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Original article

Alpha- and Gamma-Synuclein Proteins Define Complementary Metabolic and Invasive Programs in Melanoma Development, Progression, and Prognosis

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Total of Figures: 6 main figures

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Supplementary information: 8 Supplementary figures and 23 Supplementary tables

Running Title: Synuclein Paralogs Define Melanoma Cell States.

Highlights:

- Alpha- and gamma-synuclein are reciprocally expressed across independent melanoma tumors, cell lines, and single cells.
- Alpha-synuclein sustains a differentiated, metabolically active melanoma cell state.
- Gamma-synuclein drives an invasive cell state and confers resistance to multiple classes of cancer therapy.
- Melanoma cells take up extracellular synuclein proteins in a prion-like manner that promotes tumor growth.
- High gamma-synuclein expression identifies a subgroup of melanoma patients with worse clinical outcome.

Simple Summary: Synuclein proteins are best known for their role in Parkinson's disease, but they are also expressed in melanoma, one of the deadliest forms of skin cancer. This study shows that two related synuclein proteins, alpha- and gamma-synuclein, play opposite but complementary roles in melanoma cells. Alpha-synuclein supports a metabolically active, more differentiated tumor state and helps cells manage their energy production. Gamma-synuclein instead drives an invasive, drug-tolerant state, helping cells move, spread, and resist chemotherapy and targeted treatments. Tumor cells were also found to take up these proteins from their surroundings, further boosting their aggressive behavior. Importantly, tumors with high gamma-synuclein levels were linked to a worse outcome in a specific subgroup of melanoma

patients. Together, these findings identify synuclein proteins as promising new markers and potential treatment targets in melanoma.

Abstract: Melanoma, a highly aggressive skin cancer, remains a major clinical challenge due to its metastatic capacity and resistance to therapy. Synuclein proteins — alpha-synuclein (α S) and gamma-synuclein (γ S), traditionally studied in neurodegeneration — are increasingly recognized as contributors to melanoma progression, yet whether they define distinct tumor cell states or act through shared mechanisms remains unclear. Here, we show that SNCA and SNCG are robustly anti-correlated across independent transcriptomic and proteomic cohorts and at single-cell resolution, marking two complementary malignant cell states. Both synucleins support melanoma proliferation, migration, and invasion, and are internalized by melanoma cells in a prion-like manner that enhances tumor growth in vitro and in vivo. Functionally, however, α S and γ S diverge along distinct axes. α S is transcriptionally coupled to a MITF-linked mitochondrial program and functionally sensitizes complex I: its silencing improves mitochondrial bioenergetics and blunts the response to a second respiratory challenge, while exogenous fibrils impair it, alongside a corresponding shift in pigmentation. γ S instead sustains an integrin-FAK-Rac1-MAPK signaling axis and β -catenin regulation that drives invasive behavior; its silencing reduces MAPK output and confers cross-resistance to three mechanistically distinct therapeutic agents — cisplatin, vemurafenib, and paclitaxel. These opposing programs converge on a shared differentiation axis: SNCG-high cells define a dedifferentiated, MITF-low, invasive malignant subpopulation that is independently associated with shorter progression-free interval in stage IIC melanoma. Collectively, these findings establish α S and γ S as functionally distinct, mechanistically convergent regulators of a metabolic-versus-invasive cell-state dichotomy in melanoma, nominate SNCG as a candidate prognostic biomarker, and identify complex I sensitization and FAK/MAPK signaling as candidate therapeutic vulnerabilities.

Keywords: melanoma; synuclein; mitochondrial bioenergetics; FAK/Rac1 signaling; phenotype switching; drug resistance; prion-like propagation; biomarker.

1. INTRODUCTION

Melanoma, a malignancy arising from melanocytes, remains a critical public health concern due to its aggressive metastatic potential and intrinsic resistance to conventional therapies. Over the past decades, advances in the understanding of melanoma's molecular biology have driven the development of refined diagnostic methods and novel therapeutic agents, substantially improving clinical outcomes for patients afflicted with this disease^{1,2}.

Historically, melanoma diagnosis relied heavily on clinical evaluation supported by histopathological examination using criteria such as the ABCDE rule (asymmetry, border irregularity, color variation, diameter >6 mm, and evolution) to identify suspicious lesions³. However, current diagnostic workflows now integrate advanced technologies including dermoscopy, which allows enhanced visualization of skin lesion microstructures, improving diagnostic sensitivity and specificity⁴. Moreover, emerging molecular diagnostic techniques,

encompassing gene expression profiling and next-generation sequencing (NGS), facilitate more precise prognostication and personalized therapeutic decision-making, heralding a shift towards precision oncology in melanoma care⁵.

Surgical excision remains the cornerstone of treatment for early-stage melanoma, with recommendations for wide local excision based on tumor thickness and location. Adjuvant therapies, including lymph node dissection and sentinel lymph node biopsy, are employed to assess and manage regional lymph node involvement⁶. For advanced-stage or metastatic melanoma, treatment options have expanded dramatically over the past decade⁷.

The advent of targeted therapies, specifically BRAF inhibitors (e.g., vemurafenib, dabrafenib) and MEK inhibitors (e.g., trametinib, cobimetinib), has conferred significant survival benefits to patients harboring activating BRAF mutations, which are detected in approximately 50% of melanomas. These agents inhibit aberrant MAPK pathway signaling, resulting in tumor regression and extended progression-free survival. Complementarily, immunotherapies employing immune checkpoint inhibitors—most notably anti-PD-1 antibodies (nivolumab, pembrolizumab) and anti-CTLA-4 (ipilimumab)—have revolutionized treatment by unleashing T-cell-mediated anti-tumor immunity and enabling durable responses, even in metastatic disease^{7–10}.

Current combination regimens involving checkpoint inhibitors have further elevated response rates and survival, with five-year overall survival exceeding 50%—a remarkable improvement from the historically dismal 10–15% associated with chemotherapy alone¹¹. Despite this progress, intrinsic and acquired resistance remain formidable clinical barriers, alongside challenges in biomarker identification for patient stratification and management of immune-related adverse events^{12,13}.

Recent investigations have brought attention to the role of synuclein proteins in melanoma pathogenesis¹⁴. Traditionally studied in neurodegenerative disorders — most notably Parkinson's disease, where α S aggregation is pathogenic — synucleins are increasingly recognized as contributors to tumor biology beyond their canonical neuronal functions. α S has been reported as overexpressed in melanoma relative to benign nevi and normal melanocytes¹⁵, modulating proliferation, migration, and invasion¹⁶, with elevated levels correlating with advanced disease stage and poorer prognosis¹⁷. γ S, structurally related to α S but primarily studied in the context of

oncology rather than neurodegenerative diseases¹⁸, has been implicated in cytoskeletal regulation and invasive behavior in other tumor types^{14,19,20}, though its specific role in melanoma remains largely unexplored.

Despite this growing evidence, several fundamental questions remain unresolved. It is currently unknown whether SNCA and SNCG are co-regulated or instead mark distinct melanoma cell states; whether their expression reflects tumor-intrinsic biology or differences in microenvironment composition; through which molecular mechanisms each protein contributes to tumor behavior; and whether SNCG expression identifies a specific malignant subpopulation with adverse clinical outcome. Addressing these questions requires linking population-level transcriptomic associations to direct functional and mechanistic validation — a step that, to our knowledge, has not yet been undertaken for either synuclein in melanoma.

Here, we combined in vitro and in vivo functional approaches with integrated transcriptomic analyses — including multi-cohort expression correlation at the transcript and protein level, single-cell RNA-sequencing, tumor microenvironment deconvolution, and stage-stratified survival analysis — to characterize the distinct contributions of α S and γ S to melanoma biology. We show that SNCA and SNCG define reciprocal, largely non-overlapping malignant cell states, and identify the specific molecular axes through which each synuclein acts: α S engages a mitochondrial program linked to microphthalmia-associated transcription factor (MITF) which in turn sensitizes complex I to metabolic stress, while γ S sustains an integrin-FAK-Rac1-MAPK signaling module that drives invasion and confers cross-resistance to standard-of-care therapies. Notably, these two axes converge on the same differentiation program, with the γ S-associated invasive state defined by reduced expression of the master regulator MITF that also links α S to mitochondrial function. Collectively, these findings identify synuclein paralog balance as a candidate marker — and mechanistic contributor — to the metabolic-versus-invasive plasticity that underlies melanoma progression, and nominate SNCG as a prognostic biomarker with direct mechanistic grounding.

2. MATERIALS AND METHODS

2.1. Bioinformatics methods

2.1.1. *Synuclein expression in normal skin*

Gene-level RNA-seq expression values, reported as transcripts per million (TPM), for SNCA, SNCB, and SNCG in sun-exposed lower-leg skin (n = 754) and non-sun-exposed suprapubic skin (n = 651) were obtained from the Genotype-Tissue Expression (GTEx) Portal v10²¹. TPM values were log₂(TPM+1)-transformed and compared using two-sided Welch's t-tests. p-values were adjusted across the three genes using the Benjamini-Hochberg (BH) method, with FDR < 0.05 considered statistically significant. Effect sizes were reported as Hedges' g with 95% confidence intervals (CIs), together with medians and interquartile ranges (IQRs).

2.1.2. *Pan-cancer analysis of synuclein expression in tumor and normal tissues*

Expression data for SNCA, SNCB, and SNCG were obtained from TNMplot²², which integrates RNA-seq data from The Cancer Genome Atlas (TCGA)²³ and GTEx²⁴. Expression was compared between tumor and corresponding normal tissues across 22 cancer types. Differences were expressed as log₂ fold changes (log₂FC), with statistical significance assessed using FDR-adjusted p-values.

2.1.3. *Synuclein expression in normal brain and gliomas*

Raw RNA-seq counts for glioblastoma (GBM) and lower-grade glioma (LGG) were obtained from TCGA, whereas normal brain samples were retrieved from GTEx through the recount3/TCGAbiolinks interface²⁵. After harmonizing gene identifiers, count matrices were combined and processed jointly using DESeq2 v1.48.2²⁶, with study included in the design formula. Size factors were estimated using the median-of-ratios method, followed by variance-stabilizing transformation (VST; blind = FALSE).

VST-transformed expression values for SNCA, SNCB, and SNCG were compared among normal brain, LGG, and GBM using Welch's one-way ANOVA followed, when significant, by Games-Howell pairwise tests. p-values were BH-adjusted for each gene, with FDR < 0.05 considered statistically significant.

2.1.4. *Synuclein expression across skin-related tumors*

Expression data for SNCA, SNCB, and SNCG were obtained from public cohorts of cutaneous melanoma (SKCM)²⁷, acral melanoma²⁸, cutaneous squamous cell carcinoma (cSCC)²⁸, basal cell carcinoma (BCC)^{29,30}, and uveal melanoma³¹ (**Table S1**). Depending on data availability, raw

counts were converted to TPM using Ensembl gene lengths, whereas available TPM values were used directly. All values were $\log_2(\text{TPM}+1)$ -transformed.

For BCC, two independent cohorts were combined after mapping gene identifiers to HGNC symbols and retaining genes quantified in both datasets. Batch effects were corrected using ComBat (sva package)³², with cohort specified as the batch variable, and reduction of batch effects was assessed by principal component analysis (PCA). No batch correction was applied across tumor types because study origin was fully confounded with tumor type. Instead, expression values for each gene were independently Z-score standardized to enable relative cross-cohort comparisons.

Synuclein expression within each tumor type was compared using linear mixed-effects models (lme4 and lmerTest)^{33,34}, with gene as a fixed effect and sample as a random intercept. Pairwise comparisons were estimated using emmeans with Tukey-adjusted p-values³⁵. Results were interpreted as relative cross-cohort comparisons rather than absolute differences in transcript abundance.

2.1.5. Synuclein expression in normal skin, primary melanoma, and metastatic melanoma

For skin-derived samples, RNA-seq expression data for SNCA, SNCB, and SNCG were obtained from the TNMplot database³⁶, which provides harmonized gene expression values from the GTEx and TCGA projects for direct comparison across normal and tumor tissues. Harmonized expression values were downloaded for all available samples. Normal skin samples (n = 1035) originated from GTEx, whereas primary melanoma (n = 356) and metastatic melanoma (n = 444) samples were derived from TCGA, as described in the TNMplot documentation. Expression values were $\log_2$ -transformed prior to analysis. Differences among the three groups were assessed using the Kruskal–Wallis test, followed by Dunn's post-hoc test with Benjamini–Hochberg correction for multiple comparisons. Adjusted p-values < 0.05 were considered statistically significant.

2.1.6. Single-cell RNA-seq profiling of synuclein expression

Four independent single-cell RNA sequencing (scRNA-seq) datasets were analyzed to characterize cell type-specific SNCA, SNCB, and SNCG expression in normal human skin and melanoma. GSE130973³⁷, GSE115978³⁸, and GSE72056³⁹ were retrieved from the Gene Expression

Omnibus (GEO)⁴⁰, whereas E-MTAB-8142⁴¹ was obtained from ArrayExpress⁴². Each dataset was processed independently.

GSE130973 comprised 22,335 quality-filtered normal skin cells from five patients. Raw counts were normalized using SCTransform in Seurat v5.3.0. Cluster markers were identified using the Wilcoxon rank-sum test, restricted to positive markers expressed in at least 25% of cells. Markers with $FDR < 0.05$ and average $\log_2$ fold change (avg_log_2FC) > 1 were compared with canonical markers to assign cell identities, which were further curated using the ACT web server.

E-MTAB-8142 comprised 195,739 normal skin cells. SNCA, SNCB, and SNCG expression values were normalized to counts per 10,000 transcripts (CP10K) based on the total transcript count per cell, followed by $\log_{1p}$ transformation. Author-provided cell annotations were retained, with biologically related subpopulations grouped into broader cell types and compartments.

GSE115978 comprised 7,186 melanoma cells from 31 patients. Raw counts were normalized using SCTransform, and author-provided cell identities were retained.

GSE72056 comprised 4,645 malignant and non-malignant cells from metastatic melanoma tumors from 19 patients. The processed expression matrix and author-provided cell annotations were obtained from the GEO supplementary files. These annotations were used to distinguish malignant cells from major non-malignant populations; unassigned cells were excluded. No additional normalization was applied because the expression values were already normalized.

For targeted synuclein analysis, each annotated cell population was compared with all other cells within the same dataset using Seurat's Wilcoxon rank-sum test⁴³. Because only three prespecified genes were evaluated, no prefiltering by detection frequency or fold change was applied. p-values were BH-adjusted, with $FDR < 0.05$ considered statistically significant and interpreted together with average $\log_2$ fold change and detection frequency. In dot plots, dot size represents the percentage of positive cells and color indicates the average $\log_2$ fold change relative to all other cells in the corresponding dataset.

2.1.7. Synuclein protein expression in melanoma

Protein expression of SNCA, SNCB, and SNCG was evaluated in two independent public melanoma proteomic datasets: the Human Melanoma Proteome Atlas (ProteomeXchange accession PXD026086)⁴⁴ and the proteogenomic melanoma cohort PXD035206⁴⁵. Processed and normalized protein abundance matrices were obtained from the corresponding supplementary

datasets. PXD026086 provided log₂-transformed protein abundance values, whereas PXD035206 provided normalized relative abundance values centered around zero after sample median subtraction. Because the studies used different quantification and normalization pipelines, each cohort was analyzed independently without direct comparison of absolute abundance values.

Detection frequency was calculated as the number and percentage of patient-derived tumor samples with quantifiable abundance for each synuclein. Associations between SNCA and SNCG protein abundance were assessed using Spearman rank correlation in samples with matched measurements.

For PXD035206, matched transcriptomic and proteomic data were available for a subset of samples. Matrices were matched by sample identifier, and gene-wise Spearman correlations between mRNA expression and protein abundance were calculated for synucleins measured at both molecular levels. Two-sided p-values were calculated using the cor.test function in R, with P < 0.05 considered statistically significant.

2.1.8. Correlation analysis of synuclein expression

Associations between SNCA and SNCG expression were evaluated by Spearman rank correlation in the TCGA-SKCM cohort (n = 473)²⁷ and an independent validation cohort (GSE65904; n = 214)⁴⁶. Robustness was assessed after excluding observations below the 1st and above the 99th expression percentiles and by restricting the analysis to cutaneous metastatic samples (n = 74).

At the single-cell level, Spearman and Pearson correlations between SNCA and SNCG were calculated across malignant cells from GSE115978³⁸. To account for patient-level variation, pseudo-bulk expression values were also generated by aggregating malignant-cell expression within each patient before correlation analysis.

Malignant cells from GSE72056³⁹ were additionally subclustered according to markers of melanocytic differentiation (MITF, SOX10, PMEL, and MLANA) and invasive/plasticity programs (FN1, ITGA5, and ZEB1) to identify SNCG-enriched subpopulations³⁹.

2.1.9. Associations between synuclein expression and transcriptional state signatures

Single-sample Gene Set Enrichment Analysis (ssGSEA) was performed using the GSVA package (v2.4.0) to calculate per-sample enrichment scores for transcriptional state signatures. The epithelial-to-mesenchymal transition (EMT)/plasticity and oxidative phosphorylation (OXPHOS) signatures were manually curated, whereas pigmentation activity was quantified

using the Reactome Melanin Biosynthesis gene set. Associations of SNCA and SNCG expression with the EMT and OXPHOS scores, as well as the association between SNCA expression and the pigmentation score, were evaluated using two-sided Spearman rank correlations. p-values were adjusted across the five prespecified comparisons using the Benjamini–Hochberg method, with FDR < 0.05 considered statistically significant.

2.1.10. Associations between synuclein expression and tumor microenvironment cell types

Tumor microenvironment composition in TCGA-SKCM bulk RNA-seq samples was estimated using xCell2 (v1.4.0)⁴⁷ with the TMECompendium reference panel. Cell-type enrichment scores were calculated from normalized expression data using xCell2Analysis and correlated with SNCA and SNCG expression using Spearman rank correlation. p-values were BH-adjusted, with FDR < 0.05 considered statistically significant.

To assess whether SNCA expression could reflect erythrocyte contamination, additional Spearman correlations were calculated between SNCA and established erythroid marker genes.

2.1.11. Survival analysis

TPM and clinical information from TCGA-SKCM were used for survival analyses. For each synuclein, patients were dichotomized into high- and low-expression groups using the median as the cutoff. Overall survival (OS), disease-specific survival (DSS), and progression-free interval (PFI) were evaluated using Kaplan–Meier curves and log-rank tests. Hazard ratios (HRs) and 95% CIs were estimated using univariate Cox proportional hazards models.

2.1.12. Subgroup prognostic analysis of SNCG

Exploratory subgroup analyses were performed for SNCG according to major clinicopathological variables available in TCGA-SKCM. Within each subgroup, patients were classified using the global median SNCG expression of the full cohort. OS, DSS, and PFI were assessed using Kaplan–Meier and univariate Cox analyses. Only subgroups with at least 30 patients and five events were included. Significant associations were subsequently evaluated using multivariable Cox models adjusted for age at diagnosis and sex.

2.1.13. Differential expression analysis

Raw RNA-seq counts from TCGA-SKCM were retrieved using TCGAbiolinks (v2.37.1)⁴⁸, excluding normal tissue samples based on TCGA barcode annotations. Gene expression was normalized using TCGAanalyze_Normalization with the geneLength method. Differential

expression between high- and low-expression groups for each synuclein was assessed using TCGAanalyze_DEA. Differentially expressed genes (DEGs) were defined as $FDR < 0.01$ and $|\log_2FC| > 1$.

2.1.14. Pathway enrichment and GSEA

Gene identifier conversion was performed using org.Hs.eg.db (v3.21.0)⁴⁹. Over-representation analysis (ORA) of differentially expressed genes identified by TCGAanalyze_DEA was performed using clusterProfiler (v4.16.0)⁵⁰ for Gene Ontology (GO) biological process, molecular function, and cellular component categories, and ReactomePA (v1.52.0)⁵¹ for Reactome pathway enrichment (ReactomePA::enrichPathway). Gene sets containing 3–800 genes were tested, and pathways with an $FDR < 0.05$ were considered significantly enriched.

Gene Set Enrichment Analysis (GSEA) was performed using ReactomePA::gsePathway on genes pre-ranked by $\log_2$ fold-change values. Gene sets containing 5–800 genes were analyzed using 10,000 permutations, and pathways with an $FDR < 0.05$ and $|NES| > 1$ were considered significant. The same Reactome ORA procedure was subsequently applied to the upstream and downstream gene sets derived from the regulatory network analyses using identical gene-set size and significance thresholds.

2.1.15. Inference of synuclein-centered regulatory networks

Gene regulatory networks were inferred using GENIE3 (v1.24.0)⁵² to identify potential upstream regulators and downstream targets of SNCA, SNCB, and SNCG in normal skin and melanoma. Separate models were generated for each tissue and interaction direction. Interaction weights were Z-score standardized within each condition and direction, and top-ranked edges were retained using the 75th percentile for upstream and 95th percentile for downstream interactions. Inferred interactions were integrated with protein–protein interaction data from STRING (v12.5)⁵³, retaining the top 500 interactions for each synuclein, and with PrePPI⁵⁴ scores when available. All metrics were min–max normalized and combined into a prioritization score:

Priority score = $(0.5 \times \text{weight_z_scaled}) + (0.25 \times \text{string_scaled}) + (0.25 \times \text{preppi_scaled})$

Candidate genes were ranked according to this integrated score, combining transcriptomic evidence from GENIE3 with protein-interaction evidence from STRING and PrePPI.

2.1.16. MITF-adjusted associations of SNCA with pigmentation and OXPHOS programs

The association between SNCA and MITF expression was evaluated in the TCGA-SKCM cohort using two-sided Spearman correlation. Associations between SNCA expression and pigmentation or OXPHOS ssGSEA scores were assessed both before and after adjustment for MITF. Adjusted associations were estimated using partial Spearman correlations implemented in the ppcor R package (v 1.1)⁵⁵. The impact of MITF adjustment was quantified as the percentage reduction in the absolute correlation coefficient.

The same approach was applied at the gene level for genes included in the pigmentation and OXPHOS programs. MITF was excluded from adjusted gene-level analyses because it was used as the covariate. Only complete cases were analyzed. Two-sided p-values were adjusted separately for unadjusted and MITF-adjusted gene-level correlations using the Benjamini–Hochberg method, with FDR < 0.05 considered significant.

2.1.17. MITF mediation analysis

Mediation analyses were performed in TCGA-SKCM using SNCA expression as the exposure, MITF expression as the mediator, and pigmentation or OXPHOS ssGSEA scores as outcomes. Pigmentation and OXPHOS activity were quantified using the Reactome Melanin Biosynthesis and Hallmark Oxidative Phosphorylation gene sets, respectively.

All models were adjusted for tumor purity using Consensus Purity Estimate (CPE) values from the Tumor.purity dataset distributed with TCGAbiolinks⁴⁸. Five samples lacking CPE values were excluded, leaving 468 samples. SNCA, MITF, pathway scores, and tumor-purity estimates were standardized before modeling.

For each outcome, the mediator model was $MITF \sim SNCA + \text{tumor purity}$, and the outcome model was $\text{pathway score} \sim SNCA + MITF + \text{tumor purity}$. Average causal mediation effects (ACME), average direct effects (ADE), total effects, and proportions mediated were estimated using the mediation R package (v4.5.1)⁵⁶, with 5,000 nonparametric bootstrap resamples for 95% confidence intervals and p-values. Sensitivity to unmeasured mediator–outcome confounding was evaluated using medsens. Indirect-effect p-values were adjusted using the Benjamini–Hochberg procedure.

2.1.18. Serial mediation analysis of the SNCA–MITF–TYR pigmentation axis

A tumor-purity-adjusted serial mediation model was fitted using lavaan (v0.7-2)⁵⁷, including MITF and TYR as sequential mediators between SNCA and pigmentation. To avoid mathematical

overlap, the pigmentation ssGSEA score was recalculated after removing TYR from the Reactome Melanin Biosynthesis gene set.

The model estimated three specific indirect effects: SNCA $\rightarrow$ MITF $\rightarrow$ pigmentation, SNCA $\rightarrow$ TYR $\rightarrow$ pigmentation, and SNCA $\rightarrow$ MITF $\rightarrow$ TYR $\rightarrow$ pigmentation. Total indirect and total effects were derived from the corresponding path coefficients. All variables were standardized before modeling, and standardized coefficients with percentile-bootstrap 95% confidence intervals were estimated using 5,000 bootstrap resamples. Because the model was saturated, inference was based on path coefficients and bootstrap confidence intervals rather than global fit indices.

2.2. Experimental Methods

2.2.1. Expression and Purification of α S and γ S in bacterial cells

The proteins α S and γ S were obtained by over-expressing the pT-T7 α -syn or pET-30a(γ Syn_h) plasmids containing the DNA sequence of human α S and γ S in LB medium. To ensure that the overexpressed proteins were N-terminally acetylated, as they occur physiologically^{58,59}, we co-expressed the pNatB plasmid harboring the sequence of yeast NatB complex. Purification was performed according to previously described protocols^{58,59}. Protein purity was determined by SDS-PAGE analysis and quantified spectrophotometrically using an extinction coefficient of 5600 M⁻¹ cm⁻¹ (α S) and 1490 M⁻¹ cm⁻¹ (γ S) at 280 nm.

2.2.2. Generation of Oligomers and Mature Amyloid Fibrils of α S and γ S

Purified α S and γ S monomers were used for the aggregation assays. A solution of 100 μ M synuclein monomers in a final volume of 500 μ L of PBS was incubated for 5 days at 37°C under gentle mechanical agitation in amber glass vials to promote aggregation. At the end of the incubation period, the content of the vial was transferred to a low-binding Eppendorf tube (Tarson) and centrifuged at 12,000 rpm for 15 min at 4°C. The resulting supernatant, corresponding to the soluble monomeric and oligomeric fraction, was separated, while the pellet was resuspended in 200 μ L of PBS. The concentration of aggregates was estimated by measuring the absorbance of the soluble fraction at 280 nm and calculating the percentage of aggregation by subtracting this value from the initial monomer concentration. From this difference, the concentration of amyloid fibrils was estimated as the equivalent amount of aggregated monomer. The presence of the different α S and γ S species was assessed by polyacrylamide gel electrophoresis

followed by Coomassie blue staining. Formation of β -sheet rich fibrils was further confirmed by circular dichroism (CD) spectroscopy using a Jasco J-1500 spectropolarimeter. α S and γ S samples were dissolved in PBS at a concentration of 10 μ M and placed in a 1 mm path length quartz cuvette. Spectra were registered at 25°C with a 2 nm slit width and 4 accumulations. The transition from the disordered protein state to β -sheet rich aggregates is monitored by the decrease of the negative band at 200 nm and the appearance of a less intense minimum at 215 nm⁶⁰.

2.2.3. Incubation with exogenous synuclein-aggregates

Cells were incubated with exogenous α S or γ S aggregates at a final concentration of 1 μ M for 24 h, unless otherwise indicated in the corresponding section. After incubation, the relevant assays were performed as described below.

2.2.4. Cell culture

SKMEL-28 and A375 human melanoma cell lines, B16-F0 and B16-F10 C57BL/6 mice-derived melanoma cell lines, SH-SY5Y human neuroblastoma cell line, HT29 human colorectal adenocarcinoma cell line, and CT26 colon carcinoma murine cell line were purchased from the American Type Culture Collection (ATCC) through the CIQUIBIC Cell Bank (Córdoba, Argentina). Cells were cultured in DMEM (with the exception of CT26 cells cultured in RPMI) and supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific), penicillin (10 μ g/ml), streptomycin (100 μ g/ml) and L-glutamine (2 mM). Cells were incubated at 37 °C with 5% CO₂ and routinely tested for mycoplasma contamination.

2.2.5. Plasmids and transfection

Cells were seeded and incubated overnight in proliferation medium. The next day, transfection of plasmids was conducted using Lipofectamine 2000 reagent according to the manufacturer's protocol. After 48 h, the culture medium was replaced with complete medium supplemented with the corresponding selection antibiotic: Puromycin (Invitrogen; 1 μ g/mL for B16-F0 and B16-F10 cell lines, and 0.2 μ g/mL for A375 cell line) and G418 (Invitrogen; 500 μ g/mL for all cell lines). In parallel, a selection control was conducted with non-transfected cells. Selection was carried out for approximately 2 weeks and clones were isolated for cells with decreased expression of α S and γ S (the remaining resistant cells were expanded and considered as the cell pool resulting

from selection). For control cells and cells overexpressing synucleins the resulting cell pool from selection was used throughout the experiments.

Plasmids used in this work to modulate the expression of synucleins include: pLKO.1-puro (Addgene); pLKO-sh α S (MISSION shRNA, MERCK: TRCN0000003736 for human cell line; TRCN0000106639 for murine cell lines); pLKO-sh γ S (plasmid pLKO.1-puro containing an shRNA sequence to decrease the expression of human (GACCAAGGAGAATGTTGTACA) and mouse γ S (GCCAAAGAGCAAGAGGAGAAT) in eukaryotic cells, generated in our laboratory); p α S-V5 and p γ S-V5 (C-terminally tagged synucleins; kindly provided by Dr. Tiago Outeiro, Göttingen and described before⁶¹).

2.2.6. Cell proliferation

For in vitro cell proliferation assays, 1×10^3 cells were plated on 96-well plates and incubated at 37°C at a 5% CO₂ atmosphere for 24h. Proliferative capacity was assessed by counting trypsinized cells in a Neubauer chamber over five days, every 24 h. Proliferation was expressed as the number of cells counted in each well and fitted to an exponential growth curve with GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

2.2.7. Clonogenic efficiency

Cells (300/well) were seeded on 6-well plates. After attachment, cells were cultured for 7–10 days as described before⁶². After fixing the cells with 4% PBS-buffered p-formaldehyde (Anedra), colonies were stained with methylene blue (1%, Sigma) to allow quantification. Photos of the clones were taken at the end of the experiment and their size was estimated by measuring colonies areas with the ImageJ software.

2.2.8. Cell migration, invasion and gelatin zymography

Melanoma cells were plated and cultured until subconfluency. A wound was made on the cell monolayer with the help of a 200 μ l tip. To analyze cellular motility in non-proliferative conditions, cells were put on media supplemented with 0.1% FBS and maintained under these conditions throughout the assay. Migratory ability was estimated by measuring the closure of the initial wound. Photos were taken after the initial wounding and at 4, 8, 12, 16, 20 and 24 h using a Nikon Ti2E microscope. Quantification of healing was performed using the ImageJ software. Areas under the curve were determined as described before⁶².

To further assess migratory and invasive properties, transwell assays were performed using 24-well inserts with 8 μ m pore polycarbonate membranes (Thermo Scientific). Briefly, 6×10^4 cells suspended in serum-free medium were seeded into the upper chamber, while complete medium containing 10% FBS was added to the lower chamber as a chemoattractant. For invasion assays, inserts were coated with growth factor-reduced Matrigel (Corning) diluted 1:5 in serum-free medium and allowed to polymerize before cell seeding. After incubation for 24 h at 37°C, non-migrated/non-invaded cells were removed from the upper surface of the membrane. Cells that had traversed the membrane were fixed with 70% ethanol, stained with DAPI, and visualized by fluorescence microscopy. Migrated or invaded cells were quantified by counting DAPI-positive nuclei in representative microscopic fields and expressed relative to control conditions.

To analyze gelatinolytic activity, A375 melanoma cells were grown to subconfluency, and then incubated for 24 h in serum-free medium. Supernatant was collected and concentrated, using centrifugal filter devices (50 kDa, Amicon Ultra-15, Milipore). Supernatant protein concentration was determined by Lowry using a calibration curve.

Gelatin zymography was performed using a 7.5% acrylamide/bis-acrylamide resolving gel, containing 4 mg/mL gelatin, and a 4% acrylamide/bis-acrylamide stacking gel. Samples containing 40 μ g of protein were prepared with non-reducing sample buffer. After electrophoresis, the gel was washed using a washing buffer (pH 7.5) containing 2.5% Triton X-100, Tris-HCl 50 mM, CaCl_2 5 mM, and ZnSO_4 1 μ M. The buffer was then replaced with an incubation buffer (pH 7.5) containing 1% Triton X-100, Tris-HCl 50 mM, CaCl_2 5 mM, and ZnSO_4 1 μ M, and incubated for 24 h at room temperature. Then, Coomassie Blue staining was performed and gelatinolytic activity was visualized as clear bands against a dark blue background, corresponding to areas of enzymatic activity.

2.2.9. Cell Viability and Drug Sensitivity Assays

5×10^3 cells were seeded in 96-well plates in triplicate and treated with increasing concentrations (0.1 μ M, 0.5 μ M, 1 μ M, and 5 μ M) of monomers, oligomers, or fibrils of α S or γ S for 36 h. Cell viability was then assessed using the MTT assay as described⁶³.

To assess the effect of γ S silencing on drug sensitivity, sh- γ S and control A375 melanoma cells (5×10^3 /well) were seeded in 96-well plates and treated 24h later with increasing concentrations of cisplatin (0.5-100 μ M), vemurafenib (0.5 μ M-2mM), or paclitaxel (0.1nM-1 μ M) for 72h. Cell

viability was assessed by MTT assay. Dose-response curves were fitted using a non-linear regression model (GraphPad Prism 8, log[inhibitor] vs. normalized response, variable slope). IC50 values were derived from the fitted curves and compared between sh-γS and control cells using an F-test of the fitted curves and unpaired Student's t-test on log-transformed IC50 values.

To independently evaluate baseline mitochondrial metabolic rate rather than long-term cell viability, an identical cell density was seeded in parallel and incubated with the MTT reagent for a brief, sub-saturating period of 40 min, allowing the initial rate of formazan crystal formation to serve as a direct readout of real-time mitochondrial dehydrogenase activity.

2.2.10. Immunoblotting

For SDS-PAGE, cells were plated on 60mm dishes until they reached 70% of confluence and then lysed in RIPA buffer (Tris-HCl 50 mM pH: 8; NaCl 150 mM; NP40 1% P/V; DOC 0,5% P/V; SDS 0,5% P/V) supplemented with protease inhibitor cocktail (CØmplete; Roche) and phosphatase inhibitors (100 mM NaF (Sigma-Aldrich), 100 mM Na₃VO₄ (Sigma-Aldrich) and 1 M β-Glycerol phosphate (Sigma-Aldrich)) and scraped off the dish to perform protein extracts. Protein concentration was determined using the Lowry assay and gels were loaded with 40 µg protein prior to denaturation for 5 min at 95°C in SDS-PAGE protein sample buffer. Samples were separated on 10-14% SDS-polyacrylamide gels and transferred to PVDF membrane (Amersham Hybond P, GE Healthcare Life Sciences). Membranes were blocked with 5% (w/v) skim milk in TBS-Tween (TBS-T: 50 mM Tris, 150 mM NaCl, 0.05% Tween, pH 7.5) or 1% BSA in TBS-T for detection of phosphorylated proteins for 60 min at room temperature and further incubated with the indicated primary antibody overnight (ON) at 4°C. After three washes with TBS-T to remove the primary antibody, membranes were incubated with the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. Detection was done by chemiluminescence (BioLumina; Kalium Technologies, Argentina) using an Amersham Imager according to manufacturer's instructions and quantified by densitometry with ImageJ software.

2.2.11. Cell immunostaining techniques

5×10³ cells were plated on coverslips for 24 h, and then fixed in 4% paraformaldehyde, permeabilized with a 0.3% PBS-Triton solution and blocked with BSA (0.1%; Sigma) for 30 min. Cells were incubated ON with primary antibody, and then with an appropriate conjugated secondary antibody for 1 h at RT. Complementary staining with DAPI (Sigma, 1:10000) and

phalloidin-rhodamine conjugate (Invitrogen, 1:200) was carried out to visualize cells nuclei and the actin skeleton, respectively. Finally, coverslips were fixed to a slide with 4 µl of Mowiol and observed under a Nikon C2 confocal microscope.

2.2.12. Antibodies

Antibodies used for Western blot (WB) and immunocytochemistry (ICC) included α -Synuclein (BD Biosciences, 1:1000 dilution for WB and ICC), γ -Synuclein (Santa Cruz Biotechnology, dilution 1:300 for WB, 1:50 for ICC), V5 (Abcam, dilution 1:500 for WB), FAK (Santa Cruz Biotechnology, 1:50 dilution), β -catenin (BD Biosciences, dilution 1:1000 for WB), GAPDH (Santa Cruz Biotechnology, dilution 1:300 for WB), β -Actin (Cell Signaling, dilution 1:1000 for WB) and anti-active RAC1-GTP (New East Biosciences, dilution 1:250 for ICC). Alexa Fluor 488-conjugated secondary antibody (Invitrogen, 1:500 dilution) was used for ICC. For WB, anti-mouse IgG and anti-rabbit IgG (Thermo Fisher, 1:5000 dilution) were used.

2.2.13. Analysis of mRNA abundance

Total RNA was extracted from the indicated cells using TRIzol (Sigma-Aldrich) and quantitative RT-PCR performed using the 'Power SYBRTM Green RNA-to-CTTM 1-Step Kit' (Applied Biosystems) and the QuantStudio 3 System (ThermoFisher). Raw data were then analyzed using QuantStudio 3 System software. We used the abundance of the endogenous *GAPDH* mRNA as internal normalization control. Primers used for transcript quantitation included 5'-GAGTCAACGGATTTGGTCGT-3' (forward for mouse and human *GAPDH*), 5'-TTGATTTTGGAGGGATCTCG-3' (reverse for mouse and human *GAPDH*), 5'-CACTGGCTTTGTCAAGAAGGACC-3' (forward for mouse *SNCA*), 5'-CATAAGCCTCACTGCCAGGATC-3' (reverse for mouse *SNCA*), 5'-ACTGCTCCTCCAACATTTGTC-3' (forward for human *SNCA*), 5'-AGGGTGTTCTCTATGTAGGCT-3' (reverse for human *SNCA*), 5'-CGT GGT ACA AAG TGT CAC CTC AG -3' (forward for mouse *SNCG*), 5'-GACCACGATGTTTTTCAGCCTCC-3' (reverse for mouse *SNCG*), 5'-TGTGGTGAGCAGCGTCAACACT-3' (forward for human *SNCG*), 5'-TTGGATGCCACACCCTCCTGTT-3' (reverse for human *SNCG*), 5'-CGATCAGCTCTGAAACCGTCA-3' (forward for mouse and human *MAP2KI*), 5'-CTTGAAGACCACTCCACCGT-3' (reverse for mouse and human *MAP2KI*).

2.2.14. Melanin quantification

Cells were seeded in complete DMEM without phenol red and allowed to adhere overnight. Cells were then pretreated with 5 μ M α -melanocyte-stimulating hormone (α -MSH; Sigma) for 24 h. For extracellular melanin quantification, culture supernatants were collected and centrifuged at 1,500 rpm for 5 min to remove cellular debris. The clarified supernatants were transferred to a 96-well microplate, and absorbance was measured at 475 nm using a microplate reader, with complete DMEM without phenol red as blank. For intracellular melanin quantification, cells were harvested by trypsinization, centrifuged at 1,500 rpm for 5 min, and counted using a Neubauer hemocytometer. An aliquot containing 1×10^6 cells was pelleted and resuspended in 500 μ L of 1 N NaOH. Samples were incubated at 80°C for 1 h to solubilize melanin. Subsequently, aliquots were transferred to a 96-well microplate, and absorbance was measured at 475 nm using a microplate reader, with 1 N NaOH as blank.

2.2.15. Flow cytometry

Cell cycle was analyzed by flow cytometry after propidium iodide (PI) staining. Briefly, exponentially growing melanoma cells were seeded in 6-well plates, and incubated ON. Then, the cells were maintained for 24 h under serum restriction. After this time, the complete medium was restored and the cells were grown for an additional 24 h. Then, cells were collected, washed with PBS and incubated with 100 μ L of staining solution (50 μ g/mL propidium iodide (Immunostep, Spain), 0.1% Triton, and 100 μ g/mL RNase A in PBS) for 30 min. Reaction was stopped with 100 μ L of PBS, and the cells were immediately analyzed by flow cytometry (BD FACS Aria II).

Studies for apoptosis were carried out with the Annexin V Apoptosis Detection Kit (Immunostep). 5×10^4 cells were seeded in 12-well plates and harvested 48 h after incubation. The supernatant and cells were collected in the same tube. Cells were washed with PBS, resuspended in an Annexin buffer containing Annexin V and PI, and incubated in the dark at room temperature for 15 min. Apoptosis was then detected by flow cytometry (BD FACS Aria II) as described before⁶².

To evaluate mitochondrial superoxide generation, cells were seeded at a density of 3×10^5 cells per 35-mm dish and cultured for 24 h. To induce mitochondrial ROS production, cells were challenged with 10 mM metformin (Sigma-Aldrich) for 2 h prior to staining. Cells were subsequently harvested, washed with phosphate-buffered saline (PBS; Gibco), and incubated

with 1 μM MitoSOXTM Red mitochondrial superoxide indicator (Thermo Fisher Scientific) for 20 min at 37°C in the dark. Unstained cells and vehicle-treated cells (culture medium) were included as negative controls. After staining, cells were washed, resuspended in PBS, and immediately analyzed on a BD FACSMelody flow cytometer (BD Biosciences) using a 488-nm laser for excitation and detection in the PE channel.

Total intracellular reactive oxygen species (ROS) levels were determined using the general oxidative stress indicator CM-H₂DCFDA (Invitrogen, Thermo Fisher Scientific). Briefly, 1.5×10^5 cells were cultured in phenol red-free DMEM supplemented with 10% fetal bovine serum for 24 h. Cells were harvested by trypsinization, washed and incubated with 2.5 μM CM-H₂DCFDA in PBS for 30 min at 37 °C. Exposure to 50 μM hydrogen peroxide during the final 10 min of incubation served as a positive control for oxidative stress. Following incubation, cells were washed, resuspended in PBS, and immediately analyzed on the BD FACSMelody flow cytometer using 488-nm excitation and FITC-channel detection.

For evaluation of Mitochondrial Membrane Potential ($\Delta\Psi$), cells were seeded at a density of 1.5×10^5 cells per 35-mm dish in phenol red-free complete DMEM and cultured for 24 h. Where indicated, cells were treated with 10 mM metformin for 2 h as described above. Mitochondrial membrane potential was assessed using the MitoStep Apoptosis Detection Kit (Immunostep, Salamanca, Spain) according to the manufacturer's protocols. Harvested cells were washed and stained with the cationic lipophilic dye DiIC₁(5) for 15 min at 37 °C. Cells were then washed, resuspended in PBS, and immediately analyzed on the BD FACSMelody flow cytometer using a 640-nm red laser for excitation and APC-channel detection.

All flow cytometry data from the aforementioned assays were processed and quantified using FlowJo software (TreeStar).

2.2.16. Animal studies

C57Bl/6 mice were obtained from the School of Veterinary Sciences at the National University of La Plata and treated in accordance with the Canadian Council on Animal Care and ARRIVE guidelines⁶⁴. Animals were fed with commercial chow and water ad libitum, and maintained under a 12 h light/dark cycle at the CIPReB facilities (Centro de Investigación y Producción de Reactivos Biológicos, Medicine School, National University of Rosario). For all experiments, animals (n = 6–8/group) were distributed and inoculated as follows: Control cells; knock-down

synucleins cells and synuclein-pretreated cells. Animals were periodically weighed and checked for changes in skin, fur, eyes, secretions, excretions and autonomic activity (lacrimation, pilo-erection, unusual respiratory pattern, movement, etc).

2.2.17. Allograft models.

2.5×10^5 B16-F0 or B16-F10 cells were resuspended in PBS (100 μ l) and injected subcutaneously into groups of 8-week-old female mice. Tumor progression was monitored by periodic caliper measurements of tumor length (a) and width (b), and tumor volume (V) was calculated using the formula $V = 0.4ab^2$. Fitting to exponential growth was performed with the GraphPad Prism 8 software. Body weight of the mice was also recorded. After sacrifice, tumors, lymph nodes and lungs were extracted, fixed in 4% formaldehyde for 24 h and embedded in paraffin for haematoxylin/eosin (H&E) staining.

2.2.18. Metastasis models.

To examine spontaneous metastasis, subcutaneous tumors were generated using B16-F10 cells as described in the previous section ($n = 7/\text{group}$). When tumors from the different conditions reached a volume close to 1500mm³, mice were anesthetized with a mixture of acepromazine/midazolam/ketamine for surgical removal of the tumors, ensuring the muscle layer was not compromised. After 4 weeks of regular monitoring, mice were euthanized and the lungs were collected. Lungs were examined under a stereomicroscope to assess the presence of spontaneous metastases. They were then fixed in 4% formaldehyde and processed by the Pathology Department of the Faculty of Medical Sciences for histological analysis using H&E staining.

To assess experimental metastases, mice were anesthetized as described. After a small incision, the spleen was exposed to allow the injection of 1.5×10^5 B16-F10 cells ($n = 6/\text{group}$). The muscle and skin layers were then sealed with commercial adhesive. After 15 days of periodic monitoring, mice were euthanized and the spleen, liver, and lungs were collected and examined under a stereomicroscope to evaluate the presence of metastatic lesions.

2.2.19. Digital image analysis

Images from polyacrylamide gels, Western blots and immunostainings were digitally processed for cropping and adjustments (saturation, brightness, intensity) using *Canvas X 2020* software.

Microscopy images were also adjusted using the same software. All changes were applied uniformly to all images corresponding to the same experimental assay.

For the quantification of total active Rac1, confocal microscopy images of cells stained with an anti-Rac1-GTP primary antibody and a fluorophore-conjugated secondary antibody were analyzed using the NIS Elements software. To ensure reproducibility and accurate comparison, all images were acquired under identical optical and electronic settings, including exposure time, laser power, gain, and focal plane. For each cell, a region of interest (ROI) was manually delineated around the cell outline based on the corresponding phase-contrast/DAPI/phalloidin channel, and mean fluorescence intensity within the ROI was measured. Local background fluorescence, measured from an adjacent cell-free region of equivalent area, was subtracted from each ROI measurement to obtain net fluorescence values. A minimum of 10 cells across at least five independent images were analyzed per experimental condition, and statistical comparisons were performed using cell-level mean values.

2.2.20. Statistics

Statistical analyses for wet experiments were performed using GraphPad Prism 8.0, and graphical representations were generated with Canvas X 2020. Experiments were independently repeated at least three times. Results are expressed as mean $\pm$ SD or SEM, as appropriate. Statistical significance was assessed using Student's t-test (for two groups) or one-way ANOVA with multiple comparisons. A p-value < 0.05 was considered statistically significant and represented as follows: p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)

3. RESULTS

SNCA and SNCG are the predominant, differentially regulated synucleins across neural tissue, skin, and pan-cancer contexts.

To investigate the role of synucleins in melanoma, we first contextualized these proteins within their well-established functions in the brain. Synucleins (α S, β S, and γ S) are small neuronal proteins predominantly expressed at presynaptic terminals, where α S regulates synaptic vesicle trafficking and neurotransmitter release. While extensively studied in neurodegenerative disease — particularly Parkinson's disease, where α S aggregation is pathogenic — their role in cancer remains much less defined. Recent evidence identifies α S as a promoter of melanoma progression,

enhancing tumor cell proliferation, migration, and metastasis through modulation of DNA damage repair pathways and adhesion molecule trafficking^{65–67}; in vivo, α S knockout mouse models show delayed melanoma onset and reduced tumor growth, particularly in males, linked to decreased DNA damage signaling and increased apoptosis⁶⁸. In turn, γ S is aberrantly expressed across several tumor types, where it promotes invasion and metastasis via TGF- β -mediated signaling^{19,69}. Together, these reports indicate that α S and γ S act beyond their canonical neuronal functions to influence melanoma aggressiveness through distinct mechanisms, positioning both as candidate contributors to tumor progression whose relationship to one another, however, remains unexplored. Here, we asked whether this dual, paralog-specific involvement reflects a coordinated, reciprocal transcriptional relationship between SNCA and SNCG in melanoma. Given this comparatively limited characterization in cancer, we first examined synuclein expression in the tissue of origin most relevant to their canonical biology. Analysis of TCGA and GTEx datasets revealed a marked reduction in SNCA, SNCB, and SNCG transcript levels in gliomas relative to non-neoplastic brain tissue, following a progressive pattern of Normal > LGG > GBM (BH-adjusted FDR < 0.05, **Figure S1A; Table S2**), consistent with the neuron-enriched nature of synuclein expression and the glial origin of these tumors^{70,71}. Despite this overall downregulation, synuclein expression remained highly context-dependent across neuro-oncological malignancies: α S was broadly expressed in neoplasms exhibiting neuronal differentiation — gangliogliomas, medulloblastomas, neuroblastomas, primitive neuroectodermal tumors, pineocytomas/pineoblastomas, and central neurocytomas^{72,73} — whereas γ S was instead associated with poorly differentiated, aggressive tumor phenotypes, with its upregulation correlating with enhanced invasive capacity and perineural invasion⁷⁴. Despite these observations in central nervous system malignancies, the physiological distribution of synucleins outside neural tissues remains poorly characterized. To establish a baseline reference framework in the tissue of origin of melanoma, we analyzed transcriptomic profiles from normal human skin using the GTEx dataset, evaluating both sun-exposed (lower leg; n = 754) and non-sun-exposed (suprapubic; n = 651) sites. Across both anatomical regions, SNCG and SNCA exhibited the highest transcript levels, whereas SNCB expression was consistently low (**Figure S1B; Table S3**). This pattern is consistent with keratinocyte predominance over the sparse neuronal and melanocytic enrichment characteristic of bulk skin biopsies. Chronically sun-

exposed skin displayed a modest but statistically significant increase in SNCG and a negligible increase in SNCA after Benjamini–Hochberg correction, whereas SNCB expression showed no significant differences. The small effect sizes and substantial distributional overlap indicate that habitual ultraviolet exposure does not substantially alter the basal expression topography of the synuclein family in normal skin.

To assess how these baseline patterns shift during oncogenesis, we evaluated synuclein expression across 22 cancer types. SNCA and SNCG displayed the most pronounced tumor-versus-normal expression dysregulation among the three family members, showing a highly concordant pattern at the proteomic level (**Figure S1C-D**).

Finally, we compared the relative expression of SNCA, SNCB, and SNCG across five skin-related tumor types using linear mixed-effects models. SNCA was the predominant synuclein in all three melanoma subtypes, whereas SNCB showed the lowest expression in cutaneous and acral melanoma. Basal cell carcinoma was the main exception, with SNCG expressed at higher levels than both SNCA and SNCB. Pairwise comparisons confirmed these differences after correction for multiple testing (**Figure S1E; Tables S4–S6**). Together, these analyses identified SNCA and SNCG as the synucleins with the greatest transcriptomic relevance in melanoma, and subsequent analyses therefore focused primarily on these two genes.

SNCA and SNCG expression is reciprocally coupled across independent cohorts and diverges with disease progression and outcome.

We next evaluated the relationship between SNCA and SNCG across cell-line models and independent clinical cohorts. In 51 melanoma cell lines, a system devoid of tumor microenvironment contribution, their expression showed a moderate inverse correlation (Spearman's $\rho = -0.32$; $p = 0.02$), reproduced in the full TCGA-SKCM cohort ($\rho = -0.26$; $p = 9.92 \times 10^{-9}$; $n = 473$; **Figure 1A**). This association was robust to the exclusion of extreme expression values and became modestly stronger when the TCGA-SKCM analysis was restricted to cutaneous metastatic samples ($\rho = -0.32$; $n = 74$). The inverse relationship was further reproduced in the independent GSE65904 cohort ($\rho = -0.43$; $p = 4.15 \times 10^{-11}$; $n = 214$; **Figure S2A**), and further strengthened at single-cell resolution when malignant-cell expression was aggregated per patient ($\rho = -0.64$; $p = 0.001$; $n = 20$; **Figure 1A**). This inverse relationship was also detectable at the protein level in the independent PXD035206 metastatic melanoma proteogenomic cohort ($n = 137$; $\rho = -$

0.23; $p = 0.001$), in which both α S and γ S were detected in essentially all samples, whereas β S was quantified in only 42.3% (**Figure 1B**). A second, mixed primary/metastatic proteomic cohort (PXD026086) did not reproduce this correlation ($\rho = -0.009$; $p = 0.87$; **Figure S2B**), suggesting that the strength of the inverse relationship may depend on clinical composition, stage, or methodology; matched transcript-protein comparisons within PXD035206 nonetheless confirmed concordance between SNCA transcript and α S protein abundance (**Figure S2C**).

The inverse association between SNCA and SNCG at the bulk level raised the possibility that the two genes are expressed by different cell types within the tissue. To test this, we analyzed two independent scRNA-seq datasets of normal skin (E-MTAB-8142, GSE130973): SNCA was expressed predominantly in melanocytes, whereas SNCG reached its highest expression in lymphatic endothelial cells and was comparatively lower in melanocytes, while SNCB was detected in only a small fraction of cells across virtually all populations (**Figure S2D**), indicating that synuclein expression is already cell type-specific in normal skin. Extending this analysis to two independent metastatic melanoma cohorts (GSE115978, GSE72056), SNCA was enriched in malignant cells in both datasets, whereas SNCG was predominantly expressed in the endothelial compartment while remaining detectable in a smaller fraction of tumor cells (**Figure 1C**). In GSE115978, SNCA showed marked enrichment in malignant cells relative to all other populations ($\log_2FC = 4.62$), whereas SNCG showed its strongest enrichment in endothelial cells ($\log_2FC = 4.57$) but was also significantly, if more modestly, enriched within the malignant compartment itself ($\log_2FC = 2.20$; **Table S7**). Together, these results indicate that part of the SNCG-associated signal observed in bulk tumor samples reflects tumor microenvironment composition, while also revealing that a subset of malignant cells retains detectable, tumor-intrinsic SNCG expression — a subpopulation we characterize in detail below.

To determine whether SNCA and SNCG expression also tracked disease progression, we compared expression across normal skin, primary melanoma, and metastatic melanoma. SNCA and SNCG expression diverged progressively across this continuum, with SNCA increasing and SNCG showing the opposite trend toward metastatic disease, while SNCB remained comparatively stable (normal skin $n = 1035$, primary melanoma $n = 356$, metastatic melanoma $n = 444$; **Figure 1D**).

Finally, we asked whether these expression differences were reflected in patient outcome. In the overall TCGA-SKCM cohort, high SNCA expression was associated with poorer overall survival (HR $\approx$ 1.30; log-rank p = 0.03), while SNCG expression showed no association with outcome (Figure 1E). Restricting the analysis to AJCC stage IIC disease, however, revealed a specific association between high SNCG expression and shorter progression-free interval (HR = 2.66; 95% CI: 1.15-6.13; p = 0.0218; n = 64, 26 events) — a finding we return to once the cellular identity of the SNCG-high compartment has been established (Figure 6E; Table S8).

α S and γ S sustain melanoma fitness through shared and paralog-specific mechanisms in vitro and in vivo.

Having established the expression landscape of synuclein family members across melanoma datasets, we next sought to validate these findings experimentally and determine their functional relevance. RT-qPCR, Western blotting, and immunocytochemistry confirmed expression of both α S and γ S in human and murine melanoma cell lines (Figure S3A-C). Using shRNA-mediated silencing and plasmid-driven overexpression (Figure S3D-E), we found that reduction of either protein significantly impaired melanoma cell proliferation; unexpectedly, sustained overexpression of either α S or γ S also reduced proliferative capacity, associated with protein accumulation-dependent toxicity evidenced by increased apoptotic markers (Figure 2A; Figure S3F). These results indicate that synuclein expression must remain within a relatively narrow physiological range to support optimal cellular fitness.

Cell-cycle analysis showed no major phase alterations upon sustained overexpression, but a selective accumulation in G2-M was observed upon γ S knockdown (Figure 2B). α S reduction, but not γ S modulation, significantly impaired clonogenic potential (Figure 2C), while silencing of either α S or γ S significantly impaired wound-healing migration (Figure 2D), accompanied by significant changes in nuclear and cytoplasmic cell area upon γ S—but not α S—modulation (Figure S3G). Conversely, sustained overexpression of either α S or γ S was associated with increased nuclear deformability, as evidenced by a higher frequency of cells displaying elongated or kidney-shaped nuclei (Figure S3H). Extending this analysis to a three-dimensional context, Matrigel invasion assays showed that silencing of either α S or γ S significantly reduced invasive capacity, while overexpression of either paralog (α S-V5, γ S-V5) significantly increased it (Figure 2E), indicating that, unlike clonogenicity, invasive capacity is a shared property regulated by both

synucleins rather than a γ S-specific trait. Consistent with this shared regulation, immunocytochemical quantification revealed a marked reduction in GTP-bound Rac1 in both sh- α S and sh- γ S cells under basal conditions (**Figure 2F**), identifying Rac1 activation as a common downstream node engaged by both synucleins during melanoma cell migration and invasion — a point we will return to in later sections.

Having established that α S and γ S regulate multiple features of melanoma cell fitness and aggressiveness in vitro, we next investigated their contribution to tumor progression in vivo. Subcutaneous injection of B16-F0 and B16-F10 melanoma cells with reduced α S or γ S expression demonstrated a significant delay in tumor onset and reduced tumor growth kinetics compared to control cells (**Figure 2G**). Histological examination of tumors from both experimental groups revealed no noticeable morphological alterations or micrometastases in the lungs, indicating that synuclein modulation did not overtly affect tumor histopathology under these conditions (**Figure S4A**).

Because continued growth of subcutaneous melanomas rapidly approached ethical endpoint criteria before overt metastases could develop, primary tumors were surgically excised once they reached predefined volumes to allow extended observation for spontaneous metastasis. Mice bearing sh- α S tumors exhibited a lower incidence of spontaneous lung metastasis and a reduced metastatic burden, evidenced by fewer metastasis-positive mice and a decrease in the number and size of pulmonary metastatic lesions; mice bearing sh- γ S tumors showed a similar trend that did not reach statistical significance (**Figure 2H**; **Table 1**).

To disentangle metastatic dissemination from primary tumor growth constraints and surgical variables, we employed an experimental metastasis model via intrasplenic injection, which preferentially seeds metastases in the liver through the portal circulation. Mice injected with sh- α S cells showed absence of splenic tumor formation (**Figure S4B**) and developed fewer, smaller hepatic metastases relative to controls, with a similar trend for retroperitoneal lymph nodes and lungs (**Figure S4C-E**; **Table 1**). Parallel experiments with sh- γ S cells mirrored these observations at a comparatively lower magnitude, with reduced splenic tumor incidence and fewer hepatic/lymph-node metastases, though differences were only marginally significant. Notably, the effect of α S silencing on metastatic dissemination was particularly consistent across both metastasis models and generally more pronounced than the corresponding effect of γ S silencing,

consistent with a selective role for α S in sustaining the clonogenic fitness required for successful metastatic colonization, distinct from a direct effect on invasive capacity per se.

Exogenous, prion-like uptake of α S/ γ S enhances melanoma proliferative, clonogenic, and migratory capacity in vitro and tumor growth in vivo

The prion-like propagation and aggregation properties of α S have been widely characterized in neurodegenerative disorders, where distinct molecular species — monomers, oligomers, and fibrillar aggregates — can be transmitted between cells, contributing to disease progression^{75–77}.

While neuron-to-neuron transmission of α S aggregates is well documented, their uptake and functional consequences in melanoma cells remain poorly understood⁷⁸. Controlled in vitro fibrillization of purified recombinant α S successfully generated amyloid fibrils (**Figure S5A**). While WB analysis confirmed α S accumulation in challenged melanoma cells (**Figures 3A and S5B**), confocal microscopy was utilized to differentiate surface-bound from internalized protein, revealing robust intracellular localization within 4 h of exposure (**Figure 3B**).

Given the well-documented neurotoxicity of aggregated α S and the limited information available on its effects on melanoma cells, metabolic activity was assessed by MTT reduction following 36 h exposure to increasing concentrations of monomeric, oligomeric, or fibrillar α S. Human neuroblastoma SH-SY5Y cells showed the expected dose-dependent decrease in metabolic activity upon exposure to oligomeric and fibrillar α S, consistent with the established pathogenic effects of aggregated α S in neuronal systems⁷⁹ (**Figure S5C**). SK-MEL28 melanoma cells, in contrast, showed increased MTT reduction upon exposure to monomeric and oligomeric α S, with only the highest fibrillar concentrations producing a modest decrease (**Figure S5C**), revealing a striking cell type-specific response in which melanoma cells display substantially greater tolerance to, and potential metabolic benefit from, soluble α S species than neuronal cells.

Consistent with our previous findings that endogenous α S supports melanoma cell proliferation, exposure to exogenous α S further enhanced proliferative and clonogenic capacity, significantly increasing both the number and size of colonies formed by SK-MEL28 cells, with the strongest effect observed following short-term exposure to fibrillar α S (**Figures 3C-D and S5D-E**), suggesting that transient contact with amyloid assemblies is sufficient to induce durable pro-growth responses. Exogenous α S also significantly accelerated wound closure, with brief 4 h exposures sufficient to induce a migratory phenotype that persisted for at least 24 h (**Figures 3E**

and **S5F**), accompanied by cytoskeletal reorganization and increased cellular elongation, consistent with previous reports implicating synucleins in the regulation of actin and microtubule dynamics^{80,81}.

Recombinant γ S similarly underwent efficient nucleation-dependent fibrillization in vitro, confirmed by agitation-induced assembly assays and circular dichroism spectroscopy (**Figure S6A**). Uptake of exogenous monomeric γ S by melanoma cells was detected by WB (**Figures 3F and S6B**) and confocal microscopy, which confirmed predominantly perinuclear accumulation with enhanced perinuclear and nuclear-associated fluorescence relative to untreated controls, suggesting active intracellular trafficking (**Figure 3G**). Similar to the trends observed for α S, only fibrillar and high-dose oligomeric γ S impaired melanoma cell metabolic activity, while monomeric γ S did not, guiding selection of 1 μ M monomeric γ S — the highest concentration that did not induce detectable toxicity — for subsequent experiments (**Figure S6C**). Pretreatment with monomeric γ S significantly increased melanoma cell growth (**Figures 3H and S6D**) and colony-forming ability, yielding larger and more numerous colonies relative to untreated controls (**Figures 3I and S6E**), consistent with previous reports implicating γ S in tumor cell proliferation and survival^{14,82}. γ S treatment similarly enhanced migration, evidenced by accelerated wound closure relative to untreated controls (**Figures 3J and S6F**), accompanied by increased cytoplasmic and nuclear area and a higher frequency of elongated and kidney-shaped nuclei, morphologies associated with enhanced migratory behavior and metastatic competence in malignant cells (**Figure S6G-H**)⁸³⁻⁸⁵.

Collectively, these results indicate that extracellular synuclein species are not merely passively incorporated by melanoma cells but actively promote a coordinated shift toward a more proliferative, clonogenic, and motile cellular state, mirroring the phenotypes observed upon genetic modulation of endogenous α S and γ S and raising the possibility that synucleins contribute to melanoma progression through both intracellular and extracellular mechanisms.

In vivo, subcutaneous implantation of B16-F0 and B16-F10 cells pretreated with fibrillar α S or monomeric γ S resulted in accelerated tumor growth relative to vehicle-treated controls across both models (**Figure 3K**), indicating that transient exposure to extracellular synucleins is sufficient to induce durable pro-tumorigenic effects that persist following implantation in vivo. Histopathological examination revealed no major differences in tumor architecture, cellular

morphology, or differentiation status between treatment groups (data not shown). Likewise, no pulmonary metastatic lesions were detected at experimental endpoints. To determine whether this extended to metastatic dissemination, primary tumors were surgically excised once they reached predefined volumes, allowing long-term monitoring of spontaneous metastasis. Contrary to what might be anticipated from the pronounced promigratory effects observed in vitro, synuclein-treated cells did not show enhanced spontaneous pulmonary metastasis; if anything, a modest trend toward fewer metastasis-positive animals was observed, although α S-treated tumors showed a tendency toward larger metastatic lesions once established (**Figure 3L**). Consistent with this, an experimental (intrasplenic) metastasis model showed that pretreatment with extracellular synucleins did not significantly alter metastatic colonization: most animals developed splenic tumors, and metastatic lesions occurred predominantly in the liver, reflecting dissemination through the portal circulation, with no significant differences in the incidence, number, or size of hepatic, pulmonary, or retroperitoneal lymph node metastases between synuclein-treated and control groups (**Table 2**), although α S fibril-treated cells showed a modest shift toward fewer small lesions and a greater proportion of intermediate-sized metastases. Together, these observations across both metastasis models point to a dissociation between primary tumor growth/migratory phenotype and metastatic competence that we revisit in the Discussion, alongside the complementary observation that endogenous silencing, but not transient exogenous exposure, robustly impaired metastatic colonization (**Figure 2H**).

α S is transcriptionally coupled to a MITF-linked mitochondrial program and functionally sensitizes complex I in melanoma cells

To characterize the transcriptomic programs associated with SNCA, we stratified patients from the TCGA-SKCM cohort into high- and low-expression groups using median expression as the cutoff and performed differential expression analysis (2,544 differentially expressed genes for SNCA-high vs. SNCA-low; FDR < 0.01, $|\log_2\text{FC}| > 1$; **Table S9**). Over-representation analysis using Reactome showed that SNCA-high tumors were enriched for pathways related to IGFBP binding, metabolism, and extracellular matrix organization (**Figure 4A**; **Table S10**), a pattern reproduced by an independent Gene Ontology analysis, which additionally identified categories related to cytoskeletal regulation and membrane-associated function (**Figure S7A**; **Table S10**). To capture coordinated transcriptional changes not necessarily meeting differential expression thresholds,

we performed Gene Set Enrichment Analysis (GSEA) on the complete ranked gene list ($FDR < 0.05$, $|NES| > 1$): SNCA-high tumors were enriched for pathways related to mitochondrial metabolism, including respiratory electron transport, mitochondrial fatty acid β -oxidation, and mitochondrial translation, with negative enrichment of pathways related to extracellular matrix organization and immune communication (**Figure 4B**; **Table S11**). Collectively, ORA and GSEA converged on a distinct transcriptomic profile, characterizing SNCA-high tumors by a coordinated upregulation of metabolic pathways alongside the suppression of structural and invasive gene sets.

We next asked whether this population-level metabolic shift was also detectable at the single-tumor level, specifically regarding oxidative phosphorylation (OXPHOS) and epithelial-to-mesenchymal transition (EMT) programs. Single-sample GSEA (ssGSEA) confirmed a positive correlation between SNCA expression and the OXPHOS transcriptional score ($\rho = 0.23$; $p = 3.9 \times 10^{-7}$) and a negative correlation with the EMT score ($\rho = -0.31$; $p = 9.19 \times 10^{-12}$; **Figures 4C and S7B**; **Table S12**). Notably, the SNCA–OXPHOS association was highly consistent when OXPHOS activity was estimated using either the manually curated 10-gene signature or the broader 200-gene MSigDB Hallmark Oxidative Phosphorylation gene set ($\rho = 0.23$ and 0.22 , respectively; **Tables S12–S13**). These findings directly link the SNCA-high transcriptional state to a metabolic, non-invasive phenotype at the level of individual tumors.

Given the established role of MITF as the master regulator of both melanocyte differentiation and mitochondrial gene expression, we asked whether the SNCA–OXPHOS axis is mediated by this transcription factor. SNCA expression correlated positively with MITF and an array of OXPHOS-related genes (**Figure 4D**), a relationship independently validated in melanoma cell lines (**Figure 4E**), TCGA tumors at the transcript level (**Figure 4F**), and—at the protein level—between αS and MITF abundance in the PXD035206 proteomic cohort (**Figure 4G**). To determine whether the SNCA–OXPHOS link was a byproduct of shared MITF regulation, we performed partial correlation analysis controlling for MITF expression. Although adjustment attenuated most SNCA–OXPHOS associations, confirming that MITF drives a substantial component of this phenotype, correlations with the mitochondrial coactivator PPARGC1A and core respiratory-chain subunits such as UQCRC1 and NDUFS3 remained statistically significant (**Figure 4H**). These results demonstrate that while the SNCA-associated metabolic profile is partly downstream

of the MITF lineage program, α S maintains a significant, MITF-independent association with specific components of the mitochondrial oxidative machinery.

The relationship between SNCA and MITF extended beyond mitochondrial metabolism to melanogenesis: SNCA correlated strongly with a Reactome melanin biosynthesis score ($\rho = 0.52$, $p = 1.84 \times 10^{-33}$), an association that was substantially attenuated, but remained significant, after adjusting for MITF (partial $\rho = 0.32$; 38.7% reduction) (**Figure 4I**; **Table S13**).

To formally quantify MITF's contribution to both SNCA-associated programs, we performed tumor-purity-adjusted mediation analyses ($n = 468$). MITF mediated 71.6% of the SNCA–OXPHOS association, with no significant residual direct effect, indicating that this program is largely embedded within the MITF lineage state. In contrast, MITF mediated 55.7% of the SNCA–pigmentation association, leaving a significant residual direct effect (**Table S14**), suggesting an additional, MITF-independent component. Serial mediation analysis resolved this residual through TYR, a major MITF-regulated melanogenic effector: the SNCA→MITF→TYR→pigmentation pathway was significant, and once this serial route was accounted for, the residual direct SNCA–pigmentation association was no longer significant (**Table S15**) Variance inflation factors remained below 2 throughout, and sensitivity analysis indicated that the pigmentation-mediated effect was more robust to unmeasured confounding than the OXPHOS effect (**Tables S14–S16**).

Together, these results position SNCA within a broader MITF-centered differentiation state encompassing both oxidative metabolism and melanogenesis, with distinct regulatory architectures: the SNCA–OXPHOS link is predominantly MITF-dependent, while the SNCA–pigmentation link is distributed across MITF-dependent effects and downstream melanogenic effectors such as TYR.

Motivated by this convergent transcriptomic evidence, we directly tested whether α S levels modulate mitochondrial bioenergetics using stable SNCA silencing (sh- α S) and exogenous treatment with α S fibrils. Across B16-F0, A375, and SK-MEL28 melanoma cells, sh- α S increased MTT reduction, whereas exogenous α S fibrils significantly reduced it (**Figures 4J–K** and **S7C**). Consistently, sh- α S cells exhibited a significantly higher basal mitochondrial membrane potential relative to controls, while exogenous fibrils compromised this potential (**Figure 4L**), aligning with

the established capacity of fibrillar α S to impair complex I-dependent respiration and mitochondrial coupling.

To dissect whether these changes specifically reflected altered complex I engagement, we monitored mitochondrial superoxide generation (MitoSOX Red) at baseline and following a challenge with the complex I inhibitor metformin. Baseline MitoSOX signals were slightly lower in sh- α S cells than in controls. Notably, while metformin provoked a robust increase in superoxide levels in control cells, this response was markedly blunted in sh- α S cells (**Figure 4M**), indicating a reduced propensity for complex I-mediated electron leakage upon α S depletion. Concurrently, metformin decreased mitochondrial membrane potential in both groups; however, metformin-treated sh- α S cells maintained absolute potential values comparable to untreated controls (**Figure 4L**). This suggests that while the hyperpolarized basal state of α S-silenced mitochondria remains partially dependent on complex I activity, it confers superior bioenergetic resilience. Together, these functional data demonstrate that endogenous α S acts as a rheostat for mitochondrial tone, sensitizing complex I to inhibitory or proteotoxic challenge. Crucially, its depletion protects against secondary respiratory stress, providing a mechanistic rationale for the paradoxical positive SNCA-OXPHOS transcriptomic correlation—where high SNCA expression flags a metabolic state that is highly active but biologically vulnerable.

A parallel measurement of total cellular ROS (H_2DCFDA) showed higher basal signal in sh- α S cells and a further increase in control cells exposed to exogenous α S fibrils (**Figure 4N**) — the opposite basal direction from MitoSOX. Because MitoSOX is relatively selective for mitochondrial superoxide whereas H_2DCFDA reports predominantly on hydrogen peroxide and other downstream, multi-compartment species, and because superoxide is rapidly dismutated to H_2O_2 via SOD2, a state of more efficient basal electron flux — consistent with the higher $\Delta\Psi_m$ and MTT reduction in sh- α S cells — could plausibly generate superoxide that is converted to H_2O_2 more efficiently than it is subsequently neutralized. Under this interpretation, the MitoSOX and H_2DCFDA data are complementary rather than contradictory, reflecting a shift in the predominant reactive species and its subcellular origin rather than a simple increase or decrease in oxidative burden. A non-exclusive, additional contributor to this signal may be related to iron homeostasis: prior work in SNCA-knockout melanoma cells reported a coordinated dysregulation of iron-handling proteins — increased DMT1 and ferritin alongside decreased TfR1 and

ferroportin — consistent with an iron-overloaded phenotype and accompanied by elevated cellular ROS⁸⁶. If a comparable shift occurs in our sh- α S cells, iron accumulation could contribute to the elevated H₂DCFDA signal via Fenton chemistry, independently of, and in addition to, the mitochondrial-derived H₂O₂ flux discussed above. We present both explanations as plausible, non-exclusive contributing mechanisms rather than as directly tested pathways in this study; direct measurement of intracellular iron content, DMT1/ferroportin expression, and lipid peroxidation would be required to establish whether this iron-related axis is active in our system and whether it extends toward ferroptosis-related vulnerability, which we flag as a promising direction for future work.

Intriguingly, the MITF-linked pigmentation program identified transcriptomically was accompanied by a corresponding pigmentation phenotype in vitro: both sh- α S cells and cells exposed to α S fibrils consistently displayed visible changes in pigmentation relative to controls. Consistent with this, quantification of intracellular and extracellular melanin content confirmed that sh- α S cells produced significantly less melanin than controls in both compartments (**Figure 4O**), directly linking α S depletion to reduced melanogenic output. This observation was concordant with the strong SNCA association with the melanogenesis program identified in TCGA-SKCM and further resolved by the MITF/TYR mediation analyses described above.

SNCG correlates with a mesenchymal-like transcriptomic program and orchestrates an invasive, drug-sensitive FAK/MAPK signaling module in melanoma.

Applying the same differential expression, ORA/GO, and GSEA framework described above for SNCA, we identified 2,142 differentially expressed genes between SNCG-high and SNCG-low tumors ($\text{FDR} < 0.01$, $|\log_2\text{FC}| > 1$; **Table S17**). In marked contrast to the metabolic profile associated with SNCA, SNCG-high tumors were enriched for pathways related to extracellular matrix organization, collagen formation, tissue remodeling, keratinization, and immunoregulatory interactions (**Figure S7D**; **Table S18**), with GSEA additionally revealing negative enrichment of mitochondrial metabolism, mitochondrial translation, and DNA repair pathways (**Figure 5A**; **Table S19**) — the inverse of the SNCA-associated signature. An independent Gene Ontology over-representation analysis reproduced the main functional themes and additionally identified categories associated with extracellular components, cell adhesion, cytoskeletal regulation and membrane-associated functions (**Figure S7E**; **Table S18**)

At the individual-tumor level, ssGSEA confirmed that SNCG expression correlates positively with the EMT score ($\rho = 0.33$, $p = 9.56 \times 10^{-14}$) and negatively with the OXPHOS score ($\rho = -0.23$, $p = 4.39 \times 10^{-7}$; **Figures 5B and S7F; Table S12**), indicating that SNCA and SNCG mark reciprocal transcriptomic programs at the level of each patient.

Consistent with these divergent transcriptional programs, an independent analysis of synuclein-centered molecular interaction networks revealed distinct functional contexts for the two paralogs. Networks integrating GENIE3-inferred regulatory relationships with STRING and PrePPI protein-interaction evidence showed that the SNCA-associated network in melanoma was preferentially linked to metabolic regulation, protein turnover, signaling and vesicular/endosomal trafficking, whereas the SNCG-associated network was enriched for vascular development, cytoskeletal remodeling, cell migration, and invasion (**Figure S7G; Tables S20-S21**). These complementary network-level results further supported the separation between the metabolic SNCA-associated state and the invasive SNCG-associated program.

Motivated by this convergent transcriptomic evidence linking SNCG to cell plasticity and tissue remodeling, we directly examined the signaling machinery underlying γ S-driven invasiveness. Given that Rho GTPases and focal adhesion dynamics are central regulators of cytoskeletal remodeling, we evaluated whether γ -synuclein modulates Rac1 activation and focal adhesion kinase (FAK) signaling. As noted above, SNCG silencing significantly reduced basal Rac1 activation in melanoma cells (**Figure 2F**); conversely, treatment with exogenous γ S monomers markedly increased active, GTP-bound Rac1 levels (**Figure 5C**). In B16-F0 cells, genetic modulation of γ S bidirectionally regulated FAK autophosphorylation at Tyr397, where shRNA-mediated knockdown diminished and overexpression enhanced p-FAK levels. Parallely, exogenous γ S treatment of SK-MEL28 cells similarly stimulated FAK phosphorylation, with the intensified signal predominantly localized to discrete focal adhesions and leading-edge cellular protrusions (**Figure 5D**).

Concurrently, γ S levels modulated key structural components of phenotypic plasticity. Quantitative analysis revealed that sh- γ S cells displayed significantly increased total β -catenin protein expression relative to controls, whereas the exposure of control cells to exogenous γ S monomers caused a pronounced downregulation of total β -catenin (**Figure 5E-F**). While total cellular fractions do not directly isolate nuclear translocation dynamics, these results are

consistent with a modulatory shift in Wnt/ β -catenin transcriptional activity accompanying the acquisition of a mesenchymal-like phenotype. Functional validation confirmed that this signaling configuration alters matrix degradation, as sh- γ S cells exhibited significantly reduced gelatinolytic activity in conditioned media, indicating an impaired extracellular matrix-remodeling capacity (**Figure 5G**).

Because dedifferentiated, highly invasive melanoma states frequently intersect with altered mitogen-activated protein kinase (MAPK) pathway output, we next evaluated ERK phosphorylation. WB analysis revealed markedly lower levels of phospho-ERK in sh- γ S cells relative to control counterparts (**Figure 5E**). To determine whether this reflected a broader transcriptional rewiring of the cascade, we assessed transcript levels of core pathway components via qRT-PCR. SNCG-depleted cells showed a significant reduction in MAP2K1 (encoding MEK1) expression across multiple lines, whereas γ S overexpression was associated with elevated MAP2K1 transcript levels (**Figures 5H**). These data indicate that γ S regulates the baseline output of the core integrin-MAPK signaling module rather than exerting an isolated, post-translational effect on Rac1 or FAK.

Given this integrated signaling profile—characterized by attenuated FAK/MAPK output alongside a more differentiated, β -catenin-high phenotype upon γ S loss—we tested whether SNCG silencing altered sensitivity to clinically relevant therapeutic agents. Notably, sh- γ S cells demonstrated significantly reduced sensitivity to cisplatin, vemurafenib, and paclitaxel across the entire concentration ranges tested (**Figure 5I, Table 3**). The convergence of resistance across three mechanistically unrelated therapeutic classes—a DNA-damaging platinum compound, a targeted BRAF inhibitor, and a microtubule-stabilizing taxane—strongly argues against independent, drug-specific resistance pathways. Instead, these results point to a comprehensive, γ S-dependent cell-state shift toward a slower-proliferative, low-MAPK-dependent phenotype that confers multi-drug tolerance.

SNCG identifies a dedifferentiated malignant subpopulation associated with immune/stromal remodeling and poor clinical outcome.

To gain deeper resolution into the cellular distribution of SNCG within the tumor microenvironment, we extended our transcriptomic analysis to two independent single-cell RNA-sequencing (scRNA-seq) cohorts of metastatic melanoma (GSE115978 and GSE72056). In

both datasets, SNCA expression was highly enriched within malignant cells, whereas SNCG expression was predominantly localized to the endothelial compartment, though it remained clearly detectable in a discrete subset of tumor cells (**Figures 6A and 1C**). Quantitative assessment of the GSE115978 dataset revealed that SNCA exhibited a striking enrichment in malignant cells compared to all other cell populations ($\log_2FC = 4.62$), while SNCG showed its strongest enrichment in endothelial cells ($\log_2FC = 4.57$), alongside a significant and autonomous enrichment within the malignant compartment ($\log_2FC = 2.20$; **Table S7**).

To characterize the intra-tumoral heterogeneity of SNCG within the malignant compartment itself, we performed subclustering on the tumor-cell fractions in these two independent single-cell melanoma datasets. In GSE72056, this approach uncovered a molecularly distinct malignant subpopulation defined by high SNCG expression ($\log_2FC = 5.35$), representing 18.0% of the total tumor cell pool, compared to an expression frequency of just 0.4% in the remaining malignant subclusters (global FDR = 1.49×10^{-37} ; **Figure 6B**). This population showed reduced melanocytic differentiation markers together with increased expression of genes associated with plasticity and invasion. Functionally, this SNCG-high tumor cell subset was characterized by a coordinated downregulation of melanocytic differentiation markers and a concomitant upregulation of gene networks governing cell plasticity and invasion.

In GSE115978, SNCG was also significantly enriched in a multi-patient malignant subpopulation ($\log_2FC = 2.23$; 13.6% vs 7.2%; global FDR = 5.18×10^{-4}), with no single patient contributing more than 16.1% of the cluster (**Figure S8**). This population showed a relative reduction in melanocytic markers but retained substantial melanocytic identity and did not reproduce the same invasive/dedifferentiated phenotype observed in GSE72056. Thus, SNCG-associated malignant-cell heterogeneity was reproducible across datasets, whereas the associated transcriptional state was context dependent.

To determine whether these features shape the broader architecture of the tumor niche, we leveraged xCell2-derived microenvironmental scores in the bulk TCGA-SKCM cohort. SNCG expression correlated positively with endothelial ($\rho = 0.58$; FDR = 1.08×10^{-42}), fibroblast ($\rho = 0.47$; FDR = 1.19×10^{-26}), monocyte ($\rho = 0.33$; FDR = 1.16×10^{-12}), and alternatively activated macrophage ($\rho = 0.31$; FDR = 1.84×10^{-11}) signatures (**Figure 6C; Table S22**). Conversely, SNCA displayed predominantly negative correlations with these myeloid, inflammatory, and stromal

signatures. While these findings indicate that the SNCG signal captured in bulk tumor samples is partly derived from stromal and endothelial infiltration, deconvolution algorithms cannot definitively distinguish microenvironmental composition from tumor-intrinsic programs.

To isolate the tumor-cell intrinsic transcriptomic architecture from confounding microenvironmental components, we analyzed a system entirely devoid of the stroma: a panel of 51 distinct human melanoma cell lines. In this cell-autonomous context, SNCG expression remained positively correlated with hallmark markers of dedifferentiation, mesenchymal transition, and invasion, including AXL ($\rho = 0.64$), ITGA5 ($\rho = 0.57$), ZEB1 ($\rho = 0.47$), VIM ($\rho = 0.41$), TGFB1 ($\rho = 0.37$), and FN1 ($\rho = 0.29$). Concurrently, it displayed strong inverse correlations with standard melanocytic lineage markers, such as PMEL ($\rho = -0.55$), MLANA ($\rho = -0.53$), MITF ($\rho = -0.48$), and SOX10 ($\rho = -0.34$; **Figure 6D**; **Table S23**). The preservation of this opposing gene expression network in a cell-autonomous system, devoid of stromal input, strongly supports a tumor-intrinsic component to the SNCG-associated mesenchymal-like state, rather than this state simply reflecting stroma-to-malignant cellular ratios in bulk biopsies.

Finally, we evaluated whether these divergent cellular and transcriptomic configurations impact clinical outcomes. Stratification of the overall TCGA-SKCM cohort by the median expression revealed that high SNCA expression correlated with a higher risk of death as shown previously in **Figure 1E**. While SNCG was not globally predictive of survival across the unstratified cohort, an exploratory subgroup analysis based on AJCC staging revealed a potent, stage-specific clinical impact in patients with advanced stage IIC disease. Within this specific subgroup, elevated SNCG expression associated with a significantly shorter progression-free interval (PFI), conferring a 2.66-fold increased risk of disease progression (HR = 2.66; 95% CI: 1.15–6.13; $p = 0.0218$; $n = 64$, 26 events; **Figure 6E**; **Table S8**). Considered together with our previous evidence demonstrating that α S depletion drives mitochondrial efficiency while suppressing pigmentation, the finding that the SNCG-high state is defined by the suppression of MITF and the induction of mesenchymal features (**Figures 6B and D**) completes a crucial biological model. These convergent data demonstrate that the metabolic, non-invasive program governed by SNCA and the dedifferentiated, highly invasive program driven by SNCG operate as distinct molecular poles that converge, from diametrically opposite directions, on the master MITF-melanocyte differentiation axis—a conceptual paradigm we dissect further in the Discussion. The mechanistic

and phenotypic divergence between α S and γ S across all experimental axes examined is summarized in **Table 4**.

DISCUSSION

This study provides a comprehensive characterization of α -synuclein (α S) and γ -synuclein (γ S) in melanoma, establishing them as previously underappreciated regulators of tumor progression and cell-state heterogeneity. Through the integration of molecular, cellular, in vivo, and multi-cohort transcriptomic analyses, our findings reveal that α S and γ S are neither redundant nor functionally interchangeable paralogs. Instead, they delineate two complementary, mutually exclusive molecular poles that participate in alternative transcriptional and functional programs. While genetic silencing of either synuclein impairs primary tumor kinetics and metastatic colonization—confirming that endogenous expression of both family members is required for optimal tumor fitness—they orchestrate distinct cellular states. Specifically, our data position α S within an oxidative, lineage-differentiated program, and γ S within an integrin-engaged, invasive, and drug-tolerant mesenchymal-like configuration.

A central finding of this work is the robust, biologically grounded inverse correlation between SNCA and SNCG expression. This anti-correlation was reproducible across bulk clinical transcriptomics (TCGA-SKCM and GSE65904), single-cell RNA-sequencing data, malignant-cell subclustering, and independent cell lines. Deconvolution analyses indicated that this pattern is not solely attributable to differences in stromal, immune, or erythroid cellular composition, suggesting that the SNCA-SNCG relationship is, at least in part, a tumor-intrinsic feature rather than a byproduct of microenvironmental variation. This inverse relationship was also detectable at the proteomic level in one metastatic melanoma cohort (PXD035206) but not reproduced in a second, mixed primary/metastatic cohort, raising the possibility that the strength of this relationship varies with clinical stage, tumor composition, or cohort-specific methodology — a question that will require testing in additional, stage-matched proteomic cohorts. This molecular dichotomy fits within the contemporary framework of melanoma phenotype switching, where tumor cells occupy a dynamic continuum of transcriptional identities rather than a static proliferative-versus-invasive dichotomy^{87–89}. Our data position synuclein paralog balance as a candidate marker of, and potential contributor to, these alternative cell states.

Our functional and transcriptomic dissection of SNCA reveals a nuanced metabolic role that qualifies the standard interpretation of oxidative phosphorylation (OXPHOS) signatures in cancer. While strong population-level correlations between SNCA expression and mitochondrial gene sets (electron transport chain, TCA cycle, fatty acid β -oxidation) might suggest that α S acts as a direct, linear driver of OXPHOS output, our bioenergetic experiments point to a more homeostatic relationship. SNCA silencing improved basal mitochondrial parameters (MTT reduction, mitochondrial membrane potential) and blunted the depolarizing effect of a secondary complex I challenge with metformin, whereas exposure to exogenous α S fibrils — the species most consistently linked to complex I impairment in neurodegenerative models^{90,91} — instead compromised mitochondrial function. We propose that endogenous α S acts as a physiological modulator that sensitizes complex I to additional stress, rather than as a simple positive driver of respiratory output; under this model, elevated SNCA expression in OXPHOS-high tumors may reflect this sensitizing role rather than a direct causal increase in respiratory capacity, a distinction that transcriptomic correlation alone cannot resolve. This model is further supported by the loss-of-function phenotype observed upon α S depletion; the resulting reduction in cellular pigmentation suggests that α S expression is required to sustain baseline metabolic homeostasis, aligning with its proposed role as a homeostatic rheostat rather than a linear driver. Because the melanocyte lineage factor MITF coordinately governs both melanogenesis and mitochondrial biogenesis/OXPHOS gene expression in melanoma⁹², we interpret the mitochondrial and pigmentation phenotypes not as independent effects, but as related readouts of a shared, MITF-linked differentiation program — a connection further supported by our finding that SNCA correlates positively with MITF and OXPHOS-related genes across bulk, single-cell, and protein-level analyses, with a partial-correlation analysis indicating that this relationship is only in part explained by MITF itself.

γ S, in contrast, engages a coherent signaling module that anchors the opposite, invasive pole of the phenotype-switching continuum. Our data show that γ S sustains active, GTP-bound Rac1 and FAK autophosphorylation, promotes matrix-degrading gelatinolytic activity, and supports MAP2K1 transcription, alongside reduced total β -catenin expression. This regulation was bidirectional and dose-consistent: γ S silencing reduced MAP2K1 transcript levels, while γ S-V5 overexpression increased them, supporting a direct, rather than purely correlative, relationship

between γ S abundance and core MAPK pathway output. While this signaling configuration promotes mesenchymal-like migration and invasion, its genetic silencing instead shifted cells toward a low-MAPK-output, more differentiated state that showed reduced sensitivity to three mechanistically unrelated therapeutic agents — cisplatin, vemurafenib, and paclitaxel. This drug-tolerant profile is consistent with the "persister" or slow-cycling phenotypes described for dedifferentiated, low-MAPK-dependent melanoma cells^{88,89,93}, and suggests that γ S loss promotes a cell-state shift toward reduced MAPK dependence, rather than an agent-specific resistance mechanism — a possibility that will require validation across a broader panel of cell lines and direct pathway epistasis experiments to establish causality. Notably, this metabolic-to-invasive polarity converges on the same lineage program identified for α S: SNCG-high malignant cells show reduced MITF expression, the same factor that links α S-dependent mitochondrial tone to differentiation. This raises the possibility that α S and γ S act at partially overlapping nodes of the same melanocyte differentiation axis, approached from opposite molecular directions — to our knowledge, there are no previous reports linking synuclein paralog balance and this axis in melanoma.

A further notable finding is the efficiency with which melanoma cells internalize extracellular synucleins. Prion-like intercellular transfer and aggregation of α S are well-characterized features of neurodegeneration, yet their behavior in malignant contexts remains largely unexplored. Concentrations of oligomeric and fibrillar α S that impair viability in SH-SY5Y neuronal cells instead promoted melanoma cell proliferation and clonogenic capacity. This context-dependent divergence suggests that melanoma cells do not simply undergo the proteotoxic stress associated with synuclein exposure in neurons, but may instead engage extracellular synucleins as signaling or trophic cues. This interpretation is consistent with functional annotation of our protein-protein interaction networks, which identified vesicle trafficking and endosomal transport among the enriched functional categories associated with the synuclein interactome. Furthermore, these data align seamlessly with independent reports demonstrating that SNCA depletion reduces the cell-surface abundance of the adhesion molecule L1CAM⁶⁶. Mechanistically, α S acts as a critical regulator of this membrane topology, either by facilitating the anterograde transport and exocytic fusion of L1CAM-containing vesicles with the plasma

membrane, or by preventing the endosomal routing and subsequent lysosomal degradation of the internalized receptor⁶⁶.

Our in vivo data add a layer of spatial resolution to this uptake biology. While continuous endogenous expression of either synuclein was required for both local tumor growth and metastatic colonization, transient exogenous synuclein exposure stimulated primary tumor expansion but did not increase metastatic colonization under the conditions tested. This suggests that while transient extracellular exposure is sufficient to engage local proliferative and migratory programs, successful metastatic colonization instead depends on sustained, cell-intrinsic transcriptional states supported by endogenous expression⁹⁴, consistent with contemporary views of metastatic colonization as a highly regulated, rate-limiting process.

We acknowledge specific limitations that outline clear directions for future work. Several of our mechanistic links rely on total rather than compartment-specific readouts (e.g., total cellular β -catenin rather than isolated nuclear/cytosolic fractions), and formal demonstration of direct causality within the γ S-FAK-Rac1-MAPK axis awaits epistatic rescue or pharmacological inhibition experiments (e.g., a Rac1 or FAK inhibitor in γ S-overexpressing cells). Our exploratory clinical analysis in the TCGA-SKCM cohort found that while SNCG expression is not a global prognostic biomarker, its elevated expression is associated with a shorter progression-free interval specifically within the AJCC stage IIC subgroup. Given the limited size of this stage-specific subgroup (n = 64), this finding should be considered hypothesis-generating and will require validation in independent, adequately powered cohorts before any diagnostic or prognostic application can be considered.

Finally, our use of the B16-F0 model for in vivo and bioenergetic experiments was originally motivated by its suitability for syngeneic, immunocompetent studies rather than by an a priori hypothesis regarding catecholamine metabolism. Nonetheless, this choice gains additional interpretive coherence when considered alongside the shared catechol-oxidation biology underlying neuromelanin and melanin synthesis, and the well-documented epidemiological association between Parkinson's disease and melanoma risk^{95,96}. We note that this connection was not the original rationale for our experimental design, but offers a plausible framework that merits direct investigation in future work.

In conclusion, our results indicate that α S and γ S are distinct components of two complementary, partially convergent programs that contribute to melanoma cell-state heterogeneity. By integrating mechanistic experimentation with systems-level bioinformatic analyses, this study situates synuclein biology within the framework of melanoma phenotype switching, and nominates the α S/ γ S balance as a candidate marker of — and potential contributor to — the equilibrium between metabolic fitness and invasive plasticity during melanoma progression.

5. CONCLUSION AND FUTURE PERSPECTIVES

In conclusion, this study redefines the role of synucleins in oncology, demonstrating that α S and γ S are not redundant paralogs but distinct molecular rheostats that dictate opposing cell-state trajectories in melanoma progression. By operating at the intersection of the master MITF-differentiation axis and the phenotype-switching continuum, the α S/ γ S balance governs a critical trade-off between lineage-dependent mitochondrial fitness and mesenchymal-like invasive plasticity. Our findings establish that while α S maintains an active yet vulnerable oxidative program, γ S orchestrates an integrin-engaged, drug-tolerant invasive phenotype. Furthermore, the efficiency with which melanoma cells subvert and exploit extracellular synucleins underscores a highly adaptive biology that mirrors—yet functionally inverts—the mechanisms of neurodegeneration.

Future perspectives should focus on two major areas. Mechanistically, it is critical to determine the structural determinants that allow melanoma cells to turn extracellular synuclein internalization into a survival and proliferative advantage, contrasting with neuronal toxicity. Identifying the specific cell-surface receptors and chaperone networks driving this uptake will be essential. Therapeutically, our discovery that γ S depletion induces a multi-drug tolerant, slow-cycling phenotype suggests that targeting the γ S-FAK-MAPK axis must be timed carefully. Future studies should evaluate sequential treatment strategies—using γ S inhibition to force cells into a differentiated state, followed by targeting the specific metabolic or lineage-dependent vulnerabilities of that state—to effectively clear residual, drug-resistant metastatic subpopulations.

SUPPLEMENTARY MATERIAL

Figure S1-S8.

Table S1-S23.

CRedit author statement

Author Contributions: FM and LCZ performed investigation, visualization, and writing—original draft. M Mian and PSMP contributed to investigation and data curation. AA performed formal analysis and software. NCL, CF, and M Mamberto contributed to methodology, validation, and visualization. AB performed methodology and formal analysis. XRB contributed to conceptualization and formal analysis. LEA conducted software, formal analysis, visualization, conceptualization and writing—original draft. MM-M contributed to conceptualization, formal analysis, project administration, supervision, and writing—original draft. All authors read, reviewed, and approved the final manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest. All authors have read the journal's authorship agreement. The manuscript has been reviewed by and approved by all authors.

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Institutional Review Board Statement

Authors declare that all experimental protocols involving animals were approved by the “Committee for the Care and Use of Laboratory Animals (CICUAL)” of the Rosario Medical School. Mice were treated in accordance with the Canadian Council on Animal Care and ARRIVE 2.0 guidelines.

Data Availability Statement

Data presented in this study are available upon request from the corresponding author.

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LIST OF MAIN FIGURES AND TABLES

FIGURE 1. Expression landscape, co-regulatory dynamics, and prognostic relevance of alpha- and gamma-synuclein in melanoma. (A) Evaluation of SNCA and SNCG co-expression dynamics across independent experimental scales. From left to right: Pearson/Spearman correlation coefficients in a panel of melanoma cell lines, bulk transcriptomic data from the TCGA-SKCM cohort, validation in the GSE65904 dataset, and single-cell/pseudobulk resolution analysis. (B) Correlation analysis of alpha-synuclein and gamma-synuclein expression at the proteomic level utilizing the PXD035206 dataset. (C) Single-cell transcriptomic profiling of metastatic melanoma lesions derived from the integrated GSE115978, highlighting cell-type specific distribution of synuclein variants. (D) Density distribution plots illustrating the progressive shift in SNCA and SNCG expression levels across distinct stages of disease progression: normal skin, primary melanoma, and metastatic melanoma. (E) Kaplan–Meier survival curves for the unstratified complete melanoma cohort. High expression of SNCA (red) is significantly associated with compromised overall survival (Hazard Ratio, $HR \approx 1.30$), whereas SNCG expression (blue) displays no statistically significant prognostic association in bulk transcriptomic analysis. Shaded areas or ticks indicate censored events. Statistical significance was determined using the log-rank test.

FIGURE 2. Alpha- and gamma-synuclein expression modulates melanoma proliferation, invasive phenotype, and in vivo metastatic potential. (A) Growth curves depicting cell proliferation kinetics over time, quantified by direct cell counting of murine melanoma cell lines under distinct synuclein expression conditions. (B) Cell cycle phase distribution analysis (G0/G1, S, and G2/M phases) determined by flow cytometry profiling. (C) Quantification of clonogenic capacity assessed by colony formation efficiency in murine melanoma cells following alpha- or gamma-synuclein modulation. (D) Wound healing assay measuring lateral migration rates of B16-F0 and B16-F10 cells, normalized to the initial wound area at time 0. (E) Three-dimensional invasion capacity evaluated by Transwell Matrigel invasion assays under the indicated synuclein expression conditions. (F) Immunoblots and corresponding quantification of active, GTP-bound Rac1 (Rac1-GTP) levels determined by GST-PAK1 pull-down assays, reflecting small GTPase

activation status. **(G)** In vivo subcutaneous tumor growth kinetics in mice challenged with B16-F0 or B16-F10 melanoma cells transduced with control vectors or shRNAs targeting alpha-synuclein (sh-SNCA) or gamma-synuclein (sh-SNCG). Tumor volume was monitored every 2–3 days using caliper measurements. **(H)** Quantification and histopathological assessment of spontaneous pulmonary metastases. Left panel: average number of macrometastatic lung nodules per mouse; right panel: size distribution categorization of metastatic lesions (large: >1.5 mm; medium: 0.5–1.5 mm; small: <0.5 mm). Representative macroscopic images of lungs are shown, with arrows pointing to metastatic foci. Data are presented as mean $\pm$ SEM from at least three independent experiments.

FIGURE 3. Exogenous, prion-like internalization of alpha- and gamma-synuclein enhances melanoma aggressiveness in vitro and in vivo. **(A)** Western blot analysis confirming the intracellular internalization (uptake) of recombinant human alpha-synuclein (SNCA) monomers or pre-formed fibrils in SK-MEL-28 cells; PBS-treated cells served as negative controls, and β -actin was used as the loading control. **(B)** Representative confocal immunofluorescence microscopy images illustrating the intracellular localization of internalized SNCA monomers and fibrils in SK-MEL-28 cells; scale bar: 20 μ m. **(C)** Dose-dependent proliferation kinetics and cell viability of SK-MEL-28 cells exposed to SNCA monomers, oligomers, or fibrils under either continuous exposure (upper panel) or transient 24h pulse (lower panel) incubation conditions. **(D)** Clonogenic potential of SK-MEL-28 cells following exposure to SNCA fibrils, quantified by colony number and size distribution metrics. **(E)** Wound healing migration kinetics of SK-MEL-28 cells treated with exogenous SNCA monomers; data are normalized to the initial wound area at time 0. **(F)** Immunoblot validation confirming the exogenous internalization of gamma-synuclein (SNCG) monomers in human cells, with β -actin as the loading control. **(G)** Confocal laser scanning microscopy images validating the intracellular uptake and spatial distribution of SNCG monomers in SK-MEL-28 cells following incubation; scale bar: 20 μ m. **(H)** Proliferation kinetics of SK-MEL-28 melanoma cells challenged with exogenous SNCG monomer formulations. **(I)** Clonogenicity assays highlighting the impact of SNCG monomer internalization on subsequent colony-forming efficiency (total number and surface area distribution). **(J)** Lateral cell motility assessed by wound healing assays in SK-MEL-28 cells stimulated with exogenous SNCG

monomers. **(K)** In vivo subcutaneous tumor growth kinetics in mice challenged with B16-F0 or B16-F10 murine melanoma cells pretreated ex vivo with synuclein variants; tumor volume was tracked via caliper measurements every 2–3 days. **(L)** Quantitative and macroscopic profiling of spontaneous pulmonary macrometastases. Left panel: average number of secondary lung nodules per animal; right panel: lesion size stratification. Bottom: representative macroscopic photographic images of excised lungs are shown, with arrows indicating pigmented metastatic foci. Data are presented as mean $\pm$ SEM from at least three independent biological replicates. Statistical comparisons were performed using multi-way ANOVA followed by appropriate post-hoc tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4. Transcriptomic networks and functional validation position alpha-synuclein as a modulator of MITF-driven oxidative phosphorylation and mitochondrial fitness in melanoma.

(A) Reactome over-representation analysis (ORA) of differentially expressed genes (DEGs) between SNCA-high and SNCA-low tumors within the TCGA-SKCM cohort, stratified by median expression. The x-axis denotes the GeneRatio; dot size indicates gene counts mapped per pathway, and dot color represents the Benjamini–Hochberg False Discovery Rate (FDR)-adjusted p-value. **(B)** Reactome Gene Set Enrichment Analysis (GSEA) performed utilizing complete gene lists ranked by $\log_2$ fold-change from differential expression profiling, showing separate positively enriched (activated) and negatively enriched (suppressed) pathways for SNCA. The x-axis indicates absolute normalized enrichment scores ($|NES|$); dot size reflects gene-set size, and dot color represents the FDR-adjusted p-value. **(C)** Scatter plot illustrating the correlation between SNCA transcript abundance and the oxidative phosphorylation (OXPHOS) single-sample GSEA (ssGSEA) signature score across the TCGA-SKCM cohort. Solid black lines represent linear regression fits with 95% confidence intervals; Spearman correlation coefficients (ρ) and p-values are indicated. **(D)** Transcriptomic correlation analyses in a panel of human and murine melanoma cell lines, validating the coordinated expression between SNCA, MITF, and key OXPHOS-associated gene networks. **(E–G)** Inter-patient expression concordance showing that SNCA correlates significantly with MITF levels in cell lines (E), primary and metastatic melanoma lesions at both the transcriptional level (F) and the mature protein level (G). **(H)** Mediation and partial correlation analysis evaluating whether the statistical association between

SNCA expression and OXPHOS-related transcript networks is mechanistically dependent on their shared regulatory relationship with MITF. **(I)** SNCA expression is positively associated with Reactome melanin biosynthesis score in the TCGA-SKCM cohort (n=473; Spearman correlation coefficient and p-value are indicated in the figure). **(J,K)** Cell viability and metabolic activity measured by MTT assays in B16-F0 lines (comparing vehicle-control vs. sh-SNCA vectors and exogenous SNCA challenge) (J) and across human A375 and SK-MEL-28 cell lines (K) subjected to exogenous SNCA internalization. **(L)** Mitochondrial membrane potential ($\Delta\phi_m$) fluorometric measurements in control versus sh-SNCA cells, under vehicle conditions or challenged with exogenous SNCA variants, including functional rescues evaluating sh-SNCA kinetics in the presence of the complex I inhibitor Metformin. **(M)** Mitochondrial superoxide production quantified by MitoSOX Red fluorescence under basal conditions or following complex I disruption via Metformin treatment in control versus sh-SNCA melanoma models. **(N)** Fluorometric quantification of intracellular reactive oxygen species (ROS) accumulation across the indicated experimental conditions. **(O)** Intracellular content and 24h extracellular accumulation of melanin in B16-F0 cells measured as Absorbance at 475nm. Data are presented as mean $\pm$ SEM from at least three independent biological replicates. Statistical analyses were performed using appropriate parametric tests or multi-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 5. Transcriptomic networks and biochemical validation position gamma-synuclein as a driver of EMT signaling, matrix remodeling, and chemoresistance in melanoma. **(A)** Reactome Gene Set Enrichment Analysis (GSEA) performed utilizing complete gene lists ranked by log₂ fold-change from differential expression profiling, showing separate positively enriched (activated) and negatively enriched (suppressed) pathways for SNCG. The x-axis indicates absolute normalized enrichment scores (|NES|); dot size reflects gene-set size, and dot color represents the FDR-adjusted p-value. **(B)** Scatter plot illustrating the correlation between SNCG transcript abundance and the Epithelial-to-Mesenchymal Transition (EMT) single-sample GSEA (ssGSEA) signature score across the TCGA-SKCM cohort. Solid black lines represent linear regression fits with 95% confidence intervals; Spearman correlation coefficients (ρ) and p-values are indicated. **(C)** Immunoblots and corresponding quantification of active, GTP-bound Rac1

(Rac1-GTP) levels determined by immunocytochemical (ICC) detection, comparing vehicle-control cells against cells challenged with exogenous SNCG monomer formulations. **(D)** ICC detection and quantification of active, phosphorylated Focal Adhesion Kinase (p-FAK) spatial distribution, comparing B16-F0 vehicle-control cells, sh-SNCG silenced models (sh- γ S), ectopically overexpressing cells (γ -V5), and SK-MEL-28 cells subjected to exogenous γ S internalization. **(E)** Western blot and corresponding immunocytochemical analysis of total β -catenin protein accumulation and immunobiological detection of ERK1/2 phosphorylation status (p-ERK) across B16-F0 control, sh- γ S, γ S -V5 (upper panel), and β -catenin protein levels by WB in control and exogenous γ S-treated SK-MEL-28 cells (bottom panel). **(F)** β -catenin immunocytochemistry detection on control and exogenous γ S-treated SK-MEL-28 cells. **(G)** Gelatin zymography assessing the matrix metalloproteinase gelatinolytic activity, comparing vehicle-control conditions against sh- γ S and γ S-V5 engineered cell lines. **(H)** Quantitative RT-PCR analysis of MAP2K1 transcript expression levels in control versus sh- γ S and γ S -V5 settings, validated across an extended panel of distinct human and murine melanoma cell lines. **(I)** Dose-response cell viability curves evaluating drug sensitivity profiles to Cisplatin, Vemurafenib, and Paclitaxel, comparing control cells against sh-SNCG knockdown models. Data are presented as mean $\pm$ SEM from at least three independent biological replicates. Statistical comparisons were performed using multi-way ANOVA followed by appropriate post-hoc tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGURE 6. Single-cell dissection of synuclein variants in skin compartments, transcriptomic state alignment, and stage-specific prognostic relevance in melanoma. **(A)** Dot plot showing the expression of SNCA, SNCB, and SNCG across major cell populations in the GSE72056 melanoma single-cell RNA-seq dataset. Dot color represents log₂FC relative to all remaining cell types, and dot size indicates the percentage of positive cells. **(B)** Dot plot aligning synuclein expression with established melanoma lineage differentiation, invasive behavior, extracellular matrix (ECM) architecture, and active proliferation markers across discrete malignant-cell transcriptional states within the GSE72056 scRNA-seq dataset. Dot color represents the mean expression density, and dot size indicates the percentage of expressing cells per marker. **(C)** Heatmap profiling the correlation networks between SNCA or SNCG expression levels and microenvironmental

immune-cell enrichment scores, computationally derived via the xCell2 deconvolution framework across the TCGA-SKCM cohort. Color intensity scales with the strength and directional magnitude (Spearman correlation coefficients) of the specific cellular infiltration associations. **(D)** Interspecies correlation matrices evaluating SNCG expression against validated master markers governing invasive/dedifferentiated (AXL, ITGA5, and ZEB1) versus melanocytic differentiation (MITF, MLANA, and PMEL) programs across an extensive panel of melanoma cell lines. Each dot depicts an individual cell lineage; solid lines represent linear regression fits, with corresponding Spearman correlation coefficients (ρ) indicated. **(E)** Kaplan–Meier survival analysis tracking the progression-free interval (PFI) according to SNCG expression thresholds within the clinically defined AJCC Stage IIC patient subgroup of the TCGA-SKCM cohort. Patients were dichotomized into high- and low-expression arms utilizing the median expression level as the stratification cutoff. Log-rank p-values, hazard ratios (HR) with 95% confidence intervals, and cohort sample sizes (n) are explicitly detailed.

SUPPLEMENTARY FIGURE 1. Comparative transcriptomic landscape and transcriptional dysregulation of the synuclein gene family across pan-cancer and dermatological malignancies.

(A) Transcriptomic analysis utilizing integrated TCGA and GTEx datasets showing a step-wise reduction in SNCA, SNCB, and SNCG transcript levels in low-grade gliomas (LGG) and glioblastoma multiforme (GBM) compared with non-neoplastic brain tissues, demonstrating a progressive Normal > LGG > GBM expression pattern (Benjamini–Hochberg adjusted False Discovery Rate, FDR < 0.05). **(B)** Heatmap showing the mean expression of SNCA, SNCB, and SNCG in sun-exposed lower-leg skin and non-sun-exposed suprapubic skin from GTEx. Expression is reported as transcripts per million (TPM), and hierarchical clustering summarizes similarities among genes and tissue types. **(C)** Differential expression of SNCA, SNCB, and SNCG across TCGA cancer types with available tumor and matched normal samples. Dot color represents the $\log_2$ fold change between tumor and normal tissue, whereas dot size represents statistical significance as $-\log_{10}$ of the FDR-adjusted p-value. Positive values indicate higher expression in tumors and negative values indicate lower expression. TCGA project abbreviations

are shown on the x-axis. **(D)** Relative protein-expression scores for α -synuclein, β -synuclein, and γ -synuclein across normal human tissues, based on immunohistochemistry data from the Human Protein Atlas. Protein expression was classified as high, medium, low, or not detected. **(E)** Comparative analysis of relative SNCA, SNCB, and SNCG expression across five distinct skin-related malignancies modeled via linear mixed-effects frameworks. SNCA represents the predominant synuclein variant across all three cutaneous melanoma subtypes, whereas SNCB consistently exhibits the lowest expression levels in cutaneous and acral melanoma. Basal cell carcinoma displays a divergent profile characterized by SNCG enrichment exceeding both SNCA and SNCB levels. Brackets indicate significant pairwise contrasts after rigorous correction for multiple testing.

SUPPLEMENTARY FIGURE 2. Proteomic validation cohorts, proteogenomic concordance, and single-cell resolution of synuclein variants in normal skin and melanoma. **(A)** Correlation between SNCA and SNCG expression in an independent melanoma cohort, GSE65904 (left) and in cutaneous melanoma metastases from TCGA-SKCM (right). Each dot represents one tumor sample. Black lines indicate linear regression fits with 95% confidence intervals. Spearman correlation coefficients (ρ), p-values, and sample sizes are shown. **(B)** Left, correlation between α -synuclein and γ -synuclein protein abundance in the Human Melanoma Proteome Atlas (PXD026086), comprising primary and metastatic melanoma samples. Each dot represents one tumor sample. Black lines indicate linear regression with 95% confidence intervals. Spearman correlation coefficients (ρ) and p-values are shown. Right, detection frequency of α -, β -, and γ -synuclein proteins in the same cohort. **(C)** Correlation between SNCA mRNA expression and α -synuclein protein abundance in the independent proteogenomic melanoma cohort (PXD035206). Each dot represents one tumor sample. Black lines indicate linear regression with 95% confidence intervals. Spearman correlation coefficient (ρ) and p-value are shown. **(D)** Dot plots showing the expression of SNCA, SNCB, and SNCG across major cell populations in two independent healthy human skin single-cell RNA-seq datasets (E-MTAB-8142 and GSE130973). Dot color represents the $\log_2$ fold change ($\log_2FC$) of each gene relative to all remaining cell types, and dot size

indicates the percentage of cells expressing the corresponding gene within each cell population. Cell types are grouped into major tissue compartments.

SUPPLEMENTARY FIGURE 3. Molecular validation of synuclein modulation and phenotypic characterization. (A) Western blot analysis of endogenous alpha-synuclein (SNCA, left panel) and gamma-synuclein (SNCG, right panel) expression across human and murine melanoma cell lines, compared against neuronal (SH-SY5Y) and colorectal adenocarcinoma (HT29) positive controls, respectively. β -actin served as the loading control. (B) Quantitative RT-PCR assessment of endogenous SNCA and SNCG mRNA transcript levels in melanoma cell lines, normalized against murine brain or HT29 positive control tissues. (C) Immunocytochemical localization of alpha-synuclein (left) and gamma-synuclein (right). The actin cytoskeleton is counterstained in red, and nuclei are visualized with DAPI (blue). Scale bar: 50 μ m. (D) Validation of shRNA-mediated silencing efficiency for SNCA (protein level via Western blot) and SNCG (transcript level via RT-qPCR) in murine melanoma cells. (E) Validation of SNCA and SNCG ectopic overexpression in stable lines, detected via anti-V5 epitope tag immunoblotting, with β -actin as the loading control. (F) Flow cytometry-based quantification of apoptotic cell populations following alpha- or gamma-synuclein expression modulation. (G-H) Quantitative morphometric profiling of cellular surface area, nuclear area (G), and nuclear architecture metrics (H) across the indicated experimental groups.

SUPPLEMENTARY FIGURE 4. In vivo histopathological and metastatic evaluation of synuclein-modulated tumors. (A) Representative hematoxylin and eosin (H&E) stained histopathological sections of subcutaneous tumors derived from modified B16 cells. Scale bar: 50 μ m. (B) Incidence and macroscopic quantification of visible tumor formation in the spleens of mice challenged in vivo with engineered B16-F10 melanoma cells. (C) Penetrance analysis showing the percentage of mice developing macro-metastatic lesions across multiple anatomical sites, including the liver, lungs, and regional lymph nodes. (D) Cumulative metastatic burden quantified as the total number of secondary metastatic nodules per mouse across experimental groups. (E) Size

distribution dynamics of metastatic foci classified according to lesion diameter metrics. Data represent mean $\pm$ SEM from at least three independent biological replicates.

SUPPLEMENTARY FIGURE 5. Biophysical properties, multi-lineage functional screening, and extended in vivo metastatic profiling of exogenous alpha-synuclein species. (A) Biophysical and structural characterization of recombinant alpha-synuclein (SNCA) variants, assessed by far-UV circular dichroism (CD) spectroscopy to monitor secondary structure transitions from random coil (monomeric) to high β -sheet (fibrillar) conformations. (B) Western blot analysis confirming the intracellular internalization (uptake) of exogenous SNCA species across an extended panel of melanoma cell lines, including human A375 and murine B16-F0 and B16-F10 lines, with β -actin as the loading control. (C) Dose-dependent cell viability and cytotoxicity screening determined by MTT assays in human neuroblastoma (SH-SY5Y) and melanoma (SK-MEL-28) cells exposed to varying concentrations of diverse structural SNCA species (monomers, oligomers, and fibrils). (D–F) High-throughput functional evaluation of exogenous SNCA challenge across A375, B16-F0, and B16-F10 cell lines, characterizing its impact on (D) long-term proliferation kinetics, (E) clonogenic potential quantified by colony number and surface area, and (F) lateral migration velocity assessed by wound healing assays.

SUPPLEMENTARY FIGURE 6. Biophysical properties, multi-lineage functional screening, and extended in vivo metastatic profiling of exogenous gamma-synuclein species. (A) Biophysical and structural characterization of recombinant gamma-synuclein (SNCG) variants, assessed by far-UV circular dichroism (CD) spectroscopy to monitor secondary structure transitions from random coil (monomeric) to high β -sheet (fibrillar) conformations. (B) WB analysis confirming the intracellular internalization (uptake) of exogenous SNCG species across an extended panel of melanoma cell lines, including human A375 and murine B16-F0 and B16-F10 lines, with β -actin as the loading control. (C) Dose-dependent cell viability and cytotoxicity screening determined by MTT assays in human neuroblastoma (SH-SY5Y) and melanoma (SK-MEL-28) cells exposed to varying concentrations of diverse structural SNCG species (monomers, oligomers, and fibrils). (D–F) High-throughput functional evaluation of exogenous SNCG challenge across A375, B16-

F0, and B16-F10 cell lines, characterizing its impact on (D) long-term proliferation kinetics, (E) clonogenic potential quantified by colony number and surface area, and (F) lateral migration velocity assessed by wound healing assays. (G) Morphometric analysis quantifying shifts in total cellular surface area and nuclear cross-sectional area in SK-MEL-28, B16-F0, and B16-F10 cells following incubation with SNCG monomers. (H) High-content nuclear architectural profiling mapping the percentage of cells displaying rounded, elongated, or kidney-shaped nuclear phenotypes within the cell lines described in (G).

SUPPLEMENTARY FIGURE 7. Extended functional ontologies, epithelial-to-mesenchymal transition dynamics, and pigmentation program correlation networks of synuclein variants. (A) Gene Ontology (GO) over-representation analysis (ORA) of differentially expressed genes (DEGs) statistically associated with SNCA expression within the TCGA-SKCM cohort. Left panels provide a comprehensive summary of enriched signaling cascades, whereas right panels delineate the highest-ranking over-represented functional terms compartmentalized by Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) ontologies. Dot size scales proportionally with the number of genes assigned per term; the x-axis represents the GeneRatio, and dot color corresponds to the statistical significance of enrichment ($-\log_{10}$ p-value). (B) Transcriptomic correlation profiles demonstrating a significant negative association between SNCA expression and established Epithelial-to-Mesenchymal Transition (EMT) gene signature scores in human melanoma specimens. (C) Representative macroscopic or microscopic optical images of colorimetric cell viability assays (MTT), illustrating localized cell density variations and metabolic reduction profiles across the designated experimental groups. (D) Reactome over-representation analysis (ORA) of differentially expressed genes (DEGs) between SNCG-high and SNCG-low tumors within the TCGA-SKCM cohort, stratified by median expression. The x-axis denotes the GeneRatio; dot size indicates gene counts mapped per pathway, and dot color represents the Benjamini-Hochberg False Discovery Rate (FDR)-adjusted p-value. (E) Gene Ontology (GO) over-representation analysis (ORA) of differentially expressed genes (DEGs) statistically associated with SNCG expression within the TCGA-SKCM cohort. Left panels provide a comprehensive summary of enriched signaling cascades, whereas right panels delineate

the highest-ranking over-represented functional terms compartmentalized by Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) ontologies. Dot size scales proportionally with the number of genes assigned per term; the x-axis represents the GeneRatio, and dot color corresponds to the statistical significance of enrichment ($-\log_{10}$ p-value). (F) Negative correlation between SNCG expression and OXPHOS transcriptional state signature in the TCGA-SKCM cohort. (G) Functional composition of the integrated molecular interaction and regulatory networks associated with SNCA and SNCG in healthy skin and melanoma lesions. Networks were computationally generated by integrating upstream regulatory interactions inferred via the GENIE3 framework with physical protein–protein interactions (PPI) derived from the STRING and PrePPI databases, followed by downstream functional enrichment analytics. Donut charts summarize the relative percentage distribution of manually curated functional categories among the significantly enriched pathways for each specific network, with colors corresponding to the functional metadata categories indicated in the legend.

SUPPLEMENTARY FIGURE 8. Reproducible SNCG-associated malignant heterogeneity with context-dependent transcriptional states. Single-cell analysis of dataset GSE115978 reveals significant SNCG enrichment in a multi-patient malignant subpopulation ($\log_2\text{FC}=2.23$; 13.6% vs 7.2%; global FDR = 5.18×10^{-4}), with well-distributed patient representation (maximum individual contribution <16.1%). This population exhibits reduced melanocytic markers while retaining substantial lineage identity.

LIST OF TABLES

TABLE 1

A. Mice with spontaneous lung metastasis

control		sh- α S		sh- γ S	
N _{met} /N _{tot}	%	N _{met} /N _{tot}	%	N _{met} /N _{tot}	%
9/10	90	5/8	62.5	7/10	70

B. Mice with splenic tumors

control		sh- α S		sh- γ S	
N _{met} /N _{tot}	%	N _{met} /N _{tot}	%	N _{met} /N _{tot}	%
4/6	66.67	0/6	0	2/6	33

C. Mice with liver metastasis

control		sh- α S		sh- γ S	
N _{met} /N _{tot}	%	N _{met} /N _{tot}	%	N _{met} /N _{tot}	%
5/6	83.33	4/6	66.67	3/6	50

Table 1. Metastasis development.

(A) Spontaneous metastasis development following surgical excision of primary tumors at a volume of 1,500 mm³. N_{met}: number of mice with metastasis; N_{tot}: total number of animals. (B) Number of splenic tumors in an experimental metastasis model induced by intrasplenic injection of melanoma cells. (C) Hepatic metastases arising after intrasplenic injection of melanoma cells.

TABLE 2

A. Mice with spontaneous lung metastasis

control		+ α S		+ γ S	
$N_{\text{met}}/N_{\text{tot}}$	%	$N_{\text{met}}/N_{\text{tot}}$	%	$N_{\text{met}}/N_{\text{tot}}$	%
9/10	90	7/10	70	4/6	66.6

B. Mice with splenic tumors

control		+ α S		+ γ S	
$N_{\text{met}}/N_{\text{tot}}$	%	$N_{\text{met}}/N_{\text{tot}}$	%	$N_{\text{met}}/N_{\text{tot}}$	%
4/6	66.6	5/6	83.33	4/6	66.6

C. Mice with liver metastasis

control		+ α S		+ γ S	
$N_{\text{met}}/N_{\text{tot}}$	%	$N_{\text{met}}/N_{\text{tot}}$	%	$N_{\text{met}}/N_{\text{tot}}$	%
5/6	83.33	6/6	100	6/6	100

Table 2. Metastasis development after incubation of cells with synucleins.

(A) Spontaneous metastasis development following surgical excision of primary tumors at a volume of 1,500 mm³. N_{met} : number of mice with metastasis; N_{tot} : total number of animals. (B) Number of splenic tumors in an experimental metastasis model induced by intrasplenic injection of melanoma cells. (C) Hepatic metastases arising after intrasplenic injection of melanoma cells.

TABLE 3

Cell line	control			sh- γ S		
Drugs	IC50	LogIC50	R squared	IC50	LogIC50	R squared
Vemurafenib (μ M)	31.608	4.500	0.9775	52.616	4.721	0.9736
Paclitaxel (nM)	1.076	3.032	0.9873	4.865	3.687	0.9888
Cisplatin (μ M)	4.501	3.653	0.9687	7.055	3.848	0.9860

Table 3. IC50 values for antitumor drugs in melanoma cells.

Control and γ S-downmodulated cells were exposed to increasing concentrations of cisplatin (0.5-100 μ M), vemurafenib (0.5 μ M-2mM), or paclitaxel (0.1nM-1 μ M) for 72h, and cell viability was assessed by MTT assay. Dose-response curves were fitted using a non-linear regression model. IC50 values were derived from the fitted curves and compared between sh- γ S and control cells using an F-test of the fitted curves and unpaired Student's t-test on log-transformed IC50 values.

TABLE 4

Biological Axis / Process	α -Synuclein (α S / SNCA) Driven Program	γ -Synuclein (γ S / SNCG) Driven Program	Phenotypic & Translational Convergence / Context
Dominant Cell State & Lineage Alignment	Differentiated / Lineage-anchored Pole: positively coupled to MITF and SOX10; enriched in highly proliferative, non-mesenchymal bulk and single-cell tumor fractions (<i>Fig. 1A–C; Fig. 4A–I</i>).	Dedifferentiated / Neural Crest Plasticity Pole: negatively coupled to MITF; high-level enrichment in mesenchymal-like, AXL/ZEB1/ITGA5-high cell populations (<i>Fig. 1A–C; Fig. 6A</i>).	Phenotype Switching Framework: paralog balance acts as a molecular rheostat governing the sliding scale between differentiated and invasive states.
Genetic Depletion Impact (shRNA)	Impairs in vitro proliferation & clonogenicity (<i>Fig. 2A–C</i>). Suppresses metastatic colonization in vivo (<i>Fig. 2G–H</i>).	Impairs proliferation with selective G2/M phase arrest (<i>Fig. 2B</i>). Blunts in vitro matrix degradation (gelatinolytic activity) (<i>Fig. 5G</i>).	Shared Fitness Dependency: basal levels of both paralogs are mandatory to sustain absolute growth kinetics and baseline in vivo tumorigenic potential.
Core Intracellular Signaling Modules	Mitochondrial Homeostasis Rheostat: direct regulation of specific respiratory components (PPARGC1A, UQCRC1, NDUFS3) (<i>Fig. 4A–I</i>).	Integrative Adhesion–MAPK Cascade: orchestrates the Integrin–FAK(Y397)–Rac1–MAPK signaling axis (<i>Fig. 5C–E</i>); transcriptionally regulates MAP2K1/MEK1 (<i>Fig. 5H</i>).	Divergent Hubs: α S commands metabolic tone and electron flux sensitivity; γ S dictates structural remodeling and mitogenic output.
Exogenous Conformational Variant Toxicity (MTT)	Monomers/Oligomers: well tolerated, act as metabolic/trophic signals. Fibrils (β -sheet-rich): selectively impair real-time dehydrogenase kinetics (<i>Fig. 4J–K</i>).	Monomers: highly tolerated; induce nuclear expansion and architectural alterations (<i>Fig. S6C and S6G–H</i>). Oligomers/Fibrils: cytotoxic exclusively at highly elevated concentrations (<i>Fig. S6C</i>).	Malignant vs. Neuronal Dichotomy: melanoma cells display extreme resilience to proteotoxic amyloid species compared to neuroblastoma lines (SH-SY5Y).
Exogenous Internalization Outcomes (In Vitro Pulse)	Different species promote proliferation, reorganize the actin cytoskeleton and accelerate lateral cell migration.	Monomers trigger immediate Rac1-GTP loading (<i>Fig. 5C</i>), FAK hyperphosphorylation (<i>Fig. 5D</i>), and β -catenin downregulation (<i>Fig. 5E–F</i>).	Prion-like Feed-forward Loop: both protein variants are rapidly internalized, subverting extracellular pools into direct mechanical and signaling advantages.
Therapeutic Sensitivity & Resistance Profiles	Metabolic Vulnerability: α S depletion hyperpolarizes baseline mitochondrial membrane potential ($\Delta\psi$), safeguarding against secondary complex I stress / metformin (<i>Fig. 4L</i>).	Multi-Drug Tolerance Driven by State Shift: γ S silencing yields a low-MAPK, high- β -catenin state, conferring wide cross-resistance to cisplatin, vemurafenib and paclitaxel (<i>Fig. 5I; Table 3</i>).	Clinical Paradox: the invasive γ S-high subpopulation represents a lethal lineage, but its therapeutic targeting requires sequential strategies due to drug-tolerant persister induction upon immediate loss.
Clinical Progression & Prognostic Value	Levels escalate progressively from normal skin to advanced metastatic niches; associated globally with shorter overall survival in bulk disease cohorts (HR = 1.30; log-rank p = 0.03; <i>Fig. 1E</i>).	Abundant in tumor endothelial networks, but marks reprimed aggressive single tumor cells (<i>Fig. 6A</i>); structurally tied to Stage IIC progression and shorter progression-free interval (PFI) (HR = 2.66; 95% CI: 1.15–6.13; <i>Fig. 6E</i>).	Independent Stratification: α S serves as a global biomarker of high metabolic burden, while γ S acts as a stage-specific hazard marker for localized high-risk progression.

Table 4. Functional and Mechanistic Divergence of Synuclein Paralogs and Structural Variants in Melanoma State Plasticity.

Values summarize findings from shRNA-mediated silencing, exogenous protein exposure, and clinical cohort analyses described throughout the manuscript (see corresponding figure and table citations within each cell). α S, alpha-synuclein; γ S, gamma-synuclein; $\Delta\psi$, mitochondrial

- 1897 membrane potential; HR, hazard ratio; CI, confidence interval; PFI, progression-free interval;
1898 MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

SUPPLEMENTARY TABLES

Supplementary Table 1

Tumor type	Dataset code	n samples
cSCC	GSE284467	53
acral	GSE190113	105
SKCM	TCGA-SKCM	473
uveal	TCGA-UVM	80
BCC1	GSE125285	25
BCC2	GSE156171	28

Table S1. Synuclein expression in skin-related tumors.

Datasets selected for the comparative analysis of synuclein expression across skin-related tumor types. For each dataset, the tumor type, accession code, and number of samples included are indicated. cSCC, cutaneous Squamous Cell Carcinoma; SKCM, Skin Cutaneous Melanoma; BCC, Basal Cell Carcinoma.

Supplementary Table 2

Gene	Comparison	Mean 1	N1	Mean 2	N2	Δ mean	95% CI	p. adj
SNCA	GBM vs. Normal Brain	8.61	175	12.31	387	-3.71	(-3.97, -3.45)	6.40E-14
SNCA	LGG vs. Normal Brain	9.44	534	12.31	387	-2.87	(-3.06, -2.68)	8.50E-14
SNCA	GBM vs. LGG	8.61	175	9.44	534	-0.84	(-1.13, -0.54)	2.00E-10
SNCB	GBM vs. Normal Brain	8.89	175	12.76	387	-3.87	(-4.27, -3.46)	1.20E-13
SNCB	LGG vs. Normal Brain	10.32	534	12.76	387	-2.44	(-2.73, -2.15)	< 1e-16
SNCB	GBM vs. LGG	8.89	175	10.32	534	-1.43	(-1.85, -1.01)	1.20E-12
SNCG	GBM vs. Normal Brain	7.69	175	11.77	387	-4.09	(-4.4, -3.78)	1.10E-12
SNCG	LGG vs. Normal Brain	9.18	534	11.77	387	-2.6	(-2.84, -2.36)	< 1e-16
SNCG	GBM vs. LGG	7.69	175	9.18	534	-1.49	(-1.81, -1.17)	< 1e-16

Table S2. Pairwise comparisons of variance-stabilized (VST) expression values of SNCA, SNCB, and SNCG in normal brain, lower-grade glioma (LGG), and glioblastoma (GBM).

Raw RNA-seq count data from TCGA and GTEx were jointly normalized using DESeq2 and transformed using the variance-stabilizing transformation (VST). Pairwise comparisons were performed using Games–Howell tests following Welch's ANOVA. The table reports mean VST expression values, sample sizes, mean differences, 95% confidence intervals, and Benjamini–Hochberg-adjusted p-values.

Supplementary Table 3

Gene	Sun-exposed (n = 754) Median (IQR)	Non-sun- exposed (n = 651) Median (IQR)	Hedges' g (95% CI)	Effect size	t	Adjusted P (BH)
SNCA	2.24 (0.49)	2.18 (0.55)	0.14 (0.04–0.25)	Negligible	2.65	0.01
SNCB	0.34 (0.41)	0.33 (0.42)	0.04 (–0.06–0.15)	Negligible	0.82	0.41
SNCG	5.02 (0.83)	4.90 (0.71)	0.25 (0.15–0.36)	Small	4.75	6.6×10^{-6}

Table S3. Comparison of synuclein expression between sun-exposed and non-sun-exposed normal skin.

Median $\log_2(\text{TPM} + 1)$ expression values and interquartile ranges (IQR) of SNCA, SNCB, and SNCG in GTEx normal skin samples. Group comparisons were performed using Welch's two-sample t-test. The table reports Hedges' g effect sizes with 95% confidence intervals, t statistics, and Benjamini–Hochberg-adjusted p-values.

Supplementary Table 4

tumor	F value	df1	df2	p-value
acral	614.679	2	312.000	5.96E-109
BCC	709.100	2	104.000	2.50E-61
cSCC	126.330	2	104.000	1.47E-28
SKCM	770.294	2	1416.000	4.29E-227
uveal	8.463	2	158.000	3.23E-04

Table S4. Linear mixed-effects ANOVA of synuclein expression by tumor type.

Results of the lmer-ANOVA testing overall differences in synuclein gene expression (term = gen) across individual skin-related tumor cohorts. The table reports the F statistic (F_value), numerator and denominator degrees of freedom (df1, df2), and raw p-values. BCC, Basal Cell Carcinoma; cSCC, cutaneous Squamous Cell Carcinoma; SKCM, Skin Cutaneous Melanoma.

Supplementary Table 5

tumor	gene	EMM	SE	95% CI
acral	SNCA	5.79	0.12	(5.554,6.018)
acral	SNCB	0.49	0.12	(0.254,0.718)
acral	SNCG	1.01	0.12	(0.781,1.244)
bcc	SNCA	2.49	0.10	(2.288,2.701)
bcc	SNCB	2.95	0.10	(2.746,3.16)
bcc	SNCG	6.27	0.10	(6.059,6.473)
cSCC	SNCA	3.01	0.12	(2.783,3.245)
cSCC	SNCB	0.43	0.12	(0.203,0.665)
cSCC	SNCG	1.42	0.12	(1.19,1.651)
skcm	SNCA	5.73	0.07	(5.594,5.873)
skcm	SNCB	1.93	0.07	(1.791,2.069)
skcm	SNCG	2.93	0.07	(2.795,3.074)
uveal	SNCA	3.22	0.16	(2.903,3.531)
uveal	SNCB	2.84	0.16	(2.526,3.154)
uveal	SNCG	2.32	0.16	(2.004,2.632)

Table S5. Estimated marginal means of synuclein expression within each tumor type.

Estimated marginal means (EMMs) obtained from the linear mixed-effects model comparing the expression of SNCA, SNCB, and SNCG within each tumor type. The model included synuclein gene as a fixed effect and sample as a random intercept. Reported values correspond to the estimated marginal mean (EMM), standard error (SE), and 95% confidence interval (CI) of $\log_2(\text{TPM} + 1)$ expression. These estimates were used to perform the post-hoc pairwise comparisons reported in Supplementary Table S6.

Supplementary Table 6

tumor	contrast	estimate	SE	df	t ratio	p adj	95% CI	Cohen d
acral	SNCA - SNCB	5.30	0.17	208	31.82	0.01	(4.907,5.694)	4.39
acral	SNCA - SNCG	4.77	0.17	208	28.66	0.01	(4.381,5.167)	3.96
acral	SNCB - SNCG	-0.53	0.17	208	-3.16	0.01	(-0.92,-0.133)	-0.44
BCC	SNCA - SNCB	-0.46	0.11	104	-4.20	1.69E-04	(-0.718,-0.199)	-0.82
BCC	SNCA - SNCG	-3.77	0.11	104	-34.51	3.51E-14	(-4.031,-3.512)	-6.70
BCC	SNCB - SNCG	-3.31	0.11	104	-30.31	3.51E-14	(-3.573,-3.053)	-5.89
cSCC	SNCA - SNCB	2.58	0.16	104	15.75	3.51E-14	(2.191,2.97)	3.06
cSCC	SNCA - SNCG	1.59	0.16	104	9.73	6.39E-14	(1.204,1.983)	1.89
cSCC	SNCB - SNCG	-0.99	0.16	104	-6.02	7.85E-08	(-1.376,-0.597)	-1.17
SKCM	SNCA - SNCB	3.80	0.10	944	37.87	0.01	(3.568,4.039)	2.46
SKCM	SNCA - SNCG	2.80	0.10	944	27.87	0.01	(2.564,3.035)	1.81
SKCM	SNCB - SNCG	-1.00	0.10	944	-10.00	0.01	(-1.24,-0.768)	-0.65
uveal	SNCA - SNCB	0.38	0.22	158	1.72	0.20	(-0.142,0.897)	0.27
uveal	SNCA - SNCG	0.90	0.22	158	4.10	1.97E-04	(0.38,1.419)	0.65
uveal	SNCB - SNCG	0.52	0.22	158	2.38	0.048	(0.003,1.042)	0.38

Table S6. Pairwise Tukey post-hoc comparisons of synuclein expression within tumor types.

Results of Tukey-adjusted pairwise contrasts from the linear mixed-effects model, comparing log₂-transformed expression among SNCA, SNCB, and SNCG within each tumor cohort. For every tumor type, the table reports the estimated mean difference (estimate), standard error (SE), degrees of freedom (df), t-ratio, Benjamini–Hochberg–adjusted p-value (p_adj), 95 % confidence interval (ci_low–ci_high), and Cohen’s d effect size. BCC, Basal Cell Carcinoma; cSCC, cutaneous Cquamous Cell Carcinoma; SKCM, Skin Cutaneous Melanoma.

1959 **Supplementary Table 7**

gene	cell_type	avg_log2FC	pct.1	pct.2	FDR_global
SNCA	Malignant	4.62	73.8	8.1	0.000
SNCA	Endothelial	-0.11	34.6	27.3	0.2
SNCA	Cancer-Associated Fibroblast	-1.1	17.9	27.6	0.019
SNCA	Macrophage	-1.66	22.4	27.7	0.00064
SNCA	CD8+ T cell	-3.56	6.4	34.6	3.7E-119
SNCA	T cell (unspecified)	-3.94	5	30	8.8E-46
SNCA	B cell	-4.16	6.5	30.2	4.2E-49
SNCA	CD4+ T cell	-5.02	4.7	30.6	1.5E-58
SNCA	Natural Killer cell	-7.26	6.5	27.7	0.0000037
SNCB	Malignant	3.85	2.9	0.2	4.7E-25
SNCB	Cancer-Associated Fibroblast	1.97	0.9	1	0.97
SNCB	Endothelial	1.67	1.9	1	0.35
SNCB	Natural Killer cell	-2.3	0	1	0.35
SNCB	CD4+ T cell	-3.35	0.2	1.1	0.022
SNCB	Macrophage	-3.56	0.2	1	0.13
SNCB	CD8+ T cell	-3.76	0.1	1.3	0.000031
SNCB	B cell	-4.02	0.1	1.1	0.011
SNCB	T cell (unspecified)	-5.38	0	1.1	0.0076
SNCG	Endothelial	4.57	43.3	2.8	7.7E-114
SNCG	Malignant	2.2	7.9	1.5	5.6E-40
SNCG	Cancer-Associated Fibroblast	1	11.3	3.3	0.000016
SNCG	CD8+ T cell	-3.52	0.4	4.4	2.3E-15
SNCG	CD4+ T cell	-4.1	0.2	3.9	0.00000013
SNCG	B cell	-4.29	0.4	3.8	0.00000082
SNCG	Macrophage	-4.64	1	3.6	0.0063
SNCG	Natural Killer cell	-6.63	0	3.4	0.086
SNCG	T cell (unspecified)	-6.9	0.1	3.8	0.000001

1960

1961 **Table S7. Differential expression of SNCA, SNCB and SNCG across cell populations in the**
1962 **GSE115978 melanoma single-cell RNA-seq dataset.**

1963 Each cell type was compared against all remaining cells using Seurat FindMarkers (Wilcoxon
1964 rank-sum test). The table reports the average log2 fold change (avg_log2FC), the percentage of
1965 cells expressing the gene within the cell type of interest (pct.1) and in all other cell types (pct.2),
1966 and the Benjamini–Hochberg adjusted p-value (FDR_global). Positive log2FC values indicate
1967 enrichment in the indicated cell type, whereas negative values indicate lower expression relative
1968 to the remaining cells.

Subgroup variable	Subgroup	Endpoint	HR	95% CI	p-value	n	Events
AJCC pathological stage	Stage IIC	PFI	2.66	1.15–6.13	0.022	64	26
AJCC pathological N stage	NX	DSS	0.1	0.01–0.83	0.033	35	12
AJCC pathological N stage	NX	PFI	0.37	0.13–1.08	0.068	35	20
AJCC pathological T stage	T2a	DSS	2.31	0.91–5.86	0.077	31	18
AJCC pathological T stage	T2a	OS	2.23	0.90–5.51	0.083	31	19
AJCC pathological stage	Stage IIIC	OS	0.55	0.27–1.13	0.105	67	31
AJCC pathological N stage	NX	OS	0.34	0.09–1.28	0.111	35	14
AJCC pathological stage	Stage IIIC	DSS	0.61	0.29–1.27	0.187	67	29
Sex	MALE	PFI	0.83	0.62–1.10	0.195	282	197
AJCC pathological stage	Stage IIB	OS	0.36	0.08–1.69	0.196	28	13
AJCC pathological T stage	TX	DSS	0.53	0.20–1.40	0.199	44	19
AJCC pathological stage	Stage IIIB	PFI	1.66	0.74–3.73	0.219	46	30
Sex	FEMALE	PFI	1.26	0.87–1.84	0.222	173	110
AJCC pathological stage	Stage IIB	PFI	0.51	0.16–1.59	0.245	28	17
AJCC pathological stage	Stage IIIB	OS	1.83	0.66–5.06	0.246	46	21
AJCC pathological stage	Stage IIIC	PFI	0.71	0.39–1.29	0.259	67	48
AJCC pathological T stage	T3b	DSS	1.81	0.64–5.14	0.263	37	19
AJCC pathological N stage	N1b	PFI	0.64	0.29–1.42	0.271	38	27
AJCC pathological stage	Stage IIB	DSS	0.43	0.09–2.03	0.289	28	12
AJCC pathological T stage	TX	OS	0.61	0.24–1.56	0.302	44	20
AJCC pathological T stage	T4b	PFI	1.29	0.78–2.14	0.324	112	61
AJCC pathological T stage	T2a	PFI	1.53	0.65–3.60	0.328	31	23
AJCC pathological T stage	T4b	OS	1.31	0.74–2.32	0.354	112	50
AJCC pathological stage	Stage IIC	OS	1.51	0.63–3.64	0.357	64	20
AJCC pathological stage	Stage IIIB	DSS	1.67	0.53–5.30	0.384	46	16
AJCC pathological stage	Stage III	DSS	0.63	0.22–1.82	0.393	41	14
AJCC pathological N stage	N3	OS	0.71	0.33–1.55	0.394	54	26
Primary tumor ulceration	NO	DSS	1.23	0.76–1.98	0.399	145	68
AJCC pathological T stage	T2	OS	1.55	0.55–4.36	0.407	30	16
AJCC pathological stage	Stage II	DSS	1.52	0.56–4.10	0.41	30	16
Primary tumor ulceration	NO	OS	1.21	0.76–1.91	0.422	145	73
AJCC pathological T stage	T3a	PFI	1.32	0.64–2.72	0.453	39	31
AJCC pathological T stage	T4a	DSS	1.61	0.43–6.10	0.48	25	9
AJCC pathological N stage	N3	DSS	0.76	0.35–1.68	0.499	54	25
AJCC pathological stage	Stage IB	PFI	1.35	0.55–3.28	0.51	29	23
AJCC pathological T stage	T2	PFI	0.76	0.34–1.74	0.523	30	24
AJCC pathological stage	Stage I	PFI	0.77	0.34–1.72	0.525	30	26
AJCC pathological T stage	T3b	OS	1.38	0.50–3.80	0.53	37	21
AJCC pathological stage	Stage I	OS	1.38	0.50–3.84	0.532	30	17

Subgroup variable	Subgroup	Endpoint	HR	95% CI	p-value	n	Events
AJCC pathological stage	Stage IB	DSS	1.43	0.46–4.38	0.535	29	15
AJCC pathological stage	Stage II	OS	1.34	0.53–3.41	0.539	30	18
AJCC pathological N stage	N1b	DSS	0.7	0.22–2.21	0.541	38	13
AJCC pathological T stage	T2	DSS	1.38	0.48–3.99	0.553	30	15
AJCC pathological N stage	N1b	OS	0.74	0.26–2.09	0.565	38	16
AJCC pathological T stage	T3a	OS	0.79	0.33–1.88	0.596	39	22
Sex	MALE	DSS	0.91	0.64–1.30	0.613	282	126
Primary tumor ulceration	NO	PFI	1.1	0.76–1.60	0.624	145	111
AJCC pathological stage	Stage IB	OS	1.3	0.45–3.75	0.634	29	16
AJCC pathological stage	Stage III	OS	0.81	0.32–2.07	0.664	41	18
Primary tumor ulceration	YES	DSS	0.9	0.55–1.48	0.683	166	66
Sex	MALE	OS	0.94	0.68–1.31	0.717	282	144
AJCC pathological stage	Stage I	DSS	1.21	0.42–3.47	0.722	30	16
AJCC pathological stage	Stage IIC	DSS	1.18	0.44–3.16	0.747	64	16
AJCC pathological T stage	T3a	DSS	0.87	0.36–2.10	0.754	39	21
AJCC pathological T stage	T4b	DSS	1.1	0.59–2.06	0.758	112	41
AJCC pathological T stage	T3b	PFI	0.92	0.39–2.14	0.842	37	25
Primary tumor ulceration	YES	OS	0.96	0.61–1.51	0.845	166	77
AJCC pathological N stage	N0	DSS	1.04	0.69–1.56	0.847	224	96
AJCC pathological N stage	N0	OS	1.03	0.70–1.52	0.872	224	106
AJCC pathological T stage	T4a	PFI	1.08	0.40–2.88	0.884	25	17
AJCC pathological N stage	N3	PFI	1.05	0.57–1.94	0.887	54	42
Sex	FEMALE	OS	1.03	0.64–1.65	0.903	173	70
AJCC pathological T stage	T4a	OS	1.07	0.32–3.55	0.914	25	11
AJCC pathological N stage	N0	PFI	1.01	0.73–1.40	0.937	224	150
AJCC pathological T stage	TX	PFI	1.02	0.51–2.05	0.96	44	35
AJCC pathological stage	Stage II	PFI	1.02	0.43–2.39	0.969	30	23
Sex	FEMALE	DSS	1.01	0.61–1.65	0.982	173	63
Primary tumor ulceration	YES	PFI	1	0.67–1.50	0.988	166	100
AJCC pathological stage	Stage III	PFI	1	0.44–2.26	0.997	41	25

Table S8. Exploratory subgroup survival analysis of SNCG expression in the TCGA-SKCM cohort. Cox proportional hazards models were fitted independently within each clinicopathological subgroup to evaluate the association between high versus low SNCG expression and overall survival (OS), disease-specific survival (DSS), or progression-free interval (PFI). Hazard ratios (HR), 95% confidence intervals (CI), nominal p-values, subgroup sample size (n), and the number of events are shown. Rows are ordered by increasing p-value. The analysis was exploratory and no correction for multiple subgroup comparisons was applied.

Supplementary Tables 9-11

Due to their size, **Supplementary Tables S9-S11** are not included in this PDF and are provided as separate Microsoft Excel (.xlsx) files accompanying the online supplementary material. The legend for each one is detailed below.

Table S9. Differentially expressed mRNAs between high- and low-SNCA expression patient groups.

The table lists Ensembl gene ID (mRNA), log₂ fold change (logFC), Benjamini–Hochberg false discovery rate (FDR), absolute difference in normalized counts (Delta), median expression in high and low groups (High, Low), and genomic coordinates (start–end, hg38).

Table S10. Pathway over-representation analyses for the SNCA-high vs SNCA-low contrast based on differentially expressed genes.

Analyses were performed using the Gene Ontology (GO) database—Biological Process (BP), Molecular Function (MF), and Cellular Component (CC)—and curated pathways (PAT). Additionally, enrichment using the Reactome database was computed. p-values were adjusted by Benjamini–Hochberg (reported as FDR and $-\log_{10}(\text{FDR})$). Columns include: Rank; ontology/pathway ID and term/description; FDR; $-\log_{10}(\text{FDR})$; gene-set size (ng); overlap size (ncommon); and the contrast label (“Comparison”). Reactome tables additionally report GeneRatio, BgRatio, pvalue, p.adjust, qvalue, overlapping genes (geneID), and overlap count (Count). Analyses were conducted in R using clusterProfiler/ReactomePA with the human annotation package org.Hs.eg.db.

Table S11. Gene set enrichment analysis of Reactome database in SNCA-high group.

The table contains two sheets: ACTIVATED (positively enriched in SNCA-high; NES > 0) and SUPPRESSED (negatively enriched in SNCA-high; NES < 0, i.e., enriched in SNCA-low). Columns: GENESET NAME (MSigDB Reactome term), SIZE (genes in set after filtering), ES (enrichment score), NES (normalized ES), NOM p-val, FDR q-val (multiple-testing adjusted), FWER p-val, RANK AT MAX (rank position at maximum ES), and LEADING EDGE (core enrichment metrics: tags/list/signal percentages).

2007 **Supplementary Table 12**

Gene	Signature	Gene_set_source	N	Spearman_rho	p_value	FDR
SNCA	EMT	Manually curated EMT signature (12 genes)	473	-0.31	9.19E-12	1.84E-11
SNCG	EMT	Manually curated EMT signature (12 genes)	473	0.33	9.56E-14	3.82E-13
SNCA	OXPHOS	Manually curated OXPHOS signature (10 genes)	473	0.23	3.89E-07	4.39E-07
SNCG	OXPHOS	Manually curated OXPHOS signature (10 genes)	473	-0.23	4.39E-07	4.39E-07

2008

2009 **Table S12. ssGSEA associations of SNCA and SNCG with manually curated OXPHOS and EMT**
2010 **signatures in the TCGA-SKCM cohort.**

2011 Single-sample gene set enrichment analysis (ssGSEA) was used to calculate EMT and OXPHOS
2012 scores from manually curated 12-gene and 10-gene signatures, respectively. Associations between
2013 SNCA or SNCG expression and signature scores were assessed using Spearman correlation.
2014 Nominal p-values were adjusted jointly across the four comparisons using the Benjamini–
2015 Hochberg method.

Supplementary Table 13

Due to its size, **Supplementary Table S13** is not included in this PDF and is provided as separate Microsoft Excel (.xlsx) file accompanying the online supplementary material. The legend for this Table is detailed below.

Table S13. Unadjusted and MITF-adjusted associations of SNCA with pigmentation and OXPHOS programs in TCGA-SKCM.

Spearman correlations were calculated between SNCA expression and pathway-level transcriptional scores or individual genes associated with pigmentation and oxidative phosphorylation. The pigmentation score was calculated from the Reactome Melanin Biosynthesis gene set, whereas the OXPHOS score was derived from the MSigDB Hallmark Oxidative Phosphorylation gene set; only genes represented in the TCGA-SKCM expression matrix were included in score calculation. Partial Spearman correlations were subsequently calculated after adjustment for MITF expression. Nominal p-values were corrected for multiple comparisons using the Benjamini–Hochberg method separately within each analysis family. The percentage reduction in correlation magnitude was calculated as $100 \times (|\rho_{\text{unadjusted}}| - |\rho_{\text{adjusted}}|) / |\rho_{\text{unadjusted}}|$.

2032 **Supplementary Table 14**

Pathway	Effect	Unadjusted	Unadjusted p	Purity-adjusted	Adjusted p
Pigmentation	Indirect (via MITF)	0.357 [0.277, 0.442]	<0.001	0.339 [0.254, 0.422]	<0.001
Pigmentation	Direct	0.269 [0.185, 0.357]	<0.001	0.269 [0.184, 0.365]	<0.001
Pigmentation	Total	0.626 [0.530, 0.717]	<0.001	0.608 [0.505, 0.708]	<0.001
OXPHOS	Indirect (via MITF)	0.171 [0.078, 0.249]	<0.001	0.140 [0.053, 0.217]	0.004
OXPHOS	Direct	0.081 [-0.044, 0.235]	0.216	0.055 [-0.065, 0.201]	0.401
OXPHOS	Total	0.252 [0.150, 0.359]	<0.001	0.195 [0.089, 0.303]	<0.001

2033

2034 **Table S14. Comparison of unadjusted and tumor-purity-adjusted mediation analyses of the**
2035 **association between SNCA and pigmentation or OXPHOS through MITF.**

2036 Mediation analyses were performed in the TCGA-SKCM cohort using SNCA expression as the
2037 exposure, MITF expression as the mediator, and either the pigmentation or OXPHOS
2038 transcriptional score as the outcome. Indirect, direct, and total effects are shown for models fitted
2039 without covariate adjustment and after adjustment for tumor purity using the Consensus Purity
2040 Estimate (CPE). Estimates are reported with 95% bootstrap confidence intervals and p-values
2041 based on 5,000 bootstrap resamples.

Supplementary Table 15

Effect_type	Path	Standardized beta [95% CI]	p
Direct path	SNCA -> MITF	0.642 [0.532, 0.753]	<0.001
Direct path	tumor_purity -> MITF	0.122 [0.052, 0.190]	<0.001
Direct path	MITF -> TYR	0.511 [0.380, 0.630]	<0.001
Direct path	SNCA -> TYR	0.359 [0.246, 0.478]	<0.001
Direct path	tumor_purity -> TYR	-0.059 [-0.115, -0.002]	0.04
Direct path	TYR -> pigmentation_noTYR	0.522 [0.410, 0.632]	<0.001
Direct path	MITF -> pigmentation_noTYR	0.218 [0.097, 0.328]	<0.001
Residual direct path	SNCA -> pigmentation_noTYR	0.056 [-0.027, 0.149]	0.213
Direct path	tumor_purity -> pigmentation_noTYR	0.061 [-0.005, 0.126]	0.068
Indirect effect	SNCA -> MITF -> Pigmentation	0.140 [0.062, 0.217]	<0.001
Indirect effect	SNCA ->TYR -> Pigmentation	0.188 [0.122, 0.263]	<0.001
Serial indirect effect	SNCA -> MITF ->TYR -> Pigmentation	0.172 [0.113, 0.239]	<0.001
Total indirect effect	All indirect paths	0.499 [0.407, 0.594]	<0.001
Total effect	SNCA -> Pigmentation	0.555 [0.461, 0.651]	<0.001

Table S15. Serial mediation analysis of the SNCA–MITF–TYR–pigmentation axis in TCGA-SKCM.

Standardized path coefficients and indirect effects were estimated using a tumor-purity-adjusted serial mediation model in which MITF and TYR were evaluated as sequential mediators of the association between SNCA expression and a pigmentation score recalculated after excluding TYR. The model additionally allowed direct SNCA→TYR, MITF→pigmentation, and SNCA→pigmentation paths. Standardized estimates, percentile-bootstrap 95% confidence intervals, and p-values were obtained from 5,000 bootstrap resamples. The reported indirect effects include mediation through MITF alone, TYR alone, and the serial SNCA→MITF→TYR→pigmentation pathway.

Supplementary Table 16

Pathway	Model	Predictor	VIF
Both	Mediator model: MITF	SNCA	1.10
Both	Mediator model: MITF	Tumor purity	1.10
Pigmentation	Outcome model	SNCA	1.88
Pigmentation	Outcome model	MITF	1.90
Pigmentation	Outcome model	Tumor purity	1.12
OXPPOS	Outcome model	SNCA	1.88
OXPPOS	Outcome model	MITF	1.90
OXPPOS	Outcome model	Tumor purity	1.12

Table S16. Collinearity diagnostics for tumor-purity-adjusted MITF mediation models. Variance inflation factors (VIF) were calculated for all predictors included in the mediator and outcome regression models used in the tumor-purity-adjusted mediation analyses. The mediator model included SNCA and tumor purity as predictors of MITF expression, whereas the outcome models included SNCA, MITF, and tumor purity as predictors of either pigmentation or OXPPOS scores. All VIF values were below 2, indicating no evidence of problematic multicollinearity among the variables included in the models.

Supplementary Tables 17-21

Due to their size, **Supplementary Tables S17-S21** are not included in this PDF and are provided as separate Microsoft Excel (.xlsx) files accompanying the online supplementary material. The legend for each one is detailed below.

Table S17. Differentially expressed mRNAs between high- and low-SNCG expression patient groups.

The table lists Ensembl gene ID (mRNA), log₂ fold change (logFC), Benjamini–Hochberg false discovery rate (FDR), absolute difference in normalized counts (Delta), median expression in high and low groups (High, Low), and genomic coordinates (start–end, hg38).

Table S18. Pathway over-representation analyses for the SNCG-high vs SNCG-low contrast based on differentially expressed genes.

Analyses were performed using the Gene Ontology (GO) database—Biological Process (BP), Molecular Function (MF), and Cellular Component (CC)—and curated pathways (PAT). Additionally, enrichment using the Reactome database was computed. p-values were adjusted by Benjamini–Hochberg (reported as FDR and $-\log_{10}(\text{FDR})$). Columns include: Rank; ontology/pathway ID and term/description; FDR; $-\log_{10}(\text{FDR})$; gene-set size (ng); overlap size (ncommon); and the contrast label (“Comparison”). Reactome tables additionally report GeneRatio, BgRatio, pvalue, p.adjust, qvalue, overlapping genes (geneID), and overlap count (Count). Analyses were conducted in R using clusterProfiler/ReactomePA with the human annotation package org.Hs.eg.db.

Table S19. Gene set enrichment analysis of Reactome database in SNCG-high group.

The table contains two sheets: ACTIVATED (positively enriched in SNCG-high; NES > 0) and SUPPRESSED (negatively enriched in SNCG-high; NES < 0, i.e., enriched in SNCG-low). Columns: GENESET NAME (MSigDB Reactome term), SIZE (genes in set after filtering), ES (enrichment score), NES (normalized ES), NOM p-val, FDR q-val (multiple-testing adjusted), FWER p-val, RANK AT MAX (rank position at maximum ES), and LEADING EDGE (core enrichment metrics: tags/list/signal percentages).

Table S20. Inferred regulatory interactions involving SNCA and SNCG across healthy skin and melanoma.

Sheets report SNCA_interactors and SNCG_interactors. Columns: regulatoryGene, targetGene, weight (raw edge score), weight_z (z-score), direction (upstream: regulator→synuclein; downstream: synuclein→target), condition (Healthy Skin or Melanoma), in_STRING (whether the pair is supported in STRING), and reg_target (readable arrow notation).

Table S21. Pathway over-representation analyses of inferred protein-protein interactors of SNCA and SNCG in healthy skin and melanoma.

Each sheet reports GO or Reactome terms with: Cluster (condition), ID (GO/Reactome), Description (term), GeneRatio (overlap/input), BgRatio (set size in the universe), pvalue, p.adjust (Benjamini–Hochberg FDR), qvalue, geneID (overlapping genes), and Count (number of overlapping genes).

2102 **Supplementary Table 22**

rho	p_value	cell_type	FDR	gene
0.581	0.000	endothelial cell	0.000	SNCG
0.473	0.000	fibroblast	0.000	SNCG
0.331	0.000	monocyte	0.000	SNCG
0.314	0.000	alternatively activated macrophage	0.000	SNCG
-0.307	0.000	endothelial cell	0.000	SNCA
-0.293	0.000	monocyte	0.000	SNCA
0.285	0.000	macrophage	0.000	SNCG
0.284	0.000	myeloid cell	0.000	SNCG
-0.272	0.000	fibroblast	0.000	SNCA
-0.265	0.000	alternatively activated macrophage	0.000	SNCA
-0.262	0.000	macrophage	0.000	SNCA
-0.258	0.000	myeloid cell	0.000	SNCA
-0.241	0.000	inflammatory macrophage	0.000	SNCA
0.219	0.000	inflammatory macrophage	0.000	SNCG
0.218	0.000	neutrophil	0.000	SNCG
-0.210	0.000	plasma cell	0.000	SNCA
0.207	0.000	T-helper 17 cell	0.000	SNCG
-0.200	0.000	neutrophil	0.000	SNCA
0.180	0.000	B cell	0.000	SNCG
0.177	0.000	Non plasma B cell	0.000	SNCG
0.176	0.000	T cell	0.000	SNCG
-0.176	0.000	B cell	0.000	SNCA
-0.174	0.000	T cell	0.000	SNCA
0.166	0.000	regulatory T cell	0.001	SNCG
-0.159	0.001	Non plasma B cell	0.001	SNCA
-0.159	0.001	CD8-positive, alpha-beta T cell	0.001	SNCA
0.158	0.001	CD4-positive, alpha-beta T cell	0.001	SNCG
-0.156	0.001	CD8+ T cell PD1 low	0.001	SNCA
-0.152	0.001	regulatory T cell	0.002	SNCA
-0.144	0.002	CD4-positive, alpha-beta T cell	0.003	SNCA
0.144	0.002	T-helper 1 cell	0.003	SNCG
0.144	0.002	helper T cell	0.003	SNCG
-0.139	0.002	CD8+ T cell PD1 high	0.004	SNCA
0.138	0.003	CD8-positive, alpha-beta T cell	0.004	SNCG
0.127	0.006	CD8+ T cell PD1 low	0.008	SNCG
0.114	0.013	CD8+ T cell PD1 high	0.017	SNCG
0.105	0.022	plasma cell	0.028	SNCG
-0.105	0.022	T-helper 1 cell	0.031	SNCA
-0.093	0.044	natural killer cell	0.057	SNCA

rho	p_value	cell_type	FDR	gene
-0.085	0.065	T follicular helper cell	0.082	SNCA
0.084	0.069	T-helper 2 cell	0.082	SNCA
-0.067	0.147	helper T cell	0.167	SNCA
0.064	0.163	T-helper 2 cell	0.194	SNCG
-0.062	0.178	T follicular helper cell	0.202	SNCG
0.051	0.264	natural killer cell	0.287	SNCG
0.037	0.420	malignant cell	0.438	SNCG
-0.030	0.514	malignant cell	0.558	SNCA
0.027	0.561	gamma-delta T cell	0.561	SNCG
0.014	0.766	gamma-delta T cell	0.798	SNCA
0.008	0.857	T-helper 17 cell	0.857	SNCA

Table S22. Correlation between SNCA and SNCG expression and xCell2-derived cell-type enrichment scores in the TCGA-SKCM cohort.

Spearman correlation coefficients (ρ), nominal p-values, and Benjamini–Hochberg false discovery rate (FDR)-adjusted p-values are shown for the association between SNCA or SNCG expression and the enrichment scores of 25 cell-type signatures estimated using xCell2. Positive correlations indicate higher gene expression in tumors enriched for the corresponding cell-type signature, whereas negative correlations indicate lower expression. Results are ordered by correlation coefficient for each gene.

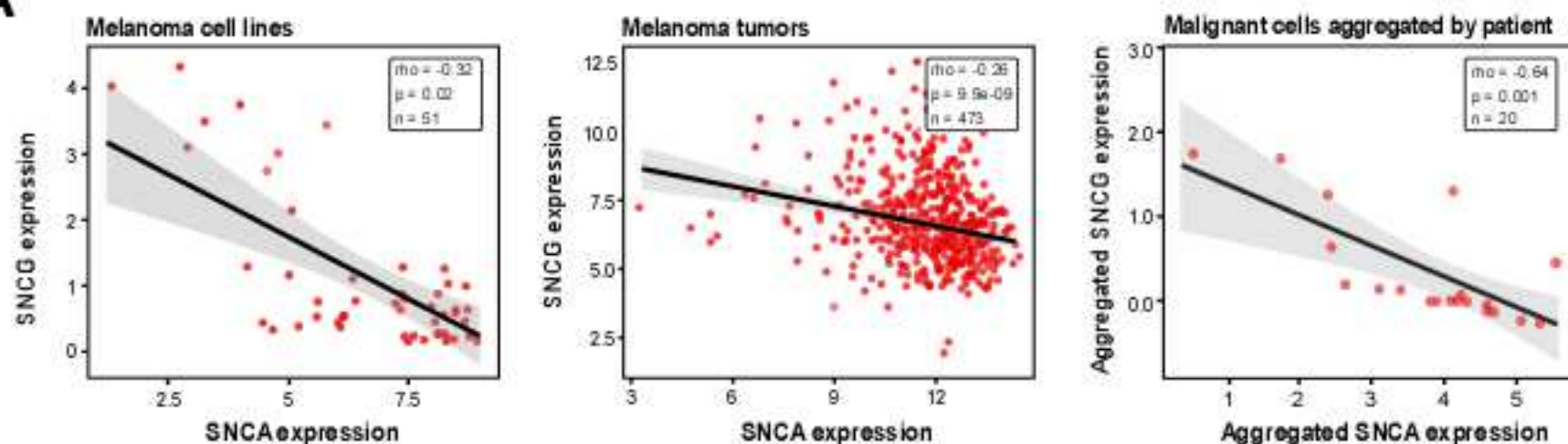
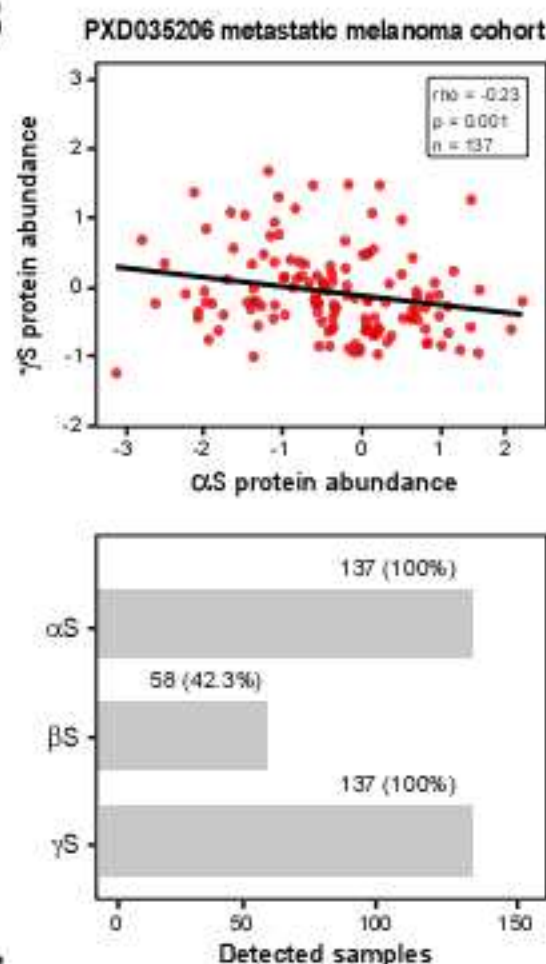
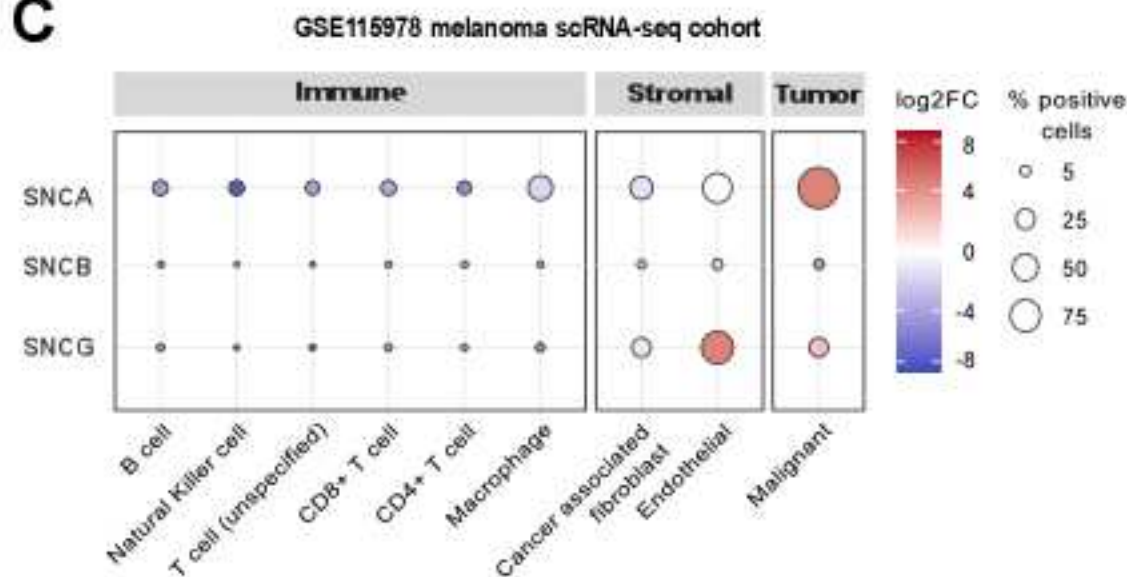
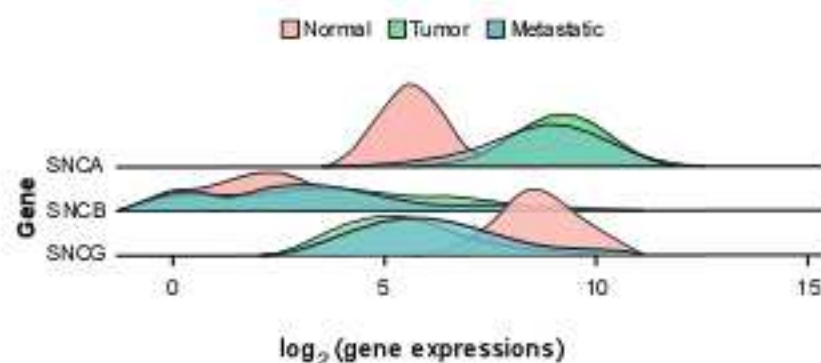
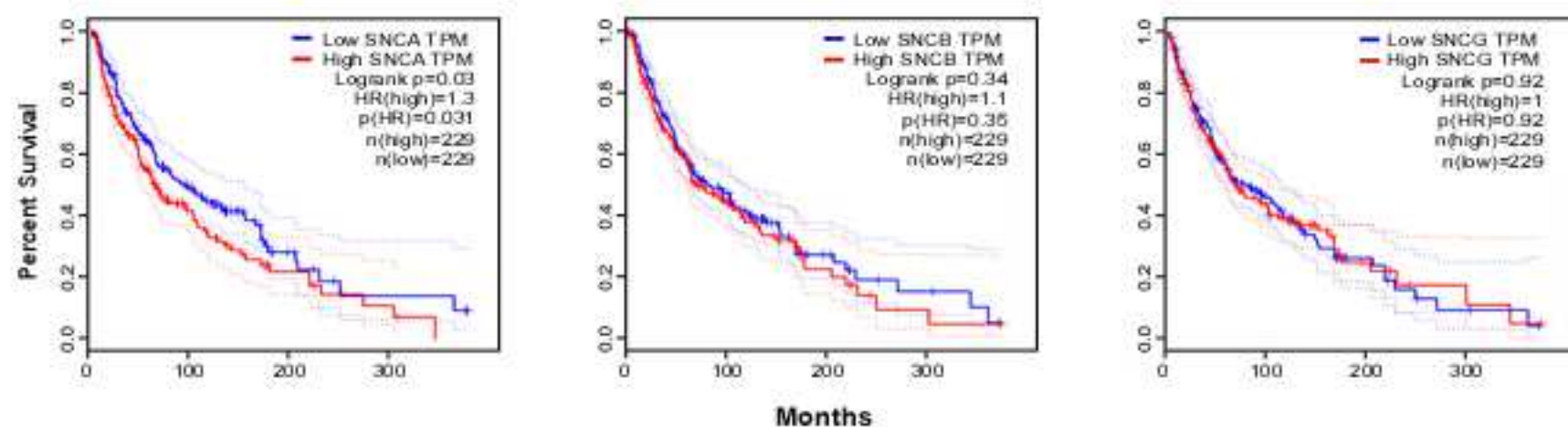
2112 **Supplementary Table 23**

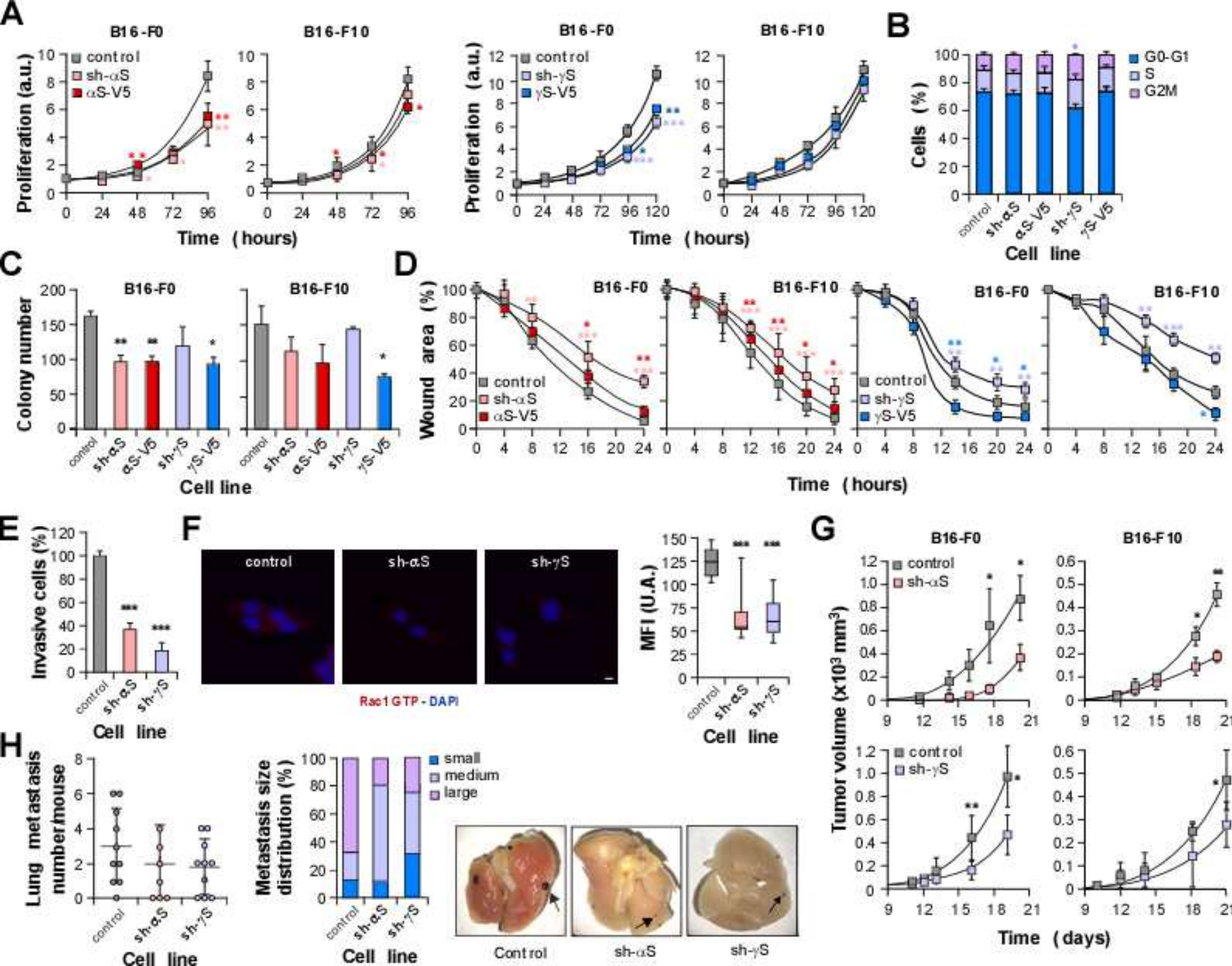
Gene	Spearman's ρ	<i>p</i> -value
AXL	0.639	4.49×10^{-7}
ITGA5	0.573	1.12×10^{-5}
PMEL	-0.546	3.43×10^{-5}
MLANA	-0.533	5.74×10^{-5}
MITF	-0.477	3.99×10^{-4}
ZEB1	0.473	4.54×10^{-4}
VIM	0.408	2.98×10^{-3}
TGFB1	0.373	7.10×10^{-3}
SOX10	-0.343	1.38×10^{-2}
FN1	0.290	3.88×10^{-2}
TOP2A	0.273	5.26×10^{-2}
MKI67	0.139	3.31×10^{-1}

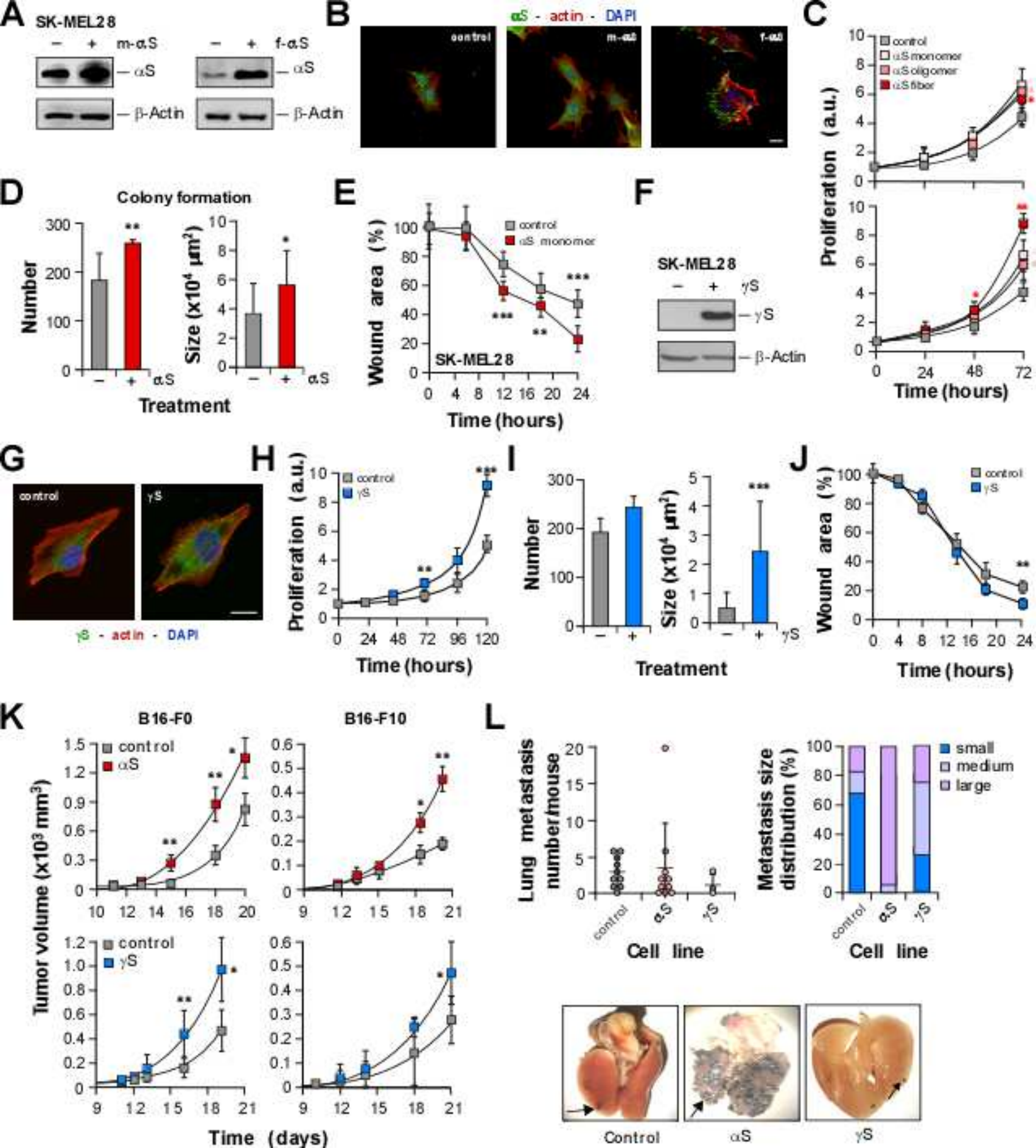
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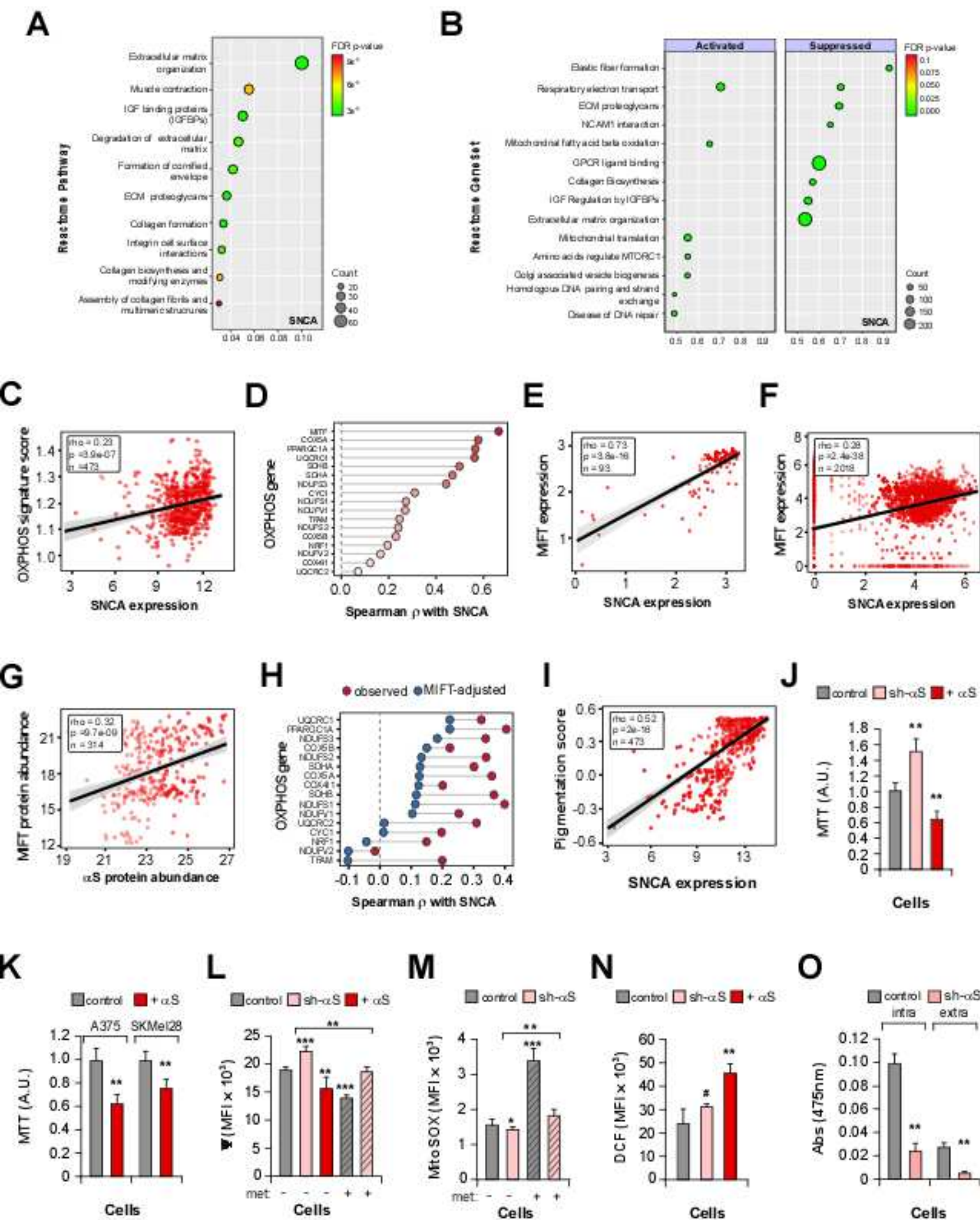
2114 **Table S23. Correlation between SNCG expression and melanoma differentiation, invasion, and**
2115 **proliferation markers across melanoma cell lines.**

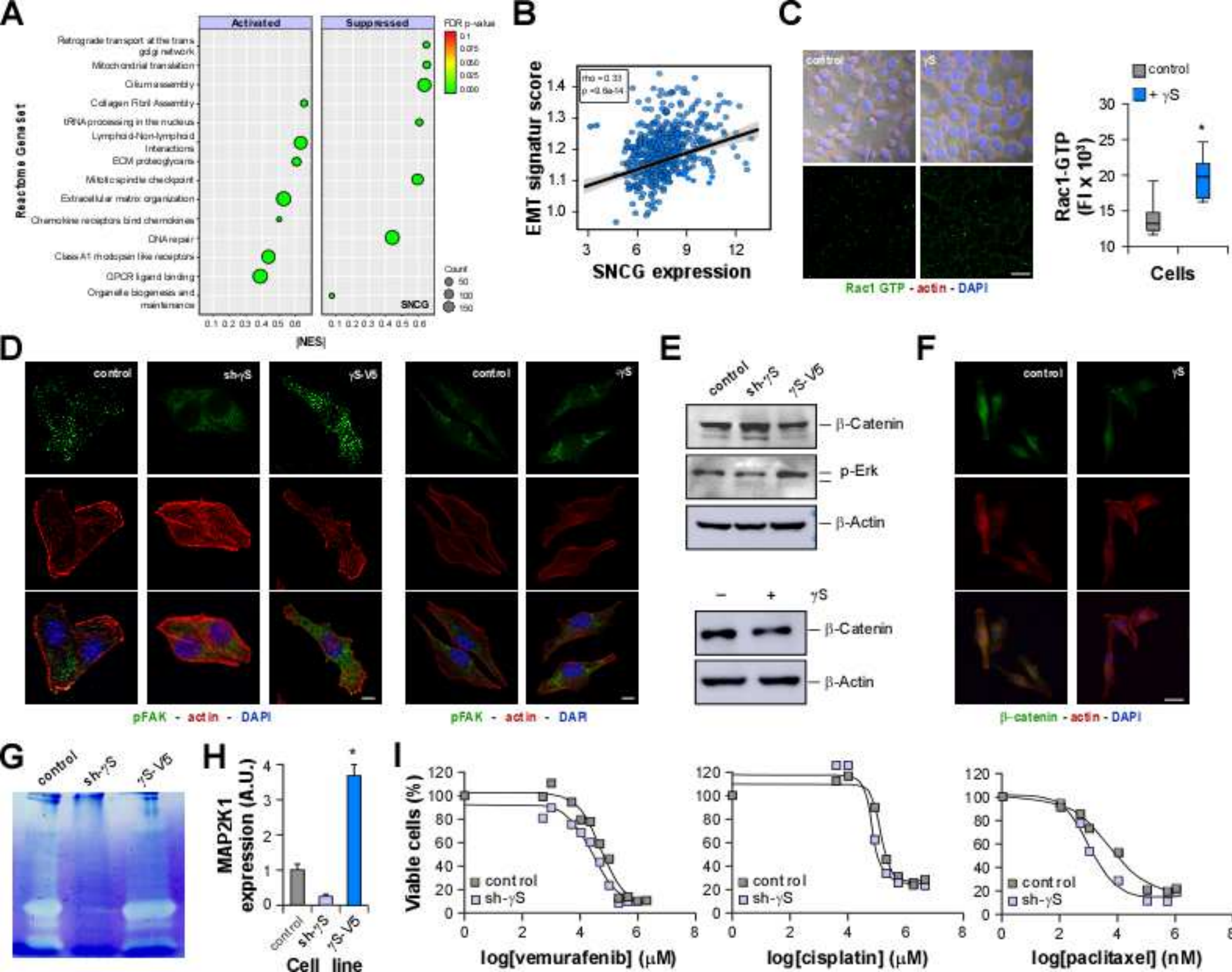
2116 Spearman correlation coefficients (ρ) and corresponding p-values are shown for the association
2117 between SNCG expression and the expression of representative genes associated with
2118 melanocytic differentiation (PMEL, MLANA, MITF, SOX10), invasive/dedifferentiated states
2119 (AXL, ITGA5, ZEB1, VIM, TGFB1, FN1), and proliferation (TOP2A, MKI67) across 51 melanoma
2120 cell lines. Positive correlations indicate increased expression of the corresponding marker in
2121 SNCG-high cell lines, whereas negative correlations indicate reduced expression.

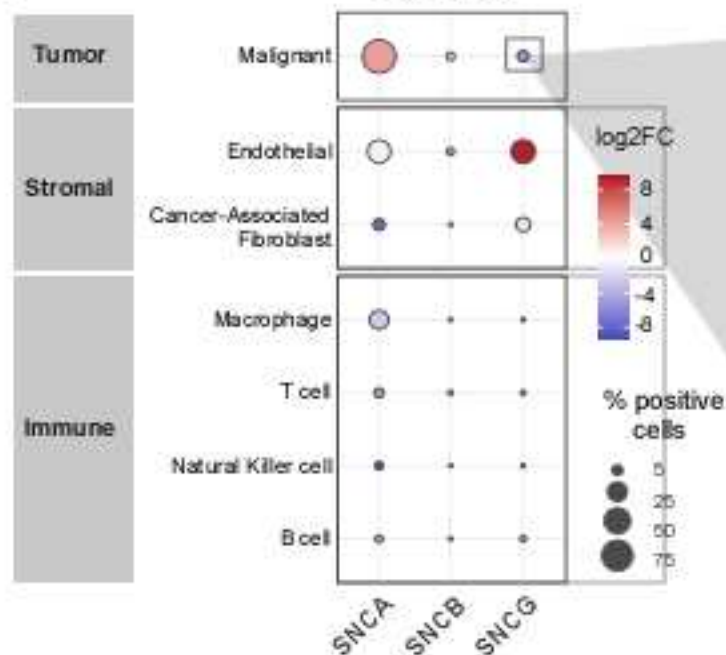
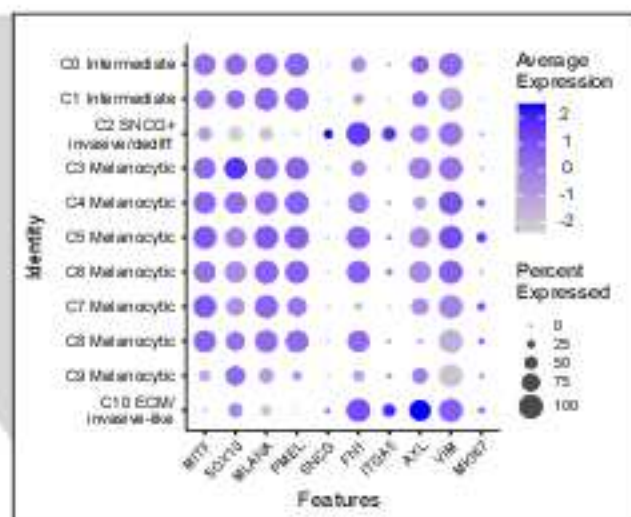
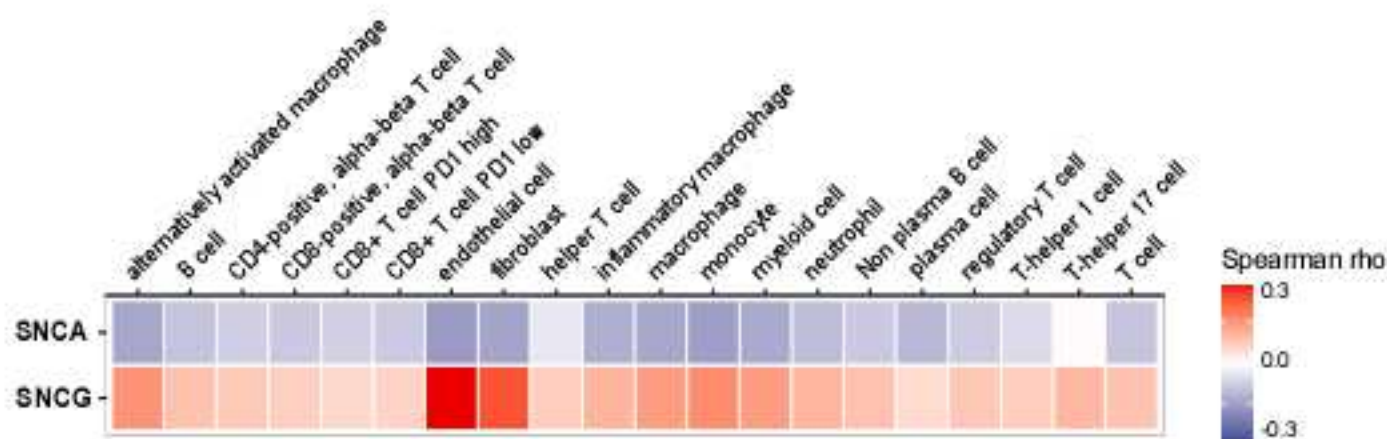
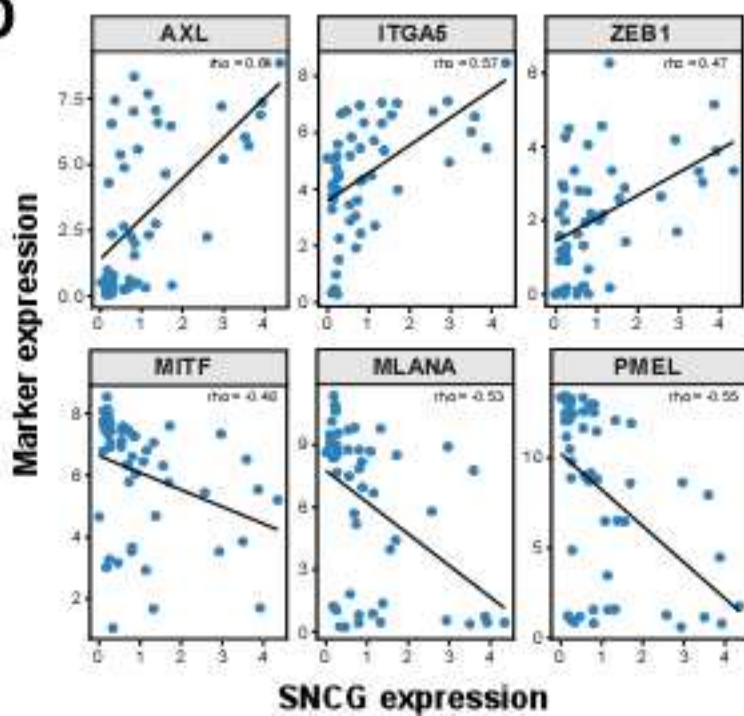
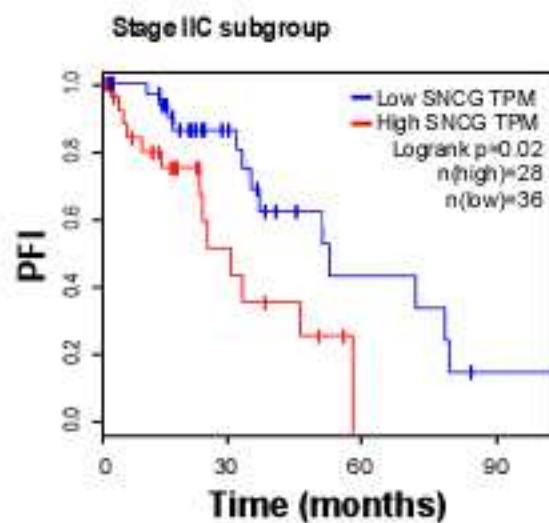
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