

Supplementary Information

Supplementary Figure Legends

Supplementary Figure 1: TDP-43 dimerization is not significantly different in Ctrl cells vs HIPK2i SH-SY5Y cells.

(a) Western blot analysis of TDP-43 protein levels in Ctrl cells and HIPK2i SH-SY5Y cells treated or untreated with 200 μ M DSG to cross-link TDP-43 dimers. Two different acquisitions of the same blot are shown in the upper and lower panel to better detect the TDP-43 dimeric and monomeric forms, respectively. The molecular weight of protein markers is indicated. (b) Densitometric analysis of the TDP-43 dimer/monomer ratio in the DSG-treated Ctrl cells and HIPK2i samples shown in a.

Supplementary Figure 2: HIPK2-WT but not HIPK2-K221R restores TDP-43 subcellular localization in HIPK2i SH-SY5Y cells.

Representative images of TDP-43 immunostaining in SH-SY5Y cells stably transfected with scrambled (Ctrl) or specific anti-HIPK2 (HIPK2i) shRNA. Plasmid encoding either an HIPK2 WT (+ HIPK2 WT) or a kinase-inactive HIPK2 mutant (+ HIPK2-K221R) were transiently transfected in HIPK2i cells. Scale bars, 5 μ m.

Supplementary Figure 3: Co-immunoprecipitation assay of TDP-43 interactors.

Ctrl cells and HIPK2i SH-SY5Y cells were lysed with NP-40 2% lysis buffer (20 mM Tris-HCl, pH 7.5; 120 mM NaCl; 2% NP-40) supplemented with protease/phosphatase inhibitor cocktail. After pre-clearing, 2 mg of lysates were immunoprecipitated with non-specific polyclonal immunoglobulins (IP IgG) or with a specific anti-TDP-43 antibody (IP anti-TDP43). 50 μ g of non-immunoprecipitated total protein lysates (Lys) were used as a control of protein expression. Samples were revealed by Western blot analysis using specific anti-TDP-43, Rad50, XPO5 antibodies. The molecular weight of protein markers is indicated.

Supplementary Figure 4: Trametinib affects TDP-43 subcellular localization in HIPK2i SH-SY5Y cells in dose- and time-dependent manner.

(a-b) HIPK2i SH-SY5Y cells were treated or not with 5, 10, 50, 100 or 200 nM Trametinib for 48 hours and then subjected to Western blot (a) or immunofluorescence analysis (b). (a) Representative immunoblot of p-ERK1/2 (T202 and Y204) and total ERK1/2 protein levels; GAPDH levels were used as loading control; the molecular weight of protein markers is indicated. (b) Representative images of TDP-43 immunostaining are shown; scale bar, 6 μ m. (c) HIPK2i SH-SY5Y cells were treated with Trametinib at low and high dose (50 and 200 nM, respectively) for 12, 24 and 48 hours and TDP-43 was labelled as in b; scale bar, 5 μ m. The percentage of cells exhibiting TDP-43 aggregates upon the different conditions is shown in b and c. The bars show relative mean values $\pm$ SD of two independent experiments; n > 30 cells.

Supplementary analysis of proteomics data

Targeted Analysis of TDP-43 Post-Translational Modifications

Phosphorylation, ubiquitination, and acetylation events on TDP-43 were investigated using a targeted extracted ion chromatogram (XIC)-based approach in Thermo Scientific Xcalibur software. Tryptic peptides derived from TDP-43 were first generated *in silico* using MD-Digest, applying the same parameters used for experimental digestion. Briefly, trypsin was selected as the proteolytic enzyme, allowing up to five missed cleavages. Fixed modification was set to carbamidomethylation of cysteine residues, while variable modifications included Gln→pyro-Glu (N-terminal Q), oxidation (M), and pyro-carbamidomethylation (N-terminal C). This *in silico* analysis generated the theoretical pool of tryptic peptides used to guide peptide selection for PTMs analysis.

For phosphorylation analysis, theoretical m/z values of candidate peptides were calculated considering a mass shift of +79.966 Da on serine, threonine, and tyrosine residues. Extracted ion chromatograms (XICs) were generated at the MS1 level using a mass tolerance of ±5 ppm. Multiple charge states up to +3 were considered. Peptide detection was evaluated based on accurate mass, isotopic pattern distribution, and chromatographic co-elution. A total of 26 peptides were selected for phosphorylation monitoring (reported in Table 1), excluding peptides that were excessively short or long due to poor chromatographic performance or unreliable detection.

Peptide Sequence	Position (aa)	Monoisotopic Mass (Da)
-MSEYIR(V)	1-6	798.3815
(R)YRNPV S QCMR(G)	43-52	1310.6092
(R)YRNPV S QCMRGVR(L)	43-55	1622.8002
(R)NPV S QCMR(G)	45-52	991.4448
(R)NPV S QCMRGVR(L)	45-55	1303.6358
(K)RKMD E TDA SS AVK(V)	83-95	1437.7002
(R)KMD E TDA SS AVK(V)	84-95	1281.5991
(R)KMD E TDA SS AVKVK(R)	84-97	1508.7625
(K)MD E TDA SS AVK(V)	85-95	1153.5041
(K)MD E TDA SS AVKVK(R)	85-97	1380.6675
(K)MD E TDA SS AVKVKR(A)	85-98	1536.7686
(K) T TEQDLK(E)	115-121	834.4203
(K) T TEQDLKE YFST FGVLMVQVK(K)	115-136	2592.2902
(K) T TEQDLKE YFST FGVLMVQVKK(D)	115-137	2720.3851
(K)GFGFVRF TEYET QVK(V)	146-160	1807.9014
(K)GFGFVRF TEYET QVKVMSQR(H)	146-165	2409.2020
(R) FTEYET QVK(V)	152-160	1144.5521
(R) FTEYET QVKVMSQR(H)	152-165	1745.8527
(R) FTEYET QVKVMSQRHMIDGR(W)	152-171	2455.1857
(K)LPN S K(Q)	177-181	558.3246
(K)LPN S K S QDEPLR(S)	177-189	1511.7812
(K)LPN S K S QDEPLR S R(K)	177-191	1754.9144
(K)HNSNRQLER(S)	264-272	1153.5821
(K)HNSNRQLER S GR(F)	264-275	1453.7367
(K)HNSNR(Q)	264-268	627.2957
(R)QLER S GR(F)	269-275	845.4588

Table 1. TDP-43 tryptic peptides monitored for phospholytation analysis.

For ubiquitination analysis, theoretical m/z values of candidate peptides, reported in Table 2, were evaluated by monitoring the characteristic diGly remnant as universal marker, corresponding to a mass shift of +114.043 Da on lysine residues.

Peptide Sequence	Position (aa)	Monoisotopic Mass (Da)
(K)RKMDETDASSAVK(V)	83-95	1437.7002
(K)RAVQKTSDLIVLGLPWK(T)	98-114	1924.1378
(K)TSDLIVLGLPWKTTEQDLK(E)	103-121	2157.1802
(K)LPNSKQSQDEPLR(S)	177-189	1511.7812
(R)AFAFVTFADDQIAQSLCGEDLIHKGISV HISNAEPKHNSNR(Q)	228-268	4513.2569

Table 2. TDP-43 tryptic peptides monitored for ubiquitination analysis.

Literature data have reported ubiquitination sites for TDP-43 including K79, K84, K95, K102, K114, K121, K160, K176, K181, and K263 [<https://doi.org/10.3389/fcell.2022.931968>]. In our experiment, based on technical considerations, we monitored K84, K102, K114, and K181, as peptides containing K79, K95, K160, K176, and K263 did not exhibit a consistent or reliable isotopic pattern in at least one of the two experimental conditions (CTRLi and HIPK2i). Monitoring these residues in a single condition was considered non-informative for comparative purposes. K121 was excluded due to the unfavorable size of the corresponding tryptic peptide.

Regarding lysine acetylation, theoretical m/z values were calculated including a mass shift of +42.011 Da on lysine residues. Among previously reported lysine residues (K79, K82, K84, K136, K142, K145, and K192) [<https://doi.org/10.1038/ncomms6845>, <https://doi.org/10.1038/s41467-022-28822-7>], we selected K82, K84, K145, and K192 based on peptide detectability for LC-MS/MS analysis (Table 3), while the remaining residues were excluded due to technical limitations.

Peptide Sequence	Position (aa)	Monoisotopic Mass (Da)
(R)LVEGILHAPDAGWGNLVYVVNYPKDN KR(K)	56-83	3137.6531
(K)RKMDETDASSAVK(V)	83-95	1437.7002
(K)TGHSKGFGFVR(F)	141-151	1192.6222
(R)SRKVFVGR(C)	190-197	948.5738

Table 3. TDP-43 tryptic peptides monitored for acetylation analysis.