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1 **Long non-coding RNA NEAT1\_1 ameliorates TDP-43 toxicity in *in vivo***  
2 **models of TDP-43 proteinopathy**

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39

40 **Abstract**

41 Pathological changes involving TDP-43 protein (“TDP-43 proteinopathy”) are typical for  
42 several neurodegenerative diseases, including frontotemporal lobar degeneration (FTLD).  
43 FTLD-TDP cases are characterized by increased binding of TDP-43 to an abundant lncRNA,  
44 NEAT1, in the cortex. However it is unclear whether enhanced TDP-43-NEAT1 interaction  
45 represents a protective mechanism. We show that accumulation of human TDP-43 leads to  
46 upregulation of the constitutive NEAT1 isoform, NEAT1\_1, in cultured cells and in the brains  
47 of transgenic mice. Furthermore, overexpression of NEAT1\_1 is protective against TDP-43  
48 toxicity in *Drosophila* and yeast models of TDP-43 proteinopathy. Thus NEAT1\_1  
49 upregulation may be protective in TDP-43 proteinopathies affecting the brain. Approaches to  
50 boost NEAT1\_1 expression in the CNS may prove useful in the treatment of these  
51 conditions.

52

53 **Key words:** TDP-43; NEAT1; FUS; FTLD; frontotemporal dementia; Alzheimer’s disease;  
54 ALS; neurodegeneration; proteinopathy; *Drosophila*; yeast

55

## 56 Introduction

57 TDP-43 is an abundant, ubiquitously expressed RNA-binding protein [1] whose dysfunction  
58 is tightly linked to and/or causative of neurodegenerative diseases amyotrophic lateral  
59 sclerosis (ALS), frontotemporal lobar degeneration (FTLD), and Alzheimer's disease [2, 3].  
60 Over 50 mutations have been described in the TDP-43 encoding gene, *TARDBP*, which are  
61 responsible for ~2% of ALS cases [1, 4]. However, in the vast majority of ALS and FTLD  
62 patients, non-mutated TDP-43 is mislocalized from its normal nuclear location and is  
63 deposited in a form of pathological inclusions in neurons and glial cells in the affected CNS  
64 regions – a condition termed “TDP-43 proteinopathy”. TDP-43 proteinopathy is typical for  
65 ~98% of sporadic and up to 50% of familial ALS cases, including those caused by *TARDBP*  
66 and *C9ORF72* mutations; for the majority of FTLD cases with tau-negative, ubiquitin-positive  
67 inclusions (FTLD-U, or FTLD-TDP); and for ~45% of Alzheimer's disease cases [2-6]. TDP-  
68 43 is often post-translationally modified in the above diseases, with the most common  
69 modifications being its ubiquitination, phosphorylation and N-terminal truncation [2-6]. Both  
70 loss and gain of TDP-43 function likely underlie TDP-43 proteinopathy however the relative  
71 contribution of the two mechanisms is still hotly debated. Studies in transgenic mouse  
72 models showed that even moderate overexpression of non-mutated, full-length human TDP-  
73 43 in the CNS is highly toxic [7-10].

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

85 Two NEAT1 isoforms sharing their 5' end have been described, the constitutive short  
86 isoform (NEAT1\_1) and the stress-inducible long isoform (NEAT1\_2) [16]. NEAT1 is one of  
87 the strongest TDP-43 interactors; TDP-43 protein binds along the entire length of NEAT1  
88 transcripts [17-19]. Furthermore, NEAT1 isoforms are structural elements of nuclear RNP  
89 granules paraspeckles, and TDP-43 was identified as a paraspeckle component [20, 21].  
90 Crosslinking and immunoprecipitation (CLIP) studies showed that NEAT1 is the RNA with  
91 the most significant increase in TDP-43 binding in the brain of FTLD-TDP patients, as  
92 compared to control individuals [19]. Recently, we and others showed that TDP-43 regulates  
93 the NEAT1 isoform ratio, where its loss of function leads to NEAT1\_2 upregulation [18, 22].  
94 Loss of TDP-43 function is likely responsible for NEAT1\_2 accumulation in spinal motor  
95 neurons of ALS patients [22, 23]. However, we failed to detect NEAT1\_2 transcript in the  
96 cortex of FTLD patients using RNAScope® ISH (Figure S1), which might be due to  
97 differences in transcript regulation in spinal and brain neurons. The constitutive NEAT1  
98 isoform, NEAT1\_1, may therefore play a more prominent role in TDP-43 proteinopathies  
99 affecting the brain such as FTLD and Alzheimer's disease.

100 In the current study, we examined the interplay between TDP-43 and NEAT1\_1 in cultured  
101 cells and in transgenic *in vivo* models.

102

## 103 Materials and methods

### 104 *SH-SY5Y cells, plasmids, transfection and immunofluorescence*

105 SH-SY5Y cells were maintained in a 1:1 mixture of DMEM and F12 medium supplemented  
106 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).

#### 114 *Mouse tissue analysis*

115 Hemizygous TDP-43<sub>PrP</sub> 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<sub>PrP</sub>  
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'-TGCTTAGGTTCCGGCATTGGAT-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.

#### 133 *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 +4°C 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).

#### 146 *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 TGTGTGTGTAAGAGAGAGAAGTTGTGG-3'; FUS, 5'-GGAAGTCACTCAACTCCCCA-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'.

### 163 *Western blotting*

164 2xLaemmli buffer was used to lyse cells or for direct homogenisation of tissue, followed by  
165 denaturation at 100°C 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).

### 173 *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: *MATa 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 30°C. The transformants were also grown in liquid  
185 plasmid selective galactose media for 2 days in a 30°C shaking incubator. Viable and dead  
186 cells were counted following Trypan Blue staining of dead cells.

### 187 *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<sub>2</sub> 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 western blot analysis, heads of 5-day-old flies were dissected and lysed in Laemmli sample  
201 buffer for SDS-PAGE containing 2% SDS. Commercial 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'-TCAACGCCCAAGTTATTTTC-3'.

### 209 *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.

## 219 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<sub>PrP</sub> 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<sub>PrP</sub> 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<sub>PrP</sub> 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<sub>PrP</sub> 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.

## 305 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 FTL  
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-



314 43. Previously, a yeast suppressor screen led to the identification of intronic lariats as RNA  
315 species that bind and sequester TDP-43 thereby reducing its toxicity [42]. Given the  
316 abundance of NEAT1\_1, this lncRNA may also act as a “sponge” that prevents unwanted  
317 interactions of TDP-43 with other RNAs in the nucleus. Studies in a number of cellular and *in*  
318 *vivo* models demonstrated that TDP-43 toxicity is dependent on its RNA binding ability [26,  
319 35, 43, 44]. When engaged with certain RNA targets, accumulated/mutant TDP-43 can gain  
320 toxic functions, e.g. in splicing [45]. Therefore, titration of TDP-43 from its numerous target  
321 RNAs by NEAT1\_1 may play an important protective role early in disease; in this scenario,  
322 increased demand for NEAT1\_1 would lead to its upregulation. Recently, it has been shown  
323 that binding to RNA prevents the cytotoxic liquid-liquid phase separation (LLPS) of TDP-43  
324 [46]. Thus, NEAT1\_1 may also play a role in antagonising TDP-43 toxicity by reducing its  
325 LLPS associated with toxic species formation.

326 Interestingly, NEAT1\_1 co-expression was not able to rescue the toxicity of another  
327 ALS/FTLD-linked protein, FUS, in *Drosophila* models. This was true even though FUS  
328 strongly binds to NEAT1 transcripts (mainly in the 5' region shared by NEAT1\_1 and  
329 NEAT1\_2) [17]. Aggregation of non-mutated FUS protein is typical for a FTLD subtype  
330 without TDP-43 pathology, FTLD-FUS [5]. Our results point to a different role for NEAT1\_1  
331 in FTLD-TDP *vs.* FTLD-FUS.

332 The ability to modulate TDP-43 and FUS toxicity was previously reported for *Drosophila*  
333 ncRNAs such as Hsrw [47-49]. In particular, Hsrw depletion in a *Drosophila* model of TDP-  
334 43 proteinopathy was shown to partially rescue TDP-43-induced retinal degeneration.  
335 Furthermore, a proposed functional orthologue of Hsrw in humans, SatIII RNA, was found to  
336 be upregulated in TDP-43 overexpressing cells in culture and in the cortex of FTLD patients  
337 [47]. Interestingly, Hsrw transcripts are the primary RNA components of ‘omega speckles’  
338 bearing structural and functional similarities to paraspeckles [50]. To the best of our  
339 knowledge, NEAT1\_1 is the first lncRNA reported to have a protective effect against TDP-43  
340 toxicity. Further studies are needed to identify other protective and maladaptive lncRNAs in  
341 TDP-43 proteinopathies.

342 Importantly, we show that overexpression of NEAT1\_1 does not result in toxicity *in vivo*, in  
343 transgenic yeast or *Drosophila* models. In line with this, we recently found that neuronal  
344 (Thy1 promoter driven) NEAT1\_1 overexpression is not associated with any deleterious  
345 effects in mice (manuscript in preparation). Approaches to boost NEAT1\_1 expression in the  
346 CNS and thereby increase the levels of “sponge” RNA to neutralize surplus/abnormal TDP-  
347 43 may prove useful in the treatment of human TDP-43 proteinopathies affecting the brain,  
348 such as FTLD-TDP and Alzheimer’s disease. NEAT1\_1 accumulation can be induced  
349 pharmacologically, for example, using HDAC inhibitors [22]. However, the latter class of  
350 compounds is known to have multiple non-specific effects, therefore further drug discovery  
351 efforts are needed to develop more targeted compounds for modulation of NEAT1\_1 levels.

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## 357 **Authors’ contributions**

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

## 362 **Disclosure statement**

363 No potential conflict of interest was reported by the authors.

364

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520 **Figure legends**

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

523 (A) TDP-43 species used in the study: full-length (FL) human TDP-43 and C-terminal TDP-  
524 43 fragment (CT, aa. 192-414).

525 (B,C) FL TDP-43 but not TDP-43 CT binds to NEAT1 in cultured cells. RNA  
526 immunoprecipitation (IP) was performed using GFP®Trap beads in SH-SY5Y cells  
527 transfected to express GFP-tagged TDP-43 FL or TDP-43 CT. For input, 1/10 of the cell  
528 lysate was used. Asterisk indicates non-specific or cleavage fragments detected by the anti-  
529 GFP antibody. Black and grey arrowheads indicate GFP-tagged FL and CT TDP-43,  
530 respectively, and double arrowhead – GFP (B). The presence of NEAT1 (NEAT1 total:  
531 NEAT1\_1 and NEAT1\_2 isoforms combined; and NEAT1\_2 only) in IP samples and cell  
532 lysates was detected by RT-PCR. Quantification of band intensities in IP samples is also  
533 given (mean±SEM, n=3). A known TDP-43 mRNA target, FUS, was included as a positive  
534 control. PCR fragment sizes are as follows: NEAT1 total, 91 nt; NEAT1\_2, 141 nt; FUS, 145  
535 nt (C). Representative western blot and PCR gels are shown.

536 (D,E) Overexpression of full-length TDP-43 but not TDP-43 lacking NLS (dNLS) upregulates  
537 NEAT1 in a stable cell line. SH-SY5Y cells were analysed 24 h post-transfection with a  
538 respective construct. TDP-43 was tagged with either GFP or Flag. Vector corresponds to  
539 pEGFP-C1. In D, subcellular localisation of GFP-tagged TDP-43 variants and a  
540 representative western blot with an anti-TDP-43 antibody are shown. Arrowhead indicates  
541 the endogenous TDP-43 band. Scale bar, 10 µm. In E, qRT-PCR results for total NEAT1  
542 and NEAT1\_2 levels are shown; data represent mean±SEM, n=4, \*p<0.05, \*\*p<0.01 (Mann-  
543 Whitney *U* test).

544 (F,G) NEAT1 upregulation in the cortex of TDP-43<sub>PrP</sub> mice. Increased TDP-43 level in the  
545 cortex of homozygous 4-week old TDP-43<sub>PrP</sub> mice [7], as compared to their non-transgenic  
546 (NT) littermates was confirmed by immunostaining and western blot (F). Note that high levels  
547 of TDP-43 (green) are detected in neurons (dim DAPI signal) but not in glial cells (bright  
548 DAPI signal). Scale bar, 100 µm. In G, NEAT1\_2 and total NEAT1 levels were measured by  
549 qRT-PCR in the spinal cord and cortex lysates; data represent mean±SEM, n=8, \*p<0.05  
550 (Mann-Whitney *U* test).

551

552 **Figure 2. NEAT1\_1 is a supressor of TDP-43 toxicity in a yeast model of TDP-43**  
553 **proteinopathy.**

554 (A) NEAT1\_1 co-expression ameliorates TDP-43 toxicity in yeast in a plate-based spot  
555 assay. L1749 yeast were simultaneously transformed with p2195, pAG413 GAL1-TDP43-  
556 EYFP, *HIS3*, *CEN* (TDP-43) or p2257, pAG413 GAL1-ccdB-EYFP, *HIS3*, *CEN* (v1) and  
557 p2454, pAG426 GAL1-hNEAT1, *URA3*, *CEN* (NEAT1\_1) or p2039, pAG426-GAL-ccdB,  
558 *URA3*, 2µ (v2) and were maintained on plasmid selective glucose (SD-His-Ura) or galactose  
559 media (SGal-His-Ura). 10-fold serial dilutions of transformants were spotted. Middle and right  
560 panels show double transformants on plasmid selective galactose media expressing both  
561 TDP-43-EYFP and NEAT1\_1, after 5 days (middle) or 8 days (right) of incubation at 30°C. In  
562 total, 16 sets of transformants of each type were analysed, and images of 4 representative  
563 sets of transformants are shown, two on each of the two independent plates.

564 (B) NEAT1\_1 co-expression reduces cell death of TDP-43 overexpressing yeast grown in  
565 liquid culture. For quantification of cell death, 3 independent transformants, of each type  
566 shown in A, were grown in liquid plasmid selective galactose media. Viable and dead cells  
567 were counted after 1 and 2 days of growth. 300-700 cells from each of 3 transformants were  
568 included in the analysis. Data represent mean±SE; \*p<0.05 (Student's *t* test).

569 **Figure 3. Overexpression of NEAT1\_1 ameliorates retinal degeneration induced by**  
570 **human TDP-43 in *Drosophila*.**

571 (A,B) Overexpression of human NEAT1\_1 does not affect retinal photoreceptor cells in  
572 *Drosophila melanogaster* and partially rescues retinal thinning induced by overexpression of  
573 human WT TDP-43. Representative images of H&E-stained retinal sections (A) and  
574 quantification of retinal thickness (B) for transgenic and double-transgenic 5-day-old flies are  
575 shown. Two independent NEAT1\_1 (N1\_1) transgenic lines (#1 and #4) differing in the  
576 levels of NEAT1\_1 expression and with the transgene insertion on different chromosomes  
577 were used. In B, data represent mean±SEM; retinas from 10 flies per genotype were  
578 analyzed. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 (two-way ANOVA with Tukey-Kramer test). Scale  
579 bar, 50 µm. Also see Figure S2.

580 (C) Overexpression of human NEAT1\_1 improves the “rough eye” phenotype in mutant  
581 TDP-43 G298S transgenic flies. Representative images of external head surface (top) and  
582 H&E stained retinal sections (bottom) of 5-day-old transgenic and double-transgenic flies are  
583 shown. Scale bar, 100 µm.

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