A long nuclear-retained non-coding RNA regulates synaptogenesis by modulating gene expression

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A growing number of long nuclear-retained non-coding RNAs (ncRNAs) have recently been described. However, few functions have been elucidated for these ncRNAs. Here, we have characterized the function of one such ncRNA, identified as metastasis-associated lung adenocarcinoma transcript 1 (Malat1). Malat1 RNA is expressed in numerous tissues and is highly abundant in neurons. Its expression is active in embryonic cells, whereas its over-expression results in a cell-autonomous increase in synaptic density. Our results reveal that Malat1 modulates the recruitment of SR family pre-mRNA-splicing factors to the transcription site of a transgene array. DNA microarray analysis in Malat1-depleted neuroblastoma cells indicates that Malat1 controls the expression of genes involved not only in nuclear processes, but also in synapse function. In cultured hippocampal neurons, knock-down of Malat1 decreases synaptic density, whereas its over-expression results in a cell-autonomous increase in synaptic density. Our results suggest that Malat1 regulates synapse formation by modulating the expression of genes involved in synapse formation and/or maintenance.

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Introduction

A large portion of the eukaryotic genome is transcribed as non-coding RNAs (ncRNAs) of various sizes ranging from ~20 nucleotides to ~100 kb (reviewed in Mercer et al., 2009; Wilusz et al., 2009). Despite the increasing number of long ncRNAs (incRNAs), very few have thus far been assigned a specific function (for review, see Mercer et al., 2009). Whether some of these ncRNAs represent transcriptional noise or are involved in important cellular functions remains a matter of debate (Mattick and Makunin, 2006). However, several observations support the assertion that IncRNAs are likely to have functions in cells (Chamberlain and Brannan, 2001; Willingham et al., 2005; Feng et al., 2006; Mancini-Dinardo et al., 2006; Martinez et al., 2007; Rinn et al., 2007; Yang and Kuroda, 2007; Hirota et al., 2008; Mariner et al., 2008; Nagano et al., 2008; Wang et al., 2008; Yu et al., 2008; Chen and Carmichael, 2009; Clemson et al., 2009; Khalil et al., 2009; Mallik and Lakhotia, 2009; Sasaki et al., 2009; Sunwoo et al., 2009). For instance, large-scale studies have shown that many IncRNAs are conserved, dynamically regulated during differentiation and exhibit tissue-specific expression patterns (Ravasi et al., 2006; Dinger et al., 2008; Mercer et al., 2008; Guttman et al., 2009). In addition, several IncRNAs have been shown to be misregulated in various diseases including cancer and neurological disorders (for reviews, see Costa, 2005; Prasanth and Spector, 2007; Tait et al., 2009; Gupta et al., 2010). In many cases, the subcellular localization of ncRNAs has been determined which has provided significant insight into their functions. For example, Xist/XIST RNA, 15–17 kb in mouse and 19 kb in human, coats the inactive X chromosome from which it is transcribed. This represents part of the mechanism by which transcriptional silencing is achieved (for reviews, see Plath et al., 2002; Heard andDISTE, 2006; Payer and Lee, 2008). Recently, MEN epsilon/beta nuclear-retained ncRNAs (also known as nuclear-enriched autosomal transcript1—NEAT1) were shown to be enriched in nuclear paraspeckles, a novel subnuclear domain that preferentially localized on the periphery of nuclear speckles or interchromatin granule clusters (IGCs). These ncRNAs have a critical function in the establishment and maintenance of paraspeckles (Chen and Carmichael, 2009; Clemson et al., 2009; Sasaki et al., 2009; Sunwoo et al., 2009).

Metastasis-associated lung adenocarcinoma transcript 1 (Malat1) ncRNA was initially characterized as a long polyadenylated ncRNA that is over-expressed in various cancers (Ji et al., 2003; Lin et al., 2006). Recent analysis has shown that the primary transcription products of the Malat1 locus include a 6.7 kb nuclear-retained Malat1 ncRNA, and through processing by the tRNA biogenesis machinery, a cytoplasmic 61 nt tRNA-like ncRNA referred to as mascRNA (Wilusz et al., 2008). Phylogenetic analysis indicates that Malat1 is highly conserved among mammals, up to 90% identity between human and mouse in the last 5 kb of the RNA (data not shown). Such conservation among mammals is indicative of important, yet unknown function(s) of
Malat1 ncRNA. The long Malat1 transcript has been localized to nuclear speckles in several cell lines (Hutchinson et al., 2007; Clemson et al., 2009). Nuclear speckles contain a large number of nuclear proteins that are involved in several aspects of mRNP processing, including pre-mRNA splicing and RNA transport (reviewed in Lamond and Spector, 2003). Nuclear speckles are not sites of transcription or pre-mRNA splicing, but represent storage/modification and/or assembly sites of various splicing factors from where pre-mRNA processing factors are recruited to active sites of transcription (reviewed in Lamond and Spector, 2003). A population of poly(A)⁺ RNA was previously reported to localize to nuclear speckles (Carter et al., 1991; Visa et al., 1993; Huang et al., 1994). Malat1 is the first example of an lncRNA that is specifically enriched in these nuclear domains.

The interesting sub-localization of the abundant Malat1 transcript, as well as its restricted evolutionary conservation among mammals, prompted us to investigate the potential function of Malat1 ncRNA in the mammalian cell nucleus. Here, we provide evidence that the nuclear speckle-enriched Malat1 ncRNA modulates synapse formation in neurons by regulating the expression of genes involved in synaptogenesis.

**Results**

**Malat1 localizes to nuclear speckles in a transcription-dependent manner**

We characterized the expression of the long Malat1 ncRNA in mouse tissues by northern analysis. The ~6.7 kb Malat1 transcript was detected in all tissue samples examined. We detected the highest levels of Malat1 ncRNA in heart, kidney and brain and a minimum level in spleen and skeletal muscle (Figure 1A; Supplementary Figure 1). As Malat1 shows elevated levels of expression in the brain, we further characterized its expression pattern by RNA fluorescence in situ hybridization (RNA-FISH) to adult mouse brain sections. We used a probe that specifically recognized the nuclear-retained 6.7 kb Malat1 RNA. We found elevated levels of Malat1 transcripts in pyramidal neurons of the hippocampus, Purkinje cells of the cerebellum, neurons of the substantia nigra and motoneurons (Figure 1B; Supplementary Figure 2). Non-neuronal cells in brain sections showed extremely low levels of Malat1 ncRNA (Figure 1C–E; arrow and open arrowheads). Interestingly, in all tissues examined, Malat1 ncRNA RNA-FISH signal displayed a punctate nuclear distribution (Figure 1C), suggesting that Malat1 ncRNA is enriched in a nuclear sub-compartment(s) that appears similar to nuclear speckles (Hutchinson et al., 2007; Clemson et al., 2009). In wild-type mouse embryonic fibroblasts (wt-MEFs) and in mouse cultured hippocampal neurons, Malat1 ncRNA signal (F, H) co-localizes with SF2/ASF (G) or CC3 immunoreactivity (I), respectively. (J) DAPI staining. Scale bar, 10 μm.

Figure 1 Malat1 is a neuron-enriched nuclear-retained ncRNA that is localized to nuclear speckles. (A) Northern blot analysis of various mouse tissues indicating that Malat1 is detected as a single ~6.7 kb band with elevated levels in heart, kidney and brain. (B) Malat1 expression is restricted to neurons in the adult mouse hippocampus. RNA-FISH shows the strongest expression in pyramidal neurons of CA1 and CA3 and in granular neurons of the dentate gyrus (dg). Weaker expression can be detected in neurons of the cortex (cx) and in hippocampal interneurons (arrows). Insets show FISH signal with a sense probe (lower) and DAPI staining (upper) in CA3 region. Scale bar, 100 μm. (C–E) Triple labelling of CA3 region showing Malat1 ncRNA by in situ hybridization (C), neuron-specific NeuN immunoreactivity (D) and DAPI labelling of cell nuclei (E). Closed arrowheads indicate cells double positive for Malat1 and NeuN staining. Open arrowheads indicate non-neuronal cells. In a few cases (arrow), weak Malat1 ncRNA signal could be detected in NeuN negative cells. Scale bar, 25 μm. (F–J) Malat1 ncRNA is enriched in nuclear speckles. In wild-type mouse embryonic fibroblasts (wt-MEFs) and in mouse cultured hippocampal neurons, Malat1 ncRNA signal (F, H) co-localizes with SF2/ASF (G) or CC3 immunoreactivity (I), respectively. (J) DAPI staining. Scale bar, 10 μm.

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The localization of Malat1 ncRNA to nuclear speckles suggests that it may execute its function in relation to gene expression (reviewed in Lamond and Spector, 2003). Next, we examined the behavior of Malat1 ncRNA upon inhibition of RNA polymerase II transcription using α-amanitin (Figure 2B and C) or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Figure 2H–K). Both drugs efficiently inhibit RNA pol II-mediated transcription. However, α-amanitin-mediated transcription inhibition is irreversible, whereas transcription in the DRB-treated cells can be reactivated upon removal of the drug from the medium. Both treatments resulted in the re-distribution of Malat1 ncRNA from nuclear speckles to a homogenous nuclear localization. However, real-time RT–PCR showed little to no turnover of Malat1 ncRNA.

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ncRNA even after prolonged treatment of cells with z-amaminit (Figure 2C) or DRB (data not shown). As previously reported (Bubulya et al., 2004), SF2/ASF localized around the nucleoli in RNA pol II transcription-inhibited cells and relocalized back to the nuclear speckles upon transcription reactivation (Supplementary Figure 3). DRB recovery kinetics revealed that within 15 min of drug removal from the culture medium, SF2/ASF could be detected within nuclear speckles, whereas Malat1 ncRNA continued to show a diffuse nuclear distribution (Figure 2L–O). However, within 30 min of recovery, Malat1 ncRNA was once again enriched in nuclear speckles in which it co-localized with SF2/ASF (Figure 2P–S). Together, these data show that Malat1 ncRNA is a very stable nuclear transcript that associates with nuclear speckles in a transcription-dependent manner.

**Malat1 modulates the recruitment of SR proteins to a transcriptionally active locus**

As Malat1 ncRNA is enriched in nuclear speckles, we were interested in addressing whether it has a function in regulating the recruitment of pre-mRNA-splicing factors to transcription sites. To assess such a function to a specific transcription site, we used a previously characterized U2OS cell line in which the transcription site of an inducible transgene array, as well as its mRNA and protein products, can be visualized and quantified (Janicki et al., 2004). As expected from previous experiments using this U2OS cell line (Janicki et al., 2004), the transgene locus, visualized by LacI-mCherry fluorescence, was actively decondensed upon transcriptional induction with doxycycline (compare Figure 3C and G). Upon transcriptional induction of the reporter locus in control cells that were transfected with scrambled oligonucleotides, SF2/ASF was recruited to the actively transcribing gene locus as shown by the increased number of cells displaying SF2/ASF co-localization at the transcription site (Figure 3A–H and Q). Interestingly, only ~40–45% of the Malat1-depleted cells continued to show a speckled distribution of SF2/ASF. In the rest of the cells, SF2/ASF displayed increased homogenous nuclear distribution (data not shown). Strikingly, in a significant population of Malat1-depleted cells that continue to show speckle localization of SF2/ASF, SF2/ASF recruitment to the induced transcription site was severely compromised (Figure 3I–Q). Importantly, this was not due to a general disruption of the recruitment of the gene expression machinery as the recruitment to the activated transcription site of the rTa transcriptional activator, CDK9, a component of the pTEF-B kinase complex that phosphorylates the C-terminal domain of RNA pol II, and RNA pol II large subunit was unaffected by knock-down of Malat1 ncRNA (Supplementary Figure 4). To determine whether Malat1 was involved in the recruitment of other splicing factors to the induced transgene, we analysed the recruitment of SC35, another member of the SR family of splicing factors. Similar to that observed for SF2/ASF, the recruitment of SC35 was also affected by the knock-down of Malat1 (Supplementary Figure 5).

Furthermore, Malat1 knock-down did not alter the transcriptional activity of the locus as observed by RT–PCR using primers against the reporter RNA (Supplementary Figure 6). These results show that Malat1 ncRNA modulates the recruitment of SR-type pre-mRNA-splicing factors to/at active transcription sites. Importantly, knock-down of the 6.7 kb Malat1 transcript does not affect the level of mascRNA (Wilusz et al., 2008), indicating that the effect we observed is specifically due to the depletion of the long nuclear-retained Malat1 ncRNA.

**Malat1 ncRNA controls the mRNA levels of genes involved in the induction/function of synapses**

As Malat1 ncRNA is highly expressed in the brain, we investigated a possible function of Malat1 in neuronal function. We first characterized the cellular processes and components that were most impacted by Malat1 deficiency using DNA microarrays. Malat1 ASO or scrambled oligodeoxynucleotides were transfected into Neuro2A neuroblastoma cells and mRNAs were subsequently hybridized to 44K agilent DNA arrays (G_4122F). We then identified the Gene Ontology (GO) groups that were significantly enriched in the population of genes whose expression were impacted upon Malat1 depletion (Tables I and II; Supplementary Tables I–III). The GO groups related to the organization and the function of the nucleus were the most significantly over-represented in Malat1-depleted cells. Interestingly, we also found that the GO groups related to synapse (cellular component; GO:0045202; 1.9-fold enrichment; \(P = 1.40 \times 10^{-7}\)) and dendrite development (biological process; GO:0016358; 4.3-fold; \(P = 6 \times 10^{-8}\); see Table I) were also affected upon Malat1 depletion. No other neuron-specific GO group was significantly enriched (\(P < 0.05\)). Our DNA microarray analysis shows that in neuronal cells, Malat1 preferentially regulates a subset of genes with a significant involvement in dendritic and synapse development.

**Malat1 RNA levels in cultured neurons influence synapse formation**

On the basis of the DNA microarray analysis, we further investigated whether Malat1 might be involved in the generation of synapses. We first examined the developmental expression of Malat1 ncRNA. In the hippocampus as well as in Purkinje cells (data not shown), Malat1 is first detected between post-natal day 0 (P0) and P7 and its level increases until P28 (Figure 4A–E). In rodent brain, the first post-natal weeks are known to be periods of intense synaptogenesis on dendrites (Figure 4F–J) (Steward and Falk, 1986, 1991; DeFelipe et al., 1997).

We then evaluated the relationship between Malat1 and synapses. Neuro2a cells express genes related to synaptic function, but do not generate synapses per se (McGee, 1980; Spoerri et al., 1980). We, therefore, used well-characterized primary hippocampal neuron cultures for examining the function of Malat1 in synapse formation or development. We examined the effect of Malat1 knock-down or over-expression on synapse density in dendrites of cultured hippocampal neurons (Supplementary Figure 7). We used accumulation of synapsin I immunoreactivity as a marker of synapses because it is expressed from the earliest stages of synapse formation (Ahmari et al., 2000) and, therefore, identifies nascent and mature synapses (Graf et al., 2004). In addition, Malat1 knock-down does not regulate the level of synapsin I mRNA (ratio = −0.026). As shown in Figure 4K, a significant reduction in synaptic density was observed in cultured neurons that were transfected with two independent Malat1 antisense oligonucleotides, as compared with control transfections (\(−33.5 \pm 3.8\%\); \(P < 0.0001\); \(n = 3\)).
Conversely, over-expression of Malat1 resulted in an increased presynaptic bouton density on dendrites (+24.7 ± 5.6% as compared with EGFP vector over-expression; \( P = 0.0008; \ n = 3; \) Figure 4L). Interestingly, in neurons neighbouring Malat1 over-expressing cells, synaptic density was unchanged (93.2 ± 7.2% as compared with EGFP vector over-expression; \( P = 0.49; \ n = 3; \) Figure 4M). These data show that the control of synaptogenesis by Malat1 ncRNA is cell autonomous and post-synaptic.

As Malat1 ncRNA controls synapse density in cultured hippocampal neurons, the effect of its knock-down was further investigated on a subset of genes controlling the formation and/or function of synapses (Figure 4N). Neuriligin1 (NLGN1) and synaptic cell adhesion molecule 1 (SynCAM1) post-synaptic proteins are able to drive the recruitment of the presynaptic release machinery through interactions with presynaptic cell adhesion molecules (Chih \ et al, 2005; Levinson and El-Husseini, 2005; Sara \ et al, 2005). We measured the effect of decreasing Malat1 ncRNA level on Nlgn1 (Chih \ et al, 2005) and SynCAM1 (Biederer \ et al, 2002) mRNAs. Cultured primary mouse hippocampal neurons transfected with two independent Malat1 antisense oligonucleotides
showed a significant decrease in the levels of Nlgn1 (−44.3 ± 1.2% of control; \( P < 0.03; n = 4 \)) and SynCAM1 (−22.7 ± 1.2%; \( P < 0.05; n = 3 \)) transcripts measured by quantitative RT–PCR as compared with cells that were transfected with control oligonucleotides (Figure 4N). In the DNA microarray experiment, a comparable decreased expression of SynCAM1 was observed in the Malat1-depleted cells (−29.8 ± 1.3%). Nlgn1 was not expressed in the Neuro2A cell line. To further evaluate the specificity of Malat1 in cultured hippocampal neurons, we analysed the effect of Malat1 ncRNA knock-down on the mRNA level of the Eph receptor B2 (EphB2) and Neuronal pentraxin-2 (Narp), which control the recruitment of glutamate receptors to synaptic sites (O’Brien et al, 1999; Henderson et al, 2001). We found that knock-down of Malat1 ncRNA did not decrease the transcript levels of EphB2 (88.3 ± 1.4%; \( P = 0.51; n = 3 \)) and Narp mRNA level was either unchanged or increased depending on the Malat1 antisense oligonucleotide that was used (Figure 4N). Similarly, Malat1 knock-down did not alter the level of transcripts unrelated to synapse function such as Hypoxanthine guanine phosphoribosyl transferase (Hprt) (96.5 ± 1.5%; \( P = 0.44; n = 3 \)) or Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (93.2 ± 0.8% \( P = 0.2; n = 4 \)) (Figure 4N).

Our results showed that in primary cultured neurons, Malat1 ncRNA regulates transcript levels of Nlgn1 and to a lesser extend SynCAM1. Together, these results suggest that in neurons, Malat1 ncRNA regulates the expression efficiency of a subset of genes controlling synapse formation, which consequently results in the regulation of synaptogenesis.

### Discussion

In this study, we have shown that Malat1, a long nuclear-retained ncRNA, modulates the recruitment of SR proteins to an active transcription site of a reporter gene locus. We further showed that in neurons, where it is highly expressed, Malat1 controls the expression of a subset of genes significantly involved in nuclear and synapse function and that it regulates synaptogenesis. Malat1 is the first nuclear-retained lncRNA that has been shown to regulate synapse formation by modulating gene expression.

Within the cell nucleus, Malat1 ncRNA co-localizes with SR-splicing factors in nuclear speckles or IGCs, which are important sub-nuclear domains known to be enriched in factors that regulate transcription and pre-mRNA processing.
Table II  Gene Ontology (GO) analysis of genes up-regulated in Malat1 knock-down Neuro2A cells

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The Gene Ontology groups are indicated for the biological processes and the cellular components. Category, number of reference genes in the category; Observed, number of genes in the category that are up-regulated in Neuro2A upon Malat1 knock-down; Expected, expected number in the category; Ratio, ratio of enrichment (R), P-value from hypergeometric test.

We hypothesize that nuclear speckle-enriched Malat1 regulates gene expression by modulating the differential association or activity of SR-splicing factors. SR-splicing factors constitute a family of pre-mRNA-splicing factors that have essential functions in constitutive as well as alternative splicing of pre-mRNAs (reviewed in Long and Caceres, 2009). In general, SR proteins contain one or two RNA-recognition motifs and a serine-arginine dipeptide-rich RS domain (reviewed in Long and Caceres, 2009). In this study, we have shown that Malat1 depletion resulted in decreased recruitment of SR-splicing factors (SF2/ASF, SC35) to a stably integrated transcriptionally active gene locus. We have also recently shown that Malat1-depleted HeLa cells show increased cellular levels of dephosphorylated SF2/ASF (Tripathi and Prasanth, unpublished data). It is known that the phosphorylation of the RS domain influences the recruitment of SR proteins to transcription sites and their binding to pre-mRNA as well as for their function in the recruitment of other splicing factors to form functional spliceosomes (Cao et al., 1997; Misteli et al., 1998; Xiao and Manley, 1998; Stamm, 2008). Our results imply that inefficient recruitment of SR proteins to a transcription site, observed in Malat1-depleted cells, could be due to defects in SR-protein phosphorylation. We have also observed aberrant alternative splicing of a subset of pre-mRNAs including Camk2b kinase, a gene essential for synaptic density and maturation (Shen et al., 1998; Wu and Cline, 1998; Rongo and Kaplan, 1999; Fukunaga and Miyamoto, 2000), in Malat1-depleted HeLa cells (Tripathi, Ellis, Blencowe and Prasanth, unpublished data). These results further confirm the regulatory function of Malat1 in the control of post-transcriptional gene expression. Although Malat1 regulates a basic gene expression event, whole-genome transcription DNA microarray analysis (this study) as well as alternative splicing microarray (Tripathi, Ellis, Blencowe and Prasanth, unpublished data) analyses show that its inhibition affects the level of only a subset of gene transcripts. One hypothesis to explain the selective action of Malat1 is that the association of SR proteins with pre-mRNAs is itself selective. The binding of SR proteins to a particular pre-mRNA is directly dependent on its splice sites and exonic-splicing enhancer sequences (Cartegni et al., 2003). Moreover, different genes can be regulated by different SR proteins (reviewed in Moroy and Heyd, 2007). Therefore, in the absence of Malat1 the binding kinetics of SR proteins would be different in regard to different pre-mRNAs in a tissue-specific manner, thus affecting some transcripts such as Nlgn1 and SynCAM1 in neurons, but not all transcripts. Future analysis will determine which steps of mRNA biogenesis are altered in Malat1-depleted neuronal cells and if the effect on neuronal genes is direct. In addition, as Malat1 ncRNA is also expressed in many other tissues, it is likely to have broader regulatory functions by modulating the expression of specific genes in a tissue-specific manner. Several examples are known of genes that are ubiquitously expressed, but whose altered expression specifically affects neuron-specific functions eventually leading to neurological diseases. The survival of motoneuron protein is expressed in all cells and it is involved in pre-mRNA splicing, but mutations reducing its expression lead to the selective dysfunction of motoneurons and ultimately to...
spinal muscular atrophies (Burghes and Beattie, 2009). The fragile X mental retardation protein (FMRP) is an RNA-binding protein expressed in many tissues and involved in mRNA trafficking. The absence of FMRP leads to synaptic dysfunction and severe cognitive deficiency (Bassell and Warren, 2008). The superoxide dismutase 1 (SOD1) gene is

Figure 4 Malat1 regulates genes involved in synaptogenesis in cultured hippocampal neurons. (A–J) Developmental time course of Malat1 RNA-FISH signal (A–E) and synapsin I immunoreactivity (IR) (F–J) in pyramidal neurons (py) of the mouse hippocampus (CA3) between postnatal day 0 (P0) and P28. First punctate signals of synapsin I-IR and first Malat1-FISH signals were detected in the stratum radiatum (sr) at P7 and increases until P28. Scale bar, 25 μm. (K) Synapsin immunoreactivity in cultured hippocampal neurons transfected with Malat1 antisense oligodeoxynucleotides (AS) or control scrambled oligodeoxynucleotide (Scr). Scale bar, 10 μm. Lower panels display higher magnifications of the outlined regions in the upper panels. Histogram shows the quantification of synaptic linear density in three independent experiments. Mean ± s.e.m.; **P<0.0001, ANOVA. (L) Synapsin labelling (red) in neurons (green) transfected with control vector (Ctl) or with Malat1 over-expressing vector (Malat1). Histogram shows the quantification of synaptic linear density in three independent experiments. Mean ± s.e.m.; *P<0.0001, ANOVA. (M) Synapsin labelling in neurons neighbouring control vector or Malat1 cDNA-transfected neurons. Histogram shows the quantification of synaptic linear density in three independent experiments. Mean ± s.e.m.; P=0.49, ANOVA. (N) Quantification by quantitative RT-PCR of mRNA encoding several genes upon transfection of three independent cultures of neurons with Malat1 antisense oligonucleotides [AS1, grey bars; AS4, black bars] or with control scrambled oligodeoxynucleotide (Scr, white bars). Mean ± s.e.m. relative to Actin mRNA level. **P<0.03; *P<0.05 Mann–Whitney U-test.

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an ubiquitously expressed metalloprotein, but its mutation specifically induces the death of motoneurons and amyotrophic lateral sclerosis (Pasinelli and Brown, 2006). Therefore, understanding how Malat1 controls the expression of neuron-specific genes will give us insight into the more general question of how the alteration of the expression of a ubiquitously expressed ncRNA functions in a cell-type-specific manner.

A great diversity of long IncRNAs is expressed and regulated in the CNS, where they are thought to have fundamental functions (Mercer et al, 2008). We have identified the involvement of one such IncRNA, Malat1, in regulating genes involved in synapse formation. In the CNS, Malat1 RNA is expressed concomitantly with synaptogenesis. It is known that in neuronal culture, synapse assembly can be induced by Neuroli1 and SynCAM1 (Chih et al, 2005). We have now shown that in cultured neurons, Malat1 controls the level of Neuroli1 and SynCAM1 mRNAs and that Malat1 level modulates synapse density in cultured neurons. Our results argue in favour of the growing consensus positioning ncRNAs as essential actors in specific gene regulation during the development and function of the CNS (Mercer et al, 2008).

The number of ncRNAs in eukaryotic genomes has been shown to increase as a function of developmental complexity (Mehler and Mattick, 2006). The biology of the IncRNAs also appears to be far more complex than anticipated (reviewed in Prasanth and Spector, 2007; Willusz et al, 2009). Given the relative low number of protein coding genes in the mouse and human genomes, it has been suggested that ncRNAs may be part of the mechanism to increase the complexity and fine-tuning of gene expression and allow higher-order functions to develop during evolution (reviewed in Mattick, 2001, 2003; Prasanth and Spector, 2007). In neuronal cells in particular, the restricted expression of ncRNAs especially during development has previously been suggested to have important functions including human brain evolution (Mehler and Mattick, 2006; Pollard et al, 2006). In the brain, the expanding complexity of synaptic networks is one of the hallmarks of evolution. Therefore, one may anticipate that many additional ncRNAs regulating synaptogenesis and other regulatory functions in the nervous system will be identified and characterized (Lein et al, 2007; Mercer et al, 2008).

Materials and methods

Cell culture and drug treatments

The wt-MEFs and U2OS cells were grown in DMEM containing high glucose (Invitrogen, Carlsbad, CA), supplemented with penicillin streptomycin and foetal bovine serum. The U2OS 2-6-3 cell line was grown in DMEM supplemented with 10% foetal calf serum (Biowest, Paris). Neurons were isolated from newborn (post-natal day 0) SWISS mouse hippocampi as described (Goslin et al, 1998) and cultured for 8 days in vitro (DIV) in astrocyte-conditioned medium.

Transfection

Antisense knock-down of Malat1 mRNA in human U2OS cells was carried out using phosphorothioate-modified DNA oligonucleotides (ISIS Pharmaceuticals): ATGCAAAACATATTGTTG as described elsewhere (Prasanth et al, 2005). In Neuro2A cells, Malat1 depletion was achieved overnight using Lipofectamine 2000 (Invitrogen, Cergy Pontoise) according to manufacturer’s instructions. Neurons were transfected at 8 DIV using Lipofectamine 2000 according to manufacturer’s instructions. Neurons were incubated in serum-free medium with liposome–DNA complexes for 1 h, rinsed and incubated in astrocyte-conditioned medium for an additional 13 h. For inhibition experiments in neurons and in Neuro2A cells, mixed LNA/DNA oligonucleotides were generated (Froligo, Paris and Eurogentec, Angers) against mouse Malat1 mRNA (5'-ACGTGGCATTGCTCTGGTCA-3') as shown for other nuclear-retained RNAs (Lanz et al, 1999). For DNA microarray experiments, Neuro2A cells were transfected with SCR or AS4. For quantitative real-time RT–PCR analysis, neurons were transfected with SCR, AS1 or AS4. For synapse analyses, neurons were transfected with SCR or with AS1 + AS2. For the over-expression experiments, the full-length mouse Malat1 cDNA (Genbank accession number AY722410) was cloned into the pCAGG vector (Barthelmy et al, 2004) (kind gift from Dr CE Henderson, CNB, Columbia) and expressed in hippocampal neurons for 14 h.

Northern blot, in situ hybridization, immunohistochemistry

Northern blot: 2 µg poly A+ RNA was used in each lane. The blot (Stratagene) was then hybridized with a full-length Malat1 cDNA probe. For histological sections, mice were perfused with 4% formaldehyde in phosphate buffer pH 7.2. Brains were dissected out, fixed and stored in 4% formaldehyde in 20% sucrose in phosphate buffer pH 7.2. A total of 40 µm coronal and sagittal sections were cut on a cryostat. Neurons were fixed in 4% formaldehyde in phosphate buffer pH 7.2. In situ hybridization (ISH) was adapted from Scharen-Wiemers and Gerfin-Moser (1993) and Henrique et al (1995). Sense and antisense digoxigenin-labelled RNA probes (Roche Diagnostics, Meylan) were synthesized from a pGEMT (Promega) vector containing the 720 first nucleotides of Malat1 cDNA. For RNA-FISH, RNA probes were revealed using rhodamine-conjugated anti-digoxigenin antibody (Roche Diagnostics). For RNA-FISH to MEFs, the probes used for ISH were derived from a nick-translated full-length cDNA probe. Immunolocalization studies were performed as described elsewhere (Dansholt et al, 2004). Antibodies were mouse monoclonal anti-NeuN antibody (MAB377, Chemicon), mouse monoclonal CC3 antibody (Kindgift from Dr O Bensaud, ENS, Paris), mouse monoclonal SF2/ASF antibody (mAb103, Invitrogen), rabbit polyclonal SON antibody (Sharma et al, 2010), mouse monoclonal RNA pol II antibody (H14), rabbit CDK9 antibody (Santa Cruz Biotechnology Inc) and mouse monoclonal anti-synapsin I antibody (Synaptic Systems, Goettingen). RNA-FISH and immunolocalization studies in wt-MEFs and U2OS cells were carried out as described elsewhere (Prasanth et al, 2005).

DNA microarrays

Total RNA was extracted from three independent cultures using the RNAqueous-Micro kit (Ambion). Labelling of the RNA (2–5 µg) with Cy3 or Cy5 dye was carried out by incorporation of amino-allyl-dUTP. Hybridization to 44K agilent DNA microarray (G_4122F) was performed according to the protocol developed by the functional genomics platform at the ecole normale superieure.

Data analysis

Microarray slides were analysed using the GenePix software 6.1 (Axon). Spots flagged by the GenePix software and saturating spots, where the median foreground intensity is >60,000 in one of the two channels, were discarded from the result files. The data were


References


