

# Altered Nuclear Retention of mRNAs Containing Inverted Repeats in Human Embryonic Stem Cells: Functional Role of a Nuclear Noncoding RNA

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## SUMMARY

In many cells, mRNAs containing inverted repeats (*Alu* repeats in humans) in their 3' untranslated regions (3'UTRs) are inefficiently exported to the cytoplasm. Nuclear retention correlates with adenosine-to-inosine editing and is in paraspeckle-associated complexes containing the proteins p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$ . We report that robust editing activity in human embryonic stem cells (hESCs) does not lead to nuclear retention. p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$  are all expressed in hESCs, but paraspeckles are absent and only appear upon differentiation. Paraspeckle assembly and function depend on expression of a long nuclear-retained noncoding RNA, NEAT1. This RNA is not detectable in hESCs but is induced upon differentiation. Knockdown of NEAT1 in HeLa cells results both in loss of paraspeckles and in enhanced nucleocytoplasmic export of mRNAs containing inverted *Alu* repeats. Taken together, these results assign a biological function to a large noncoding nuclear RNA in the regulation of mRNA export.

## INTRODUCTION

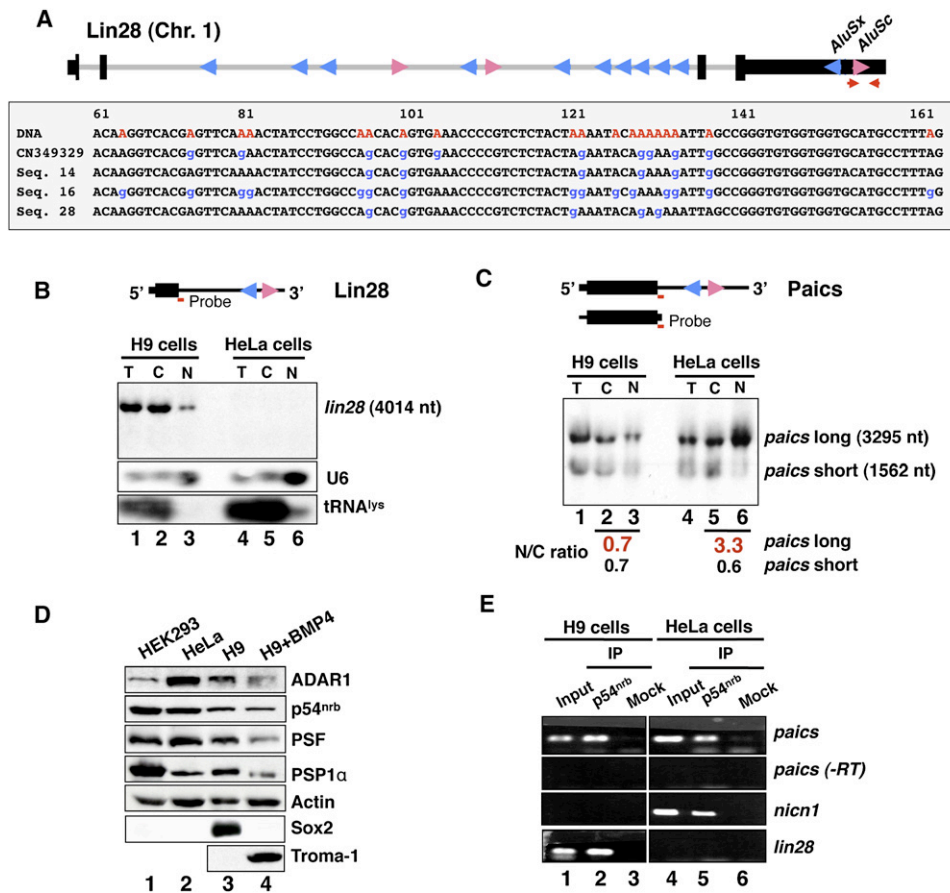
Cellular responses to double-stranded RNAs (dsRNAs) differ markedly depending on whether these molecules are found in the cytoplasm or in the nucleus. Mammalian cells rarely express dsRNA within the cytoplasm, most likely because of the ensuing dramatic effects on RNA levels, inhibition of protein synthesis, and, if prolonged, cell death (Kumar and Carmichael, 1998; Wang and Carmichael, 2004; and references therein). The primary cytoplasmic response to dsRNAs involves interferons (IFNs), protein kinase activated by dsRNA (PKR), and the 2',5'-adenylate synthesis/RNaseL pathway. In lower eukaryotes, or where these pathways are lacking or inactive, the RNA interference (RNAi) pathway provides the primary mechanism to eliminate cytoplasmic dsRNAs.

In the nucleus, dsRNAs are frequently edited by dsRNA-dependent adenosine deaminases (ADARs). ADARs are ubiquitously expressed in higher eukaryotes and catalyze the hydrolytic deamination of adenosines (A) to inosines (I) (Bass, 2002; Bass

and Weintraub, 1988; Nishikura, 1992; Polson et al., 1991). Whereas some editing directed by short dsRNA structures is site specific and leads to coding changes in mRNAs, long dsRNA regions (hairpins or sense-antisense hybrids) are edited promiscuously, with up to half of their adenosines being changed to inosines (Bass, 2002). ADAR editing is surprisingly abundant in human cells, and more than 90% of this occurs within inverted repeated *Alu* elements (*IRAlus*) (Athanasiadis et al., 2004; Blow et al., 2004; Kim et al., 2004; Levanon et al., 2004). *Alu* elements are unique to primates and account for almost all of the human short interspersed nuclear elements (SINEs), more than 10% of the genome. Their abundance leads to the frequent occurrence of inverted repeat structures in gene regions. Whereas most *IRAlus* lie within introns, we have identified 333 human genes with *IRAlus* in their 3'UTR regions (Chen et al., 2008).

What is the consequence of the presence of *IRAlus* in mRNAs? mRNAs with structured or edited 3'UTRs can be bound by a nuclear complex containing the protein p54<sup>nrb</sup>, which prevents their export to the cytoplasm (Chen et al., 2008; Prasanth et al., 2005; Zhang and Carmichael, 2001). Such nuclear retention provides a quality control mechanism that prevents inappropriate translation of promiscuously edited RNAs and might be used in other ways to regulate gene expression (Chen and Carmichael, 2008; Chen et al., 2008; Prasanth et al., 2005). p54<sup>nrb</sup> is concentrated in nuclear structures called paraspeckles, which also contain two related proteins, PSF and PSP1 $\alpha$  (Fox et al., 2002, 2005). Paraspeckles are associated not only with nuclear-retained RNAs (Chen et al., 2008; Prasanth et al., 2005), but also with an abundant nuclear-retained noncoding RNA, hNEAT1, which is 3.7 kb in length (Hutchinson et al., 2007). NEAT1 (hNEAT1 in this study) has no obvious inverted repeats that could direct editing, and no edited bases within this transcript have been reported. In agreement with recent reports (Clemson et al., 2009; Sasaki et al., 2009; Sunwoo et al., 2009), we find that hNEAT1 RNA is required for paraspeckle integrity. We further demonstrate that it influences the nuclear retention of structured or edited mRNAs.

LIN28 is a key regulatory factor in the maintenance of pluripotency (Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008; Xu et al., 2009). Because this factor is abundantly expressed in hESCs (Richards et al., 2004), we suspected that the *IRAlus* in the 3'UTR of the *Lin28* mRNA may not promote nuclear retention as is seen in other cells (Chen et al., 2008). This is indeed the case. ADAR1 activity is high in hESCs, yet the



**Figure 1. Altered Nuclear Retention in Human ES Cells**

(A) ADAR1 activity in hESCs. The upper panel shows the distribution of *Alu* elements in the *Lin28* transcript. The lower panel shows the sequence of several cDNA clones of *Lin28* showing A-to-G changes diagnostic of A-to-I editing. Red arrows indicate the primers used for amplification.

(B) *Lin28* mRNA escapes nuclear retention in H9 cells. Nuclear and cytoplasmic RNAs were isolated from H9 cells and HeLa cells and resolved on a denaturing agarose gel. The probe for the northern blot (red bar) is located upstream of the *IRAlus* from the 3'UTR of *Lin28*. tRNA<sup>lys</sup> and U6 were used as markers for nuclear and cytoplasmic RNA fractionation. Equivalent fractions of total (T), cytoplasmic (C), and nuclear (N) RNAs were loaded.

(C) *Paics* mRNA escapes nuclear retention in H9 cells, but is mostly retained in the nucleus in HeLa cells. The same samples from (B) were used. Two *Paics* isoforms are shown. The probe for the northern blot (red bar) is located upstream of the *IRAlus* from the 3'UTR of *Paics*. Bands were quantitated using ImageJ software.

(D) ADAR1 and paraspeckle-related proteins are expressed in H9 cells. Total proteins were collected from HEK293 cells (lane 1), HeLa cells (lane 2), H9 cells (lane 3), and H9 cells differentiated into trophoblasts using BMP4 (lane 4). Actin was used as loading control, Sox2 was used as a marker for human ES cells, and Troma-1 was used as a marker for trophoblasts.

(E) mRNAs containing *IRAlus* are associated with p54<sup>nrb</sup> in both H9 cells and HeLa cells. Immunoprecipitations (IP) were performed from H9 cells and HeLa cells using anti-p54<sup>nrb</sup> antibody or mock antibody (beads alone).

editing-associated nuclear retention pathway is impaired. Importantly, paraspeckles are not formed and hNEAT1 RNA is not expressed. When hESCs are induced to differentiate into trophoblasts, hNEAT1 is induced and paraspeckles appear. Finally, when hNEAT1 expression is reduced in HeLa cells, not only do paraspeckles disappear, but a number of mRNAs with *IRAlus* in their 3'UTRs are more efficiently exported to the cytoplasm.

## RESULTS

### ADAR1 Is Active in Human ES Cells

ADAR1 is responsible for most promiscuous RNA editing (Bass, 2002) and is expressed in hES H1 and H9 cells at a level com-

parable to that seen in HEK293 and HeLa cells (Figure 1D and Figure S1 available online). Further, ADAR1 expression is not significantly different when H9 cells are cultured on feeder cells or grown in feeder-free medium (Figure S1). Consistent with its expression level, ADAR1 editing activity is robust in hESCs. The LIN28 protein is an important regulator of pluripotency, and is highly expressed in hESCs (Balzar and Moss, 2007; Yu et al., 2007; Viswanathan et al., 2008; Rybak et al., 2008; Newman et al., 2008; Piskounova et al., 2008). Human *Lin28* mRNA contains a single pair of inverted *Alu* repeats in its 3'UTR (Figure 1A, top). We used RT-PCR to amplify the region spanning the *AluSc* element in the 3'UTR of *Lin28* from total RNA isolated from H9 cells. DNA sequencing of individual clones indicated

frequent A-to-I editing in this region (Figures 1A, bottom, and S2). In addition, the editing pattern is similar to sequences published in the University of California Santa Cruz genome browser (e.g., CN349329; Figure 1A) and to *Alu* editing levels seen in other cells (Chen et al., 2008). Because we examined only one of the *Alu* elements in this 3'UTR, it is likely that our results are an underestimate of the true extent of *Lin28* transcript diversity generated by editing.

### Altered Nuclear Retention Pathway in Human ES Cells

What is the fate of *IRAlus* in human ES cells? In differentiated cells, the fate of promiscuously edited RNA frequently involves nuclear retention in nuclear complexes containing p54<sup>nrb</sup> (Zhang and Carmichael, 2001; Prasanth et al., 2005; Chen et al., 2008). We recently published that edited *IRAlus* in 3'UTRs of genes can repress gene expression by sequestering mRNAs in the nucleus (Chen et al., 2008). We therefore asked whether the *Lin28* transcript is retained in the nucleus in hESCs. Northern blotting was carried out with cytoplasmic and nuclear RNAs isolated from H9 and HeLa cells. Surprisingly, we observed efficient export of *Lin28* in H9 cells (Figure 1B). In contrast, when the *Lin28 IRAlus* were inserted into a reporter construct, strong retention was seen in HEK293 cells (Chen et al., 2008). This raised the possibility that hESCs export mRNAs with *IRAlus* in their 3'UTRs more efficiently than other cells.

However, *Lin28* is a stem cell-specific transcript and might have a unique regulation in hESCs. The mRNA for Nicotin 1 contains one pair of inverted *Alu* repeats in its 3'UTR and shows strong nuclear retention in both HEK293 cells (Chen et al., 2008) and HeLa cells (data not shown). Because this mRNA is not transcribed in hESCs (Figure 1E), we sought genes that are transcribed in both hESCs and differentiated cells. From the Gladstone Microarray Data Including Stem Cell Tissue available through the UCSC genome browser, we identified several candidate genes containing *IRAlus* in their 3'UTRs. *Paics* (phosphoribosylaminoimidazole carboxylase) is one of them (Figures 1C, top, and S3), and some available EST sequences show promiscuous editing. Recent biochemical studies showed that PAICS is an important bifunctional enzyme in de novo purine biosynthesis and is especially crucial for rapidly dividing cancer cells which rely heavily on the purine de novo pathway for synthesis of adenine and guanine, whereas normal cells favor the salvage pathway (Li et al., 2007).

Northern blotting using a probe lying upstream of the inverted *Alu* repeats in its 3'UTR showed similar transcription levels of *Paics* in both H9 cells and HeLa cells (Figure 1C, lanes 1 and 4). Using equivalent amounts of RNAs from the cytoplasm and the nucleus, we observed distinct nuclear retention of the full-length *Paics* (3295 nt) in HeLa cells (Figure 1C, lanes 5 and 6, N/C ratio of 3.3), but not in H9 cells (Figure 1C, lanes 2 and 3). Notably, the *Paics* probe also detected a shorter isoform of *Paics* (1562 nt) which corresponds to the polyadenylated cDNA BC019255. This shorter isoform is mostly cytoplasmic in both cell lines, suggesting that nuclear retention in HeLa cells is mediated by the inverted *Alu* repeats, as we have seen in other cases in differentiated cells (Chen et al., 2008). Consistent with these results, we have observed that multiple mRNAs with *IRAlus* in their 3'UTRs (*Lin28*, *Paics*, and *Pccb*) are efficiently exported

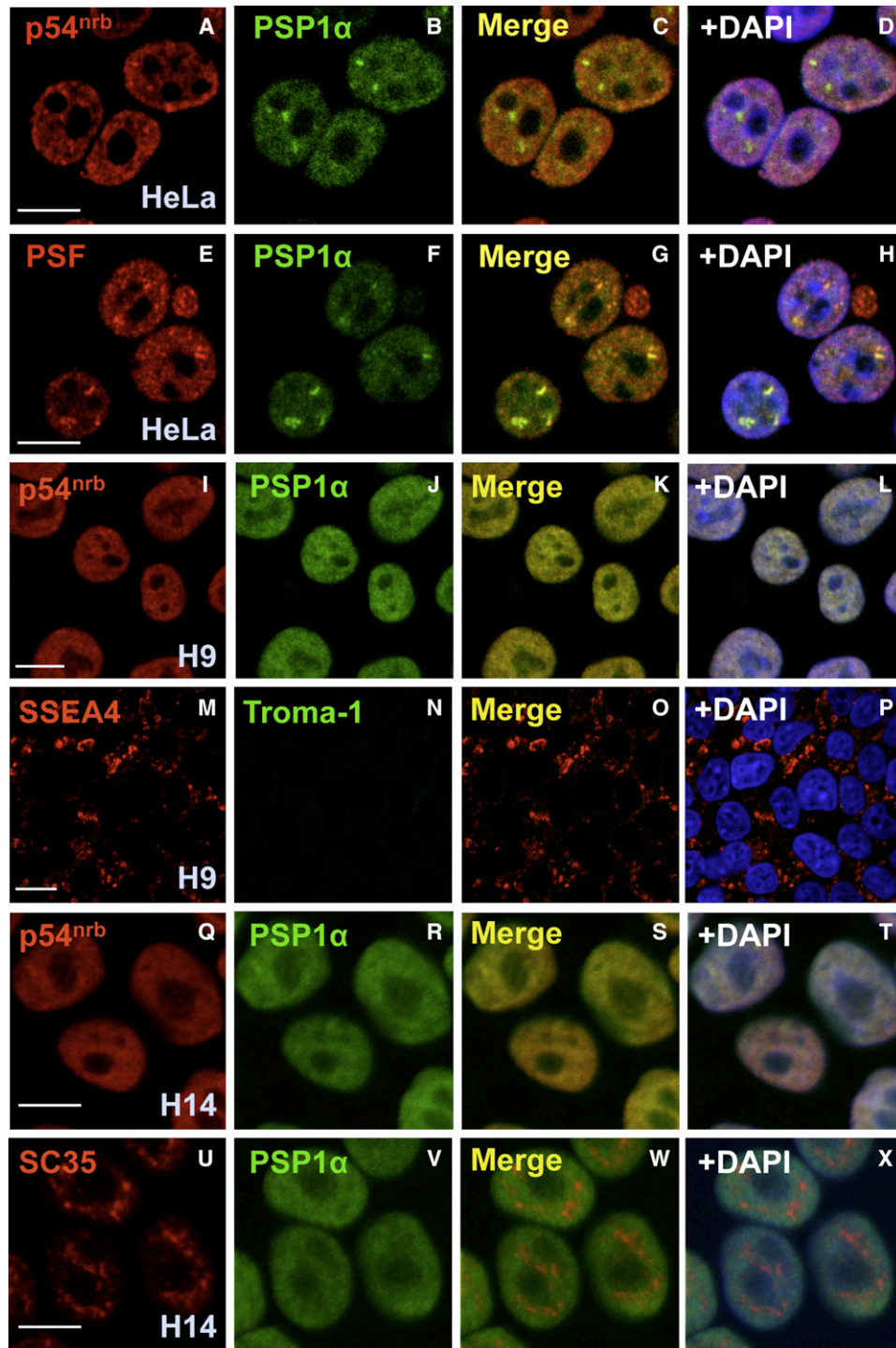
to the cytoplasm in another hESC line, H14 (Figure S4). Finally, in agreement with what we have generally observed for mRNAs containing *IRAlus* (Chen et al., 2008), *Paics*, *Nicn1*, and *Lin28* mRNAs are associated with p54<sup>nrb</sup> in vivo (Figure 1E).

### Human ES Cells Lack Paraspeckles

In mammalian cells, p54<sup>nrb</sup> and its partners, such as PSF and PSP1 $\alpha$ , likely participate in the regulation of the nuclear retention of inosine-containing RNAs (Zhang and Carmichael, 2001; Prasanth et al., 2005; Chen et al., 2008). p54<sup>nrb</sup> is a multifunctional protein and has been implicated in a variety of nuclear processes (Basu et al., 1997; Emili et al., 2002; Ishitani et al., 2003; Kameoka et al., 2004; Kaneko et al., 2007; Straub et al., 1998; Yang et al., 1993, 1997; Zhang et al., 1993; Zhang and Carmichael, 2001). It was the first RNA-binding protein described which shows high affinity for edited transcripts (Zhang and Carmichael, 2001). p54<sup>nrb</sup> contains two tandem RNA recognition motif-type RNA binding domains and a putative helix-turn-helix motif followed by a highly charged region with a predicted coiled-coil structure. These four regions together comprise the DBHS (*Drosophila* behavior and human splicing) domain, which is highly conserved among p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$  (Fox et al., 2002; Yang et al., 1993). PSF and PSP1 $\alpha$  can each form heterodimers with p54<sup>nrb</sup> (Akhmedov and Lopez, 2000; Fox et al., 2005; Myojin et al., 2004; Zhang et al., 1993). PSF is also a multifunctional protein. It binds both RNA and DNA (Zhang et al., 1993) and can act in splicing (Gozani et al., 1994; Lindsey et al., 1995; Patton et al., 1993) and transcription (Mathur et al., 2001; Urban et al., 2000). Like p54<sup>nrb</sup>, PSF also binds tightly to edited substrates (Zhang and Carmichael, 2001). PSP1 $\alpha$  is somewhat less abundant but shares about 50% sequence similarity with p54<sup>nrb</sup> and PSF in the conserved DBHS domain and has recently been found to be a marker for nuclear paraspeckles (Fox et al., 2002). Paraspeckles are cell-cycle-regulated subnuclear domains of currently unknown function that are dependent on RNA for their structural integrity (Fox et al., 2005). The edited mouse CTN-RNA was shown to at least partially localize to paraspeckles, suggesting paraspeckles could be sites of nuclear retention of at least a subset of edited dsRNAs (Prasanth et al., 2005). Our recent results on the nuclear retention of *IRAlus*-containing mRNAs are also consistent with paraspeckle association (Chen et al., 2008).

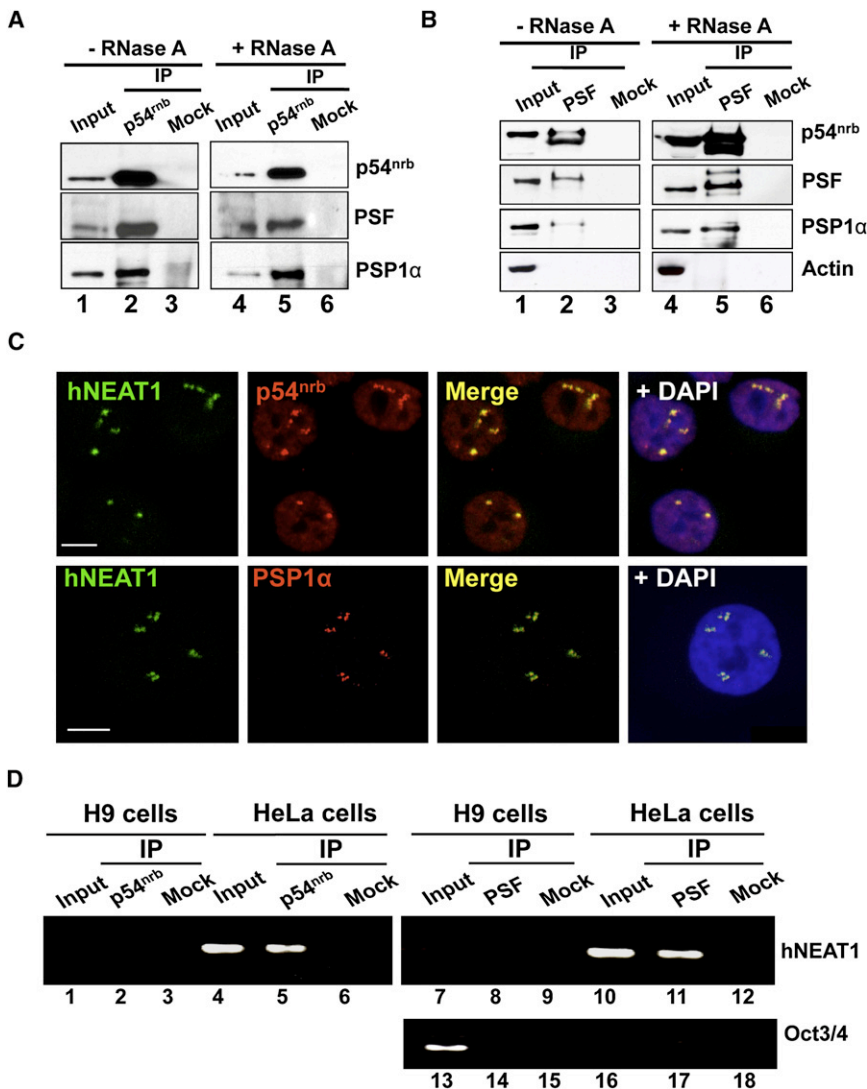
As shown in Figure 1D, p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$  are expressed at a level in H9 cells similar to that seen in HEK293 cells and HeLa cells. The same result was seen in hESC H1 cells (Figure S1B) and H14 cells (data not shown). Indeed, in HeLa cells, p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$  all colocalize in paraspeckles (Figures 2A–2H), although a fraction of all of them are found outside of these structures. Importantly, even though hESCs abundantly express these proteins, typical paraspeckles are not seen (H9 cells, Figures 2I–2L; H14 cells, Figures 2Q–2T; Figures S5 and S9). PSP1 $\alpha$  and p54<sup>nrb</sup> show an almost uniform distribution and colocalization throughout the nucleoplasm, but are excluded from nucleoli. In contrast, hESCs show no apparent defect in nuclear SC35 splicing speckles (Figures 2U–2X). Consistent with what has been observed in other cells (Akhmedov and Lopez, 2000; Fox et al., 2005; Myojin et al., 2004; Zhang et al., 1993), p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$  interact with one another in H9 cells, even in the absence of RNA (Figures 3A and 3B).





**Figure 2. Human ES Cells Lack Paraspeckles**

HeLa cells were stained with anti-p54<sup>nrb</sup> and anti-PSP1 $\alpha$  antibodies (A–D) or anti-PSF and anti-PSP1 $\alpha$  antibodies (E–H). DAPI was used to indicate DNA. Note that p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$  colocalize with each other and indicate paraspeckles. p54<sup>nrb</sup> and PSP1 $\alpha$  colocalize throughout the nucleoplasm in H9 cells, but show no apparent nuclear paraspeckles (I–L). The same batch of H9 cells was also stained with anti-SSEA4 (stem cell marker) and anti-Troma-1 (trophoblast differentiation marker) (M–P). p54<sup>nrb</sup> and PSP1 $\alpha$  also colocalize throughout the nucleoplasm in H14 cells (Q–T), but show no apparent nuclear paraspeckles. H14 cells were also stained with anti-SC35 and anti-PSP1 $\alpha$  (U–X). The scale bars represent 10  $\mu$ m.



### Noncoding hNEAT1 RNA Localizes to Paraspeckles and Is Undetectable in hESCs

RNA immunoprecipitation experiments revealed that mRNAs for Paics and Lin28 associate with p54<sup>nrb</sup> in H9 cells (Figure 1E, lane 2), as in differentiated cells (lane 5). However, these mRNAs are efficiently exported to the cytoplasm in H9 cells (Figures 1B and 1C) and H14 cells (Figure S4), indicating that binding to p54<sup>nrb</sup> is not sufficient for nuclear retention. Rather, retention correlates with the presence of paraspeckles, and these are missing in hESCs (Figures 2I–2T and S5).

What underlies the failure of paraspeckles to assemble in hESCs? Although it is known that RNA plays a role in paraspeckle formation (Fox et al., 2005), structured mRNAs such as those with IRALus cannot themselves play a crucial role because they are expressed in hESCs. Hutchinson et al. (2007) identified NEAT1 (nuclear enriched abundant transcript 1) as a large (3.7 kb) polyadenylated RNA transcript displaying striking nuclear enrichment in both human and mouse cells. In rats, NEAT1 expression can be further induced by virus infection (Saha et al., 2006).

### Figure 3. Noncoding hNEAT1 RNA Localizes to Paraspeckles and Is Undetectable in hESCs

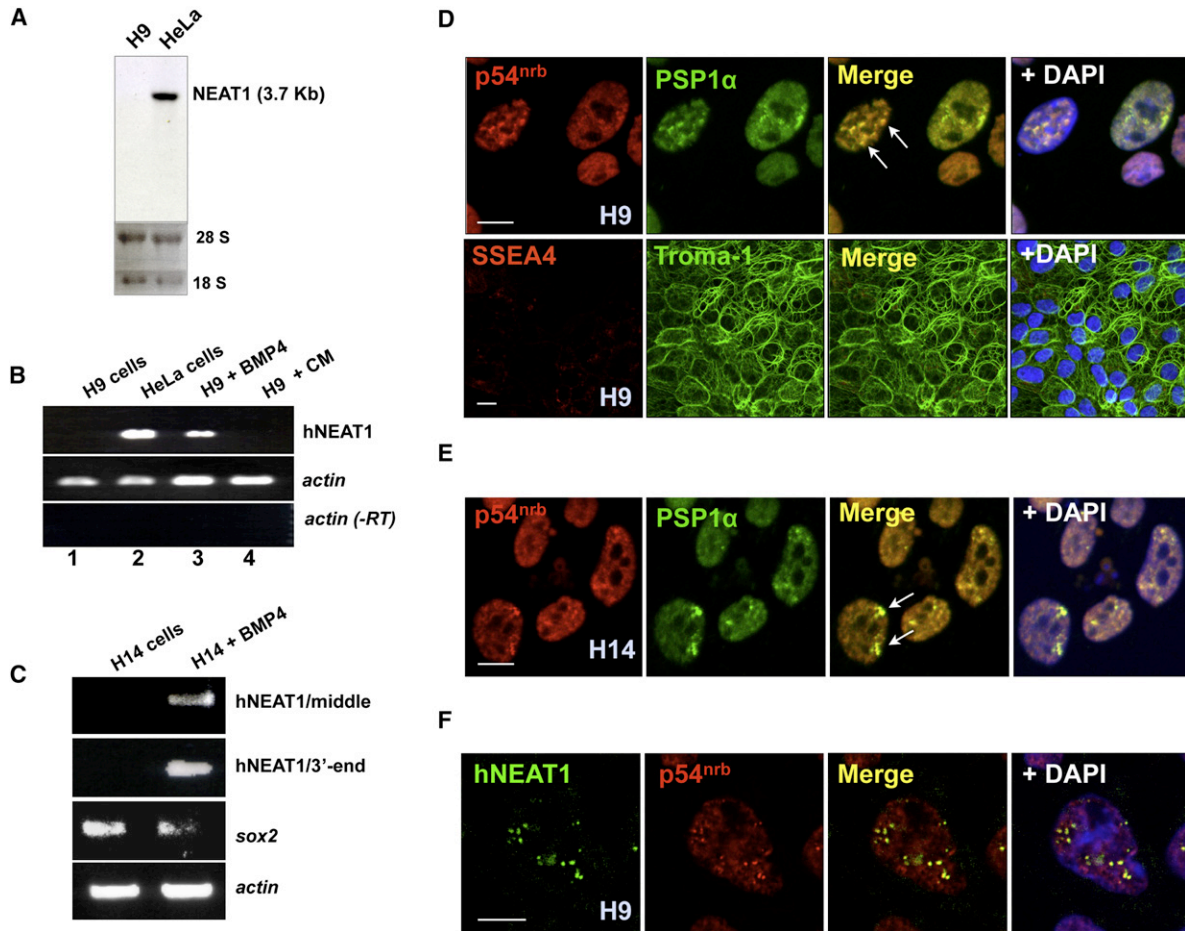
(A) RNA-independent interaction of p54<sup>nrb</sup> with PSF and PSP1α in H9 cells. Total extracts of H9 cells with or without RNaseA treatment were immunoprecipitated with p54<sup>nrb</sup> antibody or mock antibody, and then immunoblotted with anti-p54<sup>nrb</sup>, anti-PSF, and anti-PSP1α antibodies. (B) The same immunoprecipitation experiments were performed with anti-PSF antibody in H9 cells. Anti-actin was used as a negative control. (C) RNA in situ hybridization was performed with green dUTP-labeled antisense hNEAT1 probe in HeLa cells, and representative images are shown. p54<sup>nrb</sup> and PSP1α are in red. hNEAT1 colocalizes with p54<sup>nrb</sup> (upper panel) and PSP1α (lower panel). The scale bars represent 10 μm.

(D) hNEAT1 associates with p54<sup>nrb</sup> and PSF in HeLa cells. IP with anti-p54<sup>nrb</sup>, anti-PSF, or mock antibody was carried out in extracts from H9 cells or HeLa cells. RT-PCR of hNEAT1 from the IP showed amplification in HeLa cells by anti-p54<sup>nrb</sup> IP and anti-PSF IP, but not by IP with mock antibody. Oct3/4 is a marker of H9 cells.

Using in situ hybridization, we demonstrated that hNEAT1 localizes to paraspeckles in HeLa cells. As shown in Figure 3C, hNEAT1 RNA is restricted to a small number of large, distinct nuclear speckles in HeLa cell nuclei. Importantly, hNEAT1 accumulation colocalizes very well with both p54<sup>nrb</sup> and PSP1α (Figure 3C). Further confocal z section studies showed such colocalization occurs throughout the nucleus (Figures S6 and S7). These results are in complete agreement with recent work in both mouse (Sasaki et al., 2009; Sunwoo

et al., 2009) and human (Clemson et al., 2009) cells showing that NEAT1 RNA (MENε/β RNA in mouse) plays an essential role in the assembly and architecture of paraspeckles. To further examine the relationship between hNEAT1 RNA and other paraspeckle components, we carried out RNA immunoprecipitation experiments in HeLa cells. In further agreement with Clemson et al. (2009), we observed association of hNEAT1 RNA with p54<sup>nrb</sup> and PSF in HeLa cells (Figure 3D, lanes 5 and 11).

Surprisingly, hNEAT1 RNA was undetectable in all hESCs we examined (H9 cells, Figures 3D, 4A, and 4B; H14 cells, Figure 4C; H1 cells, Figure S8). This absence of hNEAT1 was further confirmed using different PCR primers across hNEAT1 sequences (Figures 4C and S8) and also using H9 cells that were cultured under different experimental conditions (Figure 4B). Curiously, the noncoding NEAT2 RNA (MALAT-1), which lies immediately downstream of NEAT1 on chromosome 11 (Hutchinson et al., 2007), is expressed at high levels in hESCs (data not shown), suggesting independent regulation of expression of these ncRNAs.



**Figure 4. hNEAT1 and Paraspeckles Appear upon hESC Differentiation**

(A) Northern blot showing absence of hNEAT1 RNA in H9 cells.  
 (B) RT-PCR showing absence of hNEAT1 RNA in H9 cells (lane 1, H9 cells cultured in mTeSR; lane 2, H9 cells cultured in CM medium) but its presence in differentiated cells (lane 3).  
 (C) RT-PCR showing hNEAT1 absence in undifferentiated H14 cells but expression in differentiated H14 cells.  
 (D) H9 cells cultured in CM medium were induced with BMP4 for 5 days and then fixed for immunostaining. Day 5 differentiated H9 cells show the trophoblast marker Troma-1 and the loss of stem cell marker SSEA4 (lower panel). p54<sup>nrB</sup> and PSP1 $\alpha$  colocalize with each other and form multiple large accumulations (white arrows), indicating paraspeckles.  
 (E) Day 4 differentiated H14 cells show p54<sup>nrB</sup> and PSP1 $\alpha$  accumulations (white arrows) in the nuclei, indicating paraspeckles.  
 (F) hNEAT1 (in situ hybridization; green) is expressed in trophoblasts derived from H9 cells and colocalizes with p54<sup>nrB</sup>.  
 The scale bars represent 10  $\mu$ m.

**hNEAT1 Is Expressed after Differentiation**

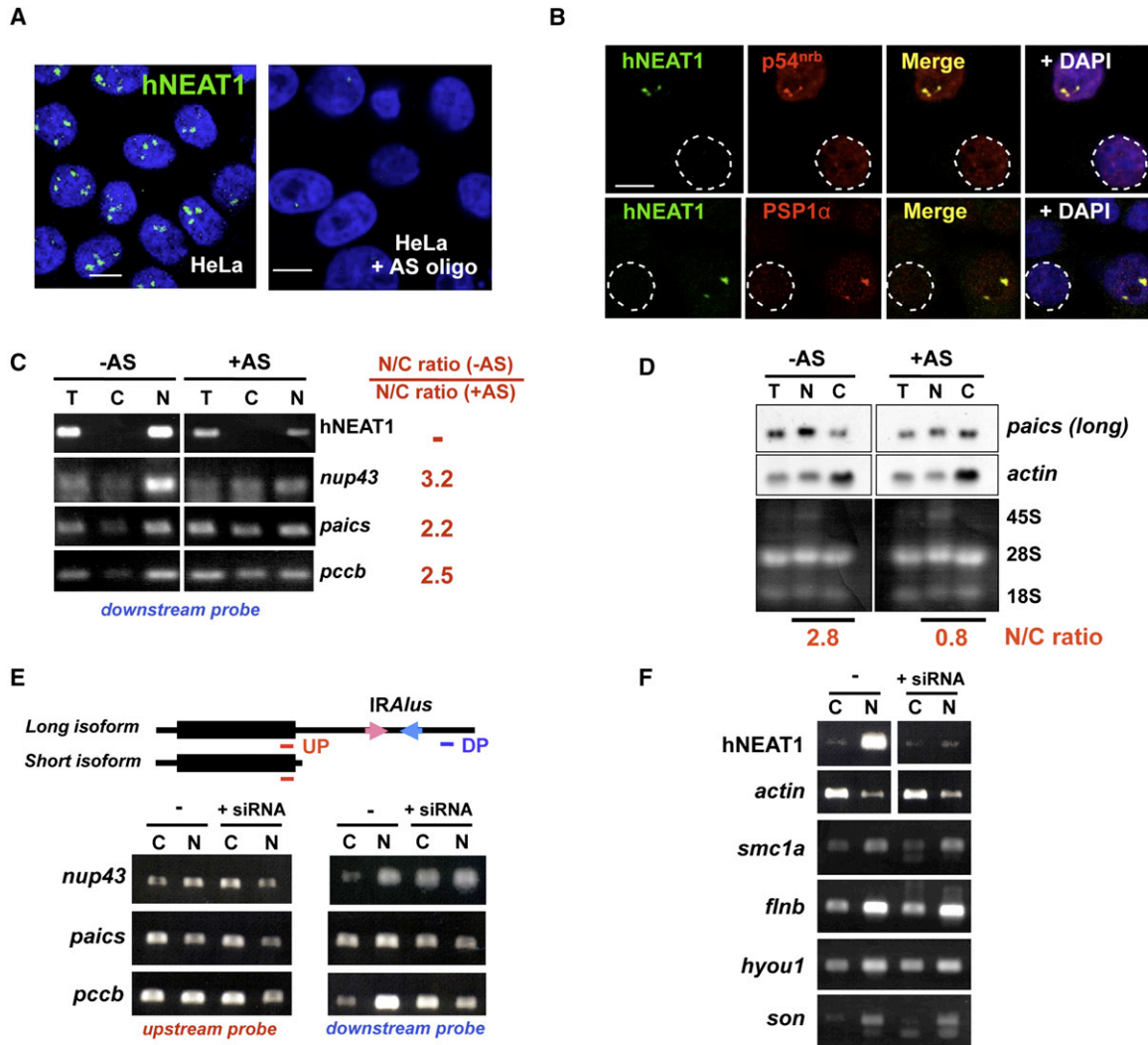
To examine whether hNEAT1 expression correlates with paraspeckle formation during hESC differentiation, we differentiated H9 cells to trophoblasts using BMP4 treatment (Xu et al., 2002, 2005). The majority of H9 cells became trophoblasts after 4–5 days of BMP4 treatment as shown by the presence of the trophoblast marker Troma-1 and the loss of the stem cell marker SSEA-4 (Figure 4D, bottom). ADAR1 and the paraspeckle-related proteins p54<sup>nrB</sup>, PSF, and PSP1 $\alpha$  were also detected in trophoblasts (Figure 1D, lane 4). Importantly, we observed the return of paraspeckles in trophoblasts derived from H9 cells (Figure 4D) and H14 cells (Figures 4E and S9). PSP1 $\alpha$  and p54<sup>nrB</sup> accumulated in numerous dots per trophoblast nucleus (Figures 4D, 4E, and S9), similar to the pattern observed in

HeLa cells (Figures 2A–2D) and other cell types (data not shown). In addition, PSF and PSP1 $\alpha$  also showed colocalization throughout the nucleus (data not shown). Importantly, the expression of hNEAT1 RNA was detected during trophoblast differentiation (Figures 4B, 4C, and 4F). Taken together, these observations led us to speculate that the lack of paraspeckles in hESCs might mechanistically underlie the weak nuclear retention for mRNAs containing inverted *Alu* repeats.

**hNEAT1 Plays a Critical Role in Both Paraspeckle Formation and Function**

Because the levels of *Lin28* or *Paics* decrease significantly (data not shown), BMP4 induction is not an ideal model to address whether the appearance of paraspeckles during differentiation





### Figure 5. Role of hNEAT1 in Paraspeckle Function

(A) hNEAT1 RNA in situ hybridization indicates efficient knockdown of hNEAT1 levels after 48 hr treatment with pooled antisense (AS) oligodeoxynucleotides. (B) hNEAT1 RNA correlates with paraspeckle formation. A typical staining pattern of hNEAT1 and p54<sup>nrb</sup> (upper panel) and of hNEAT1 and PSP1 $\alpha$  (lower panel) in hNEAT1 knockdown HeLa cells. Circled nuclei show that fewer paraspeckles are apparent in cells in which hNEAT1 has been knocked down. The scale bars represent 10  $\mu$ m.

(C) IRAlus-containing mRNAs escape nuclear retention in hNEAT1 knockdown HeLa cells. Nuclear and cytoplasmic RNAs were isolated from untreated and hNEAT1 AS-treated HeLa cells and analyzed by RT-PCR. PCR primers were located downstream of IRAlus in the 3'UTRs to detect only full-length mRNAs (Figure S3; [E], DP). In untreated HeLa cells, the majority of full-length *Nup43*, *Paics*, and *Pccb* mRNAs are retained in the nucleus (left panel), whereas more is exported to the cytoplasm in hNEAT1 knockdown cells (right panel). Bands were quantitated using ImageJ software. In each case, omission of RT led to no signal (data not shown). T, total RNA; C, cytoplasmic RNA; N, nuclear RNA.

(D) Northern blotting. Nuclear and cytoplasmic RNAs were isolated from untreated and hNEAT1-AS-treated HeLa cells and resolved on a denaturing agarose gel. In untreated HeLa cells, the majority of *Paics* mRNA is retained in the nucleus (left panel), whereas more *Paics* is exported to the cytoplasm in the hNEAT1 knockdown cells (right panel). The membrane was stripped and reprobed for *actin* mRNA, which shows no change in its cellular distribution after AS treatment.

(E) IRAlus-containing mRNAs escape nuclear retention in HeLa cells after anti-hNEAT1 siRNA treatment. The upper panel shows two isoforms of *Nup43*, *Paics*, and *Pccb*: the long isoform has one pair of IRAlus in its 3' UTR, whereas the short one lacks IRAlus. The red line indicates the location of the upstream probe (UP) which recognizes both isoforms, and the blue line indicates the location of the downstream probe (DP) which only recognizes the long IRAlus-containing isoform.

(F) Controls. The same mRNA samples from (E) were used. *Smc1a*, *flnb*, *hyou1*, and *son* are mRNAs that do not contain IRAlus or alternative poly(A) sites in their 3'UTRs but are normally enriched in the nucleus. These mRNAs show no change in their N/C distribution after hNEAT1 knockdown.

leads to the re-establishment of the nuclear retention system. However, to address the functional connection between paraspeckles and retention, we used several independent approaches to knock down hNEAT1 expression in HeLa cells.

Following treatment of cells with phosphorothioate-modified hNEAT1 antisense oligodeoxynucleotides, hNEAT1 RNA was typically reduced 60%–70% in HeLa cells after 48 hr treatment as shown by in situ hybridization (Figures 5A and S10) and

RT-PCR (Figure 5C). After treatment with antisense oligos, the nuclear hNEAT1 foci were largely reduced (Figures 5A and S10), and the appearance of PSP1 $\alpha$  and p54<sup>nrb</sup> became more uniform, showing fewer and smaller paraspeckles per nucleus. Figure 5B shows typical staining patterns of hNEAT1 and p54<sup>nrb</sup>, and of hNEAT1 and PSP1 $\alpha$ , after hNEAT1 knockdown in HeLa cells. These experiments again confirm that paraspeckle-specific accumulations of p54<sup>nrb</sup> and PSP1 $\alpha$  require the presence of hNEAT1. In other experiments, we used an siRNA directed against hNEAT1 RNA and based on one shown to be effective by Clemson et al. (2009). Figure 5F shows that siRNA is also effective at reducing hNEAT1 levels in cells.

Finally, if hNEAT1 RNA were essential for paraspeckle-associated nuclear retention, we would expect to observe that mRNAs with *IRA/Us* would have a different fate in HeLa cells after hNEAT1 knockdown. We chose three mRNAs that contain *IRA/Us* in their 3'UTRs and which are expressed well in HeLa cells (*nup43*, *paics*, and *pccb*; see Figure S3). For each of these, hNEAT1 knockdown resulted in less efficient retention in the nucleus (Figures 5C–5E), whereas the nuclear/cytoplasmic distribution of actin mRNA remained unchanged (Figures 5D and 5F). For the experiments shown in Figure 5E, we used two sets of RT-PCR primers, one specific for mRNAs with extended 3'UTRs that contain *IRA/Us* (“downstream probe”), and another set that detects both short and long isoforms (“upstream probe”). Note that the longer isoform of each of the *IRA/Us*-containing mRNA examined changes its N/C ratio after knockdown of hNEAT1 RNA, regardless of whether the knockdown was achieved using antisense DNA oligonucleotides (Figures 5C and 5D) or siRNA (Figure 5E). As the short isoforms are more efficiently exported to the cytoplasm (see Figure 1C; Prasanth et al., 2005; Chen et al., 2008), and because the upstream primers detect both long (nuclear retained) and short (more efficiently exported) mRNA isoforms, the results obtained using them show more modest effects of hNEAT1 knockdown (Figure 5E). We conclude from these experiments that noncoding hNEAT1 RNA not only orchestrates paraspeckle assembly, but also influences the nuclear retention of structured or edited mRNAs. Last, we have identified a number of mRNAs that are inefficiently exported to the cytoplasm but which do not show evidence of editing and which do not contain *IRA/Us* in their 3'UTRs. Several of these are shown in Figure 5F. For these mRNAs, hNEAT1 knockdown does not at all alter their N/C distribution, consistent again with a connection between editing-associated retention and paraspeckles.

## DISCUSSION

### Nuclear Retention of Structured or Edited mRNAs Occurs in Paraspeckles and Requires hNEAT1 RNA

There is growing evidence that gene expression can be regulated by the retention of mature mRNAs in the nucleus. Retention correlates with RNA duplex formation and with A-to-I editing. We reported earlier that nuclear dsRNAs that are promiscuously edited by ADAR can be bound by a complex containing the nuclear protein p54<sup>nrb</sup>, which displays a strong binding affinity for RNAs with inosines in them (Zhang and Carmichael, 2001). More recently, we showed that many mRNAs with inverted *Alu* repeats

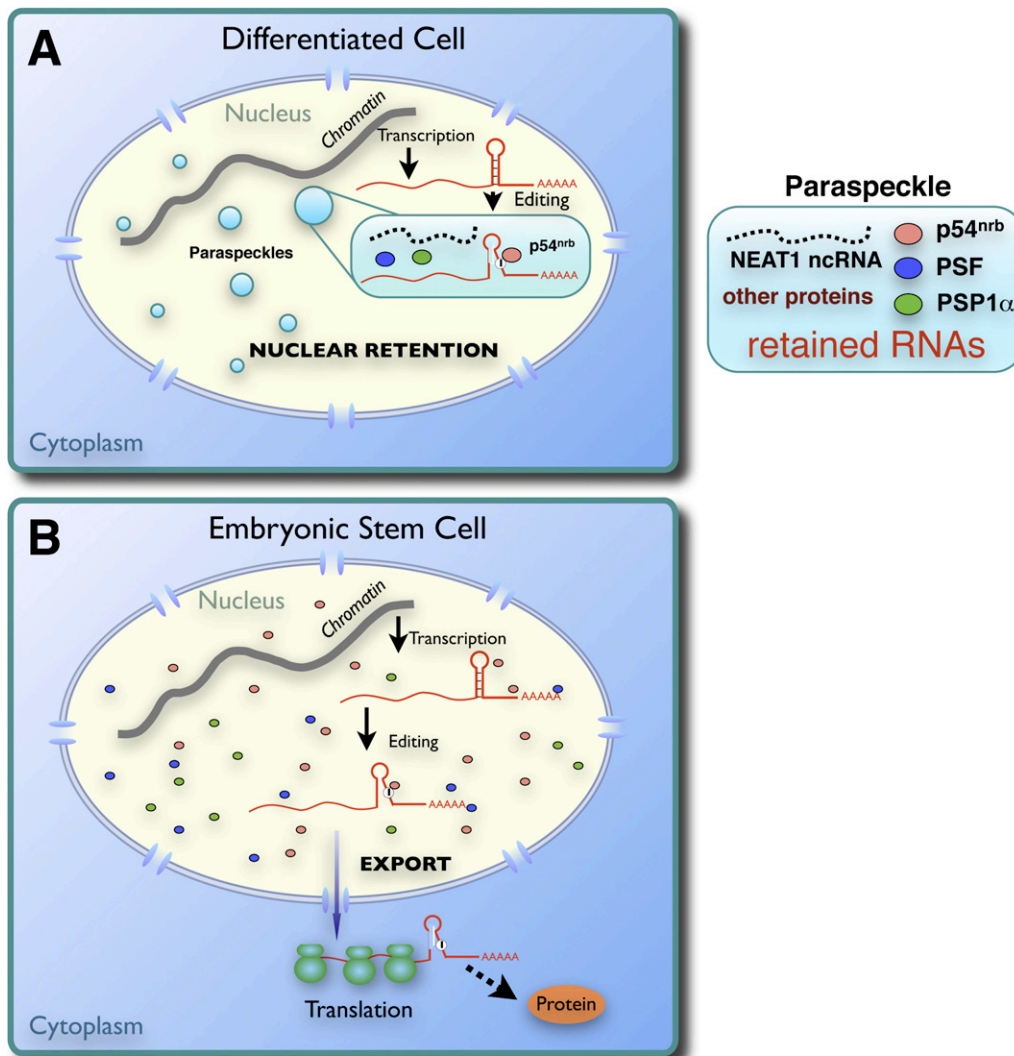
in their 3'UTRs may be inefficiently exported to the cytoplasm owing to nuclear sequestration in prominent nuclear p54<sup>nrb</sup>-containing complexes (Chen et al., 2008), which have now been shown to be paraspeckles. Such retention has been confirmed by another group, which further demonstrated that this phenomenon can provide the cell an important reservoir of mRNAs that can be mobilized for rapid export to the cytoplasm following cellular stress (Prasanth et al., 2005). However, nuclear retention is not the only fate of mRNAs with *IRA/Us*. It was recently reported that some mRNAs with *IRA/Us* could be detected in the cytoplasm, where they were associated with translating polyosomes (Hundley et al., 2008). Indeed, we have also found this to be the case for a subset of cells expressing transcripts containing *IRA/Us* (Chen et al., 2008; and data not shown). Within the same culture, there is strong nuclear retention in some cells, but only partial or even poor retention in a smaller fraction (Chen et al., 2008). Although we do not yet understand the underlying basis for this alternative fate for mRNAs with *IRA/Us*, it is possible that retention can be modulated or regulated in response to as yet obscure factors (Chen and Carmichael, 2008). Also, this variable phenomenon leads to the types of results we have shown in Figures 1C and 5C–5E, where retention is not complete.

Here we have examined in greater detail the mechanistic connection between dsRNA formation, ADAR editing, and nuclear retention. Interestingly, human embryonic stem cells express ADAR1 and the paraspeckle-associated proteins p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$ . However, paraspeckles are not formed and mRNAs with *IRA/Us* in their 3'UTRs are efficiently edited and bind to p54<sup>nrb</sup>, but are exported to the cytoplasm. These results demonstrate that retention requires more than editing and p54<sup>nrb</sup> binding. It may require paraspeckles, which are dependent on hNEAT1 for their assembly. These results suggest an important biological function for an abundant nuclear-retained noncoding RNA. Figure 6 depicts our current working model for the nuclear retention machinery in differentiated and undifferentiated hESCs.

### Paraspeckle-Associated Proteins Are Multifunctional

A role in nuclear retention most likely represents only one of a multitude of functions of the p54<sup>nrb</sup> protein. Whereas p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$  accumulate in paraspeckles, these proteins are also found elsewhere throughout the nucleoplasm and thus may have functions apart from retention. These proteins can heterodimerize with each other and, because each contains two tandem RNA recognition motifs of unknown binding specificity, it is quite possible that there are numerous important RNA targets in addition to edited RNAs. In addition, both p54<sup>nrb</sup> and PSF are also known to be DNA-binding proteins, and there have been reports of important roles for these factors in the regulation of both pre-mRNA splicing and transcription (Basu et al., 1997; Emili et al., 2002; Gozani et al., 1994; Ishitani et al., 2003; Kameoka et al., 2004; Kaneko et al., 2007; Lindsey et al., 1995; Mathur et al., 2001; Patton et al., 1993; Straub et al., 1998; Urban et al., 2000; Yang et al., 1993, 1997; Zhang et al., 1993; Zhang and Carmichael, 2001). Thus, whereas paraspeckle-associated proteins are multifunctional, NEAT1 may have only one function. Paraspeckles may represent subnuclear compartments that utilize a noncoding RNA to recruit and organize a group of





**Figure 6. A Model for Paraspeckle-Mediated Nuclear Retention in Differentiated and Undifferentiated Cells**

(A) Promiscuously edited RNAs are preferentially retained in the nuclei of differentiated cells. Noncoding hNEAT1 RNA plays a critical role in paraspeckle formation and function.

(B) Altered gene regulation in human ES cells. In these cells, edited or structured mRNAs are exported to the cytoplasm because of a defect in paraspeckle formation and function.

multifunctional nuclear proteins for a specialized function, mRNA nuclear retention. We still do not know how hNEAT1 association with these factors leads to formation of paraspeckles and results in the nuclear retention of a subset of mRNAs and edited transcripts. Because p54<sup>nrb</sup>, PSF, and PSP1 $\alpha$  form heterodimers, it is possible that if one polypeptide were to contact hNEAT1 RNA while the other were to contact an mRNA target, then retention might result from tethering to hNEAT1 RNA, which might itself be anchored in the nucleus via interactions that have not yet been elucidated. In addition, it is possible that paraspeckles may be devoid of export factors. If this were the case, RNAs directed to paraspeckles would be unable to access the export machinery. Cleavage of retained RNAs to remove the anchoring sequence(s), as described in

the work of Prasanth et al. (2005), would release the mRNAs from paraspeckles, making them available for export.

#### Human Embryonic Stem Cells Have Altered dsRNA Response Pathways

Until now, nothing has been reported regarding dsRNA response pathways in hESCs. hESCs are the only cells we have found that lack hNEAT1 RNA and efficiently export mRNAs with *IRA/lus* from the nucleus to the cytoplasm. Some of these exported mRNAs are edited, whereas others may not be. Although further studies will be required to determine the nature and abundance of cytoplasmic mRNAs with hairpin structures in hESCs, these cells are clearly unusual in that they tolerate cytoplasmic dsRNA owing to the lack of an efficient IFN/PKR response system (data not

shown). This allows the translation of mRNAs that are more restricted to the nucleus in other cells. Significantly, one such mRNA encodes LIN28. LIN28 is known to be a regulator of developmental timing in *Caenorhabditis elegans* (Ambros and Horvitz, 1984; Horvitz et al., 1983) and colocalizes with mRNP complexes, P bodies, and stress granules in pluripotent mouse P19 cells (Balzer and Moss, 2007). This protein also is one of four that together can reprogram somatic cells to pluripotent stem cells (Yu et al., 2007). In recent independent studies, this protein has been shown to affect microRNA processing in hESCs (Newman et al., 2008; Piskounova et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008) and to enhance the translation of the mRNAs for specific cell-cycle regulatory factors in mouse ES cells (Xu et al., 2009). We speculate that, like *Lin28*, there may be additional mRNAs important for hESC growth or function, whose expression may be enabled by the lack of the nuclear retention system described here. Further, because differentiation would demand that such genes be downregulated, this would be consistent with the induction of hNEAT1 expression and the formation of paraspeckles that we have observed as hESCs are induced to develop into trophoblasts.

Of potential relevance to the results described here is the fact that the chromatin of pluripotent stem cells is unusually active in transcription, including substantial sense-antisense transcription (Efroni et al., 2008). This may result at least partly from an altered state of chromatin organization, with many chromosomal domains that are condensed in differentiated cells not being silenced in ES cells (Bernstein et al., 2006; Meshorer and Misteli, 2006; Meshorer et al., 2006). Thus, hESCs may express an unusually high amount of dsRNA within the nucleus, and some of this RNA may be exported into the cytoplasm.

There may be yet another rationale for a less efficient nuclear retention system in hESCs. We previously noted that many mRNAs with *IRAlus* in their 3'UTRs undergo alternative polyadenylation to produce some mRNAs lacking *IRAlus* but others containing them (Chen et al., 2008). This phenomenon is in fact seen with the genes described in the studies reported here. mRNAs lacking *IRAlus* are efficiently exported to the cytoplasm whereas those containing *IRAlus* are largely confined to the nucleus. But the longer UTRs may contain specific microRNA binding sites that can be used for gene regulation only if these longer mRNAs reach the cytoplasm. This concept is illustrated by the *Lin28* mRNA. In hESCs, the let-7 miRNA binding sites within the *Lin28* 3'UTR are located immediately upstream of the *IRAlus*. If alternative polyadenylation were to remove not only the *IRAlus* but also the microRNA binding sites, then gene regulation in the cytoplasm would be quite different.

Finally, dsRNA editing may serve a critical but still obscure function in hESCs. *ADAR1*<sup>-/-</sup> homozygous mice die by embryonic day 11.5 with defects in erythropoiesis in the liver and with widespread apoptosis (Wang et al., 2000, 2004). Therefore, ADAR1 appears to be important for the viability and the development of nonneuronal tissues in the mouse. Recently, it has been reported that ADAR1 is essential for the maintenance of hematopoiesis and suppression of interferon signaling in the mouse (Hartner et al., 2009). In that system, ADAR1 is essential for maintenance of both fetal and adult hematopoietic stem cells, and loss of ADAR1 in hematopoietic stem cells led to global upregu-

lation of type I and II interferon-inducible transcripts, followed by rapid apoptosis. We do not yet know whether the phenotypes summarized above are related to the regulatory pathway we have described here.

In conclusion, we report here the functional role of a long nuclear noncoding RNA in posttranscriptional regulation of gene expression. The significance of this RNA retention system is further highlighted by the finding that hESCs lack it. These cells express paraspeckle protein components but little or no hNEAT1 RNA. Thus, we speculate that a low level of NEAT1 RNA might serve as a marker of pluripotency. Further studies are needed to identify not only the mRNAs that are affected by this retention system in differentiated cells but also those that are allowed to efficiently enter the cytoplasm in pluripotent stem cells.

## EXPERIMENTAL PROCEDURES

### Cell Culture, hES Cell Culture, and Transfection

HeLa and HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Human ES cells were maintained on plates coated with growth-factor-depleted Matrigel (BD Biosciences) in either serum-free, defined mTeSR medium (StemCell Technologies) or fibroblast-conditioned medium (CM) with mitotically inactivated mouse embryo fibroblasts supplemented with 4 ng/ml human bFGF (Life Technologies) (Xu et al., 2001). Cell cultures were regularly evaluated for Oct3/4 and Sox2 expression every 3–4 weeks and cells were passaged every 6–7 days. Differentiation of hESCs to trophoblasts was as previously described (Xu et al., 2002, 2005).

### RNA Nuclear Retention Analysis

Nuclear and cytoplasmic RNA isolation in HeLa cells was performed as described (Chen et al., 2008), and in hESCs was carried out with modifications detailed in Supplemental Data. In all analyses, cell-equivalent amounts of cytoplasmic and nuclear RNA samples were used.

For northern blotting, Dig-labeled *Lin28*, *Paics*, *Adar1*, and *U6* probes were made by the DIG-High Prime DNA labeling and detection starter kit (Roche); Dig-labeled antisense *tRNA*<sup>lys</sup> was made using T7 RNA polymerase with the DIG Northern Starter Kit (Roche), and the *actin* probe used was provided with the kit. Total, cytoplasmic, and nuclear RNA were isolated from 10<sup>7</sup> cells. Nuclear/cytoplasmic ratios were normalized to *actin* mRNA.

### RNA In Situ Hybridization and Immunofluorescence Microscopy

To detect hNEAT1 RNA, cells were rinsed briefly in PBS and then fixed in 3.6% formaldehyde plus 10% acetic acid in PBS (pH 7.4) for 15 min at room temperature. Cells were permeabilized in PBS containing 0.2%–0.5% Triton X-100 and 5 mM vanadyl ribonucleoside complex (Invitrogen) on ice for 5 min. Cells were washed in PBS 3 × 10 min and rinsed once in 2 × SSC prior to hybridization. Hybridization was carried out using nick-translated cDNA probes (nick-translation kit; VYSIS) in a moist chamber at 37°C for 12–16 hr as described (Spector et al., 1998). A partial hNEAT1 clone was a gift from Dr. K. Prasanth (U. Illinois). For colocalization studies, after RNA-FISH, cells were again fixed for 5 min in 2% formaldehyde, and immunofluorescence and imaging were performed as described (Chen et al., 2008). Antibodies used in immunofluorescence are listed in Supplemental Data. Images were taken with a Zeiss LSM 510 microscope.

### Immunoprecipitation and RNA-Protein Complex Immunoprecipitation

HeLa cells and H9 cells on 10 cm<sup>2</sup> dishes were rinsed twice with ice-cold PBS before harvesting in 10 ml ice-cold PBS by scraping. Cell pellets were resuspended in 1 ml IP buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.05% Igepal, 1 mM PMSF, proteinase inhibitor cocktail), and subjected to two rounds of gentle sonication and centrifuged to obtain cell extracts. For RNase treatment, 200 μg/ml RNaseA was added to cell extracts and incubated at 4°C for 2 hr. For IP, the RNaseA-treated and nontreated cell extracts were incubated with

40  $\mu$ l Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) and 2.5  $\mu$ g mouse anti-p54<sup>nrb</sup> antibody or anti-PSF antibody at 4°C for 2 hr. The beads were washed five times with IP buffer, resuspended in 2 $\times$  SDS sample buffer, and boiled for 5 min before loading on SDS-PAGE. RNA-protein complex IPs were carried out in HeLa and H9 cells as previously described (Chen et al., 2008).

#### Antisense Oligonucleotide and dsRNA Treatment

To knock down the expression of hNEAT1 RNA, phosphorothioate-modified oligodeoxynucleotides (Seq 1: 5'-GGTTCGCGAAAAGTGGTGA-3' and Seq 2: 5'-GTAACAGAATTAGTCTTACCA-3') were synthesized at University Core DNA Services, University of Calgary. In addition, hNEAT1 siRNA sense: 5'-5Phos/rGrUrGrArGrA rArGrU rUrGrC rUrUrA rGrArA rArCrU rUrUC C-3' and antisense: 5'-rGrGrA rArArG rUrUrU rCrUrA rArGrC rArArC rUrUrC rUrCrArCrUrU-3' (Clemson et al., 2009) were synthesized at Integrated DNA Technologies. Pooled DNA oligonucleotides or annealed dsRNAs were introduced to HeLa cells using Lipofectamine RNAiMax (Invitrogen). Optimal Lipofectamine RNAiMax/oligonucleotide ratios were empirically determined.

#### RT-PCR

After treatment of RNA samples with DNaseI (DNA-free kit; Ambion), cDNA was transcribed with SuperScript II (Invitrogen) using oligo (dT) or random hexamers. Primers are listed in Supplemental Data.

#### RNA Editing Analysis

Total RNAs were isolated from H9 cells and treated with DNaseI (DNA-free kit; Ambion). The *AluSc* on *Lin28* mRNA was reverse transcribed with ThermoScript (Invitrogen) with a gene-specific primer located downstream of the *IRA/Alu*. The resultant cDNA was amplified by PCR subcloned using the TOPO-TA cloning kit (Invitrogen). The editing frequency was estimated by sequencing 20 individual clones containing the appropriately sized inserts (Agencourt Bioscience).

#### SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and ten figures and can be found with this article online at [http://www.cell.com/molecular-cell/supplemental/S1097-2765\(09\)00465-1](http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00465-1).

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