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Spliceosomic profiling in pheochromocytomas and paragangliomas reveals alterations associated with clinico-molecular features and unveils RBM39 as a therapeutic target

Alejandro Ibáñez-Costa

b12ibcoa@uco.es

University of Córdoba https://orcid.org/0000-0003-4649-0095

Víctor García-Vioque

University of Córdoba

Sergio Pedraza-Arevalo

María Trinidad Moreno-Montilla

University of Córdoba

Francisco Quiñonero

University of Córdoba

Ester Arroba

Cristina Montero-Conde

Ricardo Blázquez-Encinas

Clara González-Pérez

Laura Gutiérrez-Camacho

Daniel Ruiz-Palacios

Guillermina del Río-Moreno

Virginia Albiñana

Luisa M. Botella

University of California at riverside, USA

Aura D Herrera-Martínez

María Gálvez-Moreno

Mercedes Robledo

Justo Castaño

1. Maimonides Biomedical Research Institute of Cordoba. 2. University of Cordoba. 3. Reina Sofia University Hospital. https://orcid.org/0000-0002-3145-7287

Article

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Abstract

Pheochromocytomas (PCC) and paragangliomas (PGL), collectively termed PPGL, are neuroendocrine tumors with a high metastatic potential and limited treatment beyond surgery. Alternative splicing emerged as a key regulatory layer across most cancers; however, its relevance in PPGL remains unexplored. We performed a comprehensive characterization of splicing machinery across two independent cohorts (n = 335), investigated splicing-related gene signatures associated with clinically relevant parameters, and evaluated the antitumor effects of splicing-related therapies in vitro and in vivo. The splicing machinery was broadly dysregulated in PPGL relative to normal tissue, with distinct profiles between PCC and PGL. Splicing machinery enabled accurate discrimination of molecular clusters and metastasis status. *CELF4* emerged as a discriminatory factor across all conditions analyzed. Furthermore, we identified a set of genes associated with survival, highlighting *RBM39* as an actionable candidate. In vitro studies demonstrated antitumor effects of Pladienolide B across different cell lines. *RBM39* silencing and targeted degradation with indisulam reduced proliferation and clonogenic capacity across cellular models and inhibited tumor growth in vivo. Splicing dysregulation represents a layer of biological heterogeneity in PPGL with direct clinical relevance. *CELF4* arises as a stratification biomarker, while RBM39 defines a therapeutic vulnerability, supporting splicing-targeted strategies as a therapeutic avenue for these tumors.

Background

Pheochromocytomas (PCC) and paragangliomas (PGL), collectively termed PPGL, are rare neuroendocrine tumors (NETs) originating from neural crest-derived cells of the adrenal medulla or from sympathetic and parasympathetic paraganglia, respectively [1]. These tumors are typically characterized by catecholamine hypersecretion, which leads to symptoms including the classic triad of headaches, palpitations, and diaphoresis, and may ultimately result in death [2]. Although these neoplasms usually grow slowly, they exhibit a metastatic rate of approximately 10–20%, which can reach up to 60% in PGL [3], clinical reality that underscores the urgent need for improved therapeutic strategies.

Notably, PPGL represent the human tumors with the highest degree of heritability, with approximately 40% of patients harboring germline mutations in one of the known PPGL susceptibility genes. When somatic mutations are also considered, up to 80% of patients carry alterations in these genes [4]. This genetic characterization has enabled the classification of PPGL into three molecular clusters based on the pathway in which the mutated genes participate: kinase signaling, pseudohypoxia, and Wnt signaling [5, 6].

In line with these efforts, several multi-omics studies have been conducted in recent years to improve the understanding of PPGL and their tumor microenvironment, with the goal of identifying therapeutic vulnerabilities to improve patient outcomes [7–11]. Unfortunately, these findings have not yet been effectively translated into clinical practice, and to date, surgical resection remains the only curative option [12, 13].

Given this background, the pronounced genetic and molecular heterogeneity of PPGL suggests the existence of additional regulatory layers that contribute to defining the functional and phenotypic traits of these tumors. Among these layers, alternative splicing has emerged as one of the most therapeutically promising molecular mechanisms in cancer over recent years [14–16]. It is a fundamental process in cellular homeostasis, and its dysregulation is observed across nearly all tumor types, promoting tumorigenesis and being linked to the different characteristics considered hallmarks of cancer [17]. Furthermore, splicing alterations have been shown to influence therapeutic responses and contribute to the development of resistance to anticancer treatments [18]. For these reasons, several inhibitors have been tested as anticancer therapies, with some having progressed to Phase I trials, where significant toxicity was demonstrated [14, 19, 20]. Despite this, there is no information in PPGL concerning dysregulation of this machinery beyond the identification of a few splice variants or splicing-site mutations in susceptibility genes in some patients [21–25], representing a critical knowledge gap with direct implications for patient management.

Therefore, considering the potential of spliceosomic studies for the development of therapeutic strategies in PPGL, the main hypothesis of this study is that the splicing machinery may be altered in PPGL, potentially in a tumor type-specific manner, and that this dysregulation may represent a target for the development of novel treatments against these neoplasms. Accordingly, the main objective of this study is to comprehensively characterize splicing in PPGL to enhance our understanding of their molecular landscape and, ultimately, to identify molecules whose targeting may contribute to effective interventions against this complex disease.

Methods

Patients and samples

This study includes two RNA-seq datasets from PPGL tumors. The first dataset, referred to as the CNIO cohort, comprises 162 primary PPGL samples (90 PCC and 72 PGL), processed and analyzed as previously described [8]. RNA-seq FASTQ files generated are deposited in the European Genome-Phenome Archive (EGA) under the accession EGAS00001006043 and EGAS00001006044. Request for data access will be referred directly to the Data Access Committee (DAC) of the CNIO (mrobledo@cnio.es). The access will be granted for health/medical/biomedical purposes and according to good practice recommendations.

The second dataset, referred to as the TCGA cohort, comprises 173 PPGL samples (144 PCC and 29 PGL) obtained from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/). Samples classified as cortical admixture were excluded from molecular cluster analyses. Clinical information and RSEM-normalized mRNA expression data were retrieved from the cBioPortal [6]. Gene expression data from normal adrenal gland tissue were obtained from the GTEx platform [26], to enable comparison with tumor samples. To ensure cross-dataset comparability, batch effects were corrected using the *ComBat* method [27] implemented in the *sva* R/Bioconductor package [28] v3.46.0 on $\log_2$-transformed

expression values. Clinicopathological data of all cohorts analyzed in this study are described elsewhere [29].

All experiments were conducted in accordance with the ethical principles outlined in the Helsinki Declaration and were approved by the University of Cordoba/IMIBIC and the Ethics Committees of the participating hospitals and universities (protocol code PI19/01603, v1–13/03/2019). Written informed consent was obtained from all patients.

Cell lines and reagents

For the functional assays, we employed the human female-derived neuroblastoma cell line SK-N-AS, including both wild-type (WT) and *SDHB* knocked-down (KD) (RRID:CVCL_1700) kindly provided by Dr. Massimo Manelli (University of Florence, Florence, Italy) [30]; the male rat PCC-derived cell line PC-12 Adh (RRID:CVCL_F659) generously provided by Dr. M. M. Malagon (University of Cordoba, Cordoba, Spain); and the human patient-derived cell lines PGL1 and PGL7, kindly provided by Dr. L. M. Botella (CSIC, Madrid, Spain). PGL1 was derived from a primary pelvic PGL from a 34-year-old male patient harboring an *SDHB* mutation, whereas PGL7 was derived from a metastatic cervical PGL from a 19-year-old male patient carrying an *SDHB* mutation [heterozygous 5-bp deletion in exon 2 (c.166_170delCCTCA)].

SK-N-AS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM 4.5 g/L glucose, Corning, Arizona, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Madrid, Spain), 2mM L-glutamine (Sigma-Aldrich), 1% antibiotic-antimycotic and G418 (0.2 mg/mL) (Gibco, Madrid, Spain), the latter used to maintain the selection of KD cells. The PC-12 Adh cell line was cultured in DMEM 4.5 g/L glucose supplemented with 10% FBS, 5% horse serum (HS, ThermoFisher Scientific, Waltham, MA, USA), 2 mM L-glutamine and 1% antibiotic-antimycotic. PGL1 and PGL7 were cultured in RPMI-1640 medium (Cytiva, Barcelona, Spain), supplemented with FBS at 20%, L-glutamine at 1%, antibiotic/antimycotic at 0.2% and MycoZap Plus-CL at 0.2% (Lonza, Basel, Switzerland).

Pladienolide B was purchased from Santa Cruz Biotechnology (Heidelberg, Germany), while indisulam was acquired from MedChemExpress (Sollentuna, Sweden). Both were resuspended in dimethyl sulfoxide (DMSO) and used in the 10^{-6}-10^{-10} M range. Preliminary dose-response experiments were performed at 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M for all compounds to determine the most appropriate dosage for our models, resulting in the selection of 10^{-9} or 10^{-8} M for Pladienolide B and 10^{-6} M for indisulam, chosen on the basis of concentrations associated with the most consistent and statistically significant reductions in cell viability.

RNA isolation, reverse transcription and quantitative real-time PCR (qPCR)

Total RNA was isolated from cell seeded at 250,000 cells per well in 6-well plates using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Madrid, Spain), followed by DNase treatment (Promega, Barcelona, Spain). RNA concentration and purity were assessed by NanoDrop Spectrophotometry, and 1µg of RNA was reverse-transcribed to cDNA employing the RevertAid RT Reverse Transcription Kit (ThermoFisher Scientific, K1691) in a final volume of 20 µL. Gene expression was subsequently quantified by qPCR using the Brilliant III SYBR Green-Master Mix (Stratagene, La Jolla, CA, USA) in the CFX Duet Real-Time PCR System (Bio-Rad Laboratories, Madrid, Spain), as previously reported [31]. Primer sequences are listed in Supplemental Table 1. Expression levels were normalized to two housekeeping genes (*ACTB* and *GAPDH*), using geNorm software [32].

Silencing of RBM39 in vitro

SK-N-AS WT, SK-N-AS *SDHB* KD, PGL1 and PGL7 cells, were transiently transfected with a siRNA targeting *RBM39* (ThermoFisher Scientific, Cat#4392420) to achieve gene-specific knockdown. Silencer Select Negative Control siRNA (ThermoFisher Scientific) was used as a control. Briefly, 250,000 cells were seeded in 6-well plates and transfected with 25 nM siRNA using Lipofectamine RNAiMAX reagent (ThermoFisher Scientific) at 37 ºC, following manufacturer's protocol.

Measurement of cell viability

Cell viability in response to treatment with Pladienolide B, indisulam, or *RBM39* silencing was evaluated using the Alamar Blue fluorescent assay (Thermo Fisher Scientific), as previously described [33]. Cells were seeded into 96-well plates at a density of 5,000 cells per well and maintained under serum-starved conditions for 12 h. Cell viability was then measured every 24 h up to 72 h in medium containing 5% FBS using a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA). Results were expressed as a percentage relative to control cells treated with the corresponding vehicle or Silencer Select Negative Control siRNA, depending on the experimental condition.

Measurement of colony formation

To evaluate the effect of the different treatments or *RBM39* silencing on the clonogenic capacity of the cell lines tested, 4,000 cells per well were seeded in 6-well plates following 24 h of treatment. Cells were maintained in culture for 10 days, with medium replacement every 3 days. At the end of the incubation period, the medium was removed, colonies were gently washed with PBS, and subsequently stained with a crystal violet solution (0.5% crystal violet and 6% glutaraldehyde) for 30 min, followed by air drying. Images were then analyzed using ImageJ v.1.53 (NIH, Bethesda, MD, USA, RRID:SCR_003070) to quantify the number of individual colonies.

Measurement of cell migration

The impact of drug treatment or *RBM39* silencing on the migratory capacity of the model cell lines was assessed using the wound-healing assay, as previously described [34]. To this end, 40,000 cells per well were seeded in 96-well plates and incubated for 2 h in serum-free medium once cells reached full confluence. After that, a wound was generated in each well using a sterile 100 µL pipette tip. Wells were washed with PBS, and cells were incubated for 6 h (for PGL1 and PGL7) or 24 h (for SK-N-AS and PC-12 Adh) in serum-free medium. Images of the wounds were captured using a Moticam 4000 system (Motic Europe, Barcelona, Spain) and analyzed with ImageJ software. Wound closure was quantified relative to the wound area immediately after wound formation.

Measurement of tumorspheres formation

Tumorsphere-forming capacity was evaluated following treatment with pladienolide B in SK-N-AS and PC-12 Adh cells. For SK-N-AS cells (WT and *SDHB* KD), 10,000 cells were seeded in Corning Costar ultra-low attachment 24-well plates and cultured in DMEM/F-12 medium (Gibco) for 10 days. For PC-12 Adh cells, 5,000 cells were seeded in Corning Costar ultra-low attachment 24-well plates and maintained in DMEM/F-12 medium (Gibco) supplemented with EGF (20 ng/mL), FGF (20 ng/mL), and 1% B-27 (ThermoFisher Scientific). At the end of the incubation period, images were acquired to visualize and quantify the area of the resulting tumorspheres using ImageJ software.

Analysis of alternative splicing by conventional PCR

To assess changes in alternative splicing variants in response to indisulam treatment, conventional PCR was performed using cDNA derived from SK-N-AS WT and *SDHB* KD cell lines. For each reaction, cDNA was used as template (50 ng) and amplified using the Thermo Scientific PCR Master Mix (Thermo Fisher Scientific), following the manufacturer's instructions. Primers specific for alternatively spliced variants of DNA repair genes were selected based on previously published studies [35] and are listed in Table 1. Amplification was carried out using the T100 Thermal Cycler (Bio-Rad Laboratories). PCR products were subsequently resolved by electrophoresis on 2% agarose gels containing 1:20,000 RedSafe Nucleic Acid Staining Solution (iNtRON Biotechnology, Seongnam, South Korea). Gel images were acquired using the ChemiDoc XRS+ System (Bio-Rad Laboratories) and analyzed with ImageJ (version 1.53) to quantify band intensity.

Table 1
List of the primers used in PCR experiments.

Gene	Primer sequence (forward)	Primer sequence (reverse)	Product size (bp)
ATM (exon 51–54)	TGCCTCTTATGTA CCAATTGGCT	AATTGGCTGGTCTG CTGGAA	410, 251, and 112
BRCA1 (exon 7–10)	AACTCTGAGGACA AAGCAGC	CTGTAATGAGCTG GCATGAGT	364 and 241
RAD51D (exon 3–6)	TGGCTCAGTTCTC GGCTTT	AGATGTCAAATGCA TGCACCA	354, 219 and 134
RIF1 (exon 21–23)	AGGTGGGCAAAA CTGGTCAT	TGGGTGCTCCACT ACGAAAT	316 and 223

In vivo assay

A total of 10^6 SK-N-AS WT and *SDHB* KD cells were injected subcutaneously in each flank of 7-week-old male BALB/cAnNRj-Foxn1nu mice (Janvier Labs, Le Genest Saint-Isle, France; n = 16 mice), resuspended in 100 µL of Matrigel Matrix (Corning) and DMEM 4.5 g/L glucose supplemented with 10% FBS (1:1 ratio). After four days, when the tumors became palpable, mice were randomly assigned to two homogeneous groups (n = 8) based on body weight. The control group received a daily intraperitoneal (i.p.) administration of the vehicle: 90% saline, 6.5% Tween 80 (MedChemExpress) and 3.5% DMSO (Sigma Aldrich). Meanwhile, the treated group was i.p. administered with 25 mg/kg indisulam. Both groups were daily treated for 5 consecutive days. The selected dosing regimen was based on previous reported in vivo assays [36]. Tumor growth was monitored thrice per week for 3 weeks by using a digital caliper. At the end of the experiments, animals were euthanized 24 h after completion of the treatment schedule. Body weight was also recorded throughout the study to ensure animal well-being.

All experimental procedures were carried out following the European Regulations for Animal Care, in accordance with guidelines and regulations, and under the approval of the University of Córdoba Research Ethics Committee (ref. 23/09/2022/115).

Statistic and bioinformatic analysis

Data normality was first assessed using the Kolmogorov-Smirnov test. Statistical differences between groups were evaluated using an unpaired parametric Student's t-test or the nonparametric Mann-Whitney U test, depending on data distribution. For in vivo tumor growth assay, a 2-way ANOVA mixed effects with Geisser-Greenhouse correction were used, correcting the multiple comparisons with a Tukey pos-hoc test. Partial least squares-discriminant analysis (PLS-DA) and variable importance in projection (VIP) score analyses were performed to identify the most discriminative genes among the different sample groups. These analyses, together with unsupervised hierarchical clustering heatmaps, were conducted

using MetaboAnalyst 6.0 [37]. RStudio (v4.2.2) was used for survival and relapse analysis (package *survival* v3.4.0); enrichment analyses of the RNA-seq data were performed using *fgsea* v1.24.0 package and the hallmark gene set from the Human MSigDB Collections (GSEA H gene set). At least three independent biological replicates were included for all in vitro experiments. Data are expressed as mean ± standard error of the mean (SEM), as fold change (log_2) or relative levels compared with the corresponding controls (set at 100%). Statistical significance was set at $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, $****p < 0.0001$. Statistical analyses were performed using Prism v9.0 (GraphPad, La Jolla, CA, USA, RRID:SCR_002798).

Results

Splicing machinery expression differs between PCC, PGL and healthy tissues.

Comprehensive evaluation of the splicing machinery in PPGL across two independent RNA-seq cohorts, (CNIO, n = 162; TCGA, n = 173) revealed a remarkable clustering of tumor types based solely on the expression of 25 splicing machinery-related genes (Fig. 1a), demonstrating that spliceosomic profiles alone are sufficient to distinguish these two tumor entities. PLS-DA and VIP score analyses demonstrated a substantial discriminatory capacity between PCC and PGL, with eight genes consistently identified across both cohorts as major contributors: *CELF4, SNRNP, MAGOHB, NCBP1, DDX20, SMC1A, HNRNPA1*, and *SNRPB2* (Fig. 1b). Among them, *CELF4* showed the most consistent discriminatory capacity, with significant higher expression in PCC and an area under the curve (AUC) close to 0.8 in each dataset (Figs. 1b **and c**), supporting its prioritization as a candidate biomarker. Collectively, these findings support the notion that, from a spliceosomic perspective, PCC and PGL constitute two molecularly distinct entities, warranting their separate consideration in subsequent analyses.

Comparison of splicing machinery expression between tumor tissues and healthy adrenal gland samples revealed a profound and clinically meaningful dysregulation in both tumor types. Analysis of PCC identified 26 altered components relative to normal tissue, whereas PGL exhibited 57 significant expression changes when compared with control samples derived from the GTEx dataset (Fig. 1d). Thirteen genes were consistently dysregulated across both tumor types, with strikingly opposite directions of change between PCC and PGL. This shared divergent set comprised *CELF4*, *DDX20*, *DHX38*, *DNAJC8*, *DQX1*, *HMX2*, *HNRNPA0*, *LSM6*, *RBM22*, *RNF113B*, *SNRPB2*, *SNRPN*, and *SRRM4*. Notably, *CELF4* emerged again as the most consistently altered gene across all conditions, further supporting its relevance as a potential stratification tool in PPGL.

Splicing machinery expression profiles vary across molecular PPGL clusters

Analysis of splicing machinery expression across PPGL molecular clusters revealed marked differences in spliceosomic profiles between tumor subgroups. In PCC, a clear separation was observed between samples belonging to the kinase signaling and pseudohypoxia clusters. In contrast, tumors assigned to

the Wnt signaling cluster displayed a more interspersed distribution, with no evident segregation (Fig. 2a). In PGL, for which only two molecular clusters are defined, this distinction was apparent exclusively in the CNIO cohort, likely reflecting the limited sample size compared with PCC (**Figure S1a**).

PLS-DA and VIP score analyses consistently identified *CELF4* as one of the most influential contributors to cluster discrimination in PCC, an effect observed in the CNIO cohort (Figs. 2b and S1b). When the analysis was restricted to genes exhibiting significant expression differences across all molecular groups, tumor types, and cohorts, *CELF4* emerged again as the only gene common to all conditions. In PGL, six additional genes were found to be consistently shared between cohorts, including *DHX16* and *PRPF3* (Figs. 2b and S1c).

Consistently, ROC curve analyses confirmed the strong discriminatory potential of CELF4 across virtually all pairwise comparisons in both tumor types and cohorts, with the single exception of the kinase signaling *vs*. Wnt signaling comparisons in PCC within the CNIO cohort. Remarkably, this factor displayed a strong discriminatory potential between molecular clusters, with AUC values exceeding 0.93 for comparisons between kinase signaling and pseudohypoxia groups in PCC and PGL from the TCGA cohort, as well as in PGL from the CNIO cohort, and an AUC approaching 0.96 for the comparison between pseudohypoxia and Wnt signaling clusters in PCC from the TCGA cohort (**Figures S2a** and **S2b**). These values are well above conventional thresholds for clinically useful biomarkers, positioning *CELF4* as a strong candidate for molecular stratification in PPGL.

Splicing machinery patterns are associated with metastatic status

The high metastatic potential of these tumors prompted us to examine whether splicing machinery expression could discriminate between metastatic and non-metastatic tumors, a clinically critical distinction given the limited therapeutic options available for metastatic disease. Analysis of the CNIO cohort identified gene expression signatures comprising 28 and 33 genes that discriminated with high accuracy between metastatic and non-metastatic tumors in PCC and PGL, respectively (**Figures S3a** and **S3b**). The low frequency of metastatic cases in the TCGA cohort limited the applicability of this approach in that dataset.

CELF4 emerged once again as a key contributor to group separation in PCC and as the top discriminative gene in PGL. Despite the limitations of the TCGA cohort, partial overlap was observed, with two shared genes in PCC, *PRPF6*, which was more highly expressed in non-metastatic tumors, and *USP39*, which showed increased expression in metastatic samples; and one shared gene in PGL, *CLNS1A*, also displaying higher expression in the metastatic group (**Figures S4a** and **S4b**). The recurrent identification of *CELF4* across tumor types, molecular clusters, and now metastatic status consolidates its potential as a clinically relevant stratification biomarker in PPGL.

The splicing factor RBM39 is associated with patient survival and represents a therapeutically actionable target

Having pinpointed the overt dysregulation displayed by splicing machinery in PPGL, we next assessed its prognostic relevance, a question with direct implications for patient management. An appreciable proportion of splicing-related genes showed a significant association between their expression levels and patient outcome in both PCC and PGL (Figs. 3a and 3b). Focusing on core spliceosome components or splicing factors for which pharmacological inhibitors are currently available, including *SF3B1*, *RBM39*, *PRMT1*, *PRMT5*, *BRD2*, *BRD3*, and *BRD4*. Among these candidates, *RBM39* emerged as the only factor whose higher mRNA expression was significantly associated with poorer prognosis in both tumor types (Figs. 3a, 3b, S5 and S6). Specifically, in PCC, a hazard ratio (HR) of 58.17 was observed when comparing high versus low expression ($p = 0.0024$), whereas in PGL the HR reached 101.9 under the same conditions ($p = 0.0187$), values that rank among the strongest prognostic associations reported for any single molecular marker in these tumors.

Functional enrichment analyses performed by stratifying samples according to *RBM39* expression levels further supported its biological and clinical relevance. In both PCC and PGL, high RBM39 expression was coupled to enrichment of gene sets related to the G2M checkpoint and E2F targets in PPGL, as well as mitotic spindle-associated genes in PGL. In contrast, low *RBM39* expression was accompanied by enrichment of gene sets associated with coagulation, epithelial-mesenchymal transition, inflammatory response, oxidative phosphorylation, allograft rejection, and interferon-gamma response. Together, these findings position RBM39 not only as a prognostic biomarker but as a biologically grounded therapeutic target in PPGL.

Pharmacologic inhibition of the splicing process exerts antitumor effects in PPGL in vitro models

To evaluate the therapeutic potential of splicing inhibition, we treated SK-N-AS WT, SK-N-AS *SDHB* KD, and PC-12 Adh cell lines with the splicing inhibitor Pladienolide B. Dose-response experiments resulted in highly similar outcomes across the three cell lines. Exposure to Pladienolide B concentrations of 10^{-9} M or above induced a strong, dose-dependent, reduction in cell viability across all three lines, particularly at 72 h, resulting in > 80% decrease in viability (Fig. 4a). While reductions in viability were already numerically evident at 24 h (3–40 % decrease), statistical significance at this time point was observed only in PC-12 Adh cells and at a 10^{-6} M dose in SK-N-AS *SDHB* KD cells. Importantly, the 10^{-9} M concentration of Pladienolide B emerged as the minimal effective dose across all three cell lines, with progressive and significant effects from 48 h onward in SK-N-AS WT and *SDHB* KD cells (20–30% reduction in viability) and reaching maximal effects at 72 h in all evaluated lines (approximately 40% reduction in SK-N-AS cells and 10% in PC-12 Adh) (Fig. 4b).

Consistent with the observed reductions in cell viability, we next assessed the functional consequences of splicing inhibition on clonogenic potential and migratory capacity in SK-N-AS WT, *SDHB* KD, and PC-12 Adh cells. Clonogenic potential, a functional parameter that reflects both the proliferative and stem-like properties, was markedly reduced by Pladienolide B across all three lines, with decreases of approximately 35% in PC-12 Adh, 45% in SK-N-AS WT, and 55% in SK-N-AS *SDHB* KD cells (Fig. 4c).

Likewise, Pladienolide B impaired migratory capacity in the three cell lines studied, with reductions of above 5 % in SK-N-AS WT, 0 % in PC-12 Adh, and 5 % in *SDHB* KD cells (Fig. 4d).

Interestingly, among the parameters tested, tumorsphere size, which serves as a measure of stem-like properties in cancer cells, showed the most pronounced phenotypic differences in response to treatment. Thus, after 10 days in culture, while SK-N-AS WT cells showed no significant changes compared with controls, Pladienolide B caused a 40% reduction in sphere size *SDHB* KD cells, and a 80% reduction in PC-12 Adh cells (Fig. 4e).

We next investigated whether the antitumor effects of Pladienolide B observed in established cell lines models were also observable in patient-derived PGL lines. In PGL7 cells, 10^{-9} M Pladienolide B reduced viability by 25% at 48 and 72 h, whereas PGL1 cells did not show a similar response. In contrast, 10^{-8} M Pladienolide B clearly decreased viability in both lines, causing a 25% reduction in PGL1 at 48 h that persisted at 72 h, and a marked inhibition at 24 h in PGL7, that reached 50% at 48 and 72 h (Fig. 4f). In line with the observed cell viability, evaluation of colony-forming ability indicated that 10^{-8} M Pladienolide B markedly blunted the clonogenic potential of PGL1 and PGL7 cells, reducing it by up to 80%. In contrast, although a 10^{-9} M dose of the drug did not cause any consistent, significant change, lower concentration of 10^{-10} M (which did not impact overall viability), evoked a moderate but significant reduction in clonogenic capacity, in both cell lines (Fig. 4g).

RBM39 emerges as an actionable target in PPGL

Given the strong association between *RBM39* expression and patient survival, we next sought to explore its functional role in representative PPGL disease models. Transient silencing of *RBM39* using a specific siRNA at 25 nM achieved significant and precise knockdown across all tested cell lines (Fig. 5a). As depicted in Fig. 5b, event modest reductions of *RBM39* levels (e.g. in *SDHB* KD cells) resulted in appreciable functional effects across all lines. In particular, *RBM39* silencing caused a consistent, long-lasting (up to 72 h) reduction in cell viability in both SK-N-AS WT and *SDHB* KD cells, which was also observed in patient-derived PGL1 and PGL7 cells, where, however, the effect appeared to be more transient and was not present at 72 h (Fig. 5b). Analysis of colony-forming capacity revealed a similar trend, where *RBM39* silencing led to notable reductions in SK-N-AS WT and still appreciable in *SDHB* KD cells, whereas the effect was less pronounced, but still significant, in PGL1 and was not present in PGL7 cells (Fig. 5c).

The efficacy of *RBM39* silencing prompted us to evaluate the effects of indisulam, a pharmacological inhibitor that induces RBM39 degradation and is currently under clinical investigation. Firstly, we examined its possible involvement in deregulate several DNA damage repair genes as *ATM*, *BRCA1*, *RAD51D* and *RIF*, whose splicing has been reported to be regulated by RBM39 [35]. Specifically, we evaluated whether indisulam treatment affected the splicing of these transcripts in SK-N-AS cells. Indeed, PCR analysis revealed an increase abundance of skipped exon (SE) isoforms following indisulam treatment compared with control conditions in both cell models (Fig. 5d). Interestingly, the splicing

alterations were more pronounced in the WT cell line than in *SDHD* KB cells. In WT cells, the proportion of SE isoforms was significantly increased relative to control cells for all four genes analyzed. Moreover, in each case, the proportion of SE isoforms in treated cells was approximately two-fold higher than in untreated cells, with the most marked effect observed for RIF1, where SE transcripts increased nearly five-fold. On the other hand, splicing alteration appeared less pronounced in SK-N-AS *SDHB* KD cells. While significant differences were detected for ATM, BRCA1, and RIF1, the magnitude of the increases in SE isoforms was more modest than those observed in WT cells. As in SK-N-AS WT cells, the largest change was observed for RIF1, where the proportion of SE isoforms in indisulam treated cells approximately doubled compared with control cells (Fig. 5e).

RBM39 degradation inhibits tumor growth in PPGL in vivo models

After discovering the importance of RBM39 in cellular splicing, we used the drug indisulam to test its effectiveness in vitro and in vivo. Treatment with this compound elicited appreciable effects that were variable depending on the cell line, and drug dose and exposure time tested (Fig. 6a). In SK-N-AS WT cells, indisulam induced a dose- and time-dependent reduction in viability, that was most pronounced at concentrations ≥ 10^{-7} M and after longer incubation times (72 h). In contrast, the response of *SDHB* KD cells was virtually absent, except for an isolated reduction after 24 h incubation with 10^{-10} M indisulam. In PC-12 Adh cells, all indisulam doses (excluding 10^{-9} M) significantly decrease viability in a time-dependent manner, causing a more consistently effect at 72 h. Of note, patient-derived PGL1 and PGL7 lines showed clear responses to indisulam, although the latter displayed a heterogeneous pattern. In PGL1, lower concentrations of indisulam (10^{-10}, 10^{-9} M) significantly reduced viability at 24 h, whereas doses at ≥ 10^{-8} M decreased viability more steadily and for longer periods. The response of PGL7 was more variable, with indisulam causing more pronounced, significant reductions in viability at shorter incubation times (24 h) except at the higher dose tested (10^{-6} M, 72 h).

After stablishing the potential in vitro effects of indisulam, we next sought to determine the impact of this treatment on in vivo tumor growth using mouse xenograft models derived from SK-N-AS WT and *SDHB* KD cells (Fig. 6b). Our results showed high tumor growth in the WT line (Fig. 6c), whereas growth in the *SDHB* KD tumors was significantly lower (Fig. 6d). Treatment with 5 consecutive cycles of indisulam produced marked inhibition of tumor growth, which showed no significant differences after 2 weeks in both cell models and decreased in the final days. Final tumor removal revealed significant differences in tumor weight in the WT line ($p < 0.01$), with no such statistical difference observed in the *SDHB* KD line (Fig. 6e). Furthermore, weight monitoring confirmed the absence of systemic toxicity in the mice treated with the drug (Fig. 6f).

Discussion

Alternative splicing has emerged as one of the most actively investigated regulatory processes in cancer biology, driven by the need to unravel the complex networks underlying RNA processing and to better define their role in in tumor development and progression [38, 39]. Among the diverse cancer types

wherein splicing dysregulation plays a prominent role [40–42], its contribution to PPGL biology had remained, until now, largely unexplored, a gap that this study directly addresses.

In line with this, multiple clinical trials are ongoing, mainly focused on small molecules targeting protein arginine methyltransferases and kinases involved in splicing regulation [16]. However, in contrast to these recent advances, the contribution of splicing and its potential dysregulation to the biology of PPGL remains largely unexplored.

The results presented here provide the first comprehensive characterization of the splicing machinery landscape in PPGL, revealing a previously unrecognized layer of molecular heterogeneity with direct clinical implications. Interestingly, spliceosomic profiles alone were sufficient to distinguish PCC from PGL across two independent cohorts, an aspect not commonly addressed in previous multi-omics studies, in which both tumor types have been typically analyzed as a single entity [43]. This finding is particularly significant because it suggests that the biological differences between PCC and PGL extend beyond the genomic and transcriptomic features previously described, and that splicing-based analyses can uncover robust patterns capable of capturing layers of tumor heterogeneity that are often difficult to resolve using other molecular features alone [44, 45]. The biological meaning and pathologic significance of these splicing-related differences between PCC and PGL are still unknown and warrant further investigation, but their consistency across independent cohorts already supports their potential clinical utility.

Beyond distinguishing tumor types, the expression profile of the splicing machinery was profoundly altered in both PCC and PGL relative to normal adrenal tissue, a feature widely described across multiple cancer types [17], and previously reported by our group in other types of NETs [31, 46, 47]. This global dysregulation with respect to non-tumoral tissue was not clinically silent: splicing machinery expression patterns provided high-accuracy discrimination between metastatic and non-metastatic cases, an observation that may help explain recent studies showing that metastatic PPGL are enriched for splicing-related annotations in Copy Number Alteration (CNA) analyses [8]. Actually, the involvement of splicing in metastatic progression is well documented in multiple cancers, where it can act either through widespread splicing dysregulation or via alterations in specific splicing factors that drive invasive and metastatic phenotypes [48–53]. Our findings extend this paradigm to PPGL and suggest that splicing-related features may complement current staging approaches and contribute to improved identification of patients at risk of metastatic disease.

Among the factors identified in our study, CELF4, a member of the CUG-BP and Elav-like (CELF) family of RNA-binding proteins [54], emerged as particularly relevant, displaying consistently dysregulated across both tumor types and repeatedly contributing to sample discrimination according to clinical and molecular features. Its discriminatory performance was exceptional: AUC values exceeding 0.93 in multiple comparisons and approaching 0.96 in the pseudohypoxia vs. Wnt signaling cluster comparison in PCC, place *CEL*F4 well above the thresholds conventionally considered clinically useful for a stratification biomarker. Consistent with our findings, *CELF4* has been previously described as a

diagnostic and prognostic biomarker in endometrial and colorectal cancer [55–57]. Moreover, our present observation nicely agrees with and strongly reinforces our previous studies in pancreatic NETs, where CELF4 was shown not only to be dysregulated, but also mechanistically involved in tumor aggressiveness and in the therapeutic response of these tumors to first line treatment drugs [58].

The strong discriminative capacity of *CELF4* across the different molecular clusters defined in PPGL, with particularly divergent profiles between the pseudohypoxia and kinase signaling clusters, arises an important and clinically relevant question: do these splicing differences arise predominantly as downstream consequences of the molecular alterations that define each cluster, or do they actively participate in establishing and reinforcing cluster identity? Within this framework, well-characterized examples illustrate how splicing regulation can intersect with tumor metabolism, such as the coordinated control of PKM mRNA splicing by factors including hnRNPA1/hnRNPA2, PTB/nPTB, and SRSF3 [59]. These regulators modulate the expression of PKM isoforms, favoring upregulation of PKM2 in many tumor types, a switch that promotes a metabolic shift toward aerobic glycolysis and supports tumor growth and progression [60]. Conversely, hypoxic conditions, including pseudohypoxia as observed in PPGL, have been reported to profoundly remodel alternative splicing programs, contributing to tumor cell plasticity and facilitating adaptation to metabolic and microenvironmental stress [61, 62]. These parallels suggest that the splicing differences observed between PPGL molecular clusters are unlikely to be merely epiphenomenal, and that their functional dissection may reveal novel cluster-specific therapeutic vulnerabilities.

The diverse alterations affecting the splicing machinery in PPGL identified in this study suggested that RNA splicing could represent a useful therapeutic vulnerability, and we then decided to experimentally test this notion. Indeed, treatment with the well-known SF3B1-directed splicing inhibitor Pladienolide B induced a robust reduction in cell viability at nanomolar concentrations, which are below potentially cytotoxic doses, although lower doses were not equally effective. This dose response behavior is consistent with our previous work evaluating Pladienolide B in another rare tumor entity, pseudomyxoma peritonei, supporting splicing machinery targeting reproducible and broadly applicable therapeutic strategy across rare tumor entities [34]. Pladienolide B exerted its pronounced antitumor effects by impairing key features of tumor aggressiveness, including cell proliferation, clonogenic potential, migratory capacity, and tumorsphere formation, which compares well with previous studies reporting an increased vulnerability of tumors characterized by splicing dysregulation to spliceosome targeting agents [33]. Notably, neuroblastoma, a tumor sharing a neural crest origin with PPGL, has similarly been shown to be particularly sensitive to spliceosome inhibition with Pladienolide B inducing apoptosis in neuroblastoma cell lines at comparable doses while normal cells [63], further supporting biological rationale for this approach in PPGL.

Despite the favorable results obtained with Pladienolide B, concerns regarding its safety profile and, in general, the therapeutic suitability of directly targeting core components of the spliceosome led us to explore alternative strategies, aimed at modulating splicing through auxiliary splicing factors [14]. Systematic evaluation of the relationship between patient survival and the expression of established

targets of clinically evaluated small-molecule splicing modulators, including PRMT1 [64], PRMT5 [65], BET proteins [66] and RBM39 [67], identified RBM39 as the only factor displaying a significant and reproducible association with clinical outcome across both PCC and PGL cohorts. The hazard ratios observed — 58.17 in PCC and 101.9 in PGL — are remarkable in magnitude and place *RBM39* among the strongest single-gene prognostic markers reported in these tumors. These findings align with prior reports in cholangiocarcinoma, where increased RBM39 expression was consistently associated with reduced overall survival across three independent patient cohorts [68]. Notably, RBM39 has also emerged as a high-priority therapeutic target in acute leukemia, a malignancy in which dysregulated splicing represents a well-established oncogenic vulnerability [69, 70].

Functional enrichment analyses further supported the biological and clinical relevance of RBM39 in shaping PPGL behavior, as elevated expression was coupled to proliferative, and cell cycle-dependent programs, while reduced expression was accompanied by enrichment of immune, microenvironmental and metabolic pathways, indicative of more differentiated and metabolically active tumor states. These findings are clearly supported by previous studies demonstrating that RBM39 is overexpressed in multiple tumor types, where it drives oncogenic splicing alterations in other genes, and thereby promotes cell proliferation and tumor growth [68, 71, 72]. Moreover, RBM39 degradation has been shown to alter splicing of genes involved in cell cycle and DNA repair pathways [73]. Likewise, RBM39 involvement in immune regulation has been previously reported [74], and its targeted degradation has been proposed as a potential anticancer strategy through enhancement of antitumor immunity [75, 76].

Reduction of RBM39 levels or its targeted degradation via indisulam led to decreased proliferation of the tested cell lines, with particularly pronounced effects observed at concentrations comparable to those reported in previous neuroblastoma studies [76–78]. These findings have also been confirmed in vivo, further supporting the results of our study. Although a potential limitation of RBM39 degraders is the emergence of resistance mechanisms [36, 79], recent evidence suggests that combining indisulam with other agents (e.g., PARP inhibitors) may overcome resistance and enhance therapeutic efficacy [35]. In this context, our results reveal an increase in SE events in DNA repair genes following indisulam treatment. This aberrant splicing is expected to generate frameshifts and premature stop codons, resulting in truncated transcript variants that will be predictably recognized and degraded by the NMD pathway [35]. Interestingly, SK-N-AS *SDHB* KD cells showed reduced sensitivity to indisulam compared with WT cells. *SDHB* loss enhances glutaminolysis [80,81], and elevated glutamine metabolism has been reported to confer resistance to splicing inhibition, including indisulam, by modulating *RBM39*-dependent pathways [82]. This metabolic rewiring may underlie the attenuated response of *SDHB*-deficient PPGL models, highlighting the interplay between cellular metabolism and splicing-targeted therapy.

While this study provides primary evidence of splicing dysregulation in PPGL and reveals clinically meaningful differences across tumor types, molecular clusters, and metastatic status, some aspects warrant careful consideration. The analyses are primarily based on transcriptomic data, which offer a robust overview of spliceosomic alterations but do not fully capture post-transcriptional regulation or isoform-specific protein function. In addition, although clear associations between splicing patterns and

molecular clusters were identified, the current study design does not allow to determine unequivocally whether these splicing alterations driver tumor identity or arise as downstream consequences of the underlying oncogenic programs. Functional validation was performed using in vitro models that, while representing the most widely accepted experimental systems currently available for PPGL research, may not fully recapitulate the biological diversity, tumor microenvironment, and heterogeneity that characterize PPGL in patients. However, the consistency of the observed phenotypes across multiple models and functional parameters supports the robustness and translational relevance of our findings. Future studies incorporating proteomic profiling, patient-derived organoids, and in vivo approaches will be instrumental in further dissecting the role of splicing dysregulation in PPGL.

Taken together, our results suggest that splicing dysregulation represents a previously unrecognized layer of biological heterogeneity in PPGL with potential clinical relevance. Identification of spliceosomic differences between PCC and PGL, as well as within established molecular clusters, suggests that splicing-related features may complement current classification systems and contribute to improved patient stratification. Moreover, the strong association between splicing alterations and metastatic behavior supports the exploration of splicing factors, including CELF4, as prognostic biomarkers. Finally, sensitivity of preclinical models to pharmacological splicing modulation provides a rationale for considering splicing targeted strategies as a future therapeutic avenue for PPGL.

Conclusions

This study demonstrates for the first time that PPGL are characterized by pervasive and clinically meaningful alterations of the splicing machinery that distinguish tumor types and molecular subgroups, being associated with metastatic behavior and patient prognosis. CELF4 emerges as a consistent biomarker for tumor stratification, whereas RBM39 defines a prognostic and therapeutically targetable vulnerability. Spliceosome inhibition exerts strong antitumor effects, and RBM39-directed approaches induce functionally relevant splicing perturbations in DNA repair pathways, highlighting splicing as a targetable dependency in PPGL. Collectively, these findings establish splicing dysregulation as a previously unrecognized but clinically significant dimension of PPGL biology, and provide a compelling rationale for considering splicing-targeted strategies as a future therapeutic avenue for patients with these challenging tumors.

Abbreviations

AUC (area under the curve); CAN (copy number alterations); DMEM (Dulbecco's modified eagle medium); DMSO (dimethyl sulfoxide); EGF (endothelial growth factor); FBS (fetal bovine serum); FGF (fibroblast growth factor); HS (horse serum); indi (indisulam); i.p (intraperitoneal) KD: knock-down; NES (normalized enrichment score); neuroendocrine tumors (NETs); pheochromocytomas (PCC); Pd B (Pladienolide B); PGL (paragangliomas); PLS-DA (partial least squares discriminant analysis); PPGL (pheochromocytomas and paragangliomas); ROC (receiver operating characteristic); *SDHB* (succinate dehydrogenase iron-sulfur subunit); SE (skipped exon); siRNA (small interfering RNA); wild-type (WT).

Declarations

Ethics approval and consent to participate

Experiments involving humans were approved by the University of Cordoba/IMIBIC and the Ethics Committees of the participant hospitals and universities (ref. PI19/01603). Written informed consent was obtained from all patients prior to study participation. Experiments involving mice were approved by the University of Cordoba Ethics Committee (ref. 23/09/2022/115) and mice (23/09/2022/115, 23/09/2022) and performed in accordance with the approved protocol and Declaration of Helsinki. The authors declare that the maximum tumor volume permitted by the ethics committee was 1.5 cm in diameter, equivalent to 1,767 mm^3. None of the mice included in the study reached this size, thereby complying with the pre-approved protocol.

Availability of data and materials

The datasets supporting the conclusions of this article are available in the European Genome-Phenome Archive (EGA) repository, under accession numbers EGAS00001006043 and EGAS00001006044.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors have read and approved the manuscript. Study conception and design: M.R., J.P.C. A.I.C. Material preparation and data collection were performed by V.G.V., S.P.A., M.T.M.M., F.Q., E.A., C.M.C., R.B.E., C.G.P., L.G.C., D.R.P., G.dR.M., V.A., L.M.B., A.D.H.M., M.A.G.M., M.R., J.P.C. and A.I.C. Experiments and analysis were performed by V.G.V., S.P.A., M.T.M.M., F.Q., E.A., R.B.E., C.G.P., L.G.C., D.R.P., G.dR.M.,

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