



# Electron microscopy reveals toroidal shape of master neuronal cell differentiator REST – RE1-silencing transcription factor

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## ABSTRACT

The RE1-Silencing Transcription factor (REST) is essential for neuronal differentiation. Here, we report the first 18.5-angstrom electron microscopy structure of human REST. The refined electron map suggests that REST forms a torus that can accommodate DNA double-helix in the central hole. Additionally, we quantitatively described REST binding to the canonical DNA sequence of the neuron-restrictive silencer element. We developed protocols for the expression and purification of full-length REST and the shortened variant REST-N62 produced by alternative splicing. We tested the mutual interaction of full-length REST and the splicing variant REST-N62. Revealed structure-function relationships of master neuronal repressor REST will allow finding new biological ways of prevention and treatment of neurodegenerative disorders and diseases.

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**Abbreviations:** aa, amino acid(s); bp, base pair(s); CD, circular dichroism; CoIP, coimmunoprecipitation; DLS, dynamic light scattering; EM, electron microscopy; IDRs, intrinsically disordered regions; kDa, kilodaltons; NRSE, neuron-restrictive silencer element; NRSF, neuron-restrictive silencer factor; PCNA, proliferating cell nuclear antigen; RD1/2, repressor domain 1/2; RE1, repressor element-1; REST, RE1-silencing transcription factor; REST-FL, full-length REST; REST-N62, splicing isoform of REST, also known as REST4 or REST4-S3; ZF, zinc finger

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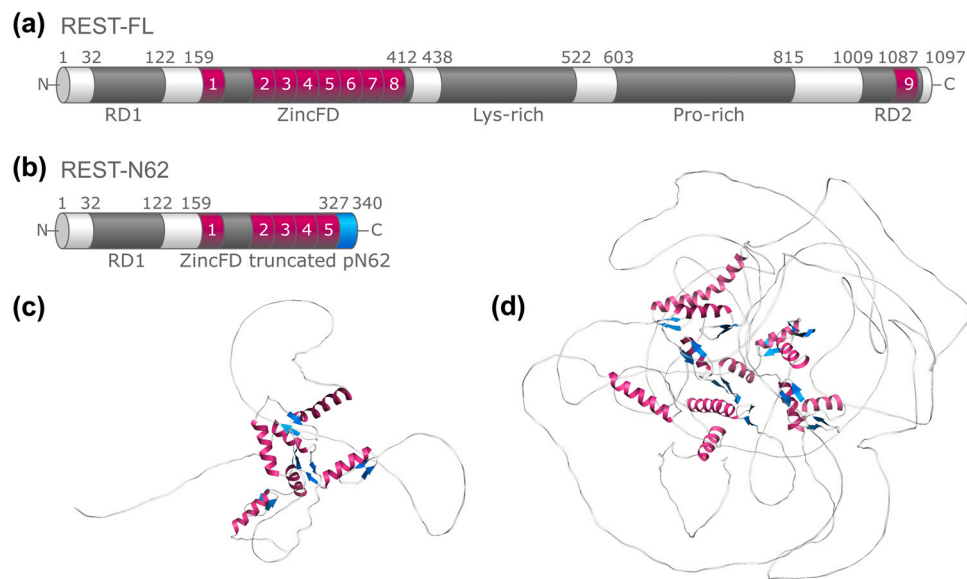
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## 1. Introduction

Human transcription factors control gene expression in each stage of cell development [1]. Neuronal cell development is tightly regulated by the RE1-silencing transcription factor that binds a 21 bp silencer element (RE1) in the type II sodium channel gene [2]. REST silences the expression of type II reporter genes in neuronal cells [3]. The RE1-silencing transcription factor is called REST, but it is also known as NRSF – neuron-restrictive silencer factor [4].

REST is widely expressed during embryogenesis and plays a critical role in the final stage of neuronal differentiation [3,4]. REST maintains neuronal stem cells in an undifferentiated state and orchestrates their stage-specific differentiation [5]. Additionally, REST is expressed in differentiated neurons during the critical time of enhanced sensitivity to plasticity in brain development [6]. REST fine-tunes genes controlling synaptic plasticity [7]. Furthermore, REST protects neurons by suppressing genes involved in the death of aging neurons [8].

Activated REST ensures normal aging in human cortical and hippocampal neurons, probably due to the prevention of oxidative stress and amyloid  $\beta$ -protein toxicity [8]. REST expression changes are



**Fig. 1.** Expression variants of human REST – domain composition and structure prediction. (a) Full-length REST (REST-FL) comprises N-terminal repression domain 1 (RD1), Zinc finger domain (ZincFD) with eight zinc fingers mediating DNA binding, lysine-rich and proline-rich domains, C-terminal repression domain 2 (RD2) with the ninth zinc finger. Magenta regions depict zinc fingers. (b) REST-N62 is a splice variant of human REST that comprises only five zinc fingers and an extra thirteen amino-acid peptide insert. The cyan region represents the insert peptide N62 (pN62) that is generated by the alternative splicing of a neural-specific exon causing premature translation termination of truncated REST variant after inserting extra 62 nucleotides encoding 13 amino acids. Predicted structures of (c) REST-N62 (AF-A0A087X1C2-F1) and (d) REST-FL (AF-Q13127-F1) deposited in the AlphaFold Protein Structure Database. The helices of zinc fingers are shown in magenta; beta sheets are in cyan.

associated with neurodegenerative disorders and diseases [6,9]. REST derepression is involved in induced neuronal death, as ischemic insults derepress REST in neurons destined to die [10]. REST is activated in selectively vulnerable mature hippocampal neurons in response to seizures [11]. In Huntington's disease, REST expression increases in the nuclei of striatal neurons [12]. In aging neurons, loss of REST is associated with the onset of Alzheimer's disease in humans [8].

The combination of bioinformatical and biochemical analyses identified 1892 putative coding and non-coding genes in the human genome that REST can target [13].

REST is a 1097 amino acid, 116 kDa protein that contains a total of nine zinc fingers (ZFs). A cluster of eight zinc fingers constitutes the zinc finger domain, which mediates specific recognition and binding to DNA (Fig. 1a). A combination of EMSA and molecular dynamics experiments suggests that ZFs 3–8 are inserted into the major groove of DNA duplex. Each ZF recognizes a triplet of canonical base pairs [14].

The function of REST as a transcriptional repressor is mediated by the N-terminal repressor domain (RD1) recruiting corepressor Sin3a/b that attracts histone deacetylases to remove acetyl groups from the core histones. The deacetylation of histones makes chromatin tightly packed, so the genes are inaccessible to the transcriptional machinery [15]. Additionally, the C-terminal repressor domain (RD2) recruits corepressor complex CoREST, including histone deacetylases and methyltransferases. Methyltransferases induce the binding of Heterochromatin protein 1 to histones leading to the transcriptionally silent state of heterochromatin [16]. Hence, REST causes gene repression through changes in the chromatin structure that prevent transcription [15,17].

Although human REST occurs in several isoforms [18], the contribution of various REST isoforms to neural induction is still under-investigated. For example, the most sequentially altered variant occurring specifically in human neurons is the REST-N62 isoform. REST-N62 (also known as REST4 or REST4-S3), which lacks four ZFs (6–9) and the C-terminal repressor domain, is generated by the alternative splicing of a neural-specific exon N located between exon IV and V (Fig. 1b). The alternative splicing produces a shortened variant of REST that contains an extra 62 nucleotide insertion in the REST mRNA that encodes 13 amino acids and bears an in-frame stop

codon that causes premature translation termination and synthesis of truncated REST variant [18].

Interestingly, REST-N62 has been shown to counteract the repressive role of full-length REST in the regulation of the target genes during neurogenesis [18–20]. Recently, it has been suggested that REST-N62 binds full-length REST and the complex formation modulates REST function by changing the number of active REST molecules [21].

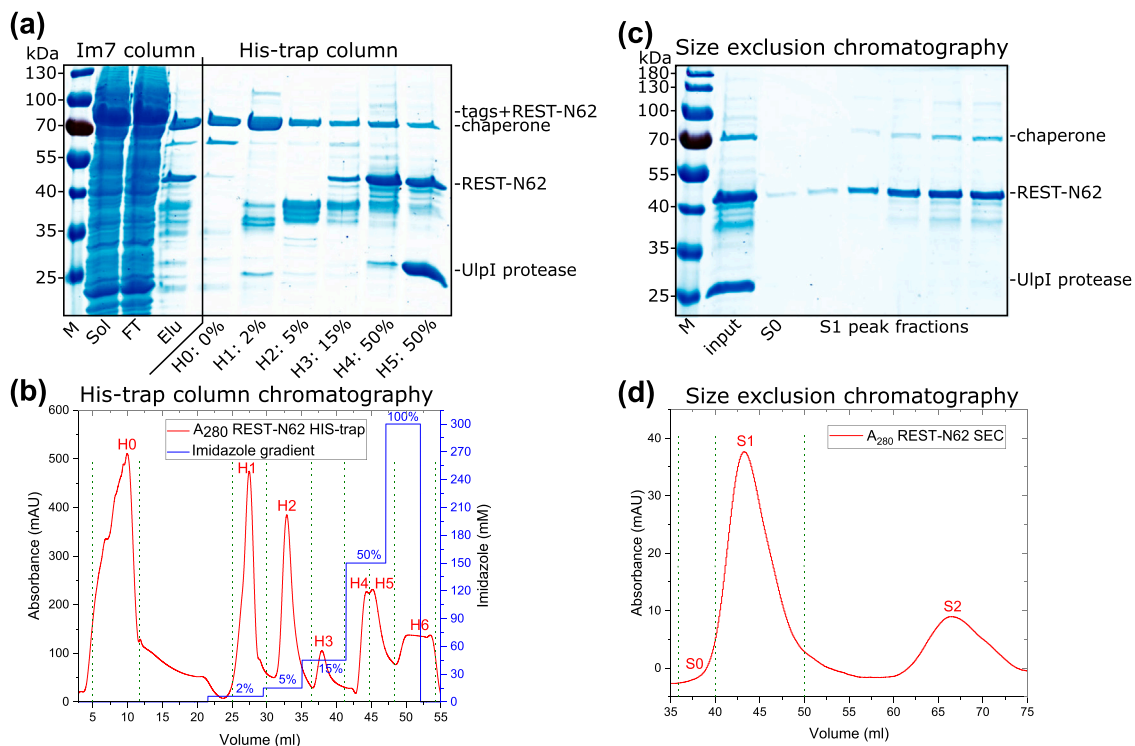
Here we addressed the following questions that are essential for further understanding the mechanism of REST function at the molecular level: 1. How to produce REST in vitro for functional and structural analyses? 2. What is the binding affinity of REST to the canonical DNA? 3. Does REST interact directly with the splicing variant REST-N62? 4. What is the structure of full-length REST? Despite being of undeniable importance, our understanding of REST function at the molecular level is still negligible. In this work, we successfully expressed and purified REST. We disclosed the first structure that helps us to understand REST spatial arrangement and provides implications for gene repression mechanisms in cells.

## 2. Results

### 2.1. Structure prediction revealed high ratio of intrinsically disordered regions

Initially, we analyzed the predicted structures of full-length REST (REST-FL) and the truncated variant produced by alternative splicing (REST-N62). We used the predicted structures of REST-FL (Q13127; AF-Q13127-F1), and REST-N62 (A0A087X1C2; AF-A0A087X1C2-F1) deposited in the AlphaFold Protein Structure Database [22,23]. To ensure the precise identification of the protein sequence and predicted models, we include unique UniProtKB and AlphaFold accession numbers, respectively.

The visualization of predictions of REST-FL, and REST-N62 shown in Fig. 1 revealed central structured regions surrounded by intrinsically disordered regions (IDRs). The structured parts belong almost exclusively to the zinc finger domains. Each zinc finger domain consists of an alpha-helix and two associated antiparallel beta-sheets. The structural prediction suggests that the structured regions occupy 19 % of the



**Fig. 2.** REST-N62 purification. (a) Analysis of Im7 and His-trap purification steps using 12 % SDS-PAGE. M – protein molecular weight marker; Sol – soluble fraction of crude extract; FT – flow-through fraction; Elu – collected elution fraction from Im7 column after cleavage. His-trap column elution samples are denoted with peak number and percent of imidazole gradient: H0 – a fraction with washed-out chaperone without imidazole. H1, H2, and H3 – fractions with 2 %, 5 %, and 15 % of imidazole, respectively. H4 and H5 – fractions with REST-N62 and UlpI protease, pooled and used as input for size exclusion chromatography. (b) His-trap column chromatography traces. His-trap column separated the chaperone and low molecular weight contaminations from REST-N62. Fraction marks correspond to the fractions analyzed in (a). Fractions H4 and H5 contained REST-N62, and UlpI protease were pooled. Imidazole absorbance is projected to the absorbance trace according to the imidazole concentration (H6 fraction). Green marks denominate collection ranges. (c) Analysis of size exclusion chromatography fractions by 12 % SDS-PAGE. M – protein molecular weight marker. Input – pooled H4 and H5 fractions from previous His-trap purification. S0 – pre-peak fraction, S1 – collected peak fractions. (d) Size exclusion chromatogram of REST-N62. SEC separated protease from REST-N62. S0 – pre-peak fraction, S1 – main peak fractions with REST-N62, S2 – peak corresponding to UlpI protease. Green dashed lines indicate collection ranges.

REST-FL sequence, while the content of structured regions in the variant REST-N62 is 30 %, suggesting that REST-N62 might be more structurally stable than the full-length protein. Taken together, both REST-FL and the truncated isoform REST-N62 show high overall structural flexibility, which may contribute to the ability of REST to recruit various factors involved in chromatin structure arrangements.

## 2.2. Establishing expression and purification procedures for recombinant REST-N62 and REST-FL

Expression and purification of the full-length REST are challenging mainly because of its large size. Additionally, the high frequency of IDRs and DNA binding capability make REST-FL an extremely challenging target for expression. Prediction studies indicated that the shorter variant REST-N62 might be more structurally stable than full-length REST. Despite our previous experience with preparing proteins, the purification procedure development for REST was challenging and lengthy. To prevent the scientific community from repeating unsuccessful ways of purification, we included a section in the discussion that describes the approaches we have tried in our effort to purify REST-N62 and REST-FL.

After considering the reasonable length of REST-N62, we decided to express REST-N62 in *E. coli*, as bacteria usually produce relatively high amounts of recombinant proteins. We used an expression construct based on vector pI21 containing CL7 affinity tag, SUMO-tag with UlpI cleavage site, and the gene for REST-N62 (340 coding amino acids) followed by a stop codon. The protein was expressed in *E. coli* strain BL21-CodonPlus® (DE3)-RIPL in a TB medium.

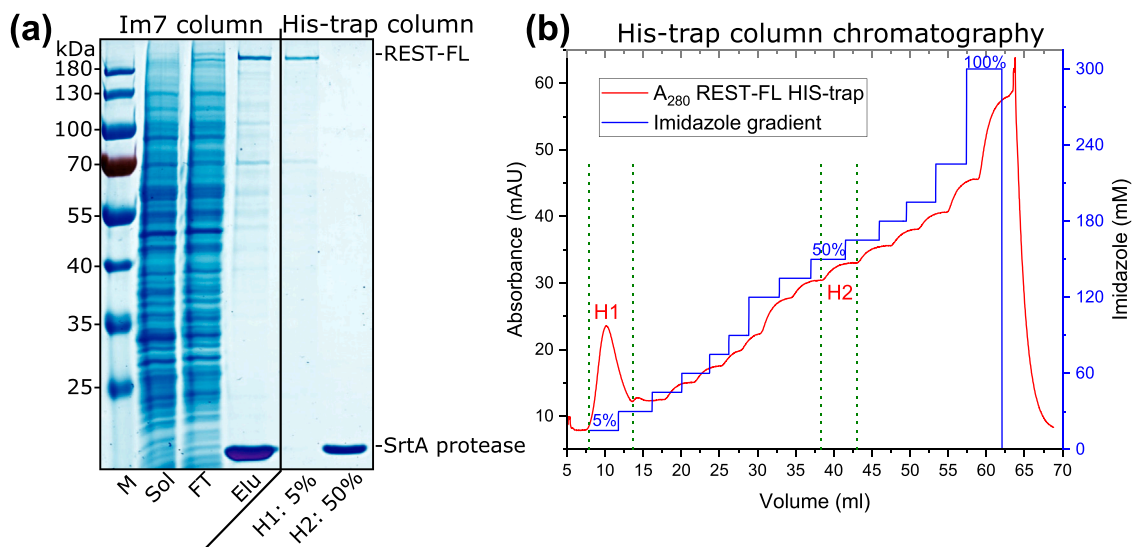
To purify bacteria-expressed REST-N62, we first loaded processed supernatant of crude extract on the ultra-high affinity chromatography column Im7, which strongly bound CL7-tagged REST-N62

(Fig. 2a, and Supplementary Figure S4a). In the second stage, after we removed the tags from REST-N62 by cleavage with UlpI protease, we loaded the protein and protease mixture on the His-trap column to separate mainly protease and chaperones from the REST-N62 (Fig. 2a and b). In the final polishing step, we achieved high purity of REST-N62 using size exclusion chromatography that separated the remaining UlpI protease (Fig. 2c and d).

After extensive trials, we decided to express REST-FL in insect cells because insect cells can easily express proteins of size over 100 kDa. An additional advantage of the expression in the eukaryotic system is the maintenance of posttranslational modifications, including phosphorylation. REST-FL can be phosphorylated at phospho-serines in a conserved phosphodegron – a short linear motif that can interact with ubiquitin ligases upon phosphorylation. The phosphodegron is located in the C-terminus of REST-FL [24]. We also tested the expression of REST-FL using the mammalian HEK293T cell line system. However, when we detected unwanted DNA contamination in REST-FL extracted from HEK cells (Supplementary Figure S2), we decided to express REST-FL in insect cells.

We have designed over thirty vector constructs for insect cell expression of REST-FL. Eventually, we found the vector construct that was the most efficient for REST-FL expression. The selected pFASTBAC1 expression vector construct contains inserts in the following order: MBP-tag, 6xHis, S-tag, CL7 affinity tag, SrtA cleavage site, REST-FL gene (1097 coding amino acids). The expression vector construct was introduced into Sf9 insect cells. The Bac-to-Bac insect cell expression system was used to generate a baculovirus that expressed REST-FL.

To purify insect-cell expressed REST-FL, in the first step, we loaded processed supernatant of crude extract on the ultra-high affinity chromatography column Im7 that specifically bound CL7-



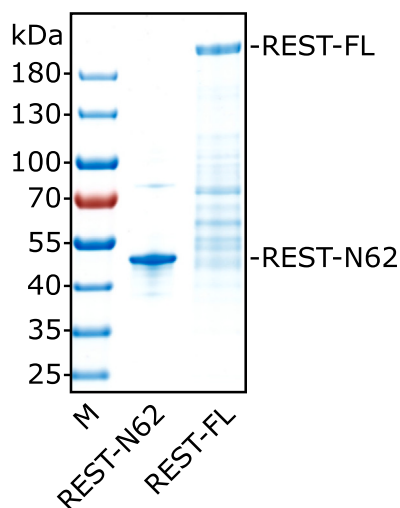
**Fig. 3.** REST-FL purification. (a) Analysis of Im7 and His-trap purification steps using 10% SDS-PAGE. M – protein molecular weight marker; Sol – soluble fraction of crude extract; FT – flow-through fraction; Elu – collected elution fraction from Im7 column after cleavage. His-trap column elution samples are denoted with peak number and percent of imidazole gradient: H1 (5% imidazole) contains REST-FL, and H2 (50% imidazole) contains SrtA protease. (b) His-trap column chromatography traces. His-trap column separated the protease and low molecular weight contaminations from REST-FL. Fraction marks correspond to the fractions analyzed in (a). Fraction H1 contained REST-FL. H2 fraction corresponds to SrtA protease. Imidazole absorbance is projected to the absorbance trace according to the imidazole concentration. Green dashed lines indicate collection ranges.

tagged REST-FL (Fig. 3a and Supplementary Figure S4b). In the second step, after cleavage with SrtA protease, we loaded the mixture of REST-FL and protease on a His-trap column to separate the protease and purified REST-FL (Fig. 3a and b).

We have described the expression and purification of REST-N62 and REST-FL in detail in the Materials and methods section.

### 2.3. Quality, homogeneity, and DNA content of recombinant REST

The quality of REST-FL and REST-N62 was analyzed by SDS-PAGE (Fig. 4). The purity values REST-N62: 95%, and REST-FL: 85% determined by densitometry analysis were sufficient for subsequent experiments. We confirmed the identity of REST-N62 by MALDI-TOF mass spectrometry. Experimentally measured molecular weight (38,370 Da) was comparable to calculated molecular weight (38,379 Da), suggesting that REST-N62 was prepared without extra tags, only with remaining glycine and serine residues at the N-terminus after Ulpl protease cleavage (Supplementary Figure S1).



**Fig. 4.** Final purity of REST constructs. Analysis of REST-FL and REST-N62 after purification using gradient SDS-PAGE. M – protein molecular weight marker.

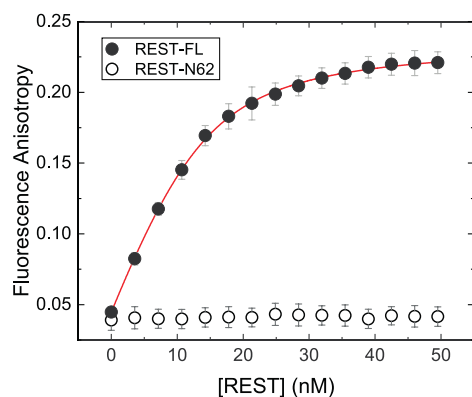
In the case of REST-FL, MALDI-TOF mass spectrometry provided suppressed signal because of the presence of molecules with lower molecular weight than REST-FL. Therefore, we used MALDI MS/MS analysis of the tryptically digested band extracted from SDS-PAGE to identify REST-FL. The MS/MS spectrum was compared to the sequence from UniProt database accession number Q13127. The MASCOT score of 12645 (Supplementary Table 1) and 70% sequence coverage of MS/MS analysis (Supplementary Figure S5) confirmed that the electrophoretic band with the expected molecular weight is formed by REST-FL.

As REST-FL serves as a transcription factor with high affinity to DNA, we measured the possible content of genomic DNA in the purified proteins before performing quantitative DNA studies.

We compared the DNA content in the REST-FL samples expressed in mammalian and insect cells by combining UV spectroscopy and gel electrophoresis. UV spectroscopy revealed an absorbance ratio  $A_{260}/A_{280}$   $1.62 \pm 0.08$  for REST-FL expressed in mammalian cells and  $0.49 \pm 0.07$  for REST-FL expressed in insect cells. The  $A_{260}/A_{280}$  ratio higher than 0.6 suggested that genomic DNA contaminated REST-FL isolated from mammalian cells. On the other hand, REST-FL isolated from insect cells showed the ratio corresponding to the absence of DNA. To confirm that REST-FL isolated from insect cells is DNA-free, we separated REST-FL and REST-N62 on an agarose gel (Supplementary Figure S2). No DNA traces were found in REST-FL from insect cells. DNA-free REST-FL from insect cells was used for subsequent binding and structural studies.

REST-N62 could be produced in bacteria in higher amounts than REST-FL in insect cells. Hence, we used more sample demanding methods to evaluate REST-N62 homogeneity and secondary structure. Dynamic light scattering (DLS) revealed that REST-N62 is homogenous with an expected average radius of 10 nm without significant aggregations (Supplementary Figure S3a). Minor content of a chaperone in the sample (Fig. 4) may contribute to the observed peak at around a 60 nm radius.

Moreover, the proper folding and secondary structure composition of REST-N62 were assessed by far-UV circular dichroism (CD). The recorded CD spectrum suggested that REST-N62 is well-folded with prevalently alpha-helical arrangements of structured regions (Supplementary Figure S3b). The observed prevalence of alpha-helices in the secondary structure is in accordance with the prediction of the REST-N62 structure (Fig. 1c). In summary, our analyses confirmed that recombinantly prepared REST-FL and REST-N62 meet the



**Fig. 5.** REST-FL and REST-N62 binding to NRSE DNA recognition sequence. REST variants were allowed to bind oligonucleotide duplex containing NRSE canonical sequence labeled by AlexaFluor 488 (7.5 nM). The dissociation constant  $K_D$  was determined by non-linear fitting using a one-site binding model (red). REST-N62 showed no significant DNA binding affinity; no fitting model was applied. Error bars represent s.d.,  $n = 3$  independent measurements.

requirements of purity, homogeneity, and DNA absence for further studies.

#### 2.4. Purified REST-FL binds NRSE with high affinity

We quantified the affinity of REST-FL and REST-N62 to canonical DNA using fluorescence anisotropy. Both REST variants were allowed to bind to an oligonucleotide duplex containing the NRSE canonical sequence (21 bp) labeled by AlexaFluor 488 (Fig. 5). We found that purified REST-FL binds NRSE with a high-nanomolar affinity. The analysis of the data using a one-site binding model revealed dissociation constant  $K_D = 5.7 \pm 0.6$  nM. Conversely, REST-N62 showed no binding capability (Fig. 5) in the same concentration range. We show that purified REST-FL actively binds canonical DNA with a nanomolar dissociation constant.

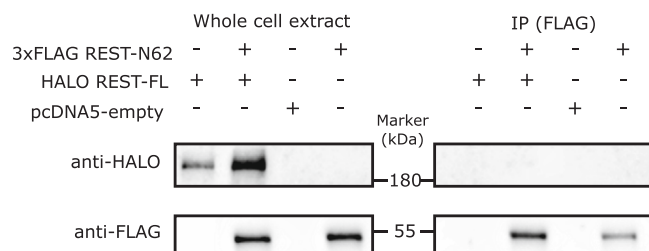
#### 2.5. Coimmunoprecipitation detected no interaction between REST-N62 and REST-FL

We wondered if REST-N62 and REST-FL would interact together. The mutual binding of REST-N62 and REST-FL was hypothesized in previous studies [19] and later suggested as a regulatory mechanism [21]. To test the hypothesis of mutual binding of REST-N62 and REST-FL, we performed coimmunoprecipitation (CoIP) of overexpressed proteins in HEK293T cells, followed by western blotting (WB) analysis. We immunoprecipitated FLAG-tagged REST-N62 using M2 FLAG magnetic beads to detect binding to REST-FL (Fig. 6). Coimmunoprecipitation results showed no detectable mutual interaction of REST-N62 and REST-FL under our conditions. Hence, our data suggest a low possibility that the shorter isoform REST-N62 can interfere with and modulate the silencing activity of REST-FL.

#### 2.6. Negative stain electron microscopy reveals the toroidal structure of REST-FL

We obtained the density map of purified recombinant human REST-FL at 18.5 Å resolution using negative stain electron microscopy. The subsequent 3D reconstruction revealed a toroidal structure of REST-FL. The reconstructed doughnut-like shape has an outer diameter of 14 nm and an inner diameter of 5 nm. The side view shows that the height of the toroid is 9 nm (Fig. 7C).

From the 2D class of top view, it might appear that the toroidal shape of REST-FL may adopt a multimer symmetry. Based on the prediction of the REST-FL monomer (Fig. 1d), we selected the partially structured N-terminal part (150–425 aa) comprising eight zinc fingers and predicted the dimer, trimer, tetramer, pentamer, and hexamer using



**Fig. 6.** Coimmunoprecipitation of REST-FL and REST-N62 showed no mutual interaction. FLAG-tagged REST-N62, and HALO-tagged REST-FL variants were transiently co-expressed in HEK293T cells. REST variants were precipitated using M2-FLAG magnetic beads. Precipitates were analyzed by western blotting with corresponding antibodies.

AlphaFold v2.1.0 multimer tool [23]. The predicted models showed no mutual interaction of monomeric subunits nor adopted any defined multiunit arrangement of REST-FL through the structured regions.

After successful analysis by negative staining, we carried out extensive cryo-EM experiments. REST-FL grids were prepared with storage buffer containing 2.5 % glycerol. Unfortunately, the glycerol content strongly affected the ice thickness, ice formation and negatively impacted the particle contrast. We attempted to prepare cryo-grids with a broad range of lower glycerol concentrations. In all cases, we observed substantially more pronounced aggregation of protein mass on the cryo-grids compared to negative staining. Thus, we preferentially concentrated our effort on precisely analyzing negative stain electron microscopy grids.

### 3. Discussion

We revealed the electron microscopy structure of master neuronal cell differentiator RE1-Silencing Transcription factor (REST). Additionally, we developed protocols for the expression and purification of full-length REST and its splicing variant REST-N62. We quantified REST binding to canonical NRSE sequence. Furthermore, our findings suggest no mutual direct binding of REST-FL and REST-N62. Our studies opened new horizons to extensive studies with full-length REST and its splicing variant REST-N62. We thoroughly discuss our findings and their implications below. We divided the discussion into three sections covering the preparation, binding, and structure of REST.

#### 3.1. Challenges in expression and purifications – dead ends to avoid

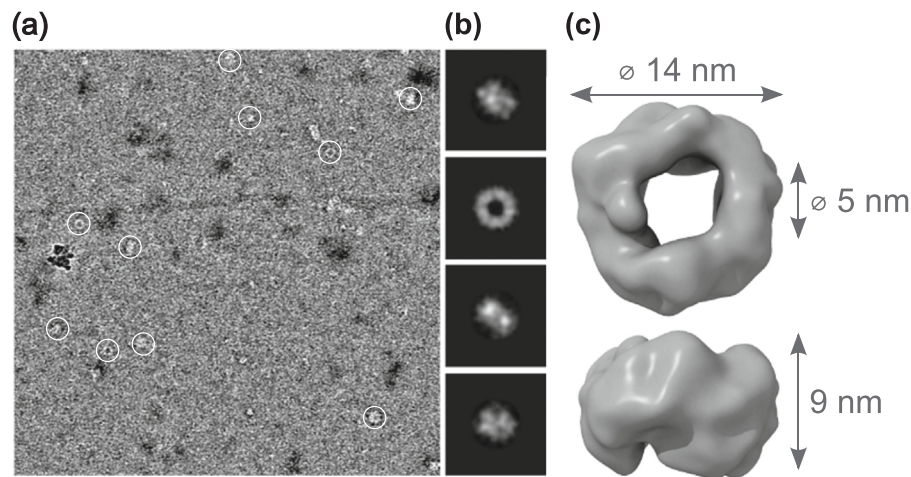
This section is dedicated to all colleagues scientists trying to optimize REST production. We described our experimental approaches, including unsuccessful ones, to avoid repeating the ineffective ways of REST-FL and REST-N62 production.

When we were preparing expression vector constructs containing the REST-FL gene, we observed that the gene comprises several palindromic sequences. The repetitive sequences, along with frequent off-frame hexahistidine sequences, might cause cloning difficulties.

We tested many possible combinations of tags for expression, solubility improvement, and affinity purification, including 6xHis, S-tag, GFP, HALO, SUMOstar, 3xFLAG, thioredoxin, CL7, and MBP. Surprisingly, we found that HRV 3C protease, which we use preferentially to cleave expression tags, cleaves REST-FL nonspecifically at amino acid region 654–661: MEVVQEGP. Therefore, the initial use of HRV 3C in our expression trials caused a low yield of REST-FL.

We consequently tried other proteases such as TEV, WELQut, Ulpl-star, and SrtA (SortaseA), with the last being the best for our purposes. Additionally, we tried different purification sorbents compatible with the previously mentioned affinity purification tags and corresponding purification procedures.

Initially, we tried REST-FL expression in mammalian cells HEK293T, but we observed that the purified protein contained



**Fig. 7.** Negative stain electron microscopy of REST-FL. (a) Representative electron micrograph of REST-FL stained with 2% uranyl acetate. Protein particles are highlighted by white circles. (b) Representative results from the reference-free 2D classification of the REST particles. (c) 3D model of REST at 18.5 Å resolution (EMD-15858). Two different views of the 3D reconstruction rotated by 90° around a horizontal axis. The arrows indicate the size of the REST structure. The outer diameter of the model is approximately 14 nm, the diameter of the inner pore is approximately 5 nm, and the height is approximately 9 nm.

genomic DNA (Supplementary Figure S2). When we added benzonase at the initial stage of purification, we observed the low DNA binding ability of REST-FL to NRSE DNA sequence (data not shown). Our inability to produce active REST-FL in mammalian cells drove our attention to the insect cell expression system.

During the optimization of REST-FL production in insect cells, we tested several bac-to-bac and multibac vectors with different promoters such as polyhedrin, p10, hr5, and IE1 with corresponding terminators, and different competent cells for bacmid production such as DH10Bac, DH10MultiBac, and DH10EmBacY, before we found the optimal combination listed in the Materials and methods.

The choice of the final purification step is essential for the resulting REST-FL activity. As REST-FL contains nine zinc-finger domains that require Zinc cations for structure stabilization, we tested if charging the immobilized metal affinity column with Ni<sup>2+</sup> or Zn<sup>2+</sup> ions affects REST-FL activity. We observed no significant effect of the ion substitution on the DNA binding activity or structure of REST-FL (data not shown).

In general, we found that the purification of recombinant REST-FL produced in insect cells is suitable for *in vitro* analyses, including electron microscopy, because insect cells contain no NRSE DNA sequence that could naturally bind and block the activity of REST-FL.

REST-N62 (38.2 kDa) is significantly smaller than REST-FL (121.9 kDa), so we found REST-N62 expression and purification less complicated. We optimized the preparation of REST-N62 in *E. coli* via different expression setups, including various solubility and purification tags – 6xHis, S-tag, 3xFLAG, thioredoxin, Sumo, and CL7 together with proteases HRV 3C or Ulp1. Thioredoxin usually improves solubility; however, in this case, the thioredoxin tag induced immediate precipitation of REST-N62 after the lysate sonication.

The optimal combination of temperature and aeration during REST-N62 expression was essential for proper protein production without excessive chaperone expression.

For example, when we used a different media:air volume ratio than the optimal 0.5 L TB medium in 2 L Erlenmeyer's flasks, the DnaK chaperone from *E. coli* was overexpressed. Similarly, when we reduced the temperature from 37 °C to 20 °C before the induction of expression, we discovered that cooling must be finished within one hour to ensure that the minimum amount of chaperone DnaK is produced. DnaK presence in samples was confirmed by MS analysis (data not shown). This chaperone can be observed on SDS-PAGE gel (Fig. 2) at the level of 70 kDa protein marker.

Despite all before mentioned challenges, we successfully optimized protocols to recombinantly produce active REST-FL and

well-folded REST-N62 *in vitro*. We present the final and optimal expression and purification protocols in the Materials and methods section.

### 3.2. REST-FL binds avidly to DNA but is reluctant to interact with REST-N62

As REST is a transcription factor that binds specifically canonical sites on DNA, we might expect that REST-FL shows DNA binding affinity similar to proteins that bind and recognize specific DNA sequences [25,26]. Human transcription factors that can interfere with genomic DNA usually bind with  $K_D$  in the nanomolar range. The observed REST-FL  $K_D$  value is in the range that is common for human transcription factors [27].

On the contrary, REST-N62 showed no DNA binding capability (Fig. 5) in the same concentration range as REST-FL. Even though REST-N62 shares the same 1–5 zinc finger motifs on its N-terminus (Fig. 1), the missing zinc fingers 6–9 seems to be the critical part for high-affinity binding to DNA. Indeed, Tang et al. showed that zinc fingers 7, and 8 contribute significantly to recognizing DNA with NRSE sequence. According to previously published results of combined electromobility shift assay and molecular dynamics with zinc fingers 1–5, mainly zinc fingers 4 and 5 contribute to DNA binding [14]. Hence, REST-N62 may still bind DNA through zinc fingers 4 and 5 if the spatial orientation is preserved as in REST-FL. The binding via only two zinc fingers may explain why we observed no significant fluorescence anisotropy increase during the addition of REST-N62 to the canonical NRSE DNA (Fig. 5).

Additionally, we tested the binding of REST-N62 to NRSE DNA motif at concentrations over 1.2 mM (data not shown), but we observed no significant binding of REST-N62 either. However, we cannot exclude that REST-N62 may still bind NRSE DNA with substantially lower affinity with a corresponding  $K_D$  value several orders of magnitude higher than full-length REST.

Finally, to investigate the possible mechanism of REST regulation on a molecular level, we tested the previously suggested hypothesis that REST-N62 isoform binds REST-FL to block the ability of REST to interact with DNA [21]. In our conditions, coimmunoprecipitation experiments in mammalian cell lysate showed no detectable interaction between human REST-N62 and REST-FL (Fig. 6, Supplementary Figure S6). We suggest that the splicing variant REST-N62 does not interact directly with full-length REST.

### 3.3. The toroidal structure of REST allows central DNA binding that is common for transcription factors

Here we described the first structure of full-length human REST obtained by negative staining electron microscopy.

The 3D reconstruction suggests a toroidal shape of REST. A strikingly large number of the proteins involved in DNA metabolism adopt a toroidal structure. Hingorani & O'Donnell suggest that ring-forming proteins may have been selected during evolution for advantages conferred by the toroidal shape on their interactions with DNA [28]. Hence, the doughnut-like shape is typical among proteins that serve as molecular machines participating in DNA replication and RNA transcription [29]. For example,  $\beta$ -clamp polymerase processivity factor (PDB: 3BEP) and Proliferating cell nuclear antigen (PCNA) (PDB: 1AXC) also possess a doughnut-like structural arrangement. The inner diameter of REST central pore (5 nm) is comparable to the pore sizes of other toroidal DNA binding proteins (~4 nm) (Fig. 8). The initial structure prediction of REST-FL by AlphaFold revealed the prevailing content of flexible and intrinsically disordered regions. The flexible IDRs of REST-FL might become rigid upon binding to DNA by zinc fingers or upon binding to other accessory factors.

Recently, Li et al. provided several modes of functional motions that may modulate the structure of toroidal systems upon substrate binding. The toroidal motion modes include cooperative saddle-like bending, rolling, counter-rolling, stretching, contraction, breathing, and clamshell-like motions [29]. The predicted high dynamics of toroidal protein complexes enable toroids to undergo several motions simultaneously, resulting in harboring specific nucleic acid.

Our agarose gel analysis allows us to consider that REST-FL produced in insect cells contains no or a minimal amount of bound DNA (Supplementary Figure S2). Then electron microscopic reconstruction might represent the relaxed form of full-length REST structure without DNA and with a wide open central pore. We hypothesize that the pore size might be reduced upon REST binding to DNA due to the dynamic modes of motion that allow tightening of the pore and DNA stabilization within the toroid.

The toroidal complexes often bind DNA as oligomers. For instance, PCNA forms a trimer; the  $\beta$ -clamp polymerase processivity factor acts

as a dimer. Thus, we may speculate that REST is capable of forming organized multimers. We have no data available to validate the monomeric or the multimeric arrangement of REST. We employed AlphaFold v2.1.0 multimer tool [23] to predict dimeric, trimeric, tetra-, penta- and hexameric structures of REST-FL or the most structured part of REST comprising eight zinc finger domains, with no success.

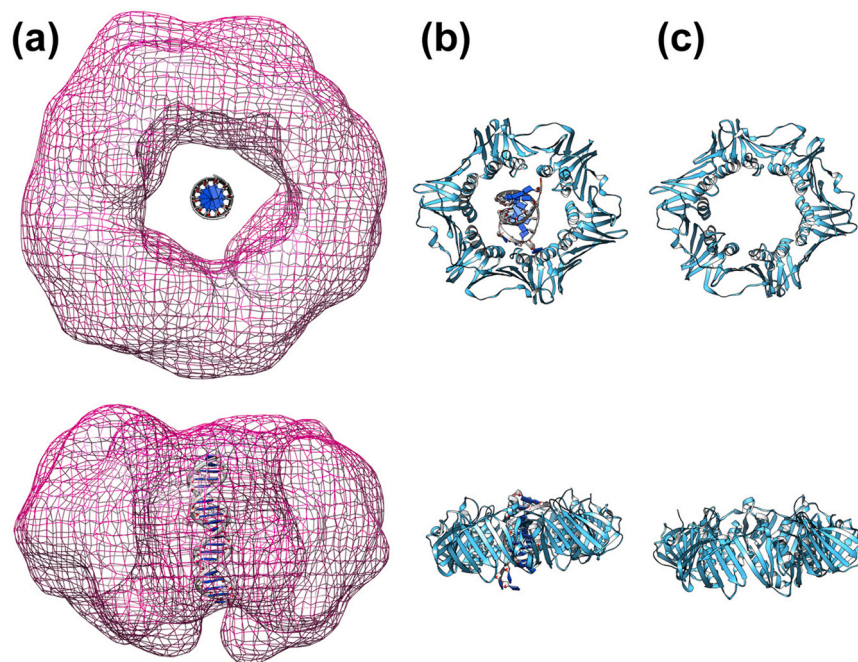
Regardless of the monomeric or multimeric REST arrangement, we speculate that the zinc finger domain may be arranged facing the inner central pore of the toroidal structure allowing zinc fingers to access and “read” the DNA sequence. Fig. 8 shows our putative model of the EM structure of REST with the canonical NRSE DNA projected in the central hole (Fig. 8a). Remarkably, the EM structure of REST-FL easily accommodates the whole 21 base pairs of NRSE DNA sequence, as the 90° rotated model demonstrates in the lower part of Fig. 8a.

To compare REST with other transcription factors, we included the known structure of toroidal  $\beta$ -clamp polymerase processivity factor with and without DNA (Fig. 8b and c). We speculate that the unstructured interacting regions RD1 and RD2 might be on the outer edge, ready to interact with REST cofactors and other binding partners. Notably, the revealed toroidal arrangement of REST allows simultaneous binding of canonical DNA and recruitment of multiple proteins.

Transactivation domains of transcription factors are often intrinsically disordered and acquire transient structure only upon binding of a partner. Additionally, the IDRs may enable REST to interact with more than two hundred different partners [30]. Specific IDRs are Lysine-rich and Proline-rich regions that could function as scaffolds for the toroidal structure. Moreover, the structure formation could be affected by posttranslational modifications.

The phosphorylation of the phosphodegron region causes the increase of local negative charge that may attract positively charged parts of REST or other interacting partners. Thus, the phosphodegron might contribute to the stable tertiary structure of REST and the long-distance recruitment of partners. The specific posttranslational modifications of IDRs might be why AlphaFold failed to predict the structure of REST.

The 3D structure determines how effectively REST finds and binds the NRSE site on DNA in the initial step of REST-mediated gene repression. The gene silencing process is a complex combination of



**Fig. 8.** Putative model of NRSE embedded inside REST-FL envelope compared with toroidal  $\beta$ -clamp polymerase processivity factor. (a) Mesh model of REST-FL obtained by negatively stained electron microscopy with the projected NRSE sequence (EMD-15858). (b)  $\beta$ -clamp polymerase processivity factor cartoon model with bound DNA in the inner pore (PDB: 3BEP) and (c) without DNA. Lower images represent the 90° rotation of models alongside the x-axis.

successive steps, including interactions with cofactors and other binding factors.

Two binding partners of REST – Sin3a/b [31] and CoREST [32] play critical roles in the recruitment of chromatin-modifying enzymes HDAC1/2, BRG1, LSD51, and G9, which affect chromatin condensation and thus inhibit gene expression. Notably, the predicted structures in the AlphaFold database show that REST-interacting regions of Sin3a (205–480 aa) and Sin3b (59–105 aa) are mostly unstructured. Similarly, CoREST is mainly flexible, and the only structured region is dedicated to recruiting LSD1 histone demethylase [33]. Generally, the interaction between two IDRs is preferred over the interaction of two structured regions [34]. Nevertheless, REST could interact with its binding partners via IDRs that are arranged to organized structures only upon complexation. The formation of a hydrophobic helix has been described for binding the N-terminal RD1 domain of REST with PAH1 domain of Sin3b [35]. Consequently, even Sin3a and CoREST interactions could be mediated by IDRs that adopt well-defined structures upon binding.

In an effort to revert REST-induced gene repression at the molecular level, Paonessa et al. developed an optometric approach to regulate REST activity via the expression of light-sensitive chimeric proteins that displaced endogenous Sin3a and the entire REST complex from target DNA [36]. A similar approach could be applied against CoREST. Our structural description of REST-FL may be further exploited in engineering new tools modulating REST transcription activity. REST-specific interfering interactions with essential binding partners or canonical DNA sequences will result in neurodevelopment activation that reverses changes in REST-controlled gene expressions [37].

We might consider several ways how REST could be regulated on a cellular level. Firstly, REST complexation with partners may initiate allosteric changes of IDRs that enhance REST affinity to the corresponding DNA sequence or release REST from DNA. Secondly, the REST function could be modulated by transporting REST from the nucleus to the cytoplasm. The example of targeted REST transport provides Htt protein which sequesters REST in the cytoplasm and thereby enables neuronal survival, neuronal plasticity, and dendritic growth [38]. Additionally, the most investigated REST regulation pathway includes phosphorylation, ubiquitination, and subsequent degradation by the proteasome. REST is probably phosphorylated by Casein Kinase 1, which makes REST available for subsequent ubiquitination. E3 ubiquitin-protein ligase  $\beta$ -TrCP adds ubiquitin units at the phosphodegron site of REST. Hence, structural changes induced by phosphorylation make the ubiquitination site on REST accessible for enzymes [39]. Finally, proteins that sterically block REST against ubiquitination might modulate the REST function. For instance, the telomeric protein TRF2 has been found to prevent REST ubiquitination [40].

Here presented results serve as a solid base for further REST investigations to answer the questions that appeared during our studies.

Future research should address the REST structure in complex with canonical and noncanonical NRSE DNA. The nucleoprotein complex REST-NRSE should be investigated further by small-angle X-ray scattering to complement our REST-FL electron microscopy density map. Moreover, dynamics of intrinsically disordered regions in REST might be studied by nuclear magnetic resonance. In addition, the stoichiometry of REST arrangement with and without DNA should be determined. The subsequent stoichiometry experiments might include recording the electron microscopy density of tagged REST-FL with specific anti-tag antibodies bound to REST. Finally, subsequent studies should describe REST interactions with cofactors essential for regulating gene repression. REST-cofactor binding description by quantitative approaches in combination with structural information might reveal new specific targets of rational drug design for molecular-based therapies of diseases in which REST is dysregulated.

## 4. Materials and methods

### 4.1. The cloning, expression, and purification of REST-N62

REST-N62, also known as REST4-S3 is a truncated variant of REST-FL achieved via alternative splicing – after Serine 327 with additional 13 amino acids: VGYGYHLVIFTRV followed by a stop codon (sequence with UniProt accession number A0A087X1C2).

The source of the REST-N62 sequence was a plasmid obtained from Addgene (#41903). Recombinant expression cassette contained inserts in the following order: CL7 Affinity Tag, SUMO-tag with UlpI cleavage site, REST-N62 (340 coding amino acids), stop codon. We found out that REST-N62 purification is baffled with the thioredoxine tag in the original pI21 plasmid, so we decided to delete thioredoxine and the short linker between CL7 and SUMO tags. Plasmid pI21 (TriAltus) was truncated by digestion with restriction enzymes *Xba*I and *Xho*I (New England Biolabs). To complete the recombinant assembly reaction NEBuilder® HiFi DNA Assembly kit (New England Biolabs) was used with the combination of three different inserts produced by PCR: the first insert contained the original pI21 vector part – the non-coding sequence before the start codon, followed by the coding sequence of CL Affinity Tag; the second insert encoded SUMO-tag; the third insert contained the REST-N62 coding sequence with flanking 13 extra amino acids followed by a stop codon. All PCR products and digested plasmid were separated by agarose gel electrophoresis and extracted from the gel with QIAquick® Gel Extraction Kit (Qiagen). After assembly, the expression vector construct was transformed into *E. coli* One Shot™ TOP10 (Invitrogen). The final construct was confirmed by sequencing, and the plasmid with the coding REST-N62 sequence was transformed into *E. coli* expression strain BL21-CodonPlus® (DE3)-RIPL (Agilent).

REST-N62 expression was carried out in 0.5 L of Terrific Broth (TB) medium containing 50  $\mu\text{g}\cdot\text{mL}^{-1}$  kanamycin and 34  $\mu\text{g}\cdot\text{mL}^{-1}$  chloramphenicol in 2 L Erlenmeyer's flask to ensure good aeration. The preheated medium was inoculated with 10 mL of overnight grown BL21-(DE3)-RIPL cells. Flasks were shaken in orbital shaker New Brunswick™ Innova® 43/43 R (Innova) at 160 rpm and 37 °C. After OD<sub>600</sub> reached 1.0, the flasks were cooled down to 20 °C within one hour. Protein expression was induced by the addition of IPTG to the final concentration of 0.1 mM. The cell culture was incubated overnight (at least 8 h) and then collected by centrifugation (8 000 g, 8 min, 4 °C). Obtained pellet was immediately used for purification (without freezing). We usually performed expression in a total 3 L TB medium and obtained around 30 g of cell pellet.

All the following purification steps were carried out at 4 °C with precooled buffers and instruments. Fresh pellet kept on ice was re-suspended in the lysis buffer (50 mM Sodium Phosphate, 500 mM NaCl, 5 % glycerol, 0.1 % Triton X-100, pH 8.0) with the addition of 0.8 mg·mL<sup>-1</sup> of lysozyme, leupeptin 1.9  $\mu\text{g}\cdot\text{mL}^{-1}$  and pepstatin A 3.1  $\mu\text{g}\cdot\text{mL}^{-1}$  (80 mL of lysis buffer was used with typical 3 L TB expression). The cell suspension was transferred into stainless steel container cooled on ice and sonicated for 12 min with a 30 % amplitude, 1-second pulse-on, 1-second pulse-off with a 60-second process time cycle with a 15-second pause for extra cooling (Misonix Qsonica S-4000). The lysate was cleared by centrifugation at 20,000g, 1 h, 4 °C, and filtered by 0.45  $\mu\text{m}$  Sterivex™ Filter Units (Merck Millipore).

REST-N62 was purified in three-step chromatography, carried out in a cold room at 4 °C with a chromatography system (NGC™ Chromatography System Quest™ 10 Plus, Bio-Rad).

Ultra-high affinity chromatography column Im7 (TriAltus) was used to capture CL7-tagged protein. Typically, a 5 mL Im7 column and a loading flow rate of 0.5 mL·min<sup>-1</sup> was used. After loading, the column was washed with Im7 Wash buffer (50 mM Sodium Phosphate, 500 mM NaCl, 5 % glycerol, pH 8.0) until the absorbance at 280 nm was stabilized, followed by a second wash with SEC buffer (50 mM Sodium Phosphate, 150 mM NaCl, 2.5 % glycerol, pH 8.0). Cleavage was carried out by premixed 6xHis-UlpI SUMO protease (recombinantly prepared in



our laboratory) to a final concentration of 35  $\mu\text{g}\cdot\text{mL}^{-1}$  in SEC buffer with the addition of DTT to the final concentration of 1 mM. The protease was loaded to the Im7 column in two steps divided by 20 min intermediate equilibration and followed up to a two-hour incubation. The cleaved protein was eluted at flow rate of 0.3  $\text{mL}\cdot\text{min}^{-1}$ . After the first affinity purification step, we typically obtained 4 mg of the protein mixture, as shown in Fig. 2a – Elu fraction.

Affinity chromatography with His-trap column (HisTrap™HP, Cytiva), typically 1 mL column volume, flow rate 1  $\text{mL}\cdot\text{min}^{-1}$ , was employed in separating chaperone and other impurities from the sample. REST-N62 partially non-specifically bound to the column matrix. Elution was done in the 0–300 mM imidazole gradient in 50 mM Sodium Phosphate, 150 mM NaCl, 2.5 % glycerol, pH 8.0. Washing by 45 mM imidazole corresponding to 15 % imidazole gradient removed impurities, as shown in SDS-PAGE Fig. 2a. REST-N62 was eluted together with Ulp1 protease at 150 mM imidazole concentration (50 % imidazole gradient) – typically total fraction volume 4 – 5 mL contained 0.8 mg of total protein amount.

Size exclusion chromatography was employed to remove the remaining Ulp1 protease contaminant and change buffer composition. SEC column HiLoad® 16/600 Superdex® 75 prep grad (Cytiva) was equilibrated in two volumes of SEC buffer. The collected sample from His-trap selected fractions was centrifugated at 15000g, 10 min, 4 °C to ensure no protein aggregation before loading to the SEC column. SEC separation was carried out at a rate of 1  $\text{mL}\cdot\text{min}^{-1}$ .

Collected samples from the first main peak were concentrated by ultrafiltration on 10 K Amicon® Ultra-4 Centrifugal Filters Units (Merck Millipore) in a swinging-bucket rotor 4000g, 4 °C, until the concentration of protein was higher than 2  $\text{mg}\cdot\text{mL}^{-1}$ . After concentrating, REST-N62 was frozen in liquid nitrogen and stored at -80 °C. Typical purification from 3 L of TB media yielded 0.3 mg of pure REST-N62. The final purity of REST-N62 is in Fig. 4. Purification was carried out within one day to preserve protein activity.

#### 4.2. The cloning, expression, and purification of REST-FL

The source of the REST-FL sequence was a plasmid obtained from Addgene (#41903). Recombinant expression cassette contained inserts in the following order: start codon, MBP-tag, 6xHis, S-tag, CL7 Affinity Tag, short 90 bp linker, SrtA site, REST-FL, stop codon. The pFASTBAC1 plasmid with polyhedrin promoter (Invitrogen) was linearized by digestion with restriction enzymes *Bam*HI and *Hind*III (New England Biolabs).

To perform the recombinant assembly reaction, NEBuilder® HiFi DNA Assembly kit (New England Biolabs) was used with the combination of three different inserts produced by PCR: the first insert contained the MBP-tag, 6xHis, and S-tag from MBP-enhanced vector pTriEx4 (Novagen); the second insert contained CL Affinity Tag followed by a 90 bp linker taken from pI21 plasmid (TriAltus); and the third contained the REST-FL coding sequence followed by a stop codon. All PCR products and digested plasmid were separated by agarose gel electrophoresis and extracted from the gel with QIAquick® Gel Extraction Kit (Qiagen). After assembly, this construct was transformed into *E. coli* One Shot™ TOP10 (Invitrogen). The recombinant gene insertion was confirmed by plasmid DNA sequencing.

Sf9 cells (Novagen) were used to generate baculovirus stocks using the Bac-to-Bac system (Life Technologies) with a combination of bacmid donor cell DH10EmBacY (Geneva-Biotech), according to the manufacturer's instructions. The titer of baculovirus stock was quantified using expressed YFP via fluorescence which serves as an infection marker.

Protein expression was carried out in sterile Vitlab Reagent bottles (VITLAB #100389) [41]. Screw caps with a drilled hole were covered in gas-permeable non-woven adhesive tape (Batist® Medical, #1320103112). Baculoviral P3 stock was used to infect Sf9 insect cells at  $1.5 \times 10^6$  cells· $\text{mL}^{-1}$  in Insect-XPRESS media (Lonza #12–730Q) supplemented with 2 % FBS (Capricorn #FBS-12A). For typical 1 L expression,

3 mL of baculoviral stock was added to 1 L of Sf9 cells. Infected cells were incubated in dark for 72 h at 27 °C and 230 rpm (New Brunswick™ Innova® 42R), then the cells were harvested by centrifugation 1500g, 20 min, 4 °C. Pellet was washed in 40 mL of 1x PBS and centrifugated in a swinging-bucket rotor 3000g, 10 min, 4 °C. After centrifugation, cells were snap-frozen in liquid nitrogen and stored at -80 °C until protein purification. From the typical 1 L expression, we have obtained 8–12 g of cells containing REST-FL.

All the following purification steps were carried out at 4 °C with precooled buffers and instruments. The frozen pellet was thawed on ice and resuspended in the lysis buffer (50 mM Sodium Phosphate, 500 mM NaCl, 5 % glycerol, 0.1 % Triton X-100, pH 8.0) with the addition of a final concentration of 1 mM PMSF and 5 mM benzamide, leupeptin 1.9  $\mu\text{g}\cdot\text{mL}^{-1}$  and pepstatin A 3.1  $\mu\text{g}\cdot\text{mL}^{-1}$ .

Typically, 30 mL of lysis buffer was used for insect cells harvested from 1 L of media. The cell suspension was transferred into stainless steel container cooled on ice and sonicated for 5 min with an amplitude 30 %, 1-second pulse-on, 1 s pulse-off with a 60-second process time cycle with a 15-second pause for extra cooling (Misonix Qsonica S-4000). The lysate was cleared by centrifugation 80000g, 1 h, 4 °C, and filtered by 0.45  $\mu\text{m}$  Sterivex™ Filter Units (Merck Millipore).

REST-FL was purified in two-step chromatography and carried out in a cold room at 4 °C with a chromatography system (NGC™ Chromatography System Quest™ 10 Plus, Bio-Rad).

Ultra-high affinity chromatography column Im7 (TriAltus) was used to capture CL7-tagged protein. We used a 1 mL Im7 column and a flow rate of 0.35  $\text{mL}\cdot\text{min}^{-1}$ . After loading, the column was washed with Im7 Wash buffer (50 mM Sodium Phosphate, 500 mM NaCl, 5 % glycerol, pH 8.0) until the absorbance at 280 nm decreased and stabilized, followed by a second wash with storage buffer (50 mM Sodium Phosphate, 150 mM NaCl, 2.5 % glycerol, pH 8.0).

Cleavage was carried out by premixed SortaseA (SrtA) protease (recombinantly prepared in our laboratory, variant  $\text{Ca}^{2+}$  independent SortaseA mutant with enhanced activity: SortaseA-6xHis [42]) to a final concentration of 90  $\mu\text{g}\cdot\text{mL}^{-1}$  in storage buffer with the addition of DTT to the final concentration of 1 mM. SortaseA has a recognition site: LPETG and leaves no extra amino acid on the N-terminal part of the protein of interest.

The protease was loaded to the Im7 column in two steps divided by 20 min intermediate equilibration and followed by a two-hour incubation. The tag-free protein was eluted at the flow rate of 0.3  $\text{mL}\cdot\text{min}^{-1}$ . After the first affinity purification step, we typically obtained 0.6 mg of protein mixture from 1 L of insect cell expression, as shown in Fig. 3a, Elu fraction.

Affinity chromatography with His-trap column (HisTrap™HP, Cytiva), 1 mL column volume, flow rate 0.5  $\text{mL}\cdot\text{min}^{-1}$ , was used to separate protease from the sample. The protein fractions were eluted in 0–300 mM imidazole gradient in a buffer of 50 mM Sodium Phosphate, 150 mM NaCl, imidazole, 2.5 % glycerol, pH 8.0. REST-FL was eluted at 15 mM imidazole concentration – typically, we obtained 0.2 mg of REST-FL in 5 – 6 mL. The remaining SrtA protease was eluted at a 150 mM imidazole as shown in SDS-PAGE Fig. 3a.

Collected samples from the first main peak were concentrated by ultrafiltration on 30 K Amicon® Ultra-0.5 Centrifugal Filters Units (Merck Millipore) in a fixed rotor 14,000g, 4 °C, until the concentration of protein was higher than 1  $\text{mg}\cdot\text{mL}^{-1}$  and imidazole in the solution was less than 5 mM (sample was diluted by storage buffer). After the final concentration, REST-FL was frozen in liquid nitrogen and stored at -80 °C. The typical yield from 2 L of insect cell culture was 0.05 mg of REST-FL with the final purity showed in Fig. 4. Purification was carried out within one day to preserve protein activity.

#### 4.3. NRSE duplex preparation

NRSE sequence, 21 bp DNA duplex was prepared by annealing a fluorescently labeled (Alexa Fluor 488) oligonucleotide 5'-TTCAGCA

CCATGGACAGCGCC-3' with its complementary unlabeled strand [14,15]. Both oligonucleotides were purchased from Eurofins Genomics. The substrate was purified on a Mono Q 5/50 GL column (GE Healthcare) in buffer 25 mM Tris-HCl, 50 mM NaCl, pH 8.0 with elution of salt gradient up to 1 M.

#### 4.4. Binding analysis using fluorescence anisotropy

The interactions of REST-FL or REST-N62 with NRSE sequence labeled by Alexa Fluor 488 were measured on a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon) at 25 °C. Fluorescence anisotropy was measured at an excitation wavelength of 496 nm and an emission wavelength of 521 nm. The slit width (both excitation and emission) for all measurements was 8 nm and the integration time was 1 s. Incubation time before the measurement was 25 min.

The cuvette contained 1.4 mL of labeled 7.5 nM NRSE DNA in a FA buffer (50 mM sodium phosphate, 150 mM NaCl, pH 8.0). The protein was titrated into the solution in the cuvette and measured after a 1 min incubation at 25 °C. Fluorescence anisotropy at each titration step was measured three times. The dissociation constant  $K_D$  was determined from non-linear fitting of the binding data using equation:  $FA = FA_{min} + FA_{max} \cdot (x + DNA_t + K_D) - ((x + DNA_t + K_D)^2 - (4xDNA_t))^{0.5} / 2DNA_t$  derived for one-site binding of REST on DNA [43];  $x$  is the concentration of added protein;  $FA_{min}$  and  $FA_{max}$  are minimal and maximal fluorescence anisotropy values;  $DNA_t$  is the total concentration of DNA in a cuvette. Experimental data were visualized, and the fit was calculated using ORIGIN® 9.9.0.225 (OriginLab).

#### 4.5. Coimmunoprecipitation and western blotting

Human cells HEK293T (kindly provided by Agnel Sfeir, MSK, USA) were cultured in DMEM (Gibco #41966) supplemented with 10 % FBS (Biosera #FB-1090), 4 mM L-glutamine (Gibco #25030), 1x non-essential amino acids (Gibco #11140), 100 units·mL<sup>-1</sup> penicillin and 100 µg·mL<sup>-1</sup> streptomycin (Sigma-Aldrich #P4333) at 37 °C with 5 % CO<sub>2</sub> for no longer than 13 passages.

3xFLAG-tagged REST-N62 (pcDNA5 vector), HALO-tagged REST-FL (pHAGE2 vector), and empty 3xFLAG-tagged pcDNA5 vector were used to transiently co-express proteins in different combinations together, as shown in Fig. 6. Transfection was carried out by polyethyleneimine – PEI in a six-well plate for 4 h. The cells continued to grow for the next 48 h and later were harvested, washed with 1x PBS. The cells were lysed in CoIP lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100, pH 7.4, and freshly added 10 mM NaF) with protease inhibitor cocktail cOmplete tablets (Roche) for 45 min in a cold room (4 °C) on a rotator. The supernatant was separated by centrifugation 15000g, 20 min, 4 °C, and mixed with Anti-FLAG® M2 magnetic beads (Millipore) – typically 900 µL of supernatant and 30 µL of preequilibrated M2 beads.

Incubation was carried out in a cold room on a rotator for 2 h. After incubation, beads were washed with 5 × 500 µL CoIP lysis buffer. To elute IP fractions, beads were incubated 1 h on ice in 30 µL of CoIP elution buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) containing 0.2 mg·mL<sup>-1</sup> 3xFLAG peptide (Sigma-Aldrich).

Samples were loaded on 10 % SDS-PAGE gel, separated, and wet transferred to a membrane (Cytiva, Amersham™ Protran®). Western blots were carried out with two different antibody setups: (i) FLAG tag was incubated with monoclonal ANTI-FLAG® M2-Peroxidase (HRP) produced in mouse (Sigma-Aldrich #A8592) diluted 1:3000, and (ii) HALO tag was incubated with primary Anti-HaloTag® Monoclonal Antibody (Promega #G9211) diluted 1:1000 with secondary antibody Anti-Mouse IgG (Fc specific) HRP (Sigma-Aldrich #A0168) diluted 1:5000. Primary antibodies were incubated in a cold room overnight. The secondary antibody was incubated for one hour at RT on a rotator. Detection was carried out with SuperSignal™

West Femto Maximum Sensitivity Substrate (Thermo Scientific™ #34095) and visualized on Fusion FX (Vilber).

#### 4.6. SDS-PAGE analyses

We employed sodium dodecyl sulfate–denaturing polyacrylamide gel electrophoresis to analyze the purity of proteins in each stage of purification. We used self-casted fixed-concentration polyacrylamide SDS gels containing 10 % or 12 % acrylamide:bis-acrylamide (37:1) for the separations of proteins with similar molecular weight. Proteins were separated on fixed-concentration SDS-PAGE at  $E = 10 \text{ V} \cdot \text{cm}^{-1}$ .

We used gradient 4–20 % Mini-PROTEAN® TGX™ Precast Protein Gels (BioRad) for separation of proteins with varied molecular range. SDS-PAGE with gradient gels was performed according to the manufacturer's protocol (200 V Standard): 15 min at 30 mA, followed by 20 min at 25 mA. We used proteins included in the molecular weight marker PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific) as standards. If needed, proteins in gels were visualized by Bio-Safe™ Coomassie Stain (BioRad).

#### 4.7. Negative stain electron microscopy

Four microliters of 0.02 mg·mL<sup>-1</sup> REST protein sample were loaded on a plasma-treated TEM grid supplemented with a homemade 12 nm thick continuous carbon layer. The sample was incubated for 30 s, washed once in a drop of 2 % uranyl acetate (UA), and then stained in the second UA drop for 60 s. Images were collected using the 200 kV Talos F200C transmission electron microscope equipped with the 16 M Ceta detector using SerialEM [44]. The overall dose was 15 e·Å<sup>-2</sup> per single image, and data were collected at the calibrated pixel size of 2.65 Å. Data analysis was carried out in CryoSparc [45].

The analyzed dataset comprised 324 micrographs. Particles from 20 micrographs were picked manually and used to generate references for template picking. Following the reference-free 2D classification, 9218 particles were selected to determine the REST structure. The CryoSparc ab initio routine was used to determine the initial model and further classify the data in 3D. Finally, 3540 particles were refined to produce the REST model at 18.5 Å global resolution.

## 5. Conclusions

Our study provides new insights into the structure-function relationships of human REST as a master regulator of neural differentiation. We presented the electron microscopy structure of full-length REST, suggesting that its toroidal shape can accommodate DNA in the central hole.

We developed protocols for the preparation and subsequent characterization of human REST and its splicing isoform REST-N62 allowing further structural and functional studies in vitro. We quantified REST binding to the canonical DNA. We excluded the high-affinity interaction of full-length REST and splicing variant REST-N62.

We discussed the implications of the new structural findings to known functions of REST.

#### CRedit authorship contribution statement

Conception and design of study: P. Veverka, T. Brom, T. Janovic, C. Hofr. acquisition of data: P. Veverka, T. Brom, T. Janovic, M. Stojaspal, M. Pinkas, J. Novacek, C. Hofr. Analysis and/or interpretation of data: P. Veverka, T. Brom, T. Janovic, M. Stojaspal, M. Pinkas, J. Novacek, C. Hofr. Drafting the manuscript: P. Veverka, T. Brom, T. Janovic, M. Stojaspal, M. Pinkas, J. Novacek, C. Hofr. Revising the manuscript critically for important intellectual content: C. Hofr. Approval of the version of the manuscript to be published: P. Veverka, T. Brom, T. Janovic, M. Stojaspal, M. Pinkas, J. Novacek, C. Hofr.

## Competing interests

None declared.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.csbj.2022.12.026](https://doi.org/10.1016/j.csbj.2022.12.026).

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