Characterization of the Human TARDBP Gene Promoter

Marco Baralle
  International Centre for Genetic Engineering and Biotechnology

Maurizio Romano (✉ mromano@units.it)
  Department of Life Sciences, University of Trieste, Via A. Valerio 28, 34127 - Trieste Italy

Research Article

Abstract

The expression of TDP-43, the main component of neuronal intracellular inclusions across a broad spectrum of ALS and FTD disorders, is developmentally regulated and studies in vivo have shown that TDP-43 overexpression can be toxic, even before observation of pathological aggregates. Starting from these observations, the regulation of its expression at transcriptional level might represent a further key element for the pathogenesis of neurodegenerative diseases. Therefore, we have characterized the human TARDBP promoter, in order to study the transcriptional mechanisms of expression. Mapping of cis-acting elements by luciferase assays in different cells outlined that the activity of the promoter seems to be higher in cell lines of neuronal origin. We have identified the first 400 nucleotides upstream from the transcription start site as the minimal region with a significant transcription activity. In addition, we tested the effects of two SNPs found in the the promoter region of ALS patients and observed no significant effect on transcription levels. Then, TDP-43 overexpression did not affect significantly the activity of its promoter, suggesting that TDP-43 does not influence its own transcription. Finally, the presence of the 5'UTR sequence and of intron-1 splicing seem to impact positively on TDP-43 expression at transcriptional level.

Introduction

TAR DNA-Binding Protein 43 (TDP-43) is an ubiquitously expressed and highly conserved nuclear protein encoded by the human TARDBP gene, and it is involved in several cellular mechanisms, including transcription, pre-mRNA processing, splicing and translation [1,2]. After initial implication in the pathogenesis of monosymptomatic forms of cystic fibrosis [3-7], it has been discovered a direct association between TDP-43 abnormalities and neurodegenerative diseases, and in particular with Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Degeneration (FTLD-U) [8,9]. Over the last years, TDP-43 immunoreactive inclusions have also been reported in 70% of patients with hippocampal sclerosis, 30% of patients with Alzheimer's disease, 33% of patients with Pick's disease and in a subset of patients with Lewy-body related disease [10-17]. Whereas many efforts have been expended in characterizing TDP-43 pathophysiological functions, a contentious debate ensued over the possible mechanisms whereby TDP-43 triggers neurodegeneration is still ongoing. It has been proposed both that cytoplasmic aggregates might be neurotoxic ("gain of function" hypothesis) and that the sequestration of the factors in aggregates might induce a functional deficiency resulting in alteration of the TDP-43-regulated processes ("loss of function" hypothesis) [18-22]. Of course, the two hypotheses are not necessarily mutually exclusive, and studies with animal models have shown that TDP-43 overexpression can be neurotoxic, even without presence of inclusions [23-28]. Recent studies have highlighted how TDP-43 levels are tightly controlled: in fact, it has been demonstrated that TDP-43 can regulate its own expression through a negative feedback loop [29-35]. It has also been suggested that it has been observed that an increase of TDP-43 protein levels might activate the aggregation process in both cytoplasmic and nuclear compartments. Altogether these observations suggest that regulation of TDP-43 expression at transcriptional level might be also implicated in the pathogenesis of neurodegenerative diseases.

The study of the TARDBP gene promoter might therefore be useful to shed further light on the pathogenesis of TDP-43 proteinopathies. Interestingly, two SNPs were found within the promoter sequence of TARDBP gene in ALS patients [36]. However, no functional studies have been so far carried out.

In this work, we have characterized the cis-acting elements important for TARDBP gene regulation and evaluated its cell-type specificity. Then, we have characterized the possible impact of some cis-acting elements (SNPs and 5'UTR), trans-acting factors (TDP-43 itself) and events (splicing) on the transcriptional activity of TARDBP promoter.

Results

Bioinformatic analysis of the *TARDBP* promoter

The characterisation of *TARDBP* gene sequence (Ensembl gene ID: ENSG00000120948) was initially performed by a bioinformatic analysis using the UCSC Genome Browser (GRCh37/hg19). The *TARDBP* gene, mapped to chromosome 1p36.22 region, spans 12870bp from position 11072679 to 11085548 on the forward DNA strand. Inspection of UCSC TARDBP transcripts matched with those retrieved from the Database of Transcriptional Start Site (DBTSS, http://dbtss.hgc.jp [37]), it was evident that most of the transcription start sites coincide with the reference sequence's one (position 11072679) (Figure 1). Apparently, there are no tissue-specific transcription start sites (TSS), since only one mRNA species seems to be predominant in Adult and Fetal Tissues, as well as in different tissues. Figure 1 shows the TARDBP promoter sequence (starting from the 1316 nucleotide upstream from the TSS of NM_007375.3 TDP-43 transcript).

Subsequently, by querying the Eukaryotic Promoter Database [38], different putative TATA-box motifs ([p-value = 0.01]: -930, -865, -606, -571, -513, -309), CG-box sequences (CCAAT-box [p-value = 0.01]: -744, -677, -572, -61) were identified, but deeper inspection revealed that the positions are fully consistent with the main mapped TSS (DBTSS) . In addition, also the analysis performed with another promoter search tool (GPMiner [39]) failed to detect the main core promoter elements.

These observations suggest that the expression of the human TARDBP gene is driven by a TATA-less promoter.

Conservation of TARDBP promoter sequence throughout evolution

In order to study the evolutionary conservation of TARDBP promoter, the 1316 nucleotides upstream of the TSS of TARDBP transcripts from human and other species were retrieved using Genbank and Ensembl Genome Browser (http://www.ensembl.org/). Alignments were performed using MUSCLE software [40].

Among primates, we compared the genomic sequences of the putative TARDBP promoter region from Hominoidea (Homo sapiens - Human, Pan paniscus - Bonobo, and Nomascus leucogenys - Gibbon), from of New World monkey (Callithrix jacchus - Common marmoset) and from Old World monkey (Macaca mulatta - Rhesus macaque; Papio anubis - Olive baboon).

In general, this comparison highlighted that the relative degree of similarity among all three species increases significantly with respect the proximity to TSS. Then, in the region spanning from nucleotide -1316 to -1000 upstream from TSS, the hominoid sequences seem to be closer to each other than with that of New and Old World monkeys. On the other hand, in the region spanning the 1000 nucleotides upstream from TSS, the sequences from all primates show a high degree of identity (Figure 2). On the other hand, the alignment of the human, mouse and rat putative TARDBP promoter sequences shows a limited degree of identity (Figure 3). Nevertheless, the region of the rodent gene promoters spanning approximately 500nt upstream from the TSS shows a higher level of similarity, suggesting that proximal region of the putative TARDBP promoter region might encompass regulatory elements conserved across primates and rodents.

Characterization of cis-regulatory sequences of TARDBP promoter

In order to identify the minimal functional sequence responsible for the transcriptional activity of the promoter of the human TARDBP gene, a luciferase assay was set up with deletion-fragments of the putative TARDBP promoter constructs cloned into the pGL4 luciferase reporter vector. Different segments of the putative regulatory region of the promoter were amplified through a PCR reaction, and eight constructs differing in length were produced: the sequences encompassed between 27 nucleotides downstream from the TSS (+1nt, Refseq NM_007375.3) to 1316, 927, 451, 380, 320, 280, 230 and 180 nucleotides upstream from the TSS (Figure 4).

The promoter fragments were subcloned into the pGL4.11 vector, transfected in cell lines of different tissutal origin (HEK293, HeLa, Neuro2A and SH-SY5Y) and the luciferase activity of each construct was normalised versus the 1316 construct (i.e., 1316 =1).

Intracellular analysis showed that most promoter activity was retained in the fragments spanning from 1316 to 451 nucleotides upstream from the TSS within all tested cell lines (Figure 4). Further deletions resulted in dramatic reduction of activity (380 and 330 constructs), with almost complete loss of activity when the promoter was shortened to 230 and 180 nucleotides upstream from the TSS, in all tested cell lines. These results show that the minimal promoter region encompasses the 451 nucleotides upstream from the TSS.

We also tried to detect any kind of tissue-specific promoter activity. To this aim, we compared the activity of the 1316, 927 and 451 promoter fragments among SH-SY5Y, Neuro2A, HeLa, and Hek293 cell lines, considering the Hek293 cells as the unitary reference. The constructs showed approximately 6-8x higher activity in the neuronal cell lines (SH-SY5Y, Neuro2A) and HeLa than in HEK293 (Figure 5).

Transcriptional effects of SNPs found within the TARDBP promoter of ALS patients

The analysis of pathogenic mutations found in the *TARDBP* gene sequence of 46 australian patients of European descent affected by sporadic Amyotrophic Lateral Sclerosis (sALS) has revealed two promoter variants (c.1-562t>c and c.1-100t>c) with a different frequency in patients than in controls (115 neurologically normal people or HapMap European and Sub-Saharan African cohorts) [36]. The c.1-562t>c single nucleotide substitution (rs9430335; NG_008734.1:g.4439C>T; NM_007375.3:c.-696C>T) was found in homozygosity (C/C) at higher frequency and in heterozygosity (T/C) at lower frequency in sALS patients, as compared to controls (0.2 vs 0.06 and 0.1 vs 0.3, respectively). On the other hand, the c.1-100t>c polymorphism (rs968545; NG_008734.1:g.4901T>C; NM_007375.3:c.-234T>C) was present only in heterozygosity (T/C) at higher frequency (0.2 vs 0.1) than in controls [36].

Therefore, we sought to test the impact of these SNPs on the transcription of TDP-43, in order to find a possible functional correlation with the manifestation of ALS. In order to analyze the SNP effects alone or in combination, we created three variants of our 927 original pGL4 construct (4439:T; 4901:T), so reproducing all the possible alleles (4439:T; 4901:C), (4439:C; 4901:C) and (4439:C; 4901:T) (Figure 6A).

The constructs were transfected in SH-SY5Y cell line. The luciferase activity of the three mutant constructs was compared to that of control (4439:T; 4901:T), used as unitary reference (Figure 6B): n*o* statistically significant differences were observed in the transcriptional activity of the three variants (Figure 6B).

TDP-43 does not influence its own transcription

After characterizing the sequence of the promoter of TARDBP gene and its activity in different cell lines, we wished to evaluate the ability of TDP -43 to influence, directly or indirectly, the synthesis of its own transcript. For transfections of these constructs have been used our inducible HEK293-TDP wt cell line [29]. This cell lines was used as it allows a more homogenous TDP-43 overexpression, in comparison to transient overexpression of this factor.

These cells were transfected with the constructs 451, 927, and 1316 (along with Renilla plasmid). TDP-43 expression was driven by Tetracycline induction (48 hrs) and its levels were probed by Western blotting (Figure 7A). The analysis of the luciferase activity 48 hrs later did not show significant differences upon TDP-43 tetracycline induced overexpression (Figure 7B). Similar results were obtained with transient transfection of SH-SY5Y of the the constructs 451, 927, and 1316 (with the Renilla reporter) after TDP-43 transient overexpression (data not shown). These results suggest that TDP-43 does not influence its own transcription.

The TARDBP 5'UTR and intron 1 splicing positively impact the luciferase expression.

The 5'UTR region of TARDBP gene encompassed exon 1 (102bp) and the first 12bp of exon 2, separated by intron 1 (972bp). In order to explore the presence of additional elements able to modulate TDP-43, the

functional impact of the 5' UTR (construct 451+Ex1Ex2) and intron 1 (construct 451+Ex1-IVS1-Ex2) of *TARDBP* was analysed by generating some variants of the 451 plasmid (Figure 7C). A first variant was containing the TARDBP 5'UTR (exon 1, 102bp and exon 2, 12bp) correctly spliced (451+Ex1Ex2). The second construct was created by inserting the region encompassing exon1 (102bp) , intron 1 (972bp) and the first 12bp of exon 2 of TARDBP gene in between the 451 promoter and the luciferase ATG codon (451+Ex1-IVS1-Ex2 wt). The third construct was a mutant of latter construct where the 3' splice site of intron 1 was distupted (451+Ex1-IVS1-Ex2 mut).

In SH-SY5Y cells, the presence of a correcly sized amplicon was observed only after transfection of the 451, 451+Ex1Ex2 and 451 Ex1-IVS1-Ex2 wt constructs (Figure 7D). Apparently, the relative luciferase activities positively correlate with the occurrence of splicing. In fact, the same constructs were transfected in SH-SY5Y cells and their luciferase activity was measured (Figure 7E): the activity of the 451+Ex1Ex2 wt (containing the pre-arranged and correctly spliced 5'UTR region) was 1.5x that of the control. The 451+Ex1-IVS1-Ex2 wt construct showed a 5x increment in luciferase activity (as compared to the control, -451 construct). On the other hand, the 451+Ex1-IVS1-Ex2 mut construct showed negligible activity when compared with the other constructs (Figure 7E).

Altogether these results suggest that the presence of the 5'UTR as well as the correct splicing event of intron 1 are elements able to modulate the luciferase expression (and, potentially, of TDP-43) at transcriptional level.

Discussion

We have experimentally characterized the promoter region of the human TARDBP gene. A previous prediction indicated that the core regions of the TARDBP promoter could be located between 500 nucleotides upstream of exon 1 and 66 nucleotides downstream of exon 1 and in two regions of intron 1 (212 and 613 nucleotides) [36]. Our predictions failed to identify the canonical TATA box and CAAT box transcriptional regulatory elements and interrogation of the database DBTSS (cataloging the positions of TSSs in the genome) outlined how, in most TDP-43 transcripts, transcription starts from the TSS indicated in the main transcript isoform NM_007375. Regarding the spatio-temporal expression pattern, TDP-43 is a developmentally regulated protein [41,42], while its distribution in adult tissues seems to be ubiquitous [6,41,42]. These observations are consistent with the hypotheses supported by recent studies reporting that TATA box is present in only a minority of promoters [43−45], often with tissue-specificity such as liver and muscle [46−48].

From an evolutionary perspective, it is interesting to note that, among primates, the greatest degree of evolutionary conservation encompasses the 600nts upstream of the TSS. On the other hand, the alignment human-rodents outlines the 200nts upstream of the TSS as the region of higher homology. These observations suggested that the region in between plays an important role in directing transcription of the TARDBP gene. Indeed, this hypothesis is consistent with the experimental mapping

that has outlined the region spanning nucleotides 451-230 as necessary for the mininal promoter activity in all the tested cell lines (Figure 4).

The comparison of the transcriptional activity in different cell lines highlights how the TARDBP promoter activity is higher in cell lines of neuronal origin (Figure 5). It is well established that neuronal TDP-43 cytoplasmic inclusions are present in several different neurodegenerative diseases, such as FTLD, ALS and Alzheimer and that the sequestration of this protein in aggregates may create gain- and loss-of-function events causing (directly or indirectly) cellular toxicity or alteration of the TDP-43-regulated gene expression. Understanding the factors that control TDP-43 expression within cells will certainly provide a better insight into disease origin and progression. Indeed, it has been observed that an increase of TDP-43 protein levels can activate the aggregation process in both cytoplasmic and nuclear compartments [49,50]. In addition, studies with animal models have shown that TDP-43 overexpression can be neurotoxic, even without the presence of aggregates [23,27,28,51,52]. Indeed, although TDP-43 can regulate its own expression through a negative feedback loop [29], some observations suggest that regulation of its expression might may also occur at transcriptional level. In particular, TDP-43 expression seems to be developmentally regulated [42], but also TDP-43 expression has been shown to decrease with age in a variety of organisms such as fruitfly [53] and mouse [41].

Looking for potential modifiers of the TARDBP promoter activity, we have focused the attention on two SNPs (c.1-562t>c, rs9430335 and c.1-100t>c, rs968545) previously identified within the TARDBP promoter of SALS patients. Although the variants do not change any transcription factor binding site, we have functionally tested their transcriptional impact. We did not find in our system statistically significant differences in the promoter activity of each haplotype, and therefore, we can infer that the potential regulatory SNPs do not lead to variation of TARDBP promoter activity.

In addition, although the TDP-43 expression undergoes a tightly autoregulated via interaction with its 3'UTR, we verified if there are transcriptional effects of TDP-43 its own promoter activity and found that this factor does not modulate its own transcription.

Considering the known ability of the 5'UTRs to affect mainly protein translation efficiency [54], we next sought to test the possible effects of the TARDBP 5'UTR and intron 1 splicing on the expression of the associated reporter gene (Figure 7). On one hand, the observation of a 1.5x (vs the 451 "control" construct) significant increase in luciferase activity associated with the presence of the TARDBP "pre-arranged" 5'UTR (construct 451+Ex1Ex2) suggests that this element can positively influence gene expression. On the other hand, recent studies have shown that approximately 35% of human 5'UTRs are annotated as harboring introns [55] and recent observations suggest that introns in UTRs may have specific regulatory functions by affecting the rate of transcription rather than transcript stability. However, the finding that insertion of the intron 1 and the occurrence of splicing (construct 451+Ex1-IVS1-Ex2wt vs 451+Ex1-IVS1-Ex2mut) was able to raise the luciferase activity up to 5.25x (vs the 451 "control" construct) lead us to hypothesize that the splicing event is per-se responsible for the increase in luciferase activity.

In conclusion, growing lines of evidence suggest that the pathophysiology of ALS, FTD and other neurodegenerative diseases might be the consequence of the convergence of multiple risk factors. TDP-43 expression can be modulated during development [42], in a cell specific fashion, and, through aging, these fluctuations are evolutionary conserved [56]. These observations will lead to a better understanding of the factors involved in controlling the activity of the TARDBP promoter and give clues to uncovering novel processes able to modify the disease-onset and/or its progression.

Methods

Constructs

The 1316 bp region upstream of the TARDBP TSS was amplified by PCR and cloned in pGEM-T easy vector. The 1316bp insert was digested with the restriction enzymes KpnI and HindIII (these sites were designed into the primers used in PCR) and subcloned into the pGL4.1 vector (Promega, Madison, WI, USA) digested with the same restriction enzymes. Other 7 deletion-mutants of TDP-43 promoter (927, 451, 380, 330, 280, 230, 180bp upstream of the TSS) were then generated by PCR using gene-specific forward primers and the pGL4-1316 construct as template. Deletion-mutant inserts were KpnI-HindIII-subcloned in the pGL4.1 vector. TARDBP promoter SNPs were generated by PCR mutagenesis. The identity of all constructs was verified by DNA sequencing. Primer sequences are avalilable on request.

Transfections and Luciferase assays

The cell lines used for transfections were the following: HEK293, HeLa, Neuro2A, and SH-SY5Y. Each construct was transfected in duplicate, 600ng of DNA was diluted in a final volume of 100µl with Opti-MEM I Reduced Serum Medium (ThermoFisher, Waltham, MA, USA). To normalize for transfection efficiency, the cells were co-transfected with 20 ng/well of pRenilla (Promega, Madison, WI, USA) plasmid along with the promoter constructs. In parallel, 98µl of Opti-MEM were added to 2µl Lipofectamine 2000 Reagent (ThermoFisher, Waltham, MA, USA). After 5 min. at room temperature, the two solutions were combined and mixed, and 20 minutes later, the growth medium was removed from the cells and replaced with fresh DMEM containing 5% FBS. Then, the transfection mix was added to each well.

Total cell lysate was prepared from cells 48 h post transfection and firefly luciferase activity was assayed using the Beetle-Juice KIT (P.J.K. GmbH, Kleinblittersdorf, Germany) and a Turner Design 20/20 Luminometer (Turner BioSystems, Sunnyvale, CA, USA). Control transfections with pGL4.1 empty vectors were used as negative controls. Data presented are mean±Standard deviation (SD) of at least three independent experiments and given as fold expression overexpression indicated in the Figures set arbitrarily at 1. Statistical analysis was performed using Student's T or ANOVA tests. In order to verify the possible influence of TDP-43 on its own promoter, we used the previously generated human stable cell line Flp-In HEK293 -TDP-43 wild type, carrying the human TDP-43 transgene locus-specific-integrated, whose expression is driven by an inducible version of the Tetracycline (Tet) promoter [29].

One day before transfection, Flp-In HEK293 -TDP-43 wild type (wt) cells were seeded at 60% confluence. Lipofectamine reagent was used for transfection with 300ng of the reporter plasmids and 20 ng of pRenilla vector. After 12h, the medium was changed, and the cells were incubated further for 24 h. Flag-tagged TDP-43 expression was induced with 1μg/ml tetracycline. The luciferase/renilla activities were then assayed and statistical analyses were performed using unpaired Student's T-test.

The relative expression of constructs 451, 451+Ex1Ex2, 451 Ex1-IVS1-Ex2 wt and 451 Ex1-IVS1-Ex2 mut constructs was performed by semi-quantitative RT – PCR, by using GAPDH as internal control. The sequences of primers were (5'-3'): GAPDH_Ex1s, CGCTCTCTGCTCCTCCTGTT; GAPDH_Ex2as, CCATGGTGTCTGAGCGATGT; TDP-43 Ex1+9 (277bp)_s, AGCTTGCGCCATTTTGTGGGAGCGA; Luc ATG+155(277bp)_as,GTAATGTCCACCTCGATATGTGCGT. The optical density of each amplicon was calculated using the ImageJ image processing program [57] and the expression levels were numerically expressed as the ratio of applicant density of the target amplicons over that of GAPDH (81 bp).

Western blot

Cells extracts were prepared in PBS containing 1x protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA). Proteins were separated by SDS-PAGE and transferred to nitrocellulose (Cytiva, formerly GE Healthcare, Chicago, IL, USA) and protein detection was carried out with standard Western blotting techniques. After transfer, membranes were incubated for 10h in blocking solution (5% nonfat dry milk in PBS containing 0.1% Tween-20, T-PBS) to prevent aspecific binding. Subsequently, membranes were incubated for 1 h at room temperature with specific primary antibodies diluted in blocking solution. Expression levels of TDP-43 was monitored by using a commercially available mouse monoclonal FLAG antibody (Sigma Aldrich, St. Louis, MO,USA). Endogenous tubulin was used as a loading control, using an in-house made mouse monoclonal antibody. Immunoblots were developed by using the ECL Star Enhanced Chemiluminescent Substrate (EuroClone, Pero, Milan, Italy).

Declarations

Aknowledgments

Author contribution statements

Study conception and design: MR; Acquisition of data: MB and MR; Analysis and interpretation of data: MB and MR; Drafting of manuscript: MR.

Competing interests
The Authors declare no competing interests.

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Figures

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-1316  aaggaaggtgggaagggagggaagggggggaaagggagggaagggaggaa  50
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  -16  ggtgggagaggacgcc                                    1316
   +1  GGTGGGCGGGGGGAGGAGGCGGCCCTAGCGCCATTTTGTGGGAGCGAAGC  1366
  +50  GGTGGCTGGGCTGCGCTTGGGTCCGTCGCTGCTTCGGTGTCCCTGTCGGG  1466
 +100  CTTCCCAGCAGCGGCCTAGCGG                              1588
```

Figure 1

Map of the humanTARDBP promoter. Proximal sequences (-1316/+122) of the human TARDBP promoter are shown. Transcript reference: NM_007375.3. The promoter sequences are numbered (left side) relative to the transcription start site (TSS, +1).

```
-1316   aaggaaggtgggaagggagggaagggggggaaagggagggaagggaggaa   50
-1266   aggaagggaaggaaagggaaagaaaggaaagaaaagatagaaaagaaaggt   100
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-1166   cttgaggccatgagttcgagatcagcctggccaacatggccaaaccctgt   200
-1116   ctctactaaaaatacaaaaattagccgggcatggtgtctttgcacgccta   250
-1066   taatcccagctatcccttgaacctaggagttggaagtggcagtgagctgc   300
-1016   agattgcgccactgcactccagcctgggggacaagagcgagactccgtct   350
 -966   caaaaaaaaaaaaaaaaaaagtgaaacaaaaagtgtgccttaaaatagat   400
 -916   gaagtactgtattattattattattaaagggctggagccagaacaagtta   450
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 -716   accgcgttcaagcaattctcctgcttcaggctcccgagtagatgggacta   650
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  -66   gagggtcatcgtggctctcgtcggtgccttccacctctggacaggcaacc   1300
  -16   ggtgggagaggacgcc                                    1316
   +1   GGTGGGCGGGGGGAGGAGGCGGCCCTAGCGCCATTTTGTGGGAGCGAAGC   1366
  +50   GGTGGCTGGGCTGCGCTTGGGTCCGTCGCTGCTTCGGTGTCCCTGTCGGG   1466
 +100   CTTCCCAGCAGCGGCCTAGCGG                               1588
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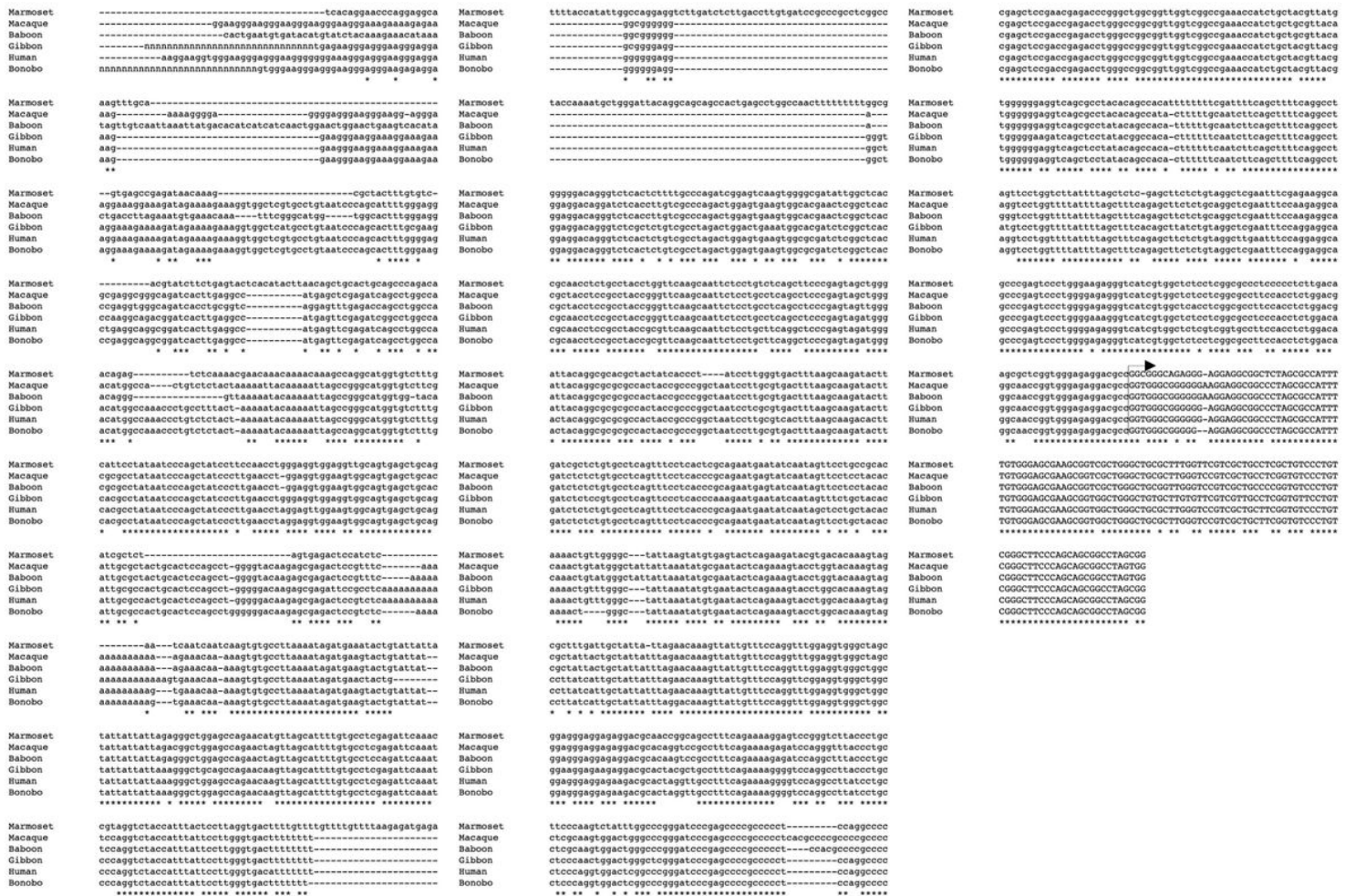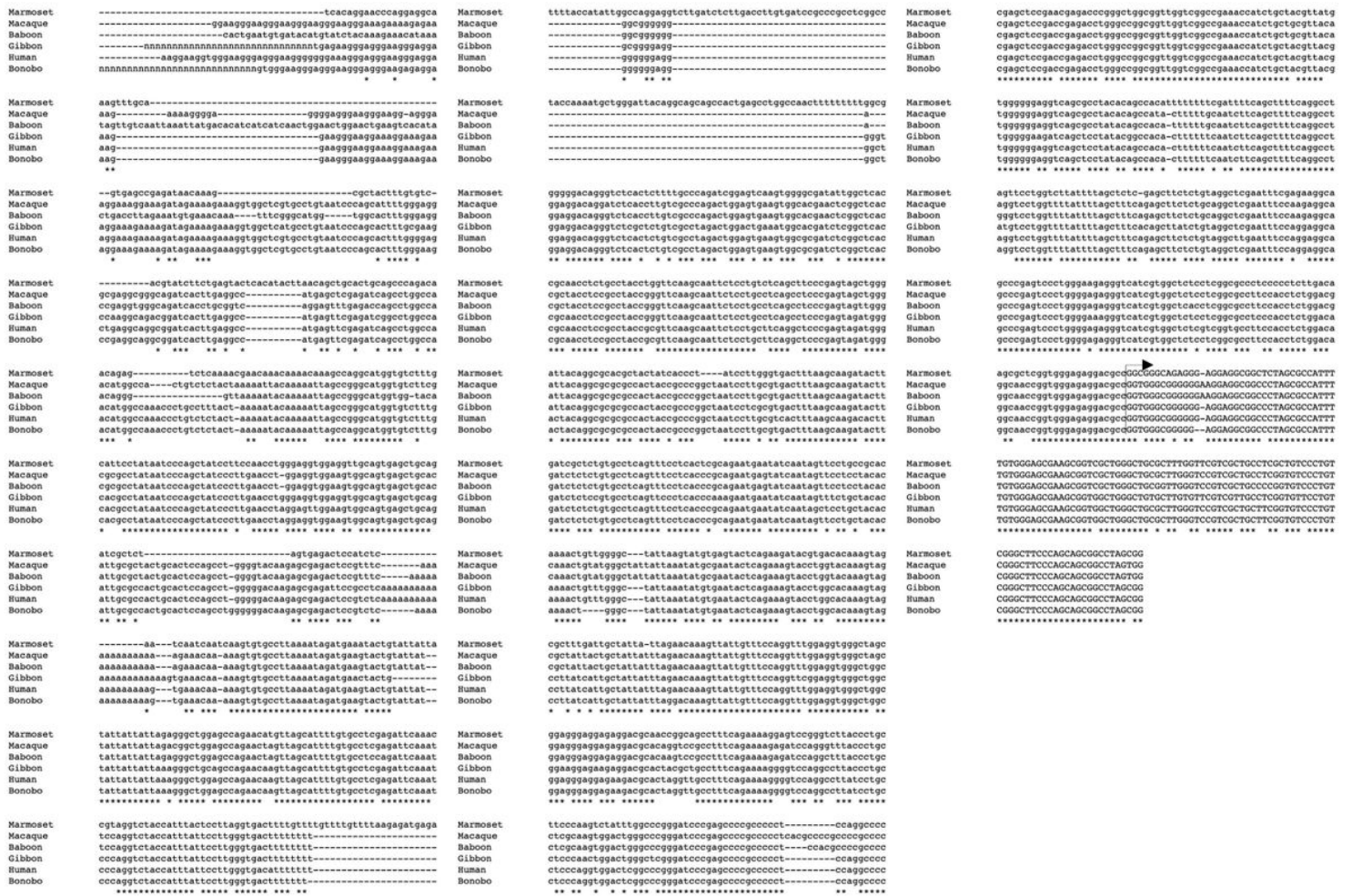
Figure 2

Genomic alignment of putative promoter regions from Homo sapiens, Pan troglodytes and Callithrix jacchus TARDBP genes. The alignment of the 1316nt – sequence upstream and 78bp dowstream of the transcription starting site of Human (Homo sapiens, ENSG00000120948, NM_007375.3) was carried out versus: Marmoset (Callithrix jacchus, ENSCJAG00000002381), Macaque (Macaca mulatta, ENSMMUG00000007456), Olive baboon (Papio anubis, ENSPANG00000017459), Gibbon (Nomascus leucogenys, ENSNLEG00000009512) and Bonobo (Pan paniscus, ENSPPAG00000039086) TARDBP transcripts, by using the MUSCLE alignment program (http://www.ebi.ac.uk/Tools/muscle/index.html).
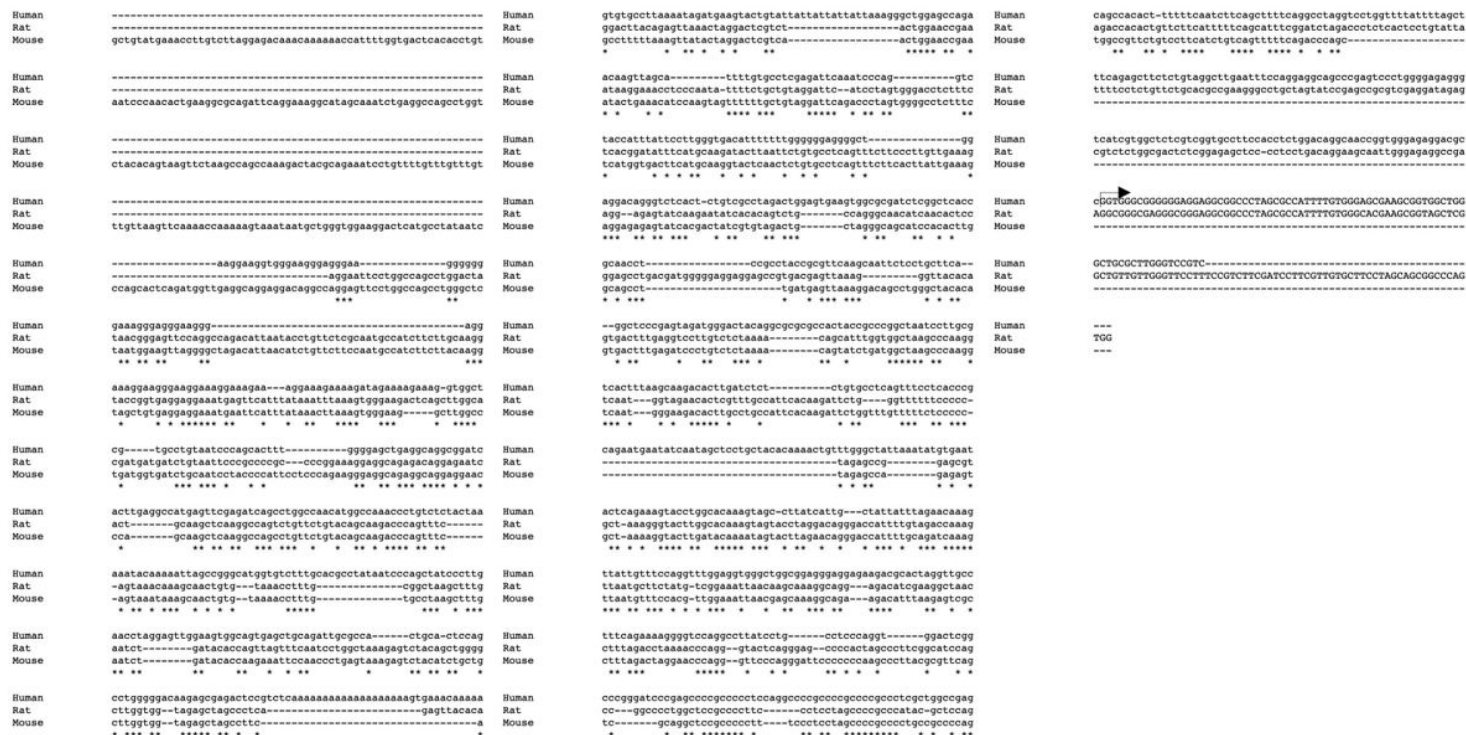
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Figure 3

Genomic alignment of putative promoter regions from Homo sapiens, Mus musculus and Rattus norvegicus TARDBP genes. The alignment of the 1316nt − sequence upstream of the transcription starting site of Human (Refseq: NM_007375.3), Mouse (ENSMUST00000084125) and Rat (ENSRNOT00000049822) TARDBP transcripts was generated with MUSCLE alignment software (http://www.ebi.ac.uk/Tools/muscle/index.html).
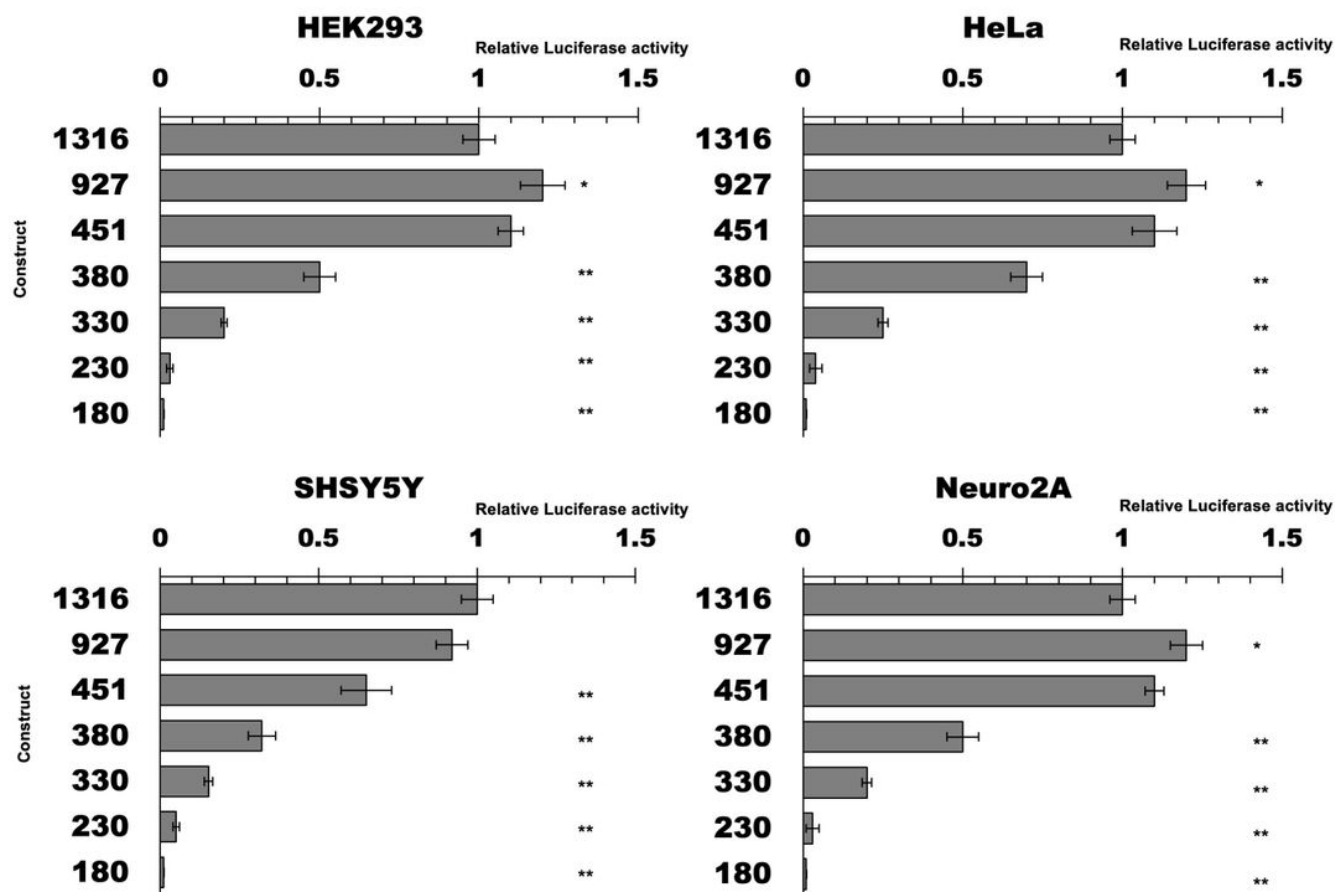
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Figure 4

Deletion analysis of the human TARDBP promoter. Firefly luciferase activity of TARDBP promoter deletants in different cell lines (HEK293, HeLa, SH-SY5Y and Neuro2A) was measured in the cell lysate and values were normalized against Renilla. Activity of deletants is expressed as-fold against the cells transfected with the 1316 construct (=1). Data are mean (±SD) of three independent assays. Significance values refer to comparisons against 1316 construct: *, p < 0.05, and **, p< 0.01.
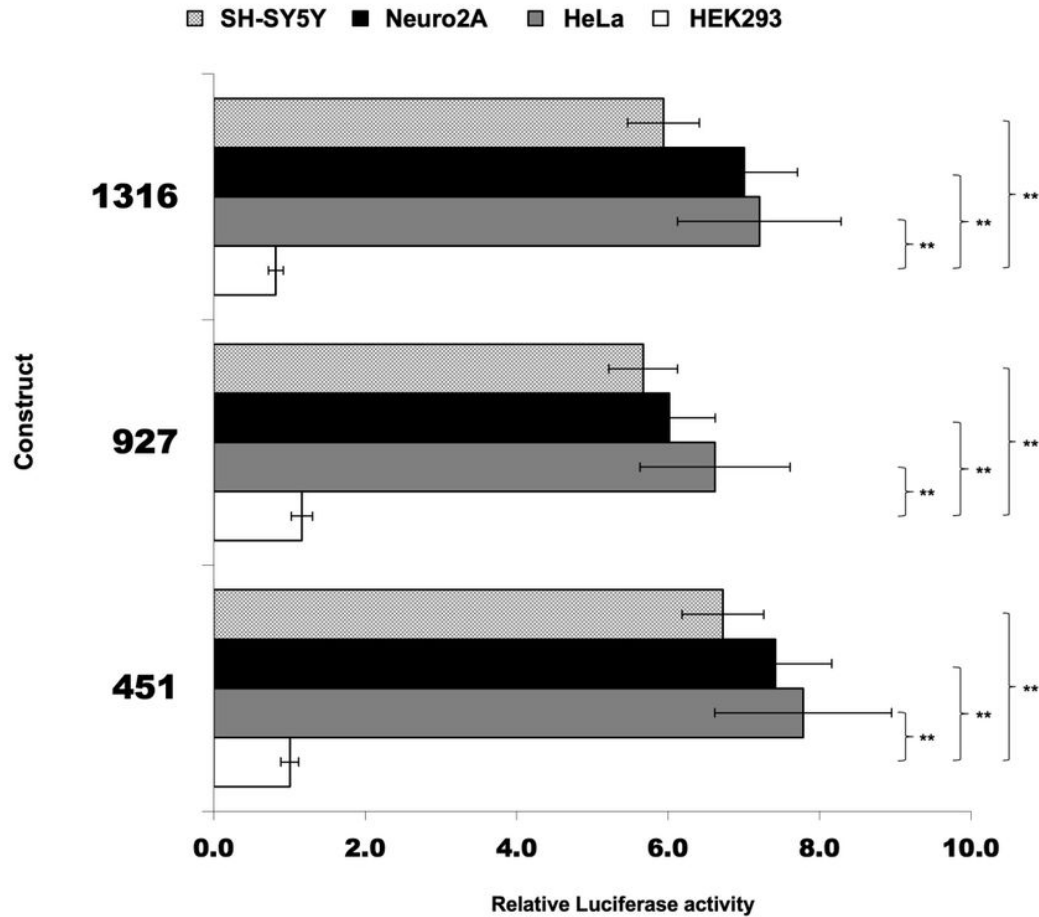
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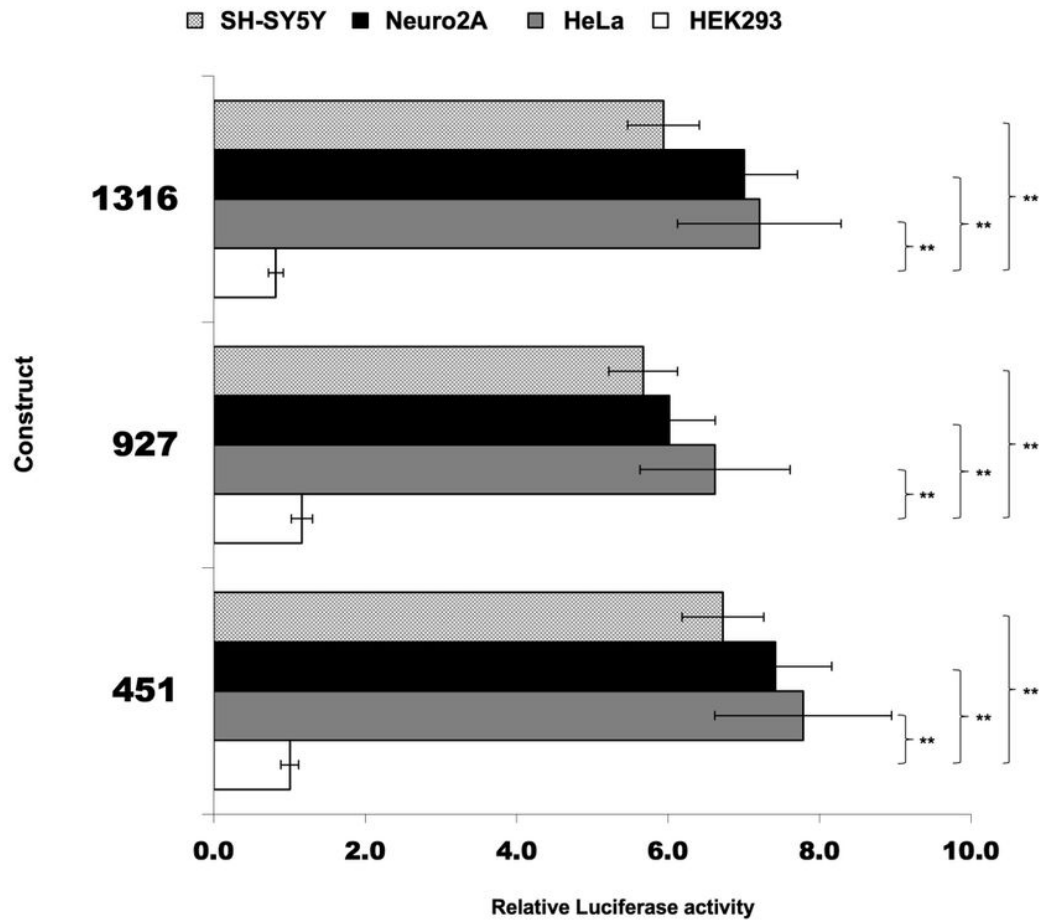
Figure 5

Comparison of the human TARDBP promoter activity in different cell lines. Firefly luciferase activity of 1316, 927 and 451 constructs was tested (normalized against Renilla) in different cell lines (HEK293, HeLa, SH-SY5Y and Neuro2A). Activity is expressed as-fold against transfections in HEK293 cells (=1). Data are mean (⊡SD) of three independent assays. Significance values refer to comparisons against HEK293 cells: *, p < 0.05, and **, p< 0.01.

Figure 5
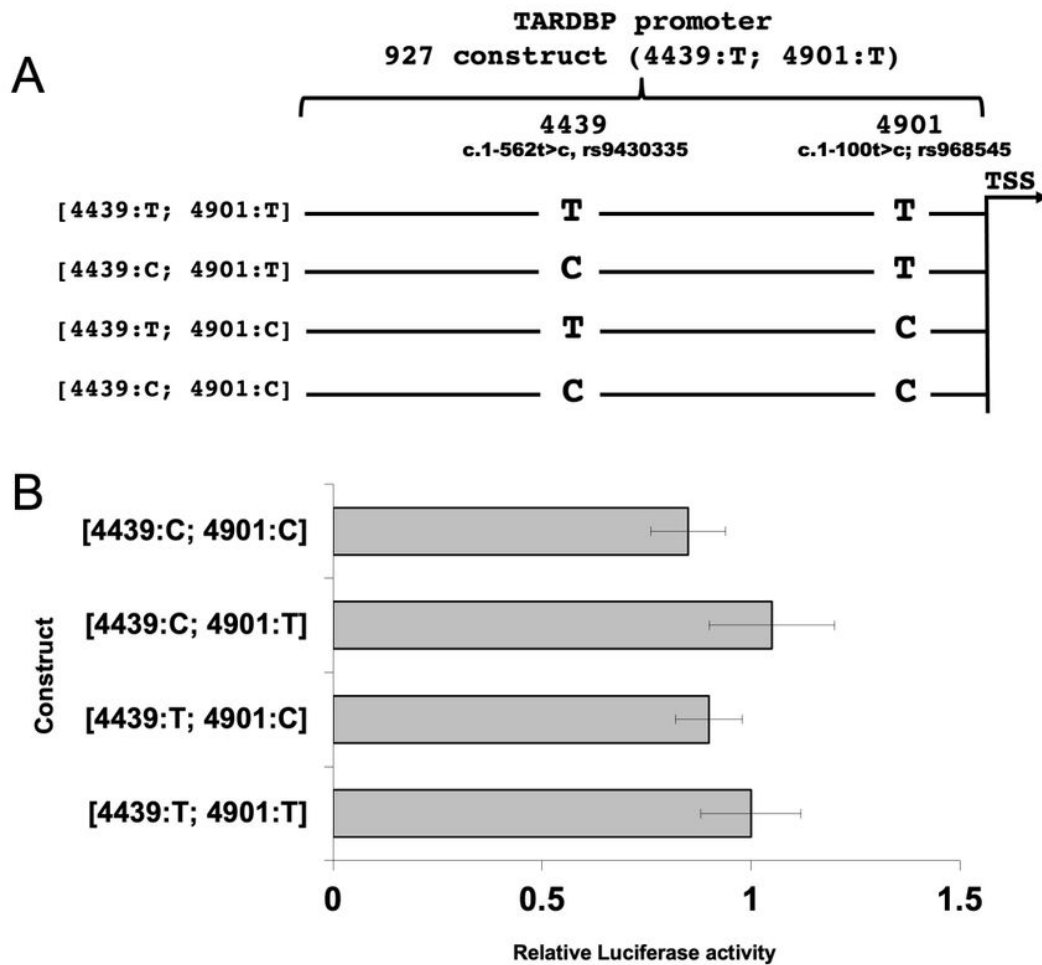
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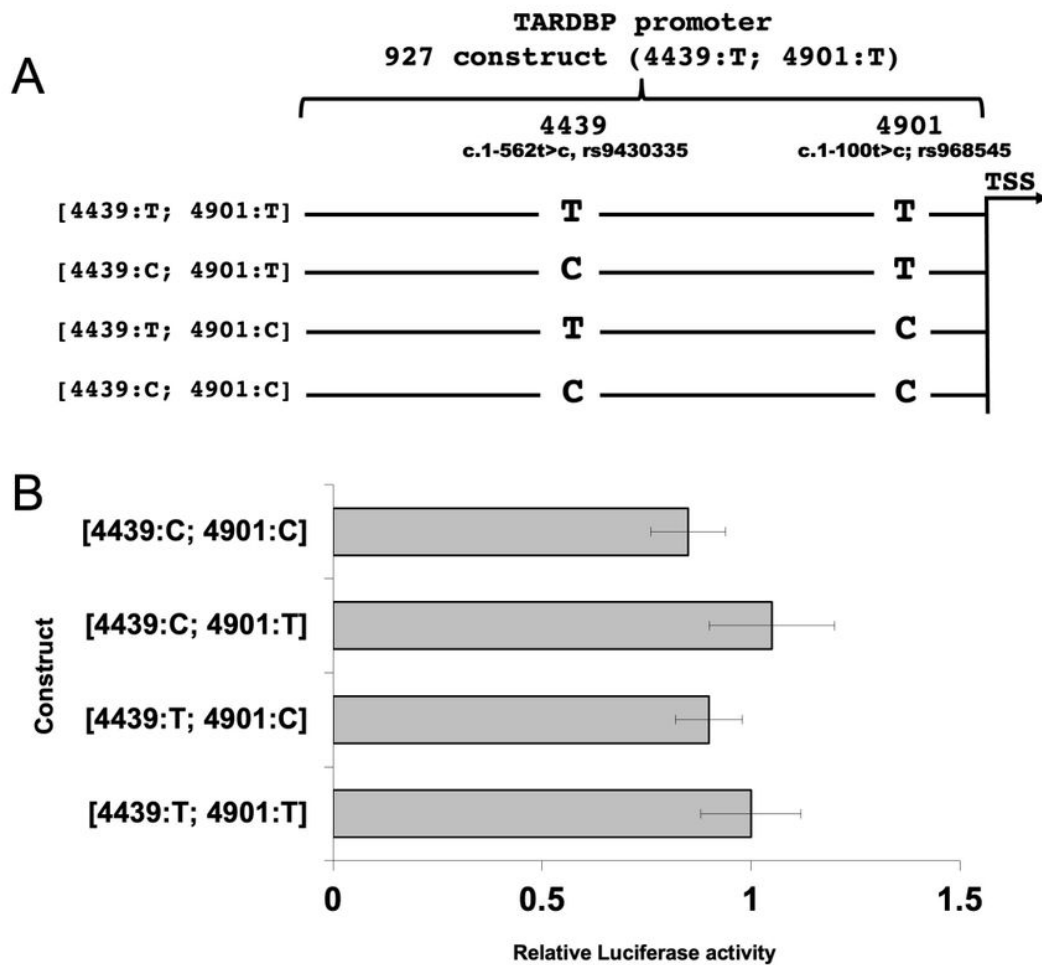
Figure 6

Effects of ALS-related SNPs on TARDBP promoter activity. A) Schematic representation of the human TARDBP promoter variants. The diagram depicts the wild type and the mutated promoter sequences used for the luciferase assay. The mutated nucleotides (and their positions) are indicated. B) Luciferase activity (normalized against Renilla) is expressed as-fold against transfections of the haplotype [4439:T; 4901:T] (=1). Data are mean (±SD) of six independent assays. Significance values refer to comparisons against the haplotype [4439:T; 4901:T]. No statistically significant differences were observed.

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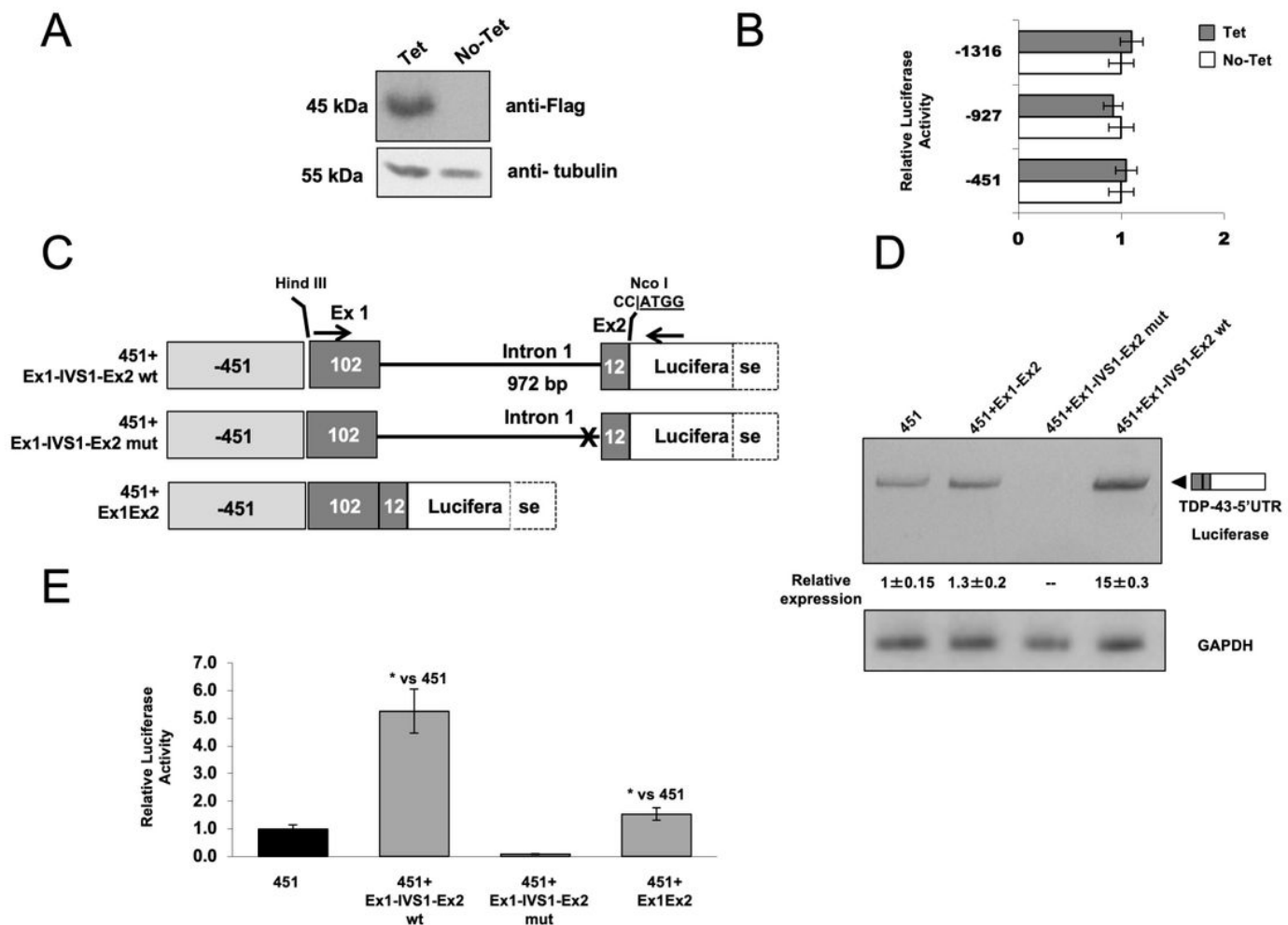
Figure 7

Effects of trans- and cis-acting elements on the transcriptional activity of the human TARDBP promoter.
A) Immunoblot analysis of TDP-43 expression after 48h-Tetracycline induction (Tet) of the HEK293-Flp-In-TDP-43 wild type stable cell line. Beta-Tubulin was used as loading control to normalize the levels of detected protein. B) Effects of TDP-43 overexpression on TARDBP promoter activity. The luciferase activity of three different constructs (1316, 927 and 451) transfected in the HEK293-Flp-In-TDP-43 wild type stable cell line was assayed without (No-Tet) or after (Tet) Tetracycline induction for 48h. No statistically significant differences were found. Data are mean (⬜SD) of three independent assays. Significance values refer to comparisons against control transfections (No-Tet). C) Diagrams of the 5'UTR TDP-43 constructs generated using the pGL-451 vector. In the 451+Ex1-IVS1-Ex2 wt construct, the 1086bp genomic region of the human TARDBP gene spanning the 5'UTR, including Exon 1 (102bp), Intron 1 (972bp) to the first 12 nt of Exon 2) was (Hind III-Nco I) cloned in between the 451nt-promoter and the firefly luciferase ORF (Luciferase). In the 451+Ex1-IVS1-Ex2 mut construct, the 3' splicing site of Intron 1 was deleted. In the 451+Ex1Ex2 construct, the fragment encompassing Exon 1 and Exon 2 (without Intron 1) was cloned in the 451 vector. D) RT-PCR of the mRNA species carrying the TDP-43 5'UTR and

luciferase ORF (upper panel). The relative quantitation of expression levels was carried out by optical densitometry of the PCR bands captured under ultraviolet light. The 451 construct was used as control (=1). Amplification of GAPDH (lower panel) was used as the endogenous control in the quantitative analysis of RT-PCR. Data are mean (☐SD) of three independent assays. Significance values refer to comparisons against control transfections (451): p <0.05. E) Luciferase activity of 5'UTR TDP-43 constructs. Transient cotransfections (luciferase and renilla) were performed in SH-SY5Y cells. After 48h, cells were assayed for firefly luciferase expression (normalized against renilla). The empty expression vector was tested and produced background levels of luciferase activity (data not shown). Data are mean (☐SD) of three independent assays. Significance values refer to comparisons against control transfections (451): p <0.05.
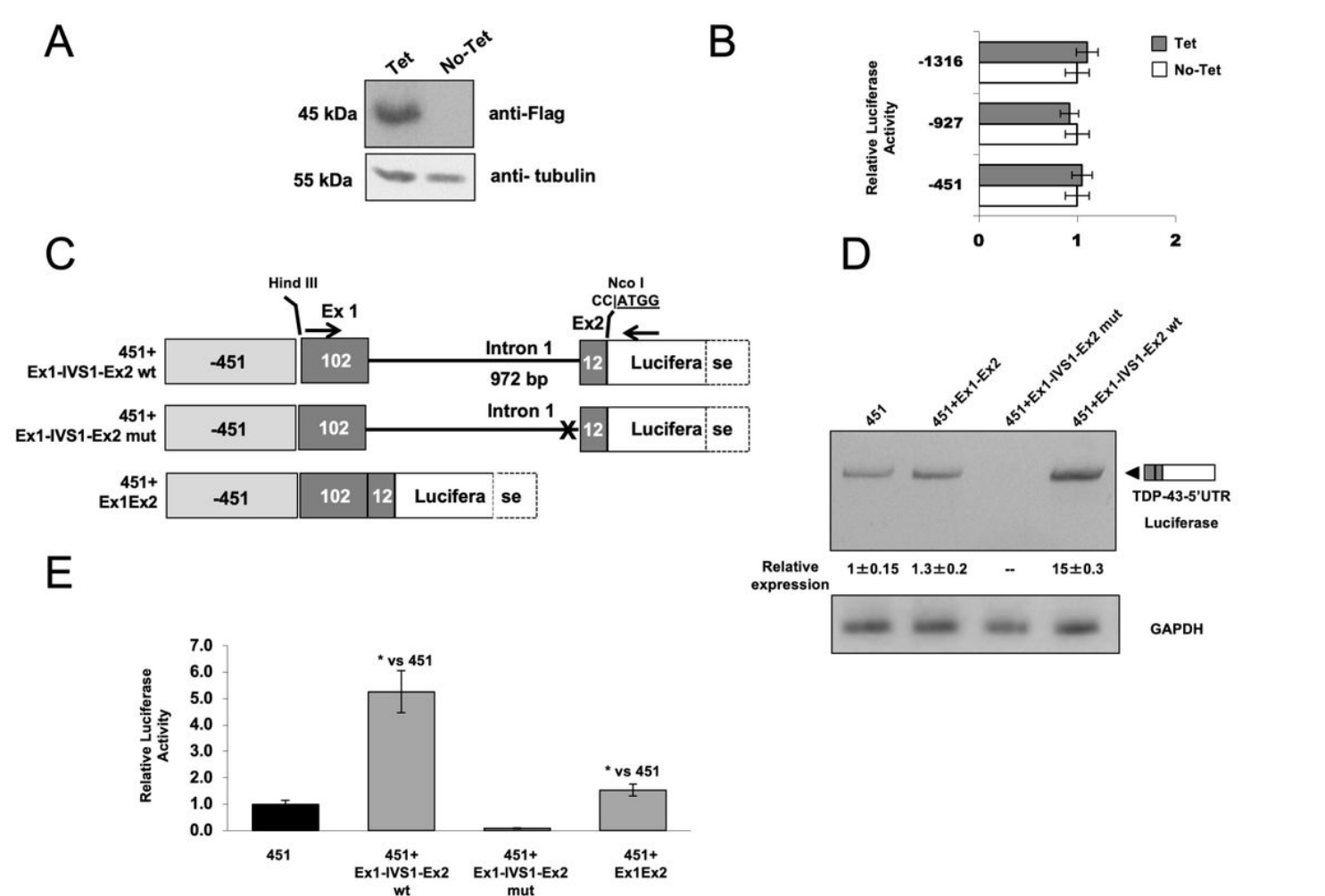

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