

14-3-3 γ S59 phosphorylation drives neuro-pathological impact and interactome remodelling

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Abstract

14-3-3 constitute a highly conserved family of proteins that participate in the regulation of essential cellular processes by establishing extensive protein-protein interactions. Consequently, perturbation of the 14-3-3s interactome can be implicated in the pathogenesis of several diseases. Phosphorylation has emerged as a key mechanism by which 14-3-3s interactome can be regulated, and aberrantly phosphorylated 14-3-3s have been observed in patients with neurological disorders, among which Parkinson's disease. Here, we specifically investigate phosphorylation of 14-3-3 γ at Ser59 and assess its impact on α -Synuclein aggregation. Consistent with observations performed by others with different 14-3-3 isoforms, we observed that phosphorylation reduces the protective abilities of 14-3-3 γ both *in vitro* and in cellular models. Importantly, this effect is also evident in cerebrospinal fluid (CSF), suggesting that phosphorylation of 14-3-3 γ may directly contribute to α -Synuclein aggregation *in vivo* and may be relevant to disease-associated mechanisms. Using bioinformatics approaches, we further examined how Ser59 phosphorylation reshapes the global 14-3-3 γ interactome, revealing novel interaction networks and pathways potentially implicated in neurodegenerative disease pathogenesis.

2. Introduction

14-3-3 proteins are an evolutionarily conserved family of dimeric regulators that control the activity, folding, interactions, and subcellular localization of client proteins through extensive protein-protein interactions (PPIs) ^{1,2}. Given their broad range of binding partners, 14-3-3 proteins function as molecular hubs involved in numerous cellular processes ³. Consequently, perturbations of the 14-3-3s' interactome can disrupt multiple signalling pathways and contribute to diverse diseases, including cancer, metabolic disorders, and neurological conditions ⁴⁻⁹. Mammals express seven 14-3-3 isoforms - β , γ , ϵ , ζ , η , σ and τ - that associate to form homo- or heterodimers, although in physiological conditions monomers also exist ^{10,11}. Phosphorylation has emerged as a key regulatory mechanism to modulate 14-3-3s dimerization ¹²⁻¹⁴. Three principal phosphorylation sites have been identified on 14-3-3s: Ser184 (present in isoforms β , ϵ , σ and ζ), Thr/Ser232/233 (present in isoforms θ and ζ) and Ser58/59 (present in all isoforms except σ and θ), with the precise residue position differing among isoforms ³. While phosphorylation at Ser184 and Thr/Ser232/233 primarily affects interactions with client proteins, phosphorylation at Ser58/59 promotes dimer dissociation, finally resulting in reduced client binding ¹⁴⁻¹⁶. For instance, phosphorylation of 14-3-3 ζ at Ser58, or the phosphomimetic S58E mutant, induces monomerization and disrupts binding to p53 ¹³. Each of these residues can serve as a substrate for specific kinases, with phosphorylation eventually occurring in an isoform and kinase dependent manner ^{15,17,18}.

It is likely that, in a given cell type or tissue, the specific combination of kinases, 14-3-3 isoforms and phosphorylation sites reflect the integration of multiple molecular signals that arise from distinct intra- and extracellular contexts ¹⁹. Phosphorylation may therefore act as a regulatory mechanism modulating 14-3-3 interactome and raises important questions regarding its relevance in disease onset.

14-3-3 proteins have attracted considerable interest in the field of Parkinson's disease (PD) due to their multifaceted connection with key pathogenic mechanisms. They share structural similarity with α -synuclein (α -Syn), co-localize with α -Syn in Lewy bodies (LBs), and interact with several PD-associated proteins, including Parkin, LRRK2, and DJ-1^{20–25}. In addition, altered expression of 14-3-3 isoforms has been consistently reported in PD models and patient-derived samples at both transcript and protein levels^{26–29}.

Functionally, 14-3-3 proteins are thought to play a protective role in PD-related pathology. In vitro studies indicate that they can interfere with early α -Syn fibril formation, while specific isoforms such as 14-3-3 θ reduce α -Syn oligomerization and intercellular spreading, thereby limiting neuronal toxicity^{30–32}. However, these protective effects are not static and can be significantly modulated by post-translational modifications. In particular, phosphorylation has emerged as a key regulatory mechanism: phosphorylation of 14-3-3 θ at Ser232 promotes α -Syn oligomer release and aggregation in neurons exposed to pre-formed fibrils (PFFs), ultimately diminishing its neuroprotective capacity^{33,34}. Similarly, endogenous 14-3-3 proteins display increased phosphorylation at Ser58/59 following rotenone exposure, although this modification appears less consistent in patient samples³³.

These observations suggest that phosphorylation may act as a molecular switch that regulate the protective potential of 14-3-3 proteins in PD.

Building on this concept, we previously demonstrated that PAK6-dependent phosphorylation of 14-3-3 γ at Ser59 destabilizes the 14-3-3 γ /LRRK2 complex and rescues pathological LRRK2 hyperactivity in neurons derived from LRRK2 G2019S mice, a model of PD¹². To better dissect the disease-related consequences of Ser59 phosphorylation, in this paper we investigated the role of Ser59 phosphorylation of 14-3-3 γ in three contexts: (1) its specific effect on α -Syn aggregation at both molecular and cellular levels, (2) its effect on α -Syn aggregation in CSF obtained from PD patients and (3) its global impact on the 14-3-3 γ interactome to identify potential client proteins involved in the onset of neurological disorders, uncovering new pathophysiological relevance to Ser59 phosphorylation of 14-3-3 γ .

3. Material and Methods

3.1 Proteins purification

Recombinant α -Syn was produced by transforming *Escherichia coli* BL21 (DE3) cells with a pET28a plasmid encoding the human α -Syn gene (pET- α Syn140). Transformed cells were plated on lysogeny broth (LB) agar plates containing kanamycin (25 μ g/mL) and incubated overnight (O/N) at 37°C. Multiple colonies were inoculated in liquid LB medium supplemented with kanamycin (25 μ g/mL) and cultured O/N at 37°C with constant shaking. The culture was then diluted to an optical density at 600 nm (OD600) of 0.1 in 1 L of LB medium (added with kanamycin 25 μ g/mL). Protein expression was induced with 100 μ M isopropyl β -D-1-thiogalactopyranoside (IPTG) when the OD600 reached 0.6, and cells were incubated for 4–5 hours at 37°C with shaking. Following induction, bacterial cells were harvested by centrifugation

at 6,000 g for 5 min at room temperature (RT) using an Allegra 25R centrifuge (Beckman Coulter) with a TA-10-250 rotor. The cell pellet was resuspended with 100 mL of osmotic shock buffer (30 mM Tris-HCl, 2 mM EDTA, 40% sucrose, pH 7.2) and incubated at RT for 10 min. Cells were pelleted again at 12,000 g for 20 min at 4°C and resuspended in 90 mL of cold deionized water with 37.5 µL of saturated MgCl₂. After 3 min on ice, samples were centrifuged at 12,000 g for 20 min at 4°C to recover the supernatant containing periplasmic proteins. The periplasmic extract was buffered with 2 mL of 1 M Tris-HCl (pH 8.0) and boiled in a water bath for 10 min, then cooled O/N at RT. After centrifugation (12,000 g for 20 min at 4°C), the supernatant was subjected to (NH₄)₂SO₄ precipitation in two steps (35% and 55% saturation). For each step, (NH₄)₂SO₄ was added to the supernatant on ice with stirring, followed by centrifugation (12,000 g for 20 min at 4°C). The protein pellet was resuspended in 5–10 mL of deionized water and dialyzed O/N using a 3.5 kDa cut-off membrane. Dialyzed protein was then centrifuged (12,000 g for 10 min at 4°C) and loaded onto a 6 mL Resource Q anion exchange column (GE Healthcare) pre-equilibrated in 20 mM Tris-HCl (pH 8.0). Elution was performed with a linear NaCl gradient (0–500 mM, with a 1 mL/min flow over 30–32 min). Eluted protein was dialyzed O/N against deionized water, snap-frozen, lyophilized using an Edwards Modulyo Freeze Dryer, and stored at -20°C.

Recombinant 14-3-3 proteins were produced by transforming *Escherichia coli* BL21(DE3) competent cells (New England Biolabs, #BC2527H) with pET28a plasmids encoding human 14-3-3γ wild type (WT), the phosphomimetic 14-3-3γ S59D and 14-3-3η. A preculture was grown O/N at 37°C with shaking. The next day the culture was diluted and induced with 500 µM IPTG O/N at 20°C under agitation. The culture was then centrifuged at 10,000g for 10 min and the cell paste weighted and lysed with BugBuster Master Mix (Novagen, #71456) complemented with protease inhibitor cocktail (Merck). The solution was then added with 100 mM Phenylmethyl Sulphonyl Fluoride (PMSF, Carl Roth #6367.2) and centrifuged at 12,000g for 20 min at 4°C. Proteins were then purified via affinity chromatography using His GraviTrap™ columns (Cytiva, #11003399). Buffer A composed of 20 mM Na₃PO₄, 500 mM NaCl and 20 mM imidazole and buffer B composed of 20 mM Na₃PO₄, 500 mM NaCl and 300 mM imidazole were used to wash and elute the proteins, respectively. Desalting was performed using PD-10 desalting columns (Cytiva, #17085101). Protein concentration and purity have been assessed using a Nanodrop spectrophotometer (Nanodrop One, Thermo Fisher Scientific) and Coomassie blue staining (SimplyBlue™ Safe Stain, Thermofisher, #LC6060). Once quantified, purified proteins have been supplemented with 3mM dithiothreitol (DTT, Carl Roth, #6367.2), aliquoted, and stored at -80°C until use.

3.2 *In vitro* kinase reactions

To obtain phosphorylated 14-3-3γ (p14-3-3γ), kinase assays were carried out as previously described³⁵. Briefly, His-14-3-3γ WT was incubated for 2 h at 37°C with PAK6 (using 1:50 14-3-3γ:PAK6 ratio) in the presence of kinase buffer (Cell Signalling, #ab189135), 2 mM DTT (Thermofisher, #R0861) and 1 mM ATP (Thermofisher, #R0441). p14-3-3γ was used immediately after preparation.

3.3 Generation of a-Syn fibrils and pre-formed fibrils

Freeze-dried α -Syn was weighed and resuspended in Dulbecco's Phosphate-Buffered Saline (DPBS; Biowest) to a concentration of 300–350 μ M. The solution was filtered through a 0.22 μ m filter and quantified at 276 nm using an Agilent 8453 spectrophotometer. Monomeric α -Syn aliquots were incubated at 37°C for 7 days in a ThermoMixer F1.5 (Eppendorf) at 1,000 rpm to promote fibrillation. A fraction of the monomeric α -Syn was reserved as a control. Fibrils were collected by centrifugation at 20,238 g for 20 min at 4°C. Fibril concentration was calculated by subtracting the residual monomer concentration in the supernatant after fibrillation from the initial monomer concentration. Fibrils were aliquoted at a final concentration of 200 μ M and assessed by transmission electron microscopy following negative staining. To obtain PFFs, α -Syn fibrils were sonicated with a Covaris S2 Ultrasonicator for a total of 40 pulses (30 sec on, 30 sec off) to produce PFFs with an average length of $\sim$ 50 nm. Quality control was performed by Transmission Electron Microscopy (TEM).

3.4 *In vitro* fibril elongation kinetics

α -Syn amplification assay was performed using a Real-Time Quaking-Induced Conversion (RT QuIC) protocol similar to ³⁶. Briefly, 2 mg/ml of monomeric α -Syn were incubated in a solution containing 170 mM NaCl (Sodium Chloride 5M, J60434.AK, Thermo Fisher Scientific), 40 mM Na₃PO₄ (Sodium Phosphate Buffer 0.5M pH 8.0, J60825K.AK, Thermo Fisher Scientific), 20 μ M ThT (T316, Sigma Aldrich) along with 6ng of PFFs in a black 96-well plate optical bottom (265301, Thermo Fisher Scientific), covered with a clear Polyolefin sealing tape (Thermo Fisher Scientific) and incubated in a BMG VANTASTAR plate reader at 45°C for 22 cycles of 1 minute double orbital shaking at 700 rpm followed by 1 minute rest. ThT fluorescence (450 nm excitation and 480 nm emission; bottom read) was recorded every 45 min for more than 4000 min. Each run was performed in triplicate. Four different α -Syn:14-3-3 molar ratios were tested, namely: 2:1, 10:1, 40:1 and 60:1. Samples without PFFs were run as negative controls.

For atomic force microscopy (AFM) experiments, samples were prepared using the same experimental conditions, but in the absence of ThT.

For RT-QuIC experiments involving cerebrospinal fluid (CSF) samples, 2 μ L were employed for each reaction, composed as mentioned above without the addition of human PFFs. Each replicate curve was fitted after baseline subtraction in OriginLab® using either a Boltzmann or Gompertz fitting function, based on the difference in the Bayesian Information criterion and Akaike Information criterion between the two models. The kinetic parameters considered were the time to reach 50% of the maximum signal intensity (T50), the time to reach the amplification threshold (lag phase) and the steepness of the amplification curve (Slope).

3.5 Atomic Force Microscopy imaging

AFM imaging was performed under ambient conditions in PeakForce mode using ScanAsyst-Air probes (BrukerAFMProbes) mounted on a Multimode 8 microscope (Bruker) equipped with a Nanoscope V controller and a JV piezoelectric scanner. For each sample, 10 μ L of solution were deposited onto freshly

cleaved mica sheets (V-1 grade Muscovite mica, Ted Pella) and allowed to adsorb for 5 min at RT (~20°C). Mica substrates were subsequently rinsed with ~ 500 µL ultrapure water (Millipore Simplicity) at the same temperature and dried under a gentle nitrogen flow. AFM micrographs were acquired at random positions on the mica substrate until several hundred individual amyloid aggregates had been detected. Morphometric analysis was carried out as follows. Particles protruding ≥ 2 nm above the mica surface were automatically assigned a minimum circumcircle radius using Gwyddion 2.69³⁷. In all samples, the length of the largest fibrillary aggregates remained well below the persistence length of mature α -Syn fibrils³⁸, allowing them to be approximated to rigid rods. Accordingly, fibril length was determined by doubling the assigned circumcircle radius. Length distributions were generated by measuring at least 250 individual fibrils pooled from multiple images of each sample. Values below 90 nm (corresponding to the PFFs length) were filtered out from the data.

3.6 Transmission electron microscopy imaging

50 µl aliquots of the 14-3-3/ α -Syn fibril co-aggregates were collected from seeding amplification assays, adsorbed onto carbon-coated copper grids, and negatively stained with 0.05% uranyl acetate. TEM images were acquired using a Tecnai G2 12 Twin microscope (FEI Company, Hillsboro, OR, USA).

3.7 Human samples and clinical data

CSF samples (obtained from 10 patients affected with PD) were supplied by Parkinson's UK Brain Bank at Imperial College London, funded by Parkinson's UK, a charity registered in England and Wales (258197) and in Scotland (SC037554). Samples were anonymous to the investigators.

3.8 Cell culture and transfection

HEK293T cells were seeded at a density of 180 cells/mm² onto 12-mm glass coverslips and transfected the following day with a total of 1.5 µg plasmid DNA using Xfect™ Transfection Reagent (Takara, #631317), according to the manufacturer's instructions. Briefly, plasmid DNA was diluted in Xfect Reaction Buffer and vortexed for 5 s, followed by the addition of Xfect Polymer at a ratio of 0.3 µL per 1 µg plasmid DNA. The mixture was vortexed for an additional 10 s and incubated at RT before being added to the cells. Cells were transfected with 1 µg pEGFP-N- α -Syn and 0.5 µg DsRed-14-3-3 γ or DsRed empty vector. 24 hours post-transfection, α -Syn PFFs were added to the cultures at a final concentration of 0.5 µM. After an additional 24 h, cells were washed twice with PBS and fixed for 20 min on ice with 4% paraformaldehyde (PFA) supplemented with 1% Triton X-100. Cells were subsequently washed twice with PBS and stained with DAPI (Carl Roth) diluted 1:10,000 in PBS. Finally, coverslips were washed with PBS and mounted onto glass slides using Fluoroshield™ mounting medium (Sigma). Images were taken using an Axio Observer 7 microscope (Zeiss) and analysed using ImageJ. α -Syn aggregates were identified by means of ROI created in imageJ as the bright green spots in proximity of the nuclei; each ROI was then checked manually to correct any potential fragmentation of the same aggregate.

3.9 HuProt protein microarrays

HuProt™ Human Proteome Microarrays v4.0 (Cambridge Protein Arrays, Babraham Research Campus, Cambridge, UK) were used to identify 14-3-3γ interacting proteins according to the manufacturer's instructions. Briefly, microarray slides were blocked O/N at 4°C with a blocking buffer consisting of 2% BSA in 1× PBS supplemented with 0.05% Tween-20 (PBST). Arrays were then incubated for 2 h at RT with gentle rocking with either recombinant 14-3-3γ or p14-3-3γ, diluted in blocking buffer to a final concentration of 1 μM. Notably, we previously demonstrated that PAK6 induces complete phosphorylation of 14-3-3γ in the presence of ATP. Although PAK6 phosphorylates two distinct sites on 14-3-3γ (Thr145 and Ser59), only phosphorylation at Ser59 promotes dissociation of the dimer into monomers, resulting in the loss of interaction with client proteins¹². A control slide was incubated with blocking buffer alone under identical conditions. Following incubation, microarrays were washed with PBS-T to remove unbound proteins and subsequently incubated for 2 h at RT with gentle rocking with a CF488A-conjugated anti-14-3-3γ antibody (1:250, orb394651-CF488A, Biorbyt) and DyLight 650-conjugated anti-GST antibody (1:1000 Columbia Biosciences, #D10-1310-100, used as technical control). Slides were then washed with PBST followed by 0.1× PBS, dried, and scanned at the Cambridge Protein Arrays Facility. The results can be found in the supplementary material (see Results_Arrays). For data analysis, values were filtered according to the following criteria: (1) fluorescent measurements with a relative standard deviation (RSD) of duplicates greater than 0.35 were excluded; (2) fluorescent measurements with a signal-to-noise ratio below 2.5 were excluded; and (3) fluorescent values flagged as artefacts were removed.

3.10 Immunoprecipitation

HEK293T cells were seeded at a density of 8×10^6 cells in 150-mm culture dishes. Upon reaching approximately 80% confluence, cells were transfected with 120 μg of plasmids encoding either YFP-14-3-3γ WT, or the phosphomimetic mutant YFP-14-3-3γ S59D using PEI, according to the manufacturer's instructions. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; 1 mM sodium orthovanadate) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich) and incubated on ice for 30 min. Lysates were clarified by centrifugation at $14,000 \times g$ for 30 min at 4°C, and the supernatants were collected. Clarified lysates were incubated with 40 μL of GFP-Trap for 2 h at 4°C under constant rotation. The 14-3-3γ/client protein complexes bound to the resin were then collected by centrifugation (1 min at 7,500 g) and washed extensively with RIPA buffer, with each wash followed by centrifugation (1 min at 7,500 g).

Finally, the immunoprecipitated complexes were incubated O/N at 4°C with 3 mg of mouse brain lysate under continuous rotation. Samples were then washed extensively, resuspended with RIPA buffer, and stored at -80°C until further analysis. Three biological runs were performed for this experiment.

3.11 Mass spectrometry analyses

Samples were digested following the Filter-Aided Sample Preparation (FASP) method³⁹. Briefly, each sample was mixed with 100 μL of buffer A (8 M urea, 100 mM Tris-HCl, pH 8.5), loaded into a centrifugal

unit with a molecular weight cutoff of 10 kDa (Vivacon 500, Sartorius, Geottingen, Germany) and centrifuged at 18,600g for 15 min. Filters were washed twice with 200 μ L of buffer A and centrifuged at 18,600g for 15 min. Reduction of protein disulfide bridges was performed by adding 200 μ L of 50 mM dithiothreitol (Fluka, Sigma-Aldrich, St. Louis, MO, USA) in buffer A and incubating the samples at 55°C for 30 min. Alkylation of free thiols was performed by adding 100 μ L of 50 mM iodoacetamide (Sigma-Aldrich) in buffer A and incubating the samples for 20 min in the dark. Filter units were washed twice with 100 μ L of buffer A and then two additional washing steps were performed with 100 μ L of 100 mM and 50 mM NH_4HCO_3 (Sigma-Aldrich), respectively.

Protein digestion was carried out at 37°C O/N with 0.2 μ g of Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) (12.5 ng/ μ L in 50 mM NH_4HCO_3). Peptides were collected by centrifugation at 18,600g for 10 min, followed by two washes with 100 μ L of 50 mM NH_4HCO_3 , acidified with 1 μ L of formic acid (FA, Sigma-Aldrich) and dried under vacuum in a speedvac system.

Peptides were dissolved in 20 μ L of 3% acetonitrile (ACN)/0.1% FA and 2 μ L of this solution were loaded into a 10 cm homemade pico-frit chromatographic column (75 μ m ID, 15 μ m tip, New Objective, Littleton, USA) packed in-house with C18 material (Aeris Peptide 3.6 μ m XB-C18, Phenomenex, Torrance, USA) and subjected to LC-MS/MS analysis with a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, USA) interfaced to a nano-HPLC Ultimate 3000 (Dionex–Thermo Fisher Scientific, Waltham, USA). Separation of peptides was performed using a linear gradient of ACN/0.1% FA from 3% to 40% in 39 min. MS and MS/MS spectra were acquired with a Top-10 data-dependent acquisition (DDA) strategy: for each cycle a full MS spectrum was acquired at high resolution (60000 nominal resolution) in the Orbitrap analyzer, while the 10 most intense ions were fragmented in the linear ion trap. Raw data files were processed with the MaxQuant software package (www.maxquant.org version 1.5.1.2) and searched against the M. musculus section of the UniProt database (UP000000589, version 2020-09-30, 55494 sequences) setting the following parameters: Trypsin/P as protease, oxidation on methionine as variable modification and carbamidomethylation on cysteine as fixed modification. Peptide tolerance was set to 20 ppm, fragment tolerance to 0.5 Da, a maximum of two missed cleavages were allowed in the database search and match between runs was enabled with a matching time window of 0.7 min. Proteins denoted as decoy hits and common contaminants were removed from MaxQuant output. Cyclic Loess normalization was performed for each condition separately using the web-based tool NormalyzerDE⁴⁰. Missing values were imputed using the impute function (MinProb method) from the DEP R package⁴¹. Differential protein expression was assessed by performing a moderated T-test (Limma) via NormalyzerDE⁴⁰ and the FDR was controlled using the Benjamini-Hochberg procedure. Proteins with adjusted P-value < 0.05 and absolute FC > 2 were considered statistically significant.

3.12 Gene Ontology analyses

Gene Ontology (GO) enrichment analysis was performed in RStudio (version 2025.09.2 + 418) using the R package clusterProfiler (version 4.18.2). Differentially regulated proteins were analyzed for over-representation of GO terms in the Biological Process (BP), Molecular Function (MF), and Cellular

Component (CC) categories of the Gene Ontology database. Gene annotation was performed using the org.Hs.eg.db package (version 3.22.0). Enrichment significance was assessed using a hypergeometric test. P-values were adjusted for multiple comparisons using the Benjamini–Hochberg method, and GO terms with adjusted p-value < 0.05 and q-value < 0.2 were considered statistically significant.

3.13 Gene set enrichment analysis (GSEA)

To assess the relevance of the merged protein list (obtained from microarray and mass spectrometry analyses) to neurodegenerative disease, a Monte Carlo permutation test was performed. The reference set of proteins associated with “Neurodegenerative disorders - adult onset” was obtained from PanelApp on 1 August 2022, comprising N = 414 entries. As the background protein pool, we used the annotated protein database provided by PINOT (Protein Interaction Network Online Tool). Enrichment analysis was conducted by randomly permuting the merged protein lists 1,000 times against this background, thereby generating a null distribution of overlaps. The observed overlap with the PanelApp neurodegenerative disease-associated proteins was then compared to this distribution to evaluate whether the enrichment was greater than expected by chance.

4. Results

4.1 Ser59 phosphorylation of 14-3-3 γ affects α -Syn aggregation *in vitro*

It has been previously shown that 14-3-3 proteins modulate the aggregation of α -Syn *in vitro*^{30,31}. We therefore investigated whether the phosphorylation of Ser59 could affect the ability of 14-3-3 γ to influence this process. To this end, we performed RT-QulC experiments to analyse the kinetics of α -Syn aggregation in the presence of either 14-3-3 γ WT or its phosphomimetic variant 14-3-3 γ S59D, which we have previously shown to effectively mimic 14-3-3 γ phosphorylation¹². We used 4 different α -Syn:14-3-3 γ molar ratios (namely 2:1, 10:1, 40:1 and 60:1) keeping a fixed amount of α -Syn in all the experiments. As positive and negative controls, we performed RT-QulC experiments in the presence or absence of human PFFs (without 14-3-3 γ), respectively. As expected, we observed a dose-dependent effect of both 14-3-3 γ WT and its S59D variant, where the α -Syn:14-3-3 γ 2:1 ratio determines the strongest decrease in α -Syn aggregation compared to the positive control (**Suppl. Figure 1A-B**). Interestingly, in the presence of the phosphomutant, the lowest ratios were all very close to the positive control, suggesting little inhibition of α -Syn aggregation (**Suppl. Figure 1B**); in the presence of 14-3-3 γ WT, instead, all the ratios showed an inhibitory effect, with their profiles deviating from the positive control (**Suppl. Figure 1A**). We also performed the same experiment using 14-3-3 η , which we have previously shown to interfere with the early phases of α -Syn aggregation in a dose-dependent manner³⁰. Similarly to the 14-3-3 γ S59D phosphomutant, we observed that only the α -Syn:14-3-3 η 2:1 ratio showed significant inhibitory effect (**Suppl. Figure 1C**).

We then investigated the potential effects of 14-3-3 γ proteins on the structural and morphological properties of α -Syn fibrils by TEM and AFM. All samples were collected at the end of the fibrillation protocol (approximately 3 days) and subsequently analysed. As expected, fibrillation in the absence of PFFs resulted in a lower abundance of fibrils, whereas the presence of PFFs led to the formation of abundant mature fibrils. Notably, a marked decrease in fibril formation was observed for both forms of 14-3-3 γ at a 2:1 ratio. Under these conditions, predominantly amorphous aggregates were detected instead of well-defined fibrils, suggesting that at near-equimolar ratios these proteins effectively inhibit fibrillation, regardless of their phosphorylation status. In contrast, at a 40:1 ratio, 14-3-3 γ WT displayed a greater inhibitory effect on α -Syn fibrillation than the S59D phosphomimetic (Fig. 1C). Likewise, qualitative AFM analyses showed that both 14-3-3 γ and the S59D phosphomimetic prevented the formation of mature fibrils. These analyses further confirmed that, at a 40:1 ratio, 14-3-3 γ exerted a stronger inhibitory effect on fibril formation than the 14-3-3 γ S59D variant, as shown in Fig. 1D. Consistently, Minimum Circumcircle analysis, used as an approximation of fibrils length, revealed that both 14-3-3 γ WT and S59D at a 2:1 ratio reduced the length of α -Syn aggregates to values comparable to, or even shorter than those observed in the absence of PFFs. Moreover, although both forms of 14-3-3 γ displayed reduced inhibitory activity at a 40:1 ratio, the addition of 14-3-3 γ WT protein still resulted in smaller fibrils compared to the S59D phosphomimetic (Fig. 1E). Similar results also confirmed the inhibitory effect of 14-3-3 η (**Suppl. Figure 1D-F**).

Overall, these results demonstrate that 14-3-3 proteins modulate α -Syn aggregation and fibril formation in a concentration-dependent manner, with phosphorylation contributing to fine-tuning their inhibitory effects *in vitro*.

Kinetic curves of α -Syn aggregation measured by RT QuIC in the presence of 14-3-3 γ WT (green line) and S59D (blue line) at α -Syn:14-3-3 γ ratios of 2:1 (**A**) and 40:1 (**B**). Aggregation of α -Syn in the absence or presence of PFFs (gray dots and black line, respectively) are reported as reference. Experiments were run as technical triplicate. Representative images of α -Syn fibrils at the end of the aggregation protocol, as visualized by TEM (**C**) and AFM (**D**). (**E**) Minimum Circumcircle Diameter (MCD) analysis of α -Syn aggregates derived from AFM images. Due to the observed very low fibril curvature, MCD values closely approximate fibrillar lengths. Crosses and lines within bars represent the mean and median values, respectively; whiskers represent the 2.5th and 97.5th percentile of the data; individual points outside this range are shown as outliers

4.2 Ser59 Phosphorylation of 14-3-3 γ affects α -Syn aggregation kinetic in CSF samples

We then examined whether the results obtained from the *in vitro* amplification assays could be replicated in a more complex and pathologically relevant setting, such as CSF. Indeed, RT-QuIC has been shown to detect α -Syn aggregates in the CSF of patients with PD or DLB with high sensitivity and specificity³⁶. Thus, we performed the RT-QuIC assay either in the absence of 14-3-3 γ or in the presence of 14-3-3 γ WT or its phosphomimetic variant. For this experiment, we selected only the 40:1 dilution ratio, as it

exhibited the greatest difference between 14-3-3 γ WT and the phosphomimetic variant in the *in vitro* assays. Curve profiles for each condition are shown in Fig. 2A-C. We observed that the kinetic curves obtained in the presence of 14-3-3 γ WT were delayed compared to those recorded in the absence of 14-3-3 γ or in the presence of the phosphomimetic variant. Consistently, both T50 -defined as the time required to reach half of the maximum fluorescence intensity- and the lag phase -representing the time needed to enter the exponential aggregation phase- were significantly increased in the presence of 14-3-3 γ WT (Fig. 2D and **F**). In agreement with these findings, the slope of the curves in the presence of 14-3-3 γ WT was lower than in the other conditions, indicating reduced steepness of the aggregation kinetics (Fig. 2E).

Kinetic curves of α -Syn aggregation measured by RT-QuIC in CSF samples in the absence of 14-3-3 γ (**A**), or in the presence of 14-3-3 γ WT (**B**) or the phosphomimetic variant 14-3-3 γ S59D (**C**). Quantitative analyses of the kinetic parameters -T50, slope, and lag phase- derived from these profiles are shown in panels **D-F**. Each patient is represented by a unique color, consistently applied across both kinetic curves and corresponding statistical plots. Each sample was run in triplicate. Outliers were assessed prior to analysis, and statistical significance was determined using a mixed-effects model followed by Tukey's multiple comparisons test. No sphericity was assumed and the Geisser-Greenhouse correction was applied. Data are represented as mean $\pm$ SD; * $p < 0.5$; ** $p < 0.1$

4.3 Phosphorylation of 14-3-3 γ affects α -Synuclein aggregation in cells

To confirm that the effect of 1433 γ phosphorylation observed *in vitro* and in CSF with recombinant proteins was also preserved in a cellular context, we co-transfected HEK293T cells with plasmids encoding pEGFP-N- α -Syn together with either the WT or the phosphomimetic S59D form of pDsRed1-N-14-3-3 γ , while cells transfected with the pDsRed1 empty vector (EV) served as controls (Fig. 3A). As shown in Fig. 3B, the proportion of HEK293T cells containing α -Syn aggregates did not differ between cells expressing WT or S59D 14-3-3 γ ; however, in both conditions, aggregate formation was reduced compared with EV-expressing cells. The expression of 14-3-3 γ WT significantly reduced the area of α -Syn aggregates compared to the control and also showed a reduction relative to the phosphomimetic variant (Fig. 3C). Additionally, we observed a significant difference in the co-localization of α -Syn with the two forms of 14-3-3 γ within the aggregates, with 14-3-3 γ WT exhibiting lower co-localization, as indicated by the Pearson coefficient quantification (Fig. 3D). Overall, these findings indicate that phosphorylation at Ser59 modulates 14-3-3 γ 's ability to influence α -Syn aggregation, suggesting a higher chaperone potential for 14-3-3 γ WT compared to the S59D phosphomimetic.

Images showing insoluble α -Syn aggregates (green) in HEK293T cells co-transfected with either an empty vector, 14-3-3 γ WT, or 14-3-3 γ S59D (red, **A**). Quantitative analyses are presented in panels **B-D**, showing the percentage of HEK293T cells containing α -Syn aggregates (**B**), the average aggregate area (**C**), and the Pearson correlation coefficient (**D**). All experiments were performed in three independent replicates and data were assumed as Gaussian. Statistical analyses were conducted using one-way

ANOVA followed by Tukey's multiple comparisons test for panels B and C, and a Welch's T-test for panel D. Data are represented as mean $\pm$ SD; * $p < 0.5$.

4.4 14-3-3 γ phosphorylation imbalance is linked to neurodegeneration

Given the high abundance of 14-3-3 proteins in the brain, we next sought to examine the impact of Ser59 phosphorylation on 14-3-3 interactome by performing immunoprecipitation from mouse whole-brain lysates, followed by mass spectrometry analysis (**Suppl. Figure 2**). As shown in Fig. 4A, a total of 39 interacting proteins were identified (showing p value < 0.05), of which 21 preferentially associated with 14-3-3 γ WT (blue dots), 8 with 14-3-3 γ S59D (red) and 10 with similar affinity for both forms. To encompass the highest number of interactors, we further expanded our analyses by employing HuProt™ Arrays as an unbiased high-throughput platform capable of screening more than 20,000 proteins, representing approximately 80% of the human proteome (<https://cambridgeproteinarrays.com>). Arrays were probed with either 14-3-3 γ WT or 14-3-3 γ phosphorylated by PAK6 (p14-3-3 γ). In this approach, which provides a more controlled setting compared to immunoprecipitation (e.g., due to the absence of phosphatases), we replaced the phosphomimetic mutant with the more physiologically relevant PAK6-mediated phosphorylation. Briefly, His-14-3-3 γ WT was incubated for 2 h at 37°C with PAK6 (using 1:50 14-3-3 γ :PAK6 ratio) in the presence of ATP. Under these conditions, in fact, we obtain a complete phosphorylation of 14-3-3 γ ³⁵. Candidate interactors were ranked according to their Z scores, with a cut-off value of 2. After applying the filtering criteria described in the Materials and Methods section, we found that 141 proteins exhibited Z scores above the threshold. Among these, 47 proteins preferentially interacted with 14-3-3 γ WT (blue dots), 46 showed higher affinity for p14-3-3 γ (red dots), and 48 interacted similarly with both forms (gray dots) (Fig. 4B).

By integrating both experimental approaches, we obtained a total of 180 proteins that passed all the filtering criteria and were identified as 14-3-3 γ interactors. To assess concordance with previously reported datasets, we compared our results with the 1,199 human 14-3-3 γ interactors curated in BioGRID, identifying 24 overlapping proteins. Furthermore, comparison with a recent study reporting 175 interactors of 14-3-3 θ revealed 17 shared proteins, despite the different isoforms analyzed. Of these, 9 were also annotated in BioGRID (**Suppl. Figure 3**).

We then conducted gene ontology analysis considering all the deregulated proteins (all the blue and red dots). As illustrated by the cnetplot and dotplot charts related to biological functions (Fig. 4C-D), the most significantly enriched node corresponded to "Regulation of protein-containing complex assembly," thus reflecting the scaffolding role of 14-3-3 proteins. Also, phosphorylation of 14-3-3 γ was associated with changes in the "Cellular response to misfolded proteins" and "Protein refolding", consistent with their chaperone-like function. In addition, by looking at the molecular function (Fig. 4E-F), we found that two of the most enriched nodes were related to "Ubiquitin ligase binding", thus suggesting that phosphorylation of 14-3-3 γ may induce a deregulation in the cellular proteostasis, which is typical of proteinopathies. Given the importance of 14-3-3 proteins in brain physiology and pathology, we

performed gene set enrichment analysis (GSEA) to identify those proteins deregulated by 14-3-3 γ phosphorylation and clinical evidence suggests a potential contribution to the onset of neurological disorders. To this end, our combined list of deregulated proteins was matched with a reference set of genes associated with “Neurodegenerative disorders - adult onset” obtained from PanelApp. The statistical significance of the observed overlap was determined by comparison with the overlap obtained by matching the same reference set of genes with a randomly generated protein list (as illustrated in Fig. 4G). This analysis identified a subset of proteins, namely Caytaxin (encoded by ATCAY), T-complex protein 1 subunit epsilon (TCP-1- ϵ , encoded by CCT5), Notch 3 intracellular domain (NICD3) and Profilin 1 (PFN1), all of which interacted more with 14-3-3 γ WT; we also identified Ataxin 3 (ATXN3) and Optineurin (OPTN) which, instead, showed higher interaction with the phosphomimetic. Among these, NICD3, PFN1 and OPTN exhibited strong evidence of disease association according to PanelApp data (Fig. 4H, highlighted in green). Finally, to evaluate the presence of potential 14-3-3 binding sites in our candidate hits, we used the 14-3-3-Pred server, a web-based tool that integrates three different classification methods to predict 14-3-3-binding phosphosites⁴². As shown in the color-coded table, NICD3 and OPTN exhibited the highest predicted probability of interacting with 14-3-3 proteins, with three binding sites consistently identified by all methods.

Collectively, these data indicate that phosphorylation of 14-3-3 γ (or its simulation obtained with the phosphomimetic) profoundly reshapes its interactome, selectively modulating interactions with proteins implicated in neuronal function and survival, and highlighting NICD3 and OPTN as high-confidence, disease-relevant candidate interactors potentially linking 14-3-3 γ signaling to neurodegenerative processes.

5. Discussion

Phosphorylation is an efficient mechanism to modulate 14-3-3s PPIs. Increasing evidence associates aberrant 14-3-3s phosphorylation with the development of neurological disorders⁴³; in this regard, a recent study reported that phosphorylation of 14-3-3 θ at Ser232 alters its interactome and disrupts vesicle trafficking in neurons⁴⁴. We previously demonstrated that phosphorylation of PAK6 has a protective effect in primary neurons derived from mice overexpressing the pathogenic LRRK2-G2019S variant. Importantly, this beneficial effect was associated with phosphorylation of 14-3-3 γ at Ser59 mediated by PAK6.¹² In line with this, we hypothesized that phosphorylation at Ser59 could alter the interaction between 14-3-3 γ and its client proteins, ultimately leading to the dysregulation of pathways potentially implicated in the pathogenesis of neurological disorders.

Consistent with previous studies, we showed that 14-3-3 γ can modulate α -Syn aggregation *in vitro*^{30,31}. Specifically, aggregates generated through seeding amplification assays and analyzed by AFM and TEM revealed the formation of smaller fibrils in the presence of 14-3-3 γ WT compared with its phosphomimetic form. We detected a dose-dependent effect, where high stoichiometric ratios (2:1) led to inhibition of α -Syn aggregation irrespective of the phosphorylation status of 14-3-3 γ . Interestingly, we

observed that the ability of 14-3-3 γ to inhibit α -Syn aggregation is preserved in a complex environment such as CSF. Specifically, the WT form of the protein delayed the aggregation kinetics, resulting in a profile characterized by a longer lag phase, higher T50, and a correspondingly less steep slope. Likewise, in cells we found that the WT protein exerted a stronger protective effect than the phosphomimetic variant, as indicated by a reduced area of α -Syn aggregates within cells.

Altogether, these findings suggest that phosphorylation may impair the ability of 14-3-3 γ to delay α -Syn aggregation. This observation aligns with recent studies reporting that phosphorylation of 14-3-3 proteins diminishes their neuroprotective capacity mainly by impacting on the chaperone properties of 14-3-3s^{33,34,44}. It is worth noting, however, that previous studies have suggested that monomeric 14-3-3 proteins exhibit enhanced chaperone activity^{45,46}. Based on this, one might therefore anticipate that phosphorylated 14-3-3 γ would be more efficient at maintaining α -Syn in a soluble conformation. Nevertheless, a more recent computational study found that the monomeric and dimeric forms of 14-3-3 proteins do not differ in their interaction with α -Syn⁴⁷. It is possible that 14-3-3 γ acts at the early stages of α -Syn aggregation³⁰. In fact, it has recently been hypothesized that the ability of 14-3-3 proteins to slow aggregate formation may arise from a molecular effect on α -Syn condensates that precede seed formation, thereby diverting α -Syn from initiating the process of fibril assembly³¹. Our data partially support this model: the increased lag phase indicates a primary effect on nucleation, delaying the formation of aggregation-competent α -Syn seeds. The higher T50 and reduced slope further suggest a global slowing of aggregation and a moderate impairment of fibril elongation. Together, these findings imply that 14-3-3 γ stabilizes α -Syn in non-aggregating states or modulates early condensates, reducing both nucleation efficiency and fibril growth.

We also investigated the impact of 14-3-3 γ phosphorylation on its interactome at a broader level by integrating mass spectrometry analyses and protein arrays. We identified a relatively limited number of interactors exceeding the established Z-score cut-off, namely 39 from immunoprecipitation-mass spectrometry analyses of brain lysates and 141 from the protein microarray data. This is consistent with a very recent study in which the authors identified 175 interactors of 14-3-3 θ using affinity purification-mass spectrometry from mouse cortical brain lysates; of these, only 32 showed preferential interaction with the non-phosphorylated form of 14-3-3 θ , while none of the interactors showed increased binding with the phosphorylated protein⁴⁴. In contrast, we also identified a few proteins displaying preferential interaction with 14-3-3 γ S59D/p14-3-3 γ .

Gene ontology analysis revealed that the deregulated proteins are primarily involved in modulating protein complex assembly and protein refolding, further supporting a potential role for 14-3-3 proteins in proteinopathies, where the misfolding and aggregation of proteins such as α -Synuclein, Tau, or Amyloid- β often underlie disease onset. In addition, terms associated with the ubiquitin system were also enriched, further implicating a possible role for 14-3-3 γ in the regulation of protein homeostasis. Finally, by comparing our list of deregulated proteins with the PanelApp list for “neurological disorders with adult onset”, we identified several proteins among the interactors of 14-3-3 γ which appear to be associated

with the development of neurological disorders. In particular, we identified Caytaxin, involved in neuronal differentiation and Cayman ataxia onset^{48,49}, TCP-1-ε, a chaperone linked to hereditary neuropathy⁵⁰; NICD3, which plays a role in cell fate determination and cerebral arteriopathy^{51,52}; and PFN1, a regulator of cytoskeletal dynamics and associated with amyotrophic lateral sclerosis (ALS)^{53,54}. In addition, we found ATXN3, associated with Machado-Joseph disease, an inherited spinocerebellar ataxia⁵⁵ and OPTN, which is involved in glaucoma and ALS onset^{56,57}.

Collectively, these results indicate that phosphorylation of 14-3-3γ could have wide-ranging effects on neuronal development and the pathogenesis of neurodegenerative diseases. They also support the idea of exploring therapeutic approaches that modulate 14-3-3γ phosphorylation. For instance, one could consider targeting kinases known to phosphorylate 14-3-3s, including PAK6, Protein Kinase A and Sphingosine-dependent protein kinase-1^{12,13,17}. In particular, targeting PAK6 and/or other members of the PAK family is especially attractive, as their preferential expression in the brain may help limit undesirable side effects⁵⁸. We have recently shown that active PAK6 mitigate α-Syn toxicity specifically in SNc dopaminergic neurons in vivo⁵⁹. In addition, we found that PAK6 regulates LRRK2 activity at multiple levels, not only by promoting its dissociation from 14-3-3γ and subsequent dephosphorylation, but also by modulating regulatory subunits of LRRK2-associated phosphatases^{12,60 61}. Interestingly, active PAK4, another member of Group II PAKs (PAK4–6), has also been reported to exert neuroprotective effects in rat models of PD, preserving neuronal and motor function while reducing α-Syn aggregation^{62,63}. However, dysregulation of PAK signaling has been strongly linked to neurodegenerative disorders, including Alzheimer's disease and PD. Consequently, the development of PAK-targeted therapies remains challenging, as precise modulation of kinase activity is required, as both insufficient and excessive PAK signaling can disrupt neuronal homeostasis^{64–67}.

An alternative approach could involve the development of small molecules that selectively favour interactions between 14-3-3γ and its target proteins^{68,69}. Such compounds could in fact preserve the neuroprotective functions of 14-3-3γ without globally interfering with its other cellular roles, offering a more targeted and potentially safer therapeutic strategy. Finally, these findings suggest that both phosphorylated 14-3-3γ and the associated pathway alterations could serve as potential biomarkers for neurological disease progression or therapeutic response.

Declarations

7. Data availability

The data obtained in this research are available from the corresponding author upon reasonable request.

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10. Contributions

L.C. and E.Gi. conceived and wrote the manuscript. Y.Z. and C.M. performed bioinformatics analyses; V.G., S.N., G.K and I.T. contributed to the production of α -Syn fibrils, kinetic assays and microarray processing. M.B. conducted AFM analyses; G.A. and G.R performed MS analyses; S.N., S.C., L.B., A.A.

and E.Gr. contributed to the experimental design, data interpretation and manuscript revision. All authors read and approved the final manuscript.

11. Ethics declarations

11.1 Competing interests

The authors declare no competing interests.

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Figures

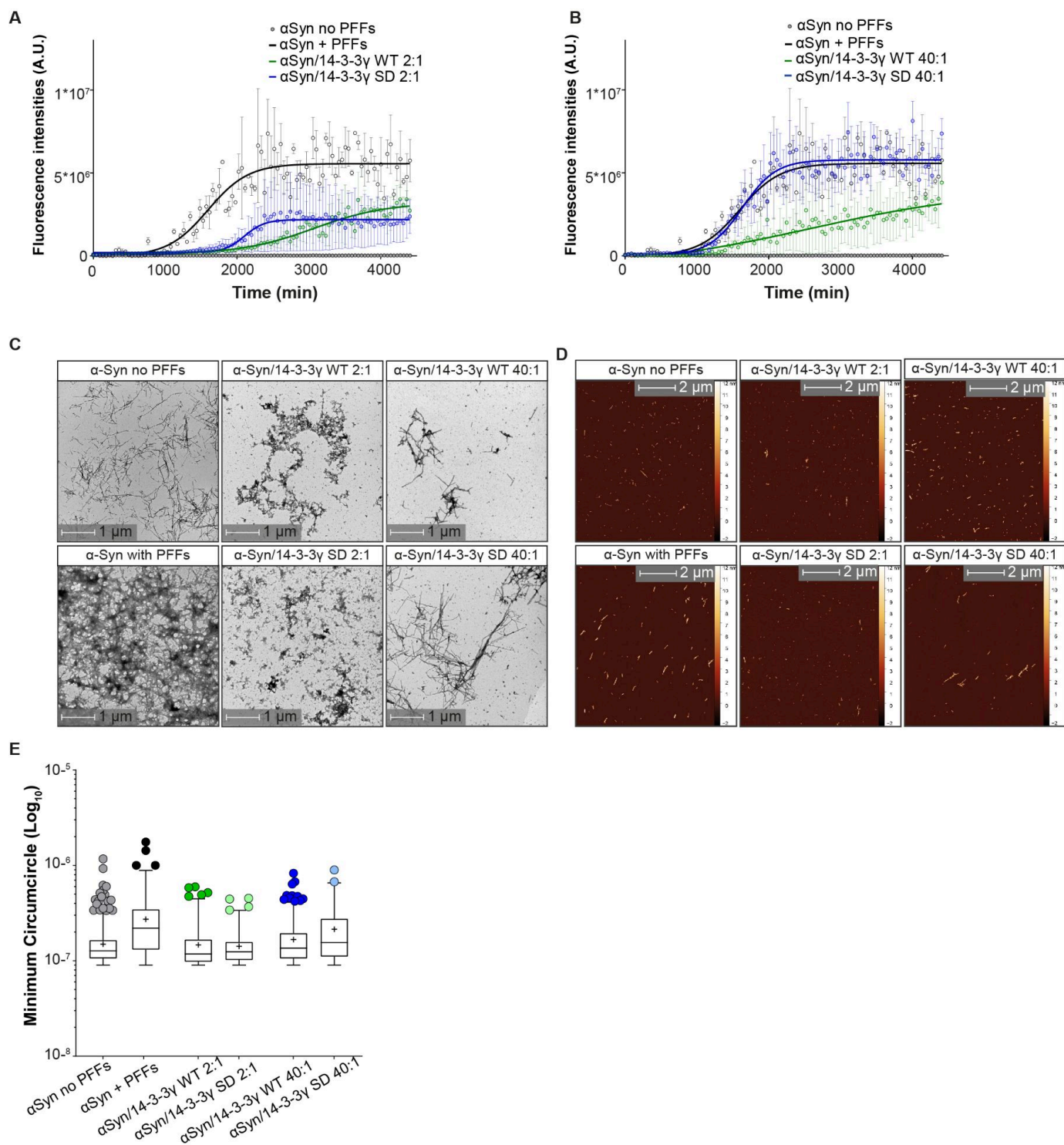


Figure 1

Effect of 14-3-3 γ WT and S59D on α -Syn Fibril Formation *in vitro*

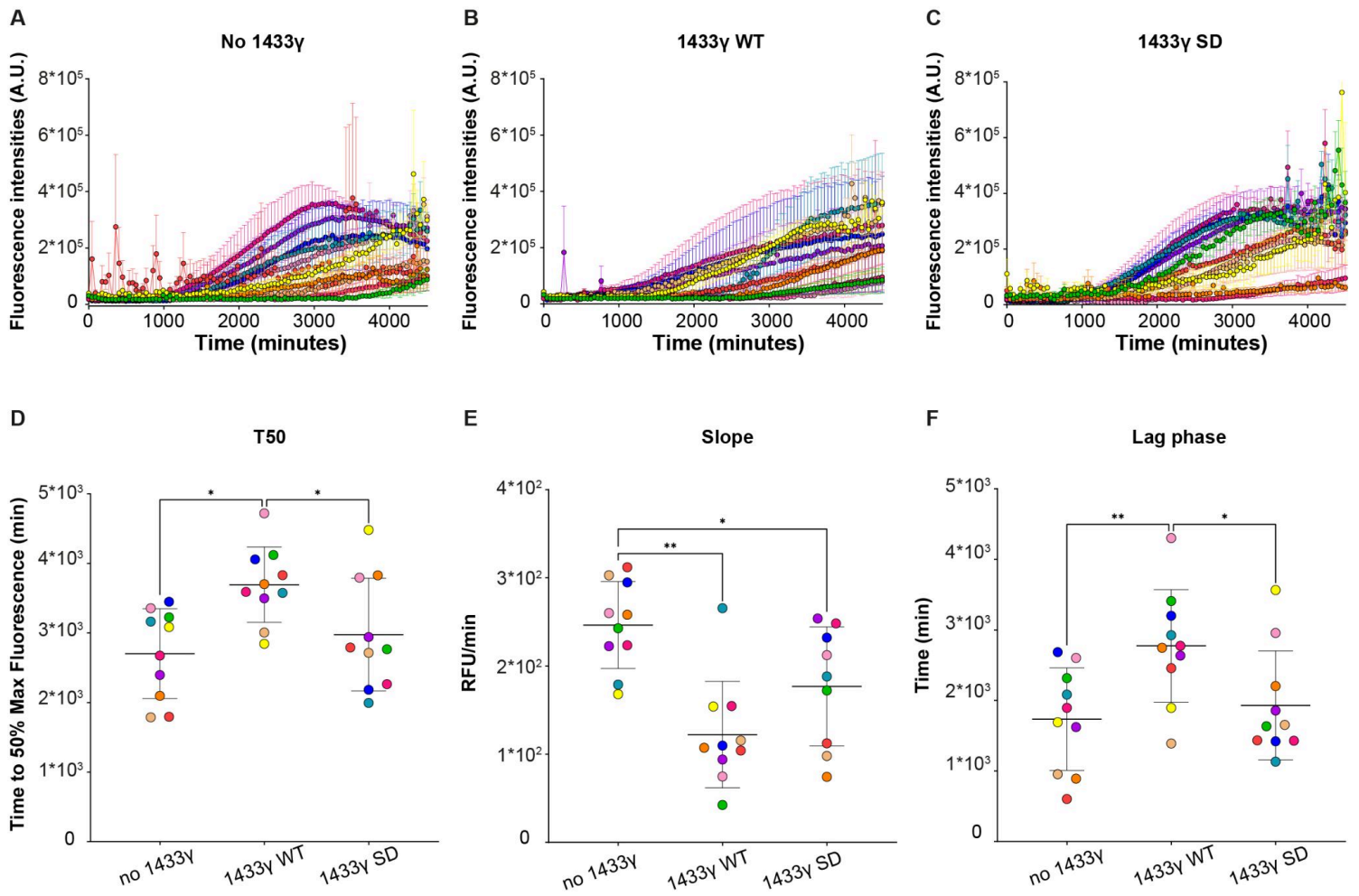


Figure 2

CSF 14-3-3 γ WT delays the kinetic of α -Syn aggregation in CSF samples

Kinetic curves of α -Syn aggregation measured by RT-QuIC in CSF samples in the absence of 14-3-3 γ (A), or in the presence of 14-3-3 γ WT (B) or the phosphomimetic variant 14-3-3 γ S59D (C). Quantitative analyses of the kinetic parameters -T50, slope, and lag phase- derived from these profiles are shown in panels D-F. Each patient is represented by a unique color, consistently applied across both kinetic curves and corresponding statistical plots. Each sample was run in triplicate. Outliers were assessed prior to analysis, and statistical significance was determined using a mixed-effects model followed by Tukey's multiple comparisons test. No sphericity was assumed and the Geisser-Greenhouse correction was applied. Data are represented as mean $\pm$ SD; *p<0.5; **p<0.1

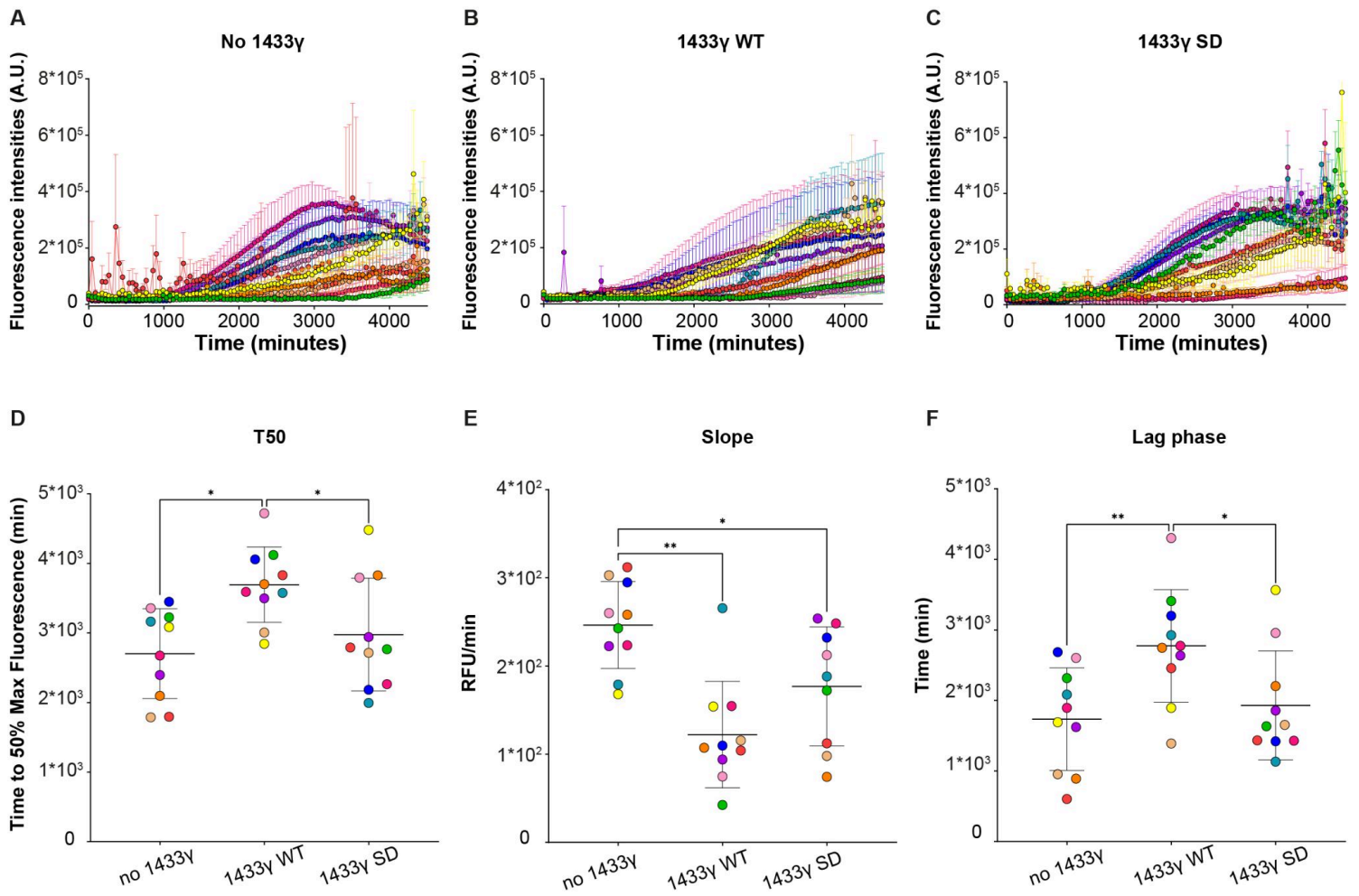


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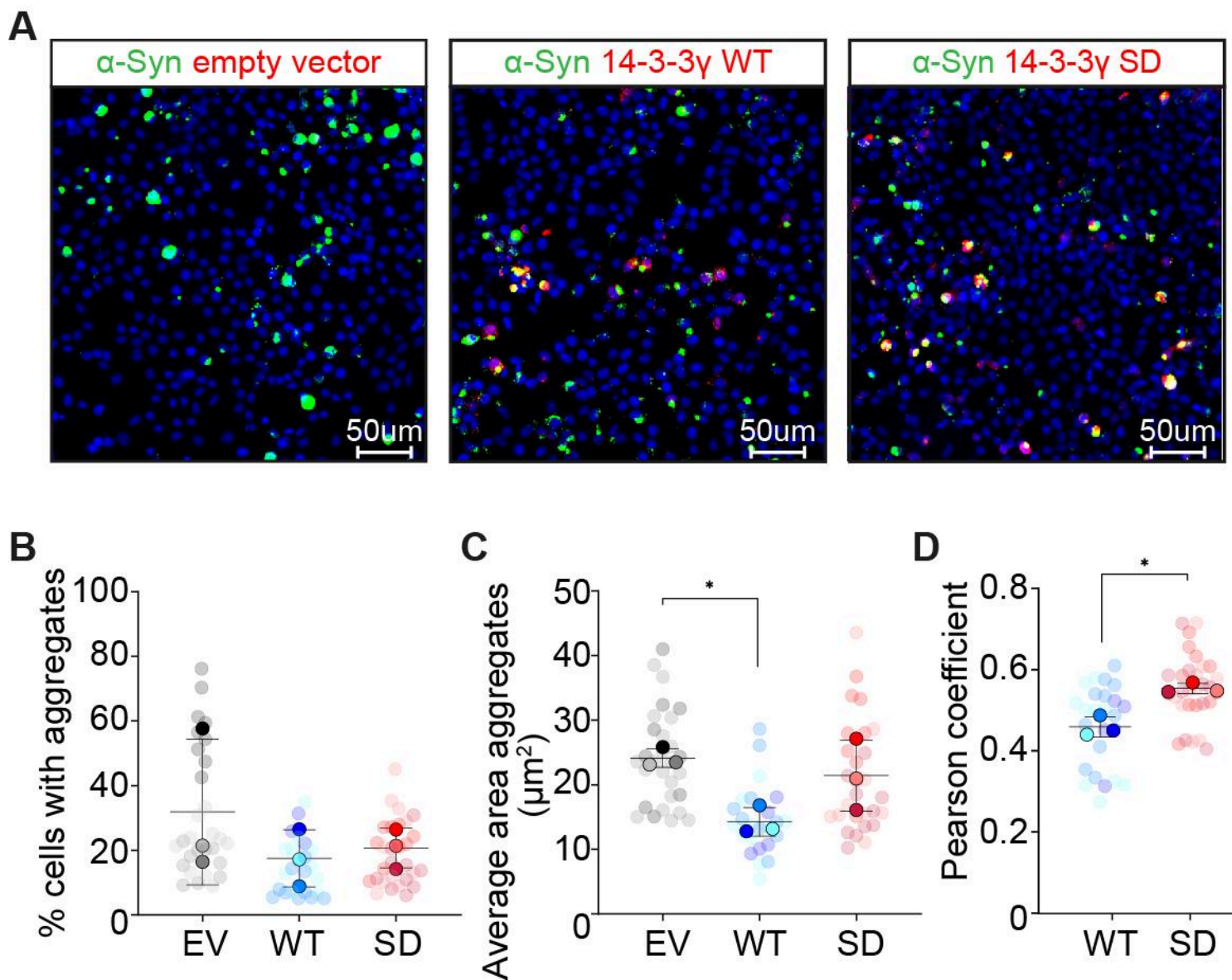


Figure 3

14-3-3γ WT affects α-Syn Fibril Formation *in cells*

Images showing insoluble α-Syn aggregates (green) in HEK293T cells co-transfected with either an empty vector, 14-3-3γ WT, or 14-3-3γ S59D (red, **A**). Quantitative analyses are presented in panels **B–D**, showing the percentage of HEK293T cells containing α-Syn aggregates (**B**), the average aggregate area (**C**), and the Pearson correlation coefficient (**D**). All experiments were performed in three independent replicates and data were assumed as Gaussian. Statistical analyses were conducted using one-way ANOVA followed by Tukey's multiple comparisons test for panels B and C, and a Welch's T-test for panel D. Data are represented as mean±SD; *p<0.5.

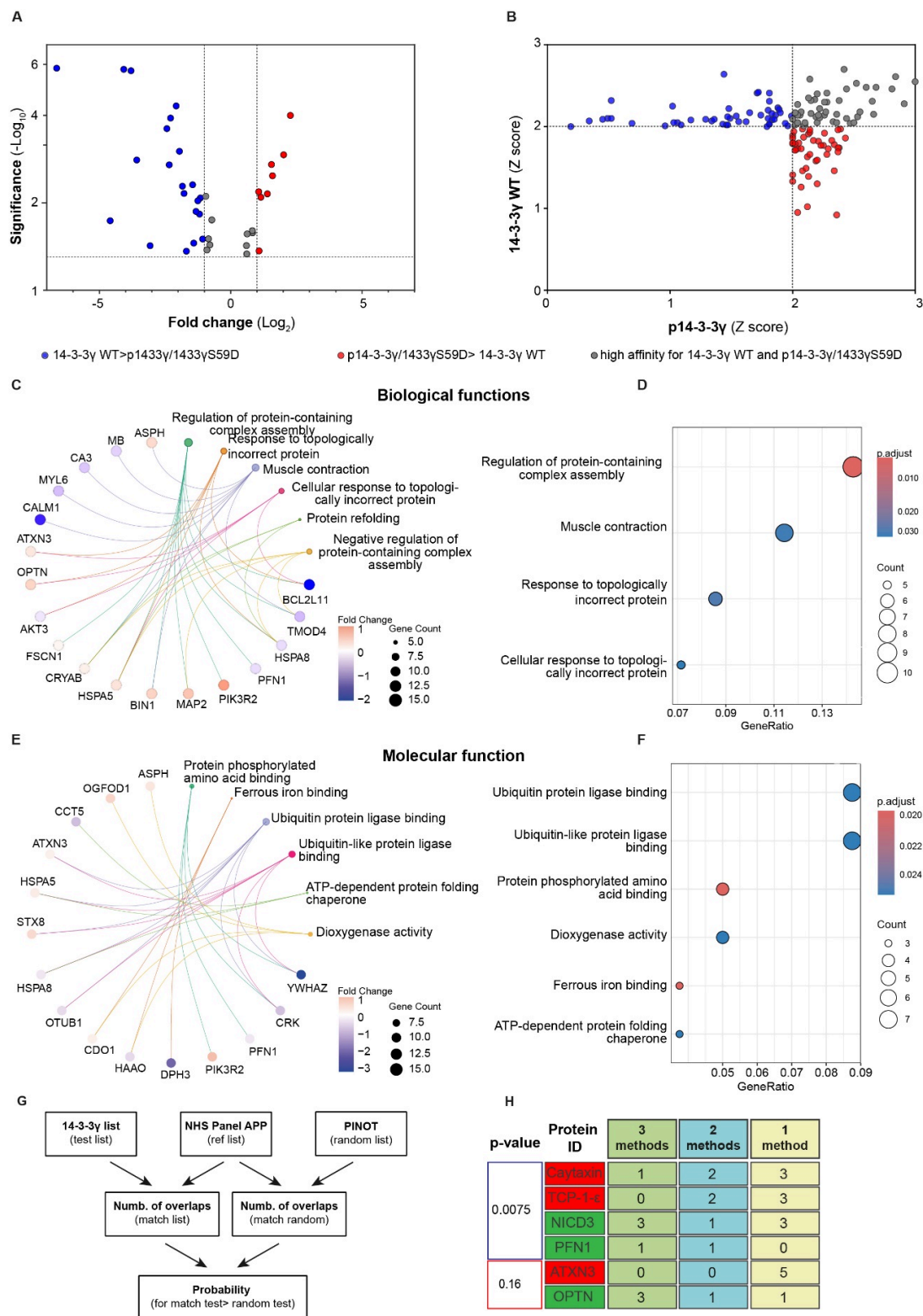


Figure 4

Phosphorylation of 14-3-3γ uncovers interactors implicated in neurodegenerative disorders

(A) Distribution of candidate interactors of 14-3-3γ WT (blue) and the phosphomimetic mutant 14-3-3γ S59D (red) identified by mass spectrometry. Candidates with p value < 0.05 and interacting with both 14-3-3γ WT and the phosphomimetic, are shown in grey. (B) Distribution of candidate interactors of 14-3-3γ

WT (blue) and phosphorylated 14-3-3 γ (red) identified using protein arrays, plotted according to their Z-scores. Candidates interacting with both WT and phosphorylated forms are shown in grey. **(C)** Cnetplot relative to “biological processes” illustrate the relationships between significantly enriched biological terms and their associated proteins. Circular nodes represent proteins which are connected to the most enriched functional categories. Gene nodes are colored according to log2 fold change, with red indicating proteins that interact preferentially with p14-3-3 γ /S59D and blue those interacting preferentially with 14-3-3 γ WT. Node size of functional categories corresponds to the number of associated proteins. **(D)** Dotplot summarizes the enrichment results for the same biological processes. Each dot represents a term, with dot size proportional to the number of associated proteins and dot color indicating the adjusted p-value. **(E)** Cnetplot showing enriched terms associated with molecular functions and their linked proteins. **(F)** Dotplot summarizing enrichment results related to molecular function. **(G)** Schematic representation of the gene set enrichment analysis (GSEA) conducted using the PanelApp knowledgebase. **(H)** List of proteins identified by GSEA, with those documented as disease-associated in PanelApp highlighted in green. Adjacent to the list, the predicted 14-3-3 binding scores for each protein are shown, as determined by 14-3-3 Pred.

Supplementary Files

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