Functional and structural deficiencies of Gemin5 variants associated with neurological disease

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ABSTRACT

Dysfunction of RNA-binding proteins are often linked to a wide range of human disease in general, and particularly with neurological conditions. Gemin5 is a member of the survival of motor neuron (SMN) complex, a ribosome-binding protein and a translation reprogramming factor. Recently, pathogenic mutations in Gemin5 protein have been reported but the functional consequences of these variants remain elusive. Here we report functional and structural deficiencies associated with compound heterozygosity variants within the *Gemin5* gene found in patients with neurodevelopmental disorders. These clinical variants are located in key domains of Gemin5, the tetratricopeptide repeat (TPR)-like dimerization module and the non-canonical RNA-binding site 1 (RBS1). We show that the TPR-like variants disrupt protein dimerization while the RBS1 variant confers protein instability. All mutants are defective in the interaction with protein networks involved in translation and RNA-driven pathways. Importantly, the TPR-like variants fail to associate with native ribosomes, abolishing the cap-dependent and selective translation control of Gemin5, and establishing a functional difference with the wild type protein. Our study provides a molecular basis of disease associated with malfunction of Gemin5 protein.

INTRODUCTION

RNA-binding proteins (RBPs) perform critical roles in RNA metabolism, regulating all steps of gene expression (Gehring et al., 2017; Diaz-Muñoz and Osma-Garcia, 2021). The modular organization of RBPs, generally consisting of RNA-binding domains (RBD) and protein-protein interaction modules (Lunde et al., 2007), provides multiple activities to these factors. Indeed, malfunction of distinct RBPs has been related to human diseases (Smith and Costa, 2021; Gebauer et al., 2021). On the other hand, dysregulation of the binding activity of a given protein can produce widespread effects on multiple RNA-dependent processes and, as a result, challenge the identification of the molecular mechanism contributing to disease.

Gemin5 is a predominantly cytoplasmic protein first described as a component of the survival of motor neurons (SMN) complex (Matera et al., 2019). In humans, the SMN complex comprises 9 members (SMN, Gemins2–8, and unr-interacting protein (Unrip)) (Otter et al., 2007). The SMN complex plays a critical role in the biogenesis of small nuclear ribonucleoproteins (snRNPs) (Lau et al., 2009; Pellizzoni et al., 2002), the components of the splicing machinery (Kastner et al., 2019). Altered levels of SMN protein causing defects in SMN complex assembly lead to spinal muscular atrophy (SMA), a severe to mild form of disease depending upon the SMN protein levels (Burghes et al., 2009). Additionally, the SMN protein affects other processes such as translation regulation (Sanchez et al., 2013; Lauria et al., 2020), muscle architecture (Rajendra et al., 2007), or transport and assembly of microRNPs or telomerase RNPs (Mourelatos et al., 2002).

Gemin5 was initially identified as the protein responsible for the recognition and delivery of small nuclear RNAs (snRNAs) to snRNPs (Battle et al., 2006; Yong et al., 2010). Nonetheless, a large fraction of Gemin5 protein is found in the cytoplasm outside of the SMN complex (Battle et al., 2007), strongly suggesting that the protein may contribute to additional cellular processes. Accordingly, Gemin5 acts as a hub for several networks performing diverse key cellular functions. This multifunctional protein has been shown to act as a regulator of translation (Pacheco et al., 2009; Workman et al., 2015; Francisco-Velilla et al., 2018), as a ribosome-interacting protein (Francisco-Velilla 2016; Simsek et al., 2017), as a reprogramming factor in zebrafish lateral line hair cells (Pei et al., 2020), as a signal recognition particle (SRP)-interacting protein (Piazzon et al., 2013), and as a trans-splicing factor (Philippe et al., 2017). In addition, Gemin5 has been identified as a member of RNP networks associated to distinct cytoplasmic aggregates (Wollen et al., 2021; Vu et al., 2021; Jiang et al. 2021). More importantly, *Gemin5* variants were recently linked with human neurodevelopmental disorders, perturbing distinct pathways as compared to defects in the SMN protein (Kour et al., 2021; Saida et al., 2021; Rajan et al., 2022). However, the molecular basis of Gemin5 dysfunction remains elusive.

The human Gemin5 protein is organized in functional domains with a distinctive structural organization (Figure 1A). The N-terminal part contains two juxtaposed seven-bladed WD40 domains (Jin et al., 2016) that recognize the Sm-site of snRNAs and the cap via base-specific interactions (Xu et al., 2016; Tang et al., 2016). The crystal structure of the central region revealed a tetratricopeptide repeat (TPR)-like domain with 17 α -helices that oligomerizes as a canoe-shaped homodimer (Moreno-Morcillo et al., 2020). Insertion of a single point substitution (A951E) at the closest position between the two subunits disrupts the assembly of the dimer. Additionally, mutation A951E confers a strong decrease in the Gemin5 interactome, and results in the loss of factors connected to RNA processing, translation regulation and spliceosome assembly, among others. Taken together, these results strongly suggest that dimerization (and perhaps, multimerization) is an evolutionary preserved trait of Gemin5.

The most C-terminal part of Gemin5 harbors a non-canonical RNA-binding site (RBS) comprising two domains, designated as RBS1 and RBS2 (Fernandez-Chamorro et al., 2014). The RBS1 moiety recognizes an internal region of *Gemin5* mRNA, stimulating its own translation and counteracting the negative effect of Gemin5 in global protein synthesis (Francisco-Velilla et al., 2018). Structural analysis of the RBS1 polypeptide shows the presence of an unfolded region (Embarc-Buh et al., 2021), a feature frequently found in intrinsically disordered regions (IDR) of proteins characterized by having multiple interactors, either RNA or proteins (Jarvelin et al., 2016).

Here we sought to establish the impact of *Gemin5* missense variants found in compound heterozygosity in patients developing neurodevelopmental disorders. Importantly, these variants are located in two separate domains of the protein, the TPR-like dimerization module and the non-canonical RBS1 domain, which are colinear on the G5₈₄₅₋₁₅₀₈ region of the protein (Figure 1A). Considering the implications of these functional domains for the multiple activities of Gemin5 (Francisco-Velilla et al., 2019) we took advantage of the known properties of the G5₈₄₅₋₁₅₀₈

fragment to analyze the impact of these clinical variants in Gemin5 protein dimerization, ribosome association, protein-protein interaction, and translation control.

We found that individual substitutions within the dimerization domain and the noncanonical RNA-binding site domain of Gemin5 lead to protein malfunction. Two nearly placed variants within the TPR-like domain impair the dimerization ability of Gemin5 protein in living cells, while the variant on the RBS1 domain decreases Gemin5 protein stability. Mass spectrometry analysis revealed that these variants abrogate the association of cellular proteins involved in RNA splicing and translation, among other cellular processes. Consistent with the reduced capacity to interact with factors involved in translation, the proteins harboring substitutions on the TPR-like domain elicit a reduced capacity to sediment with native ribosomes. Furthermore, in contrast to the enhancing activity of the wild-type (WT) G5⁸⁴⁵⁻¹⁵⁰⁸ protein on cap-dependent and selective translation, the mutants analyzed in this study failed to do so in human cells. Together, our results provide a molecular basis to understand the implication of Gemin5 variants in disease.

RESULTS

Gemin5 variants on conserved residues of the TPR-like moiety and the RBS1 domain led to disease

The exome sequencing of three patients with neurological disorders revealed the presence of biallelic variants within *Gemin5*, absent in the normal population (GnomAD database). Most interesting, the substitutions found in these patients are present in compound heterozygosity (Figure 1B). Index case 1 is one individual with a splicing variant on intron 4 in one allele (frequency 60%) that is predicted to yield a truncated protein and a missense variant in exon 22 in the other allele (frequency 50%) producing the R1016C substitution within the TPR-like domain (Table 1) (Figure 1A). Both parents and a sister with monoallelic *Gemin5* variants are healthy, reinforcing the crucial impact of compound heterozygosity in disease. A second case corresponds to a family where two kids with neurological disorders carry a D1019E missense substitution within the TPR-like moiety in one allele and a L1367P substitution within the RBS1 region in the other allele (Figure 1B) (Kour et al., 2021).

The alignment of *Gemin5* sequences obtained from vertebrate species, including mammals, birds, amphibians, reptiles and fishes (Supplementary Figure S1) shows that D1019 is fully conserved, while R1016 shows substitution to His, Leu and Glu, but never to Cys. L1367 locates within a predicted helix of RBS1, with substitutions to Val, Phe, and Cys, but not to Pro that is a helix-breaker amino acid. Therefore, each of these clinical variants affect conserved residues of the protein which is likely to affect the physiological functions.

Effect of the R1016C and D1019E mutations on the structural conformation of the TPR-like module

Understanding the involvement of the Gemin5 domains in the different activities of this protein is key to interpret malfunction of the clinical variants. Disruption of the dimerization ability interferes with critical functions of this protein (Moreno-Morcillo et al., 2020). Therefore, we sought to analyze the impact of the pathogenic variants R1016C and D1019E on the Gemin5 TPR-like dimerization module. This region forms a canoe-shaped homodimer with each subunit folding in an extended palisade of α -helices. Residues R1016 and D1019 located in a 6 aa loop between helices 12 and 13 (loop α 12-13) as well as their side chains are involved in a network of electrostatic interactions (Figure 1C). R1016 makes a salt bridge with E1018, a H-bond through the carbonyl oxygen with the amide nitrogen of D1019, and a water-mediated H-bond between the amide N and the side chain of D1019. Thus, replacing R1016 by a Cys could cause the loss of the salt bridge and a distortion of the solvent-exposed loop due to the increased hydrophobicity of this residue (Figure 1C). On the other hand, the side chain of D1019 makes a H-bond with the amide group of V1021, stabilizing the N-terminal dipole of helix 13, and a H-bond with the phenolic oxygen of Y992 in the helix 11. A glutamate replacing D1019 would clash with neighboring side chains, and only one permissive position would maintain the interactions with Y992 and with the water molecule but would not be adequate for the capping of helix 13 (Figure 1C).

To interrogate the impact of the pathogenic variants on the structure of the protein we produced the TPR-like dimerization domain bearing R1016C or D1019E substitution following the same expression and purification protocols as for the WT protein. The mutants were purified at a lower yield than the WT protein and required higher salt concentration (from 50 to 200 mM) for their solubility. Limited proteolysis analysis showed that D1019E was more susceptible to trypsin digestion than the R1016C and the WT protein (Figure 1D). However, both mutants formed stable homodimers at 200 mM salt concentration, as observed by SEC-MALS, although D1019E showed signs of instability (Figure 1E). In fact, we measured by differential scanning fluorimetry (ThermoFluor) that the melting temperature (T_m ; midpoint of the unfolding transition) of D1019E was 4°C lower than that of R1016C or WT protein (Figure 1F). These results suggest a greater flexibility of the TPR moiety carrying the D1019E substitution, while the effect of the R1016C variant was not apparent under these conditions.

Taken together, the structural changes induced by mutations on the TPR-like domain led us to investigate their potential effect on Gemin5 functions.

Gemin5 variants in the TPR-like domain decrease protein dimerization

Conserved residues in the TPR-like region are involved in interactions within the subunit and across the dimer interface (Moreno-Morcillo et al., 2020). This moiety (residues 845-1097) drives dimerization of the purified polypeptide *in vitro* as well as in human cells when is present within the G5₈₄₅₋₁₅₀₈ fragment, which encompasses both the TPR-like and the RBS1 domains (Figure 2A). Hence, we asked whether the clinical variants R1016C and D1019E in the TPR-like module would interfere with the dimerization capacity of the protein in the cell, which could ultimately impact on the functions of this multitasking protein.

To determine the recruitment of full-length Gemin5 we expressed the variants in the G5₈₄₅₋₁₅₀₈-TAP context (Figure 2A). The dimerization defective mutant A951E was used as a control (Moreno-Morcillo et al., 2020). We also generated the L1367P construct for completeness, since this mutation is present in a different domain of the protein. Variants R1016C and D1019E were expressed in HEK293 cells to a more similar extent than the WT protein and the dimerization mutant A951E, while the mutant L1367P rendered lower levels of protein (Figure 2A). Then, the intensity of co-immunoprecipitation (co-IP) of full-length Gemin5 protein was measured in protein complexes purified by TAP (Supplementary Figure S2A) using G5₈₄₅₋₁₅₀₈-WT and the mutants A951E, R1016C, D1019E, or L1367P as baits. Gemin5 and G5₈₄₅₋₁₅₀₈ were immunodetected using anti-Gemin5 (Figure 2B, top panel). In each case, the intensity of recruited Gemin5 was normalized to the intensity of purified G5₈₄₅₋₁₅₀₈. The recruitment of Gemin5 by G5₈₄₅₋₁₅₀₈-WT was 10-fold higher than that observed by the dimerization mutant A951E. Interestingly, the substitutions R1016C and D1019E impaired co-IP of the endogenous Gemin5 protein, reducing the intensity to 58 and 62% relative to the WT protein, respectively. In comparison, the construct L1367P did not have a significant effect on Gemin5 co-IP (Figure 2B, bottom panel).

Next, we identified by mass spectrometry the Gemin5 protein copurifying with G5₈₄₅₋₁₅₀₈ variants expressed in HEK293 cells (Figure 2C). After TAP purification and LC-MS/MS, PEAKS analysis of Gemin5 amino acid sequences was used to identify the presence of trypsin fragments corresponding to unique peptides of the N-terminal (1-844), and the C-terminal parts (845-1508) of Gemin5 (Supplementary Figure S2B). Then, total reads of the unique peptides within positions 1-844 and 845-1508 of Gemin5 were accounted. Relative to G5₈₄₅₋₁₅₀₈-WT, the ratio of peptide reads belonging to the N-terminal relative to the C-terminal region was diminished in R1016C and D1019E (0.77 and 0.56, respectively), revealing a reduced capacity to recruit Gemin5 (Figure 2C). In agreement with the co-IP data (Figure 2B), the dimerization mutant A951E was severely defective in Gemin5 recruitment, whereas negligible decrease in Gemin5 recruitment was observed for the L1367P construct (0.90) (Figure 2C). The mutations R1016C and D1019E within the TPR-like domain decrease the G5₈₄₅₋₁₅₀₈ recruitment ability of the Gemin5 protein, reflecting that individually these variants impair the formation of the dimer complex in a competitive cellular environment.

The Gemin5 mutants display a reduced capacity to associate factors involved in translation control and RNA metabolism

The dimerization defects associated with the substitutions present in the TPR-like module of Gemin5 prompted us to study global effects on protein-protein interactions. To this end, we identified the cellular factors associated to each TAP-tagged G5₈₄₅₋₁₅₀₈ variant (Supplementary Figure S3A,B) (Dataset 1). Of note, the identified factors revealed a closer similarity among the three clinical mutations (37%) than with the WT protein (17%) providing evidence for common features among the mutants (Supplementary Figure S3C).

Next, we sought to investigate the functional groups overrepresented within the factors copurifying with the proteins R1016C, D1019E, or L1367P relative to G5₈₄₅₋₁₅₀₈-WT by Gene Ontology (GO) annotation of the biological processes. GO classification in functional categories using BiNGO (Cytoscape platform) showed a distribution in statistically significant overrepresented nodes (Maere et al., 2005). The top GO functional networks copurifying with G5₈₄₅₋₁₅₀₈-WT are RNA-dependent processes, such as translation (2×10^{-50}), RNA metabolism (8×10^{-25}), mRNA regulation (2×10^{-5}), RNA localization (2×10^{-4}), and SMN complex (4×10^{-4}) (Figure 3A,B). In marked difference with the WT protein, the interaction networks related to translation and RNA metabolism were strongly reduced in the mutants R1016C and D1019E (Figure 3A, 3C,D). We also noticed an important decrease in the number of factors associated with the RBS1 variant L1367P (Figure 3A, 3E). The mRNA regulation network only remained in D1019E, while RNA localization was below the threshold (5×10^{-3}) in all the mutants. Loss of the SMN complex network was a marked feature of R1016C and D1019E mutants. In contrast, the L1367P mutant retained this network, although with slightly lower *p* value (3×10^{-3}) than the WT protein (4×10^{-4}) and showed a smaller number of nodes (Figure 3A,E). We also noticed that the G5₈₄₅₋₁₅₀₈-mutants copurified with members of the stress response (*p* values ranging from 2×10^{-3} - 9×10^{-4}).

The specific factors interacting with the WT and mutated variants are highlighted in Figure 3F. Most notably, the G5₈₄₅₋₁₅₀₈-WT protein specifically associated a large number of ribosomal proteins, as well as numerous RBPs and members of the SMN complex. Most of these factors remained undetected in the variants analyzed in this study. We conclude that there is a crosstalk between the ability of G5₈₄₅₋₁₅₀₈ to recruit the endogenous Gemin5 driven by the dimerization module and its capacity to associate partners of the SMN complex, as well as factors involved in translation and RNA metabolism.

The Gemin5 RBS1 variant confers protein instability

The particular combination of recessive biallelic variants found in patients -in one case, a splice variant in intron 4 with a dimerization mutant protein (R1016C), and in the other case, a dimerization mutant protein (D1019E) with a point substitution in the RBS1 domain (L1367P) (Figure 1B)- could be compatible with altered levels of Gemin5 protein. Therefore, we sought to investigate the impact of the pathogenic variants on protein stability.

For this, each variant was inserted in the Xpress-G5₈₄₅₋₁₅₀₈ construct and expressed in HEK293 cells. Twenty-four hours post-transfection, cells were treated with cycloheximide (CHX) for 16 hours, and the level of G5₈₄₅₋₁₅₀₈ protein accumulated in the presence or absence of CHX treatment was followed by immunoblotting (Figure 4A). No significant differences in the steady-state RNA levels of the WT and the mutants were observed by RTqPCR at the time of cell harvesting (Figure 4B). Quantification of the protein levels 12 and 16 h after CHX treatment relative to time 0 indicated that the relative intensity of the L1367P variant showed a strong decrease over time for G5₈₄₅₋₁₅₀₈ protein (Figure 4C).

These results prompted us to investigate whether these differences could be also observed in the stability of the Gemin5 full-length protein (Supplementary Figure S4A). No significant differences in the steady-state RNA levels were observed by RTqPCR (Supplementary Figure S4B). Cells treated with CHX for 16 h showed a moderate protein decay for Gemin5 WT, R1016C, and D1019E (average protein intensity 0.47, 0.50, and 0.38, taking 1 as the value observed at time 0). In contrast, L1367P displayed a stronger decrease of Gemin5 protein levels 12 h post-treatment (0.05), and 16 h (0.02) (Supplementary Figure S4C). Therefore, the defect in protein stability conferred by the L1367P variant is observed in both cases, presumably related to the reduced protein interactome.

Mutations in the dimerization domain negatively affect ribosome association

We have reported earlier that Gemin5 behaves as a ribosome binding protein (Francisco-Velilla et al., 2016) by using a G5₁₋₁₂₈₇ construct that includes the WD40 repeats domain and the TPR-like dimerization domain (Figure 1A). Given that G5₈₄₅₋₁₅₀₈-WT co-purified with a large number of the ribosomal proteins (Figure 3F), we asked whether this fragment interacts with the ribosome. Thus, we monitored the ribosome binding capacity using purified components, 80S ribosomal particles and His₆-tagged G5₈₄₅₋₁₅₀₈ protein (Figure 5A, left panel). The results of the binding reaction were followed by immunoblotting using anti-His for G5₈₄₅₋₁₅₀₈, and anti-P0 for the ribosome (Figure 5A, right panel). As a control, a binding reaction conducted with beads and the ribosomes yielded a rather weak signal with anti-P0, and no signal with anti-His (Figure 5A, lane 1), indicating that beads alone barely bind ribosomes. In contrast, the reaction conducted with His-G5₈₄₅₋₁₅₀₈ and 80S ribosomes was positive for both components (Figure 5A, lane 2). The purified ribosomes alone (Figure 5A, lane 3) were negative with the anti-His antibody, and positive for anti-P0. We conclude that G5₈₄₅₋₁₅₀₈ can interact directly with the ribosome in the absence of other cellular components.

We focused our attention on ribosomal proteins belonging to the 60S and 40S subunits identified by mass spectrometry (Figure 3F). GST-fusions of several ribosomal proteins were purified from bacteria and used in a pull-down assay with purified His-G5₈₄₅₋₁₅₀₈. GST alone, used as negative control of the pull-down, did not interact with His-G5₈₄₅₋₁₅₀₈ (Figure 5B). Similar results were observed with L5. In contrast, GST fusions of the ribosomal proteins L3, L4, P0, S3A, S9, and S26 showed a direct interaction with His-G5₈₄₅₋₁₅₀₈ *in vitro*. These results show that the G5₈₄₅₋₁₅₀₈ fragment can interact with various ribosomal proteins belonging to the large and small ribosomal subunits, reinforcing the conclusion that there is a direct link between Gemin5 and the ribosome.

The severe decrease noticed in the translation network of dimerization mutants (Figure 3F) prompted us to study whether these variants would impact ribosome binding. To this end, we analyzed the presence of G5₈₄₅₋₁₅₀₈ in subcellular fractions enriched in native ribosomes. HEK293 cells expressing Xpress-tagged G5₈₄₅₋₁₅₀₈-WT, -R1016C, -D1019E, and -A951E were used to prepare cytoplasmic lysates (S30) and native ribosomes (80S) (Figure 5C). The dimerization

defective mutant G5₈₄₅₋₁₅₀₈-A951E was used as a control. The Xpress-tagged G5₈₄₅₋₁₅₀₈ proteins were immunodetected in the S30 fraction (Figure 5D, upper panel). Confirming the composition of the subcellular fractions, the ribosomal protein RACK1 was detected in the S30 and 80S fractions (Rabl et al., 2011) (Figure 5D). The intensity of RACK1 indicated similar amounts of ribosome loading in all cases. However, we noticed a significant decrease in the intensity of all the mutant proteins in native ribosomes compared to the WT protein (Figure 5E), in agreement with the reduction of ribosomal proteins detected by mass spectrometry (Figure 3F). Representation of the 80S/S30 ratio readily indicated a reduced ribosome sedimentation of the TPR-like mutants (Figure 5E). Together, these results demonstrate that G5₈₄₅₋₁₅₀₈ sediments with native ribosomes, and substitutions within the TPR-like domain impair this association.

Gemin5 clinical mutants impaired in dimerization impinge on translation

The functional networks most significantly altered in the mutants analyzed in this study were RNA metabolism, mRNA regulation and RNA localization (Figure 3A). These groups of proteins are engaged in regulation of mRNA stability, splicing, translation and localization, among other processes. Beyond ribosomal proteins and eIFs, the mutants analyzed in this study were impaired in the association with several RBPs involved in translation regulation (Supplementary figure 5A). Thus, given that the R1016C and D1019E mutants showed defects in ribosome binding and in the ability to interact with factors related to translation regulation, we set up a functional assay in HEK293 cells co-expressing Xpress-tagged G5₈₄₅₋₁₅₀₈ proteins and a luciferase reporter (Figure 6A). The results revealed that expression of Xpress-G5₈₄₅₋₁₅₀₈-WT enhanced cap-dependent translation of luciferase relative to control cells, as expected (Moreno-Morcillo et al., 2020). In contrast, similar levels of expression of the proteins R1016C and D1019E failed to do so. In all cases, no significant differences in the steady-state levels of luciferase RNA were observed by RTqPCR at the time of cell harvesting (Figure 6B). Thus, we conclude that the variants found in patients, which reduce the ribosome association of Gemin5, abrogate translation stimulation driven by G5₈₄₅₋₁₅₀₈.

We then asked whether these variants could affect the translation efficiency of selective mRNAs. For this, we measured the levels of selected mRNAs previously shown associated to Gemin5 (Francisco-Velilla et al., 2018) in translationally active polysomal fractions. The presence of RACK1 in the fractions of the gradient confirmed a similar loading of ribosomes in the samples prepared from cells expressing the WT and the protein variants R1016C and D1019E (Figure 6C). The lower density fractions of the gradient show a similar amount of G5₈₄₅₋₁₅₀₈ proteins. However, a moderate reduction of R1016C and D1019E in 80S and polysome fractions was observed relative to the WT protein, reinforcing the idea of a lower capacity to associate with ribosomes (Figure 6C). Then, measurement of association to polysomes of selected mRNAs - the H12 region of Gemin5, the AGO2, and PCBP1 mRNAs - indicated that Gemin5 mRNA (H12) was significantly enriched in the polysomal fractions with G5₈₄₅₋₁₅₀₈-WT sample relative to the Input, while this enrichment was not observed with the R1016C and D1019E variants. In contrast,

the AGO2 mRNA was enriched in polysomal fractions both in the WT and mutant samples relative to the control. However, no significant differences were noticed in the association to polysomes of the mRNA encoding PCBP1, as well as in the mRNAs encoding the splicing factor SF3B3 and the RNA helicase DHX15 (Supplementary Figure S5B).

We conclude that the defective mutants on the dimerization domain of Gemin5 impair the binding to polysomes of a specific mRNA, further reinforcing the involvement of this multitasking protein in selective translation.

DISCUSSION

We show in this study that *Gemin5* pathogenic variants mapping in the protein dimerization module (TPR-like) and the non-canonical RNA-binding domain (RBS1), impair the function of this multitasking protein in central cellular processes, including ribosome binding, translation regulation, and protein-protein association. Of note, the clinical disorders associated with these *Gemin5* variants differ from those linked to SMN protein dysfunction (Kour et al., 2021; Saida et al., 2021). Consequently, these results led us to propose that variants causing *Gemin5* failure result in protein malfunction, and, hence, are at the basis of the disease.

The protein Gemin5, which is expressed in all human tissues (Kim et al., 2014; Uhlen et al., 2015), is emerging as a multifunctional factor linked to human disease. A recent study reported *Gemin5* biallelic variants among patients presenting neuro-developmental disorders (Kour et al., 2021). The presence of *Gemin5* variants clustered in conserved residues of the dimerization domain, which provides a platform for protein–protein/RNA interactions (Moreno-Morcillo et al., 2020), reinforces the biological relevance of the TPR-like module for Gemin5 function. Fully consistent with this hypothesis, *Gemin5* biallelic variants have been recently associated with cerebellar atrophy and spastic ataxia in human patients (Rajan et al., in press). These data sum up the observation that a null KO mouse is embryonic lethal, as it also happens in flies (Gates et al., 2004; Borg et al., 2015).

The properties unveiled in our study of Gemin5 shed new light on this still poorly characterized protein. Combined, the TPR-like module and the RBS1 domain integrate key roles of the G5₈₄₅₋₁₅₀₈ fragment: Gemin5 dimerization and selective RNA-binding. In turn, this supports the notion that the C-terminal region of the protein has different functions to the N-terminus. We show here that point mutations found in each of these motifs are detrimental for Gemin5 function. First, mutations R1016C and D1019E within the TPR-like domain impair endogenous Gemin5 recruitment in living cells. Second, mutation L1367P, a helix breaker residue, in a predicted helix of RBS1 induces protein instability, reducing its ability to interact with cellular RBPs but partially retaining the interaction with the SMN complex (Figure 7).

The high sequence conservation of the TPR-like sequence strongly suggests that the dimerization module plays a fundamental role for the architecture and activity of Gemin5. R1016 and D1019 form part of the loop α 12-13 that forms a protrusion at the surface of the canoe-shaped

dimer (Figure 1C). The presence of two highly conserved Pro residues (P1017 and P1020) in this loop and the network of electrostatic interactions involving residues R1016 and D1019 suggest that the conformation of this region is important for the stabilization of the dimer. We show that in the competitive environment of living cells, the full-length Gemin5 is recruited by the G5₈₄₅₋₁₅₀₈ protein, while mutations R1016C and D1019E hamper this interaction (Figure 2B,C). Furthermore, the TPR-like module bearing mutation D1019E shows increased flexibility and lower stability (Figure 1E,F), although we did not detect a similar effect for mutation R1016C under the *in vitro* conditions tested. These results could be explained by the difference in the position of these residues within the structure: while R1016 protrudes to the surface, D1019 forms part of the internal part of this loop presumably contributing to a higher extent to a rigid conformation than R1016 (Figure 1C). However, several evidences support the relevance of R1016 in Gemin5 function. The mutant R1016C in the individual of case 1 is found in compound heterozygosity with a 5' splice variant in intron 4 that is predicted to produce a truncated protein, suggesting that the R1016C mutation individually is crucial for the activity of Gemin5 in the cellular environment. In addition, the R1016C mutation has been detected in unrelated patients (Rajan et al., in press), supporting an overall negative effect in protein function. Thus, the observed destabilizing effect of mutant D1019E *in vitro*, together with the negative effects of R1016C in living cells, strongly suggest that substitutions in the loop α 12-13 of the TPR-like moiety have a negative impact on protein dimerization. Hence, it is plausible that additional variants found in patients with similar disorders would also affect the Gemin5 dimerization properties.

Ribosome binding was initially reported for the extended N-terminal fragment (G5₁₋₁₂₈₇) protein. This protein shares with G5₈₄₅₋₁₅₀₈ the residues 845-1287, including the entire dimerization domain. Considering that G5₈₄₅₋₁₅₀₈ binds the ribosome using purified components (Figure 5A) and that this version of the protein sediments with native ribosomes (Figure 5C), we conclude that one of the key activities of Gemin5 depends on its interaction with the ribosome. Defects in the dimerization module impair the capacity to sediment with native ribosomes, and also the association of G5₈₄₅₋₁₅₀₈ with ribosomal proteins. In turn, these defects impinge on translation. We and others have shown that Gemin5 is involved in translation control (Pacheco et al., 2009; Workman et al., 2015; Francisco-Velilla et al., 2018; Garcia-Moreno et al., 2019). However, while G5₈₄₅₋₁₅₀₈-WT enhances translation (Figure 6A), the mutants R1016C and D1019E fail to do so, in full agreement with the defective dimerization mutant A951E (Moreno-Morcillo et al., 2020).

Beyond protein stability and dimerization, the cellular processes negatively affected by the substitutions on the TPR-like and the RBS1 domains include protein-protein association (Figure 7). These processes are linked to RNA-driven pathways, involving the contribution of more than one functional domain of the Gemin5 protein. Further insight into the relative orientation and interconnection of the distinct functional domains awaits the structural resolution of the entire Gemin5 protein, which should also aid in predicting the damaging defect of new clinical variants.

In summary, our study shows that biallelic *Gemin5* variants found in patients within the self-dimerization module and the non-canonical RNA-binding domain are linked to protein malfunction, reinforcing the critical role of Gemin5 in RNA-related pathways. The identification of molecular features associated with compound heterozygosity variants in *Gemin5* opens new avenues for rational design of therapeutic strategies. Additionally, these results provide evidence for the relevance of preserving balanced levels of Gemin5 protein to sustain gene expression regulation.

MATERIALS AND METHODS

Constructs

The construct expressing G5₈₄₅₋₁₅₀₈-WT (pcDNA3-Xpress-G5₈₄₅₋₁₅₀₈) and Xpress-Gemin5-WT (pcDNA3-Xpress-G5) polypeptides were described (Fernandez-Chamorro et al., 2014, Francisco-Velilla et al., 2016). Likewise, the constructs pcDNA3-CTAP-G5₈₄₅₋₁₅₀₈, pcDNA3-CTAP-G5₈₄₅₋₁₅₀₈-A951E, pcDNA3-Xpress-G5₈₄₅₋₁₅₀₈-A951E, and pOPINM_G5_TPR were reported (Moreno-Morcillo et al., 2020). Constructs pcDNA3-Xpress-G5-R1016C, pcDNA3-Xpress-G5-D1019E, pcDNA3-Xpress-G5-L1367P, pcDNA3-Xpress-G5₈₄₅₋₁₅₀₈-R1016C, pcDNA3-Xpress-G5₈₄₅₋₁₅₀₈-D1019E, pcDNA3-Xpress-G5₈₄₅₋₁₅₀₈-L1367P, pcDNA3-CTAP-G5₈₄₅₋₁₅₀₈-R1016C, pcDNA3-CTAP-G5₈₄₅₋₁₅₀₈-D1019E, pcDNA3-CTAP-G5₈₄₅₋₁₅₀₈-L1367P, pOPINM_G5_TPR-R1016C, and pOPINM_G5_TPR-D1019E were generated by QuickChange mutagenesis (Agilent Technologies) using specific primers (Supplementary Table S1). pGEXKG plasmids expressing GST fusions of S3A, RACK1, L3, L4, L5, and P0 were previously described (NAR 2016). Likewise, the constructs pGEXKG-S9 and pGEXKG-S26 were generated using standard procedures. See Supplementary Table S1 for oligonucleotides sequences used for PCR. All plasmids were confirmed by DNA sequencing (Macrogen).

Protein expression

The Gemin5 TPR-like domain was expressed and purified as previously reported (18). Briefly, *E. coli* BL21-Rosetta (DE3) pLysS cultures transformed with the plasmid pOPINM_G5_TPR (encoding the TPR-like domain 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, washed extensively with buffer A supplemented with 35 mM imidazole, and eluted by 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 ultracentrifugation using an Amicon device with 10 kDa cutoff and loaded onto a size exclusion chromatography (SEC) HiLoad Superdex 200 16/60 column (Cytiva) equilibrated in buffer B. The sample eluted in a single peak and was concentrated as before. The sample was supplemented with 20% glycerol and flash-frozen in liquid nitrogen and stored at -80°C until use. Sample purity was evaluated by SDS-PAGE and Coomassie staining. Final protein purification yield was ~12 mg/L of cells. The R1016C and D1019E mutants were expressed and purified as the WT, except for increasing the NaCl concentration in the SEC buffer to 0.2 M to favor protein solubility.

His-G5845-1508 purified protein from *S. cerevisiae* was obtained as described (Fernandez-Chamorro et al., 2014).

SEC coupled to multi-angle light scattering (SEC-MALS)

250 µl of purified TPR-like domain at 1–3 mg/ml were fractionated by SEC using a Superdex 200 10/300 column (Cytiva) equilibrated in 20 mM Tris pH 6.8, 0.2 M NaCl, 5% glycerol and 1 mM DTT and an ÄKTA purifier (Cytiva) at a flow rate of 0.5 ml/min. The eluted samples were characterized by measuring the refractive index and multi-angle light scattering (MALS), using Optilab T-rEX and DAWN 8⁺ (Wyatt). Data were analyzed using the Astra6 software (Wyatt) to obtain the molar mass of each protein and represented using GraphPad Prism 9.

Trypsin limited proteolysis

4 µg of total protein in 20 µl of buffer B were mixed with 5 µl of trypsin at 0.02 to 0.32 µg/mL and incubated at 22°C for 30 min. The reaction was stopped by adding SDS-PAGE sample loading buffer and the samples were analyzed on 15% SDS-PAGE. The 30 kDa band corresponding to the non-cleaved protein band was quantified with program ImageJ.

Thermal stability assays (Thermofluor)

Protein stability was measured by differential scanning fluorimetry (Niesen et al., 2007) using a 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) and a MicroAmp optical 96-well reaction plate sealed with film. The samples, prepared in a final volume of 20 µl, contained 2.5 µM TPR-like protein in buffer 20 mM Tris-HCl pH 6.8, 0.2 M NaCl, 5% glycerol and 1 mM DTT, and 5x SYPRO orange (Thermo Fisher Scientific). Fluorescence changes were monitored from 20°C to 85°C with increments of 1°C/min. Data were processed and analyzed with program GraphPad Prism 9.

Protein complexes isolation by Tandem Affinity Purification (TAP)

HEK293 human cells were cultured in Falcon® 6-well plates with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% (v/v) Fetal Bovine Serum (FBS). Cells at 80% of confluency were transfected using Lipofectamine LTX (Thermo Fisher Scientific), and were harvested 24 hpt. The complexes associated to the TAP-tagged proteins were purified as described (Francisco-Velilla et al., 2015). 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 at 4°C overnight, pelleted at 14000 g for 15 min at 4°C, washed three times with 1 ml of acetone and dissolved in SDS-loading buffer. An aliquot (20 %) was analyzed on silver stained SDS-PAGE gels to visualize the purification of proteins associated to G5₈₄₅₋₁₅₀₈-TAP polypeptides.

In-Gel Digestion and Mass Spectrometry Analysis

Two independent biological replicates of TAP samples obtained for G5₈₄₅₋₁₅₀₈-R1016C-TAP, G5₈₄₅₋₁₅₀₈-D1019E-TAP, and G5₈₄₅₋₁₅₀₈-L1367P-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 destained in acetonitrile:water (ACN:H₂O, 1:1), were reduced and alkylated, and then digested in situ with sequencing grade trypsin (Promega, Madison, WI) (Shevchenko et al., 1996). The gel pieces were dried and re-swollen in 50 mM ammonium bicarbonate pH 8.8 with 60 ng/μl trypsin at 5:1 protein:trypsin (w/w) ratio. The tubes were kept in 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 (on-line) by reverse phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Thermo Fisher Scientific), and then separated using a 0.075mm x 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 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 ID 30 μm (Proxeon) interface. The Orbitrap resolution was set at 30,000. Peptides were detected in survey scans from 400 to 1600 amu (1 μscan), followed by twenty 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 seconds periods.

Peptide identification from raw data was carried out using PEAKS Studio X (Zhang et al., 2012) search engine (Bioinformatics Solutions Inc). Database search was performed against UniProt-Homo sapiens FASTA (decoy-fusion database). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 20 ppm for precursor ions and 0.6 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. False discovery rates (FDR) for peptide spectrum matches (PSM) were limited to 0.01. Only those proteins with at least two distinct peptides being discovered from LC/MS/MS analyses were considered reliably identified (Dataset 1).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD028959 and 10.6019/PXD028959.

BiNGO and STRING analysis

The Biological Networks Gene Ontology application (BiNGO) was used to assess the overrepresentation of proteins associated with G5₈₄₅₋₁₅₀₈-TAP variants, and to determine the statistical significance of overrepresented proteins relative to a complete human proteome (Maere et al., 2005). Nodes overrepresented on the proteins associating with these variants relative to a whole human proteome belong to functional networks. The results were visualized on the Cytoscape platform (Shannon et al., 2003). The biological processes nodes were classified according to a hypergeometric test in the default mode, FDR <0.05. *P*-values for the overrepresented nodes were used to compute the average statistical significance of the network.

STRING software was used to depict the physical and functional interactions among the factors belonging to Translation, RNA metabolism, and SMN complex networks (<https://string-db.org>).

Protein stability assays

HEK293 human cells were transfected with the pcDNA3-Xpress-G5₈₄₅₋₁₅₀₈ or pcDNA3-Xpress-G5 constructs. For cycloheximide (CHX) chase experiments, CHX (100 µg/ml) (Merck) was added to stop translation at 12 h or 24 h post-transfection (hpt) for Xpress-G5₈₄₅₋₁₅₀₈ or Xpress-Gemin5 proteins, respectively. Cells were harvested immediately (time 0), and 12 h and 16 h post-CHX treatment. Cell lysates were prepared in 100 µl lysis buffer (50 mM Tris-HCl pH 7.8, 100 mM NaCl, NP40 0.5%) and the total protein concentration was determined by Bradford assay.

RNA quantification

To measure the mRNA steady-state levels, total RNA was isolated from lysates prepared from cells harvested 24 hpt expressing the corresponding plasmid as described (Francisco-Velilla et al., 2018). On the other hand, the total RNA from cytoplasmic lysates (Inputs) and polysome-bound RNA from the polysomal fraction 9 were extracted using trizol (Thermo Fisher Scientific), isopropanol precipitated, and resuspended in RNase-free H₂O. Reverse transcriptase reaction was performed to synthesize cDNA from equal amounts of the purified total RNA samples using SuperScript III (Thermo Fisher Scientific) and hexanucleotide mix (Merck) as primer. For quantitative polymerase chain reaction (qPCR), the pair of oligonucleotides 5'Luciferase/3'Luciferase (Francisco-Velilla et al., 2018) were used. The pairs of primers Xpress-s/Xpress-as, AGO2-s/AGO2-as, PCBP1-s/PCBP1-as, SF3B3-s/SF3B3-as and DHX15-s/DHX15-as (Supplementary Table S1) were designed (Primer3 software, <http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) and tested for amplification efficiency. qPCR was carried out using NZYSupreme qPCR Green Master Mix (NZytech) according to the manufacturer's instructions on an CFX-384 Fast Realtime PCR system (Bio Rad). Values were normalized against the constitutive MYO5A RNA (Francisco-Velilla et al., 2018). The comparative cycle threshold method (Schmittgen et al., 2008) was used to quantify the results.

Purification of 80S ribosomes

Fractions corresponding to 80S peak from polysome profiles loaded with HEK293 cell lysates were collected and pooled at 48000 rpm using a T865 rotor 2 h, 4°C. 80S pellet was resuspended

in 10 mM HEPES pH 7, 10 mM MgCl₂, 50 mM KCl, 5 mM β-ME and stored at -70°C. Ribosome concentration was calculated as 1 A₂₆₀ unit = 20 pmol/ml 80S ribosome (Chen et al., 2014).

Ribosome binding assay

His-tagged G5_{845–1508} (His-G5_{845–1508}) (4 pmol) were coupled to Ni-agarose resin (25 µl of beads suspension) (Qiagen) during 1 h, at 4°C in binding buffer (RBB) (50 mM TrisOAc pH 7.7, 50 mM KOAc, 5 mM Mg (OAc)₂, 10 mM DTT, 30 µg/ml tRNA). Unbound protein was removed by 3 washes with RBB, spinning at 14000 g 3 min at 4°C. Beads-protein complexes, resuspended in 100 µl of RBB, were incubated with 80S ribosomes (0.7 pmol) during 1 h, at 4°C. After three washes of the beads-complexes with RBB supplemented with NP40 0.05%, spinning at 14000 g 3 min at 4°C, beads-bound proteins were dissolved in SDS-loading buffer, heated at 92°C 3 min, resolved by SDS-PAGE and detected by WB.

GST-pull down assay

The ribosomal proteins of interest were prepared as GST-fusions as described (Francisco-Velilla et al., 2015). For binding, the GST-fusion protein (4 µg) bound to the glutathione resin (Cytiva) was incubated with His-G5_{845–1508} purified protein, in 5 volumes of binding buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 2 mM DTT, 2 mM MgCl₂, 0.5% Igepal CA-630, 10% glycerol) 2 h, at 4°C in a rotating wheel. Beads were pelleted at 3000 g 2 min at 4°C and washed three times with 5 volumes of binding buffer, rotating the reaction tube 5 min at 4°C. Finally, the beads were boiled in SDS-loading buffer, proteins were resolved by SDS-PAGE and were detected by WB.

Subcellular fractionation (S30 and native ribosomes)

HEK293 cells grown to 70-80 % confluence in 2 P100 dishes, were transfected with Xpress-G5_{845–1508} constructs. 24 hours later cells were washed with ice cold PBS, and lysed in buffer 1 (15 mM Tris-HCl pH 7.4, 80 mM KCl, 5 mM MgCl₂, 1% Triton-X-100, Protease inhibitors (Complete mini, Roche)). Cell debris was discarded by spinning at 14000 g 10 min 4°C. The supernatant (S30 fraction) was ultracentrifuged at 95000 rpm during 1.5 h using the TLA100.2 rotor yielded the S100 fraction (supernatant), and the native ribosomes (ribosomes plus associated factors). The pellets corresponding to native ribosomes were resuspended in 100 µl of buffer 1. The total protein content in S30 fractions was measured by the Bradford assay; the ribosome concentration was determined as 14 units A₂₆₀ = 1 mg/ml.

Luciferase activity assays

HEK293 cells were cotransfected with plasmids expressing luciferase in cap-dependent way (pCAP-luc) (Lozano et al., 2018), and the Xpress-G5_{845–1508} constructs or the empty vector. Cell lysates were prepared 24 h post-transfection in 100 µl lysis buffer. The protein concentration in the lysate was determined by Bradford assay. Luciferase activity (RLU)/µg of total protein was internally normalized to the value obtained with the empty vector performed side by side.

Polysome profiles, RNA isolation and analysis

Polysome profiles were prepared from HEK293 cells (about 1×10^7 per gradient) transfected with the constructs expressing the Xpress-G5₈₄₅₋₁₅₀₈ proteins as described (Francisco-Velilla et al., 2016). Briefly, cells were washed with ice-cold PBS containing 100 µg/ml cycloheximide to block ribosomes in the elongation step. Then, cells were lysed with buffer A (15 mM Tris-HCl pH 7.4, 80 mM KCl, 5 mM MgCl₂, 100 µg/ml cycloheximide), supplemented with 1% (v/v) Triton X-100, 40 U/ml RNase OUT (Thermo Fisher Scientific), protease inhibitors (Complete mini, Roche). Cytoplasmic lysates obtained by centrifugation at 14000 g 10 min at 4°C, were loaded into a linear 10–50% (w/v) sucrose gradient in buffer A, centrifuged at 39000 rpm in a SW40 Ti rotor 2 h 15 min at 4°C. Gradients were fractionated by upward displacement with 87% (v/v) glycerol using an ISCO density-gradient fractionator, monitoring A260 continuously (ISCO UA-5 UV monitor). Fractions (12 fractions of 1 ml) were collected from gradients.

Immunodetection

The immunodetection of G5₈₄₅₋₁₅₀₈ proteins were performed with different antibodies depending on the protein tag (anti-CBP (Abcam) for G5₈₄₅₋₁₅₀₈-TAP, anti-Xpress (Thermo Fisher Scientific) for Xpress-G5₈₄₅₋₁₅₀₈, or anti-His (Merck) for His-G5₈₄₅₋₁₅₀₈). The anti-Gemin5 (Novus) antibody was used to detect the endogenous Gemin5 protein. The endogenous proteins tubulin, RACK1, and P0 were immunodetected with anti-Tubulin (Merck), anti-RACK1 (Santa Cruz), and 3BH5 (anti-P0) (Vilella et al., 1991) antibodies. Finally, GST-fusion proteins were detected with the anti-GST antibody (Santa Cruz).

Statistical analyses

Statistical analyses for experimental data were performed as follows. Each experiment was repeated independently at least three times. Values represent the mean $\pm$ SEM. We computed P-values for a difference in distribution between two samples with the unpaired two-tailed Student's t-test. Differences were considered significant when $P < 0.05$. The resulting P-values were graphically illustrated in figures with asterisks as described in figure legends.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD028959 and 10.6019/PXD028959. All data generated or analyzed during this study are included in the manuscript and supporting files.

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AUTHOR CONTRIBUTION

R.F-V, S.R-M and E.M-S designed the experiments, R.F-V, A.E-B, F.C-O, J.F-C, S.A, M.V, and S.R-M conducted the experiments, S.A, A. F-G, S.K, D.R. and U.B.P evaluated the genetic and clinical data. E.M-S wrote the paper with comments from all authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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Table 1. Patient whole exome sequencing¹

Chr.	Position	Gene	Intron/exon	Nucleotide change	Amino acid change	Mutation type	Variant frequency	Sequencing Deep
5	154311139	Gemin5	4	NM_015465.4: c.662-2A>G	-	5'splice site	60%	145
5	154278839	Gemin5	22	NM_015465.4: c.3046C>T	p.Arg1016Cys	missense	50%	68

¹ Female, compound heterozygosity, intellectual disability, autism disorder, delay staturo-ponderal growth, microcephaly, mild dysmorphic features, no motor problems.

FIGURES

Figure 1. Gemin5 protein organization, pathogenic mutants, and structural properties of the variants on the TPR-like domain. (A) Schematic of functional domains of Gemin5. The regions encompassing the WD40 domain, the TPR-like dimerization module, and the non-canonical RBS1 domain are plotted. The position of amino acids flanking each domain is indicated at the top. A thick black line depicts the region included in the protein G5₈₄₅₋₁₅₀₈. An asterisk marks the last residue of the truncated protein (about 220 residues) predicted by the splice variant mutation. **(B)** Summary of *Gemin5* biallelic variants found in the exome sequencing of patients developing neurodevelopmental disorders. **(C)** Cartoon representation of Gemin5 TPR-like homodimer with each subunit shown in different color. The numbers for the helices are indicated. Dashed lines indicate the flexibly disordered loop α 2-3. The spheres indicate the position of mutated residues. The enlarged area shows a zoom view of the loop α 12-13 with residue R1016 (yellow) and D1019 (green). Dashed lines indicate electrostatic interactions. Models of the loop α 12-13 bearing mutations R1016C or D1019E are shown below. **(D)** Limited protease digestion of the WT and mutant proteins. Equal amounts of purified Gemin5 TPR-like proteins (WT, R1016C, and D1019E) were treated with serial dilutions of trypsin (0 - 0.32 μ g/ml) and subjected to SDS-PAGE. A representative example of a Coomassie blue stained gel is shown. Quantification of the 30 kDa protein band shows differences in the sensitivity to trypsin cleavage. Analysis of the WT, R1016C and D1019E proteins by SEC-MALS **(E)** or differential scanning fluorimetry **(F)**. Values are represented as the mean $\pm$ SD from six independent measurements for each protein.

Figure 2. Mutations in the TPR-like domain impair the capacity of G5₈₄₅₋₁₅₀₈ to recruit the endogenous Gemin5 protein in living cells. (A) Expression of G5₈₄₅₋₁₅₀₈-TAP proteins. HEK293 cells were transfected with plasmids expressing G5₈₄₅₋₁₅₀₈-WT-TAP or G5₈₄₅₋₁₅₀₈-A951E, R1016C, D1019E or L1367P proteins. Twenty-four hours later, protein expression in cell lysates was followed by immunoblot using anti-CBP antibody. Tubulin was used as loading control. **(B)** Coimmunoprecipitation of Gemin5 with G5₈₄₅₋₁₅₀₈-TAP purification samples obtained from HEK293 soluble cell extracts expressing G5₈₄₅₋₁₅₀₈-WT-TAP or the G5₈₄₅₋₁₅₀₈ variants were detected by WB using anti-Gemin5 antibody (left panel). The relative intensity Gemin5/G5₈₄₅₋₁₅₀₈ (mean $\pm$ SEM) is represented (right panel). **(C)** Gemin5 endogenous protein copurifying with G5₈₄₅₋₁₅₀₈-WT or the G5₈₄₅₋₁₅₀₈ mutants was identified by mass spectrometry (left panel). The number of reads of unique peptides corresponding to the N-terminal region of Gemin5 (residues 1-844) were made relative to those found in the G5₈₄₅₋₁₅₀₈ region (845-1508) (right panel). Asterisks denote *P*-values (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure 3. Functional networks of proteins associated with G5₈₄₅₋₁₅₀₈ variants. (A) Statistical difference (median *P*-values) obtained for protein networks associated to each of the G5₈₄₅₋₁₅₀₈ variant obtained with the application BiNGO (Cytoscape platform). The *P*-values of the networks obtained for each G5₈₄₅₋₁₅₀₈ variant relative to a complete human proteome are indicated. Empty lanes depict networks with *P*-values $> 5 \times 10^{-3}$. **(B-E)** Networks obtained for the G5₈₄₅₋₁₅₀₈ variants.

The area of a node is proportional to the number of proteins in the test set annotated to the corresponding GO category, and the color intensity indicates the statistical significance of the node according to the grey scale bar. Arrows indicate branched nodes. Networks are shadowed violet, orange, green, or yellow according to the functional process. **(F)** STRING protein-protein networks associated to G5₈₄₅₋₁₅₀₈-TAP. The cellular factors of TPR mutants correspond with the overlap between R1016C and D1019E samples.

Figure 4. Protein stability of G5₈₄₅₋₁₅₀₈ carrying mutations R1016C, D1019E or L1367P. (A) HEK293 cells expressing the wild type version of G5₈₄₅₋₁₅₀₈, side by side to the variants R1016C, D1019E or L1367P during 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. A long exposure is shown for L1367P protein. (B) Steady-state analysis of Xpress-G5₈₄₅₋₁₅₀₈ mRNA levels present in transfected cells at the time of harvesting determined by RTqPCR. (C) Values represent the protein intensity (mean $\pm$ SEM) of three independent experiments relatively to time 0. Asterisks denote *P*-values (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure 5. Variants within the dimerization module abrogate the ribosome sedimentation capacity of G₅₈₄₅₋₁₅₀₈. (A) Purified G₅₈₄₅₋₁₅₀₈ binds directly to the ribosome. Overview of the procedure used to isolate ribosomes 80S (left). Overview of the ribosome-binding assay. 80S ribosomes were incubated with beads alone (lane 1), or with Ni-agarose beads bound His-G₅₈₄₅₋₁₅₀₈ (lane 2). After extensive washing, His-G₅₈₄₅₋₁₅₀₈ was immunodetected using anti-His antibody. The antibody recognizing P0 was used to determine the presence of ribosomes bound to G₅₈₄₅₋₁₅₀₈. (lanes 1, 2, 3). (B) GST pull-down of G₅₈₄₅₋₁₅₀₈ with ribosomal proteins. GST protein alone was used as negative control. G₅₈₄₅₋₁₅₀₈ was immunodetected using anti-His, the GST-fusion proteins were detected using anti-GST. I denotes Input; B, Binding. (C) Overview of the procedure used to isolate native ribosomes. (D) Protein levels present in the S30 fraction (top) of HEK293 cells expressing Xpress-tagged G₅₈₄₅₋₁₅₀₈ proteins, and sedimentation of these variants with native ribosomes (bottom). G₅₈₄₅₋₁₅₀₈ proteins were detected using anti-Xpress, the ribosomes with anti-RACK1. Tubulin was used as loading control in S30 fractions. (E) Ratio of G₅₈₄₅₋₁₅₀₈ intensity in native ribosomes (80S) relative to the intensity observed in cytoplasmic lysates (S30). In all cases, values represent the mean $\pm$ SEM and asterisks denote *P*-values (**P < 0.001).

Figure 6. Gemin5 mutants affecting dimerization fail to stimulate translation. (A) Diagram of the Xpress-G5₈₄₅₋₁₅₀₈ proteins co-expressed with the luciferase reporter mRNA. Protein expression was monitored by WB using anti-Xpress, tubulin was used as loading control. Luciferase activity measured in HEK293 cell lysates expressing cap-mRNA co-transfected with Xpress- G5₈₄₅₋₁₅₀₈-WT or the indicated variants. In all cases, luciferase values are normalized to cells expressing the empty vector conducted side by side. Values represent the mean $\pm$ SEM obtained in three independent assays. Asterisks denote *P*-values ($***P < 0.01$). **(B)** Steady-state mRNA levels analysis of the reporter were determined by RTqPCR in transfected cells at the time of harvesting for luciferase assays. **(C)** Polysome profiles prepared in 10-50% sucrose gradients loaded with total lysates of HEK293 cells expressing Xpress-G5₈₄₅₋₁₅₀₈-WT, Xpress-G5₈₄₅₋₁₅₀₈-

R1016C, Xpress-G5₈₄₅₋₁₅₀₈-D1019E or the empty vector. The fractions of the gradient corresponding to 40S and 60S ribosomal subunits, 80S monosomes and polysomes are indicated. Xpress-G5₈₄₅₋₁₅₀₈ proteins, and the ribosomal protein RACK1 were analyzed along the gradient fractions (20 µl) by WB using specific antibodies. **(D)** Histograms representing the fold enrichment in polysomes relative to the input samples of selected mRNAs (Gemin5 (region H12), AGO2 and PCBP1).

Figure 7. Functions affected by pathogenic variants on the dimerization module and the RBS1 domain of Gemin5. Schematic representation of the Gemin5 protein with its functional domains: WD40, TPR-like dimerization module, and the non-canonical RBS1 domain. The spatial disposition of the domains is not known. Gemin5 dimerization variants show deficiencies in the interaction with members of the SMN complex, ribosome association, and translation stimulation. In contrast, the Gemin5 RBS1 variant retains these properties but it is less stable (shady drawing denotes reduced protein level). All variants show a reduced protein interactome. The combination of Gemin5 variants in patients, a dimerization defective mutant (R1016C or D1019E) with a protein level deficiency (L1367P), results in protein malfunction associated to disease.