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Alternative splicing events driven by altered levels of GEMIN5 undergo translation

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ABSTRACT

GEMIN5 is a multifunctional protein involved in various aspects of RNA biology, including biogenesis of snRNPs and translation control. Reduced levels of GEMIN5 confer a differential translation to selective groups of mRNAs, and biallelic variants reducing protein stability or inducing structural conformational changes are associated with neurological disorders. Here, we show that upregulation of GEMIN5 can be detrimental as it modifies the steady state of mRNAs and enhances alternative splicing (AS) events of genes involved in a broad range of cellular processes. RNA-Seq identification of the mRNAs associated with polysomes in cells with high levels of GEMIN5 revealed that a significant fraction of the differential AS events undergo translation. The association of mRNAs with polysomes was dependent on the type of AS event, being more frequent in the case of exon skipping. However, there were no major differences in the percentage of genes showing open-reading frame disruption. Importantly, differential AS events in mRNAs engaged in polysomes, eventually rendering non-functional proteins, encode factors controlling cell growth. The broad range of mRNAs comprising AS events engaged in polysomes upon GEMIN5 upregulation supports the notion that this multifunctional protein has evolved as a gene expression balancer, consistent with its dual role as a member of the SMN complex and as a modulator of protein synthesis, ultimately impinging on cell homoeostasis.

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GEMIN5; SMN complex; alternative splicing; RNA-sequencing; polysome enrichment; translation control

Introduction

The survival of the motor neuron (SMN) complex is responsible for the cytoplasmic assembly of small nuclear ribonucleoproteins (snRNPs), prior to being imported into the nucleus [1]. In higher eukaryotes, this macromolecular complex consists of the SMN, the GEMIN2–8, and the Sm proteins [2]. Previous data showed that GEMIN5 is essential for the recognition and core assembly of snRNPs [3], the building blocks of spliceosome formation. The spliceosome is a macromolecular machine composed of 5 snRNPs and over 150 proteins responsible for the removal of introns and ligation of exons of the pre-mRNA [4] that generates the mature mRNA. Importantly, the use of different splice sites in pre-mRNA produces mRNAs by a process termed alternative splicing (AS), which increases the transcriptome in various ways, in addition to generating proteome diversity [5].

GEMIN5 is a multifunctional protein involved in numerous aspects of RNA processing, including the biogenesis of snRNPs through the SMN complex [6–8] as well as the regulation of translation at a global level [9–11], in agreement with earlier data showing its binding to cellular and viral mRNAs [12–15]. GEMIN5 is a predominantly cytoplasmic protein [16,17], which has been shown to be a hub for the assembly of various macromolecular complexes [18]. Moreover, the expression of the protein is regulated through a feed-forward mechanism involving the interaction of the protein with an internal region of its mRNA [14]. This mechanism counteracts the negative effect of this protein in translation, and sustains the physiological balance on its negative and positive roles. Previous studies showed that reduced levels of this protein regulate the association of specific mRNAs to polysomes, therefore exerting a strong effect on selective translation [15]. Nonetheless, a significant effect of GEMIN5 depletion on the steady-state of total mRNAs in siRNA-treated cells compared to control ones suggested additional poorly known effects on the processing and stability of pre-mRNAs.

GEMIN5 has been shown to be essential in various organisms, as *Drosophila* and mouse KO models are embryonic lethal [19,20]. Importantly, loss-of-function mutations in *Gemin5* gene lead to neurodevelopmental syndrome among patients presenting with developmental delay, motor dysfunction, and cerebellar atrophy [20–26]. In addition, a deleterious effect was observed upon the expression of defective GEMIN5 proteins in IPSC cells [20,21]. However, the direct involvement of GEMIN5 in AS remains poorly known [24].

Here, we have analysed the effect of GEMIN5 expression in splicing through the characterization of differential AS events. We show that high expression levels of GEMIN5, but not silencing, result in an increase of AS events compared to control cells. More importantly, a large fraction of the

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mRNAs containing exon skipping events are engaged into translationally active polysomes, indicating that they undergo translation. These results, in conjunction with the influence of GEMIN5 in selective translation, emphasize the relevance of GEMIN5 balance to maintain protein homoeostasis.

Materials and methods

Cell culture and transfection

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% foetal calf serum. This cell line was previously used to analyse the role of GEMIN5 in mRNA binding and association to polysomes [14,15]. For GEMIN5 expression, monolayers grown to 80% confluency were transfected with the plasmid pcDNA3Xpress-Gemin5 [9] using Lipofectamine LTX (Thermo Fisher Scientific 15338100). Cells expressing elevated levels of GEMIN5 were harvested 24 h post-transfection. To carry out the depletion of GEMIN5, HEK293 cell monolayers 70% confluent were treated with 100 nM siRNA targeting *Gemin5* mRNA (CCUUAAUCAAGAAGAGAAAUU,_Horizon Discovery) using Lipofectamine 2000 (Thermo Fisher Scientific 11668019) and were harvested 48 h post-transfection.

Immunodetection

Cell extracts were prepared in lysis buffer C (50 mM Tris-HCl pH 7.8, 100 mM NaCl, 0.5% IGEPAL). The concentration of total protein in the lysate was determined by Bradford assay. Equal amounts of total protein were resolved on SDS-polyacrylamide gels and transferred to a PVDF membrane (0.2 µm pore, Bio-Rad) using a semi-dry electrotransfer (Bio-Rad). GEMIN5 was immunodetected using anti-Gemin5 (Novus, NB100–61049) antibody. Immunodetection of TUBA4A (Merck, T9026-100UL) was used as loading control. The appropriate secondary HRP-conjugated antibody (Thermo Fisher Scientific 32460 and 32430) was used according to the instructions of the manufacturer.

Polysomal fractionation

Polysome profiles were prepared from four independent biological samples of control cells, GEMIN5 overexpressing cells, and GEMIN5 depleted cells as described [15]. Briefly, HEK293 cells (about 10^7 per gradient) were washed with ice-cold PBS containing 100 µg/ml cycloheximide to block ribosomes at the elongation step. Then, cells were lysed in buffer A (15 mM Tris-HCl pH 7.4, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mg/ml cycloheximide) supplemented with 1% (v/v) Triton X-100, 40 U/ml RNase OUT (Thermo Fisher Scientific 10777019), and protease inhibitors (Merck 11836153001). The lysates were centrifuged 10 min at 14,000 g, 4°C and the clear supernatants were loaded into a linear 10–50% (w/v) sucrose gradient prepared previously in buffer A, and the loaded sucrose gradients were centrifuged at 39,000 rpm using a SW40 Ti rotor during 2 h 15 min at 4°C. The sucrose gradients were fractionated using a fractionation system (ISCO UA-5 UV monitor) measuring the absorbance at A260 to record the polysome profile. Twelve fractions (1 ml) were collected from each gradient.

RNA isolation

Total RNA from cytoplasmic lysates and polysomal fractions (8–12) was extracted using phenol-chloroform, isopropanol precipitated, and resuspended in 100 µl RNase-free H_2O. RNA samples were cleaned with the RNeasy kit (Qiagen 74104). RNA quality and concentration were determined using Agilent 2100 Bioanalyzer (Agilent Technologies).

cDNA libraries preparation and RNA-Seq analysis

The cDNA libraries were prepared with 1 µg of RNA and oligo dT (50 pmol) using the Illumina TruSeq Stranded mRNA (Macrogen). RNA sequencing was carried out in an Illumina NovaSeq 6000 platform. The sequencing coverage was 30 M reads per sample, and the read length was 100 bp PE.

Quality assessment

Quality analysis of individual short read Fastq files performed with FastQC software (version 0.11.7), with default parameters, demonstrates the good quality of the sequencing process. About 96.5% of the sequences had a quality score over 30, with a negligible percentage of undetermined bases and low sequence duplication levels. Contamination assessment was conducted using FastQ Screen 0.14.1 [27], with reference genomes of model organisms (*H. sapiens*, *R. norvegicus*, *M. musculus*, *D. melanogaster*, *C. elegans*, etc.), reference sets of bacterial and viral genomes, and other potential contaminants such as vectors, adapters and phage sequences. The results found no evidence of contamination.

Sequence alignment

Illumina paired-end read Fastq files were aligned with STAR 2.7.10a [28] against the Homo sapiens genomic sequence downloaded from Ensembl release 108 [29]. Default parameters were used, except for -sjdbOverhang, which was set to 100 according to the sequencing read length, and -sjdbGTFfile, which indicated the corresponding genomic annotations file in GTF format, also downloaded from Ensembl release 108.

Alternative splicing analysis

Alternative splicing detection was carried out using two methodologies, Vast-tools and ASPLI2. Splicing event detection with Vast-tools (version 2.5.1) [30] was carried out using the annotations of Homo sapiens (hg38) available in VastDB (23/06/2020) and the default parameters. Differential splicing analysis of each group was performed using the compare module, specifying the values of 15 for -min-dPSI and 5 for -min-range. Statistical significance of PSI distributions of each event was assessed with the diff module [31]. Then, events were re-annotated against the Homo sapiens genome, downloaded from Ensembl release 108 [29].

Genome-wide analysis of alternative splicing from the aligned reads was performed by applying the standard pipeline defined for the R package ASpli (version 2.4.0) [32], indicating a minimum read length of 100 bp and a maximum intron size defined by the largest intron in the reference genome. Then, differential splicing information and gene expression were assessed for each group pair with the default parameters, adding a filtering step for uniformity test on intron retention.

Motif enrichment analysis

Enrichment analysis of known human RBP-binding motifs in AS RNAs carrying ES events was performed with the rMAPS2 Motif Map webserver [33] using the default parameters, comparing the upregulated (110) and downregulated (15) events in polysomes with the background (16024) events, all those having a |dPSI| <2. Motifs with a p-value lower than 0.01 in any of the flanking exons (50 nts upstream and downstream exons) were reported (Supplementary Table 1), indicating the exon with the most significant enrichment.

Differential expression

Genes included in the annotations file downloaded from Ensembl release 108 were quantified with the function featureCounts (ignoreDup=TRUE, primaryOnly=TRUE, requireBothEndsMapped=TRUE) of Bioconductor package Rsubread [34], counting the number of reads mapping in the reversely stranded sense to each gene. Differential expression between each pair of conditions was determined using the R library DESeq2 [25] (version 1.34.0) with the specific parameters betaPrior=TRUE, cooksCutoff=F, independentFiltering=FALSE and minReplicatesForReplace=Inf. The output data produced by DESeq2 was combined with the genomic annotations of Ensembl release 108, downloaded through the BioMart [35] API, and then loaded into the FIESTA2 viewer (https://bioinfogp.cnb.csic.es/tools/fiesta2) (Oliveros, JC. 2022. FIESTA 2.0: Visualizing differential gene expression results obtained by RNA-Seq), for posterior filtering and visualization. Principal components for the individual normalized values were calculated using the variance stabilizing transformation (VST) method [36] included in the DESeq2 R package to explore associations between variables.

Reverse transcription and PCR

cDNA synthesis was performed using equal amounts of RNA extracted from lysates of control and GEMIN5 overexpressing cells (1 or 5 µg) with 0.5 µl of SuperScript III (Thermo Fisher Scientific 12574026), 125 ng of hexanucleotide mix (Merck 11277081001) and oligo dT (50 pmol) (Merck, O4387) in a final volume of 20 µl. Semiquantitative polymerase chain reaction (PCR) was carried out using 1 µl of cDNA and 0.2 µl of Taq DNA polymerase (Applied Biosystems, N8080172) in a total volume of 20 µl. Typical parameters for PCR cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final incubation of 1 min at 72°C. The sequence of primers (IDT) used for each reaction is shown in Supplementary Table 2.

Software used for data analysis

Gene Ontology analysis was performed with DAVID V6.8 [37] to identify the biological processes of the different mRNA groups. The cut-off criteria p value $< 10^{-2}$ and gene count ≥2 were applied. STRING software was used to depict the physical and functional interactions among the selected genes, using default parameters with a minimum required interaction score of 0.4 [38]. The results were visualized on the Cytoscape platform [39].

Results

GEMIN5 expression levels regulate the steady state of mRNAs

To identify the mRNAs affected by the expression of GEMIN5 we prepared independent biological samples of HEK293 cells expressing elevated levels of GEMIN5 (G5) and control cells (Ct). GEMIN5 overexpression was verified by western blot (Figure 1A). Then, individual cell lysates were subjected to polysomal fractionation. In each case, the mRNAs present in cytoplasmic cell lysates (Input) and polysomes (Polysomes) were extracted and analysed by RNA-Seq (Figure 1B). Principal component analysis (PCA) showed minimal variations among the individual biological replicates for each input condition (Figure 1C), confirming the reproducibility of the assay.

The statistical analysis of the mRNAs differentially altered in the mRNA pool (Input) in GEMIN5-expressing cells compared to control cells (FDR < 0.05 and $|\log_2 FC| > 1$) showed 1385 genes with significantly reduced read counts, while these were significantly enriched for 1903 genes (Figure 1D), indicating that GEMIN5 expression levels modulate the steady state of cellular mRNAs. As expected from the expression strategy, the *Gemin5* mRNA was significantly augmented in G5 lysates (Figure 1D). Detailed Gene ontology (GO) analysis of transcripts downrepresented identified networks related to Regulation of transcription, RNA splicing, mRNA processing and Cell division, among others (Supplementary Figure 1A). These networks were undetected within the overrepresented mRNAs that, instead, contained other networks, such as Translation, Mitochondrial translation, Mitochondrial respiratory chain, Ribosomal biogenesis and Nucleosome assembly, among others (Supplementary Figure 1B). We noticed that while the translation network was identified with a $-\log_{10}$(p-value) > 60, all the others ranged around 10–20. Moreover, representations of the networks with higher p-value in each case denoted that genes belonging to the regulation of transcription were distributed all along the volcano plot (Supplementary Figure 1C). In contrast, the genes corresponding to the translation network were mostly located on $\log_2 FC > 1$ (Supplementary Figure 1D).

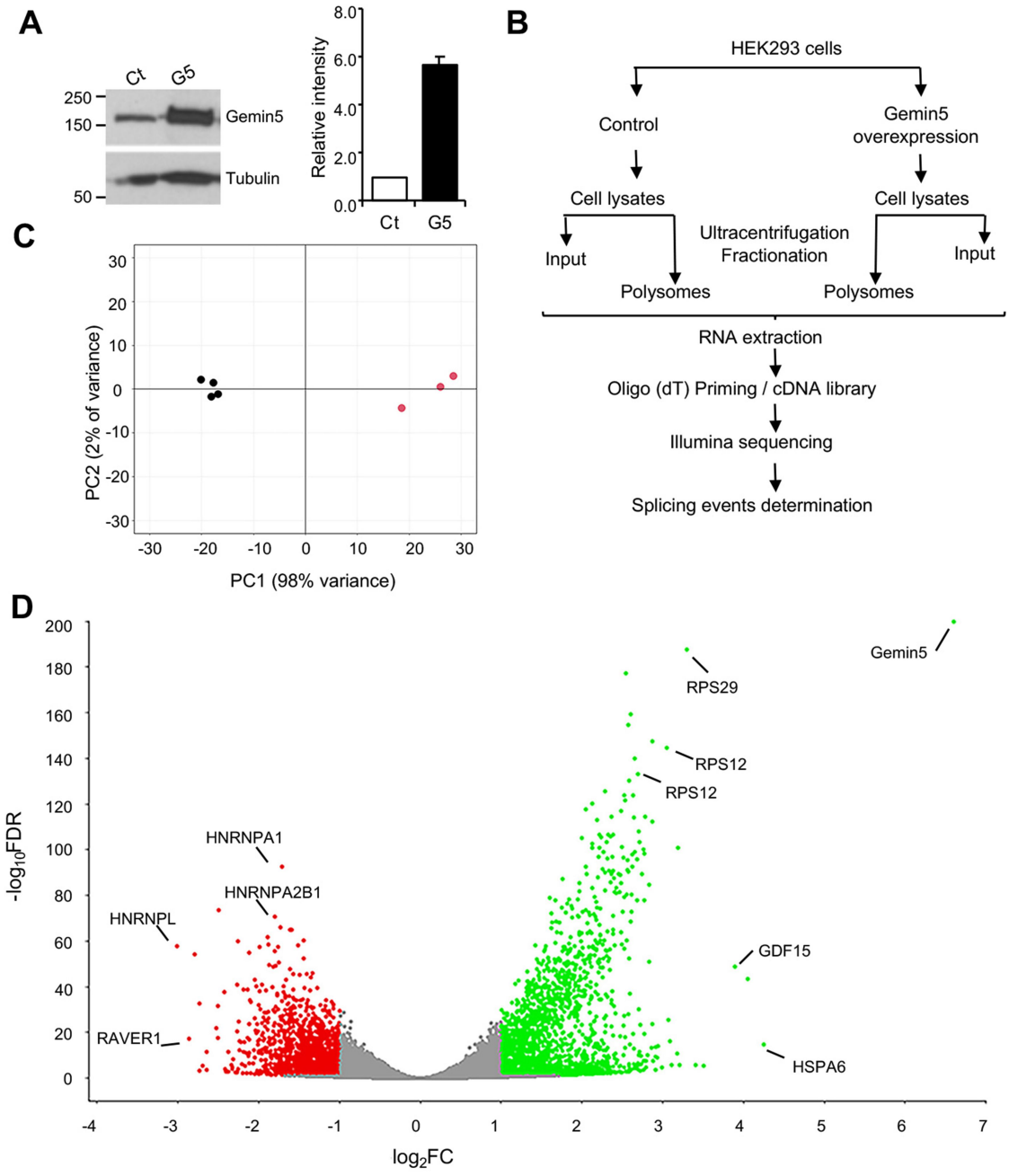

Figure 1. Analysis of the GEMIN5-dependent regulation of mRNAs steady state. A) Immunoblotting of GEMIN5 obtained from control (Ct) and GEMIN5 expressing cells (G5). Tubulin was used as loading control. A bar chart depicts the intensity of GEMIN5 relative to control cells. B) Overview of the procedure carried out to identify as events on the mRNAs present in input and polysome samples from Ct and G5 cells. Cytoplasmic lysates from 4 independent biological replicates were obtained. RNA was extracted from input samples and from the combined polysomal fractions. After oligo(dT)-primed cDNA libraries preparation, mRNAs were identified by illumina sequencing. C) Principal component analysis (PCA) of input and polysome samples prepared from Ct (black dots) and G5 (red dots) cell lysates. D) Volcano plots showing the log_2FC *versus* – $log_{10}FDR$ of each mRNA detected in the RNA-Seq analysis for the input, using as cut-off $|log_2FC| > 1$ and FDR < 0.05. For *Gemin5* RNA the $-log_{10}FDR$ is > 200. Representative examples of upregulated (green), and downregulated (red) genes are indicated.

GEMIN5 expression levels regulate alternative splicing events

Since GEMIN5 is a key factor contributing to snRNPs biogenesis [3], we investigated the alternative splicing (AS) events (Figure 2A) in cells expressing ectopically GEMIN5 (G5) compared to control cells (Ct) using Vast-tools software [30]. Vast-tools calculates the inclusion of a given differentially expressed exon as percent spliced-in (PSI) and assesses the fraction of a gene's mRNA across four main alternative splicing patterns, retained intron (IR), alternative 5' splice site (Alt), 3' alternative splice site (Alt), and exon skipping (ES) (Figure 2A). Using FDR < 0.05 and dPSI > 15%, we identified a total of 190 differential AS events. Exon skipping represented the largest number of AS events (73%), while intron retention and alternative splice site (Alt) represented 18% and 9%, respectively (Figure 2B). In addition, the software ASpli [32] that allows the identification of changes in annotated and novel alternative-splicing events (FDR < 0.05) was used for comparative sources (Supplementary Figure 2A).

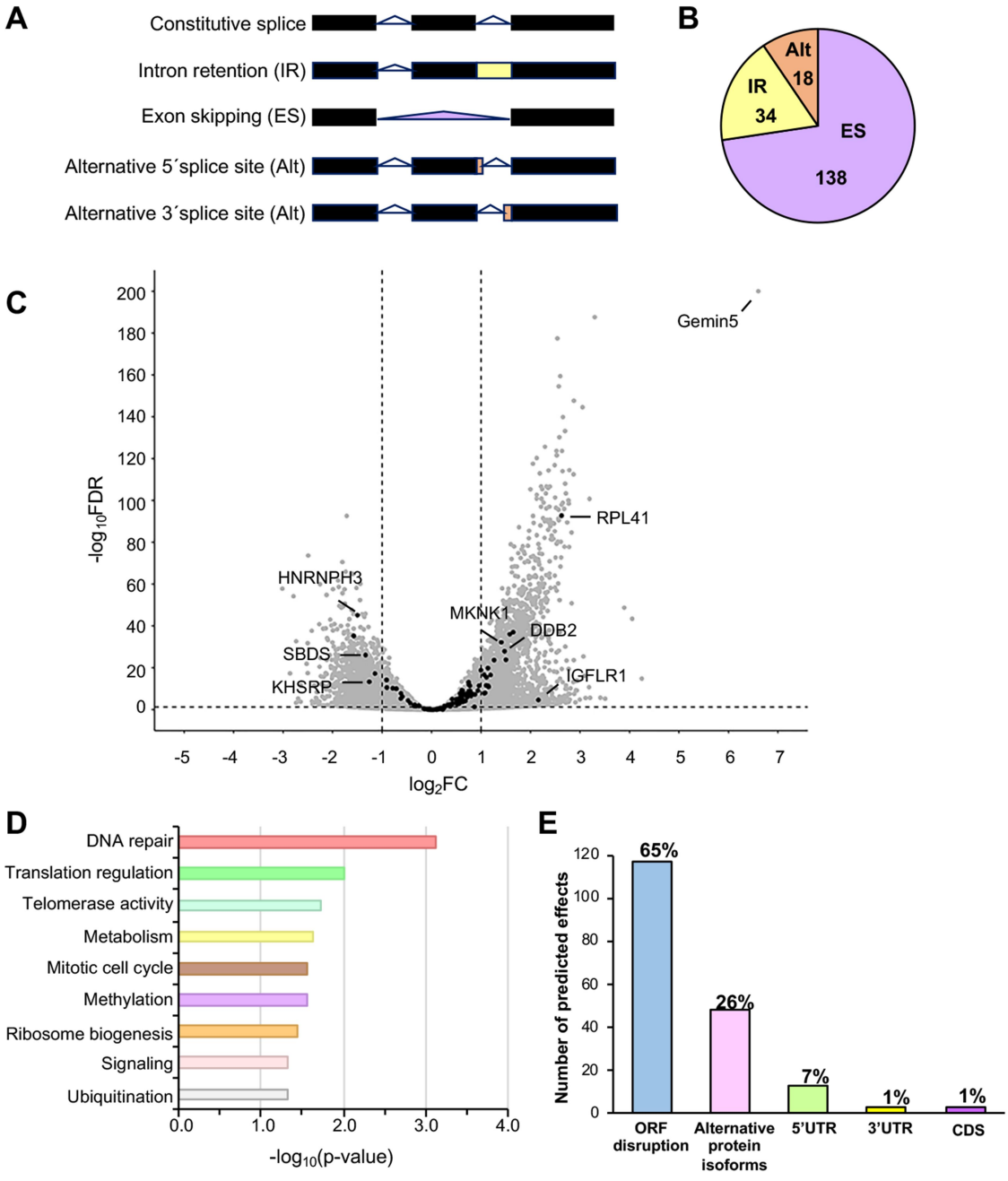

Figure 2. Determination of alternative splicing events regulated by GEMIN5 levels. A) Schematic illustrating the types of AS events considered in the analysis. B) Pie chart showing the number of exon skipping (ES), intron retention (IR), and alternative splice site (alt) events obtained with Vast-tools for the events differentially observed in G5 input versus Ct input samples. C) Volcano plots showing the distribution of genes presenting AS events (black dots) over the gene expression pattern. D) Bar chart representing the gene ontology classification for the genes comprising the differential AS events. E) histogram depicting the percentage and total number of predicted effects on the mRNAs including differential as events in G5 *versus* Ct cells.

Representation of the genes with predicted AS events over the gene expression pattern displayed a similar distribution of the AS events (Figure 2C), suggesting a lack of correlation between differential AS and RNA steady state. Next, to assess the impact of these events in biological pathways, GO term enrichment analysis was applied using as input the 134 genes that exhibited differential AS events according to Vast-tools (Figure 2D). Briefly, the most affected biological process was DNA repair, followed by Translation regulation, Telomerase activity, Metabolism, Mitotic cell cycle, Methylation, Ribosome biogenesis, Signaling, and Ubiquitination. A similar GO analysis performed with the genes with differential AS events predicted by ASpli (610) identified all the categories mentioned above, though splicing and tRNA aminoacylation were identified with higher significance ($-\log_{10}$(p-value) >10 and 8, respectively) (Supplementary Figure 2B). Notably, several GO terms were coincident with both instruments Vast-tools and ASpli (Supplementary Figure 2C), reinforcing the predictive capacity of these instruments.

Considering that AS events not only diversify the transcriptome but also generate non-functional proteins [5,40], we analysed the predicted effect on protein expression for the total set of mRNAs. Interestingly, 65% of the AS events predicted in mRNAs generated open reading frame (ORF)

disruption, 26% produced alternative protein isoforms, and only 7%, 1%, and 2% induced 5' untranslated region (UTR), 3´UTR and coding sequence (CDS) modifications, possibly altering the expression level of the corresponding ORF (Figure 2E).

Given that GEMIN5 upregulation exhibited the largest effect on exon skipping (73%) (Figure 2B), we analysed inclusion events in this category. We identified 138 events occurring in 94 transcripts that were differentially spliced in response to ectopic expression of GEMIN5 compared to control cells. First, we made use of Sashimi plots [41] to visualize the exon skipping events of selected genes, encoding the damage-specific DNA-binding protein 2 (*DDB2*) and Shwachman – Diamond – Bodian syndrome (*SBDS*) (Figure 3), encoding proteins involved in GO terms DNA repair and Ribosome biogenesis, respectively (Figure 2D). The DDB2 protein was identified as a component of the damage-specific DNA-binding heterodimeric complex involved in the repair of UV-induced DNA damage [42]. In contrast, the *SBDS* gene encodes for a conserved protein that plays an essential role in ribosome biogenesis [43]. Semiquantitative RT-PCR validation of the AS events in these examples revealed a differential exon inclusion in G5 cells relative to control cells (Figure 3). We also validated IR and Alt5´SS events in other mRNA examples (Figure 3). The deleted Azoospermia-associated protein 2 (DAZAP2) is a regulator of the DNA damage-responsive tumour suppressors (homeodomain interacting protein kinase 2, HIPK2) and specifier of the DNA damage-induced p53 response [44]. Meanwhile, the aurora kinase B (AURKB) is a serine/threonine-protein kinase and a regulator of mitosis [45].

Collectively, the diversity of functions performed by the validated examples of AS events suggests that altered levels of GEMIN5 impinge on multiple genes, possibly affecting a broad range of cellular processes.

A large fraction of the differential as events is enriched in polysomes

The data shown above prompted us to examine if mRNAs including AS events were preferentially engaged into translationally active polysomes. To this end, RNAs isolated from polysome fractions prepared from G5 and Ct cells were subjected to RNA-Seq analysis, in parallel to the corresponding Input samples (Figure 1B). This was followed by an analysis of AS events using Vast-tools. To establish a correlation between differential AS events and polysome association (Figure 4A), we analysed the translation status of the 190 differential AS events observed in cells expressing ectopically GEMIN5

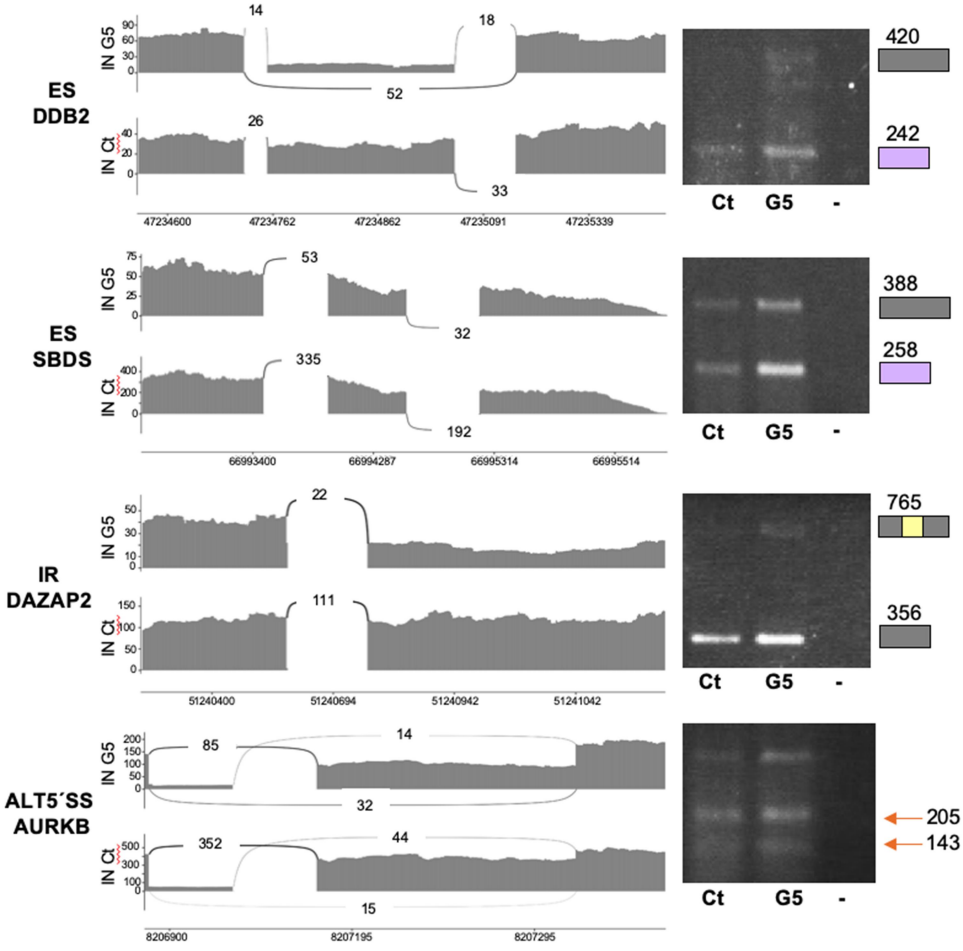

Figure 3. Validation of AS events. Sashimi plots to visualize the AS event in the indicated gene, across the Ct and G5 samples (left). Semiquantitative RT-PCR validation of selected as events (right). Colored boxes represent the expected amplification products and their size.

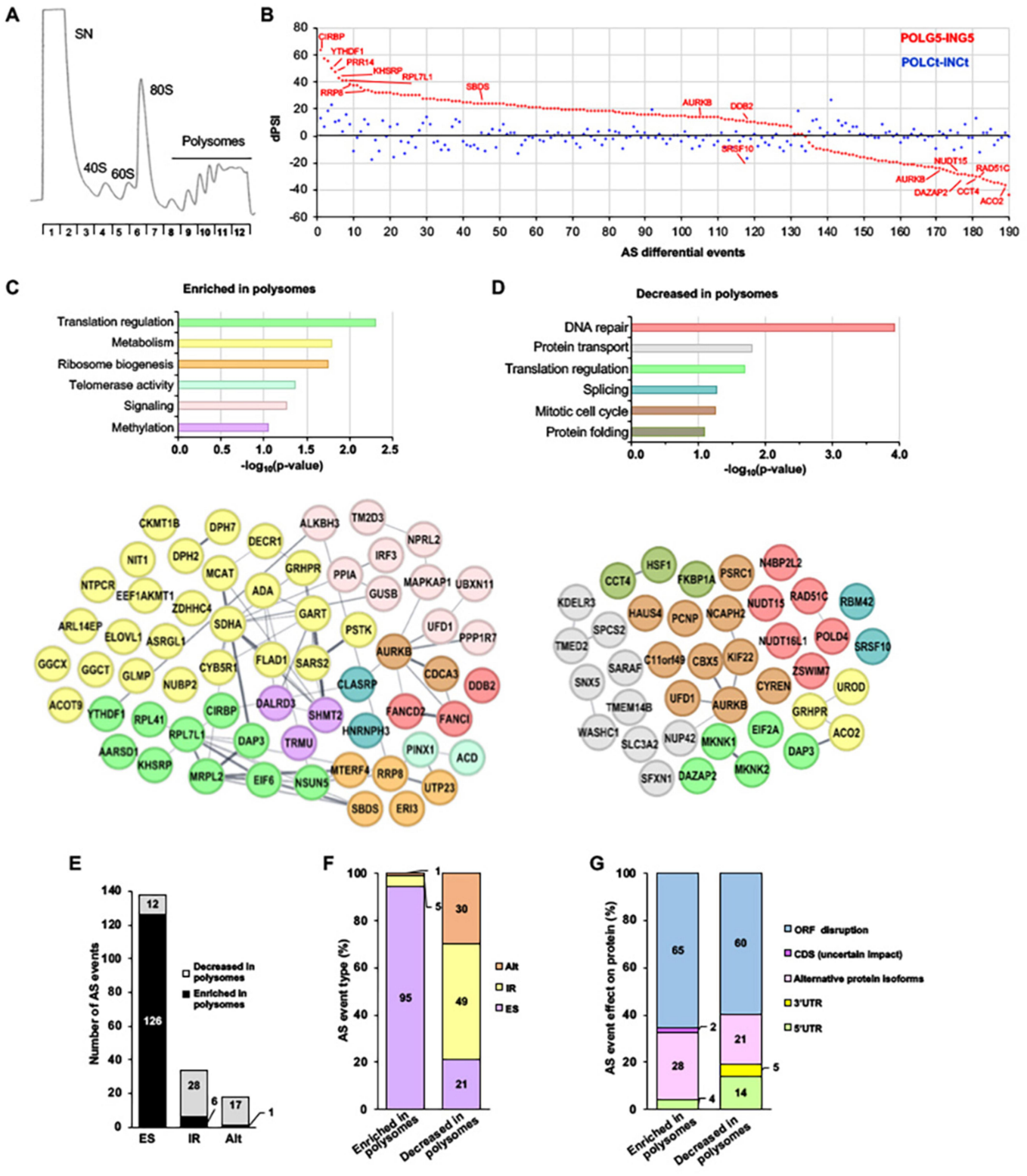

Figure 4. Analysis of AS events in mRNAs associated to polysomes. A) Representative example of a sucrose gradient profile obtained for one of the replicas used to isolate RNA from polysomes. B) PSI difference calculated for POLG5-ING5 and POLCt-INCt to determine the enrichment/decrease on polysomes of the AS differential events (190) detected in G5 vs Ct cells. Bar chart depicting the gene ontology terms and STRING protein-protein networks for the genes with AS events of the mRNAs enriched (C) or decreased in polysomes (D). E) Histogram representing the number of AS events (ES, IN, or alt) in mRNAs enriched/decreased in polysomes. Histograms depicting the percentage of each type of AS events (F) and the percentage of the predicted effects (G) in the mRNAs enriched/decreased in polysomes.

compared to control cells, computing the dPSI values obtained for POLG5 minus ING5 and for POLCt minus INCt. These data revealed that 134 AS events were preferentially enriched in polysomes (dPSI value up to 60), being CIRBP, YTHDF1, KHSRP and RRP8 among the ones showing major differences. The remaining 56 events were

downrepresented in polysome fractions compared to the Input (Figure 4B, red dots) (dPSI value down to −40), being AURKB, DAZAP2, CCT4, RAD51C, and ACO2 the ones showing major differences. In contrast, the dPSI values obtained for control samples (POLCt-INCt) ranged between −20 to +20, indicating no gross differences in any of the differential events (Figure 4B, blue dots). Thus, we conclude that the association of mRNAs containing AS events with polysomes observed after elevated expression of GEMIN5 depends on the individual RNA, exerting a positive or negative effect.

To search for patterns of sequence predicted to constitute RBPs binding sites, we analysed the presence of known human RBPs binding motifs in AS RNAs carrying ES events, comparing the upregulated (110) and downregulated (15) events in polysomes with the background events (16024), having a |dPSI| <2. The analysis returned 11 short RNA binding sites for known RBPs (splicing factors but also PABPC1 and PABPN1) which are significantly enriched (p value in flanking exons <0.01) in the upregulated ES events, and only 1 motif (for SRSF3) in the downregulated ES events (Supplementary Table 1). Next, to determine whether there was a relationship between mRNAs and AS events enriched or reduced in polysomes, we performed GO analysis. Interestingly, the polysome enriched mRNAs with AS events belong to networks related to Translation regulation, Metabolism, Ribosome biogenesis, Telomerase activity, Signaling, and Methylation (Figure 4C). In contrast, the AS events found in mRNAs with reduced levels in polysomes belong to networks of DNA repair, Protein transport, Translation regulation, Splicing, Mitotic cell cycle, and Protein folding (Figure 4D). Curiously, the Translation regulation network appears in both groups, although the genes found in each group are different. Other networks are exclusively detected in the upregulated or downregulated groups of AS events, although the same gene may be present in both cases due to the presence of several AS events, as exemplified by AURKB (Figure 4C,D).

Differential as events are engaged in polysomes depending on the type of event, irrespectively of the protein expression effect

Analysis of the types of AS events in the mRNAs associated with polysomes revealed that ES events were favoured (91%), as opposed to IR and Alt events that accumulated in the fraction decreased in polysomes (84% and 94%, respectively) (Figure 4E). Thus, a high percentage of AS events enriched in polysomes corresponds to ES, while the proportions are more similar among the types of AS events in mRNAs decreased in polysomes (Figure 4F). However, analysis of the effect produced by the AS event on protein expression (ORF disruption, alternative protein isoform, or 5′and 3 ′UTR changes) revealed different consequences. ORF disruption represented a very similar percentage in the events enriched or decreased in polysomes, 65% and 60%, respectively (Figure 4G). A similar result was observed for alternative protein isoforms (28% and 21%, respectively), while AS events affecting the 5′UTR was slightly favoured in mRNAs downrepresented in polysomes (14%) relative to those enriched in polysomes (4%). All together, these results convey the idea that AS events rendering ES, but not IR and Alt, are generally engaged in polysomes, irrespectively of the change produced in protein expression.

To deepen this observation, we calculated the difference in PSI observed for AS events in mRNAs associated to polysomes in cells overexpressing GEMIN5 with those observed in control cells (Figure 5A). In this case, there were no major differences in the AS events detected, suggesting a coordination between the negative and positive effects of the GEMIN5 protein. Furthermore, representation of the ratio for POLG5/ING5 with ING5/INCt for all the AS events showed an inverse correlation (R^2 0.847) (Figure 5B). These results reinforce the view that upregulation of GEMIN5 impacts on AS, yielding a preferential association of mRNAs containing ES events, but not IR or Alt, with polysomes.

Discussion

Our results support the idea that GEMIN5 protein levels play a role in sustaining cell homoeostasis. Consistent with this view, reduced levels of GEMIN5 confer a differential translation efficiency to selective groups of mRNAs [15], and variants reducing protein stability or inducing structural conformational changes are associated with neurological disorders [20–23]. Here, we show that elevated expression of GEMIN5 affects the inclusion of AS events, eventually rendering non-functional proteins. How the upregulation of GEMIN5 takes over this effect remains unknown. Among other possibilities, we hypothesize that the overexpression of GEMIN5 can jeopardize the assembly of the SMN complex, for instance, favouring the assembly of intermediate complexes with GEMIN3 and GEMIN4 [16]. On the other hand, the reported protein–protein interaction of GEMIN5-SMN [24] could hijack part of SMN in an unproductive complex.

GEMIN5 upregulation enhances differential as events in a variety of mRNAs

RBPs are critical determinants of the RNA-life cycle, from synthesis to degradation. Cumulative data have shown that dysregulation of a single RBP by altered levels, post-translation modifications or mutations result in mis-regulation of RNA processing, subcellular delocalization and/or anomalous aggregation of RBPs, altering the fate of multiple mRNAs and thereby leading to deficient cell growth [46,47]. We show that GEMIN5 upregulation leads to differential effects on alternative splicing, particularly affecting exon skipping events (Figure 2A). This effect was not observed after siRNA GEMIN5 silencing (Supplementary Figure 3a,b), indicating that downregulation of GEMIN5 impinges on other aspects of RNA life. Furthermore, the overexpression of GEMIN5 alters the relative abundance of mRNAs encoding SMN members (Supplementary Figure 3C), while silencing of GEMIN5 does not result in a statistically significant effect.

We noticed that the mRNAs carrying AS events generate protein variants with distinct functions, from cell fate determination to brain development. In addition, AS events can produce mRNA variants carrying pre-mature stop codons (PTCs)

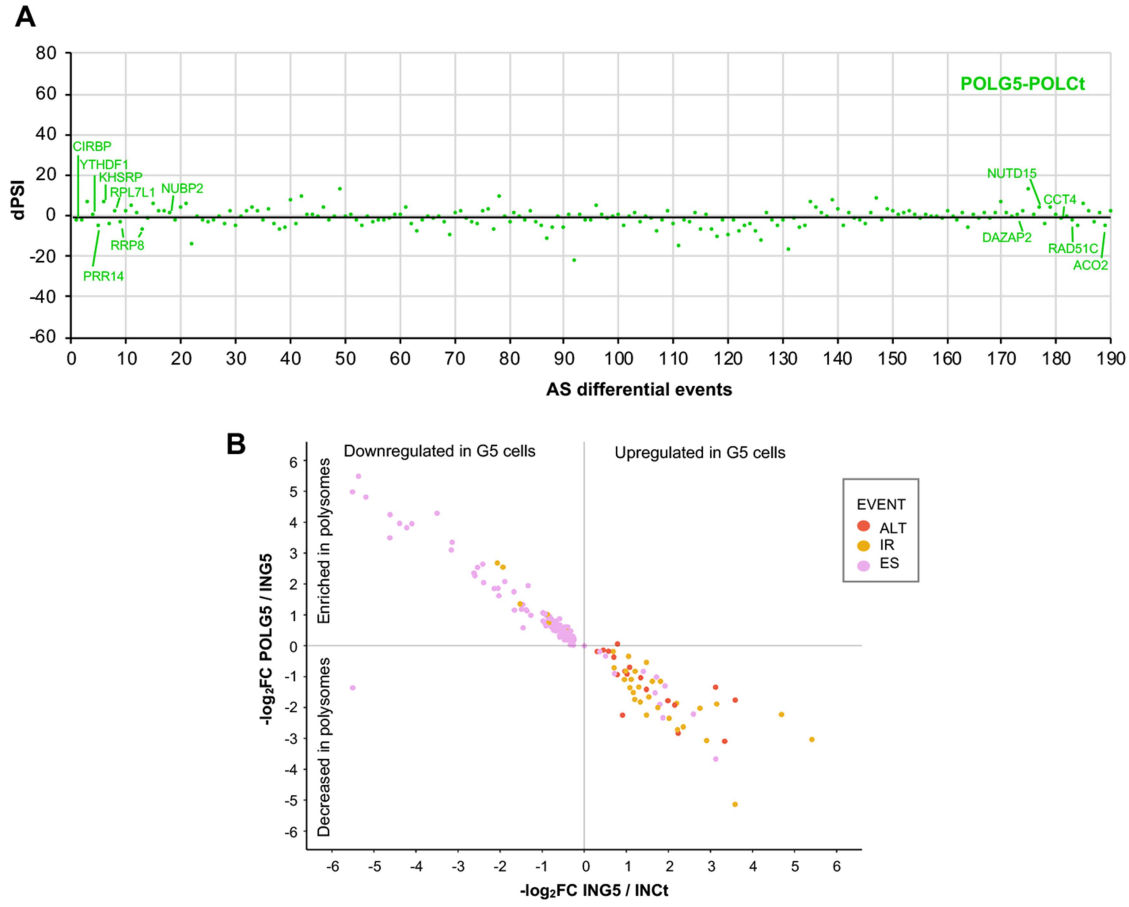

Figure 5. Comparative analysis of polysome association of mRNAs with AS differential events in cells with normal and high levels of GEMIN5. A) dPSI calculated for POLG5-POLCt of the AS differential events detected in G5 vs Ct conditions. B) PSI enrichment of AS events in polysomes depending on changes in input abundance and type of AS event.

that trigger degradation by nonsense-mediated RNA decay (NMD), preventing protein production [48]. Intron retention caused by splicing of cryptic introns or repression of splicing, leads to RNA decay by the nuclear RNA exosome [49]. Here, we show that the vast majority of the mRNAs containing differential AS events (65%) result in ORF disruption and therefore may render non-functional proteins. However, given that these AS events only take place on a fraction of the mRNA, the normal protein will be the major component in the cell lysate. Currently, the fate of the protein encoded by AS mRNAs remains unknown. A second category renders alternative protein isoforms (26%), while the predicted effects altering the 5′UTR only accounts for 7% (Figure 2E). An example of an IR event leading to alternative protein isoforms is encountered on the mRNA encoding KH-type splicing regulatory protein (KHSRP), a protein associated with worse prognosis in breast cancer patients [50]. KHSRP globally regulates the expression and AS events of MDA-MB-231 cells, specifically affecting cell cycle and DNA damage/repair pathway. Altogether, the differential AS events (either ES, IR or Alt) affect a large number of genes, many of them involved in nucleic acid metabolism, including DNA and RNA pathways.

Equally important, a significant number of AS events affect genes related to protein activation in response to different intracellular and external signals, pointing towards a key role of GEMIN5 as gene expression regulator. In support of this view, recent data showed that H913R mutant in GEMIN5 generate splicing defects presumably associated with its deleterious defect in SMN complex assembly, and thus in defective snRNPs biogenesis [24]. The latter study reported an overrepresentation of exon skipping among the total AS events, in agreement with our findings. Interestingly, it has been suggested that GEMIN5 regulates the expression of SMN [51] and *vice versa* [24], indicating that these proteins autoregulate each other, presumably keeping the balance needed to hold a permissive level of spliceosome components.

Differential as events in mRNAs engaged in polysomes encode proteins controlling cell growth

Our results show that a large fraction of mRNAs (70%) containing differential AS events are enriched in translationally active polysomes (Figure 4B). The remaining 30% are down-represented in polysomes, denoting a category of mRNAs likely silent or undergoing degradation, as reported for RNAs including retained introns that accumulate in nuclear bodies [52]. Moreover, the vast majority of the AS events enriched in polysomes correspond to ES events (Figure 5B). The observed inverse correlation between the abundance of AS events in the lysate and its polysome enrichment suggests the existence of a molecular mechanism that controls the

engagement of specific mRNA species in polysomes, consistent with an enrichment of binding sites for specific proteins in the upregulated AS RNAs (Supplementary Table 1). Consequently, the abundance of the AS events in POLG5 are comparable to those in POLCt despite the differences in the abundance of the mRNA isoforms. Nonetheless, this result reinforces the idea that GEMIN5 levels should be tightly regulated, as its downregulation compromises selective translation and its upregulation jeopardizes alternative splicing.

A group of genes showing AS events leading to ORF disruption encode proteins impacting key cellular processes, including splicing, ribosome biogenesis or RNA stability, therefore affecting distinct steps of cell proliferation. Among this group of genes, there are several networks enriched in polysomes (Figure 4C), suggesting that synthesis of the defective form of the protein may affect cell viability. These results, however, led us to hypothesize that the expression levels of *Gemin5* have been evolutionarily selected to balance the positive and negative effects on its diverse activities. Remarkably, ribosome biogenesis and mRNA transport and stability could be affected by AS events leading to ORF disruption in mRNAs encoding the ribosomal RNA processing 8 (RRp8) that silences rDNA in response to intracellular energy status [53]. Examples of alternative protein isoform are YTH N6-methyladenosine RNA binding protein 1 (YTHDF1) that recognizes the m(6)A modification site of mRNA enhancing mRNA stability [54], or the cold-inducible RNA binding protein (CIRBP), a stress-induced protein that activates the NF-κB pathway and stabilizes mRNAs encoding pro-inflammatory cytokines [55]. Other important processes affected by AS events upon GEMIN5 upregulation include metabolism, signalling, and methylation (Figure 4C).

A second group of genes containing differential AS events leading to ORF disruption appear less represented in polysomes, suggesting that the defective form of the protein will be down-represented relative to the functional version. Specifically, alternative splicing of mRNA encoding RAD51 paralog C (RAD51C) impacts on the DNA repair pathway, together with NUDT15 have implications on cell cycle and cell growth [56]. Conversely, aurora kinase B (AURKB) is a serine/threonine-protein kinase involved in the bipolar attachment of spindle microtubules to kinetochores and a regulator for the onset of cytokinesis during mitosis [57]. These results agree with the involvement of GEMIN5 in proliferation of zebrafish haematopoietic stem progenitor cells [58]. Differential AS events leading to 5′UTR changes include the chaperonin containing TCP1 subunit 4 (CCT4), a member of the chaperonin-containing T-complex (TRiC) that assists protein folding [59]. On the other hand, negative regulation of splicing may be affected via IR events of the serine/arginine splicing factor 10 (SRSF10) (Figure 4D). By controlling the alternative splicing of specific transcripts, SRSF10 has been involved in neurological processes, beyond cancer, glucose and cholesterol metabolism [60].

In summary, and despite the fact that ES events appear to be associated with ribosomes to a higher extent, there were no major differences in the percentage of genes showing ORF disruption. In addition, the broad range of mRNAs comprising AS events engaged in polysomes upon GEMIN5 upregulation supports the notion that GEMIN5 has evolved as a regulator of gene expression, consistent with its dual role as a member of the SMN complex and as a modulator of protein synthesis, ultimately impinging on cell homoeostasis.

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Author contributions

EMS and RFV designed the study. RFV performed the experiments. RFV and SA analysed the data. JAGM and JCO analysed the sequencing data and alternative splicing. EMS wrote the manuscript with comments from all authors.

Availability of data and material

The accession code for the FASTQ files and raw gene counts reported in this paper are deposited in GEO under the accession number GSE200388 and GSE261488. The data analysed during this study are included in the manuscript.

Consent to participate

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