset (Hutchinson et al., 2007). NEAT2/MALAT-1 RNA is a 8 kb, highly conserved nuclear restricted ncRNA that localizes to SC-35 domains. NEAT1 is a 4kb, unspliced, polyadenylated, nuclear restricted, noncoding transcript with a mouse homologue that shows two small regions of high conservation. NEAT1 RNA typically localizes to 10–20 bright clusters that are often confined to 1–2 nuclear regions. These very bright NEAT1 foci predominantly localize to the edge of SC-35 rich domains.

In many cases, the relationship of an RNA distribution to other structural and functional entities will provide important insights into its function, as was the case of XIST RNA's coating of the inactive X chromosome (Clemson et al., 1996). There are now known to be many discrete nuclear compartments or bodies that produce a spotted or "speckled" appearance; these are distinguished by the subsets of factors they contain and are implicated in different aspects of nuclear function (Hall et al., 2006; Lamond and Spector, 2003; Misteli, 2000). To begin to understand the potential functions of NEAT1 RNA, we investigated its relationship relative to a variety of known nuclear bodies and compartments. Here we report that NEAT1 RNA localizes specifically to paraspeckles, discrete structures that localize predominantly to the edge of SC-35 domains. Paraspeckles are seen in all cells examined throughout interphase, suggesting a ubiquitous role in fundamental nuclear function (Fox et al., 2002).

Like most nuclear substructures, neither the specific function(s) nor formation of paraspeckles is wholly understood, but their marked enrichment with certain molecular markers provides significant clues. Three human and mouse paraspeckle associated proteins: PSP1, PSF (or PSFQ), p54 (NONO) (Fox et al., 2005; Fox et al., 2002; Kuwahara et al., 2006; Myojin et al., 2004) bind both DNA and RNA and are implicated in pre-mRNA splicing, transcription regulation and nuclear retention of RNA (Kozlova et al., 2006; Xie et al., 2006). A fourth RRM-containing paraspeckle protein, PSP2/CoAA/RBM14, may couple alternative splicing to transcription (Auboeuf et al., 2004; Fox et al., 2002) The strongest evidence suggests a role for paraspeckles in mRNA regulation through nuclear retention via A-I editing: p54 is known to form complexes which can retain A-I edited RNAs in the nucleus (Zhang and Carmichael, 2001) and a specific edited nuclear transcript, Ctn, is enriched in paraspeckles and released upon stress into the cytoplasm as mCAT2 mRNA (Prasanth et al., 2005). Recently, a pre-mRNA 3′ end processing factor, cf IM68 (Cardinale et al., 2007), was found in paraspeckles, where it could play a role in cleavage and release of retained transcripts.

An RNA that is enriched in a specific structure may be present there in relation to its transcription, processing or editing, or in relation to its own function. While many localized nuclear RNAs function in RNA metabolic complexes, others could play a role in the formation of a given structure; the presence of such nuclear "architectural" RNAs has been widely speculated for some time. Results presented here show that NEAT1 RNA is the prime entity nucleating paraspeckles. This unique role for a large polyadenylated ncRNA promises to be important for understanding the biology of NEAT1 and its associated structure, paraspeckles, and at the same time provide precedence for a class of architectural ncRNAs.

Results

NEAT1 RNA Strictly Delineates Paraspeckles

Initially we investigated the potential relationship of the nuclear-restricted NEAT1 RNA to a variety of known nuclear bodies in multiple cell types including PML domains, Cajal bodies, P bodies, SMN domains, as well as replicating DNA and DMPK triplet repeat RNA foci. None of these structures showed a significant relationship with the NEAT1 RNA foci (not shown). Since the NEAT1 RNA foci localize predominantly at the edge of SC-35 domains (Hutchinson

et al., 2007) we were particularly interested in whether they associate with paraspeckles, nuclear structures that frequently abut SC35-rich speckles (Fox et al., 2002). Using antibodies to the previously defined paraspeckle markers (Fox et al., 2002), in conjunction with fluorescent in situ hybridization (FISH), we find that NEAT1 RNA associates with the intense concentrations of PSP1 and p54 that demark paraspeckles (Figure 1). Importantly, while other paraspeckle components are found diffusely throughout the nucleoplasm, NEAT1 RNA is found almost exclusively as bright tightly-bordered foci that overlap paraspeckles (linescans Figure 1D, H).

We examined the relationship of NEAT1 RNA to paraspeckle components in multiple human cells under a variety of preparation and fixation conditions and found that NEAT1 RNA foci strictly and consistently overlapped with paraspeckles under all conditions in all cells at interphase (Figure 1A–M). Though there is little sequence conservation between mouse and human NEAT1 (Hutchinson et al., 2007), the mouse homologue, Neat1 RNA, also precisely and consistently overlap with paraspeckles in all mouse cells examined (Figure 1N–P).

The correlation between NEAT1 RNA and paraspeckle markers was so striking that the overlap could be further documented by blind computer scoring of the average number and overlap of NEAT1 and PSP1 foci. Despite higher levels of nucleoplasmic protein, almost all localized PSP1 foci identified by the computer contained discrete concentrations of NEAT1 RNA (Figure S1).

Knock-down of NEAT1 RNA leads to loss of Paraspeckles

We used RNA inhibition to directly ask whether NEAT1 RNA is required for paraspeckle formation. NEAT1 RNA was significantly reduced relative to the control using RT-PCR (Figure S2) and FISH (Figure 2). In HeLa cells transfected with the control siRNA pool (Figure 2A, D) an average of 17 NEAT1 foci and 13 paraspeckle accumulations assessed by both PSP1 and p54 staining (Figure 2F) were detected. In cells transfected with the NEAT1 siRNA pool (Figure 2B, C and E), NEAT1 foci were reduced to an average of 2 per cell (Figure 2F), while accumulations of PSP1 and p54 averaged 2 and 1, respectively. The ablation of paraspeckles upon NEAT1 RNAi was also observed in HT-1080, Tig1 and 293 cells (not shown). Importantly, there was not a subset of paraspeckles that remained independent of NEAT1 RNA. The very few remaining paraspeckles (PSP-1 or p54 accumulations) overlapped with the few remaining foci of NEAT1 RNA (Figure 2B) and results further indicated that these were associated with the NEAT1 transcription site (Figure S2F). As the linescans in Figure 2 demonstrate, while NEAT1 RNA and paraspeckles are effectively eliminated through RNAi to NEAT1, the overall nucleoplasmic pool of paraspeckle proteins increased, suggesting that without NEAT1 RNA to nucleate paraspeckles, these proteins remain in the nucleoplasmic space.

Several additional controls corroborated the validity of these results. Different individual siRNA duplexes to NEAT1 RNA were used and showed a specific reduction in NEAT1 RNA levels (Figure S2). Significant effects were detected in individual cells transfected even with low concentrations (10 nM) of siRNA (Figure S2E). The depletion of NEAT1 RNA accurately predicted the depletion of paraspeckles in numerous experiments (summarized in Figure S2), indicating this was not a result of off-target effects on paraspeckles. Further, the impact of NEAT1 RNA inhibition was specifically on paraspeckles, and not on a number of other nuclear structures examined (Figure S3).

Finally, NEAT2/MALAT-1 RNA is a useful control, and an interesting comparison, in that it is also a large, nuclear restricted ncRNA, which localizes to a defined nuclear structure: SC-35 domains (Figure 3A&B and Hutchinson et al., 2007). Whether NEAT2/MALAT-1 RNA is required to maintain the structure of nuclear speckles is an important question in itself, since

the existence of a structural poly A RNA component of SC-35 domains has previously been proposed (Huang et al., 1994; Lawrence et al., 1993). We find that the structure of SC-35 domains (3C) and paraspeckles (3D) is largely unaffected in cells with reduced NEAT2/MALAT-1 expression (quantitation shown in Figure S2). These results further support that ablation of paraspeckles is specific to NEAT1 RNA reduction, but also show that the abundant NEAT2 RNA is not required to maintain nuclear speckles.

Paraspeckles form at the site of NEAT1 Transcription

In interphase nuclei, we see that both NEAT1 foci and paraspeckles often occupy a bipolar distribution (Figure 4A and B) which combines with other observations (summarized below) to suggest that paraspeckles may initially form as NEAT1 RNA is transcribed and their spread throughout the nucleus corresponds with the spread of NEAT1 RNA. Using sequential RNA/DNA hybridizations we examined the timing of NEAT1 transcription and paraspeckle formation during the progression from mitosis to newly formed daughter nuclei. While we were able to colocalize NEAT1 RNA with the NEAT1 gene (Figure S4), it was difficult to reproducibly eliminate all the abundant NEAT1 RNA so UHG (which maps near NEAT1 on 11q13) and a chromosome 11 library was used to demark the NEAT1 locus. In re-forming daughter nuclei, NEAT1 transcription occurs just prior to the accumulation of PSP1 and p54 in paraspeckles. In early G1 daughter nuclei only a few foci of NEAT1 RNA are detectable which correspond to nascent transcription sites (Figure 4C and S4). As the cell cycle progresses, NEAT1 foci distribute throughout the nucleoplasm. Paraspeckle proteins are detectable in the earliest G1 cells (Figure 4D&F), but do not concentrate in paraspeckles until approximately 60 minutes after RNA transcription (Fox et al., 2005). The earliest paraspeckles are detectable after there are at least two foci of NEAT1 RNA (Figure 4E &F); moreover, these early paraspeckles are usually located next to the NEAT1 locus (Figure 4D) and overlapping NEAT1 foci (4E, F & Figure S1).

If NEAT1 RNA nucleates paraspeckles, then it is likely that an increase in NEAT1 expression may cause a corresponding increase in paraspeckle number. To address this we stably transfected mouse cells with a construct containing the entire mNeat1 gene and quantitated the number of paraspeckles. In two cells lines expressing mNeat1 at levels above a mock infected cell line, we find marked increases in the number of Neat1 RNA foci (5A), and a corresponding increase in the number of paraspeckles. While we detected some Neat1 foci in these transfectants that were smaller than those seen in typical mouse cells, we did not see an increase in dispersed nucleoplasmic Neat1 RNA (red in 5D&E) and essentially all of the many Neat1 RNA foci contain PSP-1 and vice versa (e.g. Figure 5D). Computer quantitation demonstrated a similar several fold increase in Neat1 RNA foci and PSP1/paraspeckles (5A).

We next asked whether increased expression of PSP-1 via transfection of a full length YFP-PSP1 construct would affect paraspeckles. In a cell line expressing both the endogenous PSP1 and the YFP-PSP1 fusion protein, the number of paraspeckles is essentially unchanged upon PSP1 overexpression (Figure 5B). Additionally these experiments show that NEAT1 RNA associates with full length PSP1 protein that is ectopically expressed, and that this association occurs in paraspeckles (Figure 5F).

NEAT1 RNA Associates with Paraspeckle Proteins *in vivo* and *in vitro*

We next determined whether NEAT1 RNA and PSP1 are in the same complex using quantitative RT-PCR after co-immunoprecipitation (co-IP) (Figure 6). To address any potential non-specific binding to PSP1 RNA binding domains, we normalized NEAT1 levels to a control RNA (GAPDH) which showed weak enrichment (Figure S5). NEAT1 showed a 10 fold enrichment on the beads containing PSP1 complexes (Figure 6A, lane 3) and was not significantly enriched by PML, another nuclear body protein (lane 5). With western analysis,

we confirmed that the cellular extracts are effectively immunodepleted for PSP1 protein using the anti-PSP1 antibody (Figure 6B). Taken together with prior co-IP analyses showing that PSP1 associates with p54 (Fox et al., 2005), this indicates that NEAT1 is part of a complex with at least both these binding partners.

Using nitrocellulose binding assays, we investigated whether NEAT1 RNA can directly interact with purified paraspeckle proteins. As shown in Figure 6C, in vitro transcribed NEAT1 RNA binds with recombinant PSP1/p54. This association shows specificity as it is seen only for the sense strand in the presence of heparin and is much stronger than the weak, concentration independent interaction with the rGST control protein. RNA-protein binding curves (Figure S6) confirm that the sense strand of Neat1 RNA binds specifically to the paraspeckle proteins in a concentration dependent manner.

To further understand which parts of the PSP1 protein are important for interaction with NEAT1, we examined the distribution of YFP-PSP1 mutant fusion proteins relative to NEAT1 RNA. PSP1 protein deleted for the RNA Recognition Motifs (RRM), which can still form heterodimers with p54 (Fox et al, 2005), no longer shows an association with NEAT1 RNA or paraspeckles (Figure 7A&B). This combines with other evidence showing that the RRMs of PSP1 stabilize the interaction with p54 (Fox et al, 2005), to suggest that these proteins may bind cooperatively with NEAT1 RNA in paraspeckles. Analysis of a second PSP1 mutant deleted for the protein interaction domain (such that it can no longer form heterodimers with P54 - Fox et al, 2005) indicates that this domain is also required for the concentration of PSP-1 with NEAT1 RNA in paraspeckles (Figure 7, C–F). Importantly, since this mutant PSP1 protein does not colocalize with NEAT1 RNA (7F) or the PSP1 antibody (7C) in situ, this suggests it does not sequester and relocalize NEAT1 RNA or appear to form homodimers with the endogenous PSP1 protein.

NEAT1 RNA Is Distinct from Edited Nuclear Retained RNAs

Since some RNA editing is a mode of nuclear retention and regulation, it was of interest to determine if NEAT1 RNA itself is edited. To initially identify putative edited sites within NEAT1 we compared the sequence of multiple cDNA clones to that of genomic DNA. Candidate edited sites were examined more rigorously by comparing the Sequenom derived genotypes of pools of independently derived cDNAs from both WI38 fibroblasts and GM013130 lymphoblasts to those of genomic DNA from the same cell lines. The Sequenom method is sensitive enough to detect alleles represented in a population at frequencies as low as 2–5% (Buetow et al., 2001; Ross et al., 2000). We saw no evidence of significant G substitution in the cDNA clones, which is the hallmark of inosine substitution; nor did our sequence analysis identify any canonical ADAR enzyme targets (reviewed in Bass, 2002). Previous work suggested that nuclear editing may occur in the nucleolus not at the edge of SC-35 domains, as the RNA editing ADAR enzymes dynamically concentrate in nucleoli (Desterro et al., 2003). Additionally, we see no association of ADAR-2 with NEAT1 foci (not shown), further suggesting that active inosine substitution is not occurring in paraspeckles. While these results do not rule out editing in paraspeckles, they show that NEAT1 RNA is not itself A-I edited.

Finally, we compared the distribution of mouse Neat1 RNA to Ctn RNA, an A-I edited RNA that has been observed in paraspeckles. RNAi depletion of Ctn RNA has no effect on paraspeckle structure (Prasanth, 2005). We find that endogenous Ctn RNA localizes to a small subset (typically two) of the paraspeckles, all of which contain Neat1 RNA (Figure S7). These findings further support a unique role for Neat1 RNA in nuclear structure, distinct from edited RNAs that may localize to paraspeckles because they are acted upon or stored there.

Discussion

We find that NEAT1 RNA is highly abundant, stable, Pol II transcribed (Figure S8) and necessary for the formation of a specific nuclear structure: paraspeckles. We show that NEAT1 not only binds to PSP1 and p54 but is required for their localization to paraspeckles. Our collective results combine to show that NEAT1 RNA provides a precedent for a large, nuclear restricted RNA that is required for the structural framework of a ubiquitous, cytological-scale nuclear domain.

The sheer abundance and broad distribution of NEAT1 RNA throughout all cell types and tissues examined (this study and Hutchinson et al., 2007) suggest that it is involved in a fundamental cellular function. In interphase nuclei, NEAT1 RNA is tightly localized in paraspeckles, consistently overlapping with these structured concentrations of both PSP-1 and p54; only at mitosis when most nuclear structures are known to disassemble do these components show substantial separation (Figure S9). Not only is NEAT1 RNA highly concentrated in paraspeckles, a variety of additional evidence shows it functions to control the formation and maintenance of these discrete nuclear domains. First, NEAT1 RNA is detectable as large foci immediately upon transcription and throughout the nucleus, suggesting an inherent ability of these 4kb transcripts to form large structural complexes either on their own, or in conjunction with other factors. Second, the genesis of paraspeckles following cell division further indicates that these foci initially form as NEAT1 RNA is transcribed. The previously noted lag in paraspeckle formation following mitosis (Fox et al., 2005) closely correlates with the lag in NEAT1 transcription, and the first detectable paraspeckles emanate from the two NEAT1 loci (Figure 4). Third, expression of ectopic NEAT1 RNA from introduced transgenes induces the formation of more paraspeckles, while overexpression of PSP1 does not (Figure 5). The fourth and most conclusive line of evidence is provided by successful ablation of NEAT1 by RNAi: extensive RNAi studies demonstrate an essentially perfect relationship between depletion of NEAT1 RNA and loss of paraspeckles. Since we find no evidence for NEAT1 RNA in the cytoplasm, these results are consistent with other findings that RNA inhibition can effectively occur in the nucleus (Langlois et al., 2005; Robb et al., 2005; Valgardsdottir et al., 2005). In contrast, RNAi knockdown of NEAT2 RNA, which is abundant within SC35-enriched nuclear speckles, had no effect on paraspeckles, nor on the SC35 rich domains themselves. Finally, we show that NEAT1 RNA can be immunoprecipitated with antibodies to PSP-1, and can bind to PSP1/p54 *in vitro*, suggesting that nucleation of paraspeckles likely involves interaction with these proteins in the same molecular complex.

A-I editing is a mode of mRNA regulation that is emerging in both its scope and importance and recent results implicate paraspeckles in retaining nuclear “stored” edited RNAs. Both p54, a protein which directly binds hyper-edited RNAs (Zhang and Carmichael, 2001) and Ctn, a hyper-edited RNA (Prasanth et al., 2005) are enriched in these structures. Although the functions associated with paraspeckles are only beginning to be understood, this study clearly shows that a major functional role of NEAT1 RNA is to control paraspeckle formation. This is distinct from other nuclear ncRNAs which localize to but are not required for formation or maintenance of specific structures, such as U2 and other snRNAs in Cajal bodies (CBs), (Lemm et al., 2006; Schaffert et al., 2004). Other nuclear bodies that may be RNA-dependent are specific to certain cell types, stress conditions or disease states. For example, Gomafu RNA localizes to unique domains in some neurons (Sone et al., 2007) and the nuclear foci of mutant DMPK RNA (Mankodi et al., 2001) accumulate adjacent to SC-35 domains but do not overlap paraspeckles (Smith et al., 2007). Perhaps most relevant is Hsrω, a functional homologue to SatIII transcripts (which form nuclear stress bodies in some mammalian cells) (reviewed in Jolly and Lakhotia, 2006). Hsrω forms nuclear structures termed omega speckles in Drosophila (Arya et al., 2007; Prasanth et al., 2000). In contrast, NEAT1 RNA is expressed abundantly in all human/mouse tissues and cells examined, remains in paraspeckles upon heat shock and

interferon stimulation (not shown), associates with *all* paraspeckles in human and mouse cells, and is required for formation of paraspeckles.

Importantly, the structural role of this ncRNA is on an entirely different scale from known "scaffolding RNAs", such as telomerase or ribosomal RNA. While these RNAs are key to forming the telomerase complex or ribosomal subunits, respectively, such complexes are on a molecular scale (e.g. ribosomes are resolved at 7.5 –11.5Å Gabashvili et al., 2000; Matadeen et al., 1999) and typically contain one or two copies of a given transcript (e.g. Cohen et al., 2007). In contrast, individual paraspeckles are cytological scale structures (0.25 –1 microns) (Hutchinson et al., 2007; Cardinale, 2007). Two strong lines of evidence indicate that paraspeckles arise near the NEAT1 locus and spread into the nucleus concurrently with the spread of NEAT1 RNA. First, in early G1 cells and cells in which NEAT1 RNA/paraspeckles are clustered in just two regions of the nucleus, those clusters are consistently associated with the NEAT1 locus (Figure 1 and Hutchinson et al., 2007). Second, the only PSP-1/p54 defined paraspeckles remaining after RNAi are the few that still retain NEAT1 RNA (Figure 2B), typically near the NEAT1 transcription site (Figure S2F). The fact that the large, tightly packaged structural aggregates of NEAT1 RNA form quickly at the transcription site, and before PSP-1 or p54 are detectable within them, suggests that NEAT1 RNA may self-associate. We speculate that the 4kb, complex molecular structure of NEAT1 RNA may be well-suited to form larger-scale architectural complexes. The RNA contains many self-complementary sequences that could form complex intra-molecular structures (e.g. hairpin loops), but these same sequences can also form inter-molecular hybrids, possibly interconnecting these long, largely non-conserved transcripts to form a lattice, upon which the rest of a large nuclear body could be built. At the E.M. level, a nuclear compartment based on a foundation of RNA molecules would likely appear fibrous, and recent work concluded that paraspeckles correspond to IGAZ (Cardinale, 2007), fibrillar ultrastructural compartments that are in contrast to the distinctly granular nature of IGCs (speckles) with which they associate (Visa et al., 1993).

Since the periphery of SC-35 speckles are known sites of active gene clustering (Shopland et al., 2003), it is plausible that the ultimate broader distribution of NEAT1 RNA foci (and paraspeckles) reflects their functional association with specific loci or chromosomal regions. Functional ncRNAs serving as structural elements in nuclear architecture were proposed years ago (Herman et al., 1978) as many large non-coding hnRNAs were discovered (Salditt-Georgieff and Darnell, 1982). In particular, a putative structural poly A RNA in SC-35 speckles has long been discussed (Huang et al., 1994; Lawrence et al., 1993). Since NEAT2/MALAT-1 RNA was the first and only prime candidate for an abundant, polyA ncRNA that concentrates in speckles (Hutchinson et al., 2007), an important contribution of this study is the demonstration that NEAT2/MALAT-1 RNA is not required to maintain structure of SC35-rich domains. In contrast, our findings demonstrate that NEAT1 RNA is indeed a novel structural ncRNA, not simply localized in paraspeckles, but required to form the framework for these distinct nuclear domains. As numerous specific large ncRNAs with no known function are increasingly identified (e.g. Furuno et al., 2006), this study illustrates a new type of architectural role for large ncRNAs.

Experimental Procedures

Cells and Cell Culture

The following cell lines were used and cultured according to recommended conditions: HeLa epithelial adenocarcinoma, CCL-2 (ATCC); WI-38 normal female diploid fibroblast lung, CCL 75 (ATCC); TIG-1, AG06173A (Coriell Cell Repositories); Human EBV-transformed human lymphoblasts, GM00131 (Coriell Cell Repositories); human embryo kidney 293, CRL-1573 (ATCC); HT-1080, human male fibrosarcoma, CCL-121 (ATCC); NIH-3T3 mouse

fibroblasts, CRL-1658 (ATCC) and MEFS, primary mouse embryo fibroblasts (Cell Essentials).

Cell Preparation for In Situ Hybridization and Immunofluorescence

To prepare cells for in situ hybridization, our previously published standard protocol (Johnson et al., 1991; Lawrence et al., 1989; Tam et al., 2002) and optimized protocol for cytoplasmic preservation (Hutchinson et al., 2007) was used. For detection of paraspeckle markers the same non-ionic detergent extractions were used as described for FISH. Additionally, alternative extraction and fixation techniques (Goldenthal et al., 1985) were used to further validate our findings. Cells were extracted with either saponin (0.5% in PBS) or.002% digitonin/PBS prior to or after fixation in 4% paraformaldehyde.

In situ hybridization, Immunofluorescence and Probes

Our protocols for combined in situ RNA, DNA, whole chromosome paint and antibody detection, have been described previously in detail (Clemson et al., 2006; Clemson et al., 1996; Johnson et al., 1991; Tam et al., 2002). Antibody detection was normally carried out prior to hybridization. Antibodies used were: rabbit anti-hPSP1 dilution 1:1000 (Fox et al., 2002); mouse anti- p54 dilution 1:200 (BD Transduction Labs); anti- SC35 dilution 1:250 (Sigma) and ADAR2 dilution 1:100 (Santa Cruz).

Sequential RNA/DNA hybridizations were performed by first detecting and fixing the NEAT1 RNA signal in undenatured cells, then non-hybridized RNA is degraded via NaOH and RNAse digestion prior to heat denaturation and DNA detection. Codetection of NEAT1 RNA/ paraspeckles with Chr 11 loci: RNA FISH or antibody detection was performed prior to hybridization with Starfish Whole Chr 11 paint (Cambio) or a pool of three clones spanning 13.2 kb of the UHG locus at 11q13 (Tycowski et al., 1996). Genomic Human NEAT1, mouse Neat1 and human NEAT2/MALAT-1 probes were described in detail previously (Hutchinson et al., 2007). pBlu2SKP CTNR 3′UTR was a gift from K. Prasanth (Prasanth, 2005).

Real time quantitative PCR

For all RNA level analyses, whole cell RNA was purified using the RNeasy Mini kit (Qiagen) and reverse transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen) and random decamer primers (Ambion). Transcript levels were quantitated against a standard curve by real-time quantitative PCR analysis using an Applied Biosystems 7500 machine and POWER SYBR green master mix (ABI).

Overexpression of mouse Neat1 in NIH3T3 cells

Mouse Neat1 was amplified with iProof DNA Polymerase (BioRad) from NIH3T3 oligo dT primed cDNA with primers 3′endmNeat1 (5′-GAAGCTTCAATCTCAAACCTTTA-3′) and 5′endmNeat1 (5′-AGGAGTTAGTGACAAGGAG-3′) and TOPO cloned into pCRII-TOPO (Invitrogen). Mouse Neat1 was cloned into the KpnI and EcoRV sites of pCDNA3.1/Hygro (+) (Invitrogen), transfected into NIH3T3 cells with Lipofectamine 2000 (Invitrogen) and colonies selected with Hygromycin (200 ug/ml). To determine overall Neat1 expression levels, resistant lines were seeded at 1×10^5cells/ml in triplicate overnight and RNA purified from the subconfluent cultures.

RNA Inhibition

Using Integrated DNA Technologies RNAi design tool, siRNA duplexes targeting the 5′ and 3′ end of the human NEAT1 gene were designed and annealed prior to use, as per the manufacturer's instructions (sequences are described in the Supplement). siRNA duplexes were used separately and as a pool at final concentrations of 10nm, 50nm and 100nm. When

single transfections were performed, HeLa cells were harvested 48 hours after transfection. For double transfection, cells were retransfected 24 hours after the first transfection, and harvested after a total of 48 hours. Lipofectamine RNAimax (Invitrogen) or Dharmaffect (Dharmacon) were used according to manufacturers recommendations. Briefly: HeLa cells were seeded into each well of a 24 well plate, with 0.5 ml of DME media with 100 ug/ml Normocin transfection competent antibiotic (Amaxa) such that they would be 30% confluent in 24 hours at which time siRNA duplexes were added.

Immunoprecipitation

All RNA samples were treated with DNA-free (Ambion) and reverse-transcribed using Superscript III (Invitrogen) and oligodT primer, including no-RT controls. The cDNA was then used for PCR with primers AAATCGTGCCTTAGAAACACATC and GTTTAGAACTCAAACTTTATTTGTGC that amplify an 800bp region at the 3′ end of NEAT1, as well as a short region at the 3′ end of the human GAPDH mRNA (ATGGGGAAGGTGAAGGTCG and GGGGTCATTGATGGCAACAATA). Quantitative PCR was performed using the Quantitect SYBR-green mix (Qiagen) on a Rotorgene 3000 (Corbett Research) with primers that amplify a fragment at the 5′ end of NEAT1 (GTGGCTGTTGGAGTCGGTAT and TAACAAACCACGGTCCATGA) as well as a GAPDH fragment as described above. In each q-PCR experiment a standard curve for both sets of primers was set up with cDNA derived from HeLa total RNA and run at the same time as duplicate experimental samples. NEAT1 q-PCR values were adjusted based on the relative GAPDH signal derived for each experimental sample and the final graphs are based on analysis of three individual and identical IP-RTPCR experiments.

RNA Editing

RNA was purified from whole cell extracts from GM13130 EBV-transformed human lymphoblasts (Coriell) using the RNeasy Maxi kit (Qiagen) and reverse transcribed into cDNA using Superscript III Reverse Transcriptase (Invitrogen) and random decamer primers (Ambion). Amplified segments were gel purified, TOPO cloned into pCR4-TOPO (Invitrogen) and at least 5 separate clones sequenced. Putative edited sites were identified by as A to G substitution in cDNA as compared to both consensus sequence (March 2006 build) and GM13130 genomic sequence amplified at overlapping intervals with primer pairs described in the supplement. Putative edited sites were individually examined by primer extension assays on both genomic DNA and cDNAs (see above) from both GM13130 lymphoblasts (Coriell) and WI-38 primary human fibroblasts (ATCC). Regions containing the putative edited site were PCR-amplified with primers described in the supplement. The relative amount of the unedited versus edited base at the putative edited site was determined by primer extension of PCR products with primers described in the supplement. Detection was by matrix-assisted laser desorption ionization time-of-flight mass spectrometry on Sequenom (San Diego, CA) MassArray platform (Cowles et al., 2002; Tang et al., 1999). All samples were measured in quadruplicate.

Sequencing data for NEAT1 cDNA and genomic clones can be found as FASTA format files and Sequenom Genotyping data can be found in tab delimited format in the Supplement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Chris Lynch, John Butler, Meg Byron and Agata Sadowska for experimental help and Charles Bond, Daniel Passon and Mihwa Lee for providing purified recombinant PSP1/p54nrb proteins and intellectual input on

nitrocellulose binding assays. This work was supported in part by NIH grants GM53234, GM68138 to JBL, HD007439 fellowship to CMC, GM068711 and DC003263 to AC and NHMRC grants 402865 and 458751 to AHF and 512880 to SAS.

References

Arya R, Mallik M, Lakhotia SC. Heat shock genes-integrating cell survival and death. J Biosci 2007;32:595–610. [PubMed: 17536179]

Auboeuf D, Dowhan DH, Li X, Larkin K, Ko L, Berget SM, O'Malley BW. CoAA, a nuclear receptor coactivator protein at the interface of transcriptional coactivation and RNA splicing. Mol Cell Biol 2004;24:442–453. [PubMed: 14673176]

Bass BL. RNA editing by adenosine deaminases that act on RNA. Annu Rev Biochem 2002;71:817–846. [PubMed: 12045112]

Brockdorff N, Duthie SM. X chromosome inactivation and the *Xist* gene. Cellular and Molecular Life Sciences 1998;54:104–112. [PubMed: 9487391]

Brown SDM. XIST and the mapping of the X chromosome inactivation centre. BioEssays 1991;13(11): 607–612. [PubMed: 1772416]

Buetow KH, Edmonson M, MacDonald R, Clifford R, Yip P, Kelley J, Little DP, Strausberg R, Koester H, Cantor CR, Braun A. High-throughput development and characterization of a genomewide collection of gene-based single nucleotide polymorphism markers by chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Proc Natl Acad Sci U S A 2001;98:581–584. [PubMed: 11136232]

Cardinale S, Cisterna B, Bonetti P, Aringhieri C, Biggiogera M, Barabino SM. Subnuclear Localization and Dynamics of the Pre-mRNA 3′ End Processing Factor CF Im68. Mol Biol Cell. 2007

Clemson CM, Hall LL, Byron M, McNeil J, Lawrence JB. The X chromosome is organized into a gene-rich outer rim and an internal core containing silenced nongenic sequences. Proc Natl Acad Sci U S A 2006;103:7688–7693. [PubMed: 16682630]

Clemson CM, McNeil JA, Willard HF, Lawrence JB. XIST RNA paints the inactive X chromosome at interphase: Evidence for a novel RNA involved in nuclear/chromosome structure. JCell Biol 1996;132:259–275. [PubMed: 8636206]

Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. Protein composition of catalytically active human telomerase from immortal cells. Science 2007;315:1850–1853. [PubMed: 17395830]

Cowles CR, Hirschhorn JN, Altshuler D, Lander ES. Detection of regulatory variation in mouse genes. Nat Genet 2002;32:432–437. [PubMed: 12410233]

Desterro JM, Keegan LP, Lafarga M, Berciano MT, O'Connell M, Carmo-Fonseca M. Dynamic association of RNA-editing enzymes with the nucleolus. J Cell Sci 2003;116:1805–1818. [PubMed: 12665561]

Fox AH, Bond CS, Lamond AI. P54nrb forms a heterodimer with PSP1 that localizes to paraspeckles in an RNA-dependent manner. Mol Biol Cell 2005;16:5304–5315. [PubMed: 16148043]

Fox AH, Lam YW, Leung AK, Lyon CE, Andersen J, Mann M, Lamond AI. Paraspeckles: a novel nuclear domain. Curr Biol 2002;12:13–25. [PubMed: 11790299]

Furuno M, Pang KC, Ninomiya N, Fukuda S, Frith MC, Bult C, Kai C, Kawai J, Carninci P, Hayashizaki Y, et al. Clusters of internally primed transcripts reveal novel long noncoding RNAs. PLoS Genet 2006;2:e37. [PubMed: 16683026]

Gabashvili IS, Agrawal RK, Spahn CM, Grassucci RA, Svergun DI, Frank J, Penczek P. Solution structure of the E. coli 70S ribosome at 11.5 A resolution. Cell 2000;100:537–549. [PubMed: 10721991]

Goldenthal KL, Hedman K, Chen JW, August JT, Willingham MC. Postfixation detergent treatment for immunofluorescence suppresses localization of some integral membrane proteins. J Histochem Cytochem 1985;33:813–820. [PubMed: 3894499]

Hall LL, Lawrence JB. The cell biology of a novel chromosomal RNA: chromosome painting by XIST/Xist RNA initiates a remodeling cascade. Semin Cell Dev Biol 2003;14:369–378. [PubMed: 15015744]

Hall LL, Smith KP, Byron M, Lawrence JB. Molecular anatomy of a speckle. Anat Rec A Discov Mol Cell Evol Biol. 2006

Herman R, Weymouth L, Penman S. Heterogeneous nuclear RNA-protein fibers in chromatin-depleted nuclei. JCell Biol 1978;78:663–674. [PubMed: 701354]

Huang S, Deerinck TJ, Ellisman MH, Spector DL. In vivo analysis of the stability and transport of nuclear poly(A)+ RNA. JCell Biol 1994;126(4):877–899. [PubMed: 7519622]

Hutchinson JN, Ensminger AW, Clemson CM, Lynch CR, Lawrence JB, Chess A. A screen for nuclear transcripts identifies two linked noncoding RNAs associated with SC35 splicing domains. BMC Genomics 2007;8:39. [PubMed: 17270048]

Johnson CV, Singer RH, Lawrence JB. Fluorescent detection of nuclear RNA and DNA: Implication for genome organization. Methods Cell Biol 1991;35:73–99. [PubMed: 1723481]

Jolly C, Lakhotia SC. Human sat III and Drosophila hsr omega transcripts: a common paradigm for regulation of nuclear RNA processing in stressed cells. Nucleic Acids Res 2006;34:5508–5514. [PubMed: 17020918]

Kozlova N, Braga J, Lundgren J, Rino J, Young P, Carmo-Fonseca M, Visa N. Studies on the role of NonA in mRNA biogenesis. Exp Cell Res 2006;312:2619–2630. [PubMed: 16750525]

Kuwahara S, Ikei A, Taguchi Y, Tabuchi Y, Fujimoto N, Obinata M, Uesugi S, Kurihara Y. PSPC1, NONO, and SFPQ are expressed in mouse Sertoli cells and may function as coregulators of androgen receptor-mediated transcription. Biol Reprod 2006;75:352–359. [PubMed: 16641145]

Lamond AI, Spector DL. Nuclear speckles: a model for nuclear organelles. Nat Rev Mol Cell Biol 2003;4:605–612. [PubMed: 12923522]

Langlois MA, Boniface C, Wang G, Alluin J, Salvaterra PM, Puymirat J, Rossi JJ, Lee NS. Cytoplasmic and nuclear retained DMPK mRNAs are targets for RNA interference in myotonic dystrophy cells. J Biol Chem 2005;280:16949–16954. [PubMed: 15722335]

Lawrence JB, Carter KC, Xing X. Probing Functional Organization within the Nucleus: Is Genome Structure Integrated with RNA Metabolism? Cold Spring Harbor Sympon QuantBiol. LVIII 1993:807–818.

Lawrence JB, Taneja K, Singer RH. Temporal resolution and sequential expression of muscle-specific genes revealed by in situ hybridization. DevBiol 1989;133:235–246.

Lemm I, Girard C, Kuhn AN, Watkins NJ, Schneider M, Bordonne R, Luhrmann R. Ongoing U snRNP biogenesis is required for the integrity of Cajal bodies. Mol Biol Cell 2006;17:3221–3231. [PubMed: 16687569]

Mankodi A, Urbinati CR, Yuan QP, Moxley RT, Sansone V, Krym M, Henderson D, Schalling M, Swanson MS, Thornton CA. Muscleblind localizes to nuclear foci of aberrant RNA in myotonic dystrophy types 1 and 2. Hum Mol Genet 2001;10:2165–2170. [PubMed: 11590133]

Matadeen R, Patwardhan A, Gowen B, Orlova EV, Pape T, Cuff M, Mueller F, Brimacombe R, van Heel M. The Escherichia coli large ribosomal subunit at 7.5 A resolution. Structure 1999;7:1575–1583. [PubMed: 10647188]

Misteli T. Cell biology of transcription and pre-mRNA splicing: nuclear architecture meets nuclear function. J Cell Sci 2000;113:1841–1849. [PubMed: 10806095]

Myojin R, Kuwahara S, Yasaki T, Matsunaga T, Sakurai T, Kimura M, Uesugi S, Kurihara Y. Expression and functional significance of mouse paraspeckle protein 1 on spermatogenesis. Biol Reprod 2004;71:926–932. [PubMed: 15140795]

Prasanth KV, Prasanth SG, Xuan Z, Hearn S, Freier SM, Bennett CF, Zhang MQ, Spector DL. Regulating gene expression through RNA nuclear retention. Cell 2005;123:249–263. [PubMed: 16239143]

Prasanth KV, Rajendra TK, Lal AK, Lakhotia SC. Omega speckles - a novel class of nuclear speckles containing hnRNPs associated with noncoding hsr-omega RNA in Drosophila. J Cell Sci 2000;113 (Pt 19):3485–3497. [PubMed: 10984439]

Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY. Functional Demarcation of Active and Silent Chromatin Domains in Human HOX Loci by Noncoding RNAs. Cell 2007;129:1311–1323. [PubMed: 17604720]

Robb GB, Brown KM, Khurana J, Rana TM. Specific and potent RNAi in the nucleus of human cells. Nat Struct Mol Biol 2005;12:133–137. [PubMed: 15643423]

Ross P, Hall L, Haff LA. Quantitative approach to single-nucleotide polymorphism analysis using MALDI-TOF mass spectrometry. Biotechniques 2000;29:620–626. [PubMed: 10997276]628–629

Salditt-Georgieff M, Darnell JJE. Further evidence that the majority of primary nuclear RNA transcripts in mammalian cells do not contribute to mRNA. MolCellBiol 1982;2:701–707.

Schaffert N, Hossbach M, Heintzmann R, Achsel T, Luhrmann R. RNAi knockdown of hPrp31 leads to an accumulation of U4/U6 di-snRNPs in Cajal bodies. Embo J 2004;23:3000–3009. [PubMed: 15257298]

Shopland LS, Johnson CV, Byron M, McNeil J, Lawrence JB. Clustering of multiple specific genes and gene-rich R-bands around SC-35 domains: evidence for local euchromatic neighborhoods. J Cell Biol 2003;162:981–990. [PubMed: 12975345]

Smith K, Byron M, Johnson C, Xing Y, Lawrence JB. Defining early steps in mRNA transport: Mutant mRNA in Myotonic Dystrophy Type I is blocked at entry into SC-35 domains. Journal of Cell Biology 2007;178:951–964. [PubMed: 17846170]

Sone M, Hayashi T, Tarui H, Agata K, Takeichi M, Nakagawa S. The mRNA-like noncoding RNA Gomafu constitutes a novel nuclear domain in a subset of neurons. J Cell Sci 2007;120:2498–2506. [PubMed: 17623775]

Tam, R.; Shopland, LS.; Johnson, CV.; McNeil, J.; Lawrence, JB. Applications of RNA FISH for visualizing gene expression and nuclear architecture. Vol. 260. New York: Oxford University Press; 2002.

Tang K, Fu DJ, Julien D, Braun A, Cantor CR, Koster H. Chip-based genotyping by mass spectrometry. Proc Natl Acad Sci U S A 1999;96:10016–10020. [PubMed: 10468554]

Tycowski KT, Shu MD, Steitz JA. A mammalian gene with introns instead of exons generating stable RNA products. Nature 1996;379:464–466. [PubMed: 8559254]

Valgardsdottir R, Chiodi I, Giordano M, Cobianchi F, Riva S, Biamonti G. Structural and functional characterization of noncoding repetitive RNAs transcribed in stressed human cells. Mol Biol Cell 2005;16:2597–2604. [PubMed: 15788562]

Visa N, Puvion-Dutilleul, Francine Bachellerie J-P, Puvion E. Intranuclear distribution of U1 and U2 snRNAs visualized by high resolution in situ hybridization: revelation of a novel compartment containing U1 but not U2 snRNA in HeLa cells. EurJCell Biol 1993;60:308–321.

Wang MJ, Gegenheimer P. Substrate masking: binding of RNA by EGTA-inactivated micrococcal nuclease results in artifactual inhibition of RNA processing reactions. Nucleic Acids Res 1990;18:6625–6631. [PubMed: 2123540]

Willingham AT, Orth AP, Batalov S, Peters EC, Wen BG, Aza-Blanc P, Hogenesch JB, Schultz PG. A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. Science 2005;309:1570–1573. [PubMed: 16141075]

Xie SQ, Martin S, Guillot PV, Bentley DL, Pombo A. Splicing speckles are not reservoirs of RNA polymerase II, but contain an inactive form, phosphorylated on serine2 residues of the C-terminal domain. Mol Biol Cell 2006;17:1723–1733. [PubMed: 16467386]

Zhang Z, Carmichael GG. The fate of dsRNA in the nucleus: a p54(nrb)-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs. Cell 2001;106:465–475. [PubMed: 11525732]

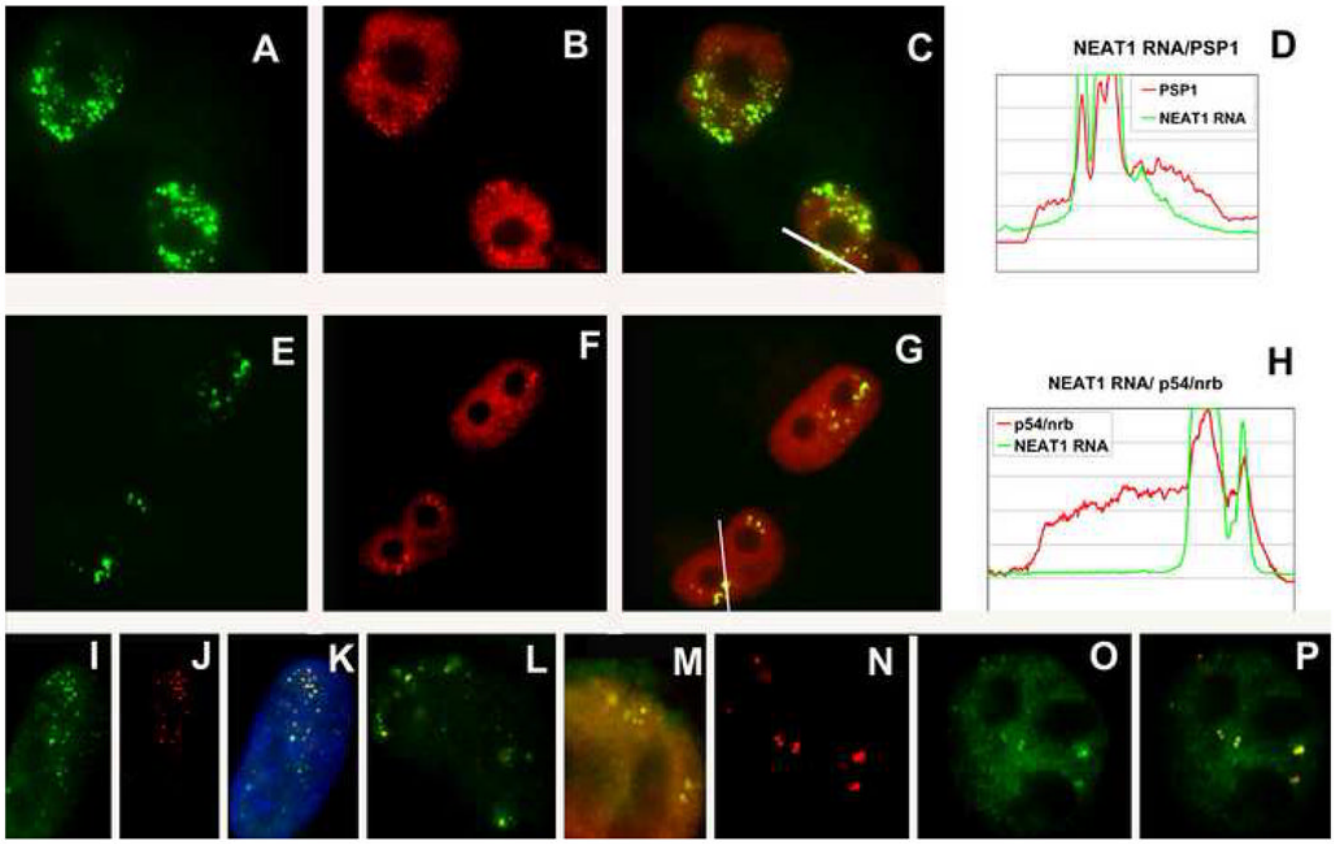

Figure 1.

NEAT1 RNA Overlaps with Paraspeckles in Multiple Human and Mouse Cells. Colocalization of NEAT1 RNA with paraspeckle proteins. A–D) NEAT1 RNA (green) localizes as bright discrete foci with little nucleoplasmic signal in HeLa cells. B) PSP1, a marker for paraspeckles (red), gives a broad nucleoplasmic signal coupled with clusters of more intense foci C) Overlap (yellow) of the numerous NEAT1 RNA foci (green) colocalize precisely with the accumulations of PSP1 antibody (red) that demark paraspeckles. E–H) NEAT1 RNA foci (E: green) also precisely align with accumulations of p54 antibody (F: red) that demark paraspeckles (G: overlap) in HeLa cells. D, H) Linescan analysis demonstrates that NEAT1 RNA (green) is primarily in paraspeckle foci, with little nucleoplasmic signal, while paraspeckle proteins (red) are found throughout the nucleoplasm and concentrate in paraspeckles. NEAT1 RNA strictly and consistently overlaps with PSP1 antibody in all human cells examined including: I–K (same cell): Tig1. I) NEAT1 RNA (green), J) PSP1 (red), K) merged: Nucleus is stained with DAPI (blue); L) HT-1080 (NEAT1 RNA - green, PSP1 antibody, red); and M) 293 (NEAT1 RNA – green, PSP1 antibody -red). N–P) Mouse cells (MEFs) display fewer Neat1 foci (panel N red) which overlap and correspond with fewer PSP1 antibody foci (panel O green – merged images panel P).

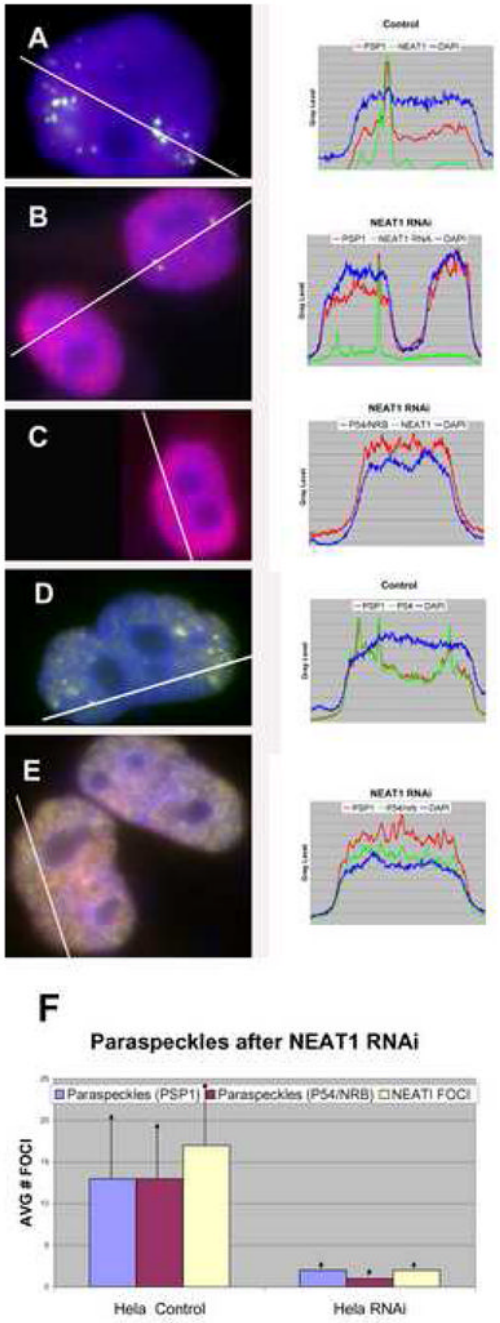

Figure 2.

Depletion of NEAT1 RNA by RNA inhibition causes Paraspeckles to Disappear

NEAT1 RNA (green) and PSP1 (red) in cells transfected twice over 48 hours with control siRNA (A) and NEAT1 RNA siRNA at a concentration of 100nM using Lipofectamine RNAimax (Invitrogen). (B). A) NEAT1 RNA is found as multiple foci throughout the nucleus unlike markers for paraspeckles (PSP1 – red) which are also found throughout the nucleoplasm (linescan). B) This representative example shows that while RNAi is successful in reducing most NEAT1 RNA (green) signal (bottom cell), occasionally a few clusters of NEAT1 signal remain (top cell), typically associated with sites of new NEAT1 transcription (Figure S2F). PSP1 enrichment in paraspeckles occurs where the NEAT1 clusters remain, while the

nucleoplasmic PSP1 (red) is elevated relative to control (linescan). *C) NEAT1 RNA (green) and p54 (red) in cells transfected with NEAT1 siRNA.* No foci of p54 are detected in NEAT1 RNA depleted cells, but the nucleoplasmic levels of p54 is elevated relative to the control (linescan). *PSP1 (red) and p54 (green) in cells transfected with control siRNA (D) and NEAT1 RNA siRNA (E):* D) PSP1 (red) and p54 (green), while found throughout the nucleoplasm, concentrate in paraspeckles. E) PSP1 and p54 no longer accumulate in paraspeckles in NEAT1 knockdowns, but nucleoplasmic levels of both proteins increase above the DAPI signal relative to control (linescan). F) Blind computer analysis and quantitation (see suppl. Methods) was used to count the number of paraspeckles using NEAT1 RNA, PSP1 or p54 antibody staining (bars represent standard error). All 3 paraspeckle markers show concomitant reduction in paraspeckle number after NEAT1 knockdown.

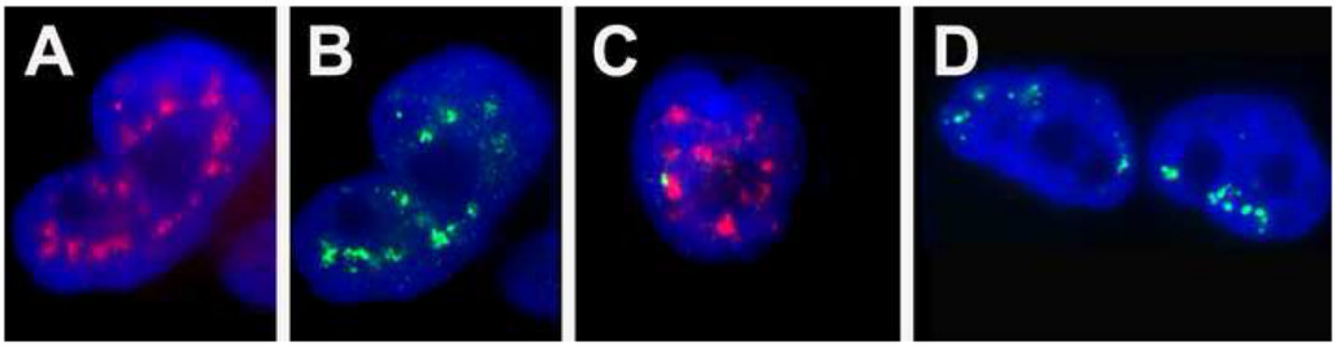

Figure 3.

NEAT2/MALAT-1 RNAi Does not affect SC-35 Domains

A) SC-35 domains (red) in HeLa cells overlap with B) NEAT2 RNA (green) in cells treated with control siRNA duplex. C) In cells inhibited for NEAT2 RNA (green), SC-35 domains (red) remain intact. Note that, similar to NEAT1 RNAi, while most of the NEAT2 RNA is effectively eliminated, occasionally some RNA associated with the site of transcription remains. D) Paraspeckles (PSP1 antibody -green) are unaffected by inhibition of NEAT2 RNA (red).

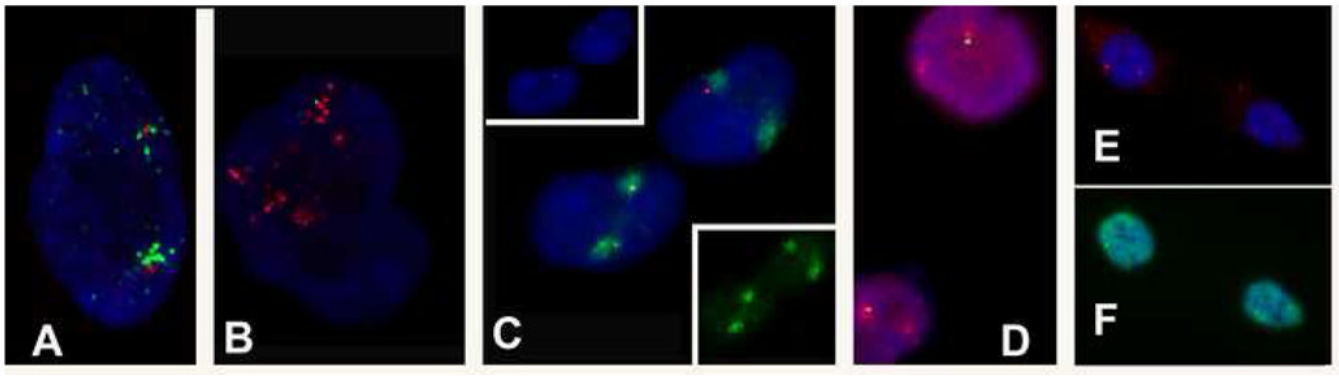

Figure 4.

Paraspeckles Form as NEAT1 Foci Spread from the Site of Transcription.

Dual NEAT1 RNA or PSP1 antibody and chr 11q13 detection. A) In interphase nuclei NEAT1 RNA foci (green) cluster near and spread from the site of transcription at 11q 13 (marked by the UHG locus–red). B) Similarly, paraspeckles (marked by PSP1 antibody – red) are often seen as a similar bipolar pattern that cluster and spread from 11q (UHG locus – green). C) In re-forming G1 daughter nuclei two large foci of NEAT1 RNA (red and top inset) are detected next to chromosome 11 (green and bottom inset). D) As G1 progresses, the earliest paraspeckles (PSP1 -red) are detected near 11q13 (UHG – green). E and F – (same cell) Later G1 daughter cells that have begun transcribing NEAT1 RNA (E: red) show bright nucleoplasmic PSP1 (F: green) signal and paraspeckle formation coincident with a single NEAT1 locus.

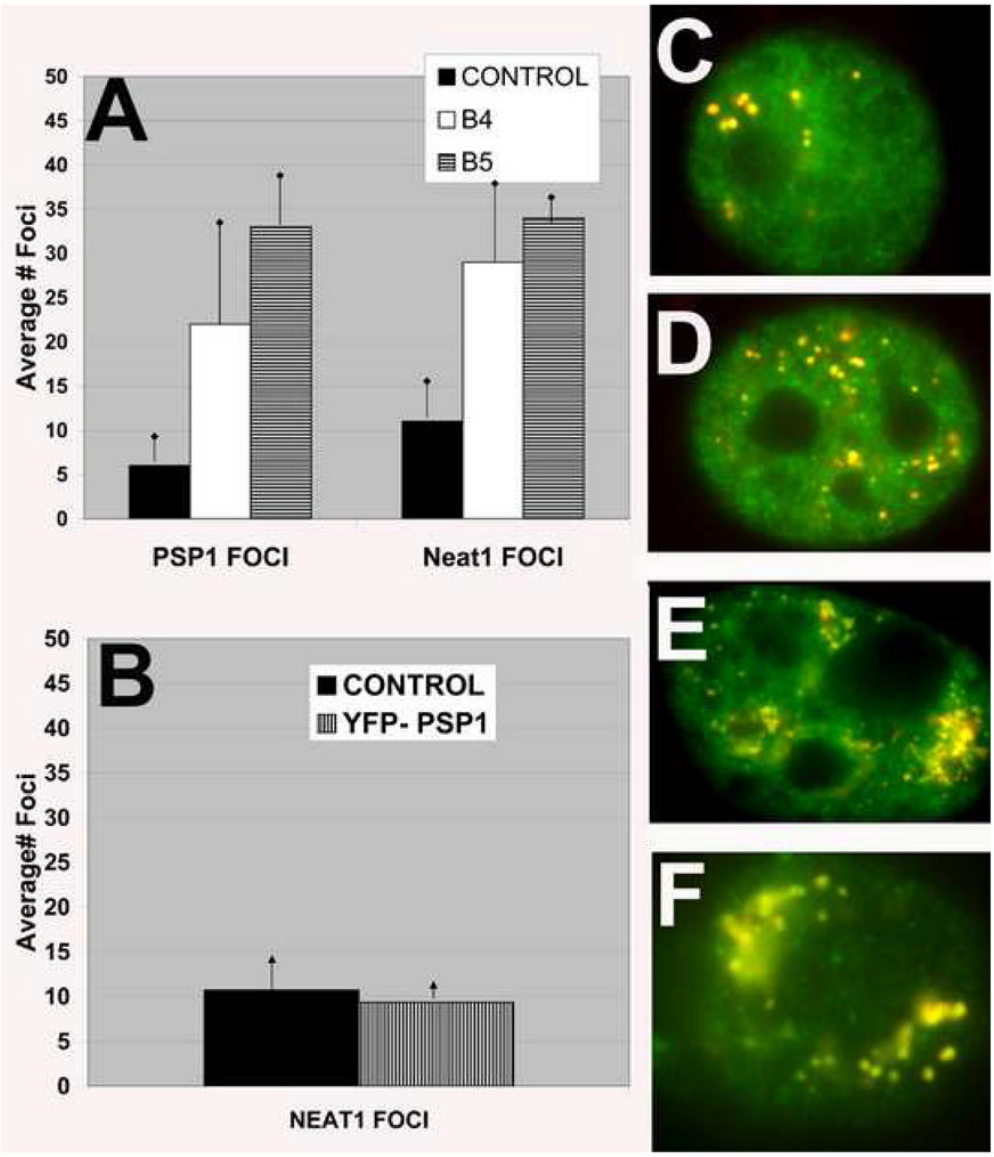

Figure 5.

Overexpression of Neat1, but not PSP1, increases the number of Paraspeckles

3T3 or HeLa cells were transfected with full length mNeat1 or hYFP-PSP1 respectively (or an empty vector control). qPCR demonstrated that cell line B4 and B5 expressed Neat1 RNA at a level 20% and 50% above the control, respectively. Immunoblotting showed that the YFP-PSP1 is expressed at a level 4X higher than endogenous PSP1 (Fox et al., 2002). Quantitation of Neat1 foci and paraspeckles was performed using blind computer scoring (see suppl Methods.). A) Increasing levels of mNeat1 expression causes a concommitant increase in paraspeckle number (as detected with anti-PSP1). B) HeLa cells overexpressing YFP-PSP1 show no change in paraspeckle number. Both NEAT1 RNA and PSP1 antibody, which detects both endogenous and transfected full length YFP-PSP1 (Fox et al., 2002), were used to mark paraspeckles with similar results (not shown). Bars represent standard error. C–E) Colocalization of Neat1 RNA (red) with PSP1 antibody (green) in: C) control; D) B4 cells and E) B5 cells. F) Colocalization of NEAT1 RNA (red) with YFP-PSP1 (green) in HeLa cells.

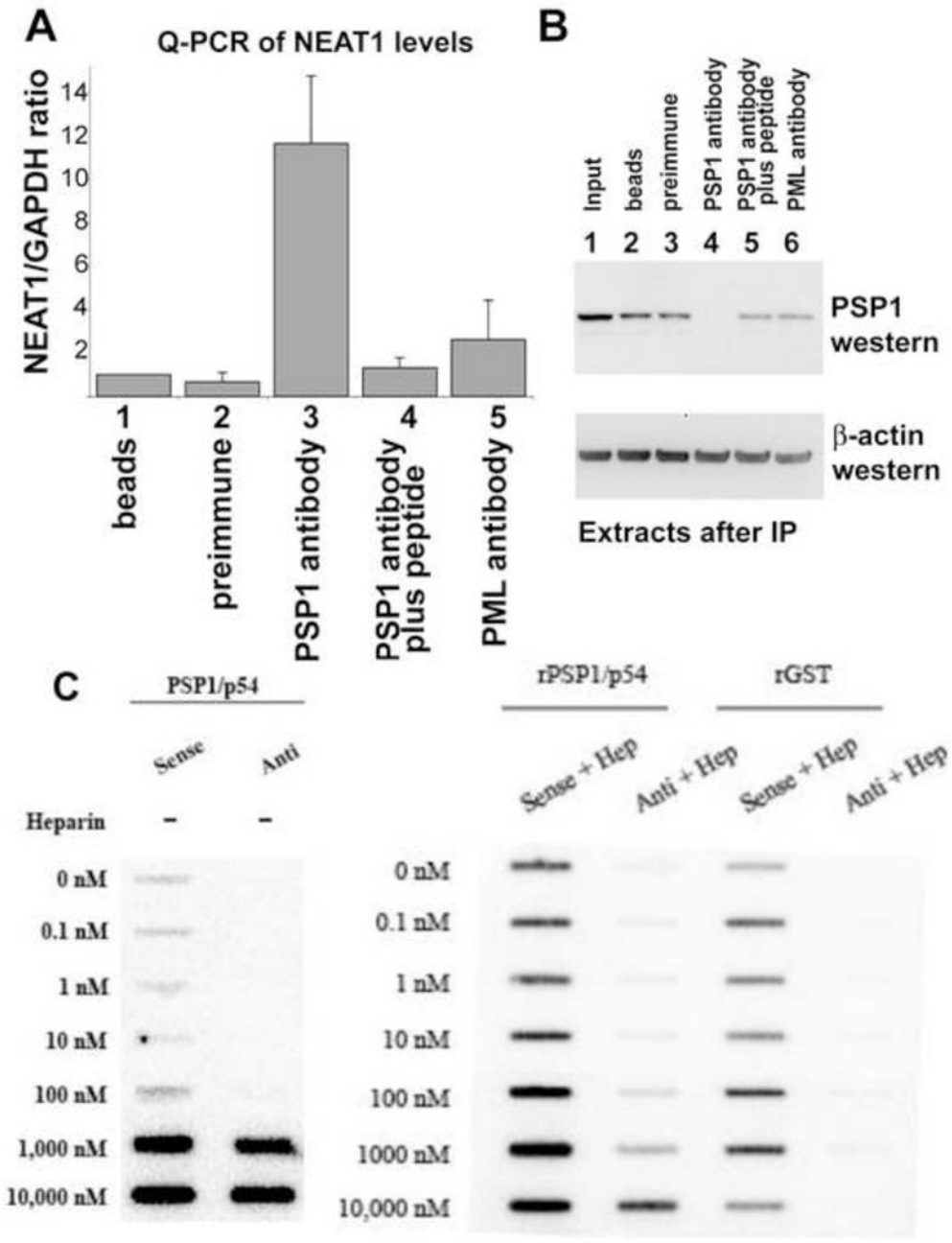

Figure 6.

NEAT1 binds with paraspeckle proteins *in vivo* and *in vitro*.

(A) NEAT1 and GAPDH RT-PCR analyzed by quantitative PCR (qPCR). Lane 1: beads alone, lane 2: rabbit preimmune serum, lane 3 anti PSP1 serum, lane 4: anti PSP1 serum pre-bound with the cognate peptide the antibody was raised against, lane 5: anti PML. The NEAT1 q-PCR data is based on amplification of a region at the 5′ end of NEAT1 (see Exptl. Proc.). The q-PCR data shown is derived from two individual IPRTPCR experiments, in which each sample was analyzed in duplicate. To control for non-specific binding, values were first normalized against the q-PCR GAPDH levels of the same samples and then adjusted such that the NEAT1 levels of the beads alone control had a value of 1. (B) Western blotting shows that only the PSP1 IP depletes PSP1 from a nuclear extract. Equal amounts of extract before (lane 1) and

after the IP (lanes 2–6) were transferred to a membrane and probed for the presence of PSP1 (upper panel) and beta-actin (lower panel). (C) Nitrocellulose filter binding assays show that *in vitro* transcribed NEAT1 RNA binds with increasing amounts of recombinant PSP1/p54 (left panel). Heparin, which abolishes non-specific RNA-protein interactions (Wang and Gegenheimer, 1990), does not affect the binding of the sense strand of NEAT1 RNA to the recombinant paraspeckle proteins, but does significantly diminish the binding by the NEAT1 RNA antisense strand. (Replicate experiments and RNA binding curves are shown in Figure S6).

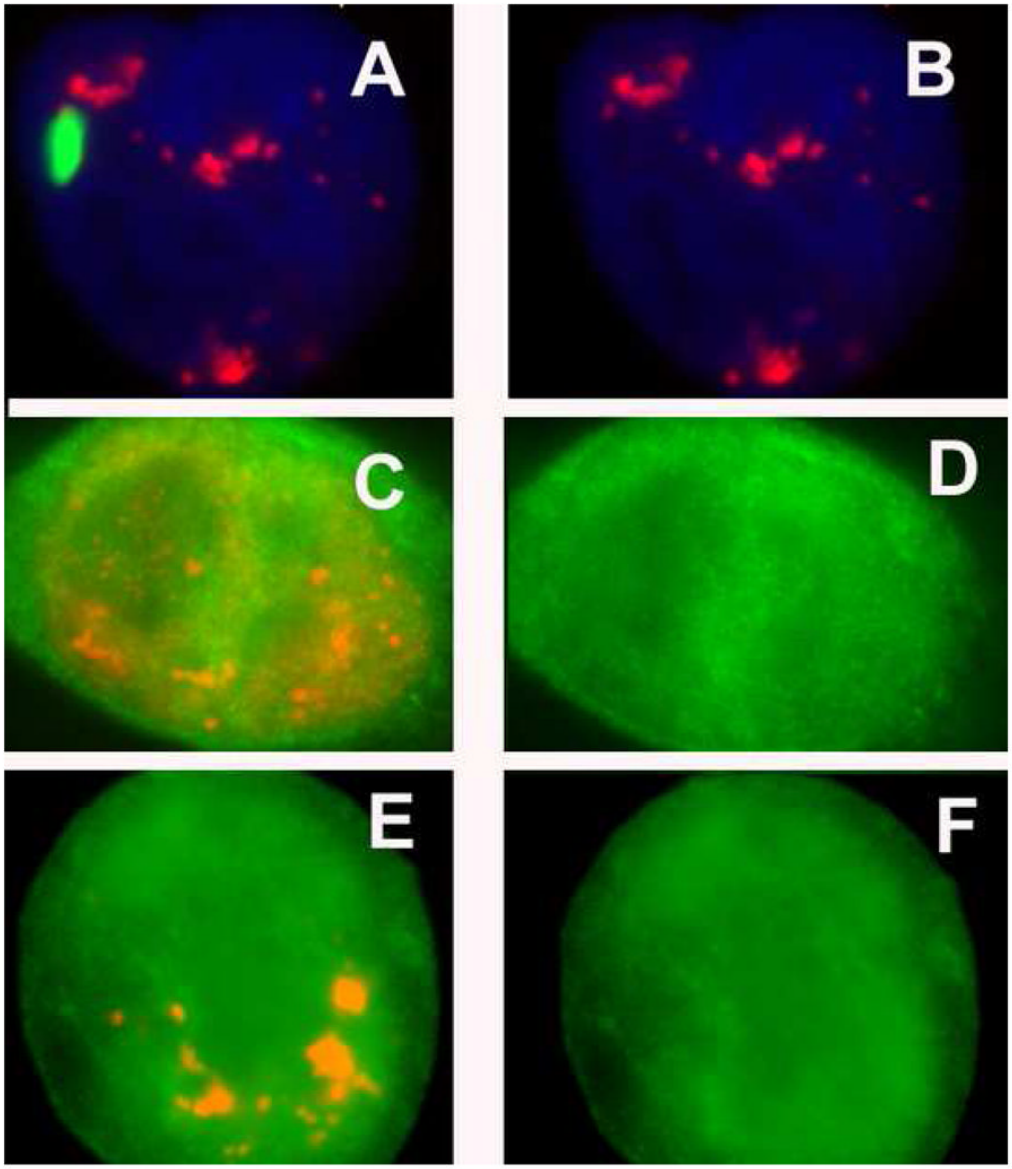

Figure 7.

Deletion of functional domains abrogates the association of PSP1 with NEAT1 RNA in Paraspeckles

A: YFP PSP1-236-523 (green) which is missing the RNA recognition motif does not localize with NEAT1 RNA (red - panel A&B) in paraspeckles. C–F: YFP PSP1 1-236 (green panel C–F) which is lacking the coiled coil domain (previously shown to be important for its interaction with p54 -Fox et al, 2005) is found diffusely throughout the nucleoplasm and does not localize to paraspeckles demarked both by PSP1 antibody (red panel C) and NEAT1 RNA (red panel E). Note: for the RRM mutant, paraspeckles are demarked by NEAT1 RNA only as the PSP1 antibody cross reacts to the PSP1-236-523 mutant protein; whereas both PSP1 antibody and NEAT1 RNA were used to demark paraspeckles with the PSP1-236-523 mutant protein as it is not detected by the PSP1 antibody (Fox et al, 2002).