Sirtuin-1 sensitive lysine-136 acetylation drives phase separation and pathological aggregation of TDP-43

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Trans-activation response DNA-binding protein of 43 kDa (TDP-43) regulates RNA processing and forms neuropathological aggregates in patients with amyotrophic lateral sclerosis and frontotemporal lobar degeneration. Investigating TDP-43 post-translational modifications, we discovered that K84 acetylation reduced nuclear import whereas K136 acetylation impaired RNA binding and splicing capabilities of TDP-43. Such failure of RNA interaction triggered TDP-43 phase separation mediated by the C-terminal low complexity domain, leading to the formation of insoluble aggregates with pathologically phosphorylated and ubiquitinated TDP-43. Introduction of acetyl-lysine at the identified sites via amber suppression confirmed the results from site-directed mutagenesis. K84-acetylated TDP-43 showed cytoplasmic mislocalization, and the aggregation propensity of K136-acetylated TDP-43 was confirmed. We generated antibodies selective for TDP-43 acetylated at these lysines, and found that sirtuin-1 can potently deacetylate K136-acetylated TDP-43 and reduce its aggregation propensity. Thus, distinct lysine acetylations modulate nuclear import, RNA binding and phase separation of TDP-43, suggesting regulatory mechanisms for TDP-43 pathogenesis.

https://doi.org/10.1038/s41467-022-28822-7

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The trans-activation response DNA-binding protein of 43 kDa (TDP-43) regulates various RNA-processing steps\(^1\) and is found in the neuropathological lesions of patients with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD)\(^2\,\,^3\,\,^4\). The TDP-43 polypeptide contains two RNA recognition motifs (RRM1 and RRM2), a nuclear localization signal (NLS) and a C-terminal glycine-rich low complexity domain (see Fig. 1a). The NLS allows active nuclear import\(^5\), but TDP-43 can also shuttle between the nucleus and cytoplasm\(^2\,\,^24\). Through its RRM1 TDP-43 binds to UG-rich regions of RNA\(^6\,\,^10\) and it is involved in RNA transport, stability, and splicing, for example, exon 9 of the cystic fibrosis transmembrane conductance regulator (CFTR) mRNA\(^11\). The C-terminal low complexity domain of TDP-43 promotes liquid-liquid phase separation (LLPS) as well as pathological protein aggregation\(^12\). Post-translational modifications (PTMs) can modulate TDP-43 functions in health and disease. Putative pathological PTMs of TDP-43 include C-terminal fragmentations and phosphorylations\(^13\), including the widely used marker pS409/410\(^14\,\,^15\). In addition, lysine modifications have been reported, including ubiquitinations at various residues\(^16\) and sumoylation and acetylation of the RRM1\(^17\,\,^18\). The putative acetyl-mimic [K145Q]TDP-43 was distributed in a stippled manner in transfected cells, eventually recapitulating pathological phosphorylation and recruitment of ALS-related factors into TDP-43 aggregates\(^19\). In addition, [K145Q]TDP-43 had reduced CFTR splicing activity. Thus, lysine modifications may be important for physiology and pathological aggregation of TDP-43. However, the genesis of TDP-43 aggregates remains elusive.

Research on LLPS is unveiling the molecular processes regulating the arrangement of membrane-less organelles. Heteronuclear ribonucleoprotein (hnRNP) LLPS is amply reported but whether and how this process is regulated in cells is poorly understood\(^20\). In the case of TDP-43, RNA binding prevents its phase separation in vitro and reduces droplet formation in vivo\(^21\). Wild-type (wt)TDP-43 is capable of going into liquid droplets but pathological mutants seem to form more resilient droplets. While the C-terminal glycine-rich domain of TDP-43 seems to play a crucial role in the formation of phase-separated droplets, the N-terminal domain may also contribute to the aggregation process\(^22\,\,^24\). Thus, the regulation of TDP-43 RNA binding could be pivotal in the pathophysiology of TDP-43. The molecular pathways regulating the disengagement from RNA are only beginning to be described.

In the present study, we discovered that acetylation of K84 within the NLS reduced nuclear import whereas acetylation of K136 in the RNA recognition domain impaired TDP-43 RNA binding and splicing capabilities. Such failure of RNA interaction triggered TDP-43 phase separation mediated by the C-terminal low complexity domain, leading to the formation of insoluble aggregates with pathologically phosphorylated and ubiquitinated TDP-43. To confirm the results from site-directed mutagenesis, we expanded the genetic code via amber suppression to introduce authentic acetyl-lysine at the identified sites. Indeed, [acK84]TDP-43 showed cytoplasmic mislocalization, and the increased aggregation tendency for [acK136]TDP-43 was confirmed. We generated antibodies selective for TDP-43 acetylated at K84 and K136. Using these tools, we found that the nuclear sirtuin SIRT1 can potently deacetylate [acK136]TDP-43. Moreover, SIRT1 reduced the aggregation propensity of [acK136]TDP-43. Thus, distinct lysine acetylations regulate nuclear import, RNA binding and phase separation of TDP-43 with possible relevance for TDP-43 pathogenesis.

**Results**

**Detection of TDP-43 lysine acetylations.** After investigating the sites of TDP-43 lysine ubiquitinations\(^16\), we became interested in how other PTMs could affect TDP-43 functionality and aggregation. Recently acetylation has been linked with TDP-43 pathology\(^17\,\,^19\), and lysine acetylation has the potential to disrupt ubiquitination by competing for the same residue. To explore this hypothesis more in depth, we re-examined the mass spectrometry data\(^16\) specifically for acetylated lysines. We found in transfected HEK293E cells that 6xHis-tagged wtTDP-43 was acetylated at lysine residues K79 and K84 around the NLS and K121 and K136 in RRM1 (Fig. 1a). We could not detect the previously published acetylations at K145 and K192\(^21\), perhaps due to slight differences in experimental conditions (see "Discussion").

**Site-directed mutagenesis of TDP-43 acetyl-lysine sites.** Each of the identified acetylated lysine residues was mutagenized to either arginine or glutamine to simulate the lack or the presence of acetylation, respectively. We first looked at the subcellular distribution of these N-terminally 6xHis-tagged TDP-43 mutants in transiently transfected HEK293E cells. Immunofluorescence (IF) staining of the 6xHis tag did not show visible deviation from the wtTDP-43 nuclear staining pattern for both acetyl-dead and acetyl-mimic mutations at residues K79 and K121 (Supplementary Fig. 1a). Likewise, the conservative [K84R]TDP-43 mutant remained mainly nuclear, but a substantial portion of acetyl-mimic [K84Q]TDP-43 was retained in the cytoplasm (Fig. 1b). Quantification showed a significant increase in the percentage of cells with cytoplasmically mislocalized [K84Q]TDP-43 (Fig. 1c). In addition, we performed two different biochemical nucleocytoplasmic fractionation assays to support these results. There was a significant increase of [K84Q]TDP-43 in the cytoplasmic fraction when compared to wtTDP-43 (Supplementary Fig. 1b–d). Nevertheless, we could still observe a sizable amount of [K84Q]TDP-43 in the nuclear fraction, as for the [ANLS]TDP-43 triple mutant. Thus, alterations at the NLS reduced but did not abolish TDP-43 nuclear import, accounting for the residual splicing activity in the nucleus (see Fig. 3e, f). By contrast, the acetyl-mimic K79Q substitution did not show evident changes in nucleocytoplasmic distribution of TDP-43, likely because K79 is localized just outside the NLS, whereas K84 is at the core of the NLS\(^5\,\,^16\).

The RRM1 mutants K136R and K136Q were predominantly localized in the nucleus, but a significant number of cells displayed a nuclear droplet-like pattern (Fig. 1b, c). This droplet-like nuclear distribution was reminiscent of the RNA-binding deficient F147L/F149L mutant TDP-43\(^23\,\,^25\). For comparison, we generated the previously described\(^17\,\,^19\) acetyl-mimic K145Q mutant in RRM1, which showed the expected stippled distribution (Supplementary Fig. 1a). As K136 is in direct contact with bound nucleic acids\(^36\,\,^27\), we assume that modifications at K136 disrupt nucleic acid binding and therefore disengage TDP-43 from hnRNP complex localizations. Such dissociated K136-modified TDP-43 may be free to phase separate and self-aggregate into inclusions.

**K136 mutant TDP-43 is pathologically ubiquitinated, phosphorylated, and insoluble.** We examined if the K136 mutant TDP-43 inclusions in cell culture also showed pathological features described for human patients\(^4\,\,^5\,\,^14\,\,^28\). 6xHis-tagged TDP-43 variants were transiently transfected into HEK293E cells with stable knockdown of endogenous TDP-43 (shTDP-43)\(^29\) to minimize interference from the endogenous wtTDP-43. To assess protein solubility, RIPA-urea solubility fractionation assays were performed. While most of the 6xHis-tagged wtTDP-43 was RIPA soluble, a larger portion of both K136R and K136Q TDP-43 mutants shifted into the RIPA-insoluble fraction (Fig. 2a). Although [F147L/F149L]TDP-43 formed similar intranuclear patches, this designed RNA-binding deficient mutant was as soluble as endogenous TDP-43 (Fig. 2a). It should be noted that
the Flag-tagged [F147L/F149L]TDP-43 was expressed at levels comparable to endogenous TDP-43 in non-silenced cells, whereas the 6xHis-tagged K136 mutants were expressed at higher levels. To check for pathological PTMs, 6xHis-tagged TDP-43 variants were transiently transfected into sh TDP-43-HEK293E cells and purified using NiNTA beads. Both K136R and K136Q TDP-43 were ubiquitinated more strongly than wtTDP-43 even after proteasomal inhibition (Fig. 2b). In addition, K136R and K136Q mutant TDP-43 showed phosphorylation at the extreme C-terminal serine residues S409/410 detected with a phospho-
Specific antibody\(^{15}\) (Fig. 2b). This pathological phosphorylation was also visible at the nuclear inclusions of K136 mutant TDP-43 by immunostaining (Fig. 2c). Thus, the nuclear inclusions formed by K136 mutant TDP-43 recapitulate PTMs found in patients.

**K136 mutant TDP-43 fails to bind RNA and lacks splicing activity.** Because K136 in the RRM1 is important for interaction with nucleic acids,\(^{26,27}\), we hypothesized that alterations at position K136 could interfere with RNA binding of TDP-43 and alter its splicing capabilities. First, we performed a RNA-protein pulldown assay of TDP-43 with its preferred binding sequence poly-(UG)\(^{12}\). While wtTDP-43 strongly bound to synthetic poly-(UG)\(^{12}\) but not the negative control poly-(UC)\(^{12}\) RNA, the acetyl-mimic [K136Q]TDP-43 showed a reduction in RNA binding (Fig. 3a). Additionally, we measured the RNA-binding strength using a two-filter trap assay using an excess of synthetic poly-(UG)\(^{12}\) as target. The first nitrocellulose membrane will capture the biotinylated RNA probe only when bound to protein and the second nylon membrane captures the unbound flow-through RNA. This assay clearly showed the concentration-dependent formation of [wt]TDP-43 protein complex with poly-(UG)\(^{12}\) RNA retained on the nitrocellulose membrane (Fig. 3b). In contrast, the acetyl-mimic [K136Q]TDP-43 had significantly reduced poly-(UG)\(^{12}\) RNA-binding strength (Fig. 2b–d).

To assess the splicing activity of TDP-43 in cells, we used the established CFTR minigene reporter assay\(^{11}\). To minimize the splicing activity of the endogenous wtTDP-43, we used shTDP-43-HEK293E cells\(^{29}\). Mutant constructs of TDP-43 were cotransfected together with a plasmid containing exons 9–11 of CFTR in a minigene. In parental, non-silenced cells exon 9 was skipped, which was severely blunted in shTDP-43 cells (Fig. 3e). Ex9 splicing could be rescued by re-transfecting [wt]TDP-43 (Fig. 3e). The nuclear [K84R]TDP-43 rescued CFTR splicing to wt levels. [K84Q]TDP-43 rescued CFTR splicing activity to a lesser extent than wt or [K84R]TDP-43 (Fig. 2h). The reduced splice activity of the nuclear import impaired [K84Q]TDP-43 was similar to that of [ΔNLS]TDP-43, suggesting that the residual nuclear TDP-43 (Fig. 1d) was sufficient to promote ex9 skipping. Indeed, in our experience, very strong reduction of TDP-43 activity is necessary to cause loss of TDP-43 splice activity in cells. In contrast, both K136R and K136Q mutants showed significantly reduced CFTR exon 9 splicing (Fig. 3e, f), linking the formation of nuclear inclusions with TDP-43 loss of function. The previously described\(^{17,19}\) K145Q mutant TDP-43 also showed some reduction of CFTR splice activity, although the effect was less pronounced than for the K136 mutants expressed at comparably high protein levels (Fig. 3e). Together, both CFTR splicing assay and RNA-protein pulldown show that alterations at position 136 severely perturb the RNA-binding and splicing capabilities of TDP-43.

It was puzzling that both the acetyl-mimic K136Q and the acetyl-dead K136R substitutions showed the same aggregation-promoting effects. The K136 residue may be in a structurally restrained position not tolerating even conservative amino acid

**Fig. 2 K136 mutations reduce the solubility of TDP-43 and trigger its ubiquitination and pathological phosphorylation.** a HEK293E shTDP-43 cells were transfected with 6xHis-tagged TDP-43 (wt, K136R and K136Q) or Flag-tagged FFLL TDP-43. RIPA soluble and insoluble fractions were separated and analyzed by Western blotting. N = 3 biologically independent samples. Source data are provided as a Source Data file. b ShTDP-43-HEK293E cells were transfected with 6xHis-tagged TDP-43 (wt, K136R and K136Q). Proteasome activity was inhibited by treatment with 20 µM MG-132 for 6 h, which caused wtTDP-43 ubiquitination (lane 3). 6xHis-tagged TDP-43 was purified with NINTA beads and analyzed on Western blots. N = 3 biologically independent samples. Source data are provided as a Source Data file. c Transfected shTDP-43-HEK293E cells were double immunostained for TDP-43 and phosphorylated TDP-43 (S409/410). Scale bar represents 10 µm. N = 3 biological replicates.
Fig. 3 K136 mutations alter the RNA binding and splicing capabilities of TDP-43. a HEK293E shTDP-43 cells were transfected with an empty plasmid, wt or K136Q TDP-43. The lysates were then incubated with biotinylated (UC)_{12} or (UG)_{12} RNA oligomers. Bound proteins were eluted and analyzed by Western blotting. \( N = 3 \) biological replicates. Source data are provided as a Source Data file. b Increasing concentrations of 6xHis purified TDP-43 proteins were incubated with biotinylated (UG)_{12} oligomers. The resulting complexes were analyzed by a filter-binding assay. Membranes were incubated with HRP-coupled streptavidin. One of the [K136Q]TDP-43 data points illustrates overload problems at high protein concentration (>875 nM), precluding firm establishment of saturation binding curves. Source data are provided as a Source Data file. c Quantification of three replicates of filter-binding assay of wt (full line) and K136Q (dashed line) TDP-43 to biotinylated (UG)_{12} RNA oligomers. **\( p < 0.01 \). Data are presented as mean values ± SD. Unpaired, two-sided t-test was used for comparison. Source data are provided as a Source Data file. d Representative Western blot of the protein levels before and after the purification of native TDP-43 via NiNTA pulldown. \( N = 3 \) biological replicates. Source data are provided as a Source Data file. e HEK293E shTDP-43 cells were cotransfected with the specified TDP-43 constructs and a plasmid containing the CFTR minigene. RNA was extracted and splicing of CFTR exon 9 was assessed via rtPCR (upper panel). Protein levels are shown in the lower panel. \( N = 3 \) biologically independent samples. f Quantification of three replicates assessing effects of mutations at different lysines of TDP-43 on the splicing of CFTR exon 9. Data are presented as mean values ± SD. Unpaired, two-sided t-test was used for comparison. **\( p < 0.01 \). Source data are provided as a Source Data file.
substitutions. Indeed, the NMR structure of the TDP-43 RRMs complexed with RNA revealed that the side chain of K136 is in close apposition to the nucleic acid and the key RNA-binding residues F147/F149 (Supplementary Fig. 2a). Moreover, K136 is within \(\approx 3\) A distance to Q134, L139 and T199, evidently forming a tight RNA-binding configuration that appears not to accommodate the larger guanidinium group of the otherwise conservative K136R substitution. Supplementary Fig. 2b highlights the K136 residue within the RRM1 bound to RNA. Modelling the acetyl-dead K136R mutation, which surprisingly caused similar aggregation propensities as the acetyl-mimicking K136Q substitution, revealed clashes with the protein backbone. Thus, classical site-directed mutagenesis might introduce local perturbations to the RRM1 structure, which may not entirely reflect the effects of authentic lysine acetylation. Indeed, the model for [acK136]TDP-43 showed hardly any clashes with the protein backbone, but a collision with the bound RNA structure. In order to distinguish mutagenesis artefacts from authentic lysine acetylation effects, we needed to introduce acK at position 136 of TDP-43.

Introduction of acetyl-lysine (acK) through amber suppression. As K136 turned out to be a very delicate residue for site-directed mutagenesis, it was important to properly validate the pro-aggregative effects for [acK136]TDP-43. We succeeded to incorporate authentic acK at the apparently regulatory sites in TDP-43 with amber suppression methodology. TDP-43 mutants were generated with a C-terminal 6xHis tag and an amber stop codon (TAG) substituting the codon at position 84 and at position 136, respectively. Each of these plasmids was cotransfected into shTDP-43-HEK293E cells, together with a plasmid containing a N-terminally Flag-tagged chimeric acetylated lysine RNA synthetase (AcRS) and four copies of tRNA\(_{\text{TAG}}\). Together the designed AcRS and the tRNA\(_{\text{TAG}}\) will incorporate acK added to the media at the introduced amber stop codon, directly incorporating an acetylated lysine site specifically during the protein translation.

In absence of acK, only truncated forms of TDP-43 were visible due to the introduction of the amber stop codons (Fig. 4a). After adding 5 mM acK in the media for 24 h, the constructs were translated beyond the amber sites such that full-length TDP-43 including the C-terminal 6xHis tag became detectable, confirming successful amber suppression (Fig. 4a). There was a reduction in the amount of truncated TDP-43 but it did not completely disappear, pointing out the slight inefficiency of the system when compared to endogenous tRNAs. The expression of amber-suppressed full-length mutant TDP-43 was not as high as that from a wtTDP-43 cDNA (Fig. 4a), but still robust. Importantly, [acK84]TDP-43 showed a similar reduction of nuclear import (Fig. 4b) as the K84Q mutant (Fig. 1b), and [acK136]TDP-43 showed the same spotty nuclear distribution (Fig. 4b) as the K136Q mutant (Fig. 1b). To check if the RNA splicing capabilities of TDP-43 were compromised after introducing an acetylated lysine at position 136 in a similar manner to the K136R and K136Q mutants, we performed the CFTR minigene assay in shTDP-43–HEK293E cells. Indeed, [acK136]TDP-43 could only partially rescue CFTR exon 9 splicing (Fig. 4c). In addition, we also introduced acetylated lysine at K79 and K121 (Supplementary Fig. 3). As expected from the point mutations (Supplementary Fig. 1a), [acK79]TDP-43 and [acK121]TDP-43 did not show any different distribution than wtTDP-43. Taken together, the acetyl-mimic lysine-to-glutamine substitutions did reflect authentic lysine acetylation properties, as confirmed by amber suppression introduction of acK directly.

We were then interested if [acK136]TDP-43 could form phase-separated droplets or even aggregates in the cytoplasm, the most common site where human neuropathological TDP-43 is found. To study this, we disrupted the NLS in the construct ΔNLS-K136TAG. [AcK136]TDP-43ΔNLS was uniformly distributed throughout the cytoplasm in most cells. However, there was a fraction of cells developing multiple cytoplasmic aggregates (Fig. 4d–f). Thus, when nuclear import is impaired, K136 acetylation may also promote aggregation of cytosolically mislocalized TDP-43. Next, we deleted the C-terminus in the construct ΔC-K136TAG because the C-terminal low complexity domain mediates LLPS and aggregation of TDP-43. [acK136]TDP-43AC showed a homogeneous nuclear distribution (Fig. 4d–f), confirming that the C-terminal part of TDP-43 plays a crucial role in phase separation and aggregation.

FRAP analysis and dynamics of K136 mutant TDP-43 aggregation. As we confirmed that [K136Q]TDP-43 showed the same properties as amber suppressed [acK136]TDP-43, we characterized in greater detail the aggregation process of a C-terminally EGFP-tagged [K136Q]TDP-43 construct. First we measured protein motility in the droplet-like inclusions by fluorescence recovery after photobleaching assay (FRAP). HEK293E cells were transfected with either wtTDP-43-EGFP or [K136Q]TDP-43-EGFP. Then rows of interest (ROI) with bleached for half a second by 488 nm laser light irradiation and allowed to recover (Fig. 5a). The wtTDP-43-EGFP showed recovery within a minute after photobleaching (Fig. 5b), consistent with the motility of TDP-43 in the nucleus. By contrast, two types of less mobile pools of [K136Q]TDP-43-EGFP were detected (Fig. 5b). Large [K136Q]TDP-43-EGFP aggregates (\(\geq 1\) µm) did not recover for up to 3 min recording (Fig. 5b), likely constituting solid aggregates. The smaller punctae (\(\approx 0.5\) µm) showed a significantly slower recovery than the wtTDP-43 with an immobile fraction not recovering at all (Fig. 5b, c), indicative of LLPS.

To investigate the kinetics of LLPS and protein aggregation, we performed long-term live imaging of [K136Q]TDP-43-EGFP. Transfected HEK293E cells were imaged for several hours and the distribution of TDP-43 was monitored. [K136Q]TDP-43 was recorded going into phase separation, forming droplets and these droplets were seen fusing with one another (Fig. 5d and Supplementary Videos 1–3). To examine the [K136Q]TDP-43 aggregate-formation kinetics in detail we quantified the size of aggregates in HEK293E cells fixed 24, 48 and 72 h after transfection. The quantification showed that after 48 h there was an increase in the percentage of TDP-43 aggregates larger than 10 µm, and the size difference became significant after 72 h (Fig. 5e). Together, these results suggest that the aggregation of TDP-43 is a dynamic process from a diffuse distribution to phase separation, eventually towards larger solid aggregates.

Generation of site-specific antibodies against lysine-acetylated TDP-43. To study the acetylated forms of TDP-43 in greater detail, we developed rat monoclonal antibodies against acK84 and acK136 TDP-43. Two clones were selected that recognized with high sensitivity their corresponding amber-suppressed TDP-43. In Western blots, with a very faint band visible in the wtTDP-43 lane. The antibodies did not recognize K84R or K136R TDP-43 mutants, respectively. Both antibodies were also targets (Fig. 6a, b) in Western blots, with a very faint band visible high sensitivity their corresponding amber-suppressed TDP-43 acK136 TDP-43. Two clones were selected that recognized with detail, we developed rat monoclonal antibodies against acK84 and TDP-43 by IF staining (Fig. 6c, d).

SIRT1 can deacetylate [acK136]TDP-43 and revert its aggregation propensity. These antibodies allowed us to check for enzymes that could deacetylate [acK136]TDP-43. In humans, there are 18 protein lysine deacetylases (HDACs) 1–8, representing class I and class II
Fig. 4 Amber suppression-mediated acetylation at K136 of TDP-43 prevents CFTR splicing, triggers the formation of nuclear droplets via its C-terminal domain. Acetylation at K84 reduces nuclear import. a HEK293E cells cotransfected with a plasmid containing AcRS and 4xT-RNA_{TAG} (E451) and with TDP-43 with an amber stop codon at different positions and a C-terminal 6xHis tag. Media was changed to 5 mM acK-containing media 24 h before lysis. Protein lysates were analyzed via Western blot. N = 3 biological replicates. Source data are provided as a Source Data file. b Cells were cotransfected with the E451 plasmid and different C-terminally 6xHis-tagged TDP-43 constructs containing amber suppression codons. Cells were fixed after 24 h in the presence of 5 mM acK. Scale bar represents 10 µm. N = 3 biological replicates. c HEK293E shTDP-43 cells cotransfected with E451, CFTR minigene, and an empty plasmid, wt or K136TAG TDP-43. After 24 h in the presence of 5 mM acK, RNA was extracted and the splicing of CFTR exon 9 was assessed via rtPCR and visualized in an agarose gel. N = 3 biological replicates. Source data are provided as a Source Data file. d HEK293E shTDP-43 cells were cotransfected with E451 plasmid and different C-terminally tagged TDP-43 constructs. Lanes 3–5 have an amber stop codon at position 136 in addition to a mutated NLS, an amber stop codon at position 84 or a deletion of the C-terminus. Cells were treated with 5 mM acK for 24 h before lysis. N = 3 biological replicates. e HEK293E shTDP-43 cells were cotransfected with E451 and different C-terminally tagged TDP-43 constructs. Cells were fixed and immunolabeled after 24 h in the presence of 5 mM acK. Scale bar represents 10 µm. N = 3 biological replicates. f Quantification of percentage of cells with 6xHis-TDP-43 protein distribution diffuse nuclear (blue bars), nuclear clumpy (grey bars), nuclear + cytosolic, diffuse (orange bars) and nuclear + cytosolic, clumpy (yellow bars). A total of 250 cells per group cotransfected with E451 plasmid and different 6xHis C-terminally tagged TDP-43 constructs. Source data are provided as a Source Data file.
deacetylases. Each of these HDACs was cotransfected into amber suppressed [acK136]TDP-43 cells. None of the HDAC1-8 effectively reduced TDP-43 K136 acetylation, (Supplementary Fig. 4a). However, the results for Myc-tagged HDAC1 and HDAC6 were somewhat inconclusive as their expression interfered with the amber suppression efficiency for [acK136]TDP-43 mutants. Three cells quantified per group. Data are presented as mean values ± SEM. Unpaired, two-sided t-test was used for comparison *p < 0.05. Source data are provided as a Source Data file. e Quantification of mobile fraction of FRAP analysis of wt and K136Q TDP-43 mutants. Three cells quantified per group. Data are presented as mean values ± SEM. Unpaired, two-sided t-test was used for comparison *p < 0.05. Source data are provided as a Source Data file.

Fig. 5 [K136Q]TDP-43 droplets have reduced mobility, growing in size and fusing over time. a Representative images of FRAP analysis of HEK293E cells transfected with C-terminally EGFP-tagged wt and K136Q TDP-43. Two different groups of [K136Q]TDP-43-EGFP droplets based on size were determined. Time in seconds. Scale Bar represents 5 µm. b Quantification of FRAP images (three cells quantified per group). The inclusions bodies are classified as follows: diffuse TDP-43 (circles), small inclusions (triangles) and big inclusions (squares). Source data are provided as a Source Data file. c Selected frames from green fluorescence live-cell imaging of cells expressing [K136Q]TDP-43-EGFP. Close-up of granules fusing (white arrows). Time in seconds. Scale bar represents 5 µm. N = 3 biological replicates. e Histogram of K136Q TDP-43 aggregate size in HEK293E fixed 24, 48 or 72 h after transfection. A total of 100 transfected cells analyzed for each time point. Different patterns represent the respective hours after transfection. Unpaired, two-sided t-test was used for comparison. Data are presented as mean values ± SEM. **p = 0.0075. Source data are provided as a Source Data file.
Discussion

In ALS only 4% of familial cases have mutations in TDP-43 and they are even rarer in FTLD (<1%)\(^36,37\). PTMs of wtTDP-43 might promote disease but it is still unknown if they are a cause or consequence of TDP-43 aggregation\(^5,17\). Here we show that TDP-43 nuclear import and RNA binding are dynamic processes that can be regulated by distinct PTMs. Specifically, we have discovered that acetylation of the core NLS residue K84 partially reduces nuclear import of TDP-43, leading to the accumulation of cytosolically mislocalized protein, which is considered as an early step of TDP-43 pathogenesis. Moreover, we identify K136 in the RRM1 as a potential regulatory site for TDP-43 RNA binding and splicing activity. Although we could confirm the stippled distribution and reduced CFTR splicing activity for the previously reported \([\text{K145Q}]\) TDP-43\(^17,19\), the effects for K136 located directly within an essential RRM1 site appeared stronger in our experimental system. Acetylation can modulate the binding to RNA of other hnRNPs and therefore likely for TDP-43 as well\(^38,39\). Such loss of functional RNA binding may lead to LLPS and eventually the formation of intranuclear TDP-43 aggregates. Our findings support this progression for \([\text{acK136}]\) TDP-43 and identify SIRT1 as a deacetylase counteracting this process. When combined with ΔNLS, \([\text{acK136}]\) TDP-43 also showed a tendency to form cytosolic aggregates, which are much more commonly found in human patients than intranuclear inclusions\(^41\). Thus, acetylation of K136 might disengage TDP-43 from functional hnRNP complexes, liberating the protein to unmix into liquid phases. Such LLPS transitions may lead to TDP-43 self-aggregation and the formation of pathological inclusions. The observation that SIRT1 can deacetylate \([\text{acK136}]\) TDP-43 indicates that this is a dynamic process that can be regulated in cells. The balance of TDP-43 protein lysine acetyltransferases and deacetylases might determine its physiological functions and when derailed, could lead to LLPS and pathological TDP-43 aggregation in disease. Modulation of this pathway could offer novel therapeutic approaches for the treatment of FTLD and ALS.

Residue K136 was recently discovered as a potential TDP-43 sumoylation site\(^18\). Consistent with this study by Maurel et al. (2020) using TDP-43 GFP fusion proteins, we found that 6xHis-tagged \([\text{K136R}]\) TDP-43 formed intranuclear inclusions. The similarities in behaviour between K136R and K136Q TDP-43 mutants were puzzling, suggesting that instead of mimicking the presence or lack of acetylation, the mutants were introducing backbone changes in TDP-43. Point mutagenesis is a broadly used approach to study the cellular effects of protein lysine acetylation but in some cases can fail to rescue protein function\(^42\).

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**Fig. 6** Antibodies against acetylated TDP-43 specifically recognize the modified forms. **a, b** HeLa293E shTDP-43 cells were co-transfected with E451 plasmid and different C-terminally 6xHis-tagged TDP-43 constructs. After 24 h in the presence of 5 mM of acK cells were lysed. Samples were Western probed with antibodies against acetylated K84 (clone 9G9) and K136 (clone 14D4), respectively. N = 3 biological replicates. Source data are provided as a Source Data file. **c, d** HeLa293E shTDP-43 cells were cotransfected with E451 plasmid and different C-terminally 6xHis-tagged TDP-43 constructs. After 24 h in the presence of 5 mM of acK cells were fixed and immunostained for 6xHis or acetylated TDP-43. Scale bar represents 10 µm. N = 3 biological replicates.
In addition, glutamine and arginine contain delocalized electrons that are critical for the formation of phase-separated entities. This provides an explanation for the stronger occurrence of phosphorylated aggregates in K136R/Q TDP-43. Amber suppression has its own disadvantages in terms of efficiency and availability of synthetic amino acids, in addition to the possibility of side effects. Nevertheless, transcriptomic analysis of stably amber-suppressed cells did not show upregulation of protein folding stress genes. Another benefit of this approach is the possibility of removing the modification in vivo via deacetylases.

We could not detect phosphorylation and ubiquitination of the amber suppressed [acK136]TDP-43, in contrast to the K136Q mutant TDP-43. Three factors might explain this negative finding. First, compared with transfected mutant [K136Q]TDP-43, expression of amber-suppressed TDP-43 is much lower (see Fig. 6a), potentially below detection limit of other PTMs. The lower concentration might also not be sufficient to seed protein aggregation, phosphorylation and ubiquitination. Second, K136-acetyl in the amber-suppressed TDP-43 can be removed by endogenous deacetylases such as SIRT1. This would attenuate any downstream effects of this acetylation. And third, as shown by Wang et al. glutamines favour the hardening of phase-separated RNA-binding molecules. While these key differences could be behind the lack of the strongest markers of TDP-43, amber suppression still could help identifying K136 as a residue crucial for RNA splicing and LLPS in TDP-43.

In our MS analysis, we did not detect the previously reported acetylations at K145 and K192 in the RNA-binding domain of TDP-43. Although our experimental setups were similar, perhaps the use of the protein aggregation optimized QBI-293 subclone in their studies made the difference, whereas our study in HEK293E cells revealed acetylation of K121 and K136 in RRM1. Moreover, the MS detection of K145 acetylation was done with ΔNLS mutant TDP-43 lacking the K84 acetylation site we detected with wtTDP-43. It is possible that differential lysine acetylation events occur in cytosolic and nuclear TDP-43 pools. Antibodies directed against [acK145]TDP-43 stained neuropathological inclusions in...
ALS but not FTLD-TDP17. Yet, MS analysis of TDP-43 PTMs in two ALS cases failed to confirm all of these lysine acetylations but instead found K82 acetylation in one ALS case45. The human disease relevance for lysine-acetylated TDP-43 remains to be further explored.

Methods

Antibodies. In this study the following antibodies were used for western blot (WB) and IF staining: rat anti-[acK84]TDP-43 (WB, 1:10, supernatant; IF: 1:2, this work); rabbit anti-K136R (WB, 1:100); rabbit anti-His (WB, 1:1000; Cell Signaling #874); anti-rabbit anti-Flag (WB, 1:1000; Sigma #A5412); rabbit anti-Flag (WB, 1:1000; Invitrogen A32727; anti-Rabbit: Cat # A32732), 647- (anti-Mouse: Cat # A32723; anti-Rabbit: Cat # A32731), 555- (anti-Mouse: Cat # A32727; anti-Rabbit: Cat # A32732), 647- (anti-Mouse: Cat # A32728; anti-Rabbit: Cat # A32733) conjugated antibodies for IF produced in goat were from Invitrogen (1:1000).

cDNA constructs. TDP-43 was cloned into pCMV 5′His6 (Clontech) via Sall/NotI. Acetyl-mimic glutamine and acetyl-dead arginine substitutions were introduced via site-directed mutagenesis and cloned into pCMV 5′His6 (Sall/NotI). The cloning and mutagenesis primers are listed in Supplementary Table 2. The mutations were introduced via a two-step PCR mutagenesis, where TDP-43 gene template for a third PCR reaction in this case amplifying the whole length of TDP-43 and introducing restriction sites at both ends.

For the EGFP-tagged constructs, pEFP c1 wtTDP-43 was used as a template40, K136R and K136Q mutations were introduced via site-directed mutagenesis and cloned into pEFP c1 via BamHI and HindIII. For the N-terminally tagged amber suppression constructs, TDP-43 was cloned from the pCMV 5′His6 vector via Sall and Bsd1201. The amber stop codons from constructs K84TAG and K136TAG TDP-43 were introduced in a two-step point mutagenesis. For the N-terminally tagged amber suppression constructs, TDP-43 was cloned from the pCMV 5′His6 vector via Sall and Bsd1201. The amber stop codons from constructs K84TAG and K136TAG TDP-43 were incorporated and expressed in HEK293E cells and characterized by North et al.47.

Nuclear-cyttoplasmic fractionation protocols. For the nuclear-cyttoplasmic fractionation protocol in HEK293E cells, wtTDP-43, with K84 acetylated (C-KDNKR(Ac)KMDETD); and the second one with TDP-43, with K84 deacetylated (C-KDNKRKMDETD) were further transfected into HEK293E cells, with K84 acetylated. The cells were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. The fractionation protocol was performed as described in Supplementary Fig. 1b, shTDP-43 HEK293E cell pellets from 5 µl of hypotonic buffer A (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.1 mM DTT and protein inhibitor cocktail (Roche) and incubated on ice for 5 min. Cell membranes were ruptured with 20 strokes in an ice-cold Dounce homogenizer and resulting samples were centrifuged at 500 g for 5 min at 4 °C. Supernatants (cytosolic fraction) were mixed with 5× RIPA buffer, centrifuged for 10 min on ice and cleared by spinning the samples at 5000 x g for 15 min at 4 °C. Pellets containing nuclei and residual cytoplasmic proteins were washed twice with hypotonic buffer A. After resuspension in 500 µl of buffer B (250 mM sucrose and MgCl2), samples were layered carefully over a 550 µl buffer C (50 mM Tris, 1 mM MgCl2, 0.5 mM EDTA, 0.1% Triton X-100 and 10% glycerol) and centrifuged at 3000 x g for 10 min at 4 °C. The supernatant was discarded, and the remaining pellet was lysed with urea buffer. DNA was sheared by passing lysates 30 times through a 23-gauge needle. The cleared supernatants in loading buffer were analyzed via Western blotting.

Cell lines, solubility fractionation, and western blotting. Cells were collected with a cell scraper and lysed in urea lysis buffer (10 mM Tris pH 8, 100 mM NaH2PO4, 8 M urea). For the sequential extraction cells were first lysed with RIPA buffer (50 mM Tris/HCl pH 8, 150 mM NaCl, 1% WPI-40, 0.5% deoxycholate, 0.1% SDS, 10 mM sodium pyrophosphate) with proteinase inhibitor cocktail (Roche). Samples were centrifuged at 5000 x g for 15 min, leaving the RIPA-soluble proteins in the supernatant. The remaining pellet was then lysed in urea buffer, and insoluble material was removed with a centrifugation step at 5000 x g for 15 min. DNA was sheared with a 23-gauge needle. Protein concentration in lysates in urea buffer was determined with a Bradford protein assay kit (Biorad) with BCA protein assay kit (Pierce). 3x Laemmli loading buffer with 100 mM DTT was added to the samples and afterwards they were boiled at 95° for 5 min. The denatured samples were subjected to western blot analysis. Denatured samples were loaded and run in polyacrylamide gels. The gels were then blotted using the wet-transfer Transblot kit. Cell lines HEK293E and 293T transfected with Hybond-P polypeptideidine difluoride membranes (Millipore). Membranes were blocked with 5% non-fat milk/TBS-T for an hour at room temperature (RT). Primary antibody incubation took place overnight at 4 °C. The membranes were washed and incubated with HRP-coupled secondary antibody for 1–2 h at room temperature. Membranes were washed and proteins were detected with Immobilon Western Chemiluminescent HRP substrate (Millipore) using the ChemiDoc XRS+ Imaging System.

Generation of monoclonal antibodies against lysine acetylated TDP-43. Two female Lou/c rats aged 12 weeks were immunized (approval by Regierung von Oberbayern AZ 55.2-1 54-25316.4-99) with 2 ovalbumin-coupled synthetic peptides at the same time: one with amino acids corresponding to residues 79-89 of TDP-43, with K84 acetylated (C-KDNKR(Ac)KMDETD); and the second one corresponding to residues 131-141, with K136 acetylated (C-LMVQV(Ac)KDKLKT). Animals were sacrificed and splenocytes were fused with mouse myeloma cells. Hybridoma supernatants were tested for binding to acetylated and non-acetylated peptides by ELISA. Those supernatants that were positive for the acetylated peptides and negative for the non-acetylated peptides were further validated on lysates of cells expressing either wt or amher suppressed TDP-43 by Western blot analysis (dilution 1:10) and IF staining (dilution 1:2). Hybridoma clones TDACA 9G9 (IgG2c) against [acK84]TDP-43, TDACB 23B3 (IgG1/x) (used only in supplementary Fig. 4) and TDACB 14D4 (IgG2b/x) against [acK136]TDP-43 were stably established by limiting dilution cloning.

Cell line and transfections. HEK293E (ATCC) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS), at 37 °C in 5% CO2. Stable TDP-43 knockdown (siTDP-43 HEK293E cells) were generated and characterized previously49. Cells were plated at different densities on coverslips corresponding to the surface of the plate for different experiments. Transfection of plasmids was done 24 h after plating using FuGENE6 (Promega) at a DNA/FuGENE ratio of 1:4.5 according to the manufacturer’s instructions.

Amber suppression. HEK293E cells were plated and cultured in DMEM with 10% FBS and transfected with FuGENE6 (Promega) 24 h later. Twenty four or 48 h after transfection media was substituted with DMEM with 1% Me Acetyl-L-lysine (A4021, Sigma). Cells were lysed or fixed after being 24h in the presence of acetylated lysine. When mentioned, Exs27 (CAS 49843-98-3, Santa Cruz Biotechnology) was dissolved in DMSO and added to the cultured cells 24 h before lysis to a final concentration of 1 or 10 µM.

Immunofluorescence staining. Cells were plated in 6-well plates and grown for 24 h. They were then transfected and 6 h after transfection they were transferred...
Native protein pulldown and 25 µl Laemmli buffer. Each RNA-protein binding reaction. After 1 h incubation proteins were eluted in oligonucleotides 5′ at 37° with 5% CO₂. Imaging was done with an Axio observer Z1.

Fluorescence recovery after photobleaching. HEK293E cells were plated on glass bottom chambered sides (Lab Tek, 154526) coated with PDL and collagen. They were transfected with C-terminally EGFP-tagged wt or K136Q TDP-43 after 24 h. Washing and photobleaching was done with a Zeiss LSM510 META confocal microscope, using a 20× objective. Cells were bleached for 1.5 min at 405 nm and a 488 nm laser at maximum power output. Cells were continuously imaged for 3 min after bleaching. Reference ROIs were measured from unbleached cells to correct for photobleaching due to fluorescence imaging.

Live cell imaging. HEK293E cells were plated on glass-bottom chambered sides coated with PDL. They were transfected with C-terminally EGFP-tagged wt or K136Q TDP-43 for 24 h. While imaging cells were kept in a temperature-controlled chamber at 37° with 5% CO₂. Imaging was done with an Axio observer Z1.

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