

Disorders of the Nervous System

Alternative *REST* Splicing Underappreciated

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As a major orchestrator of the cellular epigenome, the repressor element-1 silencing transcription factor (REST) can either repress or activate thousands of genes depending on cellular context, suggesting a highly context-dependent REST function tuned by environmental cues. While *REST* shows cell-type non-selective active transcription (Kojima et al., 2001), an N-terminal REST4 isoform caused by alternative splicing, inclusion of an extra exon (N_{3c}) which introduces a pre-mature stop codon, contributes to neurogenesis and tumorigenesis (Palm et al., 1998, 1999; Lee et al., 2000; Raj et al., 2011). Recently, in line with established epigenetic regulation of pre-mRNA splicing (Alló et al., 2010; Luco et al., 2011), we demonstrated that *REST* undergoes extensive, context-dependent alternative splicing which results in the formation of a large number of mRNA variants predictive of

multiple protein isoforms (Chen and Miller, 2013). Supported by the fact that immunoblotting/-staining with different anti-REST antibodies yield different results, alternative splicing allows production of various structurally and functionally different REST protein isoforms in response to shifting physiologic requirements, shedding light on environmental regulation of REST function. However, REST isoforms might be differentially assayed or manipulated, leading to data misinterpretation and controversial findings. For example, in contrast to the proposed neurotoxicity of elevated nuclear REST in ischemia (Noh et al., 2012) and Huntington's disease (Zuccato et al., 2003; Buckley et al., 2010), Lu et al. recently reported decreased nuclear REST in Alzheimer's disease and neuroprotection of REST in aging brain (Lu et al., 2014). Unfortunately, alternative *REST* splicing was largely neglected by Lu et al. (2014), making it necessary for a reevaluation of their findings.

As shown in Figure 1A, human *REST* gene boundary is now doubled by an alternative last exon (E_5), which is mutually exclusive to E_4 . While numerous novel alternative exons and 5'/3' ends were identified, the three constitutive exons (E_2 , E_3 and E_4) comprising the open reading frame (ORF) of *REST* can be skipped partially or completely, alone or in combination, producing at least 45 mRNA variants predictive of multiple protein isoforms (Fig. 1B; Chen and Miller, 2013). For example, REST4, which was first described in rat as a group of REST isoforms (Palm et al., 1998), is predicted by multiple mRNA variants (e.g., JX896958, JX896971, and JX896983) with E_3 followed by variable exons that introduce a pre-mature stop codon. Accordingly, like the case in rat, human REST4 is also a group of isoforms produced by variable splicing predictive of C-terminal truncated proteins that share the same functional domains (RD1 and ZFs 1–5; Fig. 1B), and it should no longer be considered as a single mRNA/protein isoform. Meanwhile, REST1, another N-terminal REST isoform, is predicted by multiple mRNA variants lacking E_3 . In addition, for the ubiquitously distributed E_2 -skipped variants (e.g.,

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Figure 1. Bioinformatics at human *REST* locus (**A**) and predicted REST protein isoforms derived from alternative splicing (**B**). Related tracks were retrieved from the UCSC Genome Browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>). *REST* gene boundary is more than doubled by an alternate last exon (E_5) which partially overlaps in opposite direction with exon 5 of *NOA1*. *REST* promoter harbors a CpG island and exhibits cell-independent active transcription as indicated by the chromatin state segmentation and H3K27Ac tracks. Predicted ORFs of the full-length and alternatively spliced REST mRNAs were briefly shown by indicating the start (blue star) and stop (red star) codons, while major domains (RD1 and RD2, repression domain 1 and 2; NLS, nuclear localization signal; and zinc fingers 1–9) of the full-length REST protein were illustrated in parallel to their coding sequences. Splice variants expressed in multiple tissues or cell lines were bolded. Locations of the mRNA and protein fragments targeted by real-time PCR primer sets (P1–P4), RNAi (shRESTa and shRESTb), and antibodies mentioned in the text were indicated. Note that only the conventional promoter is shown and that the internal region of E_4 is unconserved as indicated by the “100 vertebrate conservation” track, which supports our finding that partial skipping of E_4 is common (Chen and Miller, 2013).

XM_005265760 and JX896960) missing the conventional start codon, an in-frame AUG in E_3 may initiate translation of a C-terminal REST^C isoform (XP_005265817), which was recently described in *Rest* conditional knock-out (cKO) mice (Nechiporuk et al., 2016), while some partial E_2 -skipped

variants (e.g., JX896978 and KC117266) containing the conventional start codon are predictive of proteins missing variable regions of REST. Moreover, it was recently demonstrated that mRNAs with short ORF but previously annotated as noncoding RNAs can actually encode tiny peptides

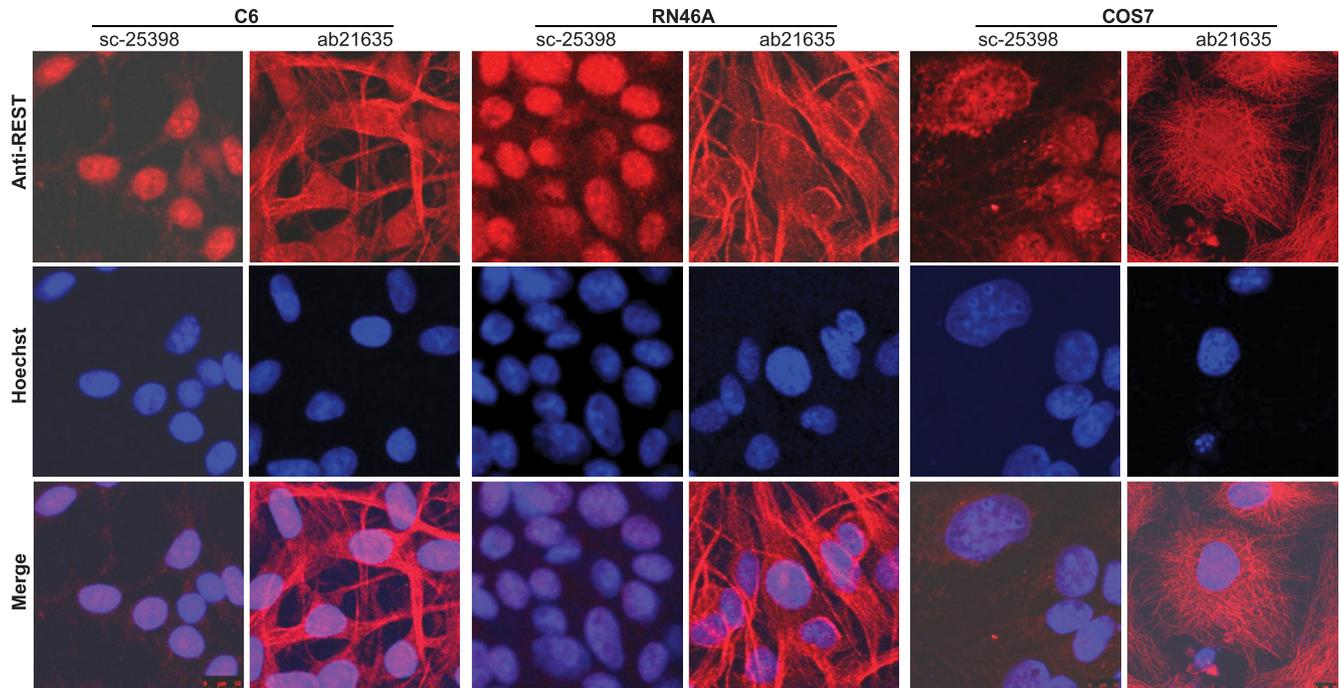


Figure 2. Immunofluorescence analysis of REST subcellular localization in different cells with two different antibodies. Immunocytochemistry (ICC) assays were performed with two anti-REST sc-25398 (Santa Cruz) and ab21635 (Abcam), which are, respectively, against N and C terminus of REST, for C6, RN46A, and COS7 cells. For each cell line, two wells of cells under the same experimental conditions were stained with sc-25398 and ab21635, respectively. Briefly, cells cultured on poly-D-lysine-coated coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and incubated with sc-25398 (1:100) or ab21635 (1:200), followed by incubation with a goat anti-rabbit secondary antibody conjugated with Alexa Fluor 568 (1:500, Invitrogen). Nuclei were stained with Hoechst-33342 (Thermo Scientific), and cells were mounted on glass slides. Confocal microscopy was performed using a Leica TCS SP5 Spectral Confocal Microscope. For each cell line, all experimental conditions were kept the same for the two antibodies. Regardless of the cell-types, ICC with sc-25398 yielded predominant localization of REST in nucleus, whereas ICC with ab21635 indicated predominant colocalization of REST with microtubule (or cytoskeleton), suggesting that REST isoforms with different subcellular localization might be differentially recognized by different antibodies.

(Magny et al., 2013; Olexiouk et al., 2015; Nelson et al., 2016), such might be the case for numerous REST variants (e.g., JX896962, JX896965, and JX896967). Taken together, REST protein isoforms caused by alternative splicing are much more complex than we expected.

Because of the existence of multiple REST mRNA and protein isoforms, it can be inferred that assay of REST expression by different primers (or probes) and antibodies may target different REST isoforms, while manipulation of REST expression by cKO or RNAi may be effective for specific but not all REST variants. In other words, REST isoforms might be differentially assayed or manipulated in different studies, leading to inconsistent results and data misinterpretation. In support of this notion, immunostaining of multiple cell lines with two widely used antibodies, sc-25398 and ab21635 raised against N and C terminus of REST, respectively, produced inconsistent results in terms of REST subcellular distribution and its colocalization with microtubule (Shimojo, 2008; Buckley et al., 2010; Fig. 2), while immunoblotting [i.e., Western blotting (WB)] with the two antibodies yielded different profiles of immunoreactive (IR) bands (Fig. 3), such is the case for some other commercial anti-REST antibodies as described by manufacturer's manual. Unfortunately, despite the mRNA

evidence, not all REST protein isoforms have been experimentally verified and normally they are not observed as expected sizes due to post-translational modifications, making it challenging to determine whether an unknown IR band is non-specific or a REST isoform. For example, REST⁴ and REST^C are predicted as 37 and 86 kDa but observed as 53 and 130 kDa, respectively (Lee et al., 2000; Nechiporuk et al., 2016), while the full-length REST has been reported as variable sizes ranging from 120 to 200 kDa (Liang et al., 2011; Zhang et al., 2011; Nechiporuk et al., 2016). So, even if detectable by WB, specific REST isoforms might be simply considered as non-specific and excluded from being presented in publication, such may explain why REST^C was not reported until recently.

In their paper describing altered nuclear REST in aging and AD brain, Lu et al. claimed that REST⁴ mRNA (N_{3c}) level in brain tissues comprised only 0.1–0.5% of REST mRNA (Lu et al., 2014), while a number of neuronal splice variants produced by ΔE_2 , ΔE_3 , and ΔE_4 (or inclusion of E_5 ; Chen and Miller, 2013), of which ΔE_3 eliminates a motif critical for nuclear targeting (Shimojo et al., 2001; Shimojo, 2006) and therefore affects nuclear REST (Chen et al., 2017) were not mentioned. It can be simply inferred

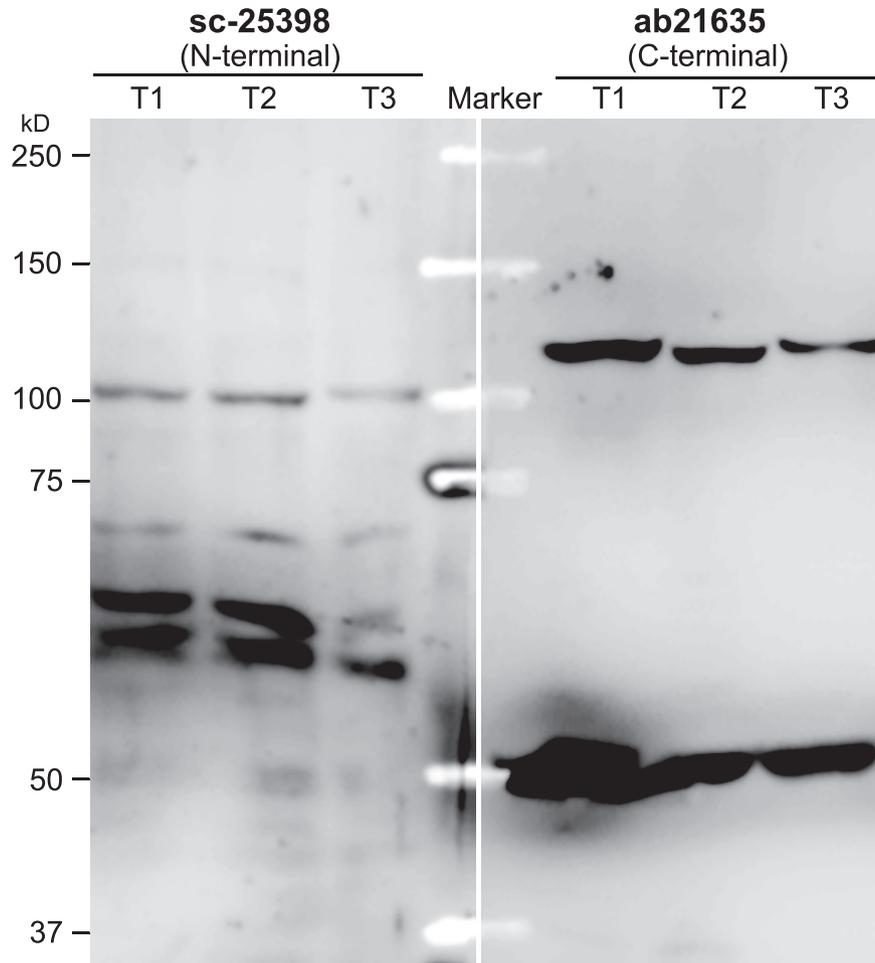


Figure 3. WB of REST expression in HEK-293T cells with two different antibodies. Two aliquots (25 μ g for each) of three different HEK293T protein samples (T1, T2, and T3), which were isolated simultaneously with RNA and DNA by TRIzol reagent (Invitrogen), along with a Kaleidoscope marker (Bio-Rad) in between, were loaded on a 7.5% PAGE-SDS gel, followed by electrophoresis and electrotranslocation onto an Immun-Blot PVDF membrane (Bio-Rad), which was then cut into two halves for incubation with sc-25398 (1:250) and ab21635 (1:500), respectively, and subsequent incubation with a goat anti-rabbit IgG (Sigma-Aldrich, 1:2500). IR signals were detected using the VisiGlo Select HRP Chemiluminescent Substrate kit (Amresco) with an ECL-based LAS-3000 image system (Fujifilm). Note that the two antibodies yielded totally different profiles of IR bands.

that if only the full-length REST mRNA exists, all segments of it should share the same level of expression; however, in accordance with the above-mentioned notion of inconsistent results yielded by different primers, qRT-PCR data in Lu et al., indicated that four primer sets (P1–P4) targeting different exons of *REST* yielded strikingly different changes in *REST* mRNA expression. Notably, patterns of this primer-dependent result varied across the aged groups. For instance, P2 assay showed the highest and lowest fold change for the 95-year and >95-year group, respectively, while some assays for aging groups (e.g., P1/P4 for 71 year, P1/P3/P4 for 95 year, and P2 for >95 year) showed mRNA expression levels similar to the 25-year group, suggesting that systematic error made minor contribution to this primer-related discrepancy, which however can be explained by individual variation in alternative *REST* splicing described in our previous study (Chen and Miller, 2013). So, qRT-PCR data presented by Lu et al. actually provided strong evidence for alternative

REST splicing, which unfortunately was not interpreted in the paper. In addition, unlike Northern blotting, which gives size information for observed mRNAs, qRT-PCR measures abundance of a specific amplicon (i.e., a segment of mRNA), which can be shared by multiple mRNA variants, such that qRT-PCR data may represent expression of multiple splice variants yielding the same amplicon but not merely the full-length *REST* mRNA. Hence, without evidence of Northern blotting, it is difficult to interpret the full-length *REST* mRNA expression level with the primer-dependent qRT-PCR data in Lu et al. Also, given that most of the previously reported mRNA variants were not tested and that the four qRT-PCR primer sets yielded different results, it is unknown how the total mRNA level and the percentage of REST4 mRNA in brain tissues were calculated. Meanwhile, Lu et al. performed a series of experiments (e.g., RNAi, ChIP-seq, and oxidative stress) using the SH-SY5Y cell line, which reportedly expresses abundant REST4 mRNA (N_{3c}) and protein (Palm et al., 1999;

Table 1. A summary of IHC assay mentioned in Lu et al., with and without the addendum

Antibody	Without addendum		With addendum			
	IHC-00141, ab28018, sc-15118		IHC-00141	ab28018	sc-15118	ab202962
Sample (n)						
Total	171	171	7 of 171	49 of 171	35 of 171	
Young	11	11	?	10	10	
Aged	77	77	?	21	14	
AD	72	72	?	18	11	
MCI	11	11	?	-	-	
Result	All presented IHC data		All presented IHC data	Data not shown	Data not shown	Data not shown

Yu et al., 2009; Chen and Miller, 2013); however, REST4 expression in SH-SY5Y was not mentioned in the paper.

At the protein level, Lu et al. assayed REST protein expression and subcellular distribution by immunoblotting/immunostaining with a total of six different antibodies, of which two (07-579 and ab52850) and three (ab28018, sc-15118, and IHC-00141) were used for WB and immunohistochemistry (IHC), respectively. As mentioned above, due to the existence of multiple REST protein isoforms, different antibodies may yield different WB/IHC results, while WB with a specific antibody may yield multiple IR bands which represent different REST isoforms sharing the same epitope. So, comparison of the WB/IHC results between different antibodies may hint about the existence of multiple REST protein isoforms; however, no such comparison was shown in Lu et al., while all the presented blots (even for the SH-SY5Y cells with REST4 expression) were maximally cropped with only the band of interest (presumably represents the full-length REST) available, making it impossible to evaluate the potential existence of multiple REST isoforms. Although Lu et al. performed immunostaining to test specificity of one IHC antibody (IHC-00141), the existence of multiple REST isoforms cannot be excluded, because isoforms sharing the same epitope can all bind to the same antibody and this binding can be eliminated by the same blocking peptide.

Notably, it was not disclosed in Lu et al. how the three IHC antibodies were assigned to samples of different groups, giving rise to the concern that nuclear REST differences between the experimental groups might be artificially generated by biased usage of the antibodies for different samples. For example, comparison of nuclear REST between young ($n = 11$), aged ($n = 77$), AD ($n = 72$), and MCI ($n = 11$) groups (Fig. 1E, imaging in Lu et al.) was presumably based on staining of the samples with three different antibodies but not a single antibody, otherwise the remaining two antibodies must have been respectively used for another two sets of samples or occasions of experiments, which however were not mentioned in the paper. So, without consideration of differences between the antibodies and disclosure of the antibody usage, the employment of multiple antibodies for IHC did not strengthen findings of Lu et al., but instead introduced an extra confounding variable which made the findings even more questionable.

In response to our doubt about the antibody usage, *Nature* published an addendum on November 16, 2016 (Lu et al., 2016). Specifically, as shown in Table 1, several occasions of IHC experiments, which had not been previously mentioned, were added to the article, making that each antibody was seemingly used on an independent occasion of IHC experiment and that the existence of the above-mentioned confounding effect by misuse of the antibodies was therefore excluded. However, this addendum also raised some concerns. For example, based on the addendum, it can be inferred that all the presented IHC data were obtained using the antibody IHC-00141 but had nothing to do with the other two antibodies (ab28018 and sc-15118) now claimed to have been employed for additional IHC experiments and yielded similar results (“data not shown”); however, all the three antibodies were referred to when the IHC-00141-related results in Figure 1D,E and Extended Data Figure 1 were mentioned in the paper. Also, if the antibodies ab28018 and sc-15118 yielded similar results as claimed in the addendum, there is no doubt that it would greatly strengthen the data obtained by IHC-00141 and make the findings more convincing; however, it is strange that results of ab28018 and sc-15118 had not been even mentioned in the original article. In other words, there was zero evidence supporting the existence of the IHC experiments that were later added in the addendum without any notification in the statement and any explanation for their absence in the original version of the paper. In addition, like the case for IHC, two antibodies (07-579 and ab52850) were previously listed for WB without disclosure of their usage for each independent experiment; however, based on the addendum, all the presented WB data were obtained using 07-579, while neither the usage nor the result information was disclosed for ab52850, raising the question why this antibody was listed in the paper.

Even if a fixed antibody was used for both IHC and WB throughout the study, expression of multiple REST isoforms caused by alternative splicing still may lead to data misinterpretation. For example, REST4, expression of which in SH-SY5Y was ignored by Lu et al., competes with the full-length REST to occupy RE-1 sites, such that it inevitably affects interpretation of REST target genes with the ChIP-seq data. Also, REST isoforms sharing the same epitope can be indiscriminately labeled by a specific antibody, and, in comparison with the full-length REST,

truncated isoforms presumably have less complexity of protein folding and three-dimensional structure which potentially masks the epitope and therefore they may be more accessible by the staining antibody. As mentioned above, test of antibody specificity by immunostaining does not help to exclude the existence of multiple REST isoforms sharing the same epitope, whose binding to the antibody can be eliminated by the same blocking peptide. So, the IHC results could not address which specific REST isoform(s) contributed to differences in nuclear REST between the experimental groups; however, with only the full-length REST having been considered, such differences were attributed to the full-length REST in Lu et al. Taken together, Lu et al. neglected previously documented REST isoforms which presumably confound experimental results and lead to data misinterpretation (e.g., qRT-PCR data), while the usage of multiple antibodies for REST protein assay is questionable, making it necessary for a reevaluation of their findings.

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