Phosphorylation of T897 in the dimerization domain of Gemin5 modulates protein interactions and translation regulation

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Abstract

Gemin5 is a multifunctional RNA binding protein (RBP) organized in domains with a distinctive structural organization. The protein is a hub for several protein networks performing diverse RNA-dependent functions including regulation of translation, and recognition of small nuclear RNAs (snRNAs). Here we sought to identify the presence of phosphoresidues on the C-terminal half of Gemin5, a region of the protein that harbors a tetratricopeptide repeat (TPR)-like dimerization domain and a non-canonical RNA binding site (RBS1). We identified two phosphoresidues in the purified protein: P-T897 in the dimerization domain and P-T1355 in RBS1. Replacing T897 and T1355 with alanine led to decreased translation, and mass spectrometry analysis revealed that mutation T897A strongly abrogates the association with cellular proteins related to the regulation of translation. In contrast, the phosphomimetic substitutions to glutamate partially rescued the translation regulatory activity. The structural analysis of the TPR dimerization domain indicates that local rearrangements caused by phosphorylation of T897 affect the conformation of the flexible loop 2–3, and propagate across the dimerization interface, impacting the position of the C-terminal helices and the loop 12–13 shown to be mutated in patients with neurological disorders. Computational analysis of the potential relationship between post-translation modifications and currently known pathogenic variants indicates a lack of overlapping of the affected residues within the functional domains of the protein and provides molecular insights for the implication of the phosphorylated residues in translation regulation.

1. Introduction

Gemin5 is a predominantly cytoplasmic RNA-binding protein (RBP) known as a component of the survival of motor neurons (SMN) complex, a molecular chaperone that plays a critical role in the biogenesis and function of snRNPs [1–3]. In humans, the SMN complex comprises 9 members, namely SMN, Gemin5–8, and unr-interacting protein (Unrip) [4]. Initially, Gemin5 was identified as the protein responsible for the recognition of the U-rich sequence known as the Sm site of small nuclear RNAs (snRNAs) and delivery to snRNPs [5,6]. However, consistent with the observation that a large fraction of Gemin5 protein is found in the cytoplasm outside of the SMN complex [7], Gemin5 acts as a hub for several networks performing diverse functions, including regulation of translation [8–10], ribosome-interaction [11,12], reprogramming factor [13], and signal recognition particle (SRP)-interacting protein [14].

Gemin5 protein is organized in functional domains with distinctive structural features (Fig. 1). The N-terminal part (G51–739) contains two juxtaposed seven-bladed WD40 domains that recognize the Sm site of snRNAs and the cap structure via base-specific interactions [15,16]. The C-terminal half (G5845–1508) encompasses

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two different domains, a dimerization domain in the central region and a noncanonical RNA-binding site at the C-terminal end [17]. Structural analysis of the central region identified a tetratricopeptide repeat (TPR)-like domain with 17 α-helices that oligomerizes as a canoe-shaped homodimer [18]. The most C-terminal part, G51297-1508 harbors a non-canonical bipartite RNA-binding site (RBS1-2) [19]. The RBS1 domain folds into a pentamer structure and a noncanonical RNA-binding site at the C-terminal end [17].

Gemin5 biallelic variants were recently linked with human neurodevelopmental disorders, perturbing distinct pathways as compared to defects in the SMN protein causing spinal muscular atrophy (SMA) [22–25]. Dimerization defects in Gemin5 variants described in patients with neurological disorders resulted in the loss of factors connected to RNA processing, translation regulation, and spliceosome assembly [25], establishing a link between the pathogenic mutations and protein malfunction. Despite this, the molecular basis of Gemin5 dysfunction remains poorly understood. In addition to the structural and functional features perturbed by specific mutations affecting the different domains of the protein, Gemin5 was identified as a phosphoprotein in the cytoplasm of human cells [26]. Furthermore, a database collecting post-translation modifications reports multiple phosphosites in Gemin5 (https://www.phosphosite.org) [27]. Therefore, it is plausible that regulatory signals affecting the phosphorylation of this protein may impact on its activities when acting individually.

Here we sought to identify the presence of phosphosites on the C-terminal half of Gemin5 (G51294-1508). This region of the protein results from the L protease cleavage in foot-and-mouth disease virus (FMDV) infected cells [28], and has been related to internal ribosome entry site (IRES)-dependent translation regulation [19]. The identified phosphosites are located in two critical domains of the protein, the TPR-like dimerization module (T897) and the non-canonical RBS1 domain (T1355). Therefore, we took advantage of the known properties of the G51294-1508 fragment to analyze the impact of these variants on Gemin5 translation regulation and protein interaction [29]. We generated a protein unphosphorylated at these residues replacing T897 and T1355 with alanine which results in decreased translation, while the phosphomimetic substitutions to glutamate partially recover the translation regulation capability of the protein. Structural analysis of the dimerization domain suggests that the phosphorylation of T897 involves local structural rearrangements that could explain the partial activity recovery in the T897E mutant. Mass spectrometry analysis revealed that variant T897A strongly abrogates the association of cellular proteins involved in the regulation of translation, among other cellular processes. Moreover, analysis of the potential relationship between post-translation modifications and pathogenic mutants appearing naturally in patients indicates a non-overlapping location of the affected residues.

2. Materials and methods

2.1. Constructs

The plasmids pcDNA3-Xpress-G51294-1508 and pcDNA3-CTAP-G51294-1508 were previously reported [18,19]. The bicistronic construct CMVpBIC [8] expresses chloramphenicol acetyl transferase (CAT) in cap-dependent manner, and luciferase (Luc) in IRES-dependent manner. Constructs pOPINM_G5_TPR-T897E, pcDNA3-Xpress-G51294-1508T897A, pcDNA3-Xpress-G51294-1508T897E, pcDNA3-CTAP-G51294-1508T897A, pcDNA3-CTAP-G51294-1508T897E, pcDNA3-Xpress-G51294-1508T1355A, pcDNA3-Xpress-G51294-1508T1355E, pcDNA3-Xpress-G51294-1508T897A/T1355A and pcDNA3-Xpress-G51294-1508T897E/T1355E were obtained by Quick-Change mutagenesis (Agilent Technologies) using specific primers (Table S1). All constructs were confirmed by DNA sequencing (Macrogen).

2.2. Protein complexes isolation by TAP

HEK293 cells were cultured with DMEM supplemented with 5 % fetal calf serum. Monolayers grown at 80 % of confluency were transfected with the constructs G51294-1508-TAP and were harvested 24 h post-treatment (hpt). The complexes associated with the TAP-tagged proteins were purified as described [11]. Briefly, the extract from the TEV protease digestion of the first IgG Sepharose (Cytiva) purification was subsequently subjected to a second calmodulin (Agilent Technologies) purification step. Purified proteins were precipitated with 10 % trichloroacetic acid, washed with acetone, and dissolved in SDS–loading buffer. An aliquot (20 %) was ana-
lyzed on silver-stained SDS–polyacrylamide (PAGE) gels to visualize the purification of proteins associated with G5845-1508–TAP polypeptides.

2.3. Identification of Gemin5 phosphorylation sites

HEK293 cell lysates expressing G5845-1508–TAP were subjected to TAP purification. The isolated protein complexes were resolved on SDS-PAGE, stained with Coomassie blue. The cropped band was in-gel digested at the IRB Proteomics Platform. The resulting peptides were reconstituted in 40 μL of buffer A (1 % formic acid in water). Samples were loaded to a C18 symmetry trap analytical column (BEH130™ C18 75 μm × 25 cm, 1.7 μm, Waters Corp.), equilibrated in buffer A at a flow rate of 15 μL/min using a nanoAcquity Ultra Performance LC/TMT chromatographic system (Waters Corp., Milford, MA). Peptides were separated using a C18 analytical column (BEH130™ C18 75 μm × 25 cm, 1.7 μm, Waters Corp.) with a 90 min run, comprising three consecutive steps with linear gradients from 1 to 35 % of buffer B (0.1 % formic acid in CH3CN) in 60 min, from 35 to 50 % B in 5 min, and from 50 % to 85 % B in 3 min, followed by isocratic elution at 85 % B in 10 min and stabilization to initial conditions (A = 0.1 % FA in water, B = 0.1 % FA in CH3CN). The column outlet was directly connected to an Advin TriVersa NanoMate (Advion) fitted on an LTQ-FT Ultra mass spectrometer (Thermo Fischer Scientific). The mass spectrometer was operated in data-dependent acquisition (DDA) mode. Survey MS scans were acquired in the FT with the resolution (defined at 400 m/z) set to 100,000. Up to six of the most intense ions per scan were fragmented and detected in the linear ion trap. The ion count target value was 1,000,000 for the survey scan and 50,000 for the MS/MS scan. Target ions already selected for MS/MS were dynamically excluded for 30 s. Spray voltage in the NanoMate source was set to 1.70 kV. Capillary voltage and tube lens on the LTQ-FT were tuned to 40 V and 120 V. The minimal signal required to trigger MS to MS/MS switch was set to 1000 and activation Q was 0.250. The spectrometer was working in positive polarity mode and singly charge state precursors were rejected for fragmentation. At least one blank run before each analysis was performed to ensure the absence of cross-contamination from previous samples.

A database search was performed with Bioworks v3.1.1 SP1 (Thermo Fischer Scientific) using the Sequest search engine and SwissProt database, which included SwissProt human (release 2013_06), Gemin5 protein, and the common repository of adventitious proteins (https://www.thegpm.org/crap/index.html). Search parameters included trypsin specificity, allowing for two missed cleavage sites, carbamidomethyl in cysteine as static modification and methionine oxidation and phosphorylation in serine, threonine, and tyrosine, as dynamic modifications. Peptide mass tolerance was 10 ppm and the MS/MS tolerance was 0.8 Da. Peptides with a q-value lower than 0.1 and an FDR < 1 % were considered positive identifications with a high confidence level (Dataset 1).

2.4. Translation assay

HEK293 cell monolayers at 80 % of confluency were cotransfected with the bicistronic construct CMVpMBIC and pcDNA3-Xpress–G5845-1508 WT or the different mutants using Lipofectamine LTX (Thermo Fisher Scientific) and were harvested 24 hpt. Cell lysates were prepared in lysis buffer C (50 mM Tris-HCl pH 7.8, 100 mM NaCl, 0.5 % IGEPA). The concentration of total protein in the lysate was determined by the Bradford assay (BioRad). IRES activity was quantified as the expression of Luciferase activity (RLU) using Berthold Sirius Single Tube Luminoimeter and normalized to the amount of protein in the lysate. CAT activity, which reported cap-dependent translation, was determined by the liquid scintillation assay [30]. Appropriate dilutions of the extracts were chosen to be in the linear range of the assay. Values represent the mean ± SEM (standard error of the mean). Differences in distribution between two samples were analyzed by paired two-tailed Student’s t-test and were considered significant when P < 0.05. The resulting P-values were graphically illustrated in figures with asterisks as described in figure legends.

2.5. Immunodetection

Equal amounts of total protein were resolved on SDS-PAGE and transferred to a 0.2 μm pore PVDF membrane (Bio-Rad) using a semi-dry electrotransfer (Bio-Rad). Xpress–G5845-1508 proteins were immunodetected using anti-Gemin5 (Novus), anti-Xpress (Thermo Fisher Scientific), or anti-CBP (Abcam) antibodies. Immunodetection of tubulin (Merck) was used as the loading control. The appropriate secondary HRP-conjugated antibodies (Thermo Fisher Scientific) were used according to the instructions of the manufacturer.

2.6. Protein stability assay

HEK293 human cells were transfected with the pcDNA3-Xpress–G5845-1508 constructs. For cycloheximide (CHX) chase experiments, CHX (100 μg/ml) (Merck) was added to stop translation at 12 h post-transfection. Cells were harvested immediately (time 0), 12 and 16 h post-CHX treatment. Cell lysates were prepared in 100 μl of lysis buffer C, and the total protein concentration was determined by the Bradford assay. Equal amounts of total protein were resolved on SDS-PAGE, the Xpress–G5845-1508 proteins were immunodetected, and the intensity of the bands was quantified. Values represent the mean ± SEM. The difference in distribution between two samples was analyzed by paired two-tailed Student’s t-test and was considered significant when P < 0.05.

2.7. TPR production and SEC analysis

Gemin5 TPR WT and the T897E mutant were expressed and purified as described [18]. Briefly, E. coli BL21–Rosetta (DE3) pLyS cultures transformed with the plasmid pOPINM–TPR (encoding the TPR fused to an N-terminal His6-MBP-tag cleavable by PreScission protease) were induced with 0.5 mM IPTG at 20 °C overnight. The cells were harvested and resuspended in 40 ml of buffer A (20 mM Tris pH 8.0, 0.5 M NaCl, 10 mM imidazole, 5 % glycerol, and 2 mM β-mercaptoethanol) supplemented with 0.5 mg/ml AEBSF [4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride] protease inhibitor. Following sonication, the clarified supernatant was loaded onto a 5 ml HisTrap HP column (Cytiva) equilibrated in buffer A, extensively washed with buffer A with 35 mM imidazole, and eluted by a stepwise increase of imidazole to 250 mM. The protein was dialyzed overnight against buffer B (20 mM Tris pH 6.8, 50 mM NaCl, 5 % glycerol, and 1 mM DTT) and GST-tagged PreScission protease (1/20th of total protein weight) was added into the dialysis bag to cleave the N-terminal tag. The sample was loaded onto a 5 ml HiTrap S HP column (Cytiva) equilibrated in buffer B, and the cleaved protein was eluted by increasing the salt concentration to 150 mM NaCl. The protein was concentrated by ultrafiltration using an Amicon device with a 10 kDa cutoff and loaded onto a Superdex 200 200 kDa increase 10/300 or Superdex 75 10/300 size exclusion chromatography (SEC) column equilibrated in buffer B. SEC columns were calibrated with standards of known molecular weight (Cytiva; ferritin, 440 kDa; aldolase, 158 kDa; conalbumin, 75 kDa; ovalbumin, 44 kDa; carbonic anhydrase, 29 kDa; ribonuclease A, 13.7 kDa; apotinin, 6.5 kDa).

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2.8. Molecular dynamics

MD simulations were performed with the GROMACS 5.0.5 software package [31] using the X-ray structure of the Gemin5 TPR-like dimer (PDB ID 6RNQ) as starting model. The phosphorylation at residue T897 and the mutation of this residue to glutamate (T897E) were introduced in both dimer subunits using the CHARMM-GUI portal (https://www.charmm-gui.org) [32]. CHARMM-GUI was also used to model the loop connecting helices 2 and 3 (loop 2–3, aa 877–883) that was not included in the crystal structure for being flexibly disordered. For MD simulations, the CHARMM36m force field was used [33]. The protein was solvated with water and counterions (150 mM KCl) were added by replacing a corresponding number of water molecules to achieve a neutral condition. Van der Waals interactions were smoothly switched off at 10–12 Å by a force-switching function and long-range electrostatic interactions were treated with the particle mesh Ewald method [34]. The simulations were performed at 310.15 K using a Nose-Hoover thermostat with \( \tau_T = 1.0 \) ps. Pressure was maintained constant at 1 bar with a Parrinello-Rahman algorithm with a semisotropic coupling constant \( \tau_p = 5.0 \) ps and compressibility \( = 4.5 \) Å \( \cdot \) 10 \(^{-5} \) bar \(^{-1} \). The LINCS method was used to constrain bond lengths. A time step of 2 fs was used for numerical integration. Coordinates were saved every 5 ps for analysis and the total simulation time was 500 ns. The trajectories were analyzed with the GROMACS suite programs and were visualized with PyMol (Schrödinger; https://pymol.org). Trajectories are available upon request.

2.9. In-gel digestion and mass spectrometry

Two independent biological replicates of TAP samples obtained for G5845–1508–T897A-TAP and G5845–1508–T897E-TAP were applied onto a 10 % SDS–PAGE gel. The protein bands concentrated in the stacking/resolving gel interface were visualized by Coomassie staining. The gel pieces were distained in acetonitrile:water (CH\(_3\)CN:H\(_2\)O, 1:1), then reduced and alkylated, and digested in situ with sequencing grade trypsin (Promega) [35]. The gel pieces were dried and re-swollen in 50 mM ammonium bicarbonate, pH 8.8, with 60 ng/μl trypsin at a 5:1 protein:trypsin (w/w) ratio. The tubes were kept on ice for 2 h and incubated at 37 °C for 12 h. Digestion was stopped by the addition of 1 % TFA. The desalted protein digest was dried, resuspended in 10 μl of 0.1 % formic acid, and analyzed by RP-LC-MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Fisher Scientific). The peptides were concentrated (online) by reverse-phase chromatography using a 0.1 × 20 mm C18 RP precolumn (Thermo Fisher Scientific) and then separated using a 0.075 × 250 mm C18 RP column (Thermo Fisher Scientific) operating at 0.3 μl/min. Peptides were eluted using a 120-min dual gradient from 5 to 25 % solvent B in 90 min followed by a gradient from 25 to 40 % solvent B over 120 min (Solvent A: 0.1 % formic acid in water, solvent B: 0.1 % formic acid, 80 % acetonitrile in water). ESI ionization was done using a Nano-bore emitters Stainless Steel 30 μm (Proxeon) interface. The Orbitrap resolution was set at 30,000. Peptides were detected in survey scans from 400 to 1,600 amu (1 μscan), followed by 20 data-dependent MS/MS scans (Top 20), using an isolation width of 2 u (in mass-to-charge ratio units), normalized collision energy of 35 %, and dynamic exclusion applied during 30 s periods. Peptides with a q-value lower than 0.1 and an FDR < 1 % were considered positive identifications with a high confidence level. Data are available via ProteomeXchange [36] with identifier PXD035227.

2.10. Proteome data analyses

Z-score value from each interactor in the overlap of G5845–1508WT-TAP, G5845–1508–T897A-TAP and G5845–1508–T897E-TAP was calculated considering the average number of peptides. Gemin5 peptides were not included in the calculations to avoid the fraction of peptides belonging to the bait. Proteins with a Z-score > 1.96 (95 % of confidence) are significantly enriched in comparison with the rest of interactors. A higher level of confidence was calculated using the CompPASS algorithm [37,38], which compares the quantity and the identity of the peptides recruited in the different affinity purifications, yielding a WD-score for each interactor. Proteins with WD-score > 1 denote interactors above 95 % confidence threshold.

The Biological Networks Gene Ontology application (BiNGO) was used to analyze the biological processes of factors recruited with G5845–1508–T897A-TAP and G5845–1508–T897E-TAP, determining the statistical significance of overrepresented proteins relative to a complete human proteome [39]. The results were visualized on the Cytoscape platform [40]. The resulting biological processes were classified according to a hypergeometric test in the default mode, FDR < 0.05. P-values were collected.

STRING software was used to depict the physical and functional interactions among the selected factors. This analysis was carried out using default parameters with a minimum required interaction score of 0.4 [41].

3. Results and discussion

3.1. Identification of Gemin5 phosphopeptides

To determine the presence of phosphorylated residues in the central and C-terminal regions of Gemin5 we expressed and purified G5845–1508 protein in HEK293 cells (Fig. S1). Two different phosphorylation sites were identified in Gemin5 (sequence coverage 78.87 %) using high confidence peptides (Fig. 1, Fig. S1, Dataset 1). The phosphopeptide FHGLFLTD (T7–ST–NL) (1087, 56749 Da), is within helix 4 of the TPR domain [18] . T897 is highly conserved (T/S) in Vertebrata, insect species, and even on the slime mold Dictyostelium discoideum (Fig. 1), suggesting a functional role in all organisms. Accordingly, residue 897 is Thr in mammals, birds and fishes, with a conservative change to Ser in insects and amphibians, and a phosphomimetic change to Glu in a green alga. Instead, the unphosphorylatable residue Gly in waps suggest that the P-state of residue 897 is not essential in this particular organism, although a compensatory effect of the flanking Ser (LLGS) is a possibility that remains unknown.

The phosphopeptide MLSTFKEKFSE (T4–ST–NL) (1457, 73416 Da), is placed in the RB51 domain at the intrinsically disordered region [42], which partially resembles an ExR-rich region modeled as a helical region in LUC7L3 [43,44]. T1355 is conserved in primates, but substitutions to A, G, and F are frequently observed in other Vertebrata (Fig. 1), indicating lower conservation than T897. Overall, this region of the protein is poorly conserved compared to the TPR module, suggesting that P-T1355 may be dispensable for some functions in insects and other invertebrate species.

3.2. Influence of T897 and T1355 phosphoresidues in translation

Since G5845–1508 is involved in translation regulation through the TPR-like and the RB51 domains [19,29], we wondered whether P-T897 and P-T1355 could affect the regulatory activity of the protein in translation. For this, cap-dependent and IRES-dependent translation assays were conducted using the bicistronic construct CMVpBIC (Fig. 2A). To analyze the potential effect of phosphorylation of T897 and T1355, we generated two different substitution mutants: a T/A rendered an unphosphorylated version of the protein, whereas a T/E was conducted to create a phosphomimetic
These constructs were expressed in HEK293 cells side by side with the wild-type (WT) protein yielding similar expression levels (Fig. 2B, C). Relative to the WT protein, the activity of the unphosphorylated T/A and the phosphomimetic T/E resulted in different effects in translation regulation. Mutations T897A and T1355A decreased IRES-dependent activity compared to the WT protein, but showed no significant effect on cap-dependent activity (Fig. 2B, C). In turn, the substitution T897E partially rescued the IRES-dependent activity and showed no statistical differences with the WT protein. Similar results were observed using the double mutants T897A/T1355A or T897E/T1355E (Fig. S2), although the IRES-dependent translation difference between T/A and T/E mutants was statistically significant only in T1355. These results show that the T/A substitution causes a functional modification resuable by the phosphomimetic mutant. Thus, we suggest that phosphorylations at residues T897 and T1355 of G5_845-1508 specifically impact on the regulation of IRES-dependent translation.

3.3. Predicted structural effect of variants T897A and T897E, and P-T897 in the TPR-like dimerization domain

Residue T897 is within the TPR dimerization domain, whose three-dimensional structure was recently solved [18]. Thereby, we wondered whether replacing T897 with Ala or Glu could alter the structural organization of the TPR dimerization module. T897 locates at the C-terminal end of helix 4, which is adjacent to helices 12, 13 and 14 in the other subunit across the dimerization interface. Mutations in residues R1016 and D1019 at the loop connecting helices 12 and 13 of the TPR were previously reported to disrupt protein dimerization and also cause gross defects in protein interactome [25].

To determine the impact of the T897 phosphorylation state on the protein structure we purified the TPR-like domain bearing T897E substitution in parallel to the WT protein. Both proteins formed stable homodimers, as observed by size exclusion chromatography (SEC) (Fig. S3).

Next, inspection of the TPR domain crystal structure shows the side chain of T897 making a H-bond with the carbonyl O of L893 within helix 4 (Fig. 3A). Replacing T897 with Ala would disrupt this H-bond (Fig. 3B) and create a void volume that could alter the conformation of loop 4–5, whereas mutation T897E would also cause the loss of the H-bond and a steric clash of the larger and negatively side chain of glutamate with the surrounding hydrophobic residues (Fig. 3C). Therefore, both mutations may cause a different rearrangement of the loop 4–5 that could propagate to nearby structural elements, including those across the dimerization interface. Similarly, modeling the modification P-T897 on the crystal structure of the WT protein shows the phosphate in an unfavorable hydrophobic environment (Fig. 3D), and thus, this posttranslational modification must involve local structural rearrangements that could explain the partial recovery of the T897E mutant. Noteworthy, the side chain of T897 is partially hidden from the solvent in the crystal structure of the dimer (Fig. 3A), and thus, presumably not accessible to kinases unless phosphorylation occurs co-translationally or at an early stage after protein synthesis before protein dimerization. Alternatively, loop 4–5 of the dimerized T897 protein could acquire a flexible conformation allowing the accessibility to kinases, such that the P-T897 and T897E fall in a similarly exposed conformation.

To validate the structural analysis, we used molecular dynamics (MD), and simulated a time frame of 500 ns for the TPR dimer (Fig. S4A) with the WT sequence, with T897 phosphorylation and with mutation T897E. MD simulations showed that both, the phosphorylation and the T897E mutation cause local changes in helix 4 and in the loop 4–5, that propagate to the adjacent helix 2 and to the helices 14–17 in the neighbor subunit (compare Fig. S4B–D). In addition, the negative charges introduced either by the phosphate or the glutamate favor the interactions with nearby positive residues that affect the conformation of the flexible loop 2–3 (Fig. S4C, D). However, the changes were stronger for phosphorylation than for the mutation to Glu, and on average, the P-T897 protein conformation was more flexible than WT and T897E (Fig. S4E–G). The phosphate is accommodated at the N-terminus of helix 5, capping the partial positive charge of the helix dipole. This causes the unfolding of helix 4, and a displacement of L893 and of the hydrophobic patch formed with L870 and A873 from helix 2 that propagates across the dimerization interface and favors the rearrangement of the C-terminal helices 14–17 in the neighbor subunit. Moreover, the side chain of residue R877 forms stable salt bridge with the phosphate, suggesting that the phosphorylation could condition the conformation adopted by the loop 2–3. In contrast, the side chain of the mutated glutamate does not interact with the N-terminus of helix 5 and only affects partially the folding of helix 4. Nonetheless, the presence of the negative side chain favors interactions with K874, R877 and R890, which determines the conformation adopted by the flexible loop 2–3, and to a lesser extent the position of the helices.

Taken together, the structural analysis and the regulatory translation activity indicated that replacing the conserved T897 residue with Ala, but not with Glu, accounts for decreased IRES-dependent translation. This effect, however, was unrelated to differences in
protein stability measured in growing cells expressing each of these proteins side by side with the WT G5_845-1508 protein (Fig. 4). Furthermore, the results obtained with the TPR-like mod-

ule also pertain to G5_845-1508 protein, where the dimer formed by two TPR units but not by the dimerization defective A951E mutant, leads to the assembly of the G5 decamer structure [20].

3.4. The protein interactome reveals a strong reduction in the translation regulation network

Previous data have shown that the C-terminal half part of Gemin5 determines the interaction with a large number of cellular factors [18]. Therefore, we sought to investigate whether mutations in T897 would also affect the Gemin5 interactome. To this end, we expressed and purified TAP-tagged versions of the WT, T897A, and T897E proteins to identify by LC-MS/MS changes in the interacting partners (Fig. S5 A–C). About 200 proteins were detected in the overlap of the replicas (Dataset 2), and the composition of recruited factors showed a higher similarity between T897A and T897E (Fig. 4).

![Fig. 4. Mutations T897A and T897E do not affect protein stability. HEK293 cells expressing the wild-type version of G5_845-1508, side by side with mutants T897A and T897E for 12 h were treated (+) or not (-) with cycloheximide (CHX) for additional 16 h. Samples were taken at 0, 12, and 16 h post–CHX treatment. The intensity of each protein at the indicated time was determined by WB. Bars represent the protein intensity (mean ± SEM) of three independent experiments relative to time 0 in each case. The differences between values were not significant in all cases.](image)

![Fig. 3. Structural modeling of T897 modifications. A-D) Cartoon representations of the TPR-like dimer (PDB ID 6RNQ) centered on the position of residue 897 with subunits colored in blue and orange. The thicker blue dashed lines represent a flexible loop between helices 2 and 3 that was not observed in the crystal structure. A) Structure of the WT protein with T897 (in yellow) and nearby residues shown in sticks. The side chain of T897 makes a H-bond (shown as a thin dashed line) with the carbonyl O of L893, being both residues in helix 4. B, C) Modeling of the mutations T897A (B) and T897E (C) on the WT crystal structure. Both mutations cause the loss of the H-bond, and the glutamate causes a steric clash of the negatively charged side chain with surrounding hydrophobic residues. In the rotamer drawn for the glutamate in panel C, the negatively charged side chain of T897E collides with the side chain of V887, and other possible rotamers would also clash against nearby elements. D) Modeling of the phosphorylation of T897 on the crystal structure of the WT protein. As for mutation T897E, the phosphate falls in a hydrophobic environment, and thus, this posttranslational modification is concomitant to local structural rearrangements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image)
T897E than to the WT (Fig. S5D, Fig. S6). Furthermore, a Venn diagram of the shared and exclusive factors associated with each of these proteins indicates that only 31% of the identified factors are shared by the three proteins (Fig. S5). Of note, the factors shared by the three proteins mostly included ribosomal proteins, SMN complex factors, and RNA-binding proteins. Instead, 26% are exclusive of the WT, 15% of T897E, and only 4% are exclusive of T897A (Fig. 5). Reinforcing these results, the connecting protein-protein networks according to STRING denote major differences in the interactome among these proteins, with T897A being the mutant that had the lowest number of exclusive factors. High confidence interactors validated using the WD-score [38] reinforced the loss of protein interactors for T897E, and particularly, for T897A (Fig. 5).

Next, Gene Ontology classification of the factors interacting with the WT, T897A, and T897E proteins indicated a barely similar composition in networks corresponding to the functional categories Translation elongation, Translation, Ribosome biogenesis, Spliceosomal snRNP assembly, and Regulation of RNA splicing (Fig. 6A). Additionally, while both mutants displayed a decrease in RNA splicing category, no prominent changes were observed in the proteome interactome related to SMN complex indicating that these mutations do not alter the interaction of Gemin5 with this complex.

Interestingly, major changes were observed in the networks Regulation of translation and RNA localization (Fig. 6A). Contrary to T897E, the T897A mutant loses the interaction with factors belonging to these Gene Ontology categories. Among the cellular factors differentially associated with T897A and T897E mutant proteins compared to the WT, we observed several RBPs known as translation regulators (Fig. 5, Fig. 6B). One of these factors is the translation initiation factor elf4E [45]. Gemin5-elf4E interaction was previously reported using GST-pull down and mass spectrometry [46]. Another study showed that Gemin5 is retained on 7-methylguanosine Sepharose resin, suggesting elf4E-binding capacity [47]. The elf4E binding site identified in Gemin5 ( YEAVELLK ) [46] resides on the TPR domain, within helix 11. Curiously, helix 11 and helix 5 from different subunits of the dimer are connected by the interposed loop 12–13, and thus, the phosphorylation site, the elf4E putative binding site, and the location of the pathogenic variants R1016C and D1019E [25] map within the same region of the dimerization domain.

The interaction between Gemin5 and the cap-binding protein elf4E [46] as well as the direct recognition of the cap structure of snRNAs by Gemin5 [15] prompted us to seek the involvement of Gemin5 in RNA cap-binding complexes. Recent studies identified Gemin5 as one of the m'G methylation-related genes, also including CYFIP1, EIF4E2, EIF4E1B, EIF4G3, NCBP2, NUDT10, NUDT4, WDR4, METTL1, and DCPs. These genes are biomarkers for predicting overall survival outcomes in hepatocellular carcinoma and gastric cancer [48,49]. Noteworthy, Gemin5 knockout has been shown to be detrimental in animal models, including mouse and drosophila [17,24]. Therefore, the potential cross-talk between Gemin5-elf4E and its translation regulatory activity awards further investigations of RNA-protein complexes impacting disease.

Multiple evidences suggest that RBPs, including Gemin5, play a key role as multifunctional gene expression regulators [50]. Additionally, cumulative data adds up in support of the role of Gemin5 outside of the SMN complex, contributing to RNA-dependent processes different than spliceosome assembly. For instance, Gemin5 has been identified in cytoplasmic aggregates under different physiological situations [51–53], opening the question of its contribution to stress granules (SGs) assembly. In agreement with this idea, the Gemin5 interactors identified in our study include many RBPs (Dataset 3, Fig. 6B), such as the markers of SGs Caprin1, G3BP2 [54], hnRNPA1, IGF2BP1, IGF2BP2, IGF2BP3, PABP, AGO2, and FXR1, typically found in RNA granules [55–57]. In comparison with the WT protein, some of these factors (IGF2BP2, IGF2BP3, AGO2, G3BP2 and FMR1) were lost in the mutant T897A more frequently than in the phosphomimetic T897E (Fig. 6B), suggesting

![Fig. 5](image_url). The protein interactome is differentially affected by T897A and T897E. Venn diagrams showing the overlap (%) of proteins identified for the WT (blue), T897A (pink), and T897E (violet). STRING protein-protein networks associated with GS5-1508 WT, T897A, and T897E depicting proteins identified in all the cases (grey border) or exclusive factors identified in GS5-1508 WT (blue border), T897A (pink border), and T897E (violet border). Interactors with WD-score > 1 are denoted by colored circles as above. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
the intervention of the phosphorylated form of Gemin5 in RNA granules formation.

3.5. Relationship between Gemin5 post-translational modification and disease

Recent studies described biallelic Gemin5 variants in patients with neurological diseases [22–25]. Thus, we wondered whether there was a relation between the residues affected in the protein variants resulting in neurological disorders with cerebellar atrophy and motor dysfunction (NEDCAM) with the Gemin5 phosphorylated residues (Fig. 7) deposited in Phosphosite [27]. The vast majority of the phosphopeptides deposited in Phosphosite derive from massive proteome studies carried out in different cell lines and physiological situations (as in response to different stresses, or in various types of tumor cells). In most of the P-sites referred to as in Gemin5 there is only one report for the observed P-site. Only 12 out of 43 phosphorylation sites (28 %) were recurrently found in more than five reports, indicating that not all the sites depicted in Fig. 7 are found simultaneously on the native protein.

It is worth noting that P-T897 and P-T1355 identified in our study were also observed in the full-length protein of human osteosarcoma (U2OS) cells [58], and human ovarian and breast cancer xenograft tissues [59], respectively, suggesting that this finding is not a particular case of G5 845-1508 region.

Comparative analysis of these data shows a non-overlapping location of naturally occurring variants in Gemin5 gene and phosphoresidues identified in the protein (Fig. 7). Phosphorylation sites are dispersed in the WD40 region of the protein. Instead, a cluster of phosphoresidues is placed in the linker region between the WD40 and the TPR-like domain, entering the N-terminal region of the TPR dimerization domain which includes the P-T897 identified in this study (Fig. 1). Three additional phosphoresidues are found at the C-terminal region of the TPR-like, flanking the naturally occurring variants described in patients (Fig. 7). Another cluster of phosphoresidues are found on the bipartite non-canonical RNA-binding site, RBS1-RBS2, including the residue T1355. Briefly, there are two main P-clusters: cluster I between residues 757–897 on the linker region between the WD40 and the TPR domains, and cluster II between residues 1355–1361 on the RBS1 domain. In support of the existence of the proposed clusters, P-T897 (RFHGLFPDRATLYR) was identified concomitant to nearby residues (S757, S778 and T807) [58], while P-T1355 (TEGRMLFtF-KELFsE) was identified in human Gemin5 together with S757, S807, S1354 and S1361 [59]. Our cluster proposal is also supported by Husedzinovic et al. [26] which identified six Gemin5 P-sites within T748-S847. Phosphorylated residues near T897 within cluster I are T859, S854, T853, T852, and S847, heavily conserved amino acids placed in the TPR-like module [18, 20]. On the other hand, phosphorylated residues near T1355 within cluster II are S1311, T1326, S1331, S1366, and S1391, the last two showing higher conservation than 1355 [20, 42]. Therefore, a compensatory function between nearby S/T residues may be plausible for T897, but it is unlikely for T1355 according to sequence conservation.

The differential location of phosphoresidues and the mutants naturally occurring in patients lead us to hypothesize that, glo-
ally, the phosphorylated residues undergo regulatory functions, while the residues mutated in biallelic Gemin5 variants mostly perturb protein functions by altering the structural conformation. The regulatory activity of proteins as a function of the phosphorylation state has been widely documented [60]. However, it remains unclear how and whether phosphorylation or another type of post-translational modification could be linked to disease. For SMN protein it has been shown that half of the potential phosphorylatable residues are phosphorylated sites, and only 10 % of these sites have been found mutated in SMA patients [61].

Previous studies identified Gemin5 within the spirophore regulated by RPS6KB1 [62], a serine-threonine kinase that responds to the mammalian target of rapamycin (mTOR) signaling and promotes protein synthesis, cell growth, and cell proliferation. The mTOR signaling pathway regulates the biogenesis of ribosomal subunits in response to phosphorylation of eIF4EBP1, −2, −3 and eIF4B. Hypo-phosphorylated eIF4EBP1 binds tightly to eIF4E and abrogates its interaction with eIF4G1, thus repressing cap-dependent translation [63,64]. On the contrary, phosphorylation of eIF4EBP1 inhibits the interaction with eIF4E, consequently stimulating the translation of mRNAs actively required for cell proliferation [65–67]. Interestingly, the Gemin5 phosphopeptide identified in mouse embryonic fibroblasts (DEPSRDPEPS) [62] is at the junction between the RB51 and RB52 domains, a region of the protein that accumulates a cluster of phosphoresidues ([Fig. 7]). Both, RB51 and RB52 have been related to translation regulation using different mRNA reporters [10,19]. A further link between Gemin5 and translation regulation is supported by recent data showing that silencing of Gemin5 in human cells results in a marked differential association of mRNAs to polysomes, demonstrating that altered levels of this protein regulate the expression of mRNAs encoding proteins with a pivotal role in cell growth, such as ribosomal proteins and histones [68].

Importantly, our data underscored that the regions of the protein harboring phosphoresidues are placed in well-defined regions, which constitute the linker between the WD40 domain and the TPR-like dimerization domain, as well as the RB51-RB52 domains, reinforcing the notion of the regulatory function for these regions. The kinase(s) regulating the phosphorylation of T897 in the TPR-like dimerization domain and T1355 in the RNA-binding site RBS1, as well as the phosphatases, remain to be elucidated. However, given the high number of phosphoresidues reported for Gemin5 under different conditions ([https://www.phosphosite.org]), we propose that beyond the mTOR signaling cascade, additional kinases are involved in Gemin5 post-translation modification regulating the activity of this multitasking protein. Whether the phosphorylation state determines the global conformation of the protein, the interaction with other cellular components (RNA or proteins), and therefore, its contribution to different cellular processes requires further studies. In additional support for the multifunctional regulatory role of this protein, Gemin5 has been recently identified in cellular complexes related to the proliferation of zebrafish hematopoietic stem progenitor cells [69], and as a differential marker in gastric cancer [70]. Dysregulation of RNA-binding proteins often results in widespread effects on the transcriptome, making it challenging to determine the underlying mechanisms that contribute to disease. Thus, the impact of post-translation modifications of Gemin5 in the activities of the protein ultimately leading to disease remains to be investigated in future works.

4. Accession codes
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD035227.

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Author contributions
EMS and RFV conceived and designed the study. RFV, AEB, SA and FdC performed the experiments and analyzed the data. SRM conducted the structural analysis and MD simulations. EMS wrote the manuscript with comments from all authors.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2022.11.018.

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