Long non-coding RNA NEAT1_1 ameliorates TDP-43 toxicity in *in vivo* models of TDP-43 proteinopathy

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Abstract

Pathological changes involving TDP-43 protein ("TDP-43 proteinopathy") are typical for several neurodegenerative diseases, including frontotemporal lobar degeneration (FTLD). FTLD-TDP cases are characterized by increased binding of TDP-43 to an abundant lncRNA, NEAT1, in the cortex. However it is unclear whether enhanced TDP-43-NEAT1 interaction represents a protective mechanism. We show that accumulation of human TDP-43 leads to upregulation of the constitutive NEAT1 isoform, NEAT1_1, in cultured cells and in the brains of transgenic mice. Furthermore, overexpression of NEAT1_1 is protective against TDP-43 toxicity in *Drosophila* and yeast models of TDP-43 proteinopathy. Thus NEAT1_1 upregulation may be protective in TDP-43 proteinopathies affecting the brain. Approaches to boost NEAT1_1 expression in the CNS may prove useful in the treatment of these conditions.

Introduction

TDP-43 is an abundant, ubiquitously expressed RNA-binding protein [1] whose dysfunction is tightly linked to and/or causative of neurodegenerative diseases amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and Alzheimer's disease [2, 3]. Over 50 mutations have been described in the TDP-43 encoding gene, *TARDBP,* which are responsible for ~2% of ALS cases [1, 4]. However, in the vast majority of ALS and FTLD patients, non-mutated TDP-43 is mislocalized from its normal nuclear location and is deposited in a form of pathological inclusions in neurons and glial cells in the affected CNS regions – a condition termed "TDP-43 proteinopathy". TDP-43 proteinopathy is typical for ~98% of sporadic and up to 50% of familial ALS cases, including those caused by *TARDBP* and *C9ORF72* mutations; for the majority of FTLD cases with tau-negative, ubiquitin-positive inclusions (FTLD-U, or FTLD-TDP); and for ~45% of Alzheimer's disease cases [2-6]. TDP-43 is often post-translationally modified in the above diseases, with the most common modifications being its ubiquitination, phosphorylation and N-terminal truncation [2-6]. Both loss and gain of TDP-43 function likely underlie TDP-43 proteinopathy however the relative contribution of the two mechanisms is still hotly debated. Studies in transgenic mouse models showed that even moderate overexpression of non-mutated, full-length human TDP-43 in the CNS is highly toxic [7-10].

Many ALS and FTLD subtypes, alongside some other neurodegenerative diseases, are characterized by altered RNA metabolism [11]. Long noncoding RNAs (lncRNAs), a class of non-protein coding transcripts longer than 200 nucleotides, are relatively new players on the neurodegenerative disease stage. Nuclear Paraspeckle Assembly Transcript 1 (NEAT1) is a highly and ubiquitously expressed nuclear-retained lncRNA with a plethora of regulatory roles. NEAT1 was originally discovered as a virus-induced lncRNA and is currently considered as one of the most dysregulated lncRNAs in cancer [12]. More recently, NEAT1 has also been implicated in normal neuronal functions as well as in the pathophysiology of neurological conditions [13-15]. In particular, altered NEAT1 levels have been reported in the CNS of patients with ALS, FTLD, Huntington's, Alzheimer's and Parkinson's diseases [reviewed in 13].

Two NEAT1 isoforms sharing their 5' end have been described, the constitutive short isoform (NEAT1_1) and the stress-inducible long isoform (NEAT1_2) [16]. NEAT1 is one of the strongest TDP-43 interactors; TDP-43 protein binds along the entire length of NEAT1 transcripts [17-19]. Furthermore, NEAT1 isoforms are structural elements of nuclear RNP granules paraspeckles, and TDP-43 was identified as a paraspeckle component [20, 21]. Crosslinking and immunoprecipitation (CLIP) studies showed that NEAT1 is the RNA with the most significant increase in TDP-43 binding in the brain of FTLD-TDP patients, as compared to control individuals [19]. Recently, we and others showed that TDP-43 regulates the NEAT1 isoform ratio, where its loss of function leads to NEAT1_2 upregulation [18, 22]. Loss of TDP-43 function is likely responsible for NEAT1_2 accumulation in spinal motor neurons of ALS patients [22, 23]. However, we failed to detect NEAT1_2 transcript in the cortex of FTLD patients using RNAScope® ISH (Figure S1), which might be due to differences in transcript regulation in spinal and brain neurons. The constitutive NEAT1 isoform, NEAT1_1, may therefore play a more prominent role in TDP-43 proteinopathies affecting the brain such as FTLD and Alzheimer's disease.

In the current study, we examined the interplay between TDP-43 and NEAT1_1 in cultured cells and in transgenic *in vivo* models.

Materials and methods

SH-SY5Y cells, plasmids, transfection and immunofluorescence

SH-SY5Y cells were maintained in a 1:1 mixture of DMEM and F12 medium supplemented with 10% foetal bovine serum (FBS), penicillin-streptomycin and glutamine (all Life

107 Technologies). Cells were transfected with plasmid DNA (200 ng/well), using
108 Lipofectamine2000 (Life Technologies) in 24-well plates. Cloning of TDP-43 WT and TDP-43
109 CT (aa. 192-414) in pEGFP-C1 vector (Clontech) was described previously [24]. Plasmid for
110 expression of TDP-43 WT Flag (in pFLAG-CMV-4 vector) was a gift of Francisco Baralle and
111 Emanuele Buratti (International Centre for Genetic Engineering and Biotechnology). Cell
112 nuclei were stained with DAPI (Sigma). Fluorescent images were taken on a BX57
113 fluorescent microscope equipped with a DP73 camera and cellSens software (Olympus).

Mouse tissue analysis

115 Hemizygous TDP-43$_{PrP}$ mice [7] were purchased from the Jacksons Laboratory (strain
116 C57BL/6-Tg(Prnp-TARDBP)3cPtrc/J) and littermate wild-type and homozygous TDP-43$_{PrP}$
117 animals were obtained by intercrossing. The following primers were used for genotyping: 5'-
118 CGGGGATGTGATGGATG-3' and 5'-CGCAATCTGATCATCTGCAA-3' (by PCR); and 5'-
119 TCAGGGCCTTTGCCTTTGTT-3' and 5'-TGCTTAGGTTCGGCATTGGAT-3' (by qRT-PCR).
120 Animals were housed using a 12 h light/12 h dark cycle, with free access to food and water.
121 All work on mice was carried out in accordance with the United Kingdom Animals (Scientific
122 Procedures) Act (1986). Mouse brains and spinal cords were dissected from 4-week old
123 mice and either fixed in 4% paraformaldehyde overnight or snap-frozen. Fixed tissue was
124 embedded in paraffin wax, cut into 8 µm thick sections and mounted on poly-L-lysine coated
125 slides (Thermo Scientific). Immunostaining was performed using anti-TDP-43 mouse
126 monoclonal antibody (R&D Systems, MAB7778) and secondary Alexa Fluor conjugated
127 antibody (1:1000, Molecular Probes, Invitrogen); nuclei were stained with DAPI (Sigma).
128 Fluorescent images were taken on a BX57 fluorescent microscope equipped with a DP73
129 camera and cellSens software (Olympus). For western blots, frozen cortex and spinal cord
130 samples were homogenized directly in 2xLaemmli buffer and processed as described below.
131 For RNA expression analysis, frozen samples were homogenized in the lysis buffer from
132 PureLink total RNA extraction kit (Life Technologies) and processed as described below.

RNA immunoprecipitation (RIP) and PCR analysis

134 SH-SY5Y cells were transfected with equal amounts of plasmids to express GFP (empty
135 pEGFP-C1 vector), TDP-43 WT GFP or TDP-43 CT GFP. After 24 h, proteins and RNA were
136 crosslinked by adding formaldehyde drop-wise to the media to a final concentration of
137 0.75%. Cells were scraped in IP buffer prepared using RNase-free water (1xPBS with 1%
138 Triton-X100 and protease inhibitors cocktail). Cells were left on ice for 10 min with periodic
139 vortexing, and the lysate was cleared by centrifuging at 13,000 rpm for 10 min. GFP-Trap®
140 beads (Chromotek) were prepared by washing in IP buffer 4 times and added directly to
141 cleared cell lysates with subsequent nutation at +4ºC for 3 h. Beads were washed 4 times in
142 IP buffer and RNA was eluted from the beads by resuspension in TRI-reagent (Sigma). RNA
143 was purified according to the manufacturer's protocol, and equal amounts of RNA were
144 taken into a cDNA synthesis reaction. PCR was run using New England BioLabs Taq DNA
145 polymerase (M0273) using specific primers (see RNA expression analysis).

RNA expression analysis

147 RNA was extracted from cultured cells or mouse brain/spinal cord using PureLink total RNA
148 extraction kit (Life Technologies) and possible DNA contamination was removed using
149 RNase free DNase kit (Qiagen). cDNA synthesis was performed on 250-500 ng of total RNA
150 using SuperScript III reverse transcriptase (Life Technologies) and random hexamers
151 (Promega) according to the manufacturer's instructions. Quantitative real-time PCR was run
152 in triplicate on an ABI StepOne™ real-time PCR instrument and data were analysed using
153 StepOne™ Software v2.0 (Applied Biosystems). GAPDH was used as a housekeeping
154 gene. Human-specific primer sequences were as follows: NEAT1 total, 5'-
155 CTCACAGGCAGGGGAAATGT-3' and 5'-AACACCCACACCCCAAACAA-3'; NEAT1_2, 5'-
156 AGAGGCTCAGAGAGGACTGTAACCTG-3' and 5'-
157 TGTGTGTGTAAAAGAGAGAAGTTGTGG-3'; FUS, 5'-GGAACTCAGTCAACTCCCCA-3'
158 and 5'-TACCGTAACTTCCCGAGGTG-3'; GAPDH, 5'-TCGCCAGCCGAGCCA-3' and 5'-

159 GAGTTAAAAGCAGCCCTGGTG-3'. Mouse-specific primer sequences were as follows:
160 Neat1 total, 5'-TGGAGATTGAAGGCGCAAGT3' and 5'-ACCACAGAAGAGGAAGCACG-3';
161 Neat1_2, 5'-AACTACCAGCAATTCCGCCA-3' and 5'-GAGCTCGCCAGGTTTACAGT-3';
162 Gapdh, 5'-TCGCCAGCCGAGCCA-3' and 5'-GAGTTAAAAGCAGCCCTGGTG -3'.

Western blotting

164 2xLaemmli buffer was used to lyse cells or for direct homogenisation of tissue, followed by
165 denaturation at 100ºC for 10 min. After SDS-PAGE on handcast gels, proteins were
166 transferred to PVDF membrane by semi-dry blotting followed by blocking in 4% milk in
167 TBST, and incubation with primary and HRP-conjugated secondary (GE Healthcare)
168 antibodies. For detection, WesternBright Sirius kit (Advansta) was used. Equal loading was
169 confirmed by re-probing membranes with antibodies against beta-actin. Primary antibodies
170 used for western blot analysis of cultured cells and mouse tissue were rabbit polyclonal
171 TDP-43 (10782-2-AP, Proteintech), mouse monoclonal GFP (sc-9996, Santa Cruz) and
172 mouse monoclonal beta-actin (A5441, Sigma).

Generation and analysis of transgenic yeast strains

174 Plasmid for the expression of human NEAT1_1 was a gift from Archa Fox (Addgene plasmid
175 #61518). The hNEAT1 gene was cut out of this vector with NotI and KpnI and then inserted
176 into the yeast expression vector pAG426-Gal-ccdB [25] using the respective sites to make
177 plasmid p2454. NEAT1_1 expression from p2454 was verified by qRT-PCR using human
178 total NEAT1 primers (see above). For L1749 (74D-694: *MAT**a** ade1–14 ura3–52 leu2–3,112*
179 *trp1–289 his3–200*) yeast transformation, p2195, pAG413 GAL1-TDP43-EYFP, *HIS3, CEN*
180 (TDP-43) [50]; p2257, pAG413 GAL1-ccdB-EYFP, *HIS3, CEN* (v1); p2454, pAG426 GAL1-
181 hNEAT1, *URA3, CEN* (NEAT1_1); and p2039, pAG426-GAL-ccdB, *URA3*, 2μ (v2) plasmids
182 were used. Yeast were grown on plasmid selective glucose (SD-His-Ura) or galactose
183 (SGal-His-Ura) media. 10-fold serial dilutions of transformants were spotted. Transformants
184 were analysed after 5 and 8 days at 30ºC. The transformants were also grown in liquid
185 plasmid selective galactose media for 2 days in a 30ºC shaking incubator. Viable and dead
186 cells were counted following Trypan Blue staining of dead cells.

Generation and characterisation of transgenic and double-transgenic Drosophila lines

188 Constructs encoding NEAT1_1, or lacZ in pUAST vector, were injected into w1118 embryos
189 to produce transgenic *Drosophila melanogaster* as previously described [26]. Several
190 independent transformant lines were analysed per construct. gmr-GAL4 and UAS-lacZ lines
191 were obtained from the Bloomington Drosophila stock center. Production of TDP-43 and
192 FUS transgenic flies was described in previous publications [26, 27]. Crosses between the
193 *Drosophila* strains were carried out at 25°C using standard procedures. For external surface
194 observation, 5-day-old flies were anesthetized with CO_2 and observed with zoom stereo
195 microscopy (Olympus SZ-PT). For histochemical analyses, heads of 5-day-old adult
196 transgenic flies were dissected, collected, briefly washed in PBS, and fixed with 4%
197 paraformaldehyde containing 0.1% Triton X-100 at room temperature for 2 h. Tissues were
198 dehydrated by graded ethanol, cleared in butanol and embedded in paraffin. Four-
199 micrometer thick coronal sections were stained with hematoxylin and eosin (H&E). For
200 wesern blot analysis, heads of 5-day-old flies were dissected and lysed in Laemmli sample
201 buffer for SDS-PAGE containing 2% SDS. Commerical antibodies against TDP-43 (rabbit
202 polyclonal, Proteintech, 10782-2-AP), FUS (rabbit polyclonal, Bethyl, A300–293A) and
203 alpha-tubulin (mouse monoclonal, Sigma, DM1A) were used. For analysis of NEAT1_1
204 expression, total RNA from fly heads was extracted using Isogen (Nippon Gene) and
205 converted to cDNA using ReverTra Ace Quantitative PCR RT Master Mix with gDNA
206 remover (TOYOBO). The primer sets used for qPCR were as follows: rp49, 5'-
207 CAGCTTCAAGATGACCATC-3' and 5'-TCAGATACTGTCCCTTGAAG-3'; NEAT1, 5'-
208 GCCTTGTAGATGGAGCTTGC-3' and 5'-TCAACGCCCCAAGTTATTTC-3'.

Analysis of human tissue samples

210 Human frontal cortex and spinal cord paraffin sections from clinically and histopathologically
211 characterised FTLD and ALS cases and neurologically healthy individuals were obtained
212 from the MRC London Neurodegenerative Diseases Brain Bank (Institute of Psychiatry,
213 Kings College, London). Consent was obtained from all subjects for autopsy,
214 histopathological assessment and research in accordance with local and national Ethics
215 Committee approved donation. For RNAscope® ISH analysis, Hs-NEAT1-long (411541)
216 probe (ACD) was used according to the manufacturer's instructions. Images were taken
217 using Leica DMRB microscope equipped with Jenoptik Progres SpeedXT core3 colour digital
218 camera and Progres CapturePro software.

Results

220 In FTLD-TDP and Alzheimer's disease, C-terminal TDP-43 fragments accumulate in
221 pathological inclusions, alongside full-length non-mutated TDP-43 [2, 28, 29]. We performed
222 RNA immunoprecipitation (RIP) using full-length TDP-43 and its 25 kDa C-terminal fragment
223 (aa.191-414) transiently expressed in neuroblastoma SH-SY5Y cells (Fig. 1A). We found
224 that although this C-terminal TDP-43 fragment retains one of the two RNA-binding motifs
225 (RRM2), it loses the ability to bind and precipitate NEAT1 (Fig. 1B,C). Therefore, full-length
226 TDP-43 is likely the primary species binding to NEAT1 in the FTLD brains.

227 TDP-43 depletion causes NEAT1_2 upregulation in cultured cells concomitant with a
228 decrease in NEAT1_1 levels [18, 22]. However, the effect of TDP-43 overabundance on
229 NEAT1 isoforms has not been examined. We measured NEAT1 levels in neuroblastoma
230 cells expressing GFP- or Flag-tagged TDP-43 by qRT-PCR and found that both total NEAT1
231 (two isoforms combined) and NEAT1_2 levels are increased upon TDP-43 overexpression
232 (Fig. 1D,E). This effect depends on the ability of TDP-43 to enter the nucleus since
233 overexpression of TDP-43 lacking the nuclear localization signal (NLS) did not affect NEAT1
234 levels (Fig. 1D,E). Even though NEAT1_1 levels could not be measured separately due to
235 the isoform overlap, NEAT1_1 is significantly more abundant than NEAT1_2 in cultured cells
236 [30,31]. In the SH-SY5Y cell line used in this study, NEAT1_1 accounts for ~75% of the total
237 NEAT1 levels (our unpublished observations). Therefore, we conclude that NEAT1_1 is
238 upregulated in TDP-43 overexpressing cells.

239 In the mammalian CNS, NEAT1_1 is the constitutive isoform, whereas basal NEAT1_2
240 expression is very low and this isoform is only induced under stress conditions [15, 32-34].
241 We measured Neat1 levels in a mouse model of TDP-43 proteinopathy with neuronal
242 overexpression of human wild-type (WT) TDP-43 under the control of PrP promoter (TDP-
243 43$_{PrP}$ mice) [7]. First, using immunohistochemistry and western blotting, we confirmed that,
244 compared to WT mice, levels of nuclear TDP-43 are increased in the cortex of symptomatic
245 4-week old homozygous TDP-43$_{PrP}$ mice (Fig. 1F). Total Neat1 (Neat1_1 + Neat1_2) levels
246 were found to be upregulated in the cortex but not in the spinal cord of TDP-43$_{PrP}$ mice, as
247 detected by qRT-PCR (Fig. 1G). Since Neat1_2 levels are very low in the brain of WT mice
248 under basal conditions [15] and remain unaltered in TDP-43$_{PrP}$ mice (Fig. 1G), the increase
249 in total Neat1 levels in the brain of this mouse model must be attributed to Neat1_1
250 upregulation. Thus, Neat1_1 becomes upregulated in the cortex of mice with neuronal
251 overexpression of TDP-43.

252 We next asked whether NEAT1_1 is capable of modulating TDP-43 toxicity *in vivo*. Yeast
253 and *Drosophila melanogaster* models of TDP-43 proteinopathy have been instrumental in
254 the studies of modifiers of TDP-43 toxicity [35, 36]. We used a yeast model expressing
255 human WT TDP-43 tagged with YFP, which forms cytoplasmic aggregates/foci and is toxic
256 [37]. We used a serial dilution spot test assay with controls on the same plate routinely
257 utilized to analyze growth inhibition in yeast. TDP-43 and NEAT1_1 expression was driven
258 by a galactose-inducible promoter. We found no difference in the growth of 10-fold serially
259 diluted yeast either containing a control plasmid (Fig. 2A rows 1 and 2) or a plasmid that
260 overexpressed human NEAT1_1 (Gal1-NEAT1) (Fig. 2A rows 3 and 4) on the galactose
261 plate. As expected, TDP-43 expression causes reduced growth (Fig. 2A rows 5-8 on

262 galactose). However, co-expression of NEAT1_1 was able to ameliorate TDP-43 toxicity as
263 evident from partial rescue of yeast growth on galactose (Fig. 2A rows 7 and 8) compared to
264 growth of control transformants without NEAT1_1 expression (Fig. 2A rows 5 and 6). In total,
265 16 independent transformants were examined and showed partial rescue by NEAT1_1. To
266 further confirm the effect of NEAT1_1 on TDP-43 toxicity, we compared the fraction of dead
267 cells in transformants expressing TDP-43 alone or together with NEAT1_1. Three
268 transformants of each type were grown in plasmid selective media and the fraction of dead
269 cells was determined after 1 and 2 days of growth. In line with the spot assay results, the
270 fraction of dead cells was larger in cultures overexpressing TDP-43 alone compared with
271 cultures also expressing NEAT1_1 (Fig. 2B). Cells expressing TDP-43 with or without
272 NEAT1_1 co-expression showed continued presence of cytoplasmic TDP-43 aggregates
273 that did not have any visible differences (data not shown).

274 We next investigated the effect of NEAT1_1 in transgenic (TG) *Drosophila melanogaster*. Six
275 independent transgenic lines overexpressing human NEAT1_1 in the retinal photoreceptor
276 neurons under the control of GMR-GAL4 driver were obtained using the GAL4-UAS system
277 with the random insertion method. We obtained three lines with the transgene insertion on
278 chromosome 2 and three lines with the insertion on chromosome 3 (Figure S2). Only one of
279 these six lines showed retinal pathology, which was likely due to disruption of an essential
280 gene by the transgene integration (Figure S2). We concluded that NEAT1_1 overexpression
281 in the fly retina is not toxic. NEAT1_1 expression varies ~3-fold in these fly strains, and two
282 strains, one with intermediate (#1) and one with high (#4) NEAT1_1 expression, were
283 selected for further studies.

284 We previously reported transgenic fly models of TDP-43 proteinopathy overexpressing
285 human WT or mutant TDP-43 in photoreceptor neurons [26]. They are characterized by
286 vacuolar degeneration and thinning of the retina, more pronounced in lines expressing TDP-
287 43 mutants. These lines were crossed with NEAT1_1 TG flies with subsequent analysis of
288 the eye phenotypes. We found that co-expression of NEAT1_1 ameliorates retinal thinning in
289 TDP-43 WT TG flies, and this effect is more pronounced in the line with higher NEAT1_1
290 expression (#4, NEAT1_1 expression 2.5-fold higher as compared to #1) (Fig. 3A,B). We
291 next crossed NEAT1_1 TG flies with a line expressing an ALS-causative TDP-43 mutant,
292 G298S. This mutation is associated with an aggressive form of the disease [38], and
293 consistent with this, TDP-43 G298S TG flies are characterized by a severe retinal phenotype
294 with nearly complete loss of photoreceptor neurons (Fig. 3C). NEAT1_1 overexpression in
295 TDP-43 G298S TG flies was nevertheless capable of visibly rescuing the "rough eye"
296 phenotype, although this effect was not quantifiable since the retina of both TG and double
297 TG flies was too thin to measure (Fig. 3C).

298 We and others previously showed that retinal expression of another ALS-linked protein,
299 FUS, is also sufficient to cause retinal degeneration in *Drosophila* [27, 39]. Overexpression
300 of human WT FUS in the fly retina, similar to WT TDP-43, results in ~30% retinal thinning
301 [27] (Figure S3). However, co-expression of NEAT1_1 failed to rescue FUS-induced retinal
302 thinning, as is evident from unaltered retinal thickness in double TG FUS/NEAT1_1 flies
303 compared to FUS TG flies (Figure S3). Therefore, NEAT1_1 is protective against TDP-43
304 toxicity but not FUS toxicity in *Drosophila* proteinopathy models.

Discussion

306 In the current study, we demonstrate that overabundance of full-length TDP-43 leads to
307 upregulation of the constitutive isoform of NEAT1, NEAT1_1, in the murine CNS and that
308 NEAT1_1 acts as a suppressor of TDP-43 toxicity in yeast and fly models.

309 TDP-43 levels are tightly autoregulated [40], and it is plausible that this autoregulatory
310 mechanism fails early during proteinopathy development, resulting in uncontrollable TDP-43
311 accumulation. Indeed, increased TDP-43 expression was reported in some ALS and FTLD
312 samples [4, 41]. Although the exact mechanisms of the protective effect of NEAT1_1 are yet
313 to be elucidated, we propose that NEAT1_1 acts to bind and neutralize the excess of TDP-

43. Previously, a yeast suppressor screen led to the identification of intronic lariats as RNA species that bind and sequester TDP-43 thereby reducing its toxicity [42]. Given the abundance of NEAT1_1, this lncRNA may also act as a "sponge" that prevents unwanted interactions of TDP-43 with other RNAs in the nucleus. Studies in a number of cellular and *in vivo* models demonstrated that TDP-43 toxicity is dependent on its RNA binding ability [26, 35, 43, 44]. When engaged with certain RNA targets, accumulated/mutant TDP-43 can gain toxic functions, e.g. in splicing [45]. Therefore, titration of TDP-43 from its numerous target RNAs by NEAT1_1 may play an important protective role early in disease; in this scenario, increased demand for NEAT1_1 would lead to its upregulation. Recently, it has been shown that binding to RNA prevents the cytotoxic liquid-liquid phase separation (LLPS) of TDP-43 [46]. Thus, NEAT1_1 may also play a role in antagonising TDP-43 toxicity by reducing its LLPS associated with toxic species formation.

Interestingly, NEAT1_1 co-expression was not able to rescue the toxicity of another ALS/FTLD-linked protein, FUS, in *Drosophila* models. This was true even though FUS strongly binds to NEAT1 transcripts (mainly in the 5' region shared by NEAT1_1 and NEAT1_2) [17]. Aggregation of non-mutated FUS protein is typical for a FTLD subtype without TDP-43 pathology, FTLD-FUS [5]. Our results point to a different role for NEAT1_1 in FTLD-TDP *vs.* FTLD-FUS.

The ability to modulate TDP-43 and FUS toxicity was previously reported for *Drosophila* ncRNAs such as Hsrω [47-49]. In particular, Hsrω depletion in a *Drosophila* model of TDP-43 proteinopathy was shown to partially rescue TDP-43-induced retinal degeneration. Furthermore, a proposed functional orthologue of Hsrω in humans, SatIII RNA, was found to be upregulated in TDP-43 overexpressing cells in culture and in the cortex of FTLD patients [47]. Interestingly, Hsrω transcripts are the primary RNA components of 'omega speckles' bearing structural and functional similarities to paraspeckles [50]. To the best of our knowledge, NEAT1_1 is the first lncRNA reported to have a protective effect against TDP-43 toxicity. Further studies are needed to identify other protective and maladaptive lncRNAs in TDP-43 proteinopathies.

Importantly, we show that overexpression of NEAT1_1 does not result in toxicity *in vivo*, in transgenic yeast or *Drosophila* models. In line with this, we recently found that neuronal (Thy1 promoter driven) NEAT1_1 overexpression is not associated with any deleterious effects in mice (manuscript in preparation). Approaches to boost NEAT1_1 expression in the CNS and thereby increase the levels of "sponge" RNA to neutralize surplus/abnormal TDP-43 may prove useful in the treatment of human TDP-43 proteinopathies affecting the brain, such as FTLD-TDP and Alzheimer's disease. NEAT1_1 accumulation can be induced pharmacologically, for example, using HDAC inhibitors [22]. However, the latter class of compounds is known to have multiple non-specific effects, therefore further drug discovery efforts are needed to develop more targeted compounds for modulation of NEAT1_1 levels.

Acknowledgments

Authors' contributions

TAS conceived research; TAS, KM, MSK, SP, SKP, SWL, TH and TI designed experiments; KM, MSK, SP, SKP, NW and TAS performed experiments and analysed data; TAS wrote manuscript with input from all authors. All authors read and approved the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Figure legends

Figure 1. Overabundance of human full-length TDP-43 leads to NEAT1 upregulation in cultured cells and in the cortex of transgenic mice.

(A) TDP-43 species used in the study: full-length (FL) human TDP-43 and C-terminal TDP-43 fragment (CT, aa. 192-414).

(B,C) FL TDP-43 but not TDP-43 CT binds to NEAT1 in cultured cells. RNA immunoprecipitation (IP) was performed using GFP®Trap beads in SH-SY5Y cells transfected to express GFP-tagged TDP-43 FL or TDP-43 CT. For input, 1/10 of the cell lysate was used. Asterisk indicates non-specific or cleavage fragments detected by the anti-GFP antibody. Black and grey arrowheads indicate GFP-tagged FL and CT TDP-43, respectively, and double arrowhead – GFP (B). The presence of NEAT1 (NEAT1 total: NEAT1_1 and NEAT1_2 isoforms combined; and NEAT1_2 only) in IP samples and cell lysates was detected by RT-PCR. Quantification of band intensities in IP samples is also given (mean±SEM, n=3). A known TDP-43 mRNA target, FUS, was included as a positive control. PCR fragment sizes are as follows: NEAT1 total, 91 nt; NEAT1_2, 141 nt; FUS, 145 nt (C). Representative western blot and PCR gels are shown.

(D,E) Overexpression of full-length TDP-43 but not TDP-43 lacking NLS (dNLS) upregulates NEAT1 in a stable cell line. SH-SY5Y cells were analysed 24 h post-transfection with a respective construct. TDP-43 was tagged with either GFP or Flag. Vector corresponds to pEGFP-C1. In D, subcellular localisation of GFP-tagged TDP-43 variants and a representative western blot with an anti-TDP-43 antibody are shown. Arrowhead indicates the endogenous TDP-43 band. Scale bar, 10 µm. In E, qRT-PCR results for total NEAT1 and NEAT1_2 levels are shown; data represent mean±SEM, n=4, *p<0.05, **p<0.01 (Mann-Whitney *U* test).

(F,G) NEAT1 upregulation in the cortex of TDP-43_PrP mice. Increased TDP-43 level in the cortex of homozygous 4-week old TDP-43_PrP mice [7], as compared to their non-transgenic (NT) littermates was confirmed by immunostaining and western blot (F). Note that high levels of TDP-43 (green) are detected in neurons (dim DAPI signal) but not in glial cells (bright DAPI signal). Scale bar, 100 µm. In G, NEAT1_2 and total NEAT1 levels were measured by qRT-PCR in the spinal cord and cortex lysates; data represent mean±SEM, n=8, *p<0.05 (Mann-Whitney *U* test).

Figure 2. NEAT1_1 is a supressor of TDP-43 toxicity in a yeast model of TDP-43 proteinopathy.

(A) NEAT1_1 co-expression ameliorates TDP-43 toxicity in yeast in a plate-based spot assay. L1749 yeast were simultaneously transformed with p2195, pAG413 GAL1-TDP43-EYFP, *HIS3, CEN* (TDP-43) or p2257, pAG413 GAL1-ccdB-EYFP, *HIS3, CEN* (v1) and p2454, pAG426 GAL1-hNEAT1, *URA3, CEN* (NEAT1_1) or p2039, pAG426-GAL-ccdB, *URA3*, 2µ (v2) and were maintained on plasmid selective glucose (SD-His-Ura) or galactose media (SGal-His-Ura). 10-fold serial dilutions of transformants were spotted. Middle and right panels show double transformants on plasmid selective galactose media expressing both TDP-43-EYFP and NEAT1_1, after 5 days (middle) or 8 days (right) of incubation at 30ºC. In total, 16 sets of transformants of each type were analysed, and images of 4 representative sets of transformants are shown, two on each of the two independent plates.

(B) NEAT1_1 co-expression reduces cell death of TDP-43 overexpressing yeast grown in liquid culture. For quantification of cell death, 3 independent transformants, of each type shown in A, were grown in liquid plasmid selective galactose media. Viable and dead cells were counted after 1 and 2 days of growth. 300-700 cells from each of 3 transformants were included in the analysis. Data represent mean±SE; *p<0.05 (Student's *t* test).

Figure 3. Overexpression of NEAT1_1 ameliorates retinal degeneration induced by human TDP-43 in *Drosophila*.

(A,B) Overexpression of human NEAT1_1 does not affect retinal photoreceptor cells in *Drosophila melanogaster* and partially rescues retinal thinning induced by overexpression of human WT TDP-43. Representative images of H&E-stained retinal sections (A) and quantification of retinal thickness (B) for transgenic and double-transgenic 5-day-old flies are shown. Two independent NEAT1_1 (N1_1) transgenic lines (#1 and #4) differing in the levels of NEAT1_1 expression and with the transgene insertion on different chromosomes were used. In B, data represent mean±SEM; retinas from 10 flies per genotype were analyzed. $*p<0.05$, $**p<0.01$, $***p<0.001$ (two-way ANOVA with Tukey-Kramer test). Scale bar, 50 μm. Also see Figure S2.

(C) Overexpression of human NEAT1_1 improves the "rough eye" phenotype in mutant TDP-43 G298S transgenic flies. Representative images of external head surface (top) and H&E stained retinal sections (bottom) of 5-day-old transgenic and double-transgenic flies are shown. Scale bar, 100 μm.

(D) Similar expression levels of normal and mutant human TDP-43 in the heads of transgenic and double-transgenic flies as determined by western blotting and subsequent quantification of band intensities (mean±SEM, n=3).

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