

Article

Mechanisms of the Antiproliferative Effects of SIRT6 Inhibition in Melanoma: A Multi-Omics Analysis

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Simple Summary

Melanoma is an aggressive skin cancer that can spread quickly if not treated early. While new treatments have improved survival, many patients still develop resistance or relapse to therapy. This highlights the need to find new targets for more effective treatment outcomes. Our study focuses on a protein called SIRT6, which helps control DNA repair and cell growth and is found at high levels in melanoma. We reduced SIRT6 in melanoma cells using genetic tools and discovered that this slowed their growth and reduced their ability to survive. Using advanced techniques to analyze genes and proteins, we found that lowering SIRT6 turned off signals that promote cancer growth and activated pathways linked to cell death. These results suggest that SIRT6 plays a key role in melanoma progression and could be an important new target for future treatments.

Abstract

Background/Objectives: Melanoma is one of the deadliest types of skin cancer due to its ability to metastasize if not treated early. While targeted- and immune- therapies have significantly improved melanoma treatment outcomes, acquired drug resistance even with combined therapeutics remain prevalent. SIRT6 is a nuclear histone deacetylase that regulates DNA repair, metabolism, and chromatin remodeling. It is overexpressed in melanoma and its inhibition in melanoma is known to have anti-proliferative response, and alterations in pathways related to cell cycle, senescence, and metastasis. **Methods:** To deepen our understanding of the role of SIRT6 in melanoma, in this study we utilized RNA sequencing, proteomics, and Ingenuity Pathway Analysis on genetically modified human melanoma cells to determine the downstream mechanism of SIRT6 in melanoma. **Results:** SIRT6 knock down (KD) in A375 and G361 melanoma cells, with CRISPR/Cas9 or shRNA techniques, resulted in a significant decrease in proliferation and clonogenic survival of the cells. SIRT6 KD caused an altered expression of multiple genes associated with cell proliferation, mitotic regulation, invasion, cell death/senescence, and immunomodulation, including *AURKB*, *ANLN*, *MYC*, *FOXM1*, *RABL6*, *E2F2*, *TP53*, *RBL1*, *OSM*, *TNF*, *IL1B*, *IL6*, and *IFNG*. Comparative analysis at both transcription and translation levels revealed coordinated downregulation of proliferation, invasion, and migration and upregulation of targets related to cell death, apoptosis, and necrosis. Multi-omics analysis also predicted downregulation of signaling networks associated with MAP3K20, MYC, MKNK, and HMGR. **Conclusions:** Given its involvement in tumorigenesis, this study underlines the importance of SIRT6 in melanoma and provides support to its potential as a novel therapeutic target for melanoma.



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Keywords: melanoma; SIRT6; MAP3K20; multi-omics analysis; MYC; cell death

1. Introduction

Melanoma is an aggressive and potentially fatal form of skin cancer that arises from melanocytes, the pigment-producing cells located in the basal layer of the epidermis. In the United States alone, approximately 104,960 new melanoma cases and 8430 deaths are projected in 2025, accounting for 6% of all new cancer cases in men and 4% in women [1]. The global incidence of melanoma has been rising over the past few decades, and solar ultraviolet (UV) radiation exposure, which induces DNA damage and drives oncogenic mutations, has been linked to melanoma as the major etiological factor [2,3]. These driver mutations have offered therapeutic insights into melanoma treatment in the past, including BRAF, NRAS, NF1, and CDKN2A [2]. Somatic mutations that activate the MAPK pathway are the most prevalent mutations of this malignancy. For instance, *BRAF*^{V600E} (c.1799T>A) and *NRAS*^{Q61} (most commonly c182A>G) can be found in up to 80% and 20% of patients, respectively [4]. Improvements in targeted therapies against these pathways and the implementation of immunotherapies have made significant impacts on the treatment strategies against this neoplasm. However, advanced melanoma remains challenging to manage due to its high metastatic potential and ability to develop therapy resistance. This compels the continued search for novel targets and therapies to combat this deadly cancer. The mammalian sirtuins (SIRT6) are a family of class III histone deacetylase proteins that have been found to be involved in a myriad of biological and cellular processes [5]. The sirtuin family members have been associated with multiple cancers, including melanoma, acting as tumor promoter as well as tumor suppressor, depending on context and cell type [6–10]. We and others have demonstrated the involvement of multiple sirtuins in melanoma progression [11–16].

This study was focused on determining the downstream mechanisms of the nuclear sirtuin SIRT6, which is known to play key roles in DNA repair, metabolism, chromatin remodeling, and gene regulation, in a variety of cell types [17–24]. Like other sirtuins, SIRT6 has also been shown to act as either a tumor suppressor or tumor promoter depending on the tissue type [25–31]. We have previously demonstrated that SIRT6 plays a pro-tumorigenic role and is associated with modulations in several key cancer and cell death pathways [32,33]. However, understanding of the molecular mechanism of SIRT6 in melanoma is far from complete. To further strengthen our understanding of the molecular mechanisms involved in the observed role of SIRT6 in melanoma, we performed RNA sequencing and label-free global proteomics studies on human melanoma cells following SIRT6 knockdown (KD). The study highlights predicted molecular targets and pathways that may interact with SIRT6 in melanoma progression and establishes a framework for future experimental validation to elucidate SIRT6's role in melanoma in greater depth.

2. Methods

2.1. Cell Culture and CRISPR/Cas9 SIRT6 Knockdown Cell Clone Generation

A375 cells were grown per recommendation from ATCC in DMEM medium (Corning, Corning, NY, USA, #10-013-CV) supplemented with 10% FBS (MilliporeSigma, Burlington, MA, USA, #F2442) and G361 cells were grown in McCoy's 5A medium (Corning, #10-050-CV) supplemented with 10% FBS. shRNA-mediated SIRT6 KD A375 cells and shNS control cells used for proteomics work were made as previously described [33], while CRISPR/Cas9-mediated SIRT6 KD A375 cell lines used for RNA-seq work were purchased from Synthego (Redwood City, CA, USA) and clones selected as described previously [32].

CRISPR/Cas9-mediated SIRT6 KD G361 cell lines were purchased from Synthego (guide sequence CUUCCGCUCCAGCUCCUCCG) and clones were isolated using the same method. Briefly, cells were diluted and plated with an average of 0.5 cells per well in a 96-well plate, then allowed to grow. Single colonies were picked and tested for SIRT6 expression and growth to select the best colonies for further experiments. Wild-type (WT) cells are the same cells used to make engineered cell pools and were provided by Synthego along with the corresponding KD cell pool. CRISPR/Cas9-mediated SIRT6 knockout in both cell lines resulted in partial decreases in SIRT6 protein, therefore we hereafter refer to both as knockdown (KD). All cells were grown under standard culturing conditions (5% CO₂ and 37 °C) and STR testing was performed for cell line verification.

2.2. Protein Expression Analysis

Proteins were isolated from G361 and A375 cell pellets using 1x RIPA buffer (Millipore-Sigma, #20-188) supplemented with Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA, USA, #87786) and PMSF (phenylmethylsulfonyl fluoride, Amresco/VWR, Radnor, PA, USA, #0754) and quantified via BCA Assay (ThermoFisher, #23225) using manufacturer's protocol and read using a BioTek Synergy H1 Multimode plate reader (Agilent Technologies, Santa Clara, CA, USA). Protein expression was determined using a capillary-based Simple Western on the ProteinSimple Jess system, utilizing a 12–240 KDa separation module and anti-rabbit secondary antibody according to the manufacturer's instructions and as described previously [11]. Briefly, primary antibody against SIRT6 (1:50, Cell Signaling Technologies, Danvers, MA, USA, #12486; 0.75 µg/µL protein per well) was used and signal was normalized to Total Protein Assay. Statistical significance was determined via one-way ANOVA using GraphPad (Boston, MA, USA) Prism software (version 10.4.2).

2.3. Cell Viability Assay

Cells were plated at a density of 250 cells/well in triplicate in 100 µL culture media in a 96-well plate and incubated for 72 h at 37 °C in a CO₂ incubator. At the indicated time, cells were incubated with XTT reagent mixture (Cayman Chemicals, Ann Arbor, MI, USA, #10010200) for 2 h using the manufacturer's recommended protocol. Absorbance was measured using a BioTek Synergy H1 plate reader (Agilent Technologies) at 450 nm. Data is representative of at least 2 biological replicates per group, with statistical significance determined via one-way ANOVA using GraphPad Prism software (version 10.4.2).

2.4. Clonogenic Survival Assay

The WT and SIRT6 KD G361 cells were plated in 6-well plates in triplicate at a seeding density of 250 cells/well. Cells were maintained under standard tissue culture conditions for 10–14 days. Cells were then stained with 0.5% crystal violet solution prepared in a 1:1 methanol:water mixture for 30 min at room temperature and destained with PBS (pH 7.4). Representative images of 3 biological replicates per group were taken after the plates were air dried.

2.5. RNA Isolation

Cells were collected during exponential growth phase, pelleted, and frozen at −80 °C until use. Total RNA was extracted from 3 biological replicates per condition using the Qiagen (Hilden, Germany) RNeasy Plus Mini Kit with QiaShredder for homogenization per manufacturer's protocol. RNA was quantified using BioTek Synergy H1 multimode plate reader with Take3 plate. To assess the integrity and quality of the RNA, we used the Qubit RNA IQ Assay by ThermoFisher (#Q33222).

2.6. Transcriptomic Analysis

CRISPR/Cas9-mediated SIRT6 KD G361 and A375 cells were used for transcriptomic analysis. Total RNA ($n = 3$ per group) was isolated as described above, quantified, and submitted to Novogene (Sacramento, CA, USA) for quality control, library preparation, and bulk RNA sequencing. The purity and integrity of total RNA were assessed by spectrophotometer and Agilent 2100 BioAnalyzer. Samples were prepared and sequenced on the Illumina NovaSeq PE150 platform using standard Novogene protocols. The original RNA-seq data presented in the study are openly available in the GEO repository (A375 accession # GSE318337, G361 data accession #GSE317488) [34,35].

Raw data reads from fastq files were processed by Pluto (Denver, CO, USA) using their standard analysis pipeline or by the University of Wisconsin Biotechnology Center in-house perl scripts. Reads containing the adapter sequences, poly-N stretches, or low-quality reads were excluded during preprocessing, and clean data was used for all subsequent analyses. Reads were mapped to the reference genome using Hisat2 v2.0.5. To count reads mapping to each gene, FeatureCounts v1.5.0-p3 was employed and FPKM for each gene was calculated based on the number of reads mapping to it and its gene length. The DESeq2Rpackage (1.20.0) was used to analyze the differential expression between our WT and the SIRT6 KD clones. The resulting p -values were adjusted using Benjamini and Hochberg's approach to calculate the false discovery rate. Genes with an adjusted p -value of ≤ 0.05 found by DESeq2 were assigned as differentially expressed.

2.7. Proteomics Analysis

Label-free global proteomics analysis was done ($n = 3$ biological replicates per group) at the University of Wisconsin School of Pharmacy Analytical Instrumentation Center as described previously [36], using the SIRT6 KD A375 cells from previously published work [33]. Briefly, freshly collected cell pellets were lysed mechanically with a 25-gauge needle and centrifuged at $10,000 \times g$ for 10 min at 4°C . Protein concentration was quantified by MicroBCA assay (ThermoFisher, Waltham, MA, USA # 23235). A total of 20 μg protein from each sample was digested with 2 μg of sequencing grade trypsin (Promega, Madison, WI, USA). After processing, samples were prepared for LC-MS/MS by C18 Zip-Tip purification according to the manufacturer's protocol (MilliporeSigma). A volume of 3 μL of each sample was injected in duplicate on a 180 min increasing acetonitrile gradient. Peptides eluting from the column were analyzed by data-dependent MS/MS on a Q-Exactive Orbitrap mass spectrometer (ThermoFisher). A top-15 method was used to acquire data. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD073999 [37,38]. Following LC-MS/MS acquisition, the raw files were searched using SEQUEST HT Proteome Discoverer 2.2 search engine (ThermoFisher), against the Uniprot Human database at a false discovery cut off $\leq 2\%$. Following protein identification, quantification of unique peptides eluting between 15 and 120 min was performed on the processed data using SIEVE 2.2 (ThermoFisher) with statistical filters set at p -value < 0.05 , and the CV raw MS intensities of the six replicates had to be within 25%. The list of unique peptides obtained was used for further analysis. GraphPad Prism (version 10.4.2) was used to make histograms for peptide/protein distribution.

2.8. Gene Ontology (GO) Enrichment Analysis

Gene Ontology enrichment analysis of differentially expressed genes (DEGs) from RNA sequencing and unique peptides from proteomics was performed using the PANTHER (Protein ANALysis THrough Evolutionary Relationships) classification system (release PANTHER 19.0) [39,40]. The Gene List Analysis tool was used to select the top 200 (for

RNA-seq data) and 300 (for proteomics data) smallest adjusted p -value genes and proteins, respectively, as these were the most significant. The datasets retrieved from the PANTHER database included the GO Biological Process (BP) and the Molecular Function (MF). The analysis was conducted using the default parameters, with a significant threshold of $p < 0.05$ after false discovery rate (FDR) correction. For graphs, the No PANTHER category assigned (UNCLASSIFIED) was removed. Microsoft Excel was used to prepare pie charts for molecular and biological functions.

2.9. Ingenuity Pathway Analysis (IPA)

The list of DEGs obtained from RNA sequencing (RNA-seq) and data were analyzed with the use of QIAGEN IPA (QIAGEN Inc., <https://digitalinsights.qiagen.com/IPA> (accessed on 30 October 2025) [41] and a Core Analysis was performed to determine pathways, biological functions and networks affected by SIRT6 KD in G361 and A375 cells. All DEGs with $p \leq 0.05$ from RNA-seq were used for IPA analysis. We also uploaded the list of identified proteins with $p \leq 0.05$ obtained from proteomics analysis of SIRT6 KD A375 (via shSIRT6) cells. IPA Core Analysis was limited to ≥ 1.2 -fold expression cut-off to assess pathways and biological functions that are brought about by 20% difference in protein expression by SIRT6 KD. The transcriptomic datasets were generated using CRISPR/Cas9-mediated SIRT6 knockdown, whereas the proteomics dataset was derived from shRNA-mediated knockdown. Although this methodological mismatch can be a confounding variable, it also provides an opportunity to identify regulatory events that are robust across distinct and independently processed datasets and therefore may be more likely to reflect true SIRT6-dependent biology. IPA Comparison Analysis was performed across the three datasets to find the top common pathways and biological functions, as well as top causal networks significantly affected by SIRT6 KD in the analyzed datasets. Activation z -scores of ≥ 2 and ≤ -2 were considered significant for predicted interactions and functions.

3. Results and Discussion

3.1. CRISPR/Cas9-Mediated SIRT6 KD Decreases Cell Proliferation and Clonogenic Survival in G361 Human Melanoma Cells

In a previous study from our lab, we demonstrated that a CRISPR/Cas9-mediated knockout of SIRT6 imparts remarkable antiproliferative response in A375 human melanoma cells in vitro and in vivo [32]. This study demonstrated that SIRT6 KD in A375 melanoma cells resulted in a significant decrease in growth, viability and clonogenic survival, and induction of G1-phase cell cycle arrest. Further, we also observed significantly decreased tumorigenicity of SIRT6 KD A375 cells in athymic nude mice [32]. To expand these findings, we used CRISPR/Cas9 to generate SIRT6 KD in the G361 human melanoma cell line, which significantly overexpresses SIRT6 both at the mRNA and protein level [33]. We found significant SIRT6 KD at the protein level in our clones as compared to wild-type (WT) cells (Figure 1A). The clones were then tested for growth inhibition and were found to have a significant decrease in proliferation and clonogenic survival (Figure 1B,C), providing further support to the hypothesis that SIRT6 has pro-proliferative effects in human melanoma.

3.2. SIRT6 KD Significantly Impacts Differential Expression of Genes in Human Melanoma Cells

To determine the downstream mechanism of SIRT6 in human melanoma, we conducted RNA-sequencing on the SIRT6 KD and WT G361 and A375 cells with 3 biological replicates each (Figure 1D) to perform differential gene expression analyses. In the SIRT6 KD G361 cells, a total of 13,968 differentially expressed genes (DEGs) were identified, out of which 5613 DEGs were statistically significant with 648 upregulated and 197 downreg-

ulated genes found with a fold change (FC) of $\text{Log}_2 \text{FC} \geq |1.5|$ (Figure 2A). To identify the biological functions associated with the DEGs in SIRT6 KD G361 cells, we performed GO enrichment analysis. Significant enrichment was observed in both biological processes (BP, Figure 2B) and molecular functions (MF, Figure 2C). The top five significant BP terms based on the term size included cellular process, biological regulation, metabolic process, response to stimulus, and localization. Similarly, the top terms in GO MF based on term size encompass binding, catalytic activity, molecular function regulator activity, transcription regulator activity, and transporter activity.

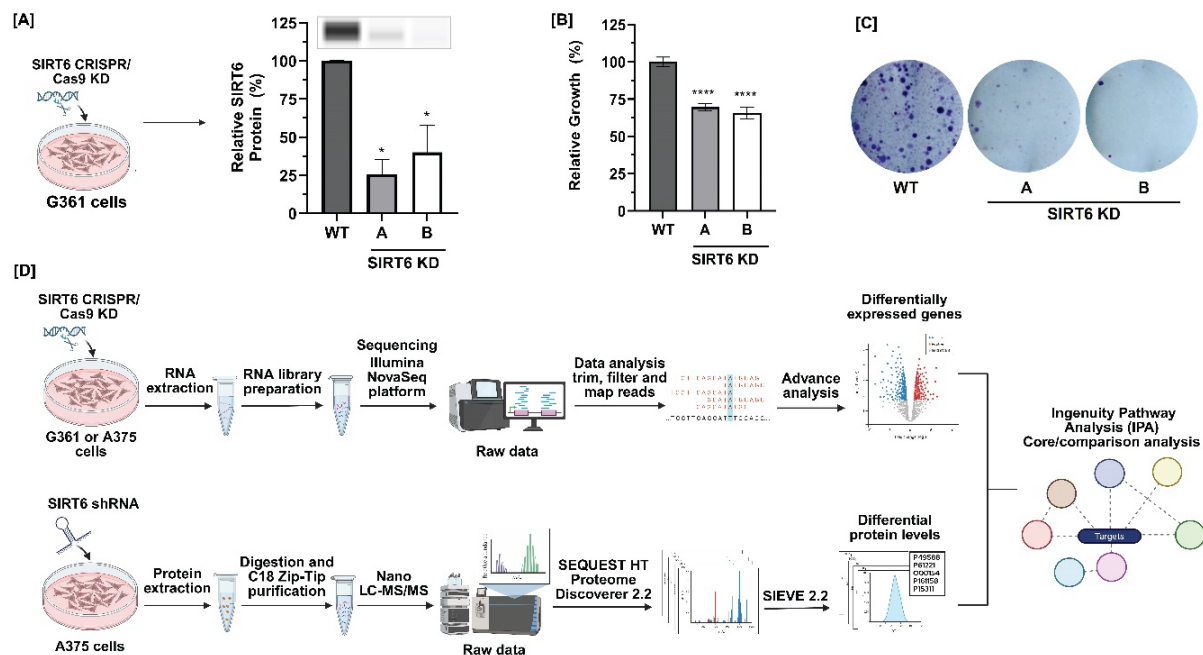


Figure 1. Characterization of SIRT6 expression in G361 knockdown (KD) cells and proteogenomics workflow in melanoma cells. (A) CRISPR/Cas9-mediated SIRT6 KD G361 cells were created, and protein expression was determined by Protein Simple automated Western blotting. SIRT6 KD reduces the (B) proliferation and (C) colony forming potential of G361 melanoma cells. Data is representative of at least 2 biological and 3 technical replicates per group, with statistical significance determined using one-way ANOVA and data shown as mean $\pm$ SEM (* $p \leq 0.05$, **** $p \leq 0.0001$). (D) Schematic representation of the experimental workflow for integrative proteogenomic analysis in A375 and G361 melanoma cells, outlining the steps from sample preparation to data integration. For RNA-seq and proteomics, data was generated from three independent biological replicates per group. For all datasets, control cell line was matched for each knockdown strategy.

The top 10 up- and downregulated genes identified in SIRT6 KD G361 dataset are involved in multiple biological aspects, including neurological/developmental processes, immune and inflammatory response, transcription regulation, cell adhesion and integrity, metabolism and reproductive function. Interestingly, many of these genes are known to be associated with various cancers, including melanoma (Table 1). For example, the top downregulated gene, *LHFPL3* ($\text{Log}_2 \text{FC} -11.16$), was reported to be overexpressed in glioma [42] and is under investigation as a potential target of regulatory microRNAs that modulate tumor pathogenesis, suggesting its involvement in glioma progression [43]. The antisense RNA *LHFPL3-AS1* has been reported to be upregulated in melanoma and promotes malignancy via the JAK2/STAT3 signaling pathway [44]. Its downregulation in SIRT6 KD cells suggests potential involvement of SIRT6 in this oncogenic pathway in melanoma, which can be validated in future studies.

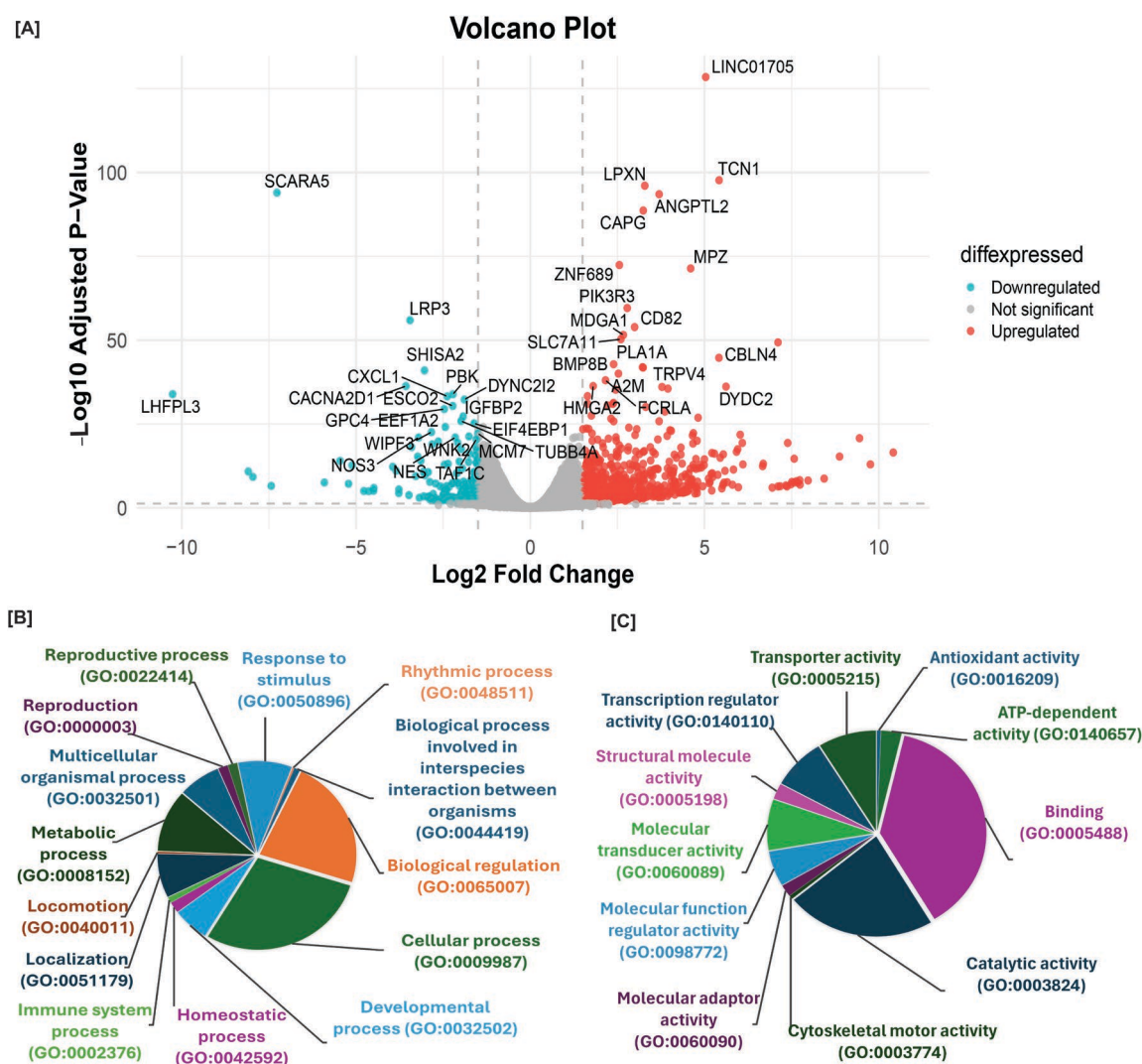


Figure 2. Transcriptomic profiling and functional enrichment analysis of SIRT6 knockdown (KD) G361 cells. **(A)** RNA-seq analysis of SIRT6 KD G361 cells identified multiple differentially expressed genes (DEGs) with the top 20 up/down regulated denoted in the volcano plot. Dashed line indicates significant genes with $\text{Log}_2 \text{FC} \geq 1.5$. Gene Ontology (GO) enrichment analysis by PANTHER of top 200 significant DEGs revealed alterations across various biological processes **(B)** and molecular functions **(C)**.

Table 1. Select cancer associations with top 10 up- and downregulated genes in SIRT6 KD G361 cells.

Gene Symbol	Log2 Fold Change	Select Associated Cancers	References
NUDT4P2	10.15	Clear Cell Renal Cell Carcinoma	[45]
		Melanoma	[46,47]
SERPINA3	9.7	Breast	[48,49]
		Colon	[50]
		Endometrial	[51]
		Glioma	[52]
		Colorectal	[53]
ZNF718	9.39	Colorectal	[54]
		Meningioma	[55]
FMC1-LUC7L2	9.32	Ovarian	[56]
ITIH6	9.06	Cholangiocarcinoma	[57]

Table 1. *Cont.*

Gene Symbol	Log2 Fold Change	Select Associated Cancers	References
AKR1B15	8.46	Hepatocellular	[58]
IL11	8.46	Melanoma Prostate Colorectal/Multiple	[59] [60] [61]
IL1RAPL1	8.24	Brain	[62]
TREM1	8.23	Lung Gastric Papillary Thyroid Colorectal	[63] [64] [65] [66]
CTSG	7.8	Non-Small Cell Lung Colorectal Head and Neck Squamous Cell Carcinoma Oral Squamous Cell Carcinoma	[67] [68] [69] [70]
ERG	−5.61	Prostate Ewing’s sarcoma Leukemia	[71] [72] [73]
LRRC4	−5.62	Epithelial Ovarian Brain Nasopharyngeal	[74] [75,76] [77]
SPATA31A7	−5.64	No known associations	-
RSC1A1	−5.68	No known associations	-
DSC3	−5.89	Colorectal Prostate Breast Lung	[78,79] [80] [81] [82]
SCARA3	−6.11	Lung Ovarian Breast Multiple Myeloma	[83] [84] [85] [86]
EYA2	−6.3	Breast Hepatocellular Pancreas Lung	[87,88] [89] [90] [91]
SCARA5	−7.3	Prostate Gastric Colorectal Melanoma	[92] [93] [94] [95]
CDX2	−7.47	Colorectal Gastric	[96,97] [98]
LHFPL3	−11.16	Glioma Gastric	[43] [99]

To get a broader idea of the effects of SIRT6 inhibition on melanoma cells, we conducted additional RNA-seq analyses using SIRT6 KD A375 cells previously generated in our laboratory [32]. A total of 18,133 DEGs were identified in this dataset, out of which 1893 were statistically significant with 262 found to be upregulated and 405 genes were downregulated at fold change (FC) $\text{Log}_2 \text{FC} \geq |1.5|$ (Figure 3A). GO enrichment analysis showed significant enrichment of BP and MF (Figure 3B,C, respectively) related to cellular processes, biological regulation, binding, catalytic activity, and molecular transducer activity based on the size of enrichment terms.

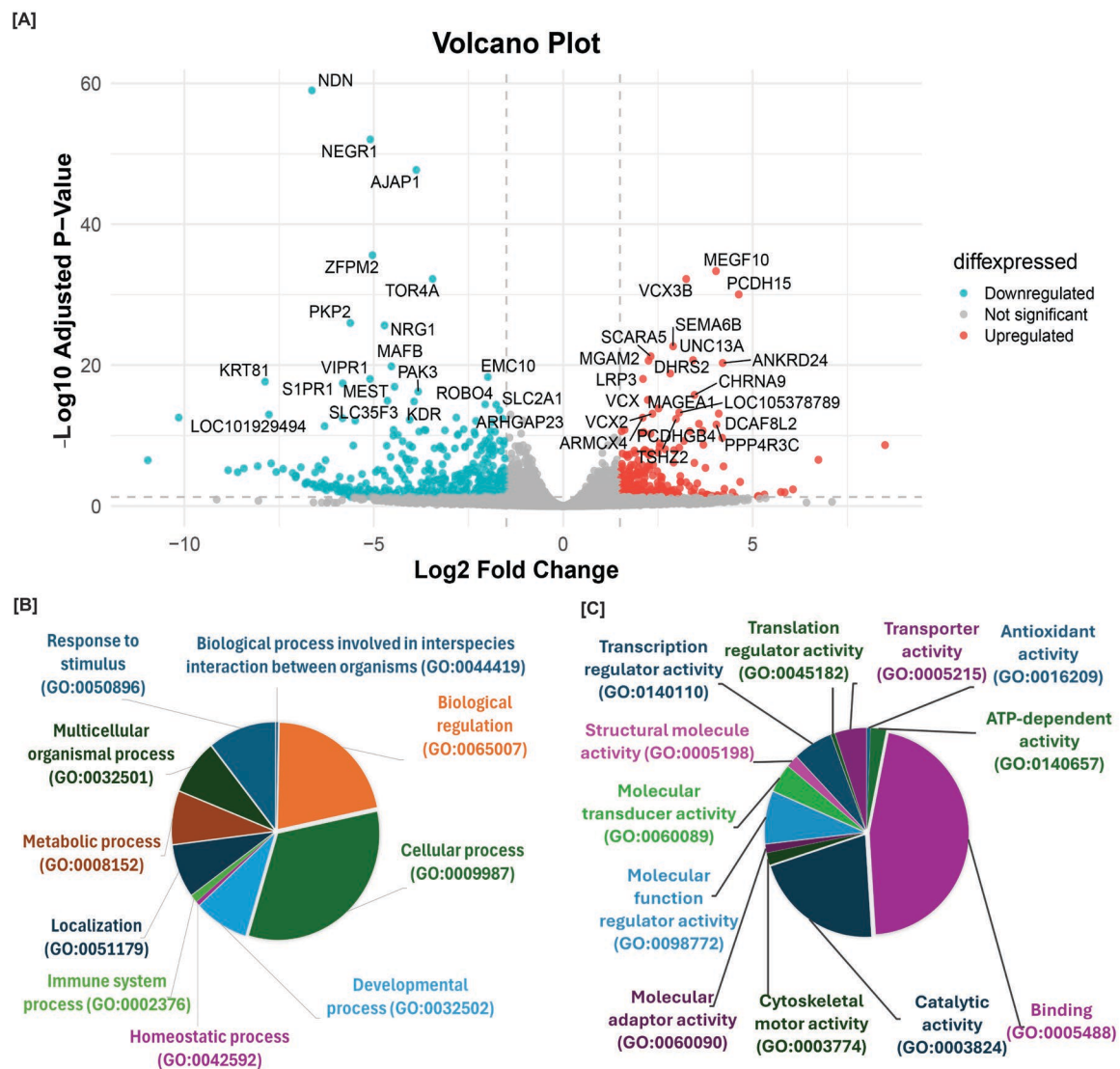


Figure 3. Transcriptomic profiling and functional enrichment analysis of SIRT6 knockdown (KD) A375 cells. (A) The top 20 up/down regulated differentially expressed genes (DEGs) identified by RNA-seq in SIRT6 KD A375 cells denoted via volcano plot. Dashed line indicates significant genes with $\text{Log}_2 \text{FC} \geq 1.5$. Subsequent Gene Ontology (GO) enrichment by PANTHER of top 200 significant DEGs found significant changes in several biological processes (B) and molecular functions (C) in SIRT6 KD A375 cells.

3.3. SIRT6 KD Is Associated with Reduced Proliferation-Associated Genes in G361 Cells

We next analyzed the DEGs from SIRT6 KD cells using Qiagen Ingenuity Pathway Analysis (IPA) to determine the overall picture of functional regulation. IPA is a powerful tool to analyze patterns and pathways present in the data as well as to predict the increase or decrease in potential downstream and upstream biological functions causally affected by transcriptome changes. We analyzed all significant DEGs from the RNA-seq datasets without applying a $\text{Log}_2 \text{FC}$ cutoff to capture even subtle transcript changes that could influence the overall direction of the biological processes. IPA revealed a multifaceted response to SIRT6 KD in the G361 melanoma cells, characterized by changes in cell proliferation, cell death, immune activation, and inflammatory response markers (Figure 4A,B). We predicted downregulation of *FOXO1* ($\text{Log}_2 \text{FC} -0.912$), *E2F2* ($\text{Log}_2 \text{FC} -1.626$), *MYC* ($\text{Log}_2 \text{FC} -0.811$), *EP400* ($\text{Log}_2 \text{FC} -0.428$), *ACTB* ($\text{Log}_2 \text{FC} -0.347$), and *RABL6* ($\text{Log}_2 \text{FC} -0.849$), which regulate chromatin remodeling, cytoskeletal integrity, and drive cell cycle progression and proliferation [100–103]. In the SIRT6 KD A375 cells, a similar downregulation was seen

in *MYC* and *ACTB* (Log2 FC of -0.552 and -0.595 , respectively). It was reported that *FOXM1* is overexpressed in metastatic melanoma and associated with poor clinical outcomes [103], supporting the hypothesis that *FOXM1* inhibition may represent a therapeutic strategy [104]. Another gene in our dataset, *MYC*, is a well-characterized proto-oncogene with higher expression reported in metastatic melanoma [102].

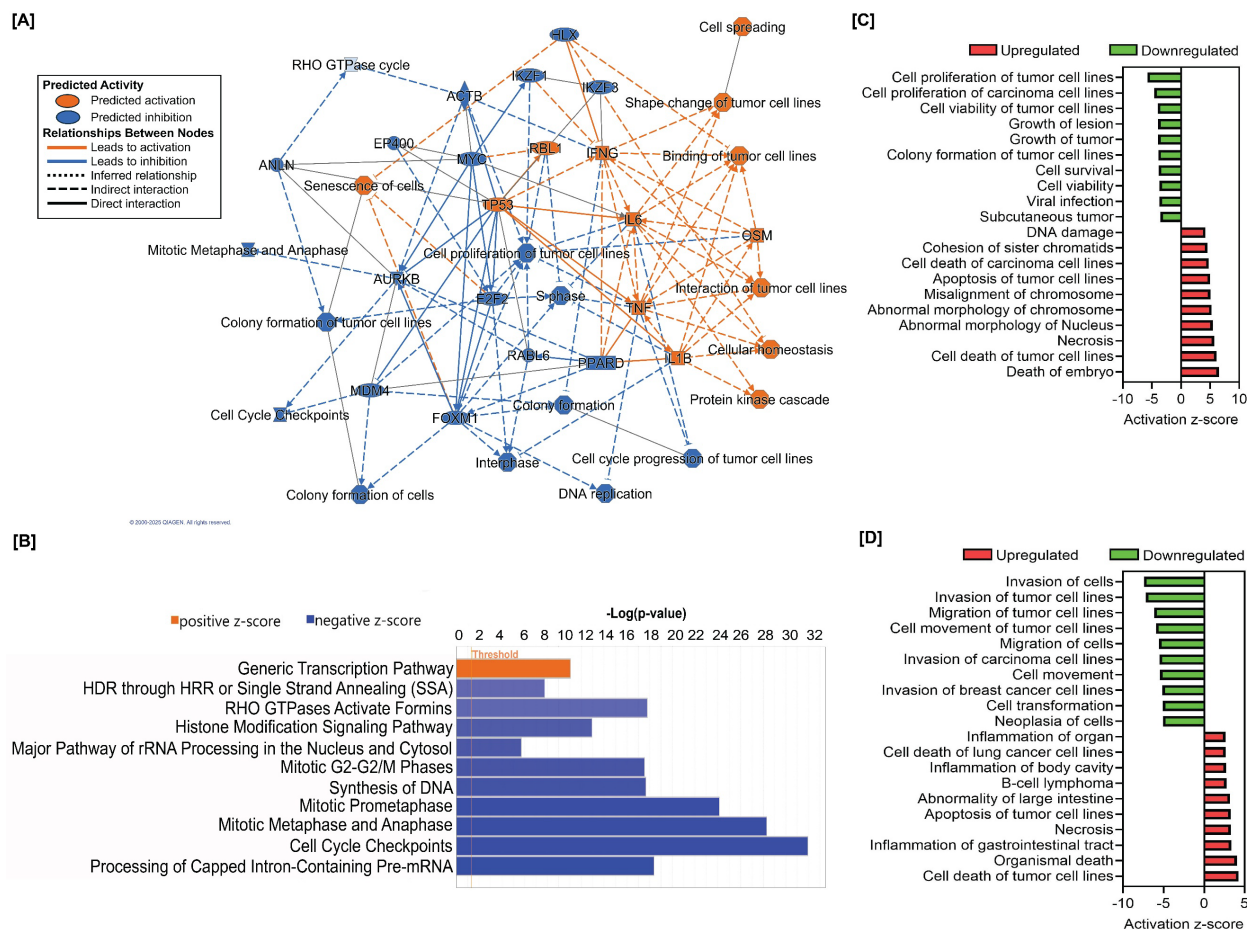


Figure 4. Functional insights from Ingenuity Pathway Analysis (IPA). (A) Graphical summary generated by Ingenuity Pathway Analysis highlighting predicted target regulation activity based on differentially expressed genes (DEGs) in *SIRT6* knockdown (KD) G361 cells. (B) Top canonical pathways affected in *SIRT6* KD G361 cells as predicted by IPA based on the transcriptomic data. IPA analysis of differentially expressed genes shows regulation of cancer-related biological functions in *SIRT6* KD G361 (C) and A375 cells (D).

ACTB is increasingly recognized for its oncogenic activity in cancer by regulating cytoskeletal dynamics and EMT [101]. A recent study has shown that *RABL6*, a GTPase involved in cell cycle regulation and oncogenic signaling, promotes squamous cell carcinoma, correlating with the poor prognosis [105]. Likewise, the literature showed that *EP400* is part of a histone chaperone complex that contributes to melanoma cell proliferation by incorporating *H2A.Z*, a gene regulator, into the promoters of cell cycle genes, thereby enhancing the transcription of E2F-regulated targets [100]. Previously, *E2F2* was reported highly expressed in cutaneous melanoma samples and its upregulation was related to lower overall survival [106]. Downregulation of these genes with *SIRT6* KD shows the potential role of *SIRT6* in regulating the expression of pro-proliferative targets in melanoma, which requires further validation.

The top cancer-associated downregulated biological functions predicted by IPA in the *SIRT6* KD G361 cells were cell proliferation (activation z-score -5.819 ; $p = 4.33 \times 10^{-110}$) and

tively. *PPARD* is a nuclear receptor involved in lipid metabolism, inflammation, and cell proliferation [107], and has been linked to carcinogenesis [108] although its role in melanoma appears to be context-dependent [109]. *PPARD* has also been associated with tumor growth and immune evasion through modulation of the tumor microenvironment and metabolic reprogramming [110]. While these predictions have not been validated, the inferred downregulation of *PPARD* in SIRT6 KD cells may suggest a shift away from metabolic programs that support proliferation, potentially contributing to the observed senescent phenotype, which we observed in our earlier publication [33]. Interestingly, *PPARD* was downregulated (Log2 FC -0.313) in the SIRT6 KD A375 dataset, suggesting a link between IPA-predicted regulation and RNA-seq-derived expression changes in SIRT6 KD melanoma cells. Furthermore, upregulation of *RBL1* and senescence-related pathways (Figure 4A) were predicted. In our datasets, we found mixed results regarding *RBL1* regulation, with SIRT6 KD in A375 cells showing a slight increase (Log2 FC 0.119) and G361 showing a slight decrease (Log2 FC -0.847). This may be due to some innate difference between the types of melanoma cells or particular signaling mechanism and warrants further investigation to determine the complete picture.

Table 2. Predicted upstream regulators affected in G361 by SIRT6 KD.

Upstream Regulator	Expr Log Ratio	Molecule Type	Predicted Activation State	Activation z-Score	p-Value of Overlap
PPARD	0.256	ligand-dependent nuclear receptor	Inhibited	-9.331	1.3×10^{-35}
MYC	-0.811	transcription regulator	Inhibited	-9.28	4.08×10^{-52}
RABL6	-0.849	other	Inhibited	-6.733	3.23×10^{-26}
CKAP2L	-0.969	other	Inhibited	-6.351	1.59×10^{-26}
CEBPB	-0.121	transcription regulator	Inhibited	-5.839	1.82×10^{-29}
TBX2	-0.304	transcription regulator	Inhibited	-5.293	6.54×10^{-14}
BMI1	0.525	transcription regulator	Inhibited	-5.205	2.89×10^{-7}
TBX3	1.177	transcription regulator	Inhibited	-5.175	7.16×10^{-21}
KDM1A	-0.631	enzyme	Inhibited	-5.049	6.31×10^{-13}
TP53	0.012	transcription regulator	Activated	8.096	3.93×10^{-52}
IGF2BP1	-0.294	translation regulator	Activated	7.226	1.85×10^{-28}
SMARCA4	-0.559	transcription regulator	Activated	6.86	2.43×10^{-16}
TNF	-	cytokine	Activated	6.765	2.55×10^{-24}
NUPR1	-1.963	transcription regulator	Activated	6.468	4.39×10^{-42}
MAGI1	-0.169	enzyme	Activated	6.438	5.5×10^{-27}
IL1B	5.154	cytokine	Activated	6.21	3.21×10^{-12}
IFNG	-	cytokine	Activated	5.913	2.52×10^{-12}
CDKN2A	0.712	enzyme	Activated	5.792	8.49×10^{-20}
STING1	0.857	ion channel	Activated	5.381	0.0307

Interestingly, *TP53* and *RBL1* are thought to help maintain cell cycle and genomic stability and their loss or inactivation contributes to pro-tumorigenic response [111,112]. Also, senescence-associated secretory phenotype (SASP) factors like *IL6* and *TNF α* can reshape the tumor microenvironment, attracting immune cells [113]. *TNF*, *IL1B*, and *IFNG* were also predicted as upstream regulators affected in SIRT6 KD G361 cells. Consistent with these predictions, several TNF receptor superfamily members were differentially expressed, accompanied by upregulation of the IFNG receptor (*IFNGR1*) and *IL1B* in the dataset. Another gene, *MDM4* (Log2 FC -1.255), was found to be downregulated in our G361 dataset and is known to bind with *TP53* and inhibit its transcriptional activity [114]. However, *MDM4* was upregulated in our A375 dataset (Log2 FC 1.519), suggesting a po-

tentially different role in that cell line. IPA also predicted upregulation of inflammation and immune related genes such as *OSM*, *TNF*, *IL1B*, *IL6*, and *IFNG* and downregulation of *HLX*, *PPARD*, and *IKZF8* (Figure 4A) in SIRT6 KD G361 cells. This trend was found to be opposite in A375 cells where there is a downregulation of immune-related genes (Figure S2C). A375 is a primary cell line where G361 is metastatic, which contributes to the potential difference in gene expression patterns in two datasets. Overall, there is a compelling shift from proliferative signaling toward immune modulation and cellular senescence in our SIRT6 KD RNA-seq datasets. In-depth analysis of SIRT6 KD in primary versus metastatic melanoma models can further delineate SIRT6's role in different pathological states of melanoma.

3.5. SIRT6 KD Is Associated with Reduction in Mitotic Regulator-Associated Genes

SIRT6 KD in G361 cells also showed decrease in mitotic regulatory genes (*AURKB*, Log2 FC -1.070 ; *ANLN*, Log2 FC -1.293) further supporting the hypothesis of reduced cell division and reduced melanoma growth, which we also observed in the cell viability and clonogenic assays (Figure 1B,C). In SIRT6 KD A375 cells, *AURKB* Log2 FC was -0.067 , and *ANLN* had a Log2 FC of -0.035 . Recent studies have shown that *ANLN*, an actin binding protein, was upregulated in multiple cancers [115,116] and can be used as an onco-immunological biomarker for tumor screening, prognosis and treatment. Likewise, *AURKB* is highly expressed in melanoma cells, and its inhibition reduces proliferation [117] and sensitizes melanoma cells to T-cell mediated toxicity [118].

Analysis of the top canonical pathways by IPA predicted upregulation of generic transcription pathway and downregulation of multiple pathways involved in cell cycle progression, DNA replication, mitosis, and oncogenic signaling (Figures 4B and S2D). The top three pathways with the highest negative z-score values are related to cell cycle check points and mitosis. This supports our previous research findings where we found effects on cell cycle with SIRT6 KD [32,33]. Further analysis of the biological functions affected by SIRT6 KD in G361 and A375 cells support our above results as most of the affected biological processes associated with cell death and DNA/chromosome damage are upregulated whereas those related to cell viability, proliferation and invasion functions are inhibited (Figure 4C,D).

The top networks of DEGs that are associated with cell cycle, cell morphology, cell-to-cell signaling and cell movement molecules in SIRT6 KD G361 and A375 cells are shown in Figure S3A,B. Overall, these effects both predicted and observed, suggest that SIRT6 KD induces cell cycle arrest and cell death pathways.

3.6. Global Proteomics Analysis of SIRT6 KD A375 Shows Significant Regulation in Key Cellular and Biological Processes

In addition to the transcriptomics work, we performed label-free global proteomics analysis [36] using previously created shSIRT6 (KD) A375 cells ($n = 3$ per group) [33]. After analysis of the proteome data, 6711 peptides were quantified with CV less than 25% and $p < 0.05$. There were 2150 proteins identified based on unique peptides, out of which 543 proteins had 2 or more peptide hits and 988 proteins had ≥ 1.2 | -fold change (Figure 6A,B). GO enrichment analysis by PANTHER showed significant enrichment of biological processes and molecular functions where top BP terms included cellular process, metabolic process, biological regulation, localization, and response to stimulus (Figure 6C). The top MF terms included binding, catalytic activity, structural molecule activity, molecular function of regulator activity, and ATP-dependent activity (Figure 6D).

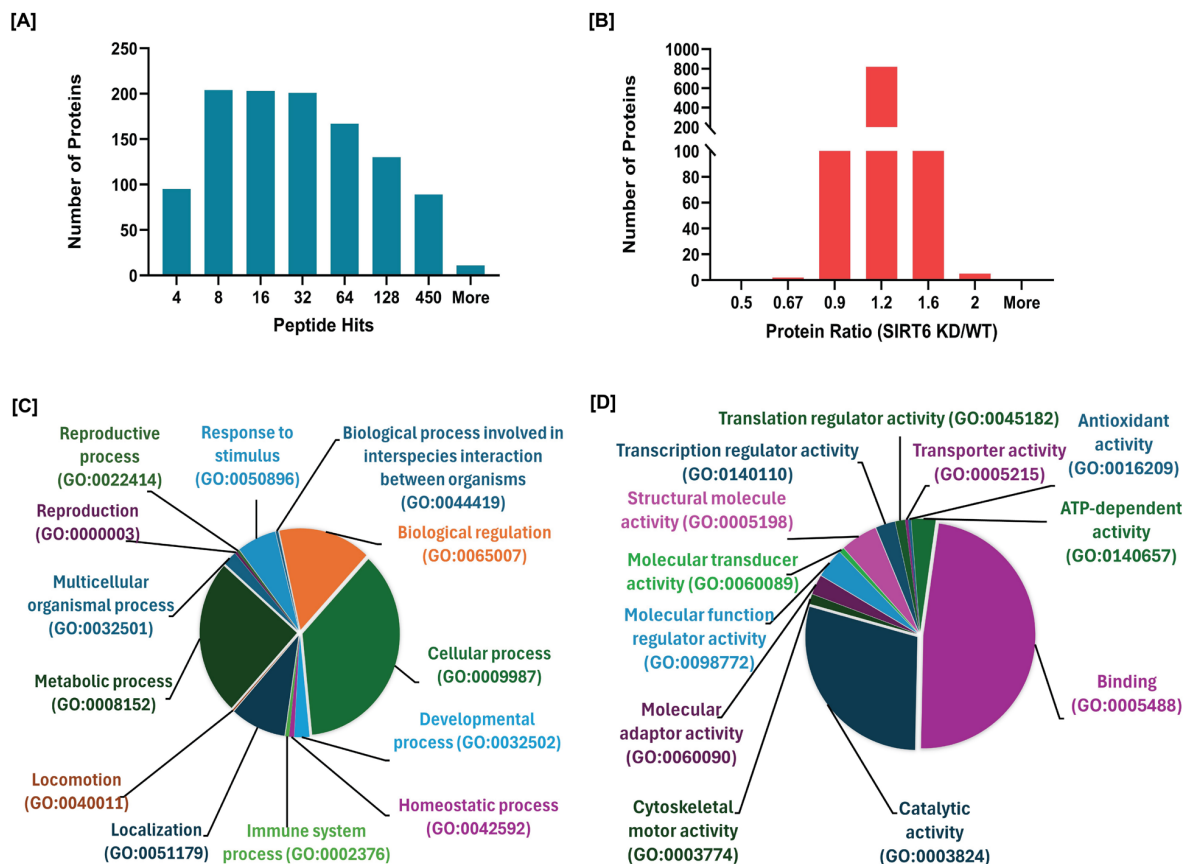


Figure 6. Proteomics analysis of SIRT6 knockdown (KD) A375 melanoma cells. Proteomic analysis of SIRT6 KD A375 cells showing peptide identifications (A) and abundance data (B) in respective proteins. Gene Ontology (GO) enrichment based on PANTHER analysis reveals regulation of multiple biological (C) and molecular (D) functions in SIRT6 KD A375 cells.

A network of proteins associated with cancer, cell proliferation, cell cycle, and cell movement in SIRT6 KD A375 cells is given in Figure S3C. The top downregulated protein in SIRT6 KD A375 cells was PTMA (FC -6.66), an important protein in regulating cell proliferation, cell death and immune responses. It was also found downregulated in RNA-seq dataset of CRISPR/Cas9 SIRT6 KD A375 (Log2 FC -0.241) and G361 (Log2 FC -0.462) cells. It was reported that overexpression of *PTMA*, among other genes, characterized tumors with a more aggressive phenotype in an in vivo melanoma model [119]. Similarly, the top upregulated protein in A375 proteomics dataset was SRPK2 (FC 2.426), a serine/arginine kinase whose overexpression is linked to increase invasive capacity in melanoma cells [120]. This was also upregulated at RNA level in A375 cells (Log2 FC 0.252). On the contrary, *SRPK2* RNA expression was reduced in metastatic SIRT6 KD G361 cells (Log2 FC -0.172), which indicates the difference in expression of certain targets attributed to the phenotypic or mutational status of the melanoma cells. This prompted us to compare the three datasets to examine overarching trends and directional shifts in functional activity at both RNA and protein levels.

3.7. Comparative Analysis of the Three 'Omics Datasets Reveal Coordinated Regulation of Cell Proliferation and Cell Death Associated Targets

We next performed a comparative analysis of the transcriptomics and proteomics datasets in IPA allowing us to pinpoint expression mismatches and regulatory nodes that could generate new mechanistic hypotheses for follow-up studies. We chose a 1.2-fold cut-off for differentially expressed protein but included all genes at the transcriptional level

based on the biological and technical differences between transcriptomics and proteomics. Using a fold-change threshold for proteins but not for transcripts allowed us to explore transcriptional regulation without prematurely excluding potentially relevant genes and identify discordant patterns, for example, genes upregulated/downregulated at RNA level but not at protein level, which may reveal post-transcriptional mechanisms.

SIRT6 has multifaceted roles in maintaining genomic stability by protecting chromosomal termini, modulating DNA damage repair via regulation of nuclear chromatin-associated proteins, regulating cellular senescence, proliferation, and migration (reviewed in [121]). The results of our comparative analysis also predicted that the SIRT6 KD inhibited the expression of canonical pathways associated with cell cycle, mitosis, DNA synthesis, and translation (Figure 7A). We observed that in all datasets biological functions associated with cancer proliferation, invasion, and migration were downregulated whereas functions related to cell death, apoptosis, and necrosis were upregulated (Figure 7B). We then generated the network of proteins that interacted to overall decrease the proliferation (Figure 7C) and increase the death (Figure 7D) of cancer cells. Similar trends were found in our RNA-seq data for most of the proteins identified by IPA. For example, the top regulated proteins related to decreased cell proliferation such as PTMA, PAWR, NFKB2, PICALM, TTN, TAF15, TJP1, and MYH14 and the top regulated proteins related to increased cell death such as PGAM2, SHC1, and H2AZ1 had expression patterns identical to the DEGs.

Next, we compared the causal networks in all datasets. The causal network generated in IPA represents the possible interaction between the upstream regulators and the target genes/proteins to control the overall biological function. It was predicted that targets associated with MAP3K20, transcription factor family, MYC genes, MKNK family, and HMGR were downregulated (Figure 8A). Likewise, PEX5L, RPS14, FBX14, and CTDPSP1 were predicted to be upregulated. However, the predicted activation z score in the proteomics dataset was between -2 to 2 , which is considered insignificant in IPA. It indicates that although the prediction is significant at the transcription level, it may not be significant at the translational level. This showed a discordant pattern, which means that not all genes, despite being transcriptionally active, may lead to significant functional activity since translational and post-translational modifications are also necessary for ultimate biological activity [122]. However, one pitfall here is that RNA-seq and proteomics were done on two different datasets where SIRT6 was knocked down by CRISPR and shRNA, respectively. Moreover, while the activation z-score may not reach statistical significance, subtle alterations in protein levels can still exert a cumulative biological effect that influences the trajectory of cellular activity.

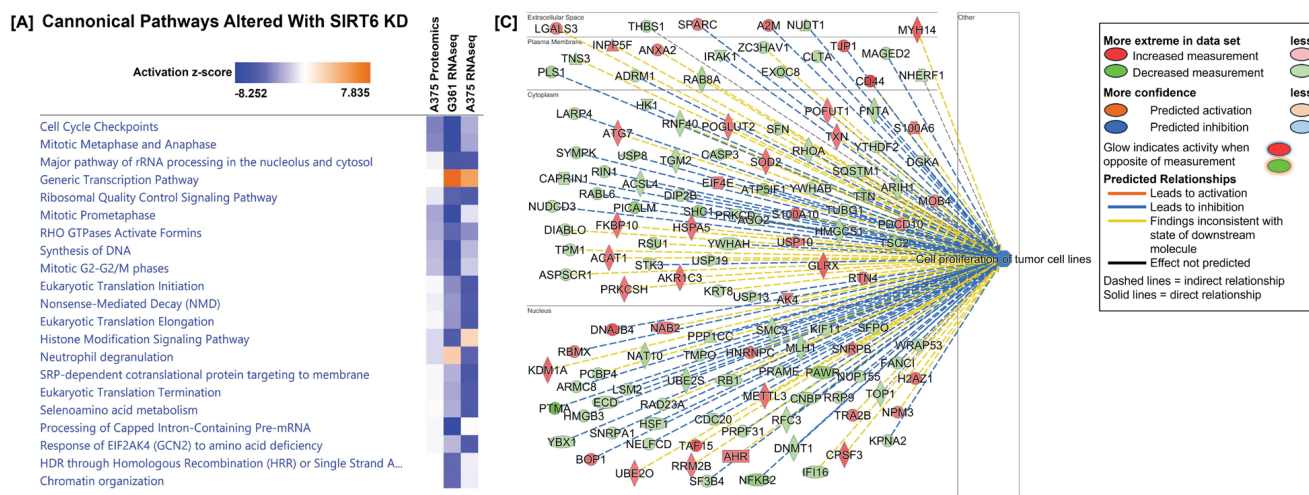


Figure 7. Cont.

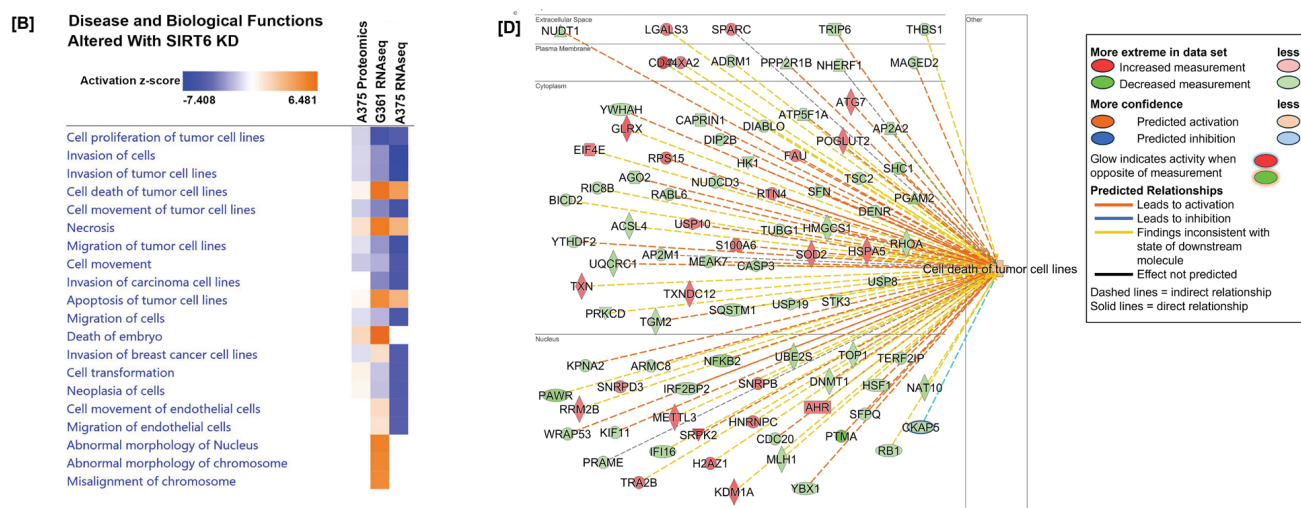


Figure 7. Comparative analysis of multi-omics data. Comparison of SIRT6 knockdown (KD) G361 and A375 cell-based transcriptomics and proteomics data by Ingenuity Pathway Analysis (IPA) predicted regulation of multiple canonical pathways (A) and biological functions (B) that were predicted to be modulated and are related to cell proliferation, division, migration, invasion, and death at RNA and protein levels. By analyzing protein modulation ≥ 1.2 -fold (red = increased levels; green = decreased levels), IPA predicted inhibition of cell proliferation (C) and activation of cell death of tumor cell lines (D) in our SIRT6 KD A375 cells as compared to the shNS control cells ($n = 3$ per group).

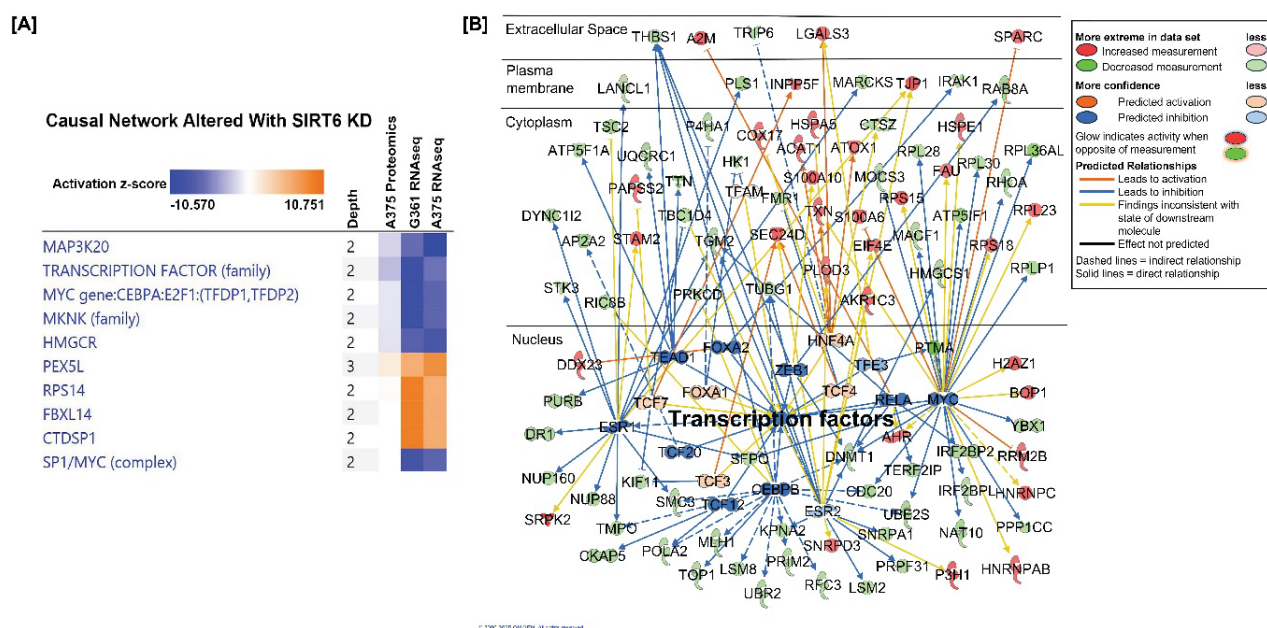


Figure 8. Causal networks from multi-omics data. Comparative analysis of multi-omics data from SIRT6 knockdown (KD) G361 and A375 cells by Ingenuity Pathway Analysis (IPA) showed predicted regulation of multiple networks. (A) Common causal networks inferred from multi-omics data, integrating upstream regulators and downstream effects. (B) SIRT6 KD in A375 cells affects multiple proteins with ≥ 1.2 -fold change ($n = 3$ per group; shNS and shSIRT6) related to transcription factor family networks, highlighting top common pathways with activation z-scores ≥ 2 in both transcriptomics and proteomics data.

Next, we generated the network of proteins from the proteomics dataset that directly or indirectly interact with “transcription factors family” in the causal network (Figure 8A) since this pathway had a significant activation z-score of ≤ -2 for all comparison datasets.

There were 95 proteins in the proteomics dataset that interacted with “transcription factors family” to form a causal network for an overall predicted decrease in its activity (Figure 8B). The transcription factors MYC, RELA, TEAD1, ESR1, TCF20, ZEB1, FOXA2, TCF12, CEBPB, ESR2, and TFE3 were predicted to be downregulated whereas TCF3, TCF7, FOXA1, TCF4, TFAM, and HNF4A were predicted to be upregulated. Comparable expression patterns were observed in our datasets for the majority of the targets. Thus, comparative analysis of DEGs and proteins showed a promising pattern, overall leading to decreased melanoma growth.

4. Conclusions

Taken together, RNA-seq and proteomics analyses of SIRT6 KD melanoma cells provide a coherent overview of the gene and protein targets modulated by SIRT6. These findings serve as hypothesis-generating observations that establish a basis for future in-depth studies aimed at delineating the mechanistic role of SIRT6 in melanoma. Despite the risk of confounding introduced by using different approaches, CRISPR/Cas9 versus shRNA-mediated SIRT6 knockdown for the transcriptomic and proteomic analyses, the use of independently processed datasets provides a valuable opportunity to identify regulatory patterns that remain consistent across platforms, thereby increasing confidence that these signatures reflect true SIRT6-dependent mechanisms. The observed expression profiles and predictions suggest a shift towards reduced melanoma cell proliferation and enhanced cell death. Importantly, the present results, together with previously published data from our lab and others, offer consistent evidence supporting SIRT6 as a promising key pathway in melanoma that may lead to additional therapeutic options for melanoma management. Nonetheless, comprehensive validation and mechanistic studies are warranted to elucidate how SIRT6 interacts with other signaling molecules in the context of melanoma management.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers18040590/s1>, Figure S1: Interactions of SIRT6 with key cell death and invasion genes. Ingenuity Pathway Analysis (IPA) evaluation of differentially expressed genes (DEGs) in SIRT6 KD A375 cells predicted SIRT6-interacting genes that are associated with cell invasion (A) or cell death (B). Line type identifies interaction, with solid lines indicating direct interactions and dashed lines indicating indirect interactions. Arrows at the end of the lines indicate that SIRT6 may affect expression of the indicated gene, while a bar at the end of the line indicates inhibition. Figure S2: Ingenuity Pathway Analysis (IPA) of differentially expressed genes (DEGs) in SIRT6 knockdown (KD) melanoma cells. (A) Interaction of DEGs in SIRT6 KD G361 cells with the top upregulated upstream target, *TP53*. (B) Interaction of DEGs in SIRT6 KD G361 cells with the top downregulated upstream target, *PPARD*. (C) Graphical summary of major predicted regulations based on DEGs in SIRT6 KD A375 cells. (D) Canonical pathways predicted to be affected based on DEGs in SIRT6 KD A375 cells. Figure S3: Top networks in multi-omics data. (A) Top network of differentially expressed genes (DEGs) associated with cell cycle, cell morphology, and cell-to-cell signaling molecules in SIRT6 knockdown (KD) G361 cells. (B) Top network of DEGs associated with cancer, cancer proliferation, cell cycle, and cellular movement in SIRT6 KD A375 cells. (C) Top network of proteins associated with cancer, cancer proliferation, cell cycle, and cellular movement in SIRT6 KD A375 cells.

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administration, M.A.N. and N.A.; funding acquisition, N.A. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data and available materials can be requested from the corresponding author per NIH/VA guidelines. The original RNA-seq data presented in the study are openly available in the GEO repository (A375 data accession #GSE318337, G361 data accession #GSE317488). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD07399.

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