The role and mechanism of sirtuin 3 (SIRT3) in melanoma

By:

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DEDICATION

This thesis is dedicated to my amazing husband Christian J. Ortiz Hernández and my immediate family: my lovely mother Marisol Pérez Pratts, my supporting father César A. Guzmán Muñoz, and my kind brother Aramis J. Guzmán Pérez. They are my greatest pillar, cheerleaders, and if I needed a shoulder to cry on, they were there for me always. They have never stopped believing in me and I owe them everything that I have and everything that I am to them.

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ABSTRACT

The role and mechanism of sirtuin 3 (SIRT3) in melanoma

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Melanoma is a potentially fatal skin malignancy, being the 5th most common cancer in the US. Although prognosis has improved due to the development of immunotherapies and targeted therapies, many melanoma patients acquire treatment resistance and even cancer recurrence. Therefore, the identification of novel melanoma-relevant targets is needed. Sirtuins are NAD+ dependent class III histone deacetylases, consisting of seven members (SIRT1-7), which regulate many proteins and consequently cellular functions which are important in cancer development and progression. Therefore, researchers have studied them in carcinogenesis, where they have been shown to play tumor suppressive or tumor promoting roles, depending on the malignancy. The mitochondrial sirtuin 3 (SIRT3), also has dichotomous roles in cancer and regulates cellular metabolism, among other processes. Particularly in melanoma, SIRT3 is overexpressed and is suggested to have proliferative functions, but more in-depth studies are needed. The main objective of this doctoral project was to explore the role and mechanism of SIRT3 and the consequences of its inhibition in melanoma. This was done by modulating SIRT3 *in vitro*, identifying SIRT3 downstream targets, and using small molecule inhibitors in a melanoma

relevant mouse model. We have found that SIRT3 expression increases with melanoma progression. Additionally, specifically inhibiting this sirtuin in A375 and G361 melanoma cells with genetic or pharmacological approaches imparts antiproliferative responses. To further elucidate the mechanisms of SIRT3 in melanoma, we performed molecular profiling after CRISPR/Cas9-mediated SIRT3 knockout (KO) where we identified multiple differentially expressed cancer genes that are involved in hallmarks of cancer. In addition, we tested 4′-bromo-resveratrol (4′-BR), a dual SIRT1 and SIRT3 inhibitor, in melanoma cells and tumors where we observed significant antiproliferative effects *in vitro* and *in vivo*. Furthermore, treatment with this compound significantly affected cancer-immune genes, most of them being involved in melanoma and having anticarcinogenic functions. Therefore, we were able to provide evidence of the therapeutic potential of 4′-BR against melanoma. From our data, it appears that targeting multiple sirtuins might result in superior clinical outcomes.

Chapter 1: Involvement of sirtuins in melanoma: therapeutics options

Status: In Progress

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SIRTUINS

Sirtuins (SIRTs) are a family of class III histone deacetylases (HDACs), which are nicotinamide adenine dinucleotide (NAD+)-dependent [1]. They were first identified in budding yeast, where the silencing information regulator 2 (Sir2) was found to be a crucial gene for transcriptional silencing of the mating type locus as well as life span extension by caloric restriction in this organism [2]. Correspondingly, sirtuins play major biological roles including regulation of aging, metabolism, cell signaling, DNA repair, apoptosis, transcription, epigenetic modifications, and signal transduction [1, 3]. Since sirtuins have essential cellular functions, it comes as no surprise that they are highly conserved through evolution and can be present in organisms ranging from bacteria, fungi, and plants, all the way to humans [4]. Furthermore, sirtuins are diverse and consequently five classes have been identified, however, class U is composed of sirtuins which have only been found in bacteria so we will be focusing on the other classes [3]. These classifications were obtained through a phylogenetic analysis, specifically by grouping them by their conserved peptide sequences in the core domain [5]. Particularly in mammals, there are seven sirtuin members (SIRT1-SIRT7) divided into four different classes (class I-IV) (***Figure 1***) [3, 5]. Class I includes mammalian sirtuins SIRT1, SIRT2, and SIRT3 as well as Sir2 (the founding member of sirtuins), whereas class IV includes SIRT6 and SIRT7, and these two classes are present only in eukaryotes [3, 6]. On the other hand, SIRT4 is part of class II sirtuins, while SIRT5 is part of class III sirtuins, and both of these classes have been found in eukaryotes as well as prokaryotes (bacteria and archeans), suggesting that may be the most ancient sirtuins [3, 6].

Sirtuins share some characteristics (e.g., NAD+ dependence and a preserved catalytic core), but differ in others, including size, subcellular location, and substrates (***Figure 1***) [3]. Briefly, SIRT1 being the most thoroughly studied, the closest ortholog to Sir2, and the largest SIRT, is mostly active in the nucleus, but can also be present in cytoplasm, and some of its main functions include regulating transcription, development, and aging as well as glucose homeostasis and DNA repair [1]. Comparatively, SIRT2 can sometimes be found in the nucleus, but it is mainly located in the cytoplasm, and it is associated with chromosome condensation and regulation of the cell cycle as well as glucose and lipid metabolism [1, 3].

On the other hand, SIRT3, SIRT4, and SIRT5 are present in the mitochondria: a metabolic hotspot [7]. Consequently, the functions of these sirtuins are mostly related to this organelle and cell metabolism. Interestingly, the subcellular localization of SIRT3 has been a topic of debate among researchers. Some have suggested that full-length SIRT3 might be found in the nucleus, yet it is targeted to the mitochondria as a stress-response mechanism where it is cleaved, and this shorter form carries out the enzymatic activity [8]. However, it has been confirmed that both mouse and human SIRT3 is solely found in the mitochondria, specifically in the matrix of this organelle; and in fact, it is one of the main mitochondrial deacetylases because its depletion results in hyperacetylation of many mitochondrial proteins [9]. Some of the roles that SIRT3 plays include energy homeostasis, protecting cells against oxidative stress, and regulating cell metabolism (e.g., urea cycle) [4]. SIRT4, being found in the mitochondrial matrix, is one of the least studied SIRTs and it is unique in its family, due to displaying a mono-adenosine di-phosphate (ADP)-ribosyl transferase functional activity, instead of deacetylation [4].

Metabolic and nutrient (e.g., fatty acid) regulation are among the main functions that SIRT4 is associated with [9]. Furthermore, SIRT5, the smallest sirtuin, has deacetylase as well as succinyl-transferase activity [10]. It is also located mitochondrial matrix where it has protective roles against metabolic stress and regulates glycolysis [4, 9]. Lastly, SIRT6 and SIRT7 are found in the nucleus, the latter being compartmented within the nucleolus [4]. These nuclear sirtuins are mainly known for their roles regarding the genome and/or ribosome, among other cellular activities [11, 12]. Particularly, SIRT6 is involved in stress responses and metabolic homeostasis [13]. On the other hand, SIRT7 has been shown to interact with RNA Polymerase complex and regulate rDNA transcription [14, 15].

SIRTUINS IN DISEASES AND CANCER

Due to the wide range of functions that SIRTs carry out and their major role in aging, scientists have been interested in further exploring them, specifically during age-related diseases, including cancer. Decreased levels of SIRT1 have been linked to hypoglycemia and hyperlipidemia, however, high expression of this sirtuin can prevent, atherosclerosis, neural cell degeneration, and inflammation [16]. In cancer, SIRT1 has been identified as both a tumor promoter and suppressor depending on the cancer type. As a potential oncogene in different cancers (e.g., lung, leukemia, prostate, and melanoma, among others) it has been shown to increase cell proliferation, target p53, and can block cell senescence [16, 17]. As a tumor suppressor, it is involved in DNA damage response which can ultimately lead to apoptosis in neoplasms like breast, bladder, and ovarian [17]. On the other hand, SIRT2 regulates glucose levels and is involved in inflammation as well as neurodegenerative diseases [18, 19]. Regarding cancer, SIRT2 can also enhance

glycolysis to increase energy within the cancer cells as well as suppress E-cadherin signaling and decrease cell-to-cell adhesion [20]. SIRT2 is upregulated in breast, pancreatic, and hepatocellular carcinoma while it is downregulated in ovarian, neck squamous cell carcinoma, and glioma [17].

SIRT3 has been directly linked to improved human life span and can even protect neural and cardiac cells against cellular damage [16]. Furthermore, considering that this protein is a major player in cell metabolism, its dysregulation has subsequently been associated with metabolic disorders like high cholesterol, insulin resistance, and obesity [10, 16]. Equal to other sirtuins, SIRT3 has been associated with anti- and pro-tumorigenic roles. Particularly, the expression levels of this protein are elevated in cancers in the digestive and excretory systems (e.g., colon, renal, esophageal) as well as melanoma [17]; while they are decreased in leukemia and breast cancers [17]. At a molecular level, SIRT3 regulates proteins such as p53, HIF-1α, or Ku70 which can support ROS levels and consequently cancer cell proliferation [21, 22]. On the other hand, SIRT4 is upregulated in Burkitt lymphoma and colorectal cancer and downregulated in stomach, thyroid, and bladder cancers [17]. Interestingly, SIRT4 activity may be used as a biomarker for coronary artery disease, yet when it is downregulated, it has been associated with insulin resistance and oxidative stress [13, 16]. The last mitochondrial sirtuin, SIRT5, has been shown to be negatively correlated with ammonia levels, but it plays protective roles from both cardiac and neurological disorders, including epilepsy [13, 16, 23]. Moreover, it is suggested that SIRT5 plays a tumor promoting role in colorectal cancer while being a tumor suppressor in lung cancer [17].

Similar to SIRT1, decreased levels of SIRT6 can lead to hypoglycemia, premature aging, and even shorten lifespan [16]. SIRT6 has been found to be upregulated in melanoma and non-melanoma skin cancers and downregulated in hepatocellular carcinoma, breast, and pancreatic cancers [17]. Lastly, there have been very limited studies regarding SIRT7 and its role in carcinogenesis. However, it has been found to regulate p53 activity and have oncogenic functions in osteosarcoma, thyroid, and ovarian cancers [17, 18]. In terms of other disorders, SIRT7 has been shown to have protective roles against heart hypertrophy, inflammation, and premature aging [13, 18].

As shown above, sirtuins have been associated with a constantly growing list of different health disorders as well as many types of malignancies. Among the hallmarks of cancer, sirtuins have been involved in carrying some of these transforming capabilities (e.g., genome instability and mutation, activating invasion and metastasis, sustaining proliferative signaling) [24]. In a similar manner, environmental factors have been linked to carcinogenesis [25]. With the skin being arguably the largest organ of the body and its main function being a barrier between the internal organs and our surroundings, it is constantly exposed to multiple environmental stressors [26]. These include, but are not limited to UV radiation, ROS, and pollutants, all of which can independently lead and/or work together with altered genetic factors to initiate skin cancer [27].

MELANOMA: THE MOST FATAL TYPE OF SKIN CANCER

Melanomagenesis and its prevalence in human populations

Cancer of the skin can be divided into 2 broad categories: melanoma and non-melanoma skin cancer, with the latter being subdivided into Basal Cell Carcinoma (BCC)

and Squamous Cell Carcinoma (SCC) [28]. Regarding the former, out of approximately 2 million Americans that will be newly diagnosed with cancer in 2022, almost 100,000 will be melanoma patients, making this the 5th most commonly diagnosed cancer in both males and females [29]. Melanoma originates in the cells found in the basal layer of the skin known as melanocytes [30, 31]. One of the main roles of these cells is the production of melanin, a photoprotective pigment found mainly in the skin, hair, and eyes [32, 33].

Briefly, melanin production is carried out through melanosomes, which are lysosome-related organelles that can be found in ocular and cutaneous melanocytes [34]. Such melanosomes will undergo a maturation process in which they increase melanin content until fully mature. However, these melanocytes are present in the basal layer of the skin. The skin darkening observed is due to the pigment transfer between these melanocytes and neighboring keratinocytes, which are the most abundant cell type in the skin and offers structural and immune support to the epithelial system [35, 36]. While the actual mechanism of how such pigmentation transfer occurs is still up for debate [37], however, it is known that the melanin is placed around the keratinocytes nucleus to prevent UV damage on the sun damaged side of the nuclei and consequently create a cap-like structure called perinuclear melanin caps [31, 38, 39]. Overall, UV stimulates melanocytes to produce melanin which safeguards the whole organism from the damaging effects that this radiation can cause. While in most cases this gives the appearance of a harmless tan, the actual consequences include damage at different levels ranging from the integumentary system to its cells and even at a molecular level (DNA) [40, 41].

However, with cumulative unprotected exposure to UV radiation, there can be either acute (e.g., erythema and inflammation from the sunburn) or chronic responses (e.g., photoaging and pathophysiological) [41, 42]. Moreover, some of the damages that UV rays can cause include cell death, dysregulation of the immune functions carried out by the skin, increase oxidative damage, and cause genetic alterations (pyrimidine dimers, photoproducts, and C to T/CC to TT shifts also known as UV signature mutations) [43-45]. Nevertheless, the most detrimental effect of UV exposure is carcinogenesis and in fact, for both melanoma and non-melanoma skin cancers, most of the cases are directly linked to UV exposure [46, 47]. Although there are multiple risk factors associated with melanoma (e.g., exposure to arsenic, radiation therapy, family history, etc.), those groups with features that are related to having lower presence of melanin and therefore less protection against UV radiation are at the highest risk (i.e., albinism, fair skin, having light hair or blue/green eyes, inability to tan, history of multiple or severe sunburns) [48-50].

Fortunately, most cases of melanoma are preventable with appropriate sun protection [51]. Additionally, most patients that are diagnosed with melanoma have favorable survival rates, but this is highly dependent on the stage where it was found. Indeed, melanoma which is localized to the epidermis has a 93% 5-year survival rate [29, 52]. Such survival odds drastically decrease as melanoma advances and even invades other skin layers, lymph nodes (5-year survival rate of 68%), or different organs, being the brain and lungs are some of the most common organs where melanoma metastasizes (5-year survival rate of 30%) [29, 52].

Current melanoma treatment options

There are multiple options for treating melanoma depending on a variety of factors such as size, tumor depth and whether it has spread to other tissues. The initial treatment for a potential melanoma lesion is a biopsy (removal) of the suspected skin lesion with a surrounding diameter of about 1 cm [53]. Such tissue is then analyzed under the microscope by pathologists and determined if the lesion is melanoma, tumor depth and if more skin needs to be surgically removed [54, 55]. While chemotherapy is a popular treatment in cancer which consists of the use of drugs to stop the proliferation of cancer cells, it is not very effective to treat melanoma. Most physicians suggest chemotherapy as a secondary treatment or as a last resource if immune or targeted therapy has not shown any effects, however, research shows a small benefit of utilizing chemotherapy drugs, such as dacarbazine, even after immunotherapies [56, 57].

Another treatment option for melanoma patients is radiation therapy which consists of the use of high-energy rays to target DNA and consequently causing cell death [58]. However, these high-energy rays lack specificity to cancer cells and therefore target normal cells as well [59, 60]. An alternative strategy has been employing a more recent treatment type known as immunotherapy. As the name suggests, immunotherapy activates the immune cells so that they carry out a response against the cancerous cells [61, 62]. Particularly, the immune cells recognize malignant cells as foreign and can reverse the mechanisms that have allowed the tumor to proliferate and grow uncontrollably [62]. One of the major disadvantages of using immunotherapies is that many patients develop resistance to the treatment [63]. Another treatment against melanoma can be adjuvant therapy which is considered more as a secondary treatment

which is less damaging to normal cells and has been shown to improve patient survival odds [64, 65]. While these newer adjuvant therapies have improved survival of patients with stage 1 or 2 melanoma, when the disease has metastasized or reached stage 3 or 4, no treatment has shown effectiveness on slowing or stopping cancer cell proliferation [66].

Moreover, an additional treatment type has made the list of options that medical professionals can provide to their melanoma patients: targeted therapy. This treatment has been developed mostly as a result of the discovery of mutations and genes that can be exploited for either their tumor promoting or suppressive roles [67, 68]. For example, the most commonly found mutated gene in melanoma patients is BRAF, particularly found as the $BRAF^{V600E}$ mutation, and dabrafenib is a BRAF inhibitor that can specifically target this mutation [69, 70]. Other mutations found in melanoma include genes in the MAPK signaling pathway like NRAS and MEK, among others [70]. Interestingly, most of these mutations can be stimulated or enhanced by UV exposure [46]. Mechanistically, targeted therapy makes use of molecular pathways involved in cell proliferation and metastasis to interfere with cancer progression [71, 72]. However, given that not all patients carry the specific mutations targeted by these therapeutics, there is a limitation in the patients that can benefit from this treatment [73]. Furthermore, similar to immunotherapy, patients that are eligible to receive targeted therapy can still develop resistance to the treatment [74].

Unfortunately, even with the multiple alternatives that have been developed over the years to treat melanoma, every treatment option has deficiencies and since there is no “one size fits all” approach, there have been efforts to use combination therapies. For example, the combination of immunotherapy and targeted therapy has been shown to

improve the effectiveness and longevity [75]. In fact, when combination therapy is used, it has been observed that patients' 5-year survival rate has increased from less than 5% to about 50% [76]. However, given that many patients still develop resistance to treatments and recurrence of the neoplasm, melanoma is still considered fatal, especially when diagnosed at advanced stages [73, 77]. This has prompted scientists and physicians to find newer and better ways to treat melanoma. Therefore, there is an urgent need of identifying novel molecular targets that could aid in the management of melanoma and eventually could even be considered as targeted therapy candidates.

SIRTUINS IN MELANOMA

Sirtuins are also known as “longevity proteins” due to their crucial role in aging and can even extend life, hence, they have been studied for their involvement during age-related diseases, including cancer. Melanoma is no exception, being the accumulation over time of UV exposure one of the main culprits of this neoplasm as it commonly arises in the long term. In fact, the probability of developing invasive melanoma dramatically increases with age, with the highest at risk being those who are 70 and older (1 in 37 for men and 1 in 84 for women in this age group) [29]. Interestingly, sirtuin activity levels have been shown to change in UV-exposed skin when compared to individuals who were protected from the sun [78]. Similarly, most sirtuins have been shown to be differentially expressed in melanoma cells and/or tissues when compared to benign counterparts [79-85]. Therefore, sirtuins have been studied for their potential roles in melanoma development and progression.

There are multiple studies regarding the involvement of sirtuins in melanoma, the first ones being less than a decade old. Below, we will introduce some of these studies, whereas in the section titled “Potential benefits of sirtuin modulation in melanoma and other skin disorders”, we will thoroughly discuss the effects of genetically and pharmacologically regulating sirtuins. With the limited information available, it is suggested that most of the seven mammalian sirtuins are overexpressed in melanoma (***Figure 2***), may play tumor promoting roles in this malignancy, and may even be involved in resistance to melanoma treatments.

SIRT1 has some of the strongest evidence of having a pro-proliferative function in melanoma. Briefly, it has been shown by our lab, alongside other researchers, that SIRT1’s expression and activity increase in melanoma cells and tissues and it is at its highest in metastatic stages [79, 80]. SIRT1 has also been shown to promote epithelial to mesenchymal transition (EMT) as well as cell migration and invasion which are indicators of metastasis [80]. Moreover, SIRT1 is positively correlated with cell proliferation and survival [78, 79, 86]. In terms of molecular mechanism, there is an inverse correlation between SIRT1 with p53 as well as the latter’s downstream target (p21) which may be an indication that SIRT1 acts through PI3K signaling pathway [79, 87].

Interestingly, there are opposing views about the role of SIRT2 in terms of resistance to melanoma treatments. It has been suggested that both its downregulation and its activation can contribute to resistance against PLX4032 (BRAF mutant inhibitor) and Dasatinib (multi-kinase inhibitor), respectively [88, 89]. However, regarding the role of SIRT2 in melanoma progression, most evidence points towards SIRT2 having a potential

tumor promotor role in melanoma. A study has shown an increase of SIRT2 expression in metastatic sites compared to early stages of melanoma [81]. Similarly, SIRT2 is associated with proliferative effects in melanoma tumors [90].

Out of the mitochondrial sirtuins, SIRT3 is the most studied regarding the function it carries out in melanoma. It is suggested that SIRT3 may have a tumor promoting role in this neoplasm, since it has been shown to be overexpressed in melanoma cells and tissues when compared to benign counterparts [83]. Like the first pair of sirtuins, SIRT3 is associated with carcinogenic effects both *in vitro* and *in vivo* [83, 91]. Interestingly, mutant p53 increases SIRT3 expression which then deacetylates and activates MnSOD, a protein that moderates reactive oxygen species (ROS) levels [92]. This supports melanoma cells in surviving and proliferating, which provides further evidence for SIRT3's potential oncogenic role in melanoma [92]. Altogether, this may provide a plausible mechanism of how SIRT3 exerts oncogenic-like features in melanoma, for example, metabolic/glycolytic shift, also referred as cell metabolism deregulation, which is another of the hallmarks of cancer [24].

Up to date, there is only one melanoma study that mentions SIRT4. Here, they observed upregulation of SIRT4 in patients after they received chemotherapy (e.g., melphalan) [82]. Another study saw increased SIRT4 levels in photoaged skin when compared to non-photoaged tissues [93]. Since melanoma usually arises in advanced aged individuals and UV-radiation is one of the main causes of its development, it can be expected that SIRT4 may also be overexpressed in melanoma, however, further studies need to be conducted.

SIRT5 has contradictory evidence about its relevance in the pathogenesis of melanoma [89]. It has been shown that SIRT5 blocks apoptosis which supports melanoma cell perseverance, however, there is another study that suggests that SIRT5 may not be crucial for melanoma tumor growth [82, 94]. The former research group used data from The Cancer Genomic Atlas (TCGA), along with loss-of-function experiments, which led them to associate the presence of proliferation markers (MITF and c-MYC) with SIRT5 expression [94]. Overall, there seems to be stronger evidence that SIRT5 might be a tumor promoter in melanoma, but in order to reach clear conclusion, other factors need to be considered including additional study models.

Our lab, together with other research groups, has conducted studies exploring how SIRT6 is involved in melanoma. Comparable to other sirtuins, SIRT6 is also overexpressed in melanoma cells (RNA and protein levels) and tissues (protein levels), indeed, the highest levels are found in metastatic tissues [84, 95]. Additionally, our lab showed that SIRT6 exerts proliferative effects both in melanoma cell lines and animal models [84, 96]. Another research group suggested that dose dependent levels of SIRT6 affect resistance to melanoma treatments (BRAF and MEK inhibitors). Particularly, SIRT6 haploinsufficiency, but not complete inhibition, supports melanoma cell survival [97]. Interestingly, SIRT6's proliferative effects in melanoma are likely due to the regulation of AKT signaling pathway as well as autophagy since SIRT6 expression is positively correlated with autophagy markers [95, 97]. In summary, with the data available up to date, it is suggested that SIRT6 may have an oncogenic role in melanoma.

For the last sirtuin member, SIRT7, the scarce publications available regarding its role in melanoma were not conducted until very recently (1-2 years ago). SIRT7 has been

found to be overexpressed in melanoma cells and tissues [85]. Additionally, there is evidence suggesting the involvement of SIRT7 in melanin production [32].

Up to this point, we have described the essential roles that sirtuins play across different diseases along with a wide range of malignancies, for instance, melanoma. Since sirtuins have controversial roles depending on the cancer type, being able to manipulate the levels and/or activity of these proteins might be favorable for patients. Correspondingly, scientists have developed sirtuin modulators in order to investigate their effects at molecular and cellular levels. On a broader spectrum, understanding the effects of sirtuin modulation, would aid in the management of many disorders including cancer and could even lead to the development of novel targeted therapeutic agents.

SIRTUIN MODULATORS IN CANCER AND OTHER DISEASES

Due to the potential breakthrough that altering sirtuin expression/activity holds, regarding human pathophysiological conditions, there have been many research groups that have been dedicated to study sirtuin modulation. In essence, there are three main approaches that can shift sirtuin levels. First, endogenous modulation including dieting (subjects restrict calorie consumption which can activate sirtuins and lead to increased lifespan) and redox state of cells (decreased NAD+/NADH ratio which affects neuron formation) [16, 98]. Second, by genetic modulation where different techniques including small interfering RNA (siRNA) or CRISPR/Cas9 can be used to inactivate sirtuins and lead to a change in cell proliferation [32, 83, 86, 94, 96]. Third, by chemical modulation where chemical compounds (e.g., pharmaceuticals) are the main drivers of sirtuin alteration.

Regarding the latter, both sirtuin activators (also referred as sirtuin-activating compounds (STACs)) and inhibitors have been identified and developed. These are recognized as compounds that alter either the strength of the link between the sirtuin(s) and their substrate or the rate at which the enzymatic reaction is carried out [98, 99]. Consequently, most modulators affect sirtuin activity and not necessarily their expression levels. Since the discovery of mammalian sirtuins, there have been hundreds of sirtuin modulators reported, most of them being categorized as inhibitors. This is likely due to structure complexities that accompany the development of activators (e.g., allosteric binding site) [100]. Interestingly, activators are usually more effective at lower doses while they are associated with fewer side effects than inhibitors [101]. Furthermore, another characteristic used to classify sirtuin modulators involves their origin, either natural or synthetic. Examples of sirtuin inhibitors and activators from both natural and synthetic sources will be presented below and are summarized in ***Table 1*** and ***Table 2*****,** respectively.

Natural sirtuin modulators and their relevance in health

Most of the naturally occurring sirtuin modulators identified can affect SIRT1. For instance, melatonin and amurensin G have been shown to inhibit SIRT1 and exert anticancer effects by decreasing tumor size and cell proliferation, and mitigating cancer cell drug resistance [102, 103]. On the other hand, vitamin D, saponins and tannins, isoliquiritigenin, piceatannol, dammarene triterpenes, and fisetin are SIRT1 activators that protect from ROS and fat accumulation, cardiovascular diseases, cancer, and even aging

[17, 100, 104-108]. Regarding SIRT2, javamide-II and lactones are natural inhibitors, the latter having antitumor effects [109, 110].

Furthermore, honokiol, a SIRT3 activator from natural origin, has been found with anticarcinogenic properties against multiple cancers (lung, prostate, breast, colon, and pancreatic cancers), and cytoprotective towards cardiomyocytes and neurons [99, 111]. Other SIRT3 activators include licoisoflavone A which is cardioprotective, while pomegraniin A has antioxidant benefits that help prevent the onset of multiple health disorders [112-114]. Kaempferol also activates SIRT3 leading to oxidative stress and consequently apoptosis in cancer cells (e.g., leukemia) [115]. Another natural SIRT3 activator is pyrroloquinoline quinone (PQQ) which has been shown to stimulate the biogenesis of mitochondria and maintain its function [116, 117]. Oroxylin A also has been shown to activate SIRT3 leading to improved cardiac function and anticarcinogenic effects in ovarian cancer [118-120].

In terms of SIRT6 natural activators, vitexin and orientin have tumor suppressive, antioxidant, anti-inflammatory, and antimicrobial properties while being protective against neurological, metabolic, endocrine, and cardiovascular diseases [121, 122]. Similarly, anthocyanidins are also SIRT6 activators which have been shown to be anticarcinogenic [123]. To our knowledge, at this moment there have not been naturally occurring sirtuin modulators identified that selectively target SIRT4, SIRT5, or SIRT7. However, there are modulators that do alter the level/activity of these proteins while also affecting other sirtuins (***Table 1***).

Among the natural sirtuin modulators that target 2 or more sirtuins simultaneously, there are bichalcones, eurochevalierine, and phloroglucinol derivatives which all inhibit

SIRT1 and SIRT2 and have anticarcinogenic potential [124-126]. Nicotinamide is a SIRT1 and SIRT3 inhibitor that can decrease cancer cell proliferation and it has even been used in a clinical trial in non-small cancer lung carcinoma [13, 127, 128]. On the other hand, silybin is a SIRT2 and SIRT3 activator which has been associated with many cellular and overall health benefits including being renoprotective, anti-fibrotic, anti-inflammatory, antioxidant, and anticarcinogenic [129]. Some natural SIRT1 and SIRT3 dual activators include dihydromyricetin, polydatin, and sesamin, the former being neuroprotective whereas the other two are cardioprotective [130-133]. Trans-(-)-e-viniferin is also a SIRT3 and SIRT1 activator found in nature which has promising evidence for treatment of Alzheimer's and diabetes as well as having antioxidant, anti-inflammatory, and antitumorigenic capabilities [134-137]. Curcumin is a widely used natural product which is cardio-, hepato-, and reno-protective and can modulate sirtuins specifically by activating SIRT1, SIRT3, and SIRT4 [138-141]. Luteolin is a SIRT1, SIRT3, and SIRT6 activator that has anti-inflammatory, antioxidant, antiallergic, and anticarcinogenic properties, while also being beneficial for metabolism (e.g., regulating insulin, fatty acid, and glucose levels) [123, 142-144]. Furthermore, D-glucose has been suggested to inhibit all 7 sirtuins and leads to antioxidant effects [145].

Interestingly, there are some compounds that have conflicting evidence regarding their mode of action towards sirtuin modulation. For example, metformin is a SIRT1 activator yet decreases SIRT5 protein levels [146]. Additionally, catechins have been identified as being inhibitors of SIRT6 as well as activators of SIRT1 and SIRT3 [100]. These compounds can aid in longevity, cancer therapy, and cardiovascular and metabolic diseases (e.g., type 2 diabetes) [99, 111]. Moreover, xanthones have been shown to be

either SIRT1 activators or SIRT2 inhibitors and have been linked to obesity management as well as cancer-related health disorders, respectively [147, 148].

In like manner, there are compounds that besides having varying roles depending on the sirtuin, they can also regulate the same sirtuin in opposite ways. For instance, berberine activates SIRT1 and SIRT3, which provides cardiac protection, whereas it can also dose-dependently inhibit SIRT3 which can be cytotoxic toward hepatocarcinoma cells [149, 150]. Likewise, some studies suggest that quercetin is a SIRT1 activator, SIRT2 inhibitor, and for SIRT6 it can be either inhibiting or activating depending on the dose levels [99, 122, 151]. Some of the benefits of this modulator, which has been part of many clinical trials, include anti-inflammation, aiding in fat metabolism, and even has the potential to treat cancer, colitis, type 2 diabetes, and high blood pressure, among others [152-154].

Resveratrol (3, 5, 4'-trihydroxystilbene) is one of the most studied natural sirtuin modulators and it is commonly recognized as a SIRT1 activator [155]. Similarly, scientists have proposed that this compound may also activate other sirtuins (SIRT1-6) [156-160]. Interestingly, there is one study suggesting that in zebra fish, resveratrol may decrease the levels and/or activities for the mitochondrial sirtuins 3 and 4 [161]. Nonetheless, the majority of the publications available regarding this natural compound and its effects in health disorders are attributed to resveratrol being a sirtuin activator (i.e., SIRT1). In fact, Curry et.al. assembled an extensive review including the many studies and clinical trials where resveratrol has been investigated [162]. Briefly, some of the many health benefits that resveratrol provides include aiding in Alzheimer’s, respiratory, cardiovascular,

inflammatory, and metabolic diseases as well as improving mitochondrial functions, being cancer preventative, and increasing lifespan [100, 111, 121, 155, 162].

Although many natural compounds have been found to modulate sirtuin activity and provide important health benefits, their specificity remains unanswered for many of them. Alternatively, scientists are taking advantage of molecular simulation and high-throughput screening to design sirtuin-specific modulators based on their chemical structure.

Synthetic sirtuin modulators and their relevance in health

There are various strategies to develop synthetic sirtuin modulators including modifying exiting natural compounds, *in silico* screening by computational chemistry, among others. Multiple researchers, including most recently Hong et.al. and Kratz et.al., have gathered some of the most relevant sirtuin modulators and evidence of their effect in multiple disorders [99, 163]. Here we will focus on selected sirtuin modulators that have been mainly associated with carcinogenesis as well as other relevant health conditions (***Table 2***).

SIRT1 modulators include inauhzin which is a SIRT1 inhibitor as well as STAC-5 and STAC-8 which activate this sirtuin. The former has been shown to inhibit cancer cell proliferation and induces senescence and apoptosis *in vitro* and in like manner reduce tumor growth *in vivo* [164]. STAC-5 and STAC-8 have the potential of being helpful in treating carcinogenesis among other health conditions like cardiovascular, autoimmune, and metabolic diseases [111]. SIRT2 has an extensive list of inhibitors with cancer-related applications. First, AF8 has antiproliferative effects in breast, lung, pancreatic and colorectal cancer cells, and for the latter malignancy, it also reduced tumor growth in mice

[165]. Second, AGK2 decreases cell proliferation while activating apoptosis and necrosis in glioma [166, 167]. Similarly, AK7 reduced glioblastoma tumor growth *in vivo*, has anti-inflammatory effects, and is neuroprotective in Huntington's and Parkinson's diseases [166, 168-170]. Furthermore, compounds 12a and 24a have antiproliferative effects in lung cancer cells while compounds 35 and 39 show the same results on leukemia and breast cancer cells [171-173]. Interestingly, compounds 3, 4, 6f, and 53 have some level of anti-breast cancer activity, but for compounds 3 and 4, it is not strong [171, 174, 175]. Likewise, the SIRT2 inhibitors RK-91230156, TM-P4-Tha, and TM also have antiproliferative effects in breast cancer *in vitro*, and the latter having additional *in vivo* evidence [176-179]. Furthermore, compounds 22 and 23, decrease cell proliferation in malignant cells [164, 180]. In the case of NH4-13, it has antiproliferative effects both *in vitro* and *in vivo* in glioblastoma, colorectal, breast, cervical, lung and pancreatic cancers [181]. In the case of NPD11033, it decreases pancreatic cell proliferation [182].

Regarding SIRT3 modulators PNU-282987, liraglutide, and adjudin activate this sirtuin [183]. PNU-282987 can suppress oxidative stress and mitochondrial dysfunction caused by angiogenesis in cardiovascular diseases [184]. Liraglutide is protective to renal cells from mitochondrial apoptosis and damage caused during diabetes [185]. Adjudin is primarily known as a potential male contraceptive, but it also exerts antioxidant properties, otoprotective effects, and can even block lung cancer cell proliferation and metastasis [186-188]. On the other hand, 2-methoxyestradiol is a potent SIRT3 inhibitor that has anticarcinogenic properties by disturbing mitochondrial biogenesis in osteosarcoma cells [189]. Another SIRT3 inhibitor that is cytotoxic to cancer cells is 4-hydroxynonenal (4-HNE) [190, 191]. To our knowledge, there have not been any SIRT4-specific modulators

developed, likely due to the lack of studies regarding this sirtuin and particularly its chemistry (e.g., structure and mechanism) [192, 193]. However, due to the importance of this sirtuin in metabolism and other cellular processes, it is safe to assume that modulators that target SIRT4 could be revolutionary in metabolic diseases including diabetes and even cancer [194-196]. Some of SIRT5's inhibitors include DK1-04e and Suramin. The latter has been used for trypanosomiasis and even potential HIV treatment [197]. Additionally, it can block angiogenesis and cancer cell proliferation as well as increase the potency of chemotherapy [198, 199]. In fact, it has been used in a clinical trial for its anticancer properties in solid tumors [200]. Similarly, DK1-04e decreases cell proliferation and tumor growth in breast cancer [201].

Regarding SIRT6 inhibitors, compounds 2, 3, 5, 8, and 11 have been found to have anticarcinogenic effects. For example, compounds 2 and 3 have synergistic effect with chemotherapeutics (e.g., gemcitabine) [202]. Moreover, compounds 5, 8, and 11 have been shown to decrease pancreatic cancer cell proliferation [202, 203]. Comparatively, the SIRT6 activator MDL-800 decreases cell proliferation and tumor growth in small lung carcinoma and hepatocellular carcinoma [204, 205]. Being SIRT7 and SIRT4 the least understood sirtuins, studies regarding SIRT7 are very limited as well. Nonetheless, recently there have been a handful of modulators developed that target this sirtuin. The first SIRT7 potent inhibitors, cyclic tripeptides 1 and 2, were not identified until 2019 [206]. However, these compounds were found to inhibit deacetylase activity in other sirtuins (SIRT1-3 and SIRT6), thus lacking specificity towards SIRT7 [206]. On the other hand, earlier this year compounds 2800Z and 40569Z were also identified as SIRT7 inhibitors, without affecting other sirtuins [207]. Markedly, these compounds additionally exhibited

anticarcinogenic effects against liver cancer by activating cell death and having synergistic effects with a chemotherapeutic (sorafenib) [207].

Throughout this report, we have provided strong evidence that sirtuins are the central players in many vital cellular functions. Together with the fact that they are localized within different cellular compartments, simultaneously affecting the levels and/or activity of more than one sirtuin, might have the most promising treatment potential. This is especially in the case of diseases where multiple sirtuins are implicated, like cancer. Presented below are some of the chemical modulators that affect multiple sirtuins and that been found to have cancer applications (***Table 2***).

Although splitomicin inhibits the Sir2 protein (Sir2p), it has weak potency in mammal sirtuins [208, 209]. However, splitomicin analogs (compound 5c, 8c and (R-8c) have been identified as SIRT1 and SIRT2 inhibitors which decrease breast cancer cell proliferation [210]. Likewise, MC2141 inhibits SIRT1 and SIRT2 and its anticarcinogenic effects are shown in leukemia, Burkitt's lymphoma, colorectal, glioblastoma, and cervical cancer cells [211, 212]. Compound 18 is also a SIRT1 and SIRT2 inhibitor which exerts antiproliferative effects *in vitro* and *in vivo* in glioma malignancies [213]. Comparatively, the dual SIRT1 and SIRT2 inhibitors, BZD9L1 and compound 3g, reduce cancer cell burden in colorectal, leukemia, and breast cancers [214-216]. Furthermore, multiple studies have shown the inhibitory effect of salermide towards SIRT1 and SIRT2 and have provided its cancer-related applications including activating apoptosis and decreasing cancer cell proliferation in the following malignancies: pancreatic cancer, lymphoma, glioblastoma multiforme, leukemia, colorectal cancer, and neuroblastoma [217-220]. On the other hand, SRT1720 is approximately 1000 times more potent than resveratrol at

SIRT1 activation and has been shown to have contradictory roles in cancer [99]. For example, it induces apoptosis in cancer cells like breast and multiple myeloma [221, 222], and conversely, it can also lead to tumor cell migration and metastasis *in vivo* [223]. Additionally, it has the potential to be used as a treatment for diabetes [224]. Interestingly, it has been suggested that this compound can also interact and even inhibit SIRT3, specifically by residing in the substrate binding site of SIRT3 and thereupon form a stable SRT1720/NAD+/SIRT3 complex [225].

Compounds 27, 30, and 64 as well as KPM-2 inhibit to some extent SIRT1, SIRT2, and SIRT3, and have anti-breast cancer activity [174, 226, 227]. Furthermore, for compounds 27 and 30 they too decrease lung cancer cell proliferation, while compound 64 has the same effect but on leukemia and prostate cancer cells [226, 227]. Regarding KPM-2, it has been shown to additionally have neuroprotective effects [174]. JH-T4 is also a SIRT1, SIRT2, and SIRT3 inhibitor that has antiproliferative effects in breast, colorectal, and lung cancer cells, however, it is cytotoxic in some normal epithelial cells [228]. Comparatively, NH4-6 is toxic when used *in vivo* likely due to targeting SIRT1-3, while NH4-13, which has a similar structure to NH4-6, is a specific SIRT2 inhibitor (see previous section) [181]. Nonetheless, NH4-6 has stronger anticarcinogenic effects in glioblastoma, breast, colorectal, cervical, lung, and pancreatic malignant cells [181]. Furthermore, MC2494 is another SIRT1, SIRT2, and SIRT3 inhibitor that decreases cell proliferation while increasing apoptosis in lymphoma and leukemia, and for the latter malignancy these effects have also been observed *in vivo* [229, 230]. YC8-02 is a SIRT1, SIRT2, and SIRT3 inhibitor that has been found to be a therapeutic target in B-cell lymphomas by having antiproliferative effects both *in vitro* and *in vivo* [231].

On the other hand, compound 1 is a SIRT2 and SIRT6 inhibitor that has chemo-sensitizing effects, disrupts autoimmune encephalomyelitis, and has therapeutic potential for both pancreatic cancer as well as diabetes [203, 232, 233]. Another sirtuin modulator, UBCS039, activates SIRT6 and has implications in inflammatory disease as well as in cancer, particularly in non-small lung carcinoma where it has induced ROS and eventually led to apoptosis [234, 235]. Additionally, activation of SIRT5 desuccinylase activity has also been observed by this compound, which needs to be further explored [235]. Cambinol is another sirtuin inhibitor, specifically targeting SIRT1, SIRT2, and to a lesser extent SIRT5 [236]. This modulator has anticarcinogenic activity *in vitro* and/or *in vivo* in multiple malignancies including hepatocarcinoma, neuroblastoma, Burkitt lymphoma, and multiple myeloma [236-241].

Notably, it is not surprising that most of the modulators presented in this section affect SIRT1 and/or SIRT2 to some degree due to the fact that these are some of the most studied sirtuins. In addition, SIRT1 and SIRT2 have the most suggestive evidence of being involved in many cancer types. Nonetheless, further elucidating the functions of sirtuins and understanding their chemical structures can provide researchers the tools to develop novel sirtuin modulators that target the other sirtuin family members. We have also provided examples of how we can take advantage of sirtuin modulators as plausible therapeutics for many health disorders. However, sirtuins play dual roles in different tissues and diseases (e.g., tumor promoters vs tumor suppressors depending on the cancer type), which can be a double edge sword. Therefore, it is important to further test the potential applications of sirtuin regulation and/or optimize the modulators that are

currently available to take advantage of their benefits while minimizing their unwanted effects.

SIRTUIN MODULATION IN MELANOMA AND OTHER SKIN DISORDERS

The identification of novel targeted therapeutic approaches is essential for cancers, and melanoma particularly. This is due to the resistance that is usually followed by current treatments, notably as the disease becomes more aggressive. Not to mention that although for many cancers the incidence has been declining over time, this is not the case for melanoma which for most years has seen a steady increase in new cases [29]. Taking this into consideration along with the promising results of sirtuin modulation, melanoma patients could also potentially benefit from treatments that make use of this approach. However, within the hundreds of sirtuin modulators developed, only a few of them have been evaluated in melanoma, so far. Therefore, we will discuss different sirtuin modulation methods that have been directly employed in melanoma and if they have additional uses in different diseases. Furthermore, we will go through the ones that have been employed in other skin disorders and may support their use in melanoma (***Table 3***).

Chemical sirtuin modulation in skin-related diseases, including melanoma

As previously mentioned, resveratrol is a SIRT1 activator that possesses an extensive list of favorable features including being an antioxidant, anti-inflammatory, and cytoprotective [99, 111, 121, 162]. Although SIRT1 has been found to be upregulated both in melanoma and non-melanoma skin cancers, these beneficial properties work together and overcome the potential tumor promoting role of SIRT1 by preventing UV-

induced damages in the skin [79, 242, 243]. In fact, resveratrol has been shown to decrease tumor burden in a mouse skin cancer model [244]. Overall, resveratrol leads to an antiproliferative response in skin cancer including altering cell cycle regulators (cyclins and p21), upregulating apoptotic markers (Smac), and downregulating proliferation markers (Survivin) [245, 246]. In fact, resveratrol is considered a photo-chemopreventive agent [244]. Comparatively, 1,4-Dihydropyridines (DHPs), for example, MC2562, can activate SIRT1 as well as SIRT2 and SIRT3 which, together with resveratrol, have been shown to improve wound healing in the skin [247-249]. Furthermore, cyanidins are naturally derived compounds that can activate SIRT6, have anti-aging properties, and have been associated with antitumorigenic effects both *in vitro* and *in vivo* in skin malignancies [123].

Regarding sirtuin inhibition in skin disorders, LC-0296 is a SIRT3 inhibitor that induces ROS in head and neck squamous cell carcinoma (HNSCC) cells and consequently suppresses malignant cell growth without affecting normal human oral keratinocytes [250]. Similarly, 3-(1H-1,2,3-triazol-4-yl) pyridine, referred to as 3-TYP inhibits SIRT3 and since this sirtuin is a major mitochondrial deacetylase, inhibiting its activity results in the downregulation of genes involved in oxidative stress as well as glucose and lipid metabolism [251, 252]. Furthermore, 3-TYP has been shown to attenuate the cytoprotective effects that some SIRT3 activators exert including curcumin, caffeine, liquiritin, luteolin, and acacetin [253-257]. Some of these sirtuin activating compounds have been shown to prevent UVA and UVB-induced skin damages like photoaging [254-257]. Although 3-TYP might not have preventative properties in the skin, it might be exploited as an antiproliferative and potentially antitumorigenic agent. For

instance, SIRT3 as a ROS regulator has been shown to protect acute myeloid leukemia (AML) cells from apoptosis [258]. However, treatment with 3-TYP can work together with chemotherapeutic agents to maximize anticancer effects *in vitro* and *in vivo* [258].

Regarding melanoma, evodiamine is a SIRT1 inhibitor that has been shown to induce apoptosis in these malignant cells [259]. Specifically, this compound can decrease SIRT1 expression while increasing the levels of the pro-apoptotic gene Bax as well as the tumor promoter p53 [259]. In contrast, treatment with silymarin, which activates SIRT1, reduces Bax expression which accordingly blocks cell death [260]. Another sirtuin modulator, EX-527, also known as selisistat, is mainly a SIRT1 inhibitor that provides many health benefits that EX-527 like neuroprotecting and mitigating conditions like anxiety, post-traumatic stress disorder (PTSD) and morphine addiction in animal models [261-264]. Interestingly, this compound has been shown to also inhibit SIRT2 and SIRT3 to a lesser extent and it has neuroprotective effects in Huntington's disease in both preclinical and during clinical trials [197, 265-267]. Regarding the effects of EX-527 in carcinogenesis, it has been shown to have strong anticancer effects against pancreatic, glioma, melanoma, endometrial, and lung cancers [87, 163, 268-271]. Moreover, it undertakes the issue of resistance to chemotherapy (e.g., cisplatin and gemcitabine) and other treatments in malignancies including leukemia, prostate, lung, and endometrial cancers [272-275]. Particularly in melanoma, our lab showed reduced melanoma cell growth after treatment with EX-527 [87]. Altogether, treatment with evodiamine, silymarin, and EX-527 validates the potential oncogenic functions that SIRT1 exerts in melanoma and that these might be through the PI3K survival pathway.

Furthermore, pharmacological inhibition of SIRT1 and SIRT2 by tenovins (Tenovin-1 and its water-soluble analog Tenovin-6) decreases tumor growth by activating p53 [276, 277]. These antineoplastic effects have been shown *in vitro* and/or *in vivo* in multiple cancer types including gastric, breast, lung, hemangiosarcoma, lymphoma, leukemia, and indeed in melanoma [78, 178, 277-281]. Regarding the latter malignancy, both tenovins can decrease aggressive melanoma cell growth and viability as well as delay the onset of tumors [282]. In fact, our lab treated multiple melanoma cell lines with Tenovin-1 which resulted in decreased cell proliferation, viability, and survival [79]. Moreover, it is important to highlight that when used *in vivo*, general toxicity is not observed meaning that these compounds can target malignant cells without affecting normal cells [282]. Interestingly, Tenovin-6 is more potent than Tenovin-1 at being cytotoxic to melanoma cells [282]. Although our focus has been on cutaneous melanoma, curiously Tenovin-6 is potent against uveal melanoma as well [283]. Overall, tenovins can be considered great therapeutic candidates for multiple cancers including melanoma.

SirReal2 is another sirtuin modulator, with most of the evidence pointing towards being a SIRT2 inhibitor, however, it is also suggested to slightly inhibit SIRT1 and SIRT6 [284]. It was shown to reduce the ability of cancer cells to proliferate, particularly in lymphoma, colorectal, cervical, lung, and gastric cancers [178, 285]. In melanoma, treatment with SirReal2 inhibited SIRT2 activity which resulted in delayed melanoma tumor progression and consequently tumor growth [90]. Additionally, the SirReal2-treated tumors had an increase of infiltrating natural killer (NK) cells [90]. In like manner, sirtinol has been shown to be a SIRT2 inhibitor, however, it can also affect SIRT1 [286, 287]. This compound has supportive evidence of exerting multiple health benefits including

being antioxidant and cardioprotective [288]. Moreover, sirtinol has synergistic effects with other cancer-therapeutic (e.g., cisplatin and camptothecin) [289, 290]. This dual inhibitor is anticarcinogenic against lung, cervical, prostate, T-cell, leukemia, lymphoma, and melanoma neoplasms [87, 286, 291-294]. Regarding the latter malignancy, treatment with sirtinol led to strong antiproliferative effects in melanoma cells [87].

Interestingly, although resveratrol is a SIRT1 activator, modifying its structure, particularly by replacing one of hydroxy groups in resveratrol with bromine, gave rise to a dual SIRT1 and SIRT3 inhibitor known as 4′-bromo-resveratrol (4′-BR) [295]. 4′-BR was able to restrict gastric cancer cell stemness which was mainly attributed to the SIRT3 inhibitory effects carried out by this compound [296]. Regarding melanoma, our lab has conducted two studies that show antiproliferative effects following *in vitro* and *in vivo* treatments with 4′-BR [297, 298]. First, we saw decreased cell proliferation, invasion, and migration, and accordingly increased caspase-mediated apoptosis and cell cycle arrest [297, 298]. In addition, this compound led to metabolic reprogramming, in particular, there was a decrease in lactate- and glucose-related markers [297]. Next, we used a melanoma-relevant mouse model, where we saw that 4′-BR treatment reduced tumor size and metastasis as well as altered the expression of proliferation and immune markers, providing a potential mechanism for sirtuin modulation in melanoma [298].

Genetic sirtuin modulation in melanoma

In addition to employing chemical modulators, sirtuins can be regulated using genetic approaches. In fact, some of the most fundamental studies where the role of sirtuins in melanoma was presented, did so by genetically manipulating these proteins.

For example, when SIRT1 is suppressed by short hairpin RNA (shRNA) or siRNA, this leads to antiproliferative effects including 1) less cell proliferation (showed by trypan blue exclusion and colony formation assays and/or MITF downregulation) , 2) more cell cycle arrest (increase in cell cycle inhibitors p15, p27, and p53), and/or 3) induction of cell senescence (senescence-associated beta galactosidase marker) [86, 299]. Interestingly, SIRT2 knockdown via shRNA was not enough to significantly affect melanoma cell growth by itself, but inhibition of SIRT1 together with SIRT2 resulted improved antiproliferative effects compared to SIRT1 inhibition only [86].

Regarding the mitochondrial SIRT3, our lab has shown in melanoma the pro-proliferative effects *in vitro* and *in vivo* (i.e., increase in cell proliferation and tumorigenesis) after overexpressing SIRT3 via plasmid transfection [83, 91]. On the other hand, an increase in cell senescence and cell cycle arrest as well as a decrease in cell proliferation and migration and suppression of tumor formation were some of the outcomes following shRNA-mediated SIRT3 knockdown in melanoma [83]. Mechanistically, similar to SIRT1, SIRT3 also affects the expression of the cell cycle regulator: p21. Particularly, SIRT3 inhibition caused, p21 levels to increase, but, contrasting to SIRT1, this was independent of p53 [83]. Due to the fact that SIRT3 is the major mitochondrial deacetylase, many of its functions have been associated with cellular metabolism. Our lab also evaluated the *in vivo* effects of SIRT3 modulation on melanoma tumor growth and metabolism [91]. As seen in the previous studies, SIRT3 inhibition leads to less tumorigenesis and tumor size, while SIRT3 overexpression showed the opposite effects [91]. Interestingly, decreasing SIRT3 levels affected the expression of 37 metabolic genes [91].

To our knowledge there have not been studies carried out where SIRT4 is genetically modulated in melanoma. Furthermore, as presented in previous sections, SIRT5 has controversial roles in melanoma, which was established by different genetic approaches. An initial study concluded that SIRT5 loss had no effect on melanoma tumor initiation and growth in a *Braf-Pten* mouse model [300]. In contrast, although another research group used a similar *Braf-Pten* mouse model as the previous study mentioned, the former showed that SIRT5 was necessary for melanomagenesis [94]. There are multiple plausible explanations for the differing results, including that the study from Moon et.al. used 1) an allele that deletes a single exon from the SIRT5 gene and 2) lower tamoxifen concentration [300]. On the other hand, the allele used by Giblin et.al. deleted most of this sirtuin's coding region and the dose for tamoxifen was more than 12 times higher [94]. Furthermore, the latter study also included additional *in vitro* models where they validated that SIRT5 inhibition both by shRNA as well as CRISPR/Cas9 led a decrease in melanoma cell viability and proliferation. [94].

Comparative to other sirtuins, shRNA-mediated SIRT6 downregulation in melanoma cells led to a decrease in cell viability and proliferation while increasing cell senescence [84]. Another study involving CRISPR/Cas9-mediated SIRT6 knockout (KO) melanoma cells, showed cell cycle arrest as well as less cell viability and proliferation when compared to wildtype cells [96]. Using an *in vivo* model, we also noticed antitumorigenic effects like diminished melanoma tumor formation [96]. In both of the studies mentioned above, the effect of SIRT6 downregulation in crucial molecular pathways was explored. Overall, SIRT6 inhibition significantly affects genes involved in cell senescence, autophagy, angiogenesis, apoptosis, and epithelial-to-mesenchymal

transition (EMT), among others [84, 96]. Regarding the last mammal sirtuin, SIRT7, there have been different genetic approaches taken to study its involvement in melanoma. First, microRNAs (miRNAs), particularly miR-148b, can decrease SIRT7 levels which leads to *in vitro* antiproliferative effects like suppressing melanoma cell proliferation, migration, and invasion [85]. In contrast, the circular RNA (circRNA), circZNF609, is negatively correlated with SIRT7 expression and therefore, has been shown to support melanoma progression by targeting DNA damage [301]. Both miRNAs and circRNAs can regulates cells and therefore have been implied in melanomagenesis as both tumor promoters or suppressors, providing an alternative avenue to modulate the activity of one or many targets of interest, including sirtuins [85, 301, 302]. Interestingly, SIRT7 CRISPR/Cas9 KOs cells showed amplified presence of melanin than normal cells [32]. Furthermore, SIRT7 is negatively correlated to the expression of crucial genes during melanogenesis including microphthalmia-associated transcription factor (MITF), tyrosinase (TYR), tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2), and tyrosine hydroxylase (TH) [32]. Therefore, SIRT7 is valuable in melanoma progression like other sirtuins and uniquely to this sirtuin is also crucial for melanin production.

In summary, in this section we have provided evidence of the potent effects of manipulating sirtuin expression and/or activity by chemical or genetic approaches in skin disorders including melanoma. However, more studies need to be conducted especially with the lesser understood sirtuins like SIRT4 in order to develop novel modulators and/or maximize the benefits of the modulators currently available.

CONCLUSIONS

Throughout this review, we have gone over the important cellular and molecular roles that sirtuins carry out including, DNA repair, regulating cell metabolism, and apoptosis among many others. Overall, sirtuins maintain cellular homeostasis which can be affected by altering the expression or activity levels of these proteins which can eventually lead to pathogenesis. However, exploiting sirtuin modulation might protect or even be able to reverse the molecular chain reaction that accompanies sirtuin disruption. Even though there are hundreds of sirtuin modulators that have been identified to this day, here we have presented the most commonly used compounds, have some of the strongest evidence of health benefits, and/or have anticarcinogenic potential. However, we recognize that with the constantly growing list of sirtuin modulators, there might be compounds that we did not cover here.

Regarding melanoma, sirtuins have been found to be dysregulated in cells and/or tissues pertaining to this malignancy. Taking this into consideration, together with the antitumorigenic effects that sirtuin modulators exert, it is important to investigate and understand how sirtuin modulation might be used to reduce melanoma burden. Here we have presented some examples of how affecting sirtuin activity and/or expression could be applied in skin diseases like melanoma. However, due to the limited information available regarding sirtuin modulation in melanoma, further studies are urgently needed which will support their use in preclinical and clinical studies. In other words, taking advance of chemical and genetic modulation of these proteins can progress into potentially effective therapeutics for melanoma as well as other diseases.

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FIGURES

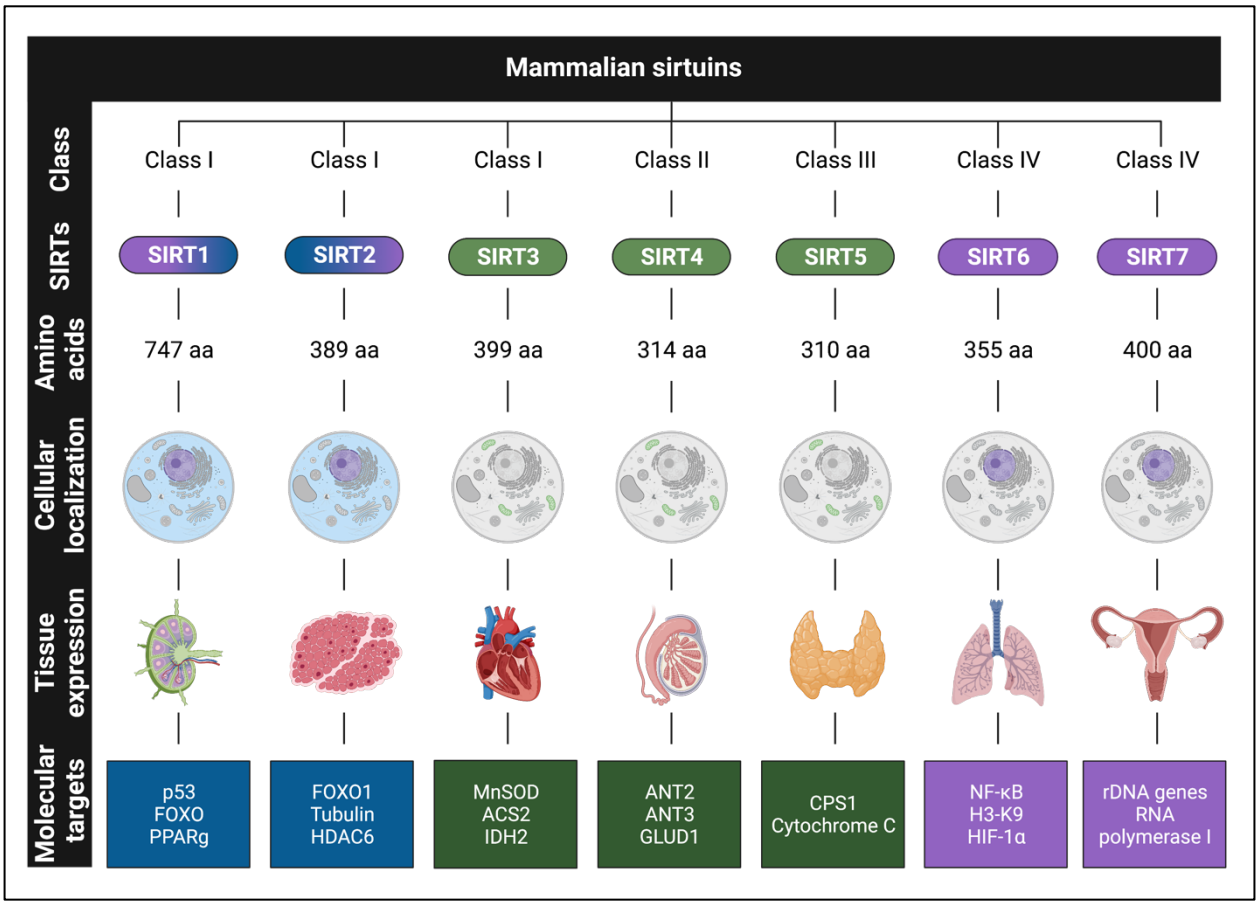

Figure 1. Mammalian sirtuins' (SIRTs) distinctive characteristics. The seven members of the sirtuin family (SIRT1-SIRT7) are presented along with their phylogenetic class, size (amino acids), cellular localization, representative human tissues where the sirtuin is highly expressed, and examples of their respective molecular targets.

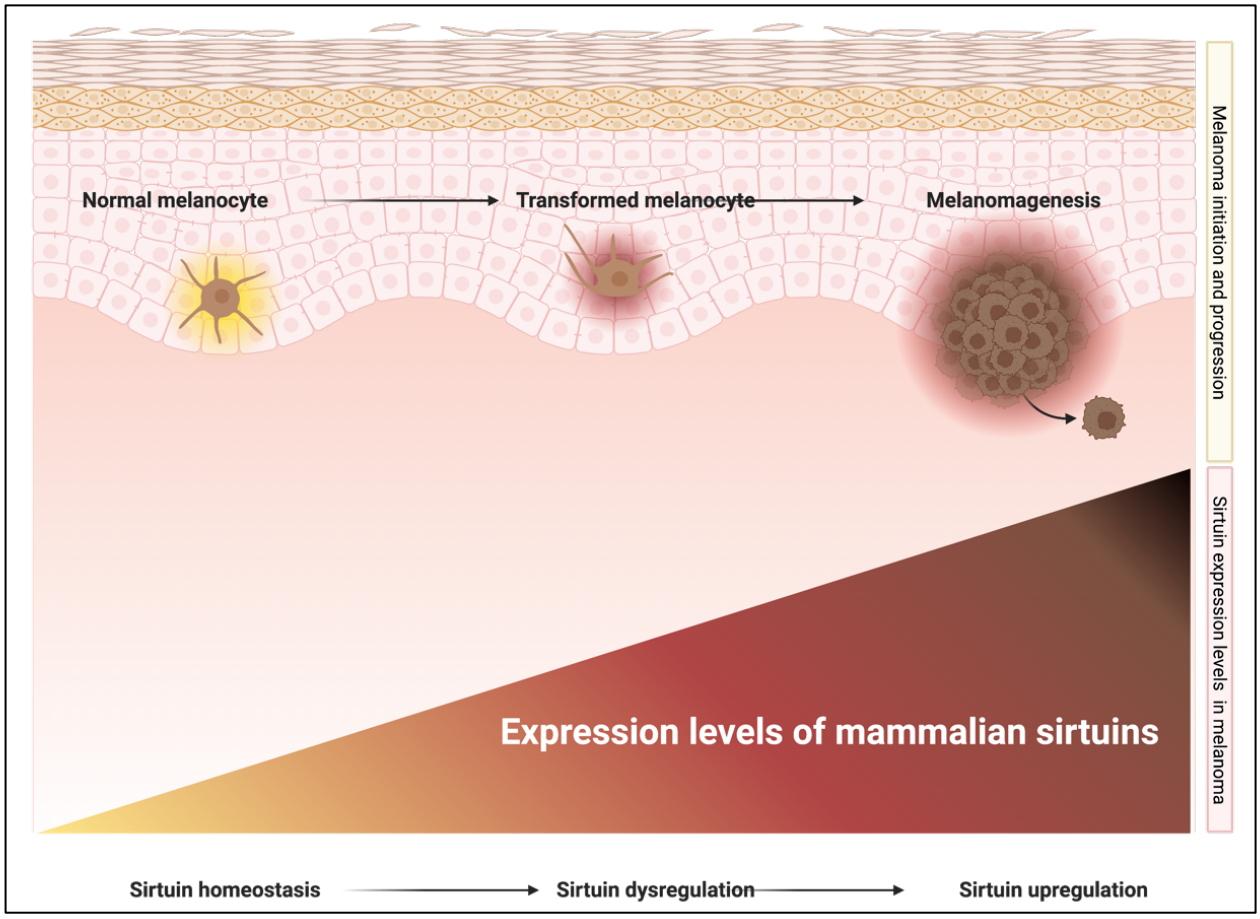

Figure 2. SIRTs expression levels are altered by melanocyte malignant transformation which support their potential tumor promoting roles in melanoma skin cancer. Most mammalian sirtuins have strong evidence of being upregulated in melanoma cells and tissues when compared to normal or benign counterparts. Accordingly, the expression for the less studied SIRTs is also expected to increase in this malignancy.

TABLES

Table 1. Natural sirtuin modulators.

Compound name	Effect on sirtuins	Applications	References
Melatonin	SIRT1 inhibitor	Anticarcinogenic.	[102, 103]
Amurensin G	SIRT1 inhibitor	Anticarcinogenic.	[102, 103]
Vitamin D	SIRT1 activator	Anticarcinogenic, protects against ROS.	[17, 104, 105]
Saponins And Tannins	SIRT1 activator	Anticarcinogenic, protects against ROS.	[17, 100, 104-108]
Isoliquiritigenin	SIRT1 activator	Anticarcinogenic, protects against ROS.	[17, 100, 104-108]
Piceatannol	SIRT1 activator	Anticarcinogenic, protects against ROS.	[17, 100, 104-108]
Dammarene triterpenes	SIRT1 activator	Anticarcinogenic, protects against ROS.	[17, 100, 104-108]
Fisetin	SIRT1 activator	Anticarcinogenic, protects against ROS.	[17, 100, 104-108]
Lactones	SIRT2 inhibitor	Antitumorigenic.	[110]
Honokiol	SIRT3 activator	Anticarcinogenic properties in lung, prostate, breast, colon, and pancreatic cancers.	[99, 111]
Licoisoflavone A	SIRT3 activator	Cardioprotective.	[112-114]
Pomegraniin A	SIRT3 activator	Antioxidant.	[112-114]
Kaempferol	SIRT3 activator	Anticarcinogenic and protects against ROS.	[115]
Pyrroloquinoline Quinone (PQQ)	SIRT3 activator	Protects mitochondria.	[116, 117]
Oroxylin A	SIRT3 activator	Anticarcinogenic effects in ovarian cancer.	[118-120]
Vitexin	SIRT6 activator	Tumor suppressive, antioxidant, anti-inflammatory, and antimicrobial.	[121, 122]
Orientin	SIRT6 activator	Tumor suppressive, antioxidant, anti-inflammatory, and antimicrobial.	[121, 122]
Anthocyanidins	SIRT6 activator	Anticarcinogenic.	[123]
Bichalcones	SIRT1 and SIRT2 inhibitor	Anticarcinogenic.	[124-126]

Eurochevalierine	SIRT1 and SIRT2 inhibitor	Anticarcinogenic.	[124-126]
Phloroglucinol derivatives	SIRT1 and SIRT2 inhibitor	Anticarcinogenic.	[124-126]
Nicotinamide	SIRT1 and SIRT3 inhibitor	Anticarcinogenic in non-small cancer lung carcinoma.	[13, 127, 128]
Silybin	SIRT2 and SIRT3 activator	Anticarcinogenic.	[129]
Dihydromyricetin	SIRT1 and SIRT3 activator	Cardioprotective.	[130, 131]
Polydatin	SIRT1 and SIRT3 activator	Cardioprotective.	[131, 132]
Sesamin	SIRT1 and SIRT3 activator	Neuroprotective.	[131-133]
Curcumin	SIRT1, SIRT3, and SIRT4 activator	Cardioprotective, hepatoprotective, and renoprotective.	[138-141]
Trans-(-)-ε-viniferin	SIRT1 and SIRT3 activator	Antitumorigenic and treatment for Alzheimer's and diabetes.	[134-137]
Luteolin	SIRT1, SIRT3, and SIRT6 activator	Anti-inflammatory, antioxidant, antiallergic, and anticarcinogenic	[123, 142-144]
Metformin	SIRT1 activator/ SIRT5 inhibitor	Cancer therapy, and cardiovascular and metabolic diseases.	[99, 111, 146]
Catechins	SRT6 inhibitors/ SIRT1 and SIRT3 activators	Cancer therapy, and cardiovascular and metabolic diseases.	[99, 100, 111]
Xanthones	SIRT1 activators/ SIRT2 inhibitors	Improving obesity and cancer -related health disorders.	[147, 148]
Berberine	SIRT1 activator SIRT3 activator/inhibitor	Cardioprotective and anticarcinogenic.	[149, 150]
Quercetin	SIRT1 activator SIRT2 inhibitor SIRT6 activator/inhibitor	Anti-inflammatory, used in metabolic disorders, anticarcinogenic.	[99, 122, 151] [152-154]
Resveratrol	SIRT1 activator	Antioxidant, anti-inflammatory, and cytoprotective.	[99, 111, 121, 162]

Table 2. Synthetic sirtuin modulators.

Compound name	Effect on sirtuins	Applications	References
Inauhzin	SIRT1 inhibitor	Antiproliferative effects.	[164]
STAC-5	SIRT1 activator	Anticarcinogenic.	[111]
STAC-8	SIRT1 activator	Anticarcinogenic.	[111]
AF8	SIRT2 inhibitor	Antiproliferative effects in breast, lung, pancreatic and colorectal cancer cells.	[165]
AGK2	SIRT2 inhibitor	Antiproliferative effects and activates apoptosis and necrosis in glioma.	[166, 167]
AK7	SIRT2 inhibitor	Antiproliferative effects in glioblastoma, anti-inflammatory, and neuroprotective in Huntington's and Parkinson's diseases.	[166, 168-170]
Compound12a	SIRT2 inhibitor	Antiproliferative effects in lung cancer cells.	[173]
Compound 24a	SIRT2 inhibitor	Antiproliferative effects in lung cancer cells.	[173]
Compound 35	SIRT2 inhibitor	Antiproliferative effects in leukemia and breast cancer	[171, 172]
Compound 39	SIRT2 inhibitor	Antiproliferative effects in leukemia and breast cancer	[171, 172]
Compound 3	SIRT2 inhibitor	Anti-breast cancer activity.	[171, 174, 175]
Compound 4	SIRT2 inhibitor	Anti-breast cancer activity.	[171, 174, 175]
Compound 6f	SIRT2 inhibitor	Anti-breast cancer activity.	[171, 174, 175]
Compound 53	SIRT2 inhibitor	Anti-breast cancer activity.	[171, 174, 175]
RK-9123015	SIRT2 inhibitor	Antiproliferative effects in breast cancer.	[176]
TM-P4-Tha	SIRT2 inhibitor	Antiproliferative effects in breast cancer.	[179]
TM	SIRT2 inhibitor	Antiproliferative effects in breast cancer.	[177, 178]
Compound 22	SIRT2 inhibitor	Antiproliferative effects in cancer.	[164, 180]
Compound 23	SIRT2 inhibitor	Antiproliferative effects in cancer.	[164, 180]
NH4-13	SIRT2 inhibitor	Antiproliferative effects in glioblastoma, colorectal,	[181]

		breast, cervical, lung and pancreatic cancers.	
NPD11033	SIRT2 inhibitor	Antiproliferative effects in pancreatic cells.	[182]
PNU-282987	SIRT3 activator	Antioxidant and cardioprotective.	[184]
Liraglutide	SIRT3 activator	Renoprotective.	[185]
Adjudin	SIRT3 activator	Potential male contraceptive and antiproliferative effects in lung cancer cells.	[186-188]
2-methoxyestradiol	SIRT3 inhibitor	Anticarcinogenic in osteosarcoma cells.	[189]
4-hydroxynonenal (4-HNE)	SIRT3 inhibitor	Cytotoxic to cancer cells.	[190, 191]
DK1-04e	SIRT5 inhibitor	Decreases cell proliferation and tumor growth in breast cancer.	[201]
Suramin	SIRT5 inhibitor	Potential HIV treatment. Blocks angiogenesis, cancer cell proliferation, and increases chemotherapy potency.	[197-199]
Compound 2	SIRT6 inhibitor	Synergistic with chemotherapeutics.	[202]
Compound 3	SIRT6 inhibitor	Synergistic with chemotherapeutics.	[202]
Compound 5	SIRT6 inhibitor	Antiproliferative in cancer cells.	[202, 203]
Compound 8	SIRT6 inhibitor	Antiproliferative in cancer cells.	[202, 203]
Compound 11	SIRT6 inhibitor	Antiproliferative in cancer cells.	[202, 203]
MDL-800	SIRT6 activator	Decreases cell proliferation and tumor growth in small lung carcinoma and hepatocellular carcinoma.	[204, 205]
2800z	SIRT7 inhibitors	Anticarcinogenic effects against liver cancer.	[207]
40569z	SIRT7 inhibitors	Anticarcinogenic effects against liver cancer.	[207]
MC2141	SIRT1 and SIRT2 inhibitor	Anticarcinogenic effects in leukemia, Burkitt's lymphoma, colorectal, glioblastoma, and cervical cancer cells.	[211, 212]

Compound 18	SIRT1 and SIRT2 inhibitor	Antiproliferative effects in glioma malignancies.	[213]
BZD9L1	SIRT1 and SIRT2 inhibitors	Anticarcinogenic in colorectal, leukemia, and breast cancers.	[214-216]
Compound 3g	SIRT1 and SIRT2 inhibitors	Anticarcinogenic in colorectal, leukemia, and breast cancers.	[214-216]
Salermide	SIRT1 and SIRT2 inhibitor	Apoptotic, decreases cancer cell proliferation in pancreatic cancer, lymphoma, glioblastoma multiforme, leukemia, colorectal cancer, and neuroblastoma.	[217-220]
SRT1720	SIRT1 activator/ SIRT3 inhibitor	Treatment for diabetes, apoptotic in breast cancer and multiple myeloma but can lead to tumor cell migration and metastasis in other malignancies.	[221-224]
Compound 27	SIRT1, SIRT2, and SIRT3 inhibition	Anticarcinogenic in breast and lung cancer.	[174, 226, 227]
Compound 30	SIRT1, SIRT2, and SIRT3 inhibition	Anticarcinogenic in breast and lung cancer.	[174, 226, 227]
Compound 64	SIRT1, SIRT2, and SIRT3 inhibition	Anticarcinogenic in breast and leukemic cancers.	[226, 227]
KPM-2	SIRT1, SIRT2, and SIRT3 inhibition	Neuroprotective.	[174]
JH-T4	SIRT1, SIRT2, and SIRT3 inhibitor	Antiproliferative effects in breast, colorectal, and lung cancer cells, however, it is cytotoxic in some normal epithelial cells.	[228]
NH4-6	SIRT2 inhibitor	Anticarcinogenic effects in glioblastoma, breast, colorectal, cervical, lung, and pancreatic malignant cells.	[181]
MC2494	SIRT1, SIRT2, and SIRT3 inhibitor	Decreases cell proliferation while increasing apoptosis in lymphoma and leukemia.	[229, 230]

YC8-02	SIRT1, SIRT2, and SIRT3 inhibitor	Antiproliferative effects in b-cell lymphomas.	[231]
Compound 1	SIRT2 and SIRT6 inhibitor	Chemo-sensitizing effects, disrupts autoimmune encephalomyelitis, and therapeutic potential for both pancreatic cancer as well as diabetes.	[203, 232, 233]
UBCS039	SIRT5 and SIRT6 activator	Implications in inflammatory disease and non-small lung carcinoma.	[234, 235]
Cambinol	SIRT1, SIRT2, and SIRT5 inhibitor	Anticarcinogenic in hepatocarcinoma, neuroblastoma, Burkitt's lymphoma, and multiple myeloma.	[236-241]

Table 3. Sirtuin modulators in skin-related diseases.

Compound name	Effect on sirtuins	Applications	References
Resveratrol	SIRT1 activator	Antiproliferative response in skin cancer.	[245, 246]
1,4-Dihydropyridines (DHPS)	SIRT1, SIRT2 and SIRT3 activators	Improves wound healing in the skin.	[247-249]
Cyanidins	SIRT6 activator	Antiaging, antitumorigenic in skin malignancies.	[123]
LC-0296	SIRT3 inhibitor	Induces ROS in head and neck squamous cell carcinoma (HNSCC) cells.	[250]
3-TYP	SIRT3 inhibitor	Protection against ROS and involved in glucose and lipid metabolism.	[251, 252]
Evodiamine	SIRT1 inhibitor	Apoptotic in malignant melanoma cells.	[259]
Silymarin	SIRT1 activator	Antiapoptotic.	[260]
EX-527 (also known as Selisistat)	SIRT1, SIRT2, and SIRT3 inhibitor	Anticarcinogenic against pancreatic, glioma, melanoma, endometrial, and lung cancers. Chemo-sensitizing effects in leukemia, prostate, lung, and endometrial cancers. Neuroprotective in PTSD, morphine addiction, and Huntington's disease.	[87, 163, 197, 261-275]
Tenovin-1	SIRT1 and SIRT2 inhibitor	Antiproliferative in melanoma cells and tumors. Antitumorigenic in gastric, breast, lung, hemangiosarcoma, lymphoma, leukemia, and melanoma.	[78, 178, 276-282]
Tenovin-6	SIRT1 and SIRT2 inhibitor	Antiproliferative in melanoma cells and tumors. Antitumorigenic in gastric, breast, lung, hemangiosarcoma, lymphoma, leukemia, and melanoma.	[78, 178, 276-282]
Sirreal2	SIRT2, SIRT1 and SIRT6 inhibitor	Antiproliferative in melanoma, lymphoma, colorectal, cervical, lung, and gastric	[90, 178, 284, 285]

		cancers. Induces immune response to tumors	
Sirtinol	SIRT2 and SIRT1 inhibitor	Antiproliferative effects in melanoma cells. Anticarcinogenic against lung, cervical, prostate, t-cell, leukemia, lymphoma, and melanoma neoplasms. Antioxidant and cardioprotective.	[87, 286-288, 291-294]
4′-bromo-resveratrol (4′-BR)	SIRT1 and SIRT3 inhibitor	Antiproliferative in melanoma cells and tumors. Affects metabolic markers.	[295, 297, 298]

Chapter 2: Sirtuin 3 (SIRT3) inhibition by pharmacological and genetic approaches leads to antiproliferative response and affects the expression of key cancer genes and pathways

Status: In Progress

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INTRODUCTION

Melanoma is the most aggressive and fatal type of skin cancer if left untreated. Recent advancements in target-based therapies as well as immunotherapies have led to promising outcomes in terms of melanoma patient survival [1]. Unfortunately, many patients still develop resistance to the treatments and recurrence of melanoma. Therefore, it is imperative to identify novel targets that could be used for melanoma management. The NAD+-dependent deacetylases known as sirtuins, are a family of seven proteins (SIRT1-SIRT7) that are found in several cell compartments including the nucleus, cytoplasm, and/or mitochondria. Mammalian sirtuins have been found to play important roles in cell cycle and chromatin regulation, DNA repair, metabolism, and aging [2]. Moreover, they are involved in several diseases including cancer, where they have been identified as both tumor promoters and tumor suppressors depending on the type of neoplasm [3]. SIRT3, a mitochondrial sirtuin, has been of interest to researchers due to its roles during apoptosis, cell proliferation, oxidative stress, insulin secretion, and transcription [4, 5]. In terms of its roles in cancer, SIRT3 has also been suggested to have both oncogenic and tumor suppressive functions depending on the cancer type [5]. Nevertheless, the role of SIRT3 in melanoma, has not been thoroughly elucidated.

Our lab has previously shown that SIRT3 is overexpressed in melanoma cells [6]. Accordingly, decreased levels of SIRT3 in melanoma resulted in antiproliferative effects both *in vitro* and *in vivo* [6]. Additionally, our lab has shown *in vitro* antiproliferative effects of a compound that inhibits SIRT3, 4′-bromo-resveratrol (4′-BR), against melanoma cells [7, 8]. However, research exploring SIRT3's mechanism in melanoma is still in its infancy, where it has been shown that SIRT3 might carry out its pro-melanoma roles by affecting

metabolism-related genes. In order to decrease this gap of knowledge, we have undertaken novel SIRT3 modulatory methods in melanoma cells, like using a SIRT3-specific inhibitor 3-(1H-1,2,3-triazol-4-yl) pyridine (referred as 3-TYP) and CRISPR/Cas9-mediated SIRT3 knockout (KO) to evaluate the cancer-related genes that interact with this mitochondrial sirtuin.

MATERIALS AND METHODS

Cell culture and SIRT3 knockout cell generation

A375 and G361 wildtype cells and SIRT3 knockout cell pools were purchased from Synthego. To create the SIRT3 KO cells, the guide sequence used for A375 cells was CUGAAGUCUGGAAUGCCACU, while the sequence for G361 cells was UUUUCUGUCAGUAUUAAAGG. To grow single clones, cell pools for A375 and G361 were respectively plated in 96-well plates at an average of 0.5 cells/well and allowed to grow until a single, discrete clone could be seen in the well. Wells with single colonies were passaged and grown using standard cell culture conditions (37°C, 5% CO_2, humidified chamber). For A375 cells, DMEM media (Corning) was used while for G361 cells, 5A McCoy's (Corning) media was used and both cell medias were supplemented with 10% FBS. Cells were authenticated and tested for mycoplasma (results were negative). Once cells achieved appropriate confluency, they were transferred from the 96-well plates to larger dishes until enough amount of cells were available for collection and further analysis. Cells were aliquoted and washed with PBS to create pellets. Afterwards, protein was isolated and used for quantitative immunoblotting to measure SIRT3 protein expression. For each cell line (A375 and G361), two SIRT3 CRISPR clones were selected for further experiments.

Drug treatments

For both drugs, wildtype A375 and G361 cells were counted, platted at appropriate density in 3 or more wells, and left to attach overnight the day before treatments. On the day of treatment, cell media was removed and fresh media with drug was added to the

cells. Cells were incubated in the drug for the appropriate amount of time depending on the assay. 3-TYP (3-(1H-1,2,3-triazol-4-yl) pyridine) was purchased from Selleckchem and dissolved in DMSO to prepare 15mM stock solution and was stored at -80ºC. Cells were divided in the following groups: DMSO (negative control) and 25uM, 50uM, 75uM, and 100uM 3-TYP-treated cells. 4'-bromo resveratrol (4'-BR) was purchased from AOBIOUS and dissolved in DMSO to prepare 25mM stock solution and was stored at -80ºC. Cells were divided in the following groups: DMSO (negative control) and 12.5uM, 25uM, 50uM, and 100uM 4'-BR-treated cells.

Protein isolation and quantification

Protein was isolated with 1X RIPA lysis buffer (Millipore) from cell lysates and quantified using Pierce BCA Protein assay kit (Thermo Scientific). The BioTek Synergy H1 plate reader was used to quantify average protein concentration in duplicates per each sample

Quantitative immunodetection analysis by ProteinSimple (Simple Western System Jess)

Protein expression was measured with automated protein capillary electrophoresis using a Simple Western system called Jess (ProteinSimple, San Jose, CA). For this purpose, protein lysates (with equal concentrations of protein per sample), antibody diluent, primary and secondary antibodies, luminol-peroxide solution, and wash buffer were dispensed into designated wells in the appropriate ProteinSimple microplate (12-230kDa). Following plate loading, default settings were used (375V; primary antibody incubation of 30min; secondary antibody incubation of 30min) to run the plate. This

system allowed separation and immunodetection via chemiluminescent signals. Primary antibodies (SIRT3, Ku70, Bax, p53, MDM2, and p21) were optimized accordingly before selecting the appropriate conditions for running the experimental immunoassay (***Supplementary Table S1***). Quantitative data analysis was done with the Compass software (ProteinSimple, San Jose, CA) which is built-in with for ProteinSimple. Peak area value of each sample represents protein expression, and this value was normalized with the total capillary area of the respective sample using the Total Protein Assay.

RealTime-Glo MT cell viability assay

To evaluate the effects of SIRT3 inhibition with via CRISPR/Cas9 and drug treatments, we used the RealTime-Glo MT cell viability assay (Promega) and followed the manufacturer's protocol. Briefly, appropriate amount of cells per well were counted and added (A375 = 750 cells, G361 = 1000 cells) to 3 or more wells in a 96-well plate and left to attach overnight. The next day, the RealTime-Glo reagents (and the SIRT3 inhibitors in the case of the drug treatments) were added to the wells. Using the BioTek Synergy H1 plate reader, luminescence was measured at 1hr, 24hrs, 48hrs, 72hrs, and/or 96hrs

Clonogenic cell survival assay

Cells were added (A375 = 250 cells, G361 = 750 cells) into 3 wells of 6-well plates after 72hrs of treatments and allowed to grow for 10-14 days. Cells were maintained under standard cell culture conditions and media was changed every 2-3 days and particularly for A375 3-TYP-treated cells, the drug was added alongside the media every 72hrs. After discrete colonies could be distinguished, cells were fixed in methanol and stained using

crystal violet (0.1% w/v, 1:1 ddH_2O:MeOH) at room temperature for 5-10min. Then, the wells were rinsed with 1X PBS three times (until background was clear) and left to air dry overnight. The following day, digital images were taken of each 6-well plate.

Tissue microarrays (TMAs)

TMA slides with from human skin tissues were purchased from US Biomax (catalog numbers: ME2082d and ME1006). Two slides were needed in order to have appropriate sample representation from benign skin tissues as well as different melanoma stages. Each TMA slide has clinical information available from each sample including sex, age, anatomic site, type of tissue (e.g., benign, malignant, metastasis), stage, as well as tumor, nodes and metastasis (TNM) grading. The ME2082d TMA consists of 192 total cores (8 normal skin tissues, 8 adjacent normal skin tissues, 112 cases of malignant melanoma, and 64 metastatic malignant melanoma) and the ME1006 TMA consists of 100 total cores (2 normal skin tissues, 14 nevus tissues, 62 cases of malignant melanoma, and 22 metastatic malignant melanoma).

TMAs staining

TMA staining was carried out in collaboration with UW Translational Research Initiatives in Pathology (TRIP) lab from the University of Wisconsin-Madison. Opal 7 color kit was used for staining of proteins of interest (i.e., p53, Acetyl-p53, p21, SIRT3, and BAX). S100A was used a melanoma marker and DAPI as nuclear staining. Each target was optimized, and appropriate conditions were used for each one (***Supplementary Table***

S2). More details regarding staining protocol can be found in **Supplementary Materials and Methods**.

TMA image acquisition (Vectra scanning) and Inform quantitative analysis

TMA slide scanning and image acquisition was carried out in collaboration with the TRIP lab via Vectra multispectral imaging system (Akoya Biosciences). Then, using the Nuance software (version 3.02; PerkinElmer) a spectral library was created and later used in the Inform software (Akoya Biosciences). This spectral library can guide the software on how to recognize different chromogens based on their unique spectral curves. Next, the Inform program (version 2.4.8) was used to create an algorithm which has many applications and can be modified depending on the goal of the project. In our case, we created an algorithm that could recognize different sections in the TMA tissue cores (e.g., tissue segments (S100 positive and S100 negative) and cell segments (i.e., nucleus and cytoplasm)). Afterwards, image analysis was carried out using the same Inform software where quantitative data was obtained for each target of interest in the individual tissue cores. Tissues that were positive to S100A were used for the quantitative analysis.

RNA isolation and quantification, cDNA preparation, and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RNA isolation was carried out using RNeasy Plus isolation kit according to manufacturer's protocols (Qiagen). RNA was then quantified for quality control (A260/280 ratio between 1.8-2.0, A260/230 ratio between 1.7-2.0). Each sample was quantified in using the BioTek Synergy H1 plate reader. cDNA was prepared (with MMLV reverse transcriptase (MMLV-

RT) and Oligo dT primers) and then used for sample cycling (QuantStudio3). RT-qPCR was conducted to measure gDNA levels , particularly with the PrimePCR DNA contamination control (Bio-Rad, catalog number: 10025352) following manufacturer's instructions. *Gapdh* and *Beta-actin* were considered endogenous controls and the delta-delta cycle threshold (ΔΔCT) method was used for analysis (results were negative for gDNA contamination). Primers used for RT-qPCR can be found in ***Supplementary Table S3***.

RT^2 Profiler PCR Array (Human Cancer PathwayFinder)

The Human Cancer PathwayFinder PCR array (catalog number: PAHS-033ZA) was carried out, following manufacturer's protocols, in order to explore the effects of CRISPR/Cas9-mediated SIRT3 KO in human melanoma cells. Particularly, 84 cancer-related genes were tested in biological and technical triplicates. The samples used were cDNA from A375 and G361 wildtype and SIRT3 CRISPR KO cells was used for the analysis. In terms of the analysis, cycle Ct values for each gene were uploaded to the Qiagen GeneGlobe Data Analysis Center and analyzed using *GAPDH* and *ACTB* as housekeeping genes. The data analysis web portal calculated fold-change using the ΔΔCT method and those genes showing ≥ 1.5-fold change and p value < 0.05 were considered significantly modulated genes.

Ingenuity Pathway Analysis (IPA)

Genes that were significantly modulated after CRISPR/Cas9-mediated SIRT3 KO Human Cancer PathwayFinder PCR Array were uploaded to the IPA software. This software was used to predict altered gene interactions, molecular pathways, and cellular functions.

NanoString Human Tumor Signaling 360 Panel Analysis

In order to create an RNA molecular profile after CRISPR/Cas9-mediated SIRT3 KO in melanoma, the nCounter Human Tumor Signaling 360 Panel (catalog number XT-CSO-H-TS360-12) was purchased from NanoString Technologies Laboratories, which consists of 780 total genes (760 tumor signaling related genes and 20 genes as internal reference controls). In collaboration with the TRIP lab, this panel was applied in the nCounter MAX system following manufacturer's protocols. Samples from isolated RNA from A375 and G361 wildtype and SIRT3 CRISPR KO cells were used in three biological and technical replicates. The expression of each of gene was normalized to endogenous genes. The ROSALIND data analysis platform from NanoString was used to carry out QC report, data normalization, and differential gene expression analysis. Additionally, the functional pathways as well as the hallmarks of cancer affected by SIRT3 inhibition in melanoma were identified. Genes with ≥ 1.5-fold change and p value < 0.05 were considered significantly modulated.

Network Analyses

In order to thoroughly study the significantly modulated tumor signaling-related genes by SIRT3 inhibition, we performed gene network analyses for both A375 and G361. We used

the STRING database (version 11.5, https://string-db.org), which predicts gene interactions based on literature evidence. Among the specifications selected to create gene network, the minimum required interaction score was set at a confidence level 0.4, inflation parameter to visualize clusters was set at 1.5 for the Markov Cluster algorithm, and network edges were defined as type of interaction between the genes. In order to visualize the gene networks, they were transferred from STRING database to Cytoscape (version 3.9.1), and final edits were done with Adobe Illustrator (2020).

Statistical Analysis

All experiments were conducted with at least three biological and three technical replicates. Statistical data analysis for RT^2 Profiler PCR Array and NanoString Panel Analysis are described in the method's description above, respectively. Data from ProteinSimple immunoassay, RealTime Glo cell viability assay, and Inform analysis was imported to GraphPad Prism software (version 9.3.1). Statistical was carried out via one-way ANOVA analysis if not specified otherwise. Exceptions include RealTime Glo data which was analyzed with 2-way ANOVA and Inform analysis where T-test was carried out. Statistical significance was considered when p value < 0.05. Experimental data is presented as mean ± standard error (SE). For expression correlation analysis in TMAs, Pearson correlation was carried out and a strong correlation was considered if R^2 value ≤ 0.7 and p value < 0.05. Experimental data is presented as individual dots with expression levels for each target of interest and slope linear regression to visualize correlation.

RESULTS AND DISCUSSION

SIRT3 protein expression increases with melanoma progression *ex vivo*

Commercially available and melanoma-relevant tissue microarrays (TMAs) were obtained from US Biomax in order to measure the protein expression of SIRT3 in skin tissues. Between the two TMA slides there almost 300 tissue cores with representative samples from benign skin tissues as well as different melanoma stages. After carrying out Inform quantitative analysis, we were able to match the data from each tissue core to its respective clinical information (i.e., tissue type and/or melanoma stage). We observed a steady increase of SIRT3 protein expression in both early and advanced melanoma stages when compared to benign tissues (***Figure 2***). This validates our previous findings that SIRT3 is overexpressed in melanoma tissues [6], and here particularly, we also noticed a stage-dependent increase of SIRT3 levels.

SIRT3 inhibition decreases melanoma cell viability and survival

In this study as well as previous publications, it has been shown that SIRT3 is overexpressed in melanoma. Additionally, our lab has demonstrated that decreasing SIRT3 levels via shRNA knockdown, leads to a decrease in cell proliferation both *in vitro* and *in vivo* [6]. To validate the effects of SIRT3 inhibition in melanoma cells, we employed novel pharmacological and genetic approaches. Specifically, for both A375 and G361 cells, we used a SIRT3-specific inhibitor (3-TYP) and generated CRISPR/Cas9-mediated SIRT3 KO single clones. Regarding the latter, we achieved significant SIRT3 protein KO in both A375 as well as G361 CRISPR KO clones (***Figure 3***). As 3-TYP inhibits SIRT3 activity rather than expression [9], we did not observe significant differences of SIRT3

protein levels in any of the doses tested, which was expected (***Supplementary Figure S1a***). First, SIRT3 inhibition via 3-TYP treatment, resulted in significant decrease of cell viability as shown by the RealTime Glo assay (***Figures 4a and 4c***). Additionally, ***Figures 4b and 4d***, present a reduction in colony formation, suggesting SIRT3 modulation can decrease clonogenic survival of melanoma cells. Overall, specific inhibition of SIRT3 activity by 3-TYP results in an antiproliferative response in melanoma cells. Interestingly, G361 cells were more responsive to 3-TYP treatment than A375 cells (***Figure 4***). Nonetheless, some caveats include that 3-TYP treatment required extended treatment time periods, applying continuous treatment, and/or using higher doses of the compound (data not shown). Furthermore, CRISPR/Cas9-mediated SIRT3 KO led to an antiproliferative response in both cell lines in all the time points measured. This can be noticed for both RealTime Glo cell viability assay and clonogenic survival assay (***Figure 5***). Altogether, these data support our previous findings, where it is suggested that SIRT3 may have tumor promoting roles in melanoma and that SIRT3 knockdown can lead to an antiproliferative response [6]. However, since high doses and continuous treatments of 3-TYP were needed to achieve significant antimelanoma effect, and due to potential loss of 3-TYP response after cell collection, we decided to continue to assess the effects of SIRT3 inhibition via CRISPR/Cas9.

SIRT3 protein expression is not correlated with levels of Ku70, Bax, p21, nor p53

One of the main goals of this study was to determine the mechanism of SIRT3 in melanoma cells and tissues. For this reason, in both melanoma tissues and cells, SIRT3

protein levels were quantified and then evaluated if in melanoma, the expression of this sirtuin correlates with other potential interactors. Particularly, Ku70, BAX, p53, acetyl-p53, and/or p21 were tested initially due to some studies connecting them with SIRT3. For example, SIRT3 can protect cells by deacetylating Ku70 which then binds to BAX (proapoptotic protein), and consequently, promote cell proliferation and prevent cell death [10-12]. Additionally, SIRT3 and BAX have been localized either permanently or temporarily in the mitochondria [13, 14]. In several types of cancer, it has been shown that SIRT3 may suppress or activate p53, and interestingly in melanoma, it was found that mutated p53 can upregulate SIRT3 expression [12, 15]. Furthermore, increased p21 levels (senescence marker) were observed after SIRT3 knockdown [6].

Taking this into consideration, we measured the protein expression of these targets in melanoma-relevant TMAs using Inform quantitative analysis. Even though SIRT3 was overexpressed in melanoma tissues compared to benign counterparts (***Figure 2***), there was no strong correlation detected between protein levels of this sirtuin and BAX, p21, p53, nor acetylated-p53 (***Figure 6a-d***). Furthermore, the effect on the potential SIRT3 interactors was assessed after CRISPR/Cas9-mediated SIRT3 inhibition. Comparatively, SIRT3 KO did not significantly affect the protein expression levels of Ku70, BAX, p53, nor p21 (***Figure 6e-h***). Therefore, the mechanism of SIRT3 in melanoma might be related to other cellular pathways not yet explored.

SIRT3 CRISPR KO affects genes and molecular pathways associated to carcinogenesis

To further elucidate the mechanism of SIRT3 in melanoma, we carried out multiple molecular profiling techniques. Specifically, we explored the differentially expressed genes between wildtype and CRISPR/Cas9 SIRT3 KO clones in both A375 and G361 melanoma cells.

First, we selected the RT^2 Profiler PCR Array, specifically the Human Cancer PathwayFinder with which we were able to analyze the expression of 84 cancer-related genes involved in different cellular processes (i.e., angiogenesis, apoptosis, cell cycle, cellular senescence, DNA damage and repair, epithelial-to-mesenchymal transition (EMT), hypoxia signaling, metabolism, and telomeres and telomerase). In A375 cells, 18 genes were significantly modulated (16 genes were downregulated and 2 upregulated), while for G361 there were 9 genes affected (3 genes were downregulated and 6 upregulated) (***Supplementary Table S4***). Using the Ingenuity Pathway Analysis (IPA) software, we were able to create gene networks with the genes that had the most interactions and presented them together with potential up- and downstream targets (***Figure 7a and 7c***). Regarding genes that were exclusively modulated in A375 melanoma cells, two genes were upregulated (*Snai2* and *Tbx2*), but their evidence in melanoma is related to supporting cell migration and proliferation, respectively [16, 17]. On the other hand, *Adm*, *Angpt2*, *Hmox1*, *Kdr*, *Mapk14*, *Mcm2*, *Pgf*, and *Skp2* were melanoma-relevant genes which were downregulated after SIRT3 inhibition in A375 cells. Most of these genes are overexpressed in melanoma, promote melanoma progression, and/or are associated with poor melanoma prognosis [18-29]. However, *Mapk14* has potential

tumor suppressive roles in melanoma [30]. Furthermore, we could not find melanoma studies where *Ppp1r15a*, *Casp2*, and *Tnks2* were explored, but we showed their downregulation after SIRT3 KO. These genes have antiproliferative as well as pro-apoptotic and -senescent properties in cancer [31-33]. Altogether, genes that were significantly modulated after CRISPR/Cas9-mediated SIRT3 KO in A375 cells were shown to mostly work together and inhibit cancer-related pathways including cell proliferation, tumor growth and invasion, and metastasis (***Figure 7b***).

In the case of G361 melanoma cells, the differentially expressed genes that were solely affected include *Flt1*, *Serpinf1*, *Lpl*, and *Snai1*. The first pair of genes were upregulated after SIRT3 inhibition are overexpressed in melanoma and associated with supporting resistance to cancer therapeutics, respectively [22, 23, 34]. *Lpl* has been suggested to have both tumor promoting and tumor suppressive roles, while *Snai1* has carcinogenic functions like promoting cell survival and invasion [35-37]. As shown in ***Figure 7d***, IPA analysis predicted that cancer-related pathways like tumor growth and metastasis were activated by the genes that were significantly modulated after CRISPR/Cas9-mediated SIRT3 KO in G361 cells. Regarding genes that were significantly modulated in A375 and G361 cells, only one gene was affected similarly in both cell lines, *Slc2a1*, which was downregulated after SIRT3 KO and is associated with worse prognosis when it is overexpressed in cancer patients [38]. In the case of *Ccl2*, *Dsp*, *Fgf2*, and *Vegfc* they are either overexpressed in melanoma or support melanoma progression [39-42]. For the last genes mentioned, they were downregulated in A375 SIRT3 KO cells while being upregulated in G361 SIRT3 KO cells.

Second, NanoString Human Tumor Signaling 360 Panel Analysis was carried out, where we were able to evaluate the effects of SIRT3 inhibition in a more extensive list consisting of 760 genes involved in tumor signaling and even hallmarks of cancer (i.e., activating invasion and metastasis, sustaining proliferative signaling, tumor-promoting inflammation, inducing angiogenesis, deregulating cellular energetics, avoiding immune destruction, evading growth suppressors, genome instability & mutation, and resisting cell death). In order to analyze the results obtained from NanoString, data was uploaded to the ROSALIND online platform (also part of NanoString) where we evaluated expression levels from the 760 genes in SIRT3 KO cells compared to expression levels in wildtype cells. As shown in ***Supplementary Table S5***, 38 genes were significantly modulated (11 genes were downregulated and 27 upregulated) in A375 cells. On the other hand, G361 cells had 10 differentially expressed genes (2 genes were downregulated and 9 upregulated) (***Supplementary Table S6***). Networks with the differentially expressed genes in A375 and G361 cells were created using STRING network, Cytoscape, as well as Illustrator, and are grouped by some commonalities in terms of cancer hallmarks (***Figure 8***). Comparative to the PCR array data, the trend observed from the significantly modulated genes in NanoString, had both tumor promoting and tumor suppressing implications.

For example, in A375 cells, *Bcl2l1*, *Cav1*, *Cdc25a*, *Cdca5*, *Il7r*, *Mcm2*, *Sdc1*, *Slc7a5*, *Tgm2*, and *Thbs1* were downregulated after SIRT3 KO and all present oncogenic-like properties in melanoma including being overexpressed, promoting metastasis, antiproliferative effects after they are inhibiting, among others [25, 43-52]. Similarly, *Ska1* was downregulated in SIRT3 CRISPR KO cells and is associated with

promoting malignant cell proliferation and migration [53]. Conversely, *Arid4a*, *Bhlhe40*, and *Pla2g4a* were upregulated and in other malignancies have been shown to decrease tumor progression, active immune responses against cancer cells, and improve survival in patients [54-56]. Within the genes that were upregulated after SIRT3 inhibition in A375 cells and have anti-melanoma effects, we can find *Dusp6*, *Fos*, *Gsk3b*, *Hla-A*, *Hla-B*, *Hla-C*, *Icam*, *Itga1*, and *Tlr4* [57-64]. In the case of *Ifnar2*, one of two subunits of type 1 interferon (IFN) receptor, was also upregulated in our NanoString analysis, but to our knowledge there are no publications found regarding this gene in the melanoma field. Instead, there were a couple of studies done with the other subunit, *Ifnar1*, mentioning its antitumorigenic activities against melanoma, how the interaction between IFN receptor and IFN can target melanoma cells, and that in fact, IFN is used as a potent melanoma treatment [65, 66]. Notwithstanding, *Atox1*, *Bcl2a1*, *Cdcp1*, *Col4a1*, *Ctla4*, *Cxxc5*, *Erbb3*, *Flt1*, *Itpr1*, *Lama4*, *P4ha2*, *Pfkfb3*, *Pik3r2*, and *Snai2* have evidence of supporting melanoma initiation, progression, and/or metastasis, but they were found to be upregulated in A375 SIRT3 KO cells [22, 67-81]. Connections between the differentially expressed genes in A375 cells are presented in ***Figure 8a***.

There were less genes found to be significantly modulated after CRISPR/Cas9-mediated SIRT3 KO in G361 melanoma cells and similar to the results in the PCR array, most of these genes were upregulated (***Figure 8b***). For instance, *Il7*, *Ldha*, *Oas1*, and *Ugdh* expression levels were increased, and these genes do not support melanoma progression [82-85]. *Epas1* was also found upregulated in G361 SIRT3 KO cells and is associated with improved prognosis in other cancers [86]. Genes that were upregulated but show conflicting evidence in melanoma include *Cd44*, *Kdr*, and *Tpd52* which are

respectively, associated with poor survival rates, downregulation after melanoma treatment, and promoting melanoma cell proliferation [21, 87, 88]. *Ash1l* and *Nuf2* were the only two downregulated genes found in G361 cells and have antiproliferative effects when downregulated, and particularly, Nuf2 is recognized as an unfavorable prognostic marker in melanoma [89-93].

Altogether, molecular profiling of A375 and G361 cells after CRISPR/Cas9-mediated SIRT3 KO, identified potential SIRT3 interactors in melanoma. However, there were discrepancies between the tested cell lines. Additionally, there was conflicting findings between the antiproliferative effects observed in these melanoma cells (***Figure 5***) and the melanoma tumor promoting literature-based evidence that some of these genes were associated with (***Figures 7 and 8***). Therefore, further validation studies should be carried out as well as incorporating other cellular pathways (e.g., ROS production, apoptosis, EMT, etc.).

CONCLUSIONS

Our data demonstrated a stage-dependent increase of SIRT3 protein expression in melanoma and showed that SIRT3 inhibition by 3-TYP as well as CRISPR/Cas9-mediated SIRT3 KO resulted in antiproliferative effects in these cells (reduced cell viability and clonogenic survival). However, in melanoma cells and tissues, we could not find correlations between SIRT3 expression levels and Ku70 BAX, p21, p53, nor acetylated-p53. Consequently, we carried out two molecular profiling techniques (PCR Array and NanoString analysis), where we assessed the effects of CRISPR/Cas9-mediated SIRT3 KO in hundreds of genes involved in tumor signaling as well as cancer-related pathways. Interestingly, we saw marked differences between the two cell lines in terms of the genes that were significantly modulated, and in some cases, the fold change of the same gene was in opposite direction for the cell lines evaluated. Although a greater part of the differentially expressed genes had supporting evidence of the antiproliferative effects observed after SIRT3 KO, there were some genes that the literature suggested the opposite. Therefore, we were able to identify potential SIRT3 interactors in melanoma cells, but these need to be further validated by techniques like qPCR or immunoblotting. Additionally, other melanoma cell lines can be evaluated as well as other cellular pathways. Taking this into consideration, SIRT3 seems to support melanoma progression, but simultaneously inhibiting this sirtuins together with other tumor promoting proteins might lead to stronger antimelanoma effects.

Our lab has carried out other studies where they have shown that 4'-bromo-resveratrol (4'-BR), which inhibits SIRT3 and SIRT1 activity, can block melanoma cell and tumor growth [7]. Therefore, we decided to use this compound to treat our same A375

and G361 cells. We also measured the effects SIRT3 expression after 4'-BR treatment in these cell lines, where we found no significant differences in SIRT3 protein levels (***Supplementary Figure S1b***). Furthermore, we were able to validate our previous results showing that 4'-BR exerts antiproliferative effects in melanoma cells (***Supplementary Figure S2***). Interestingly, these effects were observed in less time and at lower doses than 3-TYP for both cell lines, suggesting that 4'-BR could be more potent than 3-TYP. Consequently, we will be using 4'-BR for further analyses and even in melanoma-relevant *in vivo* models.

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FIGURES

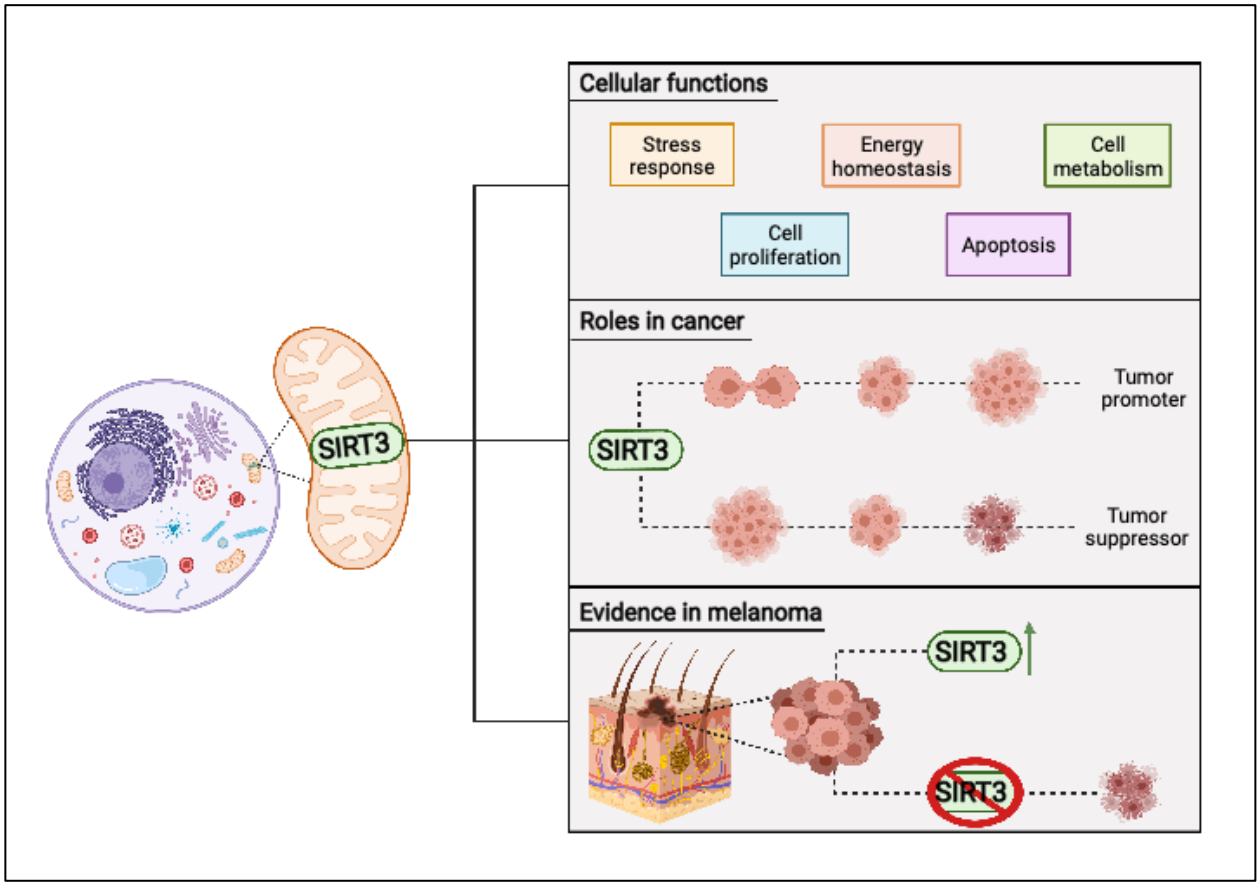


Figure 1. Mitochondrial sirtuin 3 (SIRT3) and its cellular functions, dual roles in carcinogenesis, and current evidence in melanoma.

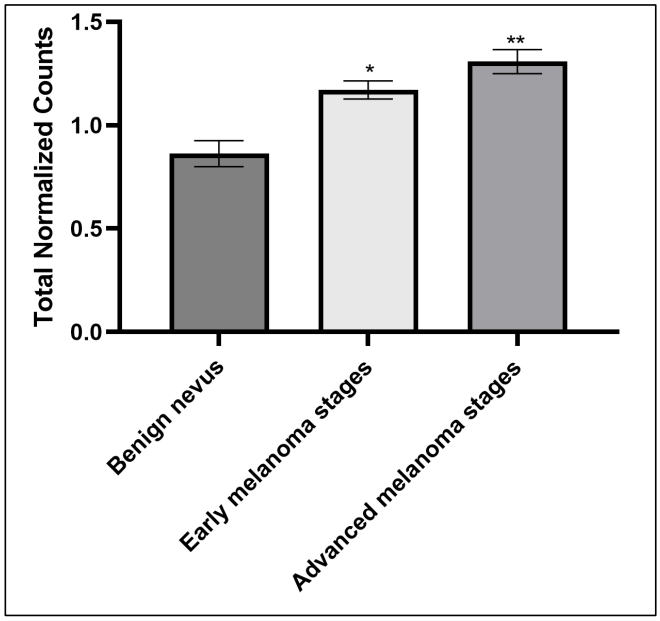


Figure 2. SIRT3 protein expression significantly increases with melanoma progression. TMAs with benign skin tissues and different melanoma stages were subjected to SIRT3 immunodetection followed by Vectra scanning and Inform analysis. Expression levels from melanoma tissues were compared to benign tissues. Between the two TMA slides there were almost 300 tissue cores analyzed. Data was normalized to pixel area of tissue core and is presented as the average of total counts for each group (which represent protein expression levels) ± SE. Statistical significance was determined by T-TEST using GraphPad Prism software (version 9.3.1) and asterisks imply p value < 0.05.

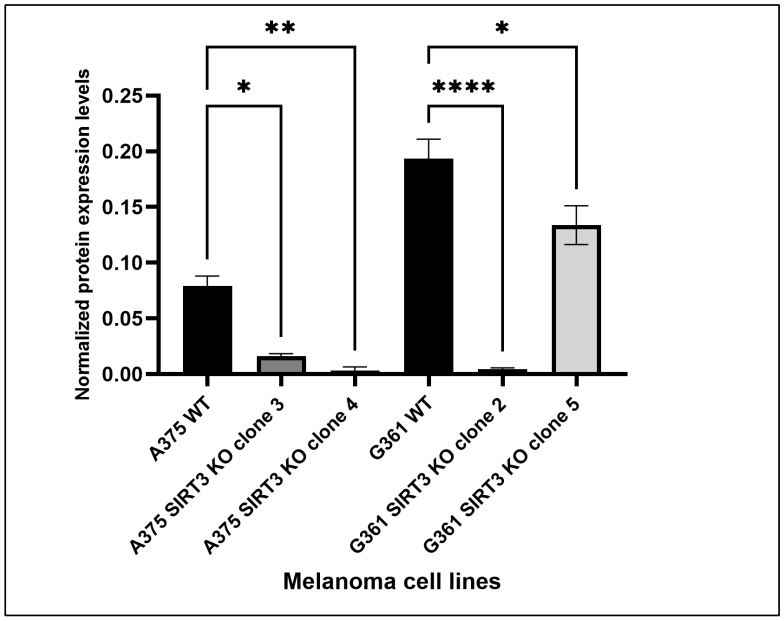


Figure 3. SIRT3 protein expression levels are significant reduced in A375 and G361 CRISPR/Cas9-mediated SIRT3 KO clones. Quantitative immunodetection via ProteinSimple Jess was carried out with triplicates of each sample. Lysates were added with equal amounts of protein concentration. Data was normalized with the ProteinSimple Total Protein Assay and is presented as mean protein expression levels ± SE. Statistical significance was determined by one-way ANOVA using GraphPad Prism software (version 9.3.1) and asterisks imply p value < 0.05.

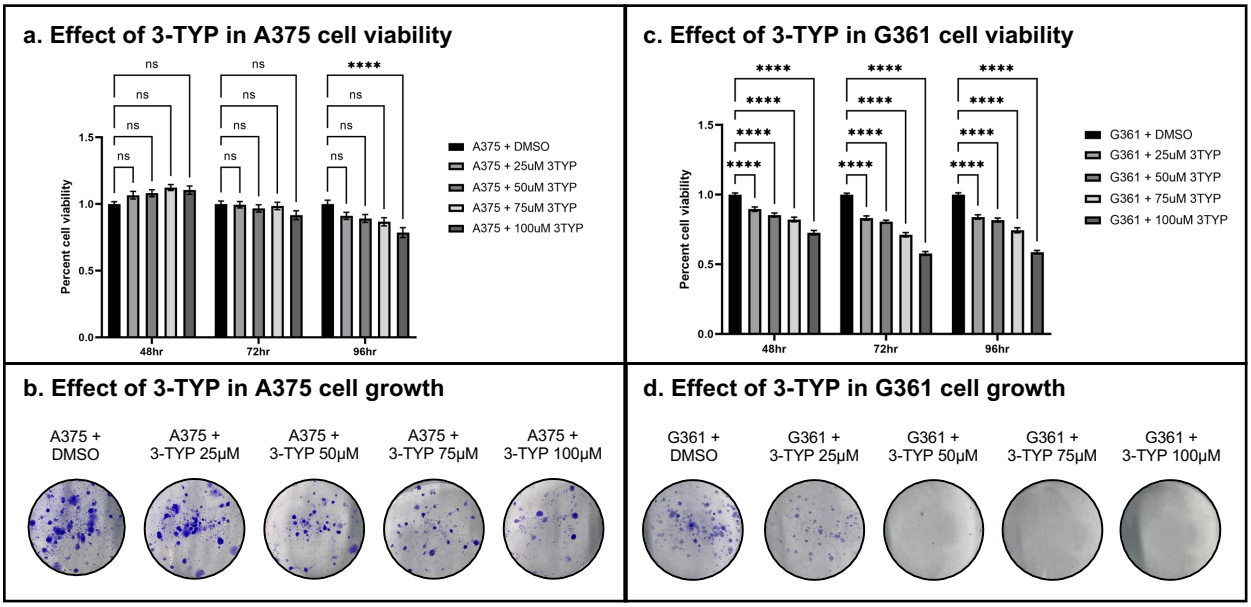


Figure 4. 3-TYP reduced cell viability and growth in human melanoma cells. (a) RealTime Glo cell viability assay in 3-TYP treated A375 wildtype cells, 48-96hrs after drug treatment. (b) Clonogenic survival assay in 3-TYP treated A375 wildtype cells, after continuously adding drug treatment every 72hrs and growing cells for 10-14 days. (c) RealTime Glo cell viability assay in 3-TYP treated G361 wildtype cells, 48-96hrs after drug treatment. (d) Clonogenic survival assay in 3-TYP treated G361 wildtype cells, after continuously adding drug treatment every 72hrs and growing cells for 10-14 days. Triplicates of each sample were tested for both cellular analyses. For RealTime Glo, data is normalized to 1hr baseline reading and presented as mean values ± SE. Statistical significance was determined by 2-way ANOVA using GraphPad Prism software (version 9.3.1) and asterisks imply p value < 0.05. For clonogenic survival assay, representative images of each treatment group are used.

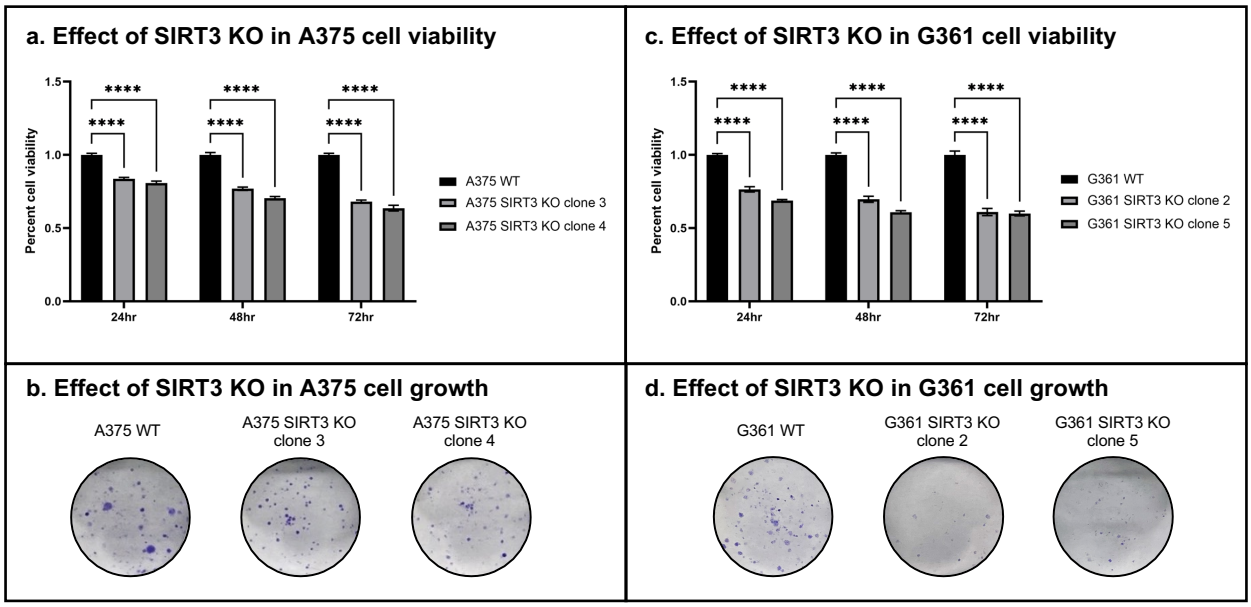
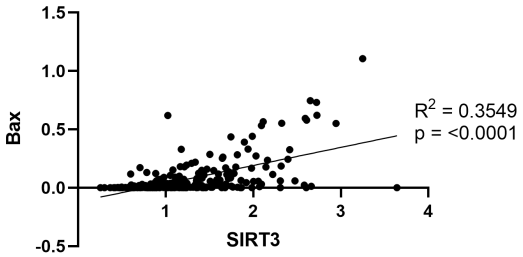
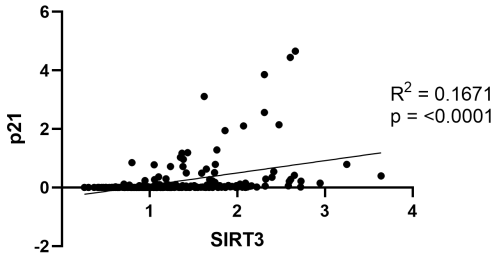


Figure 5. CRISPR/Cas9-mediated SIRT3 KO reduced cell viability and growth in human melanoma cells. (a) RealTime Glo cell viability assay in A375 WT and 2 clones with CRISPR/Cas9-mediated SIRT3 KO, 24-72hrs after seeding cells. (b) Clonogenic survival assay in A375 WT and 2 clones with CRISPR/Cas9-mediated SIRT3 KO, 10-14 days after seeding cells. (a) RealTime Glo cell viability assay in G361 WT and 2 clones with CRISPR/Cas9-mediated SIRT3 KO, 24-72hrs after seeding cells. (b) Clonogenic survival assay in G361 WT and 2 clones with CRISPR/Cas9-mediated SIRT3 KO 10-14 days after seeding cells. Triplicates of each sample were tested for both cellular analyses. For RealTime Glo, data is normalized to 1hr baseline reading and presented as mean values ± SE. Statistical significance was determined by 2-way ANOVA using GraphPad Prism software (version 9.3.1) and asterisks imply p value < 0.05. For clonogenic survival assay, representative images of each cell group are used.

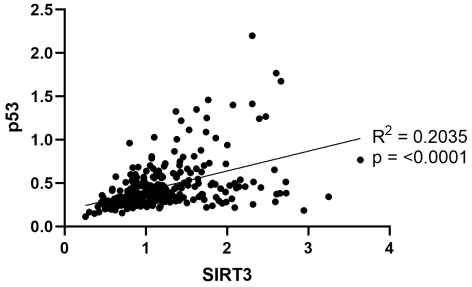
a. Expression correlation between SIRT3 and BAX in benign skin and melanoma tissues



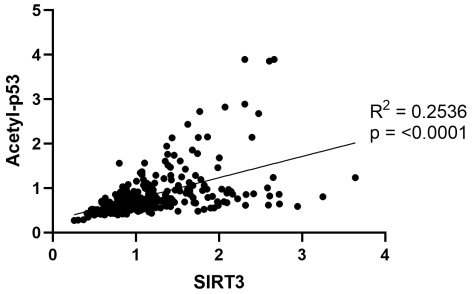
b. Expression correlation between SIRT3 and p21 in benign skin and melanoma tissues



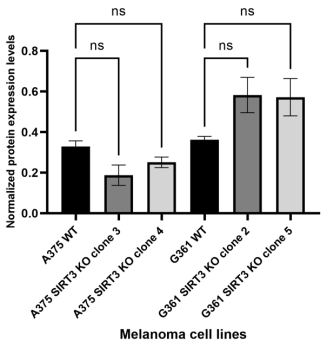
c. Expression correlation between SIRT3 and p53 in benign skin and melanoma tissues



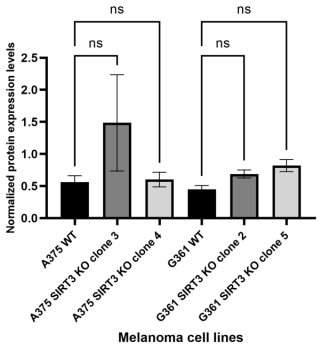
d. Expression correlation between SIRT3 and acetylated-p53 in benign skin and melanoma tissues



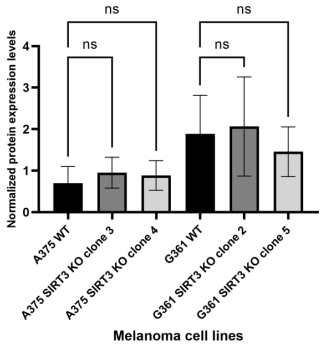
e. Effect of SIRT3 KO in BAX protein expression



f. Effect of SIRT3 KO in p21 protein expression



g. Effect of SIRT3 KO in p53 protein expression



h. Effect of SIRT3 KO in Ku70 protein expression

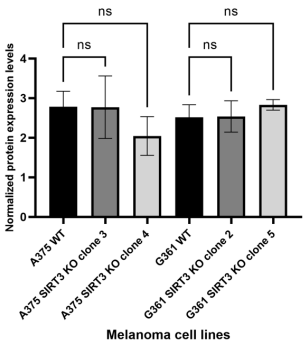
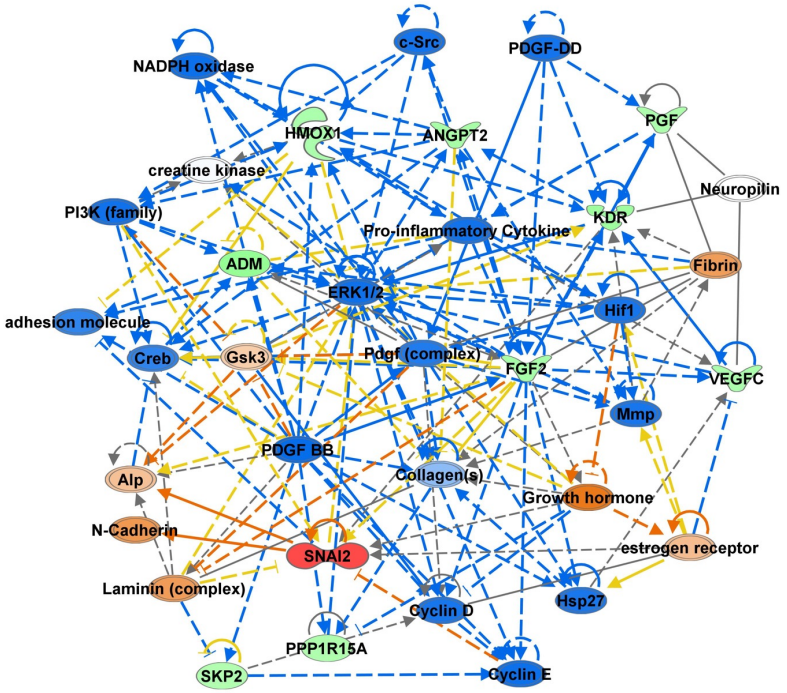
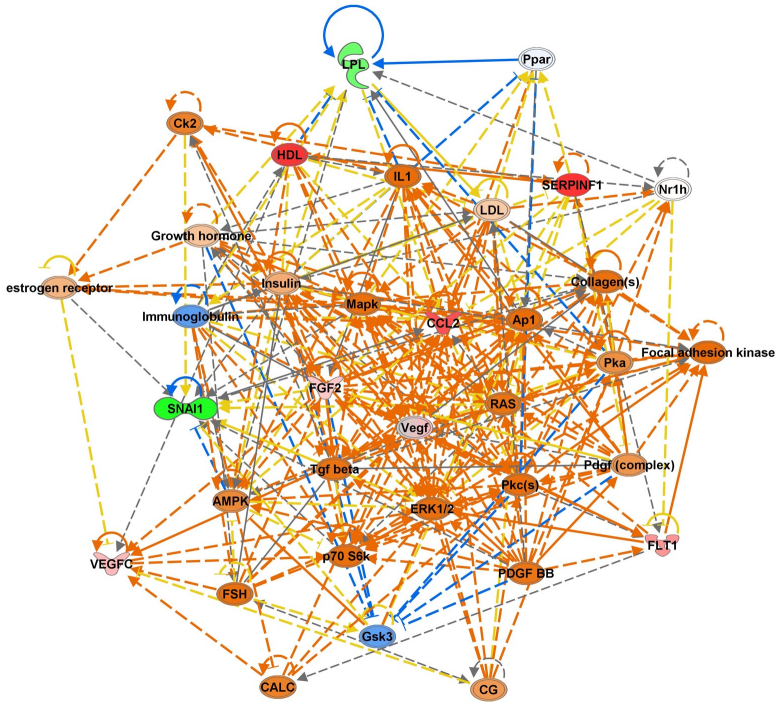


Figure 6. SIRT3 expression, at both tissue and cell levels, is not correlated with protein expression of BAX, p21, p53, acetylated-p53, nor Ku70. Correlation between SIRT3 and (a) BAX, (b) p21, (c) p53, and (d) acetylated-p53 protein expression in benign skin and melanoma. Effect of CRISPR/Cas9-mediated SIRT3 KO in (e) BAX, (f) p21, (g) p53, and (h) Ku70 protein expression. For tissue data, TMAs with benign skin tissues and different melanoma stages were subjected to multiplex immunodetection of SIRT3, BAX, p53, acetylated-p53, and p21, followed by Vectra scanning and Inform analysis. Between the two TMA slides there were almost 300 tissue cores analyzed. Data was normalized to pixel area of tissue core and is presented as individual points of total normalized counts for each protein of interest. Statistical significance was determined using Pearson correlation analysis. Strong correlation was considered when $R^2 > 0.7$ and p value < 0.05. For cell data, Quantitative immunodetection via ProteinSimple Jess was carried out with triplicates of each sample. Lysates were added with equal amounts of protein concentration. Data was normalized with the ProteinSimple Total Protein Assay and is presented as mean protein expression levels ± SE. Statistical significance was determined by one-way ANOVA using GraphPad Prism software (version 9.3.1) and asterisks imply p value < 0.05.

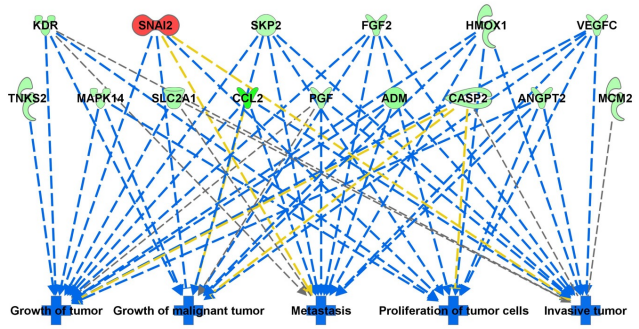
a. Molecular targets of significantly modulated genes in A375 SIRT3 KO cells



c. Molecular targets of significantly modulated genes in G361 SIRT3 KO cells



b. Cancer pathways associated with differentially expressed genes in A375 SIRT3 KO cells



d. Cancer pathways associated with differentially expressed genes in G361 SIRT3 KO cells

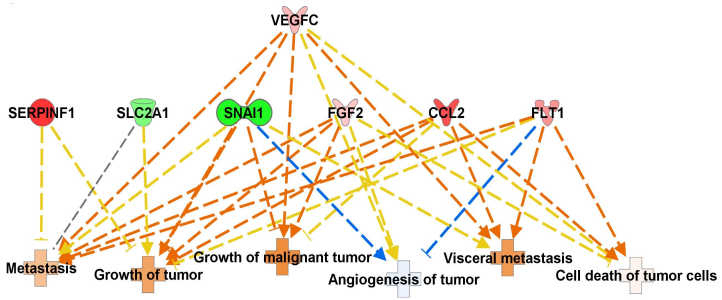


Figure 7. Effect of CRISPR/Cas9-mediated SIRT3 KO in cancer-associated genes and pathways in A375 and G361 human melanoma cells. This was evaluated using the Human Cancer PathwayFinder PCR Array followed by IPA analysis as described in Materials and Methods. Green and red icons correspond to significantly modulated genes from PCR array that were downregulated or upregulated, respectively. Blue symbols (ovals, crosses, and lines) represent downregulated genes or pathways which were predicted by IPA software as direct targets of the significantly modulated genes. Orange symbols are predicted as being upregulated. Yellow lines indicate inconsistent findings (evidence in the literature found in IPA vs results obtained after PCR array) and gray lines represent unpredicted effects. (a) A375 differentially expressed genes identified in PCR array and their up- and downstream molecular targets. (b) A375 differentially expressed genes identified in PCR array and their up- and downstream cancer pathways. (c) G361 differentially expressed genes identified in PCR array and their up- and downstream molecular targets. (d) G361 differentially expressed genes identified in PCR array and their up- and downstream cancer pathways. Fold change was calculated using ΔΔCT method. Significantly modulated genes were considered when p value < 0.05 and fold change was ≥ 1.4 in SIRT3 KO cells compared to wildtype cells.

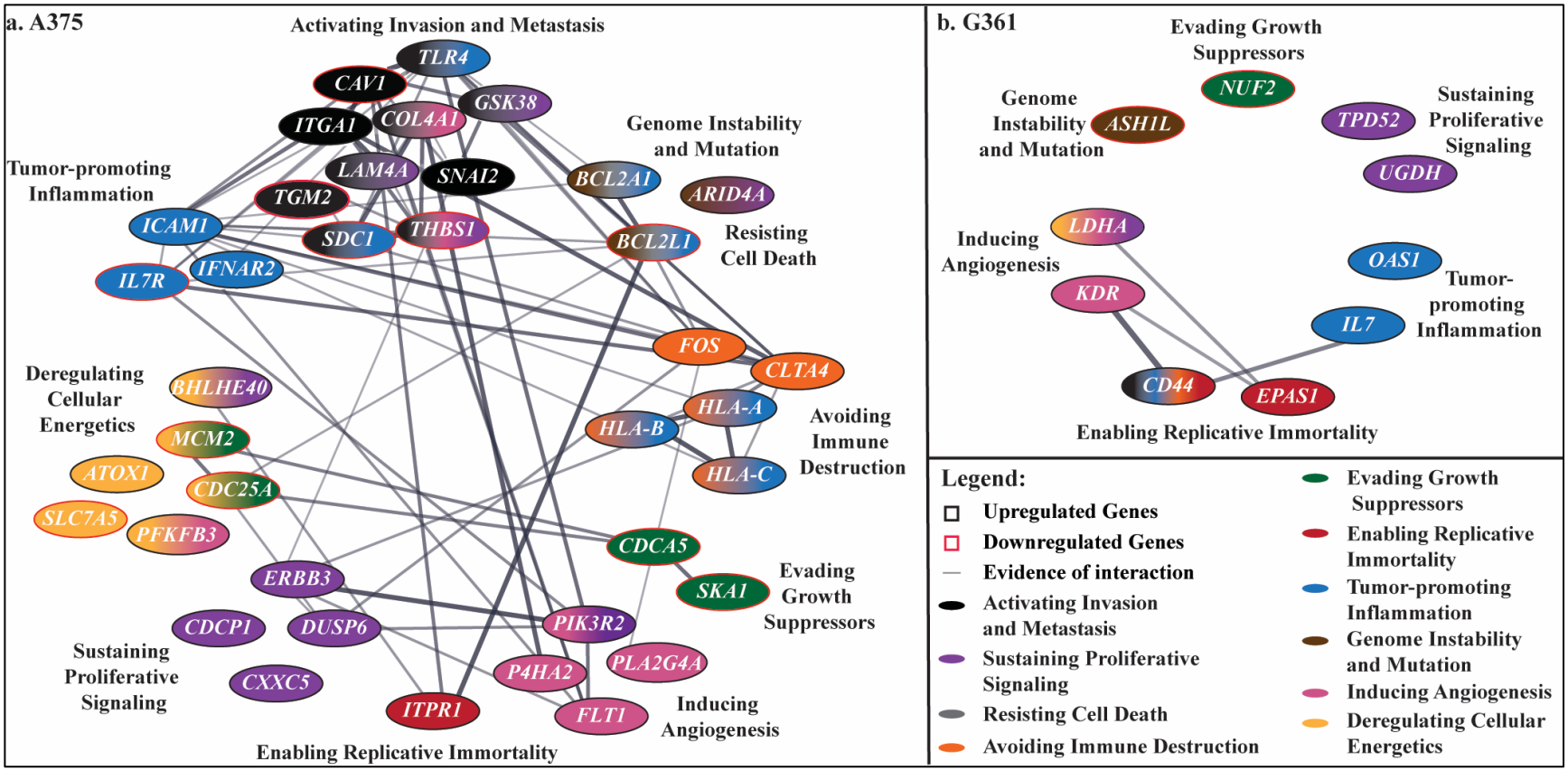


Figure 8. Effect of CRISPR/Cas9-mediated SIRT3 KO in tumor signaling genes in A375 and G361 human melanoma cells. This was evaluated using the NanoString Human Tumor Signaling 360 Panel Analysis and data was imported into STRING network, Cytoscape, and Illustrator as described in Materials and Methods. Genes are grouped by the common hallmarks of cancer they are associated with in NanoString. Hallmarks of cancer include activating invasion and metastasis (black),

sustaining proliferative signaling (purple), resisting cell death (grey), avoiding immune destruction (orange), evading growth suppressors (green), enabling replicative immortality (maroon), tumor-promoting inflammation (blue), genome instability & mutation (brown), inducing angiogenesis (pink), and deregulating cellular energetics (yellow). Upregulated genes have black outline while downregulated genes have red outline. (a) A375 differentially expressed genes identified in NanoString analysis and their interactions (grey lines). (b) G361 differentially expressed genes identified in NanoString analysis and their interactions (grey lines). Significantly modulated genes were considered when p value < 0.05 and fold change was ≥ 1.5 in SIRT3 KO cells compared to wildtype cells.

SUPPLEMENTARY MATERIALS

Supplementary Materials and Methods

TMAs staining

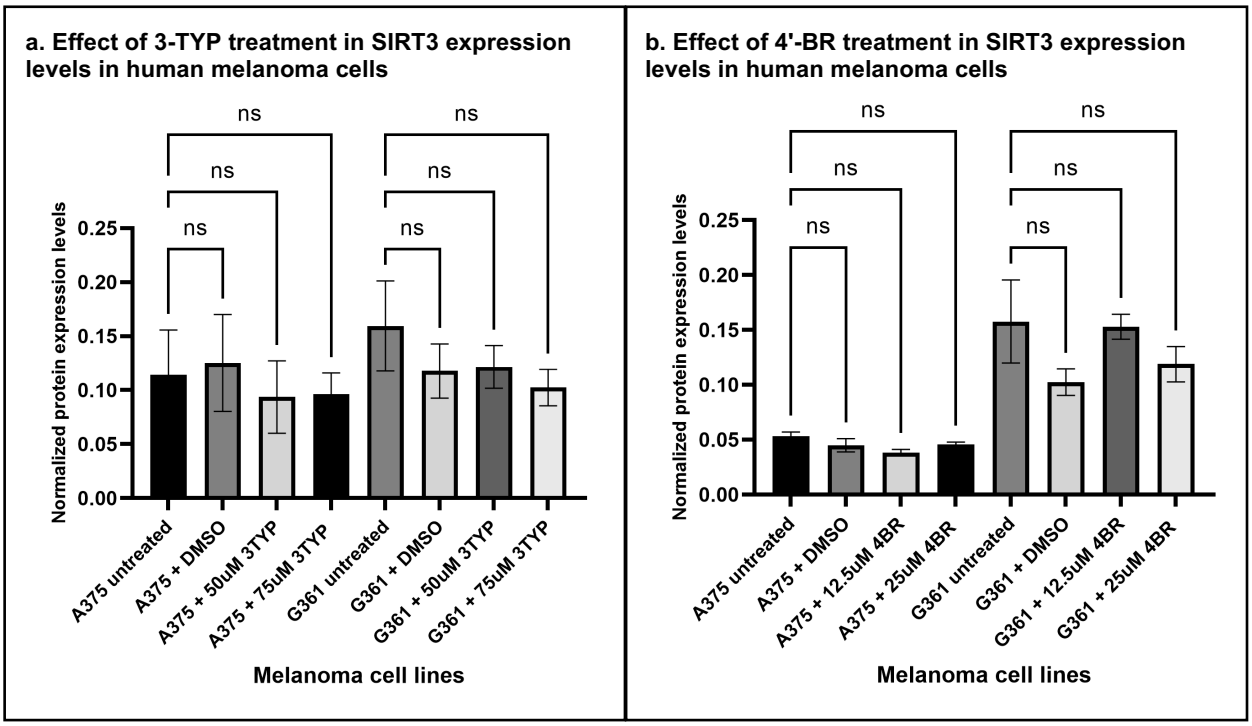
Sections were deparaffinized at 60˚C for 25min and then hydrated by following this order: 1) xylene two incubations for 10min each, 2) 100% EtOH two incubations for 5min each, 3) 95% EtOH one incubation for 5min, 4) ddH2O one incubation for 2min, and 5) TBST one incubation for 2min. Afterwards, the steps presented below were carried out (6 total cycles):

Step 1: Antigen Retrieval/Microwave treatment	Rinsed slides with AR9 solution. Placed slides in AR jar with AR9 solution and filled up to the top. Performed MWT under optimized conditions as determined previously and allowed slides to cool down to RT on the bench for at least 15 min.
Step 2: Blocking	Rinsed slides with 1X TBST. Encircled the area of tissue to be stained with a hydrophobic barrier pen. Incubated the tissue with antibody diluent for 10 min at RT.
Step 3: Primary Antibody Incubation	Removed the blocking solution and then applied the antibody solution to the tissue. Then, washed slides with 1X TBST three times for 2min each.
Step 4: Secondary Antibody Incubation	Applied Opal Polymer HRP secondary antibody solution to the tissue and incubated for 10min at RT. Washed slides with 1X TBST three times for 2min each.

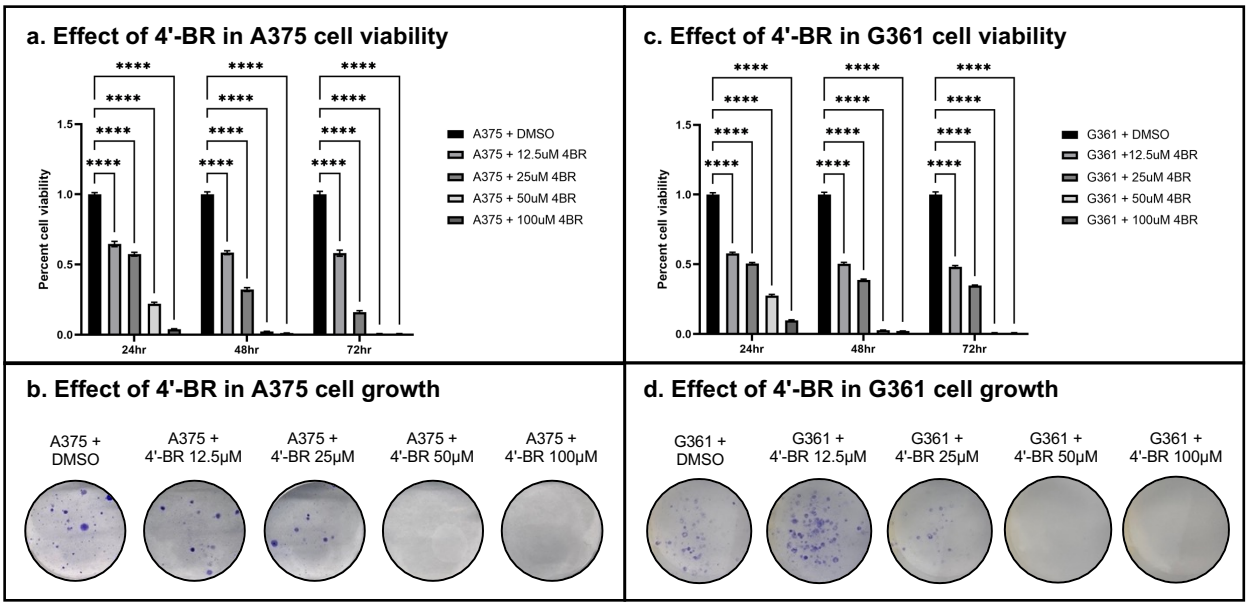
Step 5: Opal Fluorophore Incubation	Applied Opal dye diluted 1:100 in 1X amplification diluent and incubated for 10min at RT. Then, washed slides with 1X TBST three times for 2min each.
Step 6: Microwave Treatment	Rinsed slides with AR9. Placed slides in AR jar with AR9 solution and filled up to the top. Performed MWT as previously and allowed slides to cool down to room temperature on the bench for at least 15 minutes.
Step 7: 6 cycles	Carried out steps 2-6 for each protein of interest following appropriate antibody dilutions and incubation conditions. See ***Supplementary Table S2***.
Step 8: DAPI	Rinsed slides in distilled water and then in TBS. Incubated slides in DAPI solution for 10min at WT. Washed slides with TBS for 2min, and then with distilled water for 2min.
Step 9: Mount	Applied mounting medium for fluorescence microscopy and coverslip.

Abbreviations: MWT = microwave treatment, RT = room temperature, TBST = Tris-Buffered Saline with 0.1% Tween, HRP = horseradish peroxidase

Supplementary Figures



Supplementary Figure S1. Pharmacological treatment of SIRT3 inhibitors does not affect SIRT3 protein expression levels. Quantitative immunodetection via ProteinSimple Jess was carried out with triplicates of each sample. Lysates were added with equal amounts of protein concentration. Data was normalized with the ProteinSimple Total Protein Assay and is presented as mean protein expression levels ± SE. Statistical significance was determined by one-way ANOVA using GraphPad Prism software (version 9.3.1) and asterisks imply p value < 0.05.



Supplementary Figure S2. 4'-BR reduced cell viability and growth in human melanoma cells. (a) RealTime Glo cell viability assay in 4'-BR treated A375 wildtype cells, 24-72hrs after drug treatment. (b) Clonogenic survival assay in 4'-BR treated A375 wildtype cells, after adding a single drug treatment at 72hrs and growing cells for 10-14 days. (c) RealTime Glo cell viability assay in 4'-BR treated G361 wildtype cells, 24-72hrs after drug treatment. (d) Clonogenic survival assay in 4'-BR treated G361 wildtype cells, after adding a single drug treatment at 72hrs and growing cells for 10-14 days. Triplicates of each sample were tested for both cellular analyses. For RealTime Glo, data is normalized to 1hr baseline reading and presented as mean values ± SE. Statistical significance was determined by 2-way ANOVA using GraphPad Prism software (version 9.3.1) and asterisks imply p value < 0.05. For clonogenic survival assay, representative images of each treatment group are used.

Supplementary Tables

Supplementary Table S1. Lysate and antibody conditions used for ProteinSimple in a 12-230kDa Microplate.

Protein	Antibody supplier	Catalog no.	Host	Antibody dilution	Sample type	Lysate concentration
SIRT3	Cell Signaling	2627	Rabbit	1:100	Human cells	0.5µg/µL
Ku70	Santa Cruz	sc-5309	Mouse	1:250	Human cells	0.045µg/µL
Bax	Cell Signaling	2772	Rabbit	1:50	Human cells	0.25µg/µL
p53	Invitrogen	AHO0152	Mouse	1:50	Human cells	0.5µg/µL
MDM2	Invitrogen	MA1-113	Mouse	1:250	Human cells	0.95µg/µL
p21	Cell Signaling	2947	Rabbit	1:250	Human cells	0.4µg/µL

Supplementary Table S2. Antibody conditions used for immunofluorescence in tissue microarrays.

Protein	Antibody supplier	Catalog no.	Antibody dilution	Incubation	Opal dye	Opal dye dilution
p53	Invitrogen	AHO0152	1:2000	15min at RT	520	1:100
Acetyl-p53 (K382)	R&D Systems	MAB13552	1:500	30min at RT	540	1:100
S100	Cell Signaling	13018	1:1500	15min at RT	570	1:100
p21	Cell Signaling	2947	1:1000	30min at RT	620	1:100
SIRT3	Cell Signaling	2627	1:100	30min at RT	650	1:100
BAX	Abcam	ab81083	1:500	30min at RT	690	1:100

Abbreviations: RT = room temperature

Supplementary Table S3. Primer sequences for RT-qPCR.

Gene	Amplicon size (bp)	Primer orientation	Primer sequence 5′ -> 3′	Primer length (bp)	Tm (°C)	Primer Bank ID
Gapdh	101	Forward	TCCTCTGACTTCAACAGCGACAC	23	58.9	378404907c3
		Reverse	CACCCTGTTGCTGTAGCCAAATTC	24	58.6	
Beta-actin	135	Forward	CACCATTGGCAATGAGCGGTTC	22	59.3	Origene (HP204660)
		Reverse	AGGTCTTTGCGGATGTCCACGT	22	61.1	

Supplementary Table S4. Human Cancer PathwayFinder PCR Array significantly modulated genes after CRISPR/Cas9-mediated SIRT3 KO in human melanoma cells.

Gene	Description	Molecular functions	A375		G361	
			Fold change	p value	Fold change	p value
SNAI2	Snail family transcriptional repressor 2	EMT	1.56	0.00159	-	-
TBX2	T-box transcription factor 2	Cellular Senescence	1.62	0.00204	-	-
SKP2	S-phase kinase associated protein 2	Cell Cycle	-1.51	0.000351		
MCM2	Minichromosome maintenance complex component 2	Cell Cycle	-1.52	0.00125	-	-
MAPK14	Mitogen-activated protein kinase 14	Cellular Senescence	-1.57	0.00108	-	-
PPP1R15A	Protein phosphatase 1 regulatory subunit 15A	DNA Damage & Repair	-1.58	0.00427	-	-
PGF	Placental growth factor	Angiogenesis	-1.61	0.00511	-	-
TNKS2	Tankyrase 2	Telomeres & Telomerase	-1.62	0.000416	-	-
HMOX1	Heme oxygenase 1	Hypoxia Signaling	-1.63	0.0187	-	-
VEGFC	Vascular endothelial growth factor C	Angiogenesis	-1.64	0.000308	2.05	0.0139
CASP2	Caspase 2	Apoptosis	-1.68	0.00537	-	-
SLC2A1	Solute carrier family 2 member 1	Hypoxia Signaling	-1.7	0.0249	-1.53	0.00987
ANGPT2	Angiopoietin 2	Angiogenesis	-1.72	0.0183	-	-
FGF2	Fibroblast growth factor 2	Angiogenesis	-1.74	0.000883	1.61	0.00946
DSP	Desmoplakin	EMT	-1.86	0.00345	2.61	0.0035
KDR	Kinase insert domain receptor	Angiogenesis	-1.9	0.0312	-	-

ADM	Adrenomedullin	Hypoxia Signaling	-2.08	0.00295	-	-
CCL2	C-C motif chemokine ligand 2	Angiogenesis	-4.43	0.00062	5.21	0.0478
SERPINF1	Serpin family F member 1	Angiogenesis	-	-	6.14	0.000854
FLT1	Fms related receptor tyrosine kinase 1	Angiogenesis	-	-	3.19	0.0317
LPL	Lipoprotein lipase	Metabolism	-	-	-1.73	0.000464
SNAI1	Snail family transcriptional repressor 1	EMT	-	-	-2.66	0.00202

Genes are ordered by descending values of fold change. Differentially expressed genes in A375 cells appear first.

Abbreviations: EMT = Epithelial-to-Mesenchymal Transition

Supplementary Table S5. NanoString Human Tumor Signaling 360 Panel Analysis significantly modulated genes after CRISPR/Cas9-mediated SIRT3 KO in A375 human melanoma cells.

Gene	Description	Molecular functions	Fold change	p value
FOS	FBJ murine osteosarcoma viral oncogene homolog	TCR Signaling	18.8749	0.002599
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	T-cell Costimulation T-cell Exhaustion TCR Signaling	15.3333	0.001469
ITGA1	Integrin, alpha 1	Cell Adhesion & Motility ECM Remodeling & Metastasis	4.72286	0.000573
BCL2A1	BCL2-related protein A1	Apoptosis Epigenetic & Transcriptional Regulation NF-kB Signaling	3.21917	0.00162
SNAI2	Snail family zinc finger 2	EMT Hippo Signaling	3.12001	0.001392
HLA-B	Major histocompatibility complex, class I, B	Antigen Presentation Interferon Response	3.09421	4.14E-05
BHLHE40	Basic helix-loop-helix family, member e40	mTOR Signaling TGF-beta Signaling	3.08744	3.85E-06
LAMA4	Laminin, alpha 4	Cell Adhesion & Motility ECM Remodeling & Metastasis MET Signaling	2.9406	0.000467
PLA2G4A	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	VEGF Signaling	2.89076	0.000362
HLA-C	Major histocompatibility complex, class I, C	Antigen Presentation Interferon Response	2.84848	0.001147
FLT1	Fms-related tyrosine kinase 1	HIF1 Signaling VEGF Signaling	2.57823	0.00099
ITPR1	Inositol 1,4,5-trisphosphate receptor, type 1	Senescence	2.51853	0.000703
CDCP1	CUB domain containing protein 1	MAPK Signaling	2.51628	0.00133

PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Glucose Metabolism HIF1 Signaling	2.24193	0.000349
HLA-A	Major histocompatibility complex, class I, A	Antigen Presentation Interferon Response	2.01221	0.000122
ICAM1	Intercellular adhesion molecule 1	Interferon Response NF-kB Signaling	1.87478	0.001445
CXXC5	CXXC finger protein 5	Estrogen Signaling	1.87179	0.000334
TLR4	Toll-like receptor 4	EMT NF-kB Signaling	1.79533	0.004824
P4HA2	Prolyl 4-hydroxylase, alpha polypeptide II	HIF1 Signaling	1.70487	0.004907
ARID4A	AT rich interactive domain 4A (RBP1-like)	Androgen Signaling Epigenetic & Transcriptional Regulation	1.6894	0.002097
ERBB3	Erb-b2 receptor tyrosine kinase 3	MAPK Signaling	1.67568	0.003634
GSK3B	Glycogen synthase kinase 3 beta	EMT Hedgehog PI3K-Akt Signaling	1.63624	0.004628
COL4A1	Collagen, type IV, alpha 1	Cell Adhesion & Motility ECM Remodeling & Metastasis PDGF Signaling	1.62035	0.005894
ATOX1	Antioxidant 1 copper chaperone	Nrf2 & Oxidative Stress	1.5942	0.000511
DUSP6	Dual specificity phosphatase 6	MAPK Signaling	1.57103	0.001959
PIK3R2	Phosphoinositide-3-kinase, regulatory subunit 2 (beta)	PDGF Signaling PI3K-Akt Signaling	1.56727	0.003701
IFNAR2	Interferon (alpha, beta and omega) receptor 2	Interferon Response	1.54082	0.003101
CDCA5	Cell division cycle associated 5	Cell Cycle	-1.53399	0.001868
BCL2L1	BCL2-like 1	Apoptosis Epigenetic & Transcriptional Regulation NF-kB Signaling	-1.54625	0.001415
MCM2	Minichromosome maintenance complex component 2	Cell Cycle mTOR Signaling	-1.56537	0.000508

CDC25A	Cell division cycle 25A	Cell Cycle mTOR Signaling	-1.63397	0.002662
SKA1	Spindle and kinetochore associated complex subunit 1	Cell Cycle	-1.64877	0.00315
TGM2	Transglutaminase 2	Hippo Signaling	-1.84112	0.003292
SDC1	Syndecan 1	ECM Remodeling & Metastasis Interleukin Signaling	-1.97191	0.005505
SLC7A5	Solute carrier family 7 (amino acid transporter light chain, L system), member 5	mTOR Signaling	-2.07498	0.005792
CAV1	Caveolin 1, caveolae protein, 22kda	Cell Adhesion & Motility EMT	-4.19343	0.000222
IL7R	Interleukin 7 receptor	Interleukin Signaling	-4.5082	0.002916
THBS1	Thrombospondin 1	ECM Remodeling & Metastasis Hippo Signaling Myc PDGF Signaling TGF-beta Signaling	-4.85722	0.00273

Genes are ordered by descending values of fold change. Abbreviations: Nrf2 = nuclear factor erythroid 2-related factor 2, NF-kB = nuclear factor kappa B, mTOR = mechanistic target of rapamycin kinase, TGF-beta = transforming growth factor-beta, EMT= Epithelial-to-Mesenchymal Transition, MAPK = mitogen activated protein kinase, PDGF = platelet derived growth factor, TCR = T-cell receptor, HIF1 = hypoxia-inducible factor-1, VEGF = vascular endothelial growth factor, PI3K-Akt = phosphatidylinositol 3-kinase-AKT serine-threonine protein kinase, ECM = extracellular matrix, MET = MET proto-oncogene, Myc = Myc proto-oncogene.

Supplementary Table S6. NanoString Human Tumor Signaling 360 Panel Analysis significantly modulated genes after CRISPR/Cas9-mediated SIRT3 KO in G361 human melanoma cells.

Gene	Description	Molecular functions	Fold change	p value
KDR	kinase insert domain receptor	VEGF Signaling	3.77778	0.00136
IL7	interleukin 7	Interleukin Signaling	2.58336	0.00018
UGDH	UDP-glucose 6-dehydrogenase	Androgen Signaling	1.90314	6.56E-05
OAS1	2'-5'-oligoadenylate synthetase 1, 40/46kDa	Interferon Response	1.856	0.00105
TPD52	tumor protein D52	Androgen Signaling	1.74066	5.21E-07
LDHA	lactate dehydrogenase A	Glucose Metabolism HIF1 Signaling mTOR Signaling Myc	1.60941	0.00035
CD44	CD44 molecule (Indian blood group)	ECM Remodeling & Metastasis EMT Immortality & Stemness Interferon Response T-cell Exhaustion	1.55589	0.00067
EPAS1	endothelial PAS domain protein 1	Immortality & Stemness	1.54365	0.00095
ASH1L	ash1 (absent, small, or homeotic)-like (Drosophila)	Epigenetic & Transcriptional Regulation	-1.80938	2.22E-05
NUF2	NUF2, NDC80 kinetochore complex component	Cell Cycle	-2.52674	4.64E-05

Genes are ordered by descending values of fold change. Abbreviations: ECM = extracellular matrix, EMT= Epithelial-to-Mesenchymal Transition, VEGF = vascular endothelial growth factor, HIF1 = hypoxia-inducible factor-1, mTOR = mechanistic target of rapamycin kinase, Myc = Myc proto-oncogene

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Chapter 3: Antimelanoma Effects of Concomitant Inhibition of SIRT1 and SIRT3 in $Braf^{V600E}/Pten^{NULL}$ Mice

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ABSTRACT

Novel therapeutic strategies are required for the effective and lasting treatment of metastatic melanoma, one of the deadliest skin malignancies. In this study, we determined the anti-melanoma efficacy of 4′-bromo-resveratrol (4′-BR), which is a small molecule dual inhibitor of SIRT1 and SIRT3 in a BrafV600E/PtenNULL mouse model that recapitulates human disease, including metastases. Tumors were induced by topical application of 4-hydroxy-tamoxifen on shaved backs of 10-week-old mice, and the effects of 4′-BR (5-30 mg/kg b.wt.; intraperitoneally; 3d/week for 5 weeks) were assessed on melanoma development and progression. We found that 4′-BR at a dose of 30 mg/kg significantly reduced size and volume of primary melanoma tumors, as well as lung metastasis, with no adverse effects. Further, mechanistic studies on tumors showed significant modulation in markers of proliferation, survival and melanoma progression. As SIRT1 and SIRT3 are linked to immunomodulation, we performed differential gene expression analysis via NanoString PanCancer Immune Profiling panel (770 genes). Our data demonstrated that 4′-BR significantly downregulated genes related to metastasis-promotion, chemokine/cytokine-regulation, and innate/adaptive immune functions. Overall, inhibition of SIRT1 and SIRT3 by 4′-BR is a promising anti-melanoma therapy with anti-metastatic and immunomodulatory activities warranting further detailed studies, including clinical investigations.

INTRODUCTION

Recent advances in the understanding of melanoma biology have led to the development of promising targeted therapies. The BRAF inhibitors vemurafenib and dabrafenib achieved significant improvement over traditional chemotherapy and were approved for patients with metastatic melanomas harboring BRAF-mutations. More recently, the combination of dabrafenib with the MEK inhibitor trametinib demonstrated improved progression-free survival as compared to monotherapy, and has received approval from the US FDA. However, even with the combination treatment, most patients develop resistance, thereby failing to achieve lasting tumor regression [1, 2]. Interestingly, melanoma has been characterized as one of the most immunogenic tumors and immune-targeted therapeutics have been successful and approved for melanoma treatment. However, the high rates of resistance acquisition, large percentage of partial responders and lack of durable responses remain obstacles to the success of these therapies [3]. Since melanoma is notoriously resistant to treatment and current therapeutic approaches have not been able to effectively manage this neoplasm, additional novel target-based approaches are needed.

The mammalian sirtuins (SIRTs) constitute a family of seven known members (SIRT1 – SIRT7) with NAD^+-dependent protein deacetylase and/or ADP-ribosyltransferase activities [4]. SIRTs play critical roles in important cellular processes, and are shown to be involved in the pathogenesis of a variety of diseases, including cancer [5]. The role of SIRTs in cancer is extremely complex and they appear to have distinct functions depending on cell contexts [6]. The founding member of this family, the nuclear SIRT1, has been extensively studied and is linked with several health conditions

including metabolic syndrome, inflammation, and cancer [4]. We have earlier shown that SIRT1 is overexpressed in melanoma, and its inhibition imparts anti-proliferative responses against melanoma cells. [7-9]. One of the other well-studied sirtuins, SIRT3, is a mitochondrial sirtuin that coordinates global shifts in mitochondrial activity by deacetylating proteins involved in diverse mitochondrial functions [10]. SIRT3 also plays important roles in the regulation of a variety of cellular processes, including transcription, insulin secretion, and apoptosis [11]. The fact that SIRT3 can regulate several cellular processes which are critical in cancer cell proliferation makes it a promising therapeutic target for cancer management [12]. In a study from our laboratory, we have demonstrated that SIRT3 is overexpressed in melanoma and its inhibition resulted in significant anti-tumor responses against melanoma *in vitro* as well as *in vivo* [13, 14].

Recently, we discussed the potential usefulness of combined inhibition of specific sirtuins in the management of melanoma [15]. In a recent study, Nguyen et al. demonstrated that the small molecule 4′-bromoresveratrol (4′-BR) completely inhibited the activity of both SIRT1 and SIRT3 at the 0.2 mM concentration in the deacetylation assays using FdL-1 fluorophore. Further, they identified two compound binding sites and substrate competition with NAD+ as the mechanism for this inhibition [16]. Using this compound, we have previously shown that 4′-BR treatment of multiple melanoma cell lines *in vitro* resulted in decreased cell proliferation and clonogenic survival, induction of apoptosis, and inhibition of melanoma cell migration [17]. Moreover, as both SIRT1 and SIRT3 have been implicated in the regulation of cancer cell metabolism, their inhibition by 4′-BR treatment caused metabolic reprogramming by decreasing mitochondrial function, reducing glucose uptake and dampening NAD+/NADH ratio [17]. In this current

investigation, we determined the *in vivo* efficacy of 4′-BR against melanoma in a genetically engineered *Braf*^{V600E}/*Pten*^{NULL} mouse model which is known to recapitulate human melanoma's cardinal features, including lung metastases [18]. We have also analyzed potential mechanisms of action of 4′-BR against melanoma using tumors obtained from *Braf*^{V600E}/*Pten*^{NULL} mice and NanoString technology (PanCancer Immune Profiling Panel).

MATERIALS AND METHODS

Animals and materials

Animal experiments were approved by the University of Wisconsin (UW) Institutional Animal Care and Use Committee. Genetically engineered Braftm1Mmcm Ptentm1Hwu Tg(Tyr-cre/ERT2)13Bos/BosJ mice (8-9 weeks old) were purchased from The Jackson Laboratory (Stock Number: 012328; Bar Harbor, ME). Mice were maintained under defined conditions and experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and institutional guidelines. Mice were acclimatized for at least one week before start of the experiment. 5-([E]-2-[4-bromophenyl]vinyl)benzene-1,3-diol (4′-BR) was obtained from Aobious (Gloucester, MA). 4-hydroxytamoxifen (4-HT) was purchased from Cayman Chemical (Ann Arbor, MI).

In vivo melanoma development and treatments

Localized melanoma tumors were induced on the back skin of *Braf*^{V600E}/*Pten*^{NULL} mice as described previously [18]. Briefly, dorsal hair was removed and 2 µL of 5 mmol/L 4-HT dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) was topically applied in four discrete spots on 10-week-old mice for three consecutive days. Mice were monitored for overall health and the presence of cutaneous malignant melanoma. As shown in Figure 1a, after 2 weeks mice harboring established melanoma were randomly divided in 5 groups (n=6, 3 male and 3 female) and treated with 4′-BR (5, 10, 20 and 30 mg/kg b.wt.; intraperitoneal (i.p.); 3d/week for 5 weeks) or an equivalent volume of vehicle (50% DMSO + 50% polyethylene glycol (PEG)).

Tumor analysis and lung metastasis

During the study, mice were monitored for melanoma development. Tumor growth was determined using digital calipers, and tumor sizes were represented as mean±SEM (n=6). At the termination of the experiment, tumors were measured using a digital caliper and tumor volume was calculated using the formula $0.5\times length\times width^2$. Further, at the termination of experiments tumors were excised, and weighed before being divided into either formalin (for immunohistochemistry (IHC) analysis) or snap-frozen (for protein/RNA analysis). Lungs were harvested and fixed in Fekete's solution [40] to visualize metastasis. All other analyses related to metastatic markers were performed using primary melanoma tumors obtained from 4′-BR treatment and control groups. Statistical significance was determined via t-test using GraphPad Prism software.

Quantitative immunodetection analysis by ProteinSimple

Quantitative immunodetection analysis was done on protein lysates per manufacturer's protocol (see Supplementary Materials and Methods). Briefly, protein from tumor tissues were isolated in 1X RIPA lysis buffer (Millipore, Burlington, MA) with Protease Inhibitor Cocktail (Thermo Scientific, Waltham, MA) and PMSF (Amresco, Solon, OH), and quantified using Pierce BCA Protein assay kit. Protein expression was measured with automated protein capillary electrophoresis using a ProteinSimple Wes instrument (ProteinSimple, San Jose, CA). The assay was optimized for the targets of interest and tissue samples (Figure S4 and S5, Table S1).

Immunohistochemistry (IHC)

After excision, tumors were fixed in 10% formalin for 48 hours before rinsing and stored short-term in 70% ethanol. After fixation, tissues were embedded in paraffin, sectioned, and mounted on slides at the UW Translational Research Initiatives in Pathology (TRIP) lab. IHC analysis was performed as described recently [41] utilizing ABC-HRP reagent (Vector Labs, Burlingame, CA), Vector Red chromogen and hematoxylin per manufacturer's protocol. The primary antibodies used in IHC analysis are detailed in Table S2. Imaging and analysis were performed per details in Supplementary Materials and Methods. Statistical analysis was performed using two-tailed unpaired Student's t-test using GraphPad Prism software.

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

RNA was isolated using the RNeasy Plus kit and quantified using the BioTek Synergy H1 multimode plate reader. RNA from 6 mice were pooled in each group to make 3 groupings of RNA per group for further analysis. cDNA was made using M-MLV reverse transcriptase and oligo dT primers. RT-qPCR analysis was performed using QuantStudio 3 with SYBR Premix Ex Taq II with cDNA and primers (Table S3). Relative mRNA levels were analyzed using ACTB as endogenous control and ΔΔCT algorithm. Statistical significance was determined via multiple t-tests using the Holm-Sidak method to correct for multiple comparisons using GraphPad Prism software.

NanoString PanCancer Immune Profiling Panel analysis

RNA from control and 4′-BR-treated (30 mg/kg b. wt.) mice (n = 6) were used to determine the expression of 770 genes (730 immune genes and 40 housekeeping genes) via the

Mouse PanCancer Immune Profiling Panel using nCounter platform in the NanoString Technologies laboratories. nSolver software with an advanced analysis module (NanoString Technologies, Seattle, WA) was used for quality control, normalization, differential gene expression, and functional pathways score analyses in accordance with guidance provided by Nanostring. Principle component analysis (PCA) was performed to assess sample grouping (Figure S6). The expression levels of each gene were normalized to housekeeping genes. Fold changes greater than 2-fold upregulation or downregulation with p-values <0.05 between the two groups were considered significant. The heat map for differentially expressed genes in 4′-BR (30 mg/kg b. wt.) group compared to control group was plotted and analyzed using nSolver software.

Network analyses

Network analyses were carried out as described previously with a few modifications [42]. Genes with significant fold changes (≥2) were considered for network analysis. The STRING database version 11.0 (https://string-db.org) was used for the prediction of gene interactions, based on information from previous publications [43]. Since the number of genes was relatively small (49 significantly altered genes), the minimum required interaction score was set at a confidence level of ≥0.4. Using STRING, clusters were visualized by selecting the Markov Cluster (MCL) algorithm as part of the analysis, with an inflation parameter of 1.5. Furthermore, network edges (lines that connect the genes with one another) were defined in STRING as the molecular action between genes, to visualize the type and effect of mode of action. Afterward, gene networks were transferred to Cytoscape version 3.8 for visualization [44]. Nodes represent genes of interest while

lines or edges correspond to interactions between those genes. Visualizations were refined using Adobe Illustrator 2019.

RESULTS AND DISCUSSION

4′-BR significantly reduces melanoma tumor growth *in vivo* in *Braf*^{V600E}/*Pten*^{NULL} mice

In order to determine the translational value of combined inhibition of SIRT1 and SIRT3, in this study, we determined the *in vivo* efficacy of SIRT1 and SIRT3 dual inhibitor 4′-BR against melanomas in a genetically engineered *Braf*^{V600E}/*Pten*^{NULL} melanoma mouse model. This mouse model was designed to allow 4-hydroxytamoxifen (4-HT)-inducible, melanocyte-specific expression of mutant *Braf*^{V600E} and deletion of tumor suppressor gene *Pten*, which are genetic events relevant in human melanoma. 4-HT specifically induces Cre recombinase-mutated estrogen receptor fusion transgene that is under the control of melanocyte-specific tyrosinase promoter (Tyr::CreER) [18]. Due to this, topical application of 4-HT results in development of black pigmented skin lesions that progress to malignant melanoma. In our study, pigmented spots developed 2 weeks after 4-HT treatment, at which point mice were randomly assigned to treatment groups of either vehicle or 4′-BR (5, 10, 20 or 30 mg/kg b.wt.) as described in Materials and Methods and shown in Figure 1a. The treatments were continued for 5 weeks and tumor volumes were estimated weekly.

As shown in Figure 1b, melanoma tumors in *Braf*^{V600E}/*Pten*^{NULL} mice were noticeably smaller after 4′-BR treatment. Images of all mice (n=6 per group) at the end of study (17 weeks) have been shown in Figure S1. Measurement of tumor volume (Figure 1c) and tumor weight (Figure 1d) revealed that there was indeed a trend toward reduced tumor growth upon 4′-BR treatment at all tested doses in *Braf*^{V600E}/*Pten*^{NULL} melanomas. This difference was statistically significant at 30 mg/kg b. wt. (4′-BR) after 5 weeks,

therefore, tumor tissues derived from this group and control were used subsequently for molecular analyses. Further, we found no significant change in body weight after treatment (Figure 1e) and there were no noticeable adverse effects of 4′-BR treatment at the tested doses. A previous study has demonstrated that 4′-BR inhibits the activity of SIRT1 and SIRT3, by binding to the allosteric and substrate competition with NAD+ [16]. We determined the expression of these sirtuins in 4-HT-induced mouse melanomas and verify if 4′-BR modulated their expression in these tumors by performing a quantitative immunodetection analysis using ProteinSimple. Our results suggested that both SIRT1 and SIRT3 are expressed in *Braf*^{V600E}/*Pten*^{NULL} tumors validating the target expression. Furthermore, we found no significant change in protein levels of SIRT1 and SIRT3 after 4′-BR treatment, suggesting that 4′-BR does not affect the protein expression of these sirtuins (Figure S2), and the observed effects could be due to the inhibition of their activity.

Because 4′-BR has not been investigated in *in vivo* cancer models including melanoma, these are important and promising pre-clinical observations in *Braf*^{V600E}/*Pten*^{NULL} melanoma mouse model, which is an excellent model for pre-clinical evaluation of anti-melanoma agents due to its close resemblance with melanomas in human [19, 20].

4′-BR treatment modulates key markers of melanoma progression in BrafV600E/PtenNULL mice

Next, tumor samples obtained from the 30 mg/kg and control groups were analysed for Ki67 and PCNA (proliferating cell nuclear antigen) as biomarkers of cell proliferation [21].[21]. Additionally, we used survivin as a biomarker of cell survival, as it

prevents programmed cell death and is shown to be associated with melanoma progression [22].[22]. As shown in Figure 2a, 4′-BR treatment significantly reduced the percent of Ki67-positive cells in tumors, indicating decreased proliferative indices in this group. Further, our results showed a marked reduction in PCNA and survivin both at mRNA and protein levels (Figure 2b). Furthermore, we analyzed growth factor signaling markers insulin-like growth factor 1 (IGF1) a tumor-promoting growth factor, and insulin-like growth factor-binding protein 5 (IGFBP5) a tumor suppressor protein. We observed decreased *Igf1* and increased *Igfbp5* in response to 4′-BR treatment at mRNA levels as well as an increase in IGFBP5 at protein level (Figure 2b). IGF1 has been shown to promote melanoma progression by increasing proliferation, tumor cell mobility and dissemination, maintaining stemness features crucial for the immune escape, chemoresistance, and tumorigenicity [23].[23]. Further, IGFBP5 has been shown to function as an important tumor suppressor gene in melanoma tumorigenicity and metastasis using both *in vitro* and *in vivo* experiments [24].[24]. Thus, the observed decrease in Ki67, PCNA, survivin and IGF1, as well as increase in IGFBP5 by 4′-BR treatment, demonstrate anti-melanoma potential of this dual SIRT1 and SIRT3 inhibitor.

Because melanoma has been linked with dysregulated oxidative stress with deteriorated cell functioning at the mitochondrial level [25, 26], we assessed the effects of 4′-BR treatment on selected markers of oxidative stress, viz. nuclear factor-erythroid 2 related factor (NRF2) and Kelch-like ECH Associated Protein 1 (KEAP1). Using RT-qPCR, we found that 4′-BR treatment significantly up-regulated *Keap1* compared to a slight decrease in *Nrf2,* resulting in a significant decrease in the ratio of *Nrf2*/*Keap1* (Figure 2c). NRF2 is known to play an important role in cell survival and defense against

endogenous/exogenous stresses, and generally, its overexpression in cancer cells enhances the expression of cytoprotective genes, resulting in increased cell proliferation and inhibition of apoptosis [27]. KEAP1, a negative regulator of NRF2, is known to bind and restrict NRF2 activation. Recently, some reports have shown that NRF2 expression in melanoma is related to invasion, thereby worsening melanoma-specific survival [28]. Overall, our data demonstrate the ability of 4′-BR to exert anti-proliferative and anti-tumorigenic effects in a human-relevant melanoma mouse model with significant modulations in markers of cell proliferation, cell survival, tumor growth and oxidative stress.

4′-BR modulates genes associated with cancer immune pathways

Given that *Braf*^{V600E}*/Pten*^{NULL} is an inducible melanoma mouse model with an active immune system, it is a powerful tool to study the immune modulations in response to therapy. As sirtuins are linked to immunomodulation, and melanoma is an immunologically “hot” cancer, we performed differential gene expression analysis using high throughput multiplex analysis via NanoString nCounter PanCancer Immune Profiling panel, which analyzes 770 genes related to cancer-immune pathways (cancer progression, chemokines and cytokines and their receptors, and innate and adaptive immune response). nSolver software was used to analyze the data and a heat map was generated to show differential expression of 770 genes (Figure 3a). Further, using a fold cut-off of ≥2 and p-value <0.05, we observed that 4′-BR significantly modulated the expression of multiple genes as shown via volcano plot (Figure 3a). Moreover, several cancer immune pathways were significantly downregulated by 4′-BR treatment, while

senescence and T-cell functions were significantly upregulated (Figure 3b). Overall, this high throughput gene expression analysis suggested that 4′-BR treatment strongly favored systemic antitumor immunity, resulting in inhibition of tumor growth in mice.

At a more granular level, we found that 49 genes were significantly (fold cut-off of ≥2 and p-value <0.05) affected by 4′-BR treatment, the associations and interactions of which were further studied by performing network analysis. As shown in Figure 4a, we found that a majority of the 49 genes modulated by 4′-BR treatment were highly interconnected. Interestingly, out of these 49 genes, 41 were downregulated, while only 8 were upregulated after 4′-BR treatment (indicated with dashed outlines in Figures 4a and 4b). Three genes out of the 49 did not show evidence of interaction within the other genes in the network: *Colec12*, *Rae1c*, and *Prg2*. However, *Itgam* (integrin subunit alph c a M) demonstrated to have the most interactions (25 interactions with other genes), which is not unexpected as it is a member of the integrin family of transmembrane adhesion molecules that facilitate cell-cell and cell-extracellular matrix attachments. On the other hand, *Sigirr*, *Fap*, and *Jam3* had the least interactions (1 interaction each). Overall, network analyses of the genes affected by 4′-BR treatment revealed that 18 genes were involved in innate/adaptive immune functions, 14 genes were associated with cancer progression/adhesion/apoptosis, 9 genes had basic/immune cell functions, and 8 genes which were chemokines/cytokines (Figure 4a).

Furthermore, genes that had evidence of roles in melanoma were analyzed using network visualization (Figure 4b). Out of the 49 total genes that were affected by 4′-BR treatment, 30 genes have been associated with melanoma. A literature analysis of the 30 genes reported to be associated with melanoma and significantly affected by 4′-BR

treatment in *Braf*^{V600E}/*Pten*^{NULL} mice has been detailed in Table 1. Interestingly, in our network analysis, all the 30 genes showed evidence of at least one interaction with each other. *Fn1* had the most interactions (14 interactions with other genes) while *Il21r*, *Fap*, and *Jam3* had the least interactions (1 each). Importantly, in a recent study, *Fn1* (Fibronectin 1) was shown to promote melanoma proliferation and metastasis by inhibiting apoptosis and regulating epithelial to mesenchymal transition (EMT) [29], suggesting it may be directly affected by 4′-BR treatment. Of these 30 melanoma-involved genes, 23 were downregulated and 7 were upregulated after 4′-BR treatment. From the 23 downregulated genes, 21 have been shown to support melanoma progression and 2 to inhibit it. Of the 7 upregulated genes, 6 genes are known to inhibit melanoma, and 1 has been associated with melanoma development (Figure 4b).

A review of available literature revealed that 10 of these downregulated genes (*Ccr1*, *Itgam*, *S100a8*, *C1qb*, *F13a1*, *Il1b*, *Fap*, *Col3a1*, *Angpt2*, and *Spp1*) were associated with increased risk of melanoma and their expression was dysregulated in melanoma (detailed in Table 1). Additionally, their expression has been correlated with poor survival in melanoma patients, suggesting they could be explored as melanoma markers. Similarly, 6 genes (*Fn1*, *Jam3*, *Thbd*, *Angpt1*, *Ptgs2*, and *Ccr5*) downregulated following 4′-BR treatment were involved in promoting tumorigenesis, cell migration and invasion, and epithelial to EMT. Furthermore, 5 downregulated genes (*Nlrp3*, *Cd33*, *Serping1*, *Tlr2*, and *Gpr183*) have been reported to play immune-related roles, which contribute to melanoma progression. On the other hand, two genes (*IL21R* and *OSM*) downregulated after 4′-BR treatment have been reported to inhibit melanoma progression by suppressing tumorigenesis as well as blocking cell proliferation and invasion.

Interestingly, all 7 genes that were upregulated after 4′-BR treatment (*Il12rb2*, *Xcl1*, *Ccl1*, *Zap70*, *Rorc*, *Tnfsf10*, and *Ccl19*) have been reported to inhibit melanoma progression, which is consistent with the anti-melanoma effects of 4′-BR. Specifically, 4 upregulated genes (*Xcl1*, *Ccl1*, *Zap70*, and *Ccl19*) share immune-related functions which have anti-tumor effects and thus are correlated with good prognosis. The remaining 3 upregulated genes (*Il12rb2*, *Rorc*, and *Tnfsf10*) are tumor suppressor genes which are associated with prolonged survival of melanoma patients and even could be potential candidates for treatments against melanoma. Overall, our findings support the antiproliferative effects of 4′-BR and suggest that this response may occur via modulation of important tumor suppressor pathways, immune responses, and reductions in cell proliferation and invasion pathways.

4′-BR modulates protein expression of immune and inflammatory markers

Considering that melanoma has been classified as an immunogenic malignancy [30] together with the immune- and inflammatory-related roles that SIRT1 and SIRT3 carry out [31], we determined the potential immunomodulatory effects after 4′-BR treatment in melanoma tumors. Likewise, to confirm our Nanostring transcriptomic data on immunomodulatory activities of 4′-BR, we performed IHC analysis for certain immune and inflammatory markers obtained from Nanostring analysis. Using melanoma tumors from control and 4′-BR at 30 mg/kg b. wt. dose groups (n=6), we determined the potential differences in protein levels of program death-ligand 1 (PDL-1), interleukin-1 beta (IL-1β), and NOD-like receptor family pyrin domain containing 3 (NLRP3).

PDL-1, found in cancerous or infiltrating immune cells, is a ligand of the programmed death receptor 1 (PD-1), which together can provide cells the ability to evade the immune system [32]. In our Nanostring analysis, we found that the gene that encodes for PD-L1 (CD274) was slightly increased in 4′-BR treated group, although the change was not statistically significant. In our IHC analysis, we observed no significant changes in PDL-1 expression in 4′-BR when compared to control group (Figure 4c). It is possible that this slight/no change in PD-L1 levels in both assays may be due to the complexity of using PD-L1 as a biomarker, since it has been shown that PD-L1 levels widely vary across tumor tissues [33, 34].

To further evaluate the effects of 4′-BR treatment on inflammatory markers, we used IHC to confirm our results from Nanostring analysis on selected statistically significant downregulated genes after 4′-BR treatment, IL-1β and NLRP3. IL-1β is a proinflammatory cytokine that can be produced by malignant cells to increase their invasiveness [35]. NLRP3 is an inflammasome that is constitutively activated in melanoma cells and when it is inhibited this leads to decreased melanoma invasion and metastasis in vitro and in vivo [36]. Interestingly, it has been shown that melanoma cells activate NLRP3 which induces IL-1β secretion [36, 37]. Accordingly, increased levels of IL-1β and NLRP3 have been associated with melanoma development and progression [37, 38]. Using IHC, for both IL-1β and NLRP3, our results showed a clear reduction of protein levels in 4′-BR when compared to control group (Figure 4c). Altogether, these experiments provide preliminary data in terms of the immunomodulatory effects that 4′-BR treatment imparts in melanoma tumors.

4′-BR treatment reduces melanoma metastasis in *Braf^{V600E}/Pten^{NULL}* mice

The *Braf^{V600E}/Pten^{NULL}* mouse model is an excellent model as it has a high rate of metastatic disease in a manner that recapitulates the clinical course of melanoma in humans, which provides an opportunity for studying mechanisms of melanomagenesis, melanoma progression and metastasis [18]. To explore the effects of 4′-BR on melanoma metastasis, we analyzed the resected lungs from the mice at the termination of the experiment. Visually, we observed a marked decrease in lung metastasis at the end of study (17 weeks) as shown in Figures 5a. Additionally, using melanoma tumors, we also found a significant decrease in vimentin at both protein and mRNA levels following 4′-BR treatment (Figure 5b). This is an interesting data, as vimentin has been reported to be a metastatic indicator in melanoma and its higher expression in primary human melanoma tissues may indicate patients with high risk of hematogenous metastasis [39].

To further explore the mechanisms behind this reduction in metastatic potential, we validated several genes associated with melanoma progression and metastasis obtained from our network analysis of NanoString data. Using primary melanoma tumors obtained from 4′-BR treatment group, our RT-qPCR analysis showed significant downregulation of several genes promoting melanoma progression and metastasis such as *S1008a*, *Thbd, Il1b, Fap*, *Angpt1*, *Angpt2*, *Ptgs2*, *Fn1, Jam3, and Spp1* (Figure 5c). We also validated two tumor suppressor genes *Zap70* and *Rorc* which have been shown to inhibit melanoma metastasis. *Zap70* showed a significant increase, while *Rorc* showed a marked but non-significant increase in 4′-BR treated tumors (Figure 5d). Further, some of the significantly modulated genes obtained from NanoString data analysis were found either with no change or not significant in our validation by RT-qPCR such as *Osm*, *Il21r*,

Ccr5 and *Gpr183* (Figure S3). Together, these results strongly support the anti-metastatic potential of 4′-BR treatment in *Braf*^{V600E}*/Pten*^{NULL} melanoma tumors.

Collectively, our investigation demonstrates the ability of 4′-BR to inhibit melanoma in vivo with no noticeable adverse effects in a human-relevant melanoma mouse model. Additionally, 4′-BR was able to visibly reduce melanoma metastasis to the lungs, which was associated with reductions in metastasis-related genes via RT-qPCR. These results in combination with high-throughput multiplex analysis using Nanostring Mouse PanCancer Immune Profiling Panel suggest that 4′-BR, a dual SIRT-1 and SIRT-3 inhibitor, may be a promising anti-melanoma agent with anti-metastatic and immunomodulatory effects, warranting further investigations including clinical studies.

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FIGURES

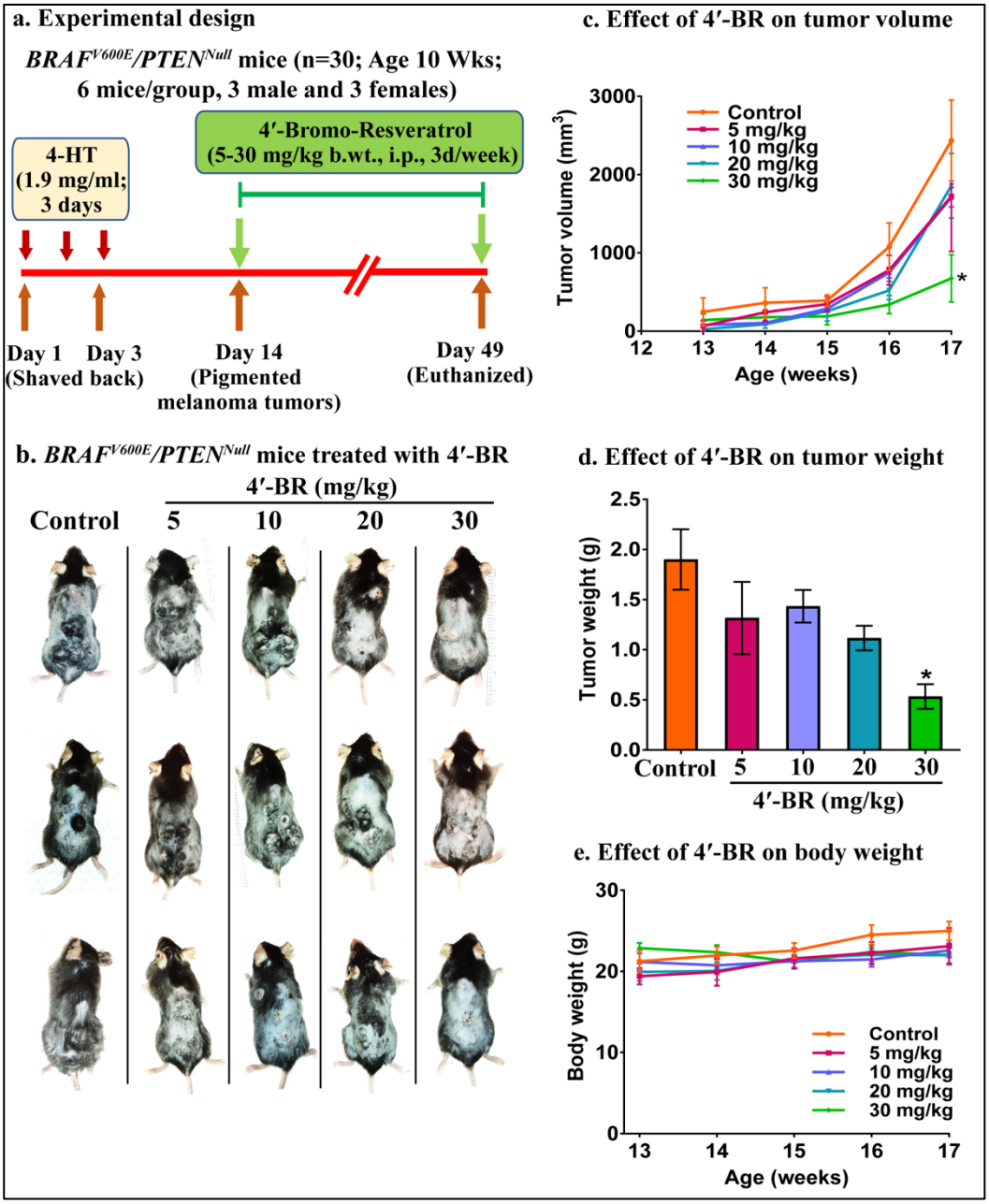


Figure 1. 4′-BR significantly reduced melanoma tumor growth in BrafV600E/PtenNULL mice. (a) Experimental design. (b) Representative images of BrafV600E/PtenNULL mice after treatments with 4′-BR (5-30 mg/kg b.wt.; intraperitoneally; 3d/week for 5 weeks) from each group at the end of the study (week 17). (c) Average tumor volume per group for

weeks 13-17. (d) Average tumor weight per group at the end of study (week 17). (e) Average body weight per group for weeks 13-17. All data are presented as mean ± standard error of the mean. *$p<0.05$.

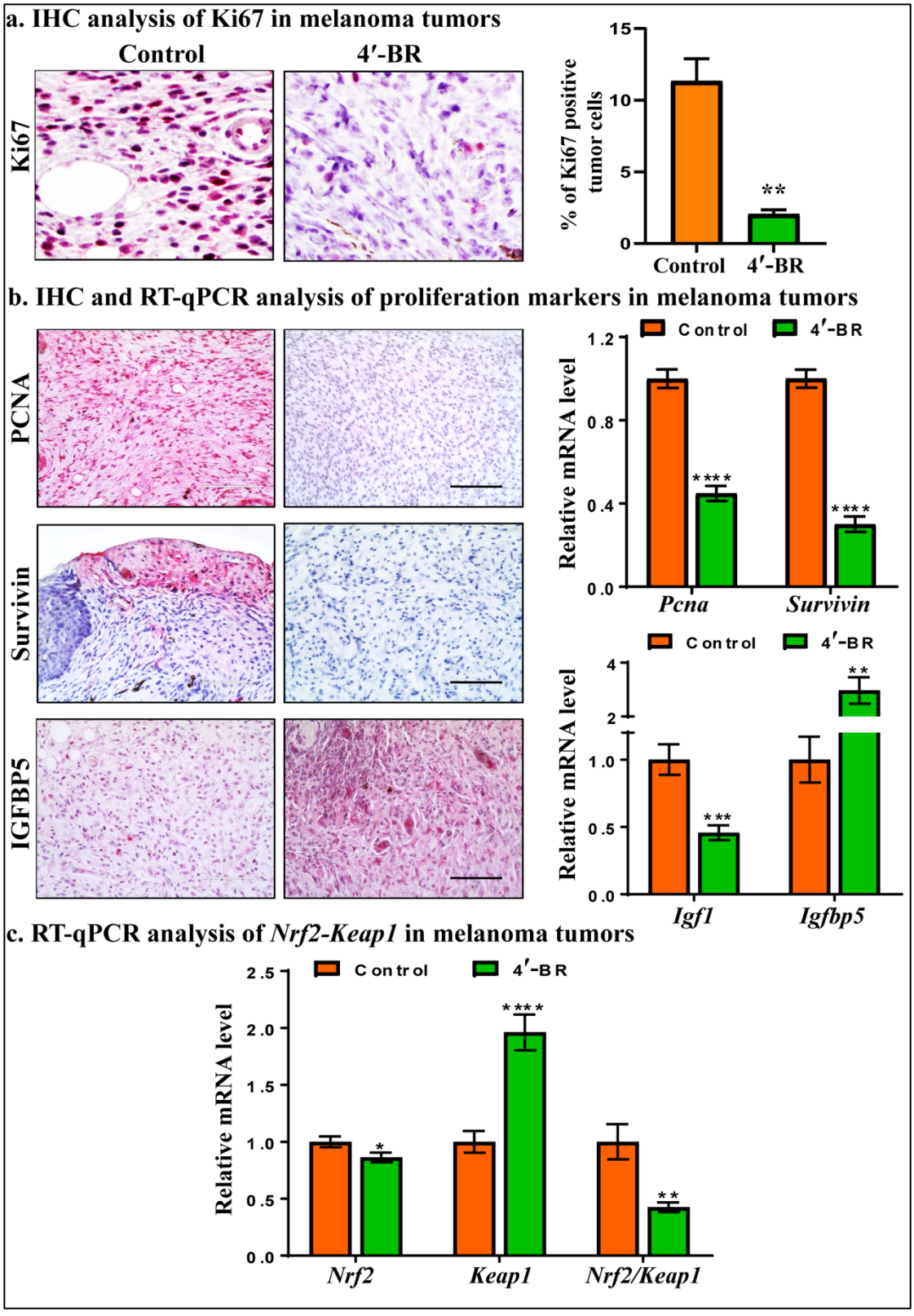

a. IHC analysis of Ki67 in melanoma tumors
Control
4′-BR
Ki67
% of Ki67 positive tumor cells
15
10
5
0
**
Control
4′-BR
b. IHC and RT-qPCR analysis of proliferation markers in melanoma tumors
PCNA
Survivin
IGFBP5
Control
4′-BR
Relative mRNA level
1.2
0.8
0.4
0.0
****
****
Pcna
Survivin
Control
4′-BR
Relative mRNA level
4
2
1.0
0.5
0.0
***
**
Igf1
Igfbp5
c. RT-qPCR analysis of Nrf2-Keap1 in melanoma tumors
Control
4′-BR
Relative mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
*
****
**
Nrf2
Keap1
Nrf2/Keap1

Figure 2. 4′-BR significantly modulated cell proliferation, survival, growth factor signaling and oxidative stress markers in *Braf*^{V600E}*/Pten*^{NULL} mice. (a) Representative images of Ki67 protein expression by IHC (40X objective and 10X ocular) on an Olympus BX53 microscope. Each image is one quadrant (25%) of dimensions of 1360 x 1024 pixels. Ki67-positive tumor cells with bright red nuclear staining were counted manually. Stromal cells were excluded from counting. The fraction of Ki67-positive cells was determined as Ki67 positive tumor cells divided by total tumor cells. (b) Representative images of PCNA, Survivin, and IGFBP5 IHC (40x magnification, scale bars = 100 μm) and relative mRNA levels of *Pcna, Survivin, Igf1*, and *Igfbp5* as determined by RT-qPCR. (c) mRNA expression analysis of activators of antioxidant response element, *Nrf2* and *Keap1* (by RT-qPCR) and their ratio. Data are presented as mean ± standard error of the mean, with statistical analysis performed using one-way analysis of variance with Tukey multiple comparisons. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Each bar represents a pool of six animals, as described in the Materials and Methods section. 4′-BR, 30 mg/kg body weight of 4′-bromo-resveratrol; IHC, immunohistochemistry.

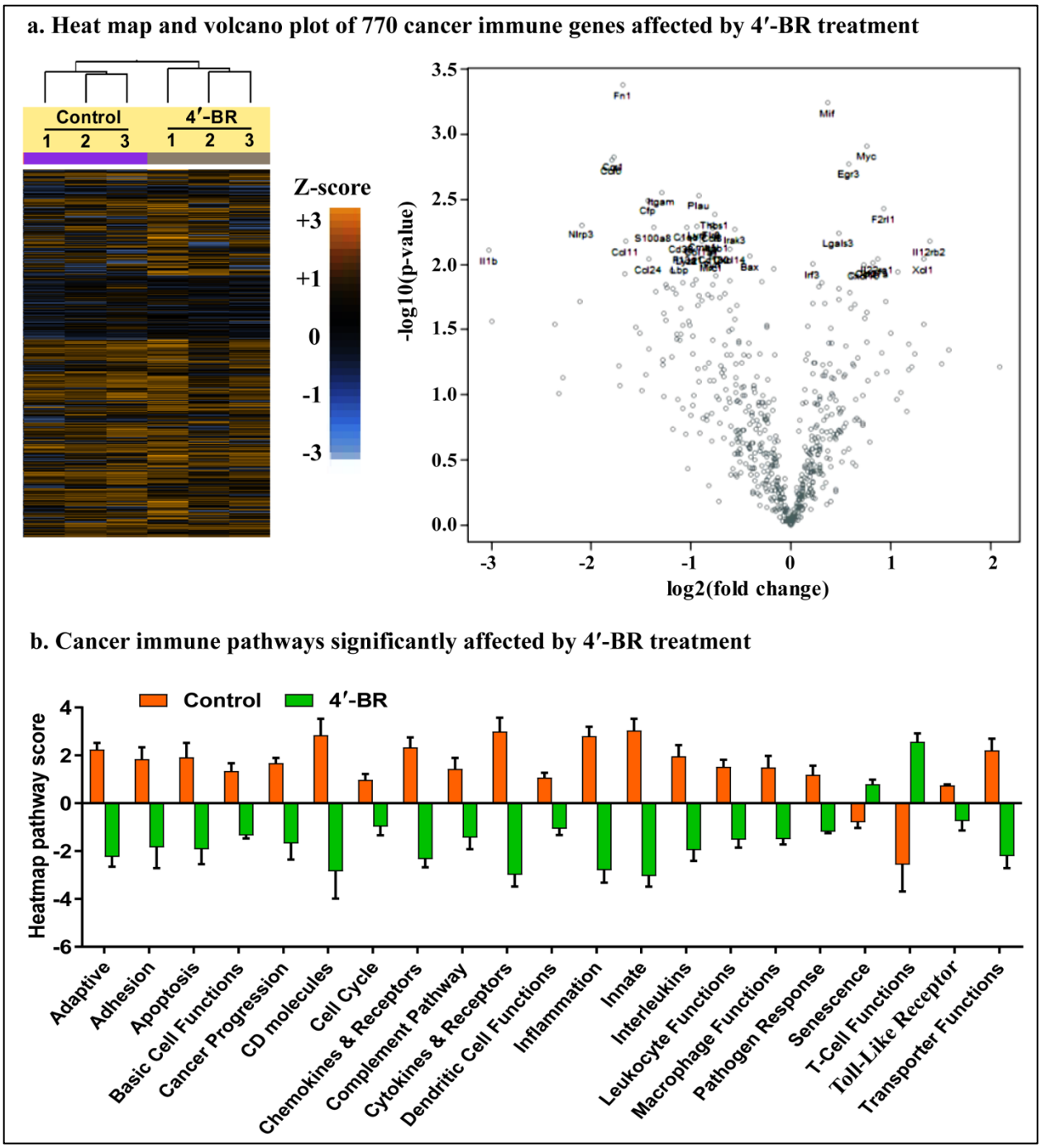


Figure 3. 4′-BR significantly modulated genes associated with cancer immune signaling, as determined using NanoString PanCancer Immune Profiling Panel. RNA from primary tumor tissues of control and 4′-BR treated groups were subjected to NanoString analysis

of 770 immune-related genes. (a) Left: Heatmap displaying each gene with color-coding for up- or down-regulation (orange and blue, respectively). Right: Volcano plots displaying each gene's -log10 (P-value) and log-two-fold change in 4′-BR treated group normalized with control group. Some of the most statistically significant genes are labeled in the plot. (b) Cancer immune pathways significantly (*P<0.05) modulated by 4′-BR treatment. Data are presented as mean ± standard error of the mean (n=6) of the pathway score.

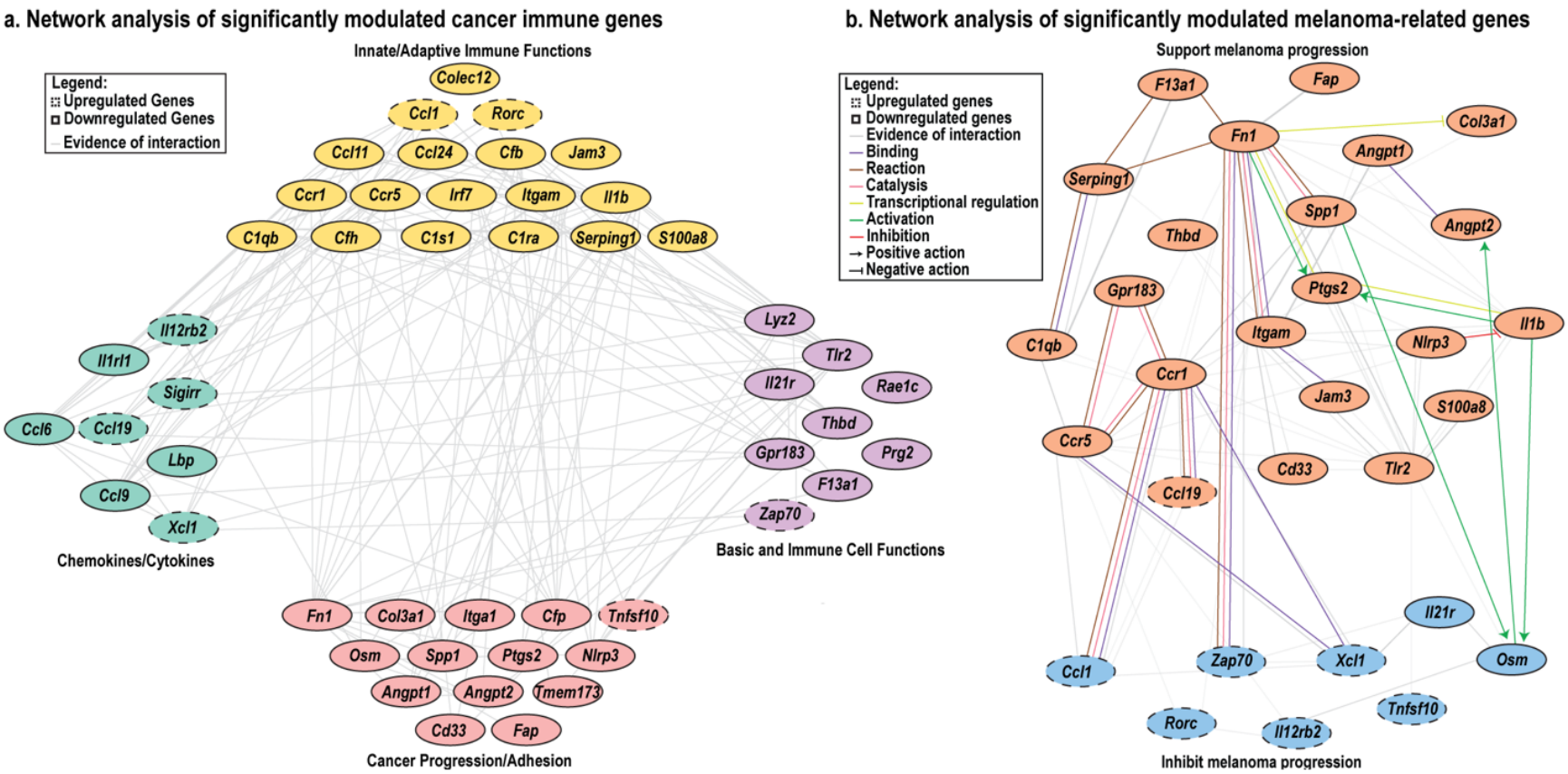
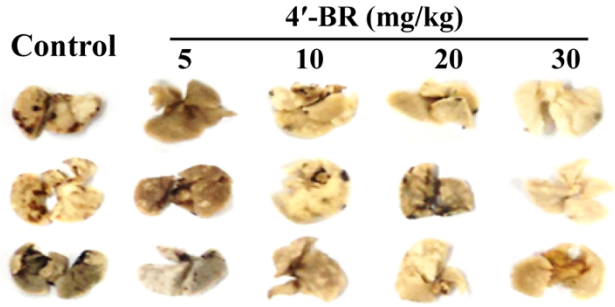


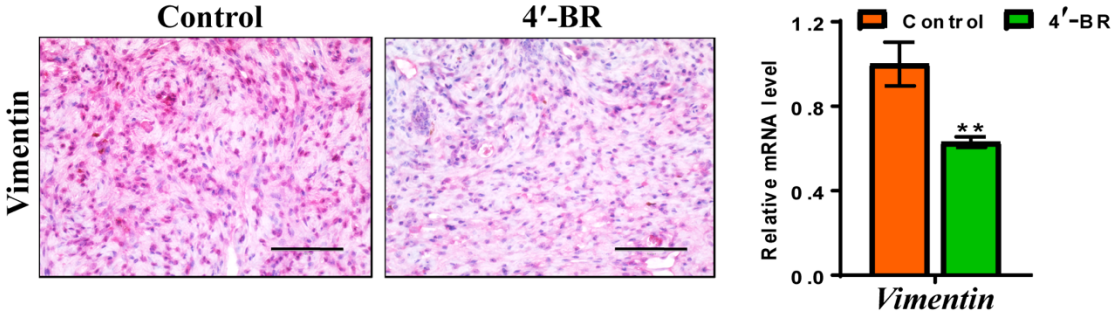
Figure 4. 4′-BR significantly modulated immune genes/proteins that are interconnected and have supporting or suppressing roles in melanoma. Network analysis and IHC analysis were carried out as described in the Materials and Methods section. (a). Representative network of all differentially expressed genes. Genes were grouped according to their main functions provided by Nanostring as well as their GO term biological process. Gene roles include innate/adaptive immune functions

(yellow/top group), basic and immune cell functions (purple/right group), chemokine/cytokines (green/left group), and cancer progression/adhesion/apoptosis (pink/bottom group). Grey lines indicate interactions. (b). Representative network of differentially expressed genes with previous evidence of being linked with melanoma. Genes were categorized according to previous evidence suggesting that they either support melanoma progression (orange) or hinder melanoma progression (blue). Downregulated genes have black solid outlines, while upregulated genes have black dashed outlines. Line color and termini indicate types of interactions: uncategorized evidence of interaction (grey), binding (purple), reaction (brown), catalysis (pink), transcriptional regulation (yellow), activation (green), inhibition (red), positive interaction (arrow), and negative interaction (bar-headed line). (c) IHC analysis of immune and inflammatory markers in 4'-BR treated BrafV600E/PtenNULL melanoma tumors. Representative images of PDL-1, IL-1β, and NLRP3 protein expression by IHC (20X magnification, scale bars = 100 μm) in a Lionheart FX automated microscope. 4'-BR: 4'-bromoresveratrol (30 mg/kg body weight).

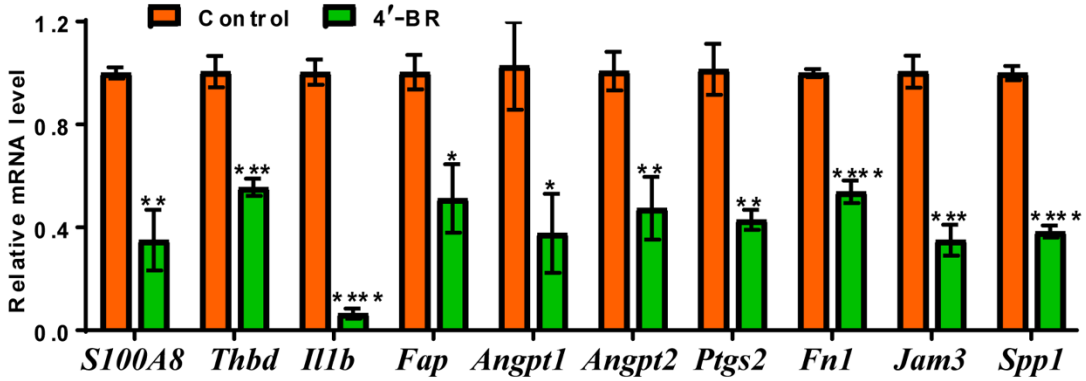
a. Lung metastasis in 4′-BR treated mice



b. IHC and RT-qPCR analysis of vimentin in 4′-BR treated melanoma tumors



c. RT-qPCR validation of melanoma metastasis promotor genes



d. RT-qPCR validation of melanoma suppressor genes

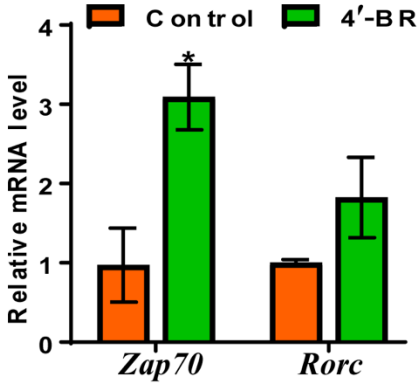


Figure 5. 4′-BR significantly reduced melanoma metastasis in *Braf*^{V600E}*/Pten*^{NULL} mice. (a) Lungs were harvested and stored in Fekete's solution at the end of study (week 17). Images of all collected lungs are shown. Data is available for 4-5 mice per group. (b) Expression of vimentin protein (by IHC, 40x magnification, scale bars = 100 μm) and mRNA (by RT-qPCR). (c) RT-qPCR validation of melanoma promotor genes identified by NanoString analysis (d) RT-qPCR validation of melanoma suppressor genes identified by NanoString analysis. Data are presented as mean ± standard error of the mean. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Each bar represents a pool of six animals, as described in the Materials and Methods section. 4′-BR, 30 mg/kg body weight of 4′-bromo-resveratrol.

TABLES

Table 1. A detailed literature analysis of the 30 genes associated with melanoma and significantly affected by 4′-BR treatment in *Braf*^{V600E}/*Pten*^{NULL} mice.

Gene	Fold change	Gene functions in melanoma
TNFSF10	3.00	TNFSF10, a proapoptotic gene, decreased in melanoma cells suggesting it contributes to better survival and growth [46]; Induces an immune response and has a very high and selective capability to kill cancer cells [47, 48].
IL12RB2	2.62	Acts as a tumor suppressor which activates immune responses and stops angiogenesis and even tumor growth [49]; In invasive cells, expression decreases due to hypermethylation, which is associated with increased tumorigenesis [50].
XCL1	2.52	High expression is correlated with good prognosis [51]; It recruits dendritic cells (DCs) to tumors [52], slow down and protect against tumor growth and even cause tumor regression [53, 54].
CCL1	2.52	Both T cells and NK cells depend on CCL1 to activate immune reactions leading to antitumor effects [55, 56].
CCL19	2.36	Presence of CCL19, an immune and inflammation-related chemokine, is significantly associated with longer survival [57]; Contributes to T cell and DCs survival as they migrate to the lymph nodes [58, 59].
RORC	2.27	Expression decreases as melanoma progress, lowest expression in advanced stages and metastasis. High expression is correlated with good prognosis [60]; A tumor suppressor which regulates local homeostasis [61, 62]. High levels lead to inhibition of melanoma *in vivo* [63].
ZAP70	2.00	An immune response gene associated with longer patient survival [64]; Activated T cells with increased levels of ZAP70 leads to T cell toxicity against melanoma [65].
FAP	-1.97	Expressed at high levels in melanoma [66, 67]; Promotes metastasis by deteriorating the ECM, which helps cancer cells disseminate and invade other tissues [68].
PTGS2	-1.97	Commonly present in advanced melanomas, increases cell proliferation, migration, and invasiveness as well as promote tumor growth and metastasis [69]; Elevated levels are

		significantly correlated with thicker melanomas as well as high proliferation and poor survival [69-71].
THBD	-2.02	Loss is associated with tumorigenesis, invasion, and metastasis [72-74]; Decreased levels are correlated with less survival [75].
F13A1	-2.04	Highly expressed in advanced tumors and functions in blood coagulation cascade and resistance to treatments [76].
C1QB	-2.06	Upregulated in blood samples of melanoma patients from all stages, especially in patient's leukocytes [77].
CD33	-2.13	$CD33^{+}$ myeloid-derived suppressor cells (MDSCs) are associated with increased risk of melanoma progression as well as less survival; A potential predictive and prognostic biomarker for advanced melanoma [78-80]
TLR2	-2.14	Expressed in melanoma cells [81]; Activates chemokines, cytokines, dendritic cells, and tumor-associated macrophages [82].
GPR183	-2.15	Upregulated in metastatic melanoma [83, 84]; High levels decrease immune responses, increases cell proliferation, and activate B-cell migration [85].
ANGPT1	-2.19	Play important role in angiogenesis and tumor development [86]; However, ANGPT1 deficient mice exhibited increase in lung metastasis of B16F10 melanoma cells [87].
SERPING1	-2.27	Forms part of a network formed by immune-related genes which in melanoma is dysregulated [88].
JAM3	-2.36	Associated with cell invasion and metastasis [89]; Expressed in metastatic melanoma cell lines [90].
COL3A1	-2.36	Mutation in *Col3a1* gene is linked to melanoma progression and less survival [91].
SPP1	-2.36	*Spp1* gene is mutated in melanoma [92]; Consistently upregulated in metastatic stages [93].
ANGPT2	-2.40	Stimulates angiogenesis [94-96]; A tumor promoter that can be used as a predictive and prognostic biomarker in melanoma; High expression is correlated with less survival; involved in resistance to treatments [97].
ITGAM	-2.43	Linked with more risk of melanoma [98].
S100A8	-2.58	Enhances melanoma carcinogenesis [99]; Supports metastatic progression [100, 101]; Higher expression in metastatic than primary melanoma [102].
CCR5	-2.68	Associated with cancer progression and EMT with higher expression in melanoma cells [103, 104]; Decreasing levels have inhibitory effects on primary tumors as well as

		metastasis [104]; Stimulates MDSCs when inflammation occurs during melanoma progression [103, 105].
FN1	-3.20	Regulates EMT and blocks apoptosis; Promotes melanoma metastasis [29, 106].
CCR1	-3.40	Present in melanocytes and melanoma cells; Expression increases with melanoma progression [107].
NLRP3	-4.25	Enhances tumorigenesis [37]; Activates IL-1β secreted by advance stage melanoma cells [108]; Inhibition diminishes migration and invasion [36].
OSM	-5.12	In both early and advanced melanoma tumors and cell lines, this cytokine has tumor suppressive functions [109, 110]; Decreases cell adhesion and invasion both *in vitro* and *in vivo* in mouse metastatic cells [111].
IL21R	-8.00	A tumor suppressor gene progressed to clinical trials in metastatic melanoma for a plausible treatment [112]; Can be used in combination with chemoimmunotherapy to improve the treatment response by increasing the tumor-specific T cells [113].
IL1B	-8.19	Expression increases with melanoma progression [35]; Upregulated in metastatic melanoma [38].

Genes are ordered by descending values of fold change.

SUPPLEMENTARY MATERIALS

Supplementary Materials and Methods

ProteinSimple assay setup

After protein isolation and quantification, the following reagents were prepared according to manufacturer's protocol: dithiothreitol (DTT), 5X fluorescent master mix, biotinylated ladder, and luminol-peroxide solution. The samples were prepared by mixing 1-part 5X fluorescent master mix and 4-parts of protein lysate diluted in 0.1X sample buffer. Next, the protein samples were denatured by heating them at 95 °C for 5 min. Primary antibodies (SIRT1 and SIRT3) were optimized following manufacturer's protocols and used at 1:10 for SIRT1 and 1:50 for SIRT3. Afterwards, the biotinylated ladder, prepared protein samples (with equal concentrations of protein per sample), antibody diluent, primary antibodies, streptavidin horseradish peroxidase (HRP), HRP-conjugated secondary antibodies, luminol peroxide solution, and wash buffer were dispensed into designated wells in a Protein simple microplate (12-230kDa). Following plate loading, default settings were used (375V; primary antibody incubation 30 minutes; secondary antibody incubation 30 minutes) to run the Wes plate. Separation and immunodetection were carried out and the resulting chemiluminescent was detected at multiple exposure times. Quantitative data analysis was carried out with the Compass for Simple Western software (ProteinSimple, San Jose, CA) which is built-in with for Protein Simple Wes. For each sample, the electropherograms showed a peak area value which represents protein expression and this value was normalized with the total capillary area of the appropriate sample using the Total Protein Assay.

ProteinSimple assay optimization

Primary antibodies (SIRT1 and SIRT3) were optimized following manufacturer's instructions before running the experimental immunoassay. Briefly, a three-by-three matrix was carried out, meaning that at least three antibody dilutions at 5-fold difference (e.g. 1:10, 1:50, and 1:250) and three different lysate concentrations at 5-fold difference (e.g. 1.2µg/µL, 0.242µg/µL, and 0.0482µg/µL) were tested. In order to select the best conditions for the lysate and antibody of interest, different factors were taken into consideration, for example, antibody saturation, baseline was less than 20% of the peak area, and luminol/peroxide signal was not burnout. After optimal conditions were selected, immunoassay was carried out alongside a total protein assay. Furthermore, since Protein simple is a quantitative assay, we validated that the peak area values in the immunoassay were within the dynamic linear range of the assay, meaning that the experimental values were between the lowest and highest values obtained during the optimization step.

Microscopy of IHC slides

Ki-67 immunostaining of tumor cells in each slide was assessed by taking a random representative Olympus DP71 photomicrograph at 400X magnification (40X objective and 10X ocular) on an Olympus BX53 microscope. Each image had dimensions of 1360 x 1024 pixels. Images were then downsampled by cropping one quadrant (25%) of the aforementioned images, and the downsized images were printed. Positive cells were considered cells with bright red nuclear staining for Ki67. Stromal cells were excluded from counting. Cells were counted manually in the entire printout, crossing out each cell as it got counted. The percent of Ki-67-positive cells was expressed as positive tumor

cells divided by total tumor cells with mean±SEM for each group (n=3). For PCNA, vimentin, survivin, and IGFBP5 we used EVOS XL microscope (ThermoFisher Scientific, Waltham, MA) and images were captured at 40X. For PDL-1, IL-1β, and NLRP3, the Lionheart FX automated microscope (BioTek, Winooski, VT) was used at 20X.

Supplementary Figures

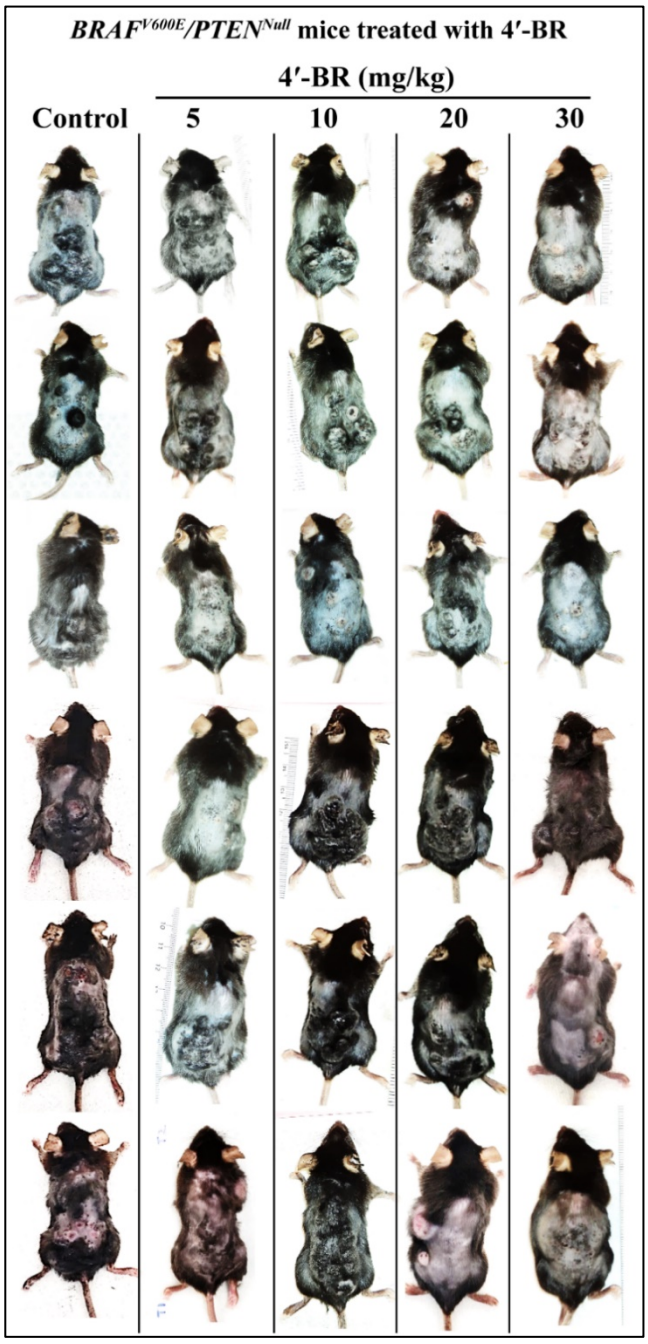


Figure S1. 4′-BR reduced melanoma tumors in *Braf*^{V600E}*/Pten*^{NULL} mice. Images of *Braf*^{V600E}*/Pten*^{NULL} mice after treatment with 4′-BR (5-30 mg/kg body weight intraperitoneally; 3d/week for 5 weeks) from each group (n=6) at the end of the study (week 17).

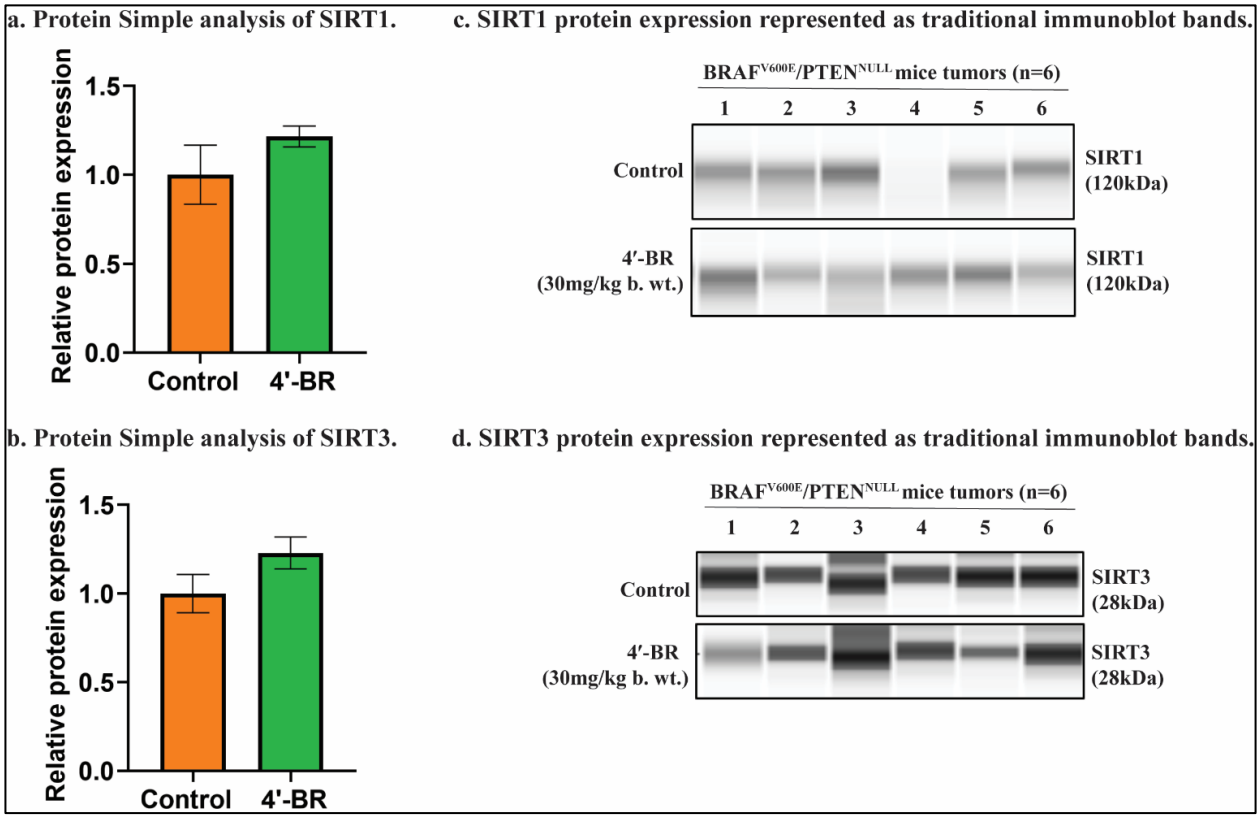


Figure S2. SIRT1 and SIRT3 protein expression is not affected by 4'-BR treatment. Quantitative immunodetection analysis of SIRT1 and SIRT3 expression by Protein Simple in treated *Braf*^{V600E}*/Pten*^{NULL} melanoma tumors. (a) SIRT1 and (b) SIRT3 relative protein expression in control and 4'-BR treated mice. (c) SIRT1 and (c) SIRT3 protein expression in control and 4'-BR treated mice represented like traditional immunoblot bands. Data are presented as mean ± standard error of the mean. Each bar represents a pool of 6 animals, as described in the Materials and Methods section. 4'-BR: 4'-bromoresveratrol (30mg/kg body weight).

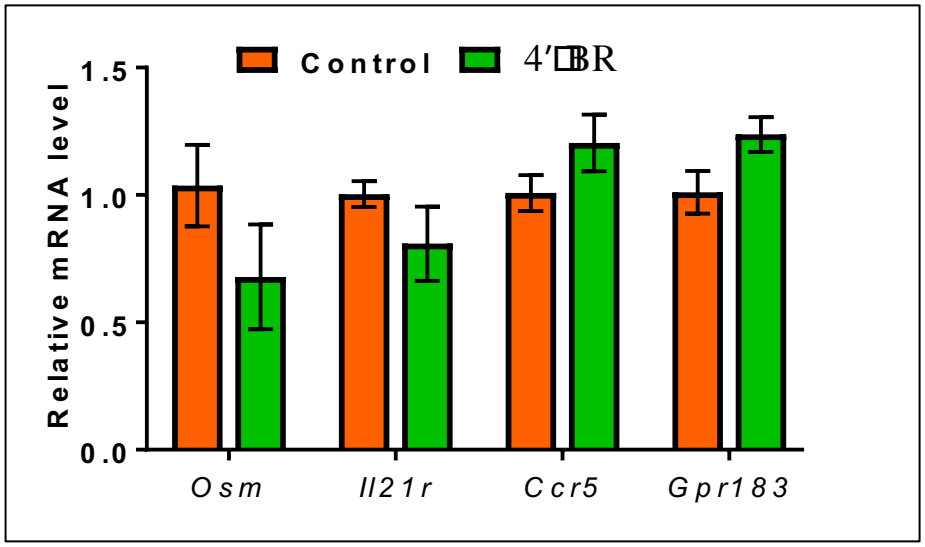


Figure S3. Non-significantly modulated gene set after 4′-BR treatment in *Braf*^{V600E}/*Pten*^{NULL} melanoma tumors. RT-qPCR analysis of four genes obtained from NanoString data of 4′-BR treated tumors showed non-significant changes in expression when compared to control tumors. Data are presented as mean ± standard error of the mean. Each bar represents a pool of six animals, as described in the Materials and Methods section. 4′-BR: 4′-bromo-resveratrol (30 mg/kg body weight).

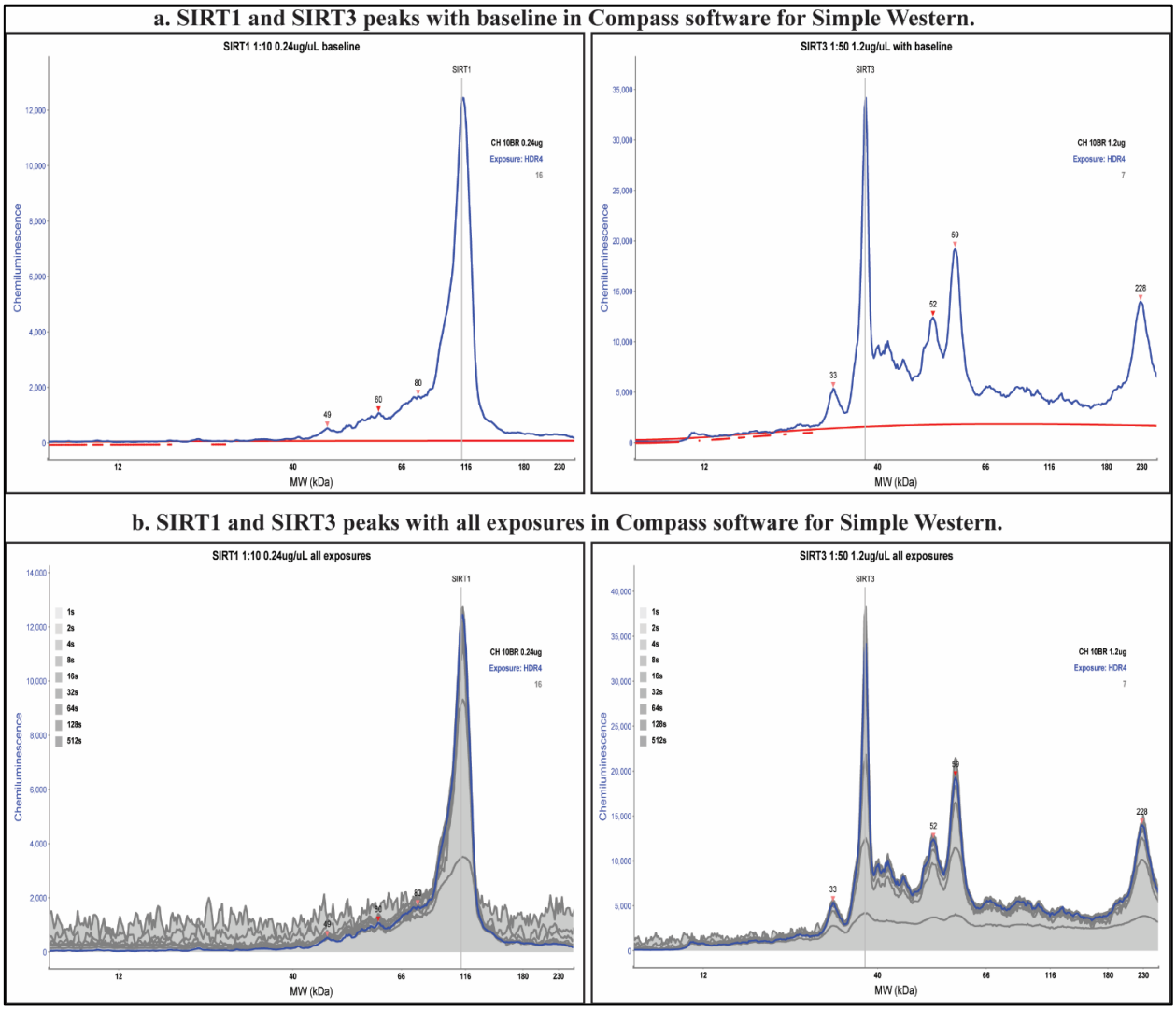


Figure S4. Protein Simple optimization for SIRT1 and SIRT3. Representative electropherograms for SIRT1 (left) and SIRT3 (right) at the indicated antibody dilutions (1:10 and 1:50) and lysate concentrations (0.24 µg/µL and 1.2 µg/µL), respectively. (a) Electropherograms showing predominant peak (blue) for SIRT1 or SIRT3 together with baseline representing antibody background (red). (b) Electropherogram showing predominant peak (blue) for SIRT1 or SIRT3 with all exposures (grey).

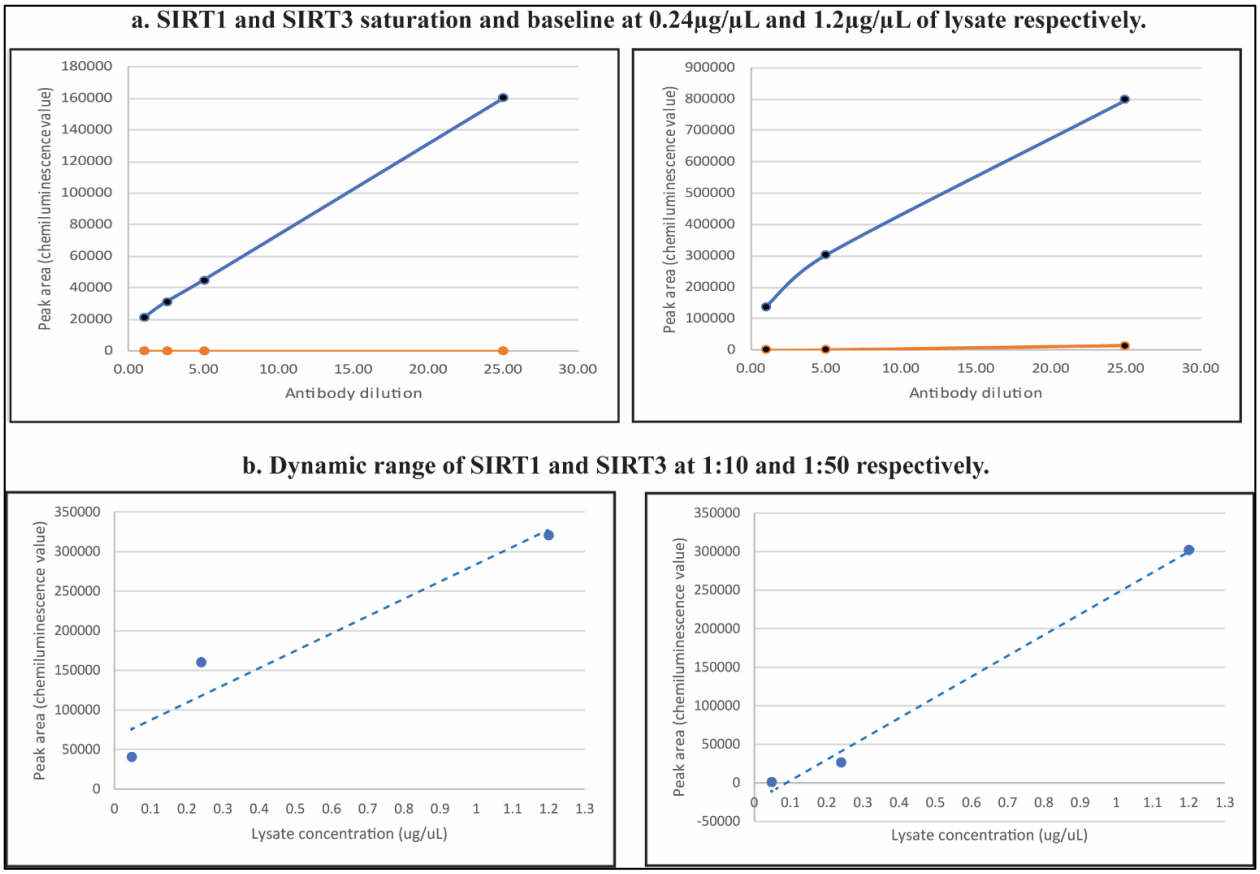


Figure S5. ProteinSimple optimization calculations for SIRT1 and SIRT3. Representative graphs of SIRT1 (left) and SIRT3 (right) at the appropriate lysate concentrations and antibody dilutions. (a) Antibody saturation and baseline of SIRT1 and SIRT3 at 0.24µg/µL and 1.2µg/µL of lysate concentration, respectively. (b) The dynamic range of SIRT1 antibody at 1:10 and SIRT3 antibody at 1:50 each with three different lysate concentrations. For graphs a and b, each dot represents an antibody dilution and are represented as 25 (1:10), 5 (1:50), 2.5 (1:100), and/or 1 (1:250). For graph c, each dot represents a lysate concentration (1.2µg/µL, 0.24µg/µL and 0.048µg/µL).

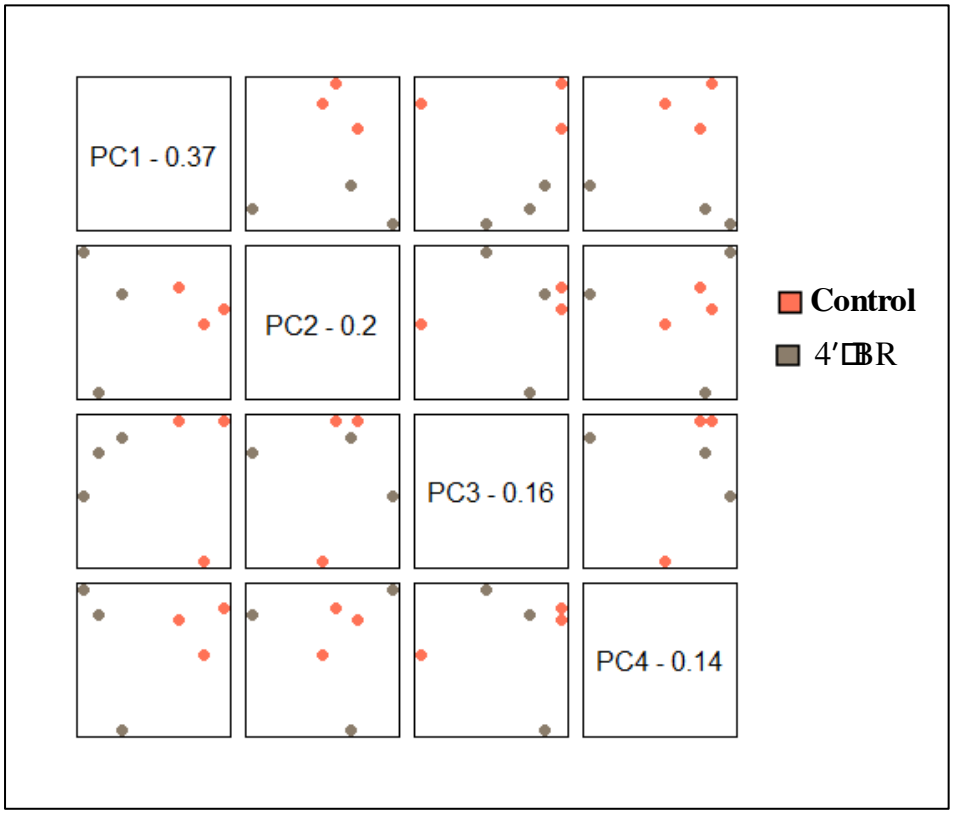


Figure S6. Principal component analysis (PCA) of the gene expression data using NanoString technology. The principal components of the gene expression data are plotted against each other and colored by the values of the selected covariate. 4′-BR: 4′-bromo-resveratrol (30 mg/kg body weight).

Supplementary Tables

Table S1. Lysate and antibody conditions used for Protein Simple in a 12-230kDa microplate.

Antibody	Supplier	Catalog Number	Antibody Dilution	Lysate Concentration
SIRT1	Cell Signaling	9475	1:10	0.24ug/uL
SIRT3	Proteintech	10099-1-AP	1:50	1.2ug/uL

Table S2. Antibodies used for immunohistochemistry (IHC).

Target protein	Supplier	Catalog Number	IHC Dilution
Ki-67	Cell Signaling	12202	1:400
PCNA	Invitrogen	PA5-27214	1:500
Survivin	Cell Signaling	2802	1:500
Vimentin	Cell Signaling	5741	1:500
IGFBP5	Invitrogen	PA5-37369	1:200
PDL-1	Proteintech	17952-1-AP	1:1200
IL-1β	Novus Biologicals	NB600-633	1:100
NLRP3	Novus Biologicals	NBP2-12446	1:50

Table S3. Primer sequences used for RT-qPCR validation.

Gene	Amplicon size (bp)	Orientation	Sequence (5'-3')	Length (bp)	Tm (°C)	Primer Bank ID
Pcna	135	F	TTTGAGGCACGCCTGATCC	19	62.3	7242171a1
		R	GGAGACGTGAGACGAGTCCAT	21	63	
Survivin	114	F	GAGGCTGGCTTCATCCACTG	20	62.6	59859886c1
		R	ATGCTCCTCTATCGGGTTGTC	21	61.1	
Igf1	142	F	GTGGATGCTCTTCAGTTCGTGTG	23	61	*Origene Gene ID 16000*
		R	TCCAGTCTCCTCAGATCACAGC	22	60.6	
Igfbp5	154	F	CCAAGCACACTCGCATTTCC	20	61.9	70909321c2
		R	CCTTGTTCGGATTCCTGTCTCAT	23	61.9	
Nrf2	153	F	TAGATGACCATGAGTCGCTTGC	22	62.1	76573877c2
		R	GCCAAACTTGCTCCATGTCC	20	61.6	
Keap1	104	F	TGCCCCTGTGGTCAAAGTG	19	62.1	7710044a1
		R	GGTTCGGTTACCGTCCTGC	19	62.7	
Vim	124	F	CGGCTGCGAGAGAAATTGC	19	61.8	31982755a1
		R	CCACTTTCCGTTCAAGGTCAAG	22	61.3	
S100a8	165	F	AAATCACCATGCCCTCTACAAG	22	60	7305453a1
		R	CCCACTTTTATCACCATCGCAA	22	60.9	
Thbd	117	F	CACAGGCAGTCAATGCGTG	19	61.7	215490000c1
		R	GAGCGCACTGTCATCAAATGT	21	61.2	
Il1b	116	F	GAAATGCCACCTTTTGACAGTG	22	60.2	118130747c1
		R	TGGATGCTCTCATCAGGACAG	21	61	
Fap	87	F	GTCACCTGATCGGCAATTTGT	21	60.9	118131069c1
		R	TCGTAGATGTAGTATGTCGCTGT	23	60.4	
Angpt1	198	F	TGCACTAAAGAAGGTGTTTTGCT	23	60.9	118130300c2
		R	CCGGTGTTGTATTACTGTCCAA	22	60	

Angpt2	123	F	CAGCCACGGTCAACAACTC	19	61	118131014c1
		R	CTTCTTTACGGATAGCAACCGAG	23	60.7	
Ptgs2	124	F	TGCACTATGGTTACAAAAGCTGG	23	61	118130137c3
		R	TCAGGAAGCTCCTTATTTCCCTT	23	60.8	
Fn1	124	F	ATGTGGACCCCTCCTGATAGT	21	61.5	1181242a1
		R	GCCCAGTGATTTCAGCAAAGG	21	61.8	
Jam3	100	F	CTGCGACTTCGACTGTACG	19	60.2	12963613a1
		R	TTCGGTTGCTGGATTTGAGATT	22	60.2	
Spp1	90	F	AGAGCGGTGAGTCTAAGGAGT	21	61.8	323668334c3
		R	TGCCCTTTCCGTTGTTGTCC	20	63	
Zap70	151	F	CGTGCGCTTCCACCATTTC	19	62	31981915c1
		R	GTTACACGGCTTACGCAGGT	20	62.5	
Rorc	137	F	GACCCACACCTCACAAATTGA	21	60.2	6755344a1
		R	AGTAGGCCACATTACACTGCT	21	60.9	
Osm	127	F	CCCGGCACAATATCCTCGG	19	62.2	257796227c3
		R	TCTGGTGTTGTAGTGGACCGT	21	62.8	
Il21r	144	F	GGCTGCCTTACTCCTGCTG	19	62.4	11230786a1
		R	TCATCTTGCCAGGTGAGACTG	21	61.5	
Ccr5	158	F	TTTTCAAGGGTCAGTTCCGAC	21	60.2	31542356a1
		R	GGAAGACCATCATGTTACCCAC	22	60.4	
Gpr183	128	F	ATGGCTAACAATTTCACTACCCC	23	60.3	33942116a1
		R	ACCAGCCCAATGATGAAGACC	21	62.1	
Actb	154	F	GGCTGTATTCCCCTCCATCG	20	61.8	6671509a1
		R	CCAGTTGGTAACAATGCCATGT	22	61.1	

DATA AVAILABILITY

Datasets related to this article (NanoString PanCancer Immune Profiling Panel) can be found at NCBI's Gene Expression Omnibus (Edgar et al., 2002)and are accessible through GEO Series accession number GSE168564. (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE168564).

CREDIT STATEMENT

Conceptualization: GC, CS, MN, NA; Data curation: GC, CS, GG; Formal Analysis: GC, CS, MN, GG, KI; Funding Acquisition and Resources: NA; Investigation: GC, CS, GG, NA; Methodology: GC, CS, MN, GG; Supervision: NA; Validation: GC, CS, GG; Visualization: GC, CS, GG; Writing - Original Draft Preparation: GC, CS, GG; Writing - Review and Editing: GC, CS, GG, MN, KI, NA.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Chapter 4: Concluding Remarks and Future Directions

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The findings of this doctoral thesis project provide insight into the role and mechanism of mitochondrial sirtuin 3 (SIRT3) in melanoma. Using Inform analysis, we validated that SIRT3 is overexpressed in melanoma tissues and additionally demonstrated that there is a stage-dependent increase of this sirtuin. Furthermore, we carried out different SIRT3 inhibitory approaches, including CRISPR/Cas9-mediated SIRT3 KO, as well as treatment with SIRT3 activity inhibitors: 3-TYP (specific to this sirtuin) and 4'-BR (dual SIRT1 and SIRT3 inhibitor). We found that both genetic and pharmacological SIRT3 downregulation leads to antiproliferative effects in melanoma cells (A375 and G361), particularly decrease in cell viability (RealTime Glo assay) and colony formation ability (clonogenic survival assay). This provides further evidence that SIRT3 may have tumor promoting functions in melanoma development and progression.

Furthermore, we wanted to further elucidate the mechanism of SIRT3 in melanoma. Therefore, we carried out immunodetection techniques in 1) melanoma tissues to measure SIRT3 protein levels as well as proposed SIRT3 interactors in other molecular settings and 2) melanoma cells after CRISPR/Cas9-mediated SIRT3 KO. We did not find any correlation between SIRT3 and Ku70, BAX, p21, p53, nor acetylated-p53. Therefore, we undertook differential gene expression analyses, using PCR array and NanoString analysis, where hundreds of cancer-related genes were evaluated to explore the effects of SIRT3 genetic inhibition in melanoma cells. We found around 60 genes that were significantly modulated after SIRT3 modulation between the two cell lines. Nonetheless, there were differing results between A375 and G361 cells, and while most

of the literature-based evidence showed that effects pointed towards being anticarcinogenic, other modulations are suggested to promote carcinogenesis.

Consequently, we selected 4'-BR to carry out additional experiments, since it targets both SIRT3 as well as SIRT1, both of which have been shown to have proliferative effects in melanoma. We found that this compound hinders melanoma cell proliferation, and these responses are observed in shorter time points than 3-TYP and SIRT3 CRISPR KO, suggesting 4'-BR might have more potent effects against melanoma. Furthermore, treatment with 4'-BR in a melanoma relevant mouse model ($BRAF^{V600E}$/$PTEN^{NULL}$) led to antitumorigenic effects include reduction in tumor size and volume as well as lower expression of proliferation markers. Moreover, we observed a decrease in metastasis to the lungs along with the levels of metastatic markers. Additionally, we carried out molecular profiling, by NanoString analysis of cancer-immune genes, to evaluate the effects of 4'-BR treatment in melanoma tumors. We found that the treated tumors downregulated genes involved in chemokine/cytokine-regulation, innate/adaptive immune functions as well as metastasis-promotion, further validating the robust therapeutic potential of SIRT1 and SIRT3 inhibition in melanoma.

To further evaluate the role and mechanism of SIRT3 in melanoma, additional studies need to be conducted. For example, validate the differentially expressed genes after CRISPR/Cas9-mediated SIRT3 KO, which were found in both PCR array and Nanostring analysis. This can be done by employing molecular techniques like RT-qPCR, immunoblotting (e.g., Western Blot, immunocytochemistry, etc.), among others. In addition, the effect of SIRT3 inhibition in melanoma can be explored in other cellular

pathways including metabolism, mitochondrial functions, production of ROS, apoptosis, EMT, among others.

Other potential experiments that can be conducted to further advance this project include exploring the *in vivo* effects of 3-TYP treatment and/or CRISPR/Cas9-mediated SIRT3 KO in melanoma-relevant animal models (e.g., $BRAF^{V600E}$/$PTEN^{NULL}$). Particularly, since we showed that 4'-BR treatment led to strong antimelanoma effects, it would be interesting to assess if this response is mainly due to SIRT1 or SIRT3 inhibition, or the combination of both. This could be carried out by monitoring melanoma development and progression in genetically engineered $BRAF^{V600E}$/$PTEN^{NULL}$ mice with SIRT1 and/or SIRT3 knockout.

Lastly, considering only a small percentage of the currently identified sirtuin inhibitors/activators have melanoma applications, a broader range of chemical sirtuin modulators should be tested both *in vitro* and *in vivo*. Our data suggested that taking an approach that simultaneously inhibits two sirtuins leads to stronger antimelanoma effects than inhibiting a single sirtuin. Therefore, compounds that can target more than one sirtuin (e.g., JH-T4, KPM-2, MC2494, YC8-02) or even a pan-sirtuin inhibitor could have the best therapeutic potential in melanoma.

Appendixes

Appendix 1: The sirtuin 6: An overture in skin cancer

Status: Published

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ABSTRACT

In the recent past, the sirtuins have been under intense investigation for their roles in biology and disease, including cancer. The sirtuin SIRT6 is comparatively a lesser studied member of this family of seven proteins. Like certain other sirtuins, SIRT6 is emerging to have an oncogenic function as well as tumor suppressor roles in cancer. Limited studies have been conducted assessing the role and functional significance of SIRT6 in melanoma and non-melanoma skin cancers. In this review, we have attempted to critically dissect the potential role and significance of SIRT6 in skin carcinogenesis. With limited available information to date, SIRT6 appears to have a pro-proliferative function in non-melanoma skin cancers (NMSCs), including squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). In addition, SIRT6 is also emerging to have an oncogenic function in melanoma. Moreover, we have provided information regarding the available SIRT6 inhibitors. Conclusively, it appears that additional comprehensive studies are needed to establish the role of SIRT6 in skin biology and skin diseases, including cancer. Further, concerted efforts are needed to characterize the stage-specific role of SIRT6 in skin cancers.

INTRODUCTION

Over the past decade, the role of Sirtuin-6 (SIRT6) in cellular processes has been rapidly evolving. Researchers have become interested in defining the role of this sirtuin in important cellular functions and disease conditions, including metabolism, aging, and cancer. SIRT6 is one of seven members of the histone deacetylase (HDAC) class III, sirtuin clan (SIRT1-SIRT7), an ancient family of proteins that have been conserved through evolution from prokaryotic to eukaryotic cell types. Sirtuins were first discovered in yeast as the Silent Information Regulator 2 (SIR2), which functioned as an essential gene silencing complex [1]. The catalytic mechanism of sirtuins differs from the rest of the HDACs due to nicotinamide adenine dinucleotide (NAD+) requirement as an enzymatic co-factor in order to catabolize the deacetylation of a given substrate [2] . The key feature of NAD+ requirement facilitates the function of sirtuins in cellular metabolism and the modulation of histones and transcription factors. Each sirtuin is characterized by an amino acid catalytic core domain of approximately 275 amino acids and also by unique N- and/or C-terminal sequences of variable length.

Interestingly, each sirtuin is biologically unique based on their cellular location, and thus, their functions (***Figure 1***). Moving from the nucleus to the cytoplasm, sirtuins participate in critical cellular functions, thereby regulating a variety of mechanisms for cellular homeostasis. In mammals, sirtuins have shown to undergo nuclear-cytoplasmic shuttling, where their functions within the cell are suggested to change depending on each tissue and cell type [3]. For instance, SIRT1, 6, and 7 are actively located in the nucleus, accompanied by the roles they each possess in DNA repair, transcription, and epigenetics[3]. SIRT1, as well as SIRT2, can function in both the nucleus and cytoplasm

[3]. SIRT2 is mostly found in the cytoplasm, where it performs key functions associated to microtubule stabilization, gluconeogenesis, and inflammatory response [4]. SIRT3, 4, and 5 are described as mitochondrial sirtuins, based on their localization and observed roles in cellular metabolism [2]. Altogether, despite the structural similarities among the sirtuins, each sirtuin has its own role, regulating critical mechanisms within the cell that help maintain homeostasis.

Extensive studies have been undertaken in order to understand the function of sirtuins in different biological systems. Nonetheless, a deeper understanding of the mechanisms underlying the functions of each sirtuin, in distinct biological processes, remained unexplored. Formerly, SIRT6 was only studied as a chromatin regulator, now as a nascent topic of study, more researchers have become interested in defining the role of SIRT6 in cancers, including skin neoplasms. It is critical to explore the mechanistic workings of SIRT6 in skin, since it may be a key regulator to engendering strategies that may have significant impact in the field of cutaneous biology and towards development of new therapeutic approaches for skin diseases and skin cancers.

ESSENTIAL BIOCHEMICAL FUNCTIONS OF SIRT6

Elucidating the specific molecular and biochemical roles of SIRT6 is challenging, mostly due to its three distinctive enzymatic activities in histone-deacetylation [5] , adenosine diphosphate (ADP)-ribosylation [6] and most recently found lysine defatty-acylation (***Figure 2***) [7]. Typically, SIRT6 has been shown to deacetylate targets sites on histone 3 (H3), specifically lysines H3K9ac, H3K18ac, and H3k56ac [5]. Observations of SIRT6 auto-mono-ADP-ribosylation were first observed when an incubation with NAD was conducted *in vitro* [6]. This was followed by reports of SIRT6 dependent mono-ADP ribosylation of poly adenosine diphosphate-ribose polymerase 1 (PARP1) polymerase and Kruppel-associated box associated protein-1 (KAP1) [8, 9]. These reports suggested that the diverse enzymatic activities conducted by SIRT6 could be specific to certain substrates as well as physiological conditions. Intriguingly, new studies in SIRT6 biology highlighted that SIRT6 can also efficiently catalyze the removal of certain long-chain fatty-acyl groups from lysine *in vitro*, suggesting additional important physiological functions conducted by SIRT6. Initial studies detected defatty-acylation activity of SIRT6 in *in vitro* assays on tumor necrosis factor alpha (TNF-α), lysine K19 or K20, and H3 peptides with 6- to 16-carbon chain fatty-acyl groups on lysine K9 [10, 11]. Recently, SIRT6 was shown to remove long chain octenoyl-groups on H3K9, H3K18 and H3K27 [12]. Nonetheless, a deeper understanding of SIRT6 substrate specific activities is still needed in order to link these processes to important cellular functions.

SIRT6 CONNECTION BETWEEN AGING AND CANCER

Because of the important biochemical functions of SIRT6 in biological systems, SIRT6 plays a critical role as a modulator of transcription factors that are associated with important cellular functions such as telomere maintenance, inflammation, metabolism, disease development and aging (e.g., nuclear factor-kappa B (NF-κB), hypoxia inducible factor 1 subunit alpha (HIF-1α), and cellular-Myelocytomatosis (c-Myc)) (***Figure 2***) [5]. Unfortunately, aging has been linked to the development of various diseases, including cancer [13]. The likelihood of developing neoplasms increases with aging. Nevertheless, aging as a process remains complex, as it encompasses a variety of factors [13]. These factors include elements that affect lifespan, cellular changes, and longevity [14]. Because of the role of SIRT6 in longevity, it may be viewed as a potential connection between aging and cancer. Throughout the years, sirtuins have been associated with epigenetic changes that maybe linked with aging and cancer. Indeed, loss of genomic stability is an important aspect of cancer. In an important study, Tennen and colleagues demonstrated a role for SIRT6 in regulating an ageing-associated epigenetic silencing process and provided evidence suggesting that SIRT6 is required for maintenance of telomere position effect in human cells [15]. In another study, Michishita and colleagues proposed that SIRT6 contributes to the propagation of a specialized chromatin state at telomeres, required for proper telomere metabolism and function. This study linked chromatin regulation by SIRT6 to telomere maintenance and organismal aging [16]. In another recent study, Tasselli *et al* demonstrated that SIRT6 deacetylates H3K18ac at pericentric chromatin to prevent mitotic errors and cellular senescence [17]. Their study unraveled a new function for SIRT6 that provides a connection between SIRT6 and aging-

and cancer- related dysfunction in mammalian cells. Moreover, Endisha *et al* showed that reduced levels of SIRT6 lead to Hutchinson-Gilford Progeria Syndrome (HGPS), a human premature aging disorder [18]. Both HGPS cells and SIRT6 deficient cells showed abnormalities in pericentric heterochromatin, aberrant transcription and dysfunction of telomeres [18]. Additionally, the group demonstrated that restoring SIRT6 expression in HGPS cells may partially impede senescence and the formation of dysmorphic nuclei. In fact, the authors implied that lamin A/C (LMNA) point mutation, the established main molecular pathology underlying HGPS, occurs downstream of SIRT6 events. In all, understanding the different mechanisms involved in SIRT6 heterochromatin dysfunction could lead to novel discoveries in aging associated diseases.

At organism levels, SIRT6 was shown to be necessary to maintain health and to prevent the development of several progeroid pathologies in mice [19]. SIRT6 deficiency in mice was shown to result in premature aging phenotypes leading to death at about four weeks of age [19]. A key factor in the regulation of lifespan is the insulin-like growth factor 1 (IGF-1) signaling pathway. Mostoslavsky and colleagues showed that serum IGF-1 levels were severely reduced in SIRT6 knockout (KO) mice, IGF-1 levels were low in 12 day old mice before any other phenotypes were detected proposing SIRT6 function is essential for glucose homeostasis and normal IGF-1 levels [19]. In contrast, overexpression of SIRT6 in male mice increased lifespan [20]. Interestingly, SIRT6 overexpression extended lifespan only in males, potentially by reducing IGF-1 signaling specifically in white adipose tissue (WAT) [20]. To understand the changes observed in IGF1 signaling, the authors analyzed different key targets of this pathway. The analysis encompassed the phosphorylation levels of protein-serine/threonine kinase (AKT)

activation sites (Thr 308 and Ser 473), FOXO1 (Thr 24) and FOXO3 (Thr 32). The most significantly decreased phosphorylation levels were observed in the perigonadal WAT of SIRT6 transgenic males in comparison to wildtype (WT) males. The levels of AKT on both activation sites, forkhead box protein O1 (FOXO1) and forkhead box protein O3 (FOXO3) in WAT were lower in the transgenic mice. The phosphorylation levels of the IGF-1 receptor (Tyr 1135) and S6 (Ser 235/236) were lower in SIRT6 transgenic males than in the WT male littermates. Interestingly, no significant change was found in the phosphorylation levels observed in female mice [20]. Overall, decrease in the phosphorylation levels of AKT and FOXO proteins in male SIRT6 mice demonstrated that lifespan was regulated by changes in IGF-1 signaling [20]. Thus, it would be interesting to further study the relationship between IGF-1 signaling and SIRT6 expression in additional animal models of both genders to determine whether the effect SIRT6 has on IGF-1 is strictly gender dependent or time dependent. In another important study, SIRT6 was shown to link H3K9 deacetylation to NF-κB-dependent gene expression and organismal life span [21]. SIRT6 regulates the signaling of an important target in charge of aging-associated changes in gene expression, namely NF-κB [21]. SIRT6 was shown to interact with v-rel avian reticulo-endotheliosis viral oncogene homolog A (RELA) subunit of NF-κB and deacetylate H3K9 at NF-κB target gene promoter site [21].

In the context of skin, a recent study conducted by Kim *et al* found a reduction in the expression of SIRT6, as well as Sirtuin-1 (SIRT1) in human dermal fibroblasts (HDF) [22]. SIRT6 was classified as a late aging biomarker since statistical correlation studies revealed that procollagen Types I and VII, fibrillin-1, along with SIRT6 had a strong correlation with the cellular stiffness of HDFs as the passage number of the cells

increased [22]. This was directly associated with key aging biomarkers found in fibroblasts [22]. The group proposed reduced levels of SIRT6 expression in older fibroblasts, which had a direct impact in age development of the cell [22]. In another study, it was found that SIRT6 knockdown in HDFs influence the synthesis and degradation of collagen by hyperactive NF-κB signaling, leading to a decrease in dermal collagen fibrils, suggesting a role of SIRT6 in skin anti-aging [23]. To further amplify these arguments, Sharma *et al* determined that human dermal fibroblasts, retrieved from an older human participant, possessed a higher resistance towards reprogramming by classic Yamanaka factors [24]. Nonetheless, older cells with increased levels of SIRT6 expression during reprogramming, permitted the cells to undergo the process more effectively. Further, increase expression of SIRT6 was found in the younger cells[24]. Suggesting, SIRT6 is essential for cellular development.

Overall, these findings support a potential role for SIRT6 regulating longevity and indicate that manipulation of SIRT6 levels could possibly lead to a new avenue of treatments for age-related conditions. Very limited information is available regarding the role of SIRT6 in skin aging and skin health. Additional detailed studies are needed to dissect the skin aging pathways modulated by SIRT6.

TUMOR SUPPRESSOR AND ONCOGENIC PROPERTIES OF SIRT6

The exact role of SIRT6 in cancer remains complex with available evidence for both oncogenic and tumor suppressor properties [25-29] (***Figure 3***)**.** SIRT6 has been shown to be dysregulated in several cancers. Based on published studies, SIRT6 has been shown to imparts its tumor suppressor properties by repressing transcriptional

activity of key pathways such as NF-κB, c-Myc, and HIF-1α in osteosarcoma, ovarian, liver, lung, colorectal, bladder, endometrial, and pancreatic cancers [30]. Wu *et al* demonstrated that inhibition of SIRT6 lead to cell invasion, proliferation and migration of adrenocortical carcinoma cells that was associated with upregulated toll-like receptor 4 (TLR4) and phosphorylation of NF-κB subunit p65 [31]. In another study, SIRT6 was shown to inhibit colon cancer progression by modulating phosphatase and tensin/AKT (PTEN/AKT) signaling [32]. Further, repression of N-cadherin by SIRT6 has been shown to prevent epithelial-mesenchymal transition linked to metastasis [33]. Liu *et al* found that SIRT6 inhibited colorectal cancer stem cell proliferation by targeting cell division cycle 25A (CDC25A) by reducing H3K9 levels [34].

In a very detailed study, Kugel and colleagues found that SIRT6 suppressed pancreatic ductal adenocarcinoma (PDAC) by modulating Lin28b, a negative regulator of the let-7 microRNA [35]. SIRT6 loss was shown to result in histone hyperacetylation at the Lin28b promoter, Myc recruitment, and induction of Lin28b and let-7 target genes, high mobility group at-hook 2 (HMGA2), insulin-like growth factor binding protein 1 (IGF2BP1), and insulin-like growth factor binding protein 3 (IGF2BP3). Overall, SIRT6 was suggested to be an important PDAC tumor suppressor [35]. Another study by Lin *et al*. found that the ubiquitin-specific peptidase 10 (USP10) antagonizes c-Myc transcriptional activation through SIRT6 stabilization to suppress tumor formation [36]. In this study, the downregulation of USP10 was found to activate SIRT6 instability and negatively control activity of the c-Myc oncogene that inhibits cell growth, cell-cycle progression, and tumor formation [36]. Additional studies suggested a tumor suppressor role of SIRT6 in ovarian cancer [37], non-small cell lung cancer [38], and glioma [39]. Wu

and colleagues demonstrated that the transcription factor E2F1 enhances glycolysis by suppressing SIRT6 transcription in cancer cells, suggesting a tumor suppressor function of this HDAC [40].

On the other hand, SIRT6 has also been shown to have oncogenic function via multiple mechanisms, in several cancers such as hepatic-, lung-, breast-, esophageal-, prostate- and skin- cancers. Huang *et al* demonstrated that SIRT6 plays an oncogenic role by downregulating genes involved in the NF-κB pathway as well as the phosphatidylinositol-3 Kinase-AKT (PI3K-AKT) pathway, which can also contribute to autophagy induction [41]. SIRT6 overexpression was shown to be required for the activation of transforming growth factor (TGF)-β1 and H_2O_2/HOCl reactive oxygen species (ROS) which help to facilitate tumorigenesis of hepatocellular carcinoma (HCC) cells [42]. Cagnetta and colleagues recently demonstrated that depletion of SIRT6 enhanced the vulnerability of acute myeloid leukemia (AML) cells to DNA-damaging agents [43].

Indeed, the role of SIRT6 is extremely complex and needs to be carefully investigated. A very interesting study by Elhanati and colleagues suggested that a reciprocal regulation between SIRT6 and microRNA-3122 (miR-122) controls liver metabolism and predicts hepatocarcinoma prognosis [44]. In this study, SIRT6 was shown to downregulate miR-122 by deacetylating H3K56 in the promoter region. In addition, miR-122 was shown to bind to three sites on the SIRT6 3' untranslated region (UTR) and reduces its levels. SIRT6 and miR-122 were shown to oppositely regulate a similar set of metabolic genes and fatty acid β-oxidation. In addition, loss of a negative

correlation between SIRT6 and miR-122 expression in hepatocellular carcinoma patients was shown to be is significantly associated with better prognosis [44].

Thus, overall SIRT6 plays critical roles in the regulation of tumorigenesis through different biological pathways and can either act as an oncogene or a tumor suppressor. Below, we have provided a discussion regarding the potential role of SIRT6 in skin cancers.

SIRT6 IN MELANOMA AND NON-MELANOMA SKIN CANCERS

Molecular and genetic alterations and environmental stress play an important role in the pathogenesis of skin cancers [45]. The most commonly studied forms of skin cancer are divided into two main categories: melanoma, and non-melanoma skin cancers (NMSC). Incidence of both melanoma and NMSC continue to increase [46]. One of the causes of skin cancer is the presence of alterations in oncogenes and/or tumor suppressor genes. Particularly, B-rapidly accelerated fibrosarcoma kinase (BRAF), PTEN, and TP53 are some of the important genes mutated in skin cancer [47]. These genetic modifications can cause cells to proliferate uncontrollably. NMSCs are derived from both cutaneous squamous cells and basal cells, and are further classified into basal cell carcinoma (BCC) or squamous cell carcinoma (SCC) [48]. The NMSCs are most common cancers and have been associated with significant morbidity. However, they rarely metastasize and are not a liked to high mortality among skin cancers.

The melanomas originate from melanocytes and are a dangerous type of skin cancers. It is estimated that by the end of this year 96,480 individuals will be diagnosed with melanoma [46]. In contrast to NMSC, melanoma can metastasize to other organs in

the body, making this a fatal skin cancer [47]. If not diagnosed at an early stage, the anticipated 5-year survival of melanoma patients is only 23% [46]. Moreover, approximately 7,230 Americans with melanoma will die in 2019 [46]. Ultraviolet (UV) radiation has been recognized as the most probable environmental causative factor of melanoma as well as NMSCs [49]. Melanin can act as a barrier against UV radiation, blocking their penetration into skin layers [50]. Thus, melanin production and content are factors in determining individual's susceptibility to melanoma. Indeed, metastatic melanomas are very difficult to treat, often acquiring resistance to chemotherapy. Therefore, new treatments for patients with melanoma are continuously evolving, including immunotherapies and targeted therapies [51]. Notwithstanding, these treatments are often associated with relapse of the malignancy. In the case of BRAF inhibitors, half of the patients have been shown to develop recurrence within less than 6 months [51]. Consequently, there is an urgent need to find new targets to effectively treat melanoma.

In the recent past, SIRT6 is being actively investigated for its potential role in skin cancers as well as its potential targetability for anti-skin cancer drug development (***Figure 4***). Unfortunately, limited research has been done regarding defining the role and functional significance of SIRT6 in skin cancer. SIRT6 has been shown to be upregulated in human SCC. Ming *et al* found that in keratinocytes, SIRT6 functions as an oncogene acting as a downstream target of AKT activation after UVB exposure [29]. AKT has been suggested to serve as a mediator of UVB-induced SIRT6 expression through c-fos [52], because AKT activation induces c-fos expression [53], and c-fos induces SIRT6 transcription [26]. UVB-induced cycloocygenase-endoperoxide synthase 2 (COX-2)

upregulation interferes with AKT signaling [29], in a manner that SIRT6 and AKT/COX-2 signaling form a network cascade leading to skin carcinogenesis.

Lefort *et al* demonstrated that microRNA-34a (miR-34a) suppresses SIRT6 in keratinocyte differentiation [27]. In this study, the expression of SIRT6 was found to be inversely correlated to mir-34a in keratinocyte-derived tumors and keratinocyte differentiation. The authors demonstrated that miR-34a is induced with keratinocyte differentiation, and inhibition in cutaneous SCCs can be justified through loss of p53 function [54]. Interestingly, miR-34a regulates neurogenic locus notch homolog protein 1 (NOTCH1) expression through p53. NOTCH1, is a transmembrane receptor known to regulate keratinocyte differentiation, suggesting pro-differentiation function associated with miR-34a is due to a crosstalk between NOTCH1 and p53 [54]. These studies suggest that SIRT6 is important role in keratinocytes and their differentiation, potentially acting as a pro-proliferative sirtuin in keratinocytic cancers.

Very limited information is currently available regarding the role of SIRT6 in BCC. In one study, Temel *et al* investigated the expression profile of all mammalian sirtuins in non-tumoral and tumor tissues with BCC, in a total of twenty-seven patients. Expression levels of SIRT6 mRNA were not altered in tumor tissues compared with non-tumor tissue samples [55]. Nonetheless, probably because of a lack of enough samples, this study did not focus of a stage-specific differences in the expression of SIRT6 [55]. However, the authors speculated that SIRT6 expression could increase with UVB-induced DNA damage repair in BCC. While it is possible that SIRT6 has tumor modulatory functions in BCC, additional studies are needed [55].

A few studies have been focused on defining the role of SIRT6 in melanoma. Recently, we demonstrated that SIRT6 is upregulated, both at mRNA and protein levels, in melanoma cell lines, as well as clinical tissue samples of human melanoma [56]. Further, a lentiviral short hairpin RNA (shRNA)-mediated transient knockdown of SIRT6 in human melanoma cells was found to induce cellular senescence, accompanied by a marked accumulation of cells in G_1 phase arrest [56]. In addition, our data showed that SIRT6 inhibition significantly alters genes and pathways related to autophagy in melanoma cells and reduced the conversion of of microtubule-associated protein 1a/1b-light chain 3 (LC3) protein from its free form LC3-I to phosphatidylethanolamine-conjugated form LC3-II, which is known to initiate autophagosome formation [56]. This study from our laboratory was supported by another study where Wang *et al* demonstrated that aberrant expression of SIRT6 contributes to melanoma growth [57]. The authors suggested that the effects of SIRT6 on autophagy in melanoma are mediated by IGF-AKT signaling. In melanoma, autophagy has been shown to suppress tumorigenesis by degrading oncogenic and toxic proteins at initial stages [58] or promote tumor development in established tumors by reducing cancer cell vulnerability to stress and maintaining tumor cells homeostasis [58]. Thus, based on these studies, there seems to be a positive association between SIRT6 expression and the levels of autophagy in melanoma [56, 57]. Wang *et al*, provides strong evidence supporting IGF-AKT as one of the intermediary players between SIRT6 and autophagy in melanoma, where the deacetylase activity of SIRT6 appears to be indispensable [57]. AKT has been shown to translocate to the plasma membrane upon PI3K activation, stimulating a wide range of downstream signaling targets in melanoma that regulate cell cycle, apoptosis, DNA

repair, glucose metabolism, cell growth, invasion and angiogenesis [53]. The main target of AKT is mTOR, which has a central role in PI3K-AKT pathway in cancer [53]. Thus, it would be interesting to explore the association between SIRT6 and PI3K-AKT-mTOR signaling pathway as it may serve as a new avenue for melanoma treatments.

Based on the limited number of studies done so far, SIRT6 seems to affect a number of important processes, which are relevant to cutaneous neoplasms, including BCC, SCC, and melanomas (***Figure 5***). Indeed, at the mechanistic level, a number of targets and processes have been linked with SIRT6 in the skin (***Figure 6 and Table 1***). Nevertheless, these published studies point towards a pro-proliferative role of SIRT6 in skin cancers, promoting cellular proliferation and survival [53, 56, 58].

The potential targetability of SIRT6 for the management of skin cancers is being actively investigated in our laboratory as well as by others. In another very interesting study, Strub *et al* demonstrated that haploinsufficiency of SIRT6 promotes insulin-like growth factor binding protein 2 (IGFBP2) expression via increased chromatin accessibility, H3K56 acetylation at the IGFBP2 locus, and consequent activation of the insulin-like growth factor 1 receptor (IGF1R) and downstream AKT signaling allowing melanoma cells to persist in the presence of mitogen activated protein kinase inhibitors (MAPKi) [59]. Using matched melanoma samples derived from patients receiving dabrafenib + trametinib, the authors found that IGFBP2 is a potential biomarker for MAPKi resistance [59]. This study identified not only an epigenetic mechanism of drug resistance, but also provided a rationale for a new combination therapy that could overcome resistance to standard-of-care therapy for $BRAF^{V600}$-mutant melanoma patients [59]. In contrast to SIRT6, Sirtuin-5 (SIRT5) does not improve sensitivity to BRAF inhibitors in

$BRAF^{V600}$-mutant melanoma animals [59, 60]. Given the targetable properties of SIRT6 in melanoma and non-melanoma skin cancers, the development of SIRT6 specific inhibitors accompanied by detailed molecular insights into the mechanistic and therapeutic tangibility of SIRT6 in skin is essential for the development of novel strategies to modulate skin cancer [59].

SIRT6 SPECIFIC INHIBITORS: AN AVENUE OF OPPORTUNITIES FOR DRUG DEVELOPMENT

Because of the potential oncogenic function of SIRT6 in certain cancers as well as its importance in other conditions, it has become an attractive target for the development of small molecule inhibitors. A broad range of therapeutic applications are foreseen for SIRT6 inhibitors, with potential applications in diabetes (as blood glucose downregulation agents), immune-mediate disorders, and cancers (including as chemosensitizers) [61]. Moreover, the development of these inhibitors could be used to complement the available genetic tools to embark on studies defining the biological importance of SIRT6 as well as its mechanisms, interactions, and downstream targets. Unfortunately, only a small number of SIRT6 inhibitors are currently available, and some of these inhibitors are not very well characterized, especially regarding their structure activity relationships. Pseudopeptides, cyclic pentapeptides, peptides, and thiomyristoyl peptides have been reported to inhibit SIRT6 activity; however, these lack a strong selectivity for SIRT6 *versus* other sirtuins [62, 63]. Some small molecule inhibitors, with salicylate-based structures that are not related to peptides, have been better characterized. These seem to hold

promise for their future use and towards the development of next-generation SIRT6 inhibitors [63, 64]. Some of the existing SIRT6 inhibitors are briefly discussed below.

Parenti *et al* synthesized and tested a total of twenty different compounds for their potential for SIRT6 inhibition, of which only three (*viz*. SYN17739303, BAS13555470, BAS00417531) achieved reasonable inhibitory effects at a low micromolar range (***Figure 7***) [63]. SIRT6, unlike the rest of the sirtuin family, lacks a co-factor binding loop that allows different conformations dependent on the ligands bound to the active site [65]. This distinguishing feature of SIRT6 requires the presence of modifications to the active site that interferes with the design of selective inhibitors [63]. Fortunately, the three compounds identified by Parenti *et al*, are quite promising, because of being structurally diverse and nonpeptide selective SIRT6 inhibitors. Additionally, the SIRT6/SIRT1 selectivity ratio for two of the compounds identified show an IC_{50} towards SIRT6 that is 17- and 19- fold lower than those toward SIRT1 [63]. This is very promising given the fact that deacetylase activity of purified SIRT6 protein *in vitro* is 1000-fold lower for SIRT6 when compared to SIRT1. Biologically, these compounds were shown to reduce TNF-α secretion, as should be expected based on studies regarding the role of SIRT6 in TNF-α production [63]. Moreover, a strong increase in glucose transporter 1 (GLUT1) expression was followed by an increase in glucose uptake in culture cells treated with these compounds, in accordance with the roles SIRT6 has shown to have in glucose homeostasis [63]. Interestingly, Sociali *et al* determined the pharmacological effects of one of these compounds, commercially known as SIRT6-inhibitor-compund-1 (SIRT6-IN-1) also called, 2,4,-dioxo-N-(4-(pyridine-3-yloxy)phenyl)-1,2,3,4-tetrahydroquinazoline-6-sulfonamide, on a mouse model for Type 2 diabetes mellitus in a in a mouse model (high-

fat-diet–fed animals) [66]. The data from this study demonstrated that SIRT6-IN-1 resulted in a significant improvement in glucose regulation, notable increase in glucose transporters and tryglycerides, as well as reduced cholesterol and levels of insulin [66]. Although SIRT6 has many significant biological roles, the biggest drawback, when developing inhibitors, is the identification of structural features that can be associated with SIRT6 specific inhibition. Evidently, successful development of SIRT6-specific inhibitors may lead to the development of new pharmacological applications.

CONCLUSIONS

Based on studies, in the recent past, sirtuins have emerged to be important in skin biology. The role of SIRT6 in skin biology and disease, including skin cancers is beginning to be explored. With limited available information to date, it appears that in skin neoplasms, including SCC, BCC, and melanomas, SIRT6 plays a pro-proliferative function. However, detailed investigations are needed to determine the stage specific behavior of SIRT6, during the process of skin carcinogenesis. This is mainly based on the evidence showing an early lower expression of SIRT6 in pre-cancerous keratinocytic neoplasms. It appears that there may be a connection between SIRT6 with aging and cancer, which needs to be explored in detail. Further, improved SIRT6 inhibitors are needed, which could be tested in robust pre-clinical and clinical studies. Addressing the knowledge gaps regarding the connections and association between SIRT6 and other driver pathways of neoplastic transformation, could be useful towards the management of both melanoma as well as no-melanoma skin cancers.

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FIGURES

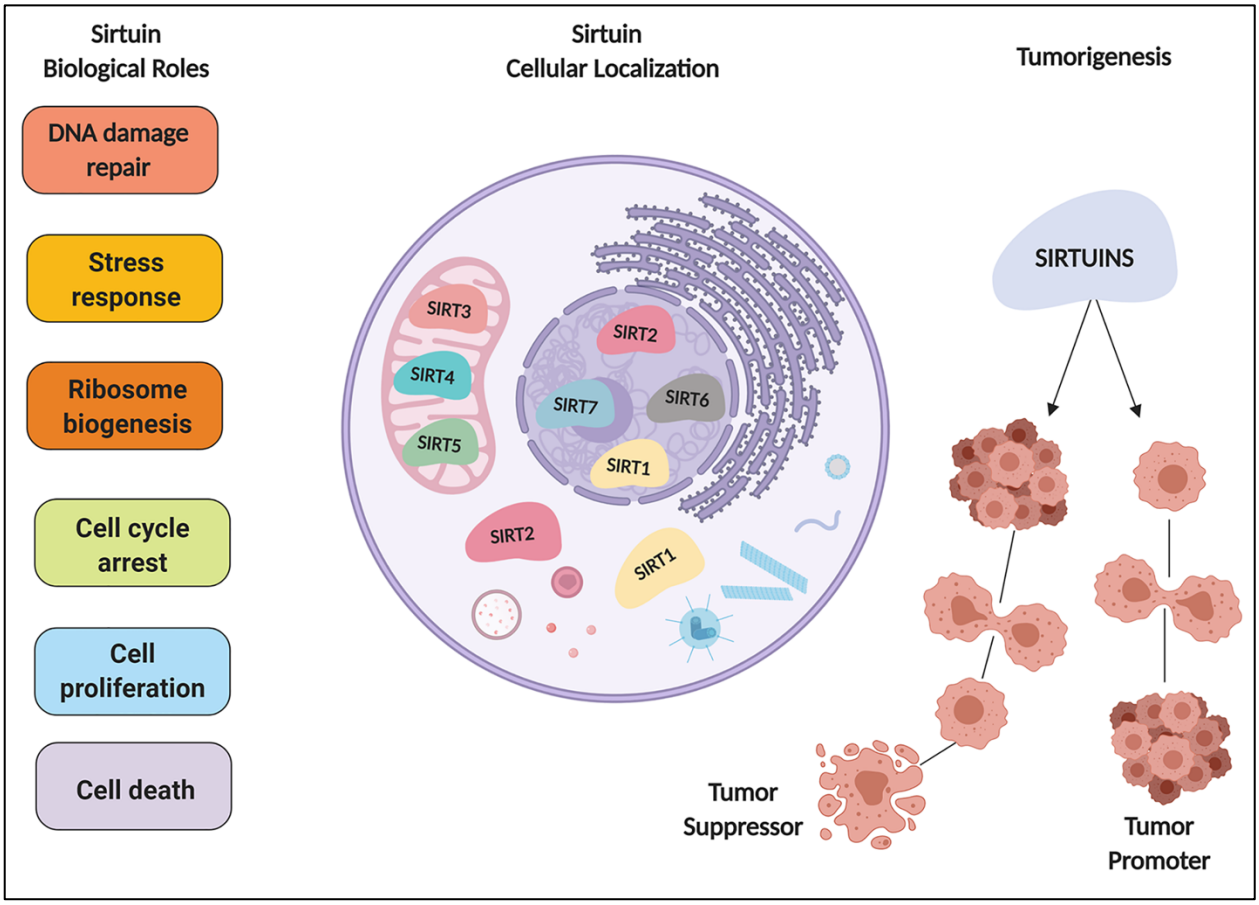


Figure 1: Sirtuin localization and function. Each sirtuin is biologically unique based on their cellular location and function. With controversial roles in cancer sirtuins possess important biological functions that are necessary for cellular homeostasis.

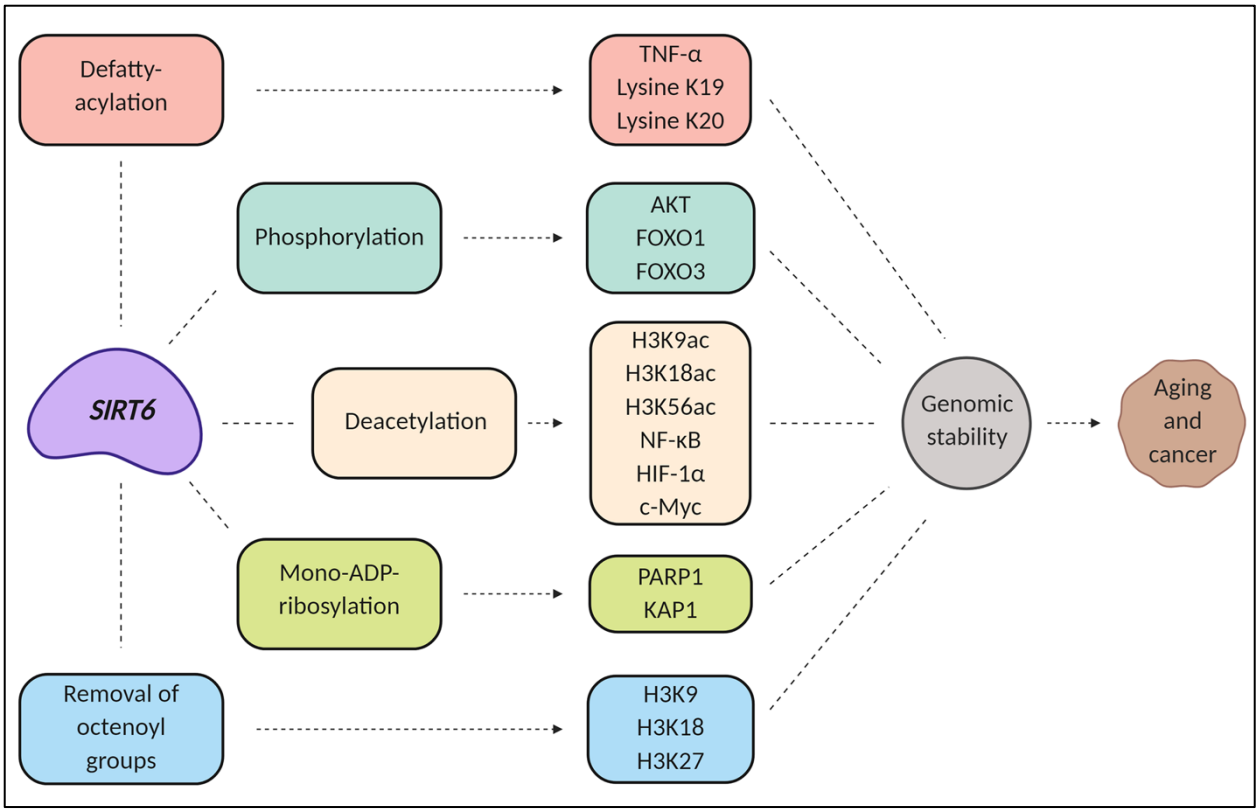


Figure 2: SIRT6 biochemical functions associated with aging and cancer. SIRT6 interacts with multiple molecular targets that are involved in aging and potentially carcinogenesis.

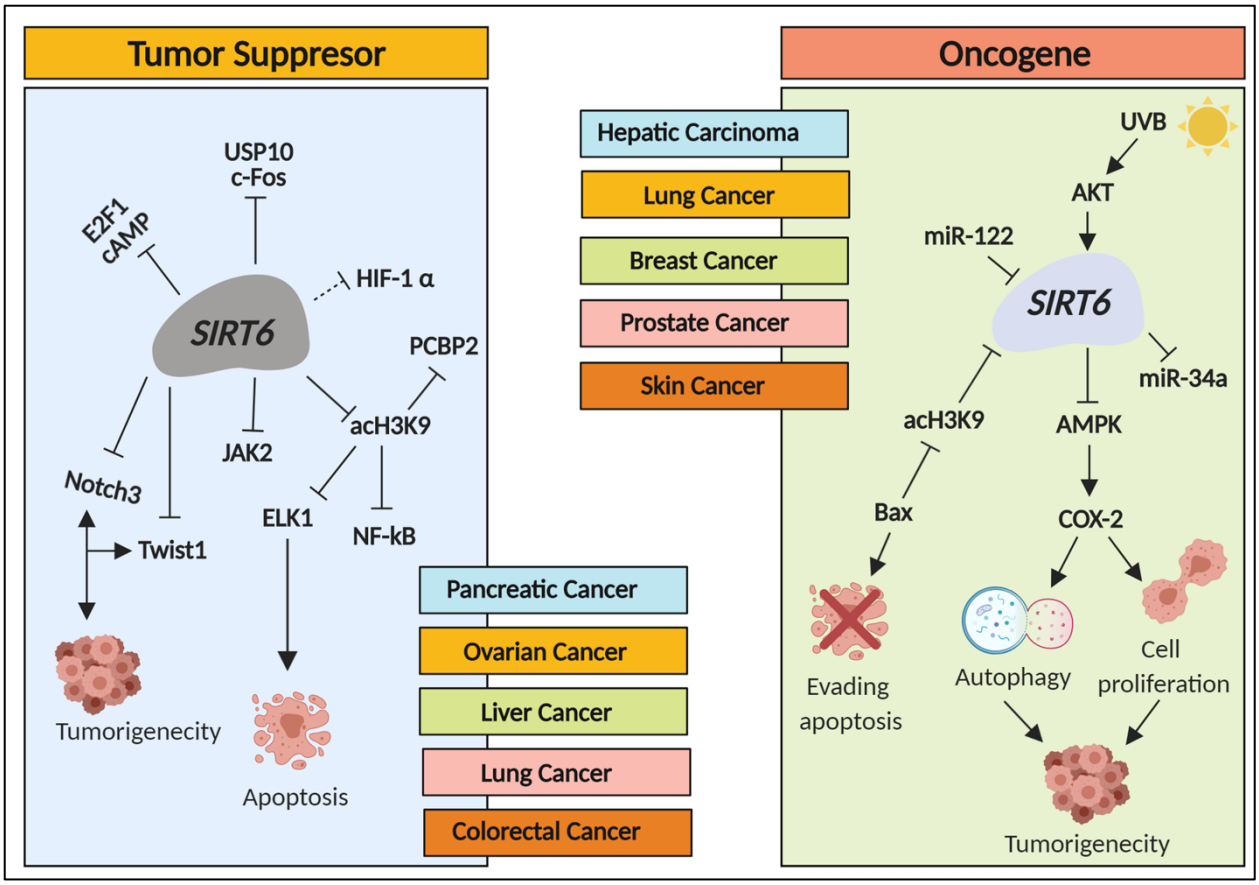


Figure 3: SIRT6 tumor suppressor and oncogenic functions. SIRT6 can act as both a tumor promoter and oncogene in cancer by targeting different cellular pathways.

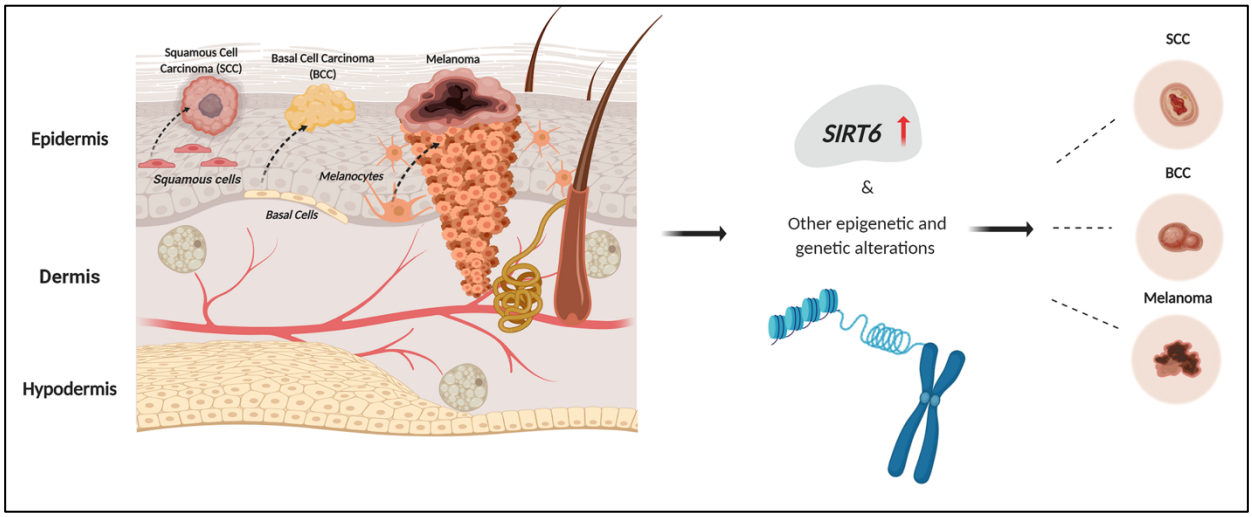


Figure 4: SIRT6 functions as an oncogene in skin cancers. The skin is divided into epidermis, dermis and hypodermis. Within the skin squamous cells, basal cells and melanocytes can give rise to different types of skin cancers. SIRT6 aids proliferation of non-melanoma and melanoma skin cancers.

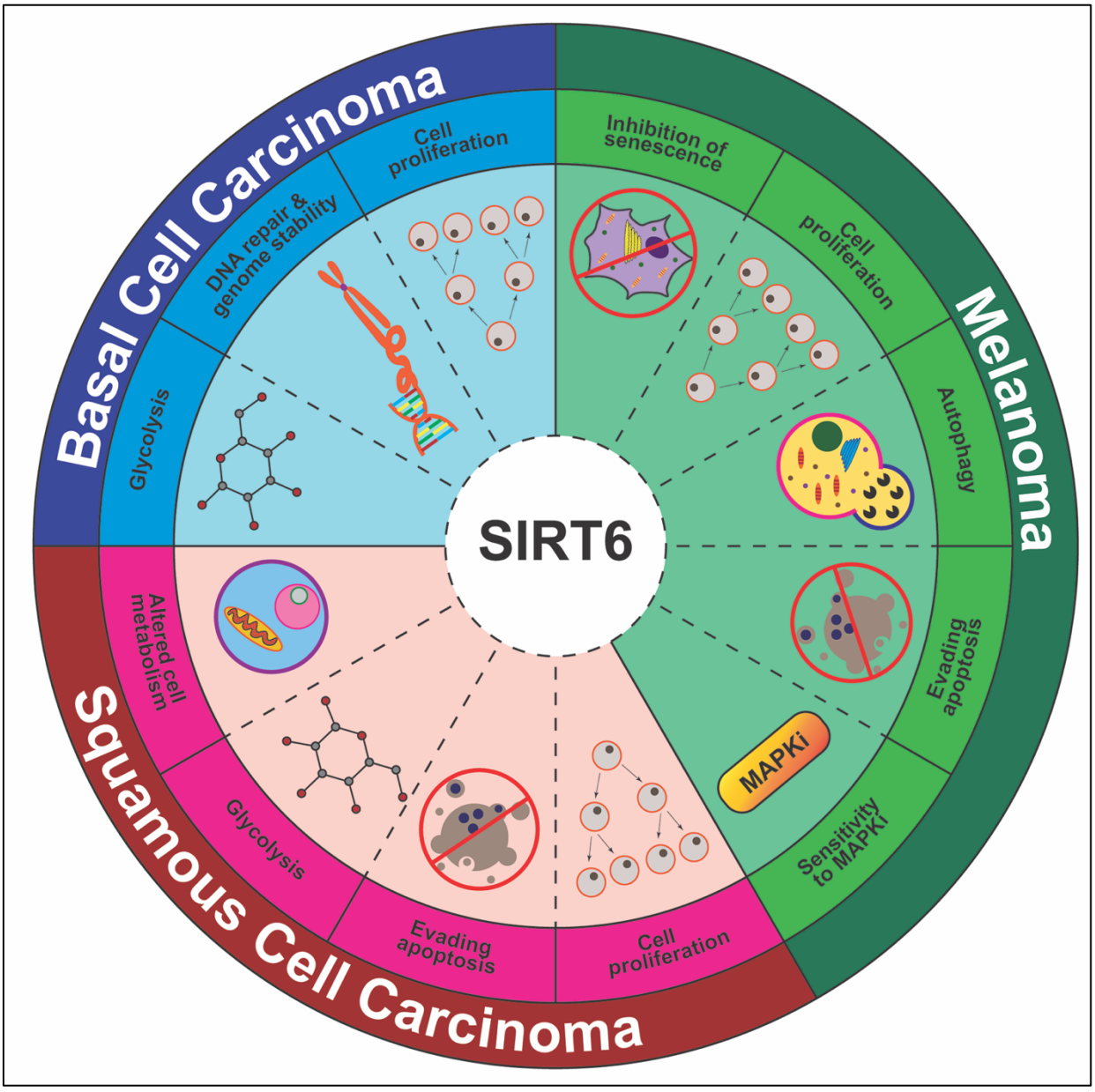


Figure 5: SIRT6 modulates cellular mechanisms and processes relevant to skin carcinogenesis. The important cellular processes which are known to be linked to SIRT6 and are relevant to development and/or progression of melanoma and non-melanoma skin cancers are depicted.

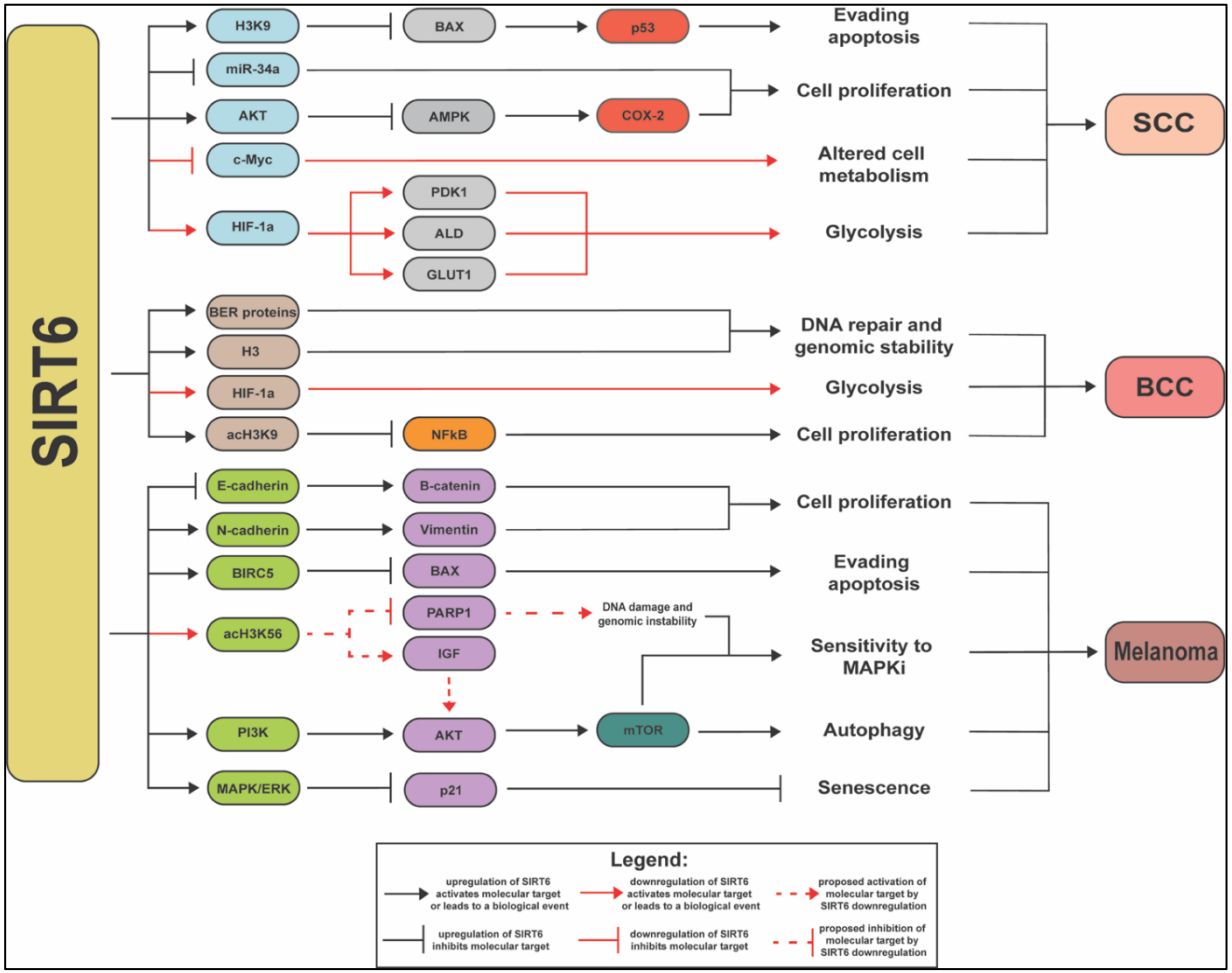


Figure 6: SIRT6 cellular functions and their impact on skin cancer. The known links between SIRT6 and other molecular targets, affecting important cellular processes, relevant to development and/or progression of melanoma, and non-melanoma skin cancers are depicted.

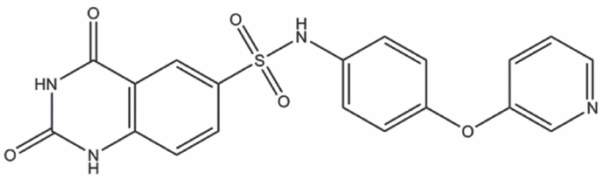
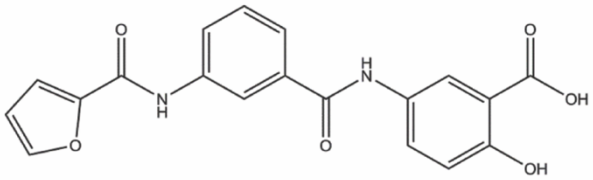
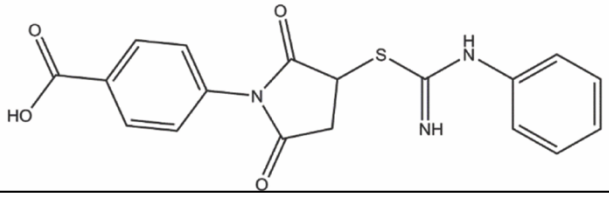
Asinex ID	Compound structure	% inhibition of SIRT6 at 200μM
SYN17739303		100±4
BAS13555470		62±7
BAS00417531		66±6

Figure 7: SIRT6 Specific Inhibitors Structures and SIRT6 Inhibition. Current SIRT6 inhibitors with inhibitory effects at a low micromolar range are depicted. It is important to note that this figure was modified from Parenti *et al*.

TABLES

Table 1. The known mechanistic information of SIRT6 in skin cancer.

Skin Cancer Type	Substrates and Targets	Function(s)
SCC	H3K9ac, BAX, p53, miR-34a, AKT, AMPK, COX-2, HIF-1α, PDK1, ALD, GLUT-1	• SIRT6 interacts with H3K9ac suppressing BAX to alter p53, avoiding apoptosis [67]. • Tp53 promotes accumulation of SIRT6, leading to SCC [54]. • SIRT6 contributes to SCC tumorigenesis by altering the differentiation of skin cells [54]. • SIRT6 upregulation decreases miR-34a levels in SCC. miR-34a controls the differentiation of squamous cells, downregulation leads to uncontrollable cell proliferation [27]. • UV radiation activates AKT pathway and AKT and SIRT6 form a positive feedback loop to inhibit Adenosine Monophosphate-Activated Protein Kinase (AMPK), which activates COX-2, leading to SCC [29]. • SIRT6 deficiency activates HIF-1α. HIF-1α can further activate glycolytic genes including PDK1, ALD, and GLUT-1 [68].
BCC	Histones (e.g. H3, H3K9ac), base excision repair (BER) proteins, HIF-1α, NF-κB	• SIRT6 interacts with histones and BER proteins, preventing genetic alterations and genomic instability along with DNA repair [55, 69]. • After UV exposure, upregulation of SIRT6 is initiated by DNA damage [55]. • Low levels of SIRT6 induce a metabolic switch, in which, the Warburg Effect takes place due to HIF-1α activation [70]. • SIRT6 deacetylates H3K9ac promoting NF-κB expression [71]. • NF-κB activation leads to cell proliferation in the skin [72].
Melanoma	E-cadherin, N-cadherin, vimentin, β-catenin,	• SIRT6 interacts with epithelial-mesenchymal transition (EMT) markers, to initiate metastasis [73]. SIRT6 blocks E-cadherin (epithelial marker) upregulating β-catenin activating N-

	BIRC5, BAX, H3K56ac, PARP1, IGF, PI3K, AKT, mTOR, p21	cadherin and vimentin, (mesenchymal markers) causing melanoma cells to proliferate uncontrollably [74]. • In advanced stages of melanoma,SIRT6 promotes BIRC5 activity by suppressing apoptosis, which helps cancerous cells to proliferate [57]. • SIRT6 activates the MAPK/ERK pathway which then blocks p21, inhibiting senescence [71]. • Evasion from apoptosis has been demonstrated by a reduction in proapoptotic proteins (e.g. Bcl-2-associated X protein (BAX)) [57]. • SIRT6 depletion increases H3K56ac inducing DNA damage [59]. • Alterations in SIRT6 can prevent PARP1 from aiding in DNA repair [8]. • Downregulation of SIRT6 upregulates IGF, activating AKT, leading to DNA damage and increase sensitivity to MAPKi [59]. • SIRT6 upregulation activates PI3K/AKT/mTOR pathway [57], initiating autophagy which promotes melanoma survival and migration [75].

ABBREVIATIONS

1. **ADP:** Adenosine Diphosphate
2. **AKT:** Protein-Serine/Threonine Kinase, also known as Protein Kinase B
3. **AML:** Acute Myeloid Leukemia
4. **AMPK:** Adenosine Monophosphate-Activated Protein Kinase
5. **BAX:** Bcl-2-Associated X Protein
6. **BCC:** Basal Cell Carcinoma
7. **BER:** Base Excision Repair
8. **BIRC5:** Baculoviral Inhibitor of Apoptosis Repeat-Containing 5, also known as, Survivin
9. **BRAF:** B-Rapidly Accelerated Fibrosarcoma Kinase, B-Raf Proto-Oncogene, Serine/Threonine Kinase
10. **CDC25A:** Cell Division Cycle 25A
11. **c-Fos:** Fos Proto-Oncogene
12. **c-Myc:** Cellular-Myelocytomatosis
13. **COX-2:** Cyclooxygenase-Endoperoxide Synthase 2
14. **E2F1:** E2F Transcription Factor 1
15. **FOXO:** Forkhead Box Protein
16. **FOXO1:** Forkhead Box Protein O1
17. **FOXO3:** Forkhead Box Protein O3
18. **GLUT1:** Glucose Transporter 1
19. **H3:** Histone 3
20. **H3K9/H3K9ac:** Histone 3 Lysine 9/Acetylated Histone 3 Lysine 9

21. **H3K18/H3K18ac:** Histone 3 Lysine 18/Acetylated Histone 3 Lysine 18
22. **H3K27:** Histone 3 Lysine 27
23. **H3K56/H3K56ac:** Histone 3 Lysine 56/Acetylated Histone 3 Lysine 56
24. **HDAC:** Histone Deacetylase
25. **HDF:** Human Dermal Fibroblasts
26. **HGPS:** Hutchinson-Gilford Progeria Syndrome
27. **HIF-1α:** Hypoxia Inducible Factor 1 Subunit Alpha
28. **HMGA2:** High Mobility Group at-Hook 2
29. **IGF:** Insulin-Like Growth Factor
30. **IGF-1:** Insulin-Like Growth Factor 1
31. **IGFBP1:** Insulin-Like Growth Factor Binding Protein 1
32. **IGFBP2:** Insulin-Like Growth Factor Binding Protein 2
33. **IGFBP3:** Insulin-Like Growth Factor Binding Protein 3
34. **IGF1R:** Insulin-Like Growth Factor 1 Receptor
35. **KAP1:** Kruppel-Associated Box Associated Protein-1
36. **KO:** Knockout
37. **LC3:** Microtubule-Associated Protein 1A/1B-Light Chain 3
38. **LMNA:** Lamin A/C
39. **MAPK/ERK:** Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase
40. **MAPKi:** Mitogen-Activated Protein Kinase Inhibitors
41. **miR-:** MicroRNA-
42. **miR-34a:** MicroRNA-34^{a}
43. **miR-122:** MicroRNA-122

44. **mTOR:** Mammalian Target of Rapamycin, also known as, Mechanistic Target of Rapamycin
45. **NAD+:** Nicotinamide Adenine Dinucleotide
46. **NF-κB:** Nuclear Factor-Kappa B
47. **NMSC:** Non-Melanoma Skin Cancer
48. **NOTCH1:** Neurogenic Locus Notch Homolog Protein 1
49. **P53:** Tumor Protein or TP53
50. **PARP1:** Poly Adenosine Diphosphate-Ribose Polymerase 1
51. **PDK1:** Pyruvate Dehydrogenase Kinase-1
52. **PI3K:** Phosphatidylinositol-3 Kinase
53. **PTEN:** Phosphatase and Tensin
54. **RELA:** V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A
55. **ROS:** Reactive Oxygen Species
56. **SCC:** Squamous Cell Carcinoma
57. **Ser:** Serine
58. **shRNA:** Lentiviral Short Hairpin RNA
59. **SIR2:** Silent Information Regulator 2
60. **SIRT1-7**: Sirtuin 1-7
61. **SIRT6-IN-1:** SIRT6-Inhibitor-Compund-1, Also Called 2,4,-Dioxo-N-(4-(Pyridine-3-Yloxy)Phenyl)-1,2,3,4-Tetrahydroquinazoline-6-Sulfonamide
62. **TGF-β1:** Transforming Growth Factor Beta 1
63. **Thr:** Threonine
64. **TNF-α:** Tumor Necrosis Factor Alpha

65. **TLR4:** Toll-Like Receptor 4
66. **UV:** Ultraviolet
67. **USP10:** Ubiquitin Specific Peptidase 10
68. **UTR:** Untranslated Region
69. **WAT:** White Adipose Tissue
70. **WT:** Wildtype

AUTHOR CONTRIBUTIONS STATEMENT

LMGP, GGP, and NA contributed to the conception and design of the manuscript.

LMGP, GGP, and CRK wrote the first draft of the manuscript.

All the authors reviewed and edited the manuscript

All authors have read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare

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Appendix 2: CRISPR/Cas9-mediated Knockout of SIRT6 Imparts Remarkable Antiproliferative Response in Human Melanoma Cells in vitro and in vivo.

Status: Published

Liz M. Garcia-Peterson[1, †], Mary A. Ndiaye[1, †], Gagan Chhabra[1], Chandra K. Singh[1], Glorimar Guzmán-Pérez[1], Kenneth A. Iczkowski[2], and Nihal Ahmad[1,3,*]

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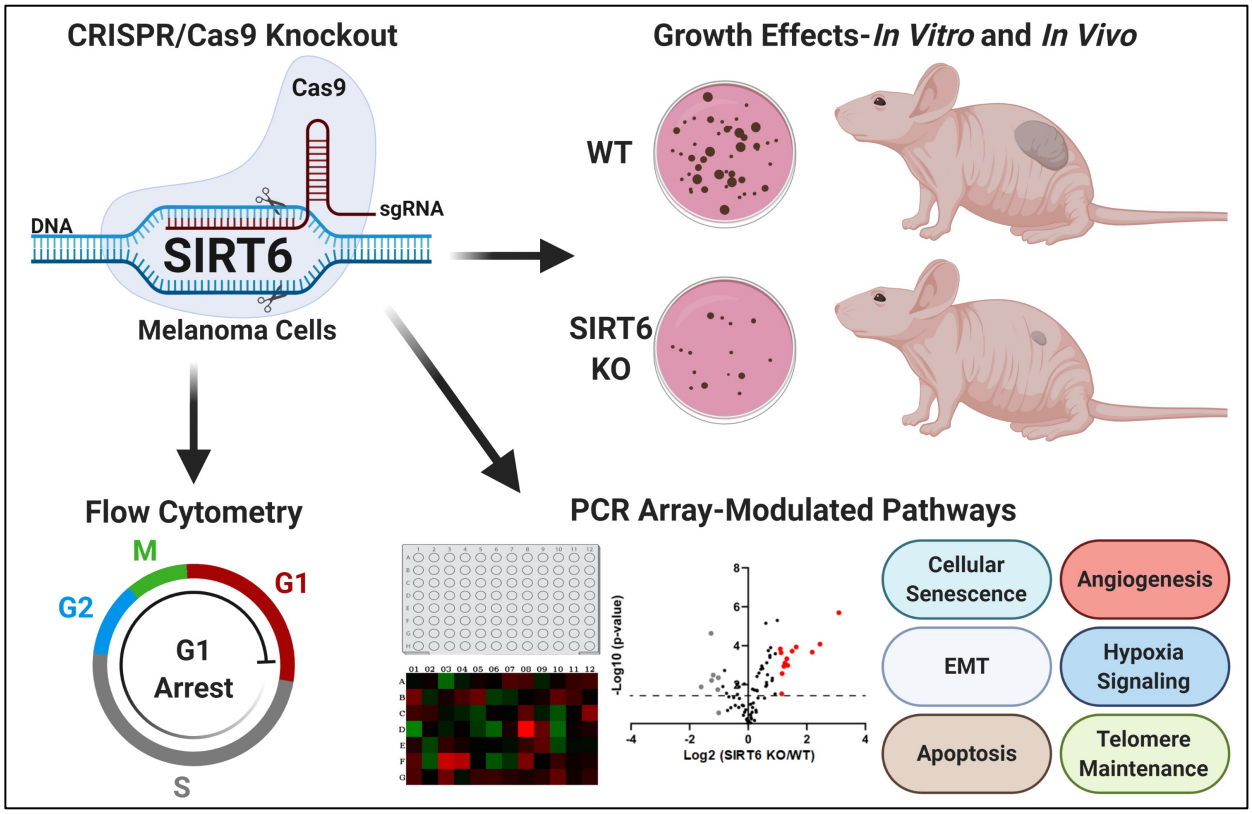

GRAPHICAL ABSTRACT

SIRT6 has been shown to act as a tumor promoter or suppressor depending on the cancer type. This study supports the tumor promoter role of SIRT6 in melanoma, one of the deadliest forms of UV-related skin cancers. We found CRISPR/Cas9-mediated SIRT6 knockout (KO) imparted marked anti-proliferative effects in human melanoma cells *in vitro* and anti-tumorigenic response *in vivo*. Additionally, we found SIRT6 KO significantly affected several cancer-associated pathways, including apoptosis, cellular senescence, and epithelial-to-mesenchymal transition, suggesting SIRT6 has an important role in

melanoma progression. Thus, SIRT6 inhibitory approaches could potentially be used in melanoma management and should be explored further.

ABSTRACT

Melanoma is one of the most aggressive, potentially fatal forms of skin cancer, and has been shown to be associated with solar ultraviolet radiation-dependent initiation and progression. Despite remarkable recent advances with targeted- and immune-therapeutics, lasting and recurrence-free survival remain significant concerns. Therefore, additional novel mechanism-based approaches are needed for effective melanoma management. The sirtuin SIRT6 appears to have a pro-proliferative function in melanocytic cells. In this study, we determined the effects of genetic manipulation of SIRT6 in human melanoma cells, *in vitro* as well as *in vivo*. Our data demonstrated that CRISPR/Cas9-mediated knockout (KO) of SIRT6 in A375 melanoma cells resulted in a significant i) decrease in growth, viability, clonogenic survival, and ii) induction of G1-phase cell cycle arrest. Further, employing a RT^2 Profiler PCR array containing 84 key transformation and tumorigenesis genes, we found that SIRT6 KO resulted in modulation of genes involved in angiogenesis, apoptosis, cellular senescence, epithelial-to-mesenchymal transition, hypoxia signaling, and telomere maintenance. Finally, we found significantly decreased tumorigenicity of SIRT6 KO A375 cells in athymic nude mice. Our data provide strong evidence that SIRT6 promotes melanoma cell survival, both *in vitro* and *in vivo,* and could be exploited as a target for melanoma management.

INTRODUCTION

Melanoma is one of the deadliest types of skin cancer, largely due to its ability to become aggressively metastatic when not diagnosed and treated at an early stage [1]. It is estimated that during the course of 2020, approximately 100,350 new cases of melanoma will be diagnosed and 6,850 deaths will occur due to this malignancy [2]. Solar ultraviolet (UV) radiation, a predominant environmental carcinogen, is a major risk factor for developing melanoma. Melanoma develops from melanin-producing melanocytes in the basal layer of the epidermis [3]. UV irradiation causes cutaneous DNA damage leading to the formation of cyclobutane–pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts, which are the primary source of mutations in UV-induced melanoma [4]. Current treatment options include surgery, cryotherapy, radiation therapy, or immunotherapies, as well as various chemotherapies, including drugs that target specific molecular pathways (i.e. BRAF or MEK inhibitors) [5-7]. However, melanoma has been associated with the tendency to develop resistance to the available treatments, which can lead to a relapse of the cancer [7, 8]. Additionally, although recently developed immunotherapies have been extremely promising, they are extremely expensive and may be associated with significant adverse effects. Therefore, it is crucial to identify new molecular targets and targeted therapies, which could be used alone or in combination with existing therapeutics for melanoma management.

The sirtuins are a family of nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylases (HDACs), with seven known members in mammals (SIRTs 1-7). Although SIRTs have structural similarities, their localization varies within the cells, influencing their unique roles [9-11]. Overall, the sirtuins have been implicated in the

regulation of several important molecular processes, including transcription, DNA repair, metabolism, and aging, [10, 12]. Interestingly, studies have shown that the SIRTs can play both oncogenic and tumor suppressor roles, depending on the cell and tissue types [13-15]. SIRT6, the focus of this study, is one of the less-studied sirtuins. It has been shown to interact with acetylated histone H3 lysines, such as acH3K9, acH3K18, and acH3K56 [16], and is generally found in the nucleus, where it plays roles in DNA repair, chromatin and transcription regulation, telomere maintenance, lipid metabolism and glycolysis [9, 17-19]. A very limited number of studies suggest a potential oncogenic role of SIRT6 in skin [20, 21]. In one such study by Ming et al, the authors showed that SIRT6 functions as an oncogene in non-melanoma skin cancer and exposure to UVB radiation leads to increased expression of SIRT6 in skin keratinocytes [21]. In our lab,e have previously shown that SIRT6 was overexpressed in human melanoma cells and tissues, and its inhibition via shRNA-mediated RNA interference resulted in a significant antiproliferative response in melanoma cells [22]. Another study by Wang and colleagues supported our findings to show that SIRT6 contributed to melanoma growth in an autophagy-dependent manner [23]. Together, these studies suggest a pro-proliferative role for SIRT6 in melanoma. However, additional investigations regarding the definitive role and mechanisms of SIRT6 in melanoma are needed to determine the therapeutic significance of SIRT6 in melanoma. This study was undertaken to determine the effects of SIRT6 KO in human melanoma cells in vitro and in vivo, and its associated downstream mechanisms.

MATERIALS AND METHODS

SIRT6 knockout cell line generation

A375 (RRID:CVCL 0132) SIRT6 knockout cell pool (and WT cells) were purchased from Synthego and maintained in DMEM supplemented with 10% FBS using standard cell culture conditions (37°C, 5% CO_2, humidified chamber). Cells were maintained at low passages and authenticated, as well as tested for mycoplasma (results were negative). The pool was generated using guide sequence CCUGAAGUCGGGGAUGCCAG. To grow single clones, the cell pool was plated in 96-well plates at an average of 0.5 cells/well and allowed to grow until a single, discrete clone could be seen in the well. Wells with single colonies were passaged and grown as standard cultures. Once enough cells were available, cells were collected and subjected to immunoblot as described below to determine SIRT6 protein expression. The clones with the best protein reduction were used in growth assays to determine the best clones for further experiments.

Trypan blue exclusion assay

Cells were plated in triplicate in 6-well plates (TPP) and allowed to grow for 24-72 hours. The cells were then trypsinized and both live and dead cells were collected. A small aliquot was mixed 1:1 with trypan blue and counted using the Countess II FL Automated Cell Counter (ThermoFisher). Statistical significance was determined with t-test using Prism software (GraphPad Software).

Colony formation

Cells were seeded at low density (300 cells per well) into 6-well plates in triplicate and allowed to grow for 8-12 days. Cells were then stained in 0.1% crystal violet in PBS:methanol (1:1), and destained using PBS rinses until background was clear before imaging.

Protein isolation and immunoblotting

Proteins were isolated from collected cell pellets and subjected to SDS-PAGE and immunoblotting as described previously [22]. Antibodies used for immunoblotting are outlined in Supplementary Materials (Table S1).

Cell cycle analysis

Cells were seeded into 6-well plates ($7x10^4$ cells per well) and allowed to grow for 24 hours. Cells were then detached from the plates using trypsin, collected (both live and dead cells), rinsed, and stained with propidium iodide before analyzing on the BD Accuri C6 flow cytometer. Cell cycle profile was analyzed using ModFit software (Verity Software House). Data was averaged from 3 separate runs performed in triplicate. Statistical analysis was done using a 2-way ANOVA with Dunnet's multiple comparison test using Prism software.

RNA isolation and RT-qPCR

RNA was isolated using the Qiagen RNeasy Plus isolation kit per manufacturer's protocol and quantified using the BioTek Synergy H1 multimode plate reader with Take3 plate. cDNA was made using MMLV-RT and Oligo dT primers (Promega) per manufacturer's

protocol and used in the qPCR array and for follow-up validations. Statistical significance was determined via multiple t-tests using the Holm-Sidak method to correct for multiple comparisons using Prism software.

PCR array

The effects of SIRT6 KO were assessed using the Qiagen Human Cancer PathwayFinder RT² Profiler PCR Array (#PAHS-033ZA) per the manufacturer's instructions. Ct values for the genes were uploaded to the Qiagen GeneGlobe Data Analysis Center and analyzed using *GAPDH* and *ACTB* reference genes. The data analysis web portal calculated fold-change using the ΔΔCT method (Table 1). Also, statistically significant selected genes from the PCR array results (>2-fold in one group and ≥1.5-fold in other) were validated using RT-qPCR analysis. Primers pairs detailed in Supplementary Materials (Table S2) were retrieved from Primer Bank [24].

Mouse xenograft studies

Animal studies were approved by the University of Wisconsin Animal Care and Use Committee and all measures were taken to reduce pain and/or discomfort to experimental animals. For cell implantation, $5x10^5$ A375 WT or SIRT6 KO cells were subcutaneously injected into the right flank of each mouse (Envigo Nu/Nu Strain #088; n=12; 6 males, 6 females per group) and monitored until tumors were palpable. Tumors were then measured twice weekly using a Biopticon TumorImager and tumor volume was calculated by TumorManager software. At the end of the study, mice were euthanized and tumors

were collected for further analysis. Statistical significance was determined via t-test using Prism software.

Immunohistochemistry

After excision, tumors were fixed in 10% formalin for 48 hours and then embedded in paraffin and sectioned. Sections were deparaffinized and rehydrated using a standard xylene/ethanol series before epitope retrieval using IHC-Tek IHC Buffer and Epitope retrieval system. After blocking endogenous peroxidase activity, slides were blocked in normal serum and incubated overnight in primary antibody as outlined in Supplementary Materials (Table S1). The following day, slides were incubated in secondary antibody, ABC-HRP reagent (Vector Labs), and then Vector Red chromogen before counterstaining with hematoxylin. Slides were then dehydrated and coverslipped using Permount (Sigma) before imaging at 40x for Ki67 analysis. Cells with bright pink nuclei were counted as positive for Ki67, while blue/light purple cells were counted as negative. Percent of Ki67-positive cells were determined in each image and mean ±SEM for each group is shown. Statistical significance was determined via t-test using Prism software.

RESULTS AND DISCUSSION

CRISPR/Cas9-mediated SIRT6 knockout (KO) inhibits cellular growth, viability, clonogenic survival, and induces G1 arrest in A375 human melanoma cells

We employed an experimental strategy of CRISPR/Cas9-mediated knockout of SIRT6 in A375 human melanoma cells to determine the functional significance of SIRT6 in melanoma. We generated single clones from a CRISPR/Cas9-mediated SIRT6 KO A375 cell pool, selecting the best KO clones for further experiments (shown in Supplementary Materials; Figure S1). As shown in Figure 1A, we achieved significant SIRT6 protein KO in our selected clones. Next, we assessed the effects of SIRT6 KO on proliferative potential of cells using trypan blue exclusion and long-term clonogenic survival assays. Our data demonstrated that SIRT6 KO significantly decreased cell growth and viability in both of the selected clones (clones #6 and #9) (Figure 1B). As seen in Figure 1C, SIRT6 KO resulted in a marked decrease in the number of colonies, suggesting that SIRT6 KO results in a significant decrease in clonogenic survival of melanoma cells. Additionally, we wanted to determine if the observed anti-proliferative response following SIRT6 KO was associated with dysregulation in the cell cycle, since SIRT6 was shown to regulate melanoma growth via the IGF-AKT signaling pathway [23], which is known to cause G1 arrest via p21, p27, and Cdk2 [25]. As measured by flow cytometric analyses (Figures 1D-E), we found that SIRT6 KO resulted in an enhanced accumulation of cells in G1 phase, which was accompanied by reductions of cell populations in S and G2/M phases. These data support our previous findings [22], further validating the pro-proliferative function of SIRT6 in melanoma.

SIRT6 modulation via CRISPR/Cas9-mediated KO significantly altered multiple cancer pathways

To obtain an insight into the molecular mechanisms of the antiproliferative response of SIRT6 KO, we performed a PCR array analysis profiling the expression of 84 key genes related to transformation and tumorigenesis using the two selected SIRT6 KO clones. Volcano plots outlining the distribution of the tested genes within the Cancer PathwayFinder qPCR Profiler Array are shown in Figure 2A. As shown by the heat maps (Figure 2B), SIRT6 KO caused a modulation in the expression of a number of important genes related to angiogenesis, apoptosis, cell cycle, cellular senescence, DNA damage repair, epithelial-to-mesenchymal transition (EMT), hypoxia signaling, metabolism, and telomere maintenance.

The cut-off criteria chosen in our experiment was for genes significantly modulated >2-fold in one clone and ≥1.5-fold in the other clone. The key genes that met these criteria were *ADM, CA9, CCL2, CFLAR, FLT1, IGFBP5, KDR, SERPINF1, SNAI2, TBX2* and *TEP1.* A graphical representation of this data is shown in Figure 3A, with more details in Supplementary Materials (Table S3). Although most of the genes appear to have similar increases/decreases in expression in both clones tested in the PCR array, *CA9* and *CCL2* appeared to be differently regulated (i.e. increased in one clone but decreased in the other). However, further validation with RT-qPCR (Figure 3B) found that both *CA9* and *CCL2,* along with *ADM,* were downregulated in both SIRT6 KO clones. This also confirmed the significant upregulation of *IGFBP5*, *FLT1* and *SNAI2.* Further, *CFLAR, KDR, SERPINF1, TBX2* and *TEP1* did not show significant modulation in either of the SIRT6 KO clone upon validation.

Interestingly, in our PCR array analysis, we found that *IGFBP5* (Insulin-Like Growth Factor Binding Protein 5) showed the maximum increase among the genes tested, with a greater than 8-fold increase in expression in both clones after SIRT6 KO. This is an important observation, since IGFBP5 is often dysregulated in human cancers [26], and a study by Wand et al. found that IGFBP5 functions as a tumor suppressor in human melanoma cells [27]. Additionally, we found that SIRT6 KO caused a decrease in *CA9* (Carbonic Anhydrase), a crucial regulator of pH that is overexpressed in several cancers. Intriguingly, a study by Martinez-Zaguilan et al. found that high pH environments significantly increased the invasiveness of human melanoma cells [28], suggesting the potential role for SIRT6 in EMT. We also found a downregulation of *CCL2* (C-C Motif Chemokine Ligand 2), a known mediator of tumorigenesis in a variety of cancer types. CCL2 has been reported to enhance the survival and invasiveness of melanoma cells and favor melanoma growth *in vivo* [29], and use of CCL2-targeting antibodies and/or BRAF inhibitors have been shown to decrease tumor growth in mouse models of metastatic melanoma [30]. Further, the observed decrease in *ADM* (adrenomedullin) in our experiments also supports the pro-proliferative function of SIRT6. ADM is expressed in tumor-associated macrophages in melanoma and plays a crucial role in both promoting angiogenesis and melanoma growth [31].

On the other hand, two of the genes found to be modulated upon SIRT6 KO did not support the pro-proliferative role of SIRT6. *SNAI2* (Snail Family Transcriptional Repressor 2; also known as SLUG) was increased in SIRT6 KO clones. This is in accordance with the study by Kunming et al. demonstrating that SIRT6 overexpression reduced the transcription levels of SNAI2 in TGF-β1-treated A549 lung carcinoma cells

[32]. However, this does not support the antiproliferative response of SIRT6 KO seen in our study. Similarly, an increased level of *FLT1* (fms related receptor tyrosine kinase 1; also known as VEGFR1) in SIRT6 KO clones was not in-line with our hypothesis that SIRT6 has a tumor-promoter function in melanoma, because FLT1 has been shown to stimulate tumor growth and metastasis [33]. Therefore, the downstream effects of SIRT6 on SNAI2 and FLT1 need to be further investigated in additional experiments. Overall, our results based on PCR array data suggest that SIRT6 possesses an important role in melanoma progression, potentially acting as a tumor promoter.

CRISPR/Cas9-mediated SIRT6 KO results in marked decrease in melanoma tumor growth in vivo

To validate our *in vitro* data to *in vivo* situations, we conducted a tumor xenograft study with SIRT6 KO melanoma cells. We implanted A375 WT and A375 SIRT6 KO cells subcutaneously onto athymic nude mice and followed their tumorigenicity over time. As shown in Figure 4A, the A375 SIRT6 KO tumors were demonstrably smaller compared to A375 WT tumors at the end of the experiment, and demonstrated significantly decreased tumorigenicity, in terms of tumor volume (Figure 4B) and tumor weight (Figure 4C).

We found similar results with an additional clone, as well as in female mice (data not shown), suggesting that SIRT6 depletion consistently and reliably inhibits xenograft tumor progression in athymic nude mice. At the termination of the experiment, the tumors were excised and stained with Ki67 to determine the effects of SIRT6 KO on proliferation. As shown in Figures 4D and 4E, the SIRT6 KO tumors showed significant reductions in the percent of Ki67-positive cells, indicating decreased proliferative indices of SIRT6 KO

tumors. These results corroborate our *in vitro* data suggesting that SIRT6 promotes melanoma growth.

Thus, our study further underscores the tumor promoter function of SIRT6 in melanocytic cells, *in vitro* and *in vivo*. In order to further dissect the role of SIRT6 in melanoma, it is important to determine the association and interaction of SIRT6 with other melanoma driver pathways, including the PI3K/AKT and MAPK/ERK pathways. Additionally, as mentioned earlier, SIRT6 has been linked to the IGF-AKT signaling pathway [23], though further studies need to be conducted in order to understand the interplay between SIRT6 and these key pathways. To date, there is no study showing an association between SIRT6 and the MAPK/ERK signaling pathway in melanoma, although recent findings have revealed that the RAS/RAF/MEK/ERK signaling pathway is involved in the modulation of autophagy [34]. This is relevant to our findings because we have shown in our previous studies that in melanoma cells, SIRT6 knockdown modulates key autophagy markers including BECN1, SQSTM1, ATG3, ATG7, ATG10 and GAA, as well as the reduced conversion of LC3 protein from its free form LC3-I [22]. These results suggest that SIRT6 may positively regulate autophagy in melanoma cells. Thus, the exploration of an association between SIRT6 and autophagy through the RAS/RAF/MEK/ERK signaling pathway could provide new mechanistic information into interactions of SIRT6 with melanoma driver pathways. Indeed, detailed studies unraveling other pathways and mechanisms affected by SIRT6 in melanocytic cells could provide new information towards the development of novel treatments against melanoma.

One limitation of this study is that we have used a single CRISPR/Cas9-mediated SIRT6 knockout melanoma cell line. Although further work is needed to validate this work

in other melanoma lines with varied genetic backgrounds, when combined with previously published data from our lab and others, there is strong evidence to support the oncogenic role of SIRT6 in melanoma. In combination with recent studies showing a potential role for SIRT6 in UV-induced DNA damage repair, our study provides a scientific basis for future investigations aimed at defining the connection between UV response and SIRT6 signaling during melanomagenesis and melanoma progression.

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FIGURES

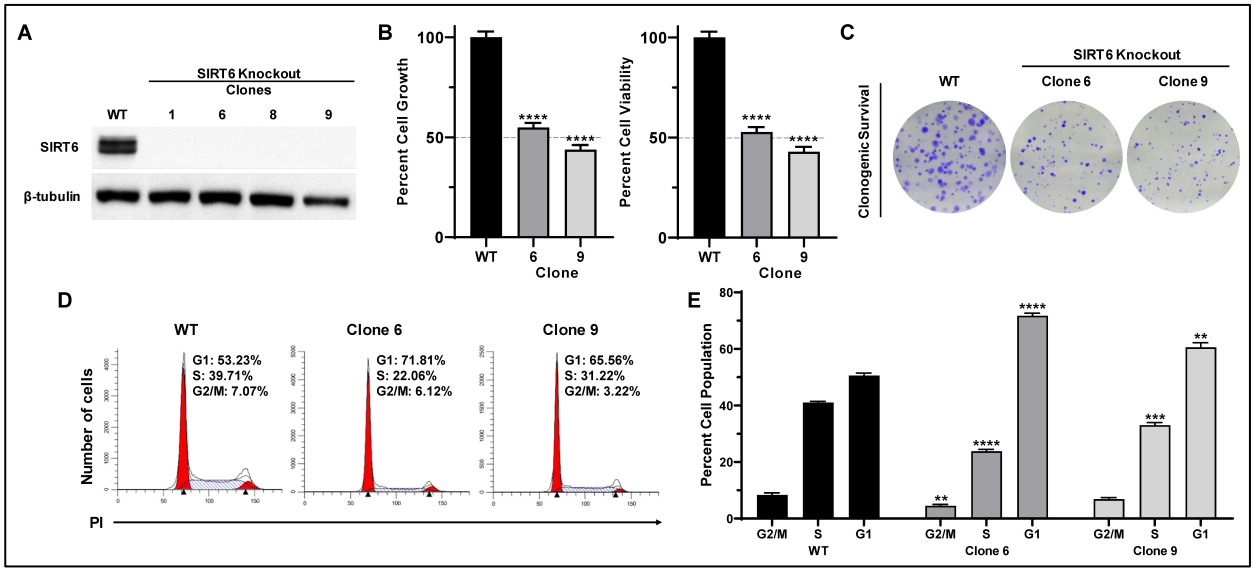

Figure 1. CRISPR/Cas9-mediated SIRT6 knockout inhibits growth and clonogenic survival, induces G1-phase arrest in human melanoma cells. A375 SIRT6 WT and KO clones were seeded and analyzed after 72 h, with different assays. (A) Cell lysates (with equal amount of protein) were subjected to immunoblot analysis to confirm SIRT6 KO. β-tubulin was used as a loading control. (B) Cell growth and viability were determined by trypan blue exclusion assay. Results are expressed as percentage of viable or total SIRT6 KO cells compared to WT. (C) To determine the clonogenic survival, an equal number of viable cells were plated into 6-well plates at low density. After ~10 days, cells were fixed and stained with crystal violet. The images shown are representative of three experiments with similar results. (D) Cell cycle analysis was performed using propidium iodide (PI) staining, and data were analyzed with ModFit software, representative histograms are shown. (E) Mean percent of cells in each phase of the cell cycle are shown. The data are expressed as mean ±SEM of three experiments, done in triplicate. Statistical significance

is determined by two-way ANOVA analyzed via GraphPad Prism Software and is denoted as **P≤0.01, ****P≤0.0001.

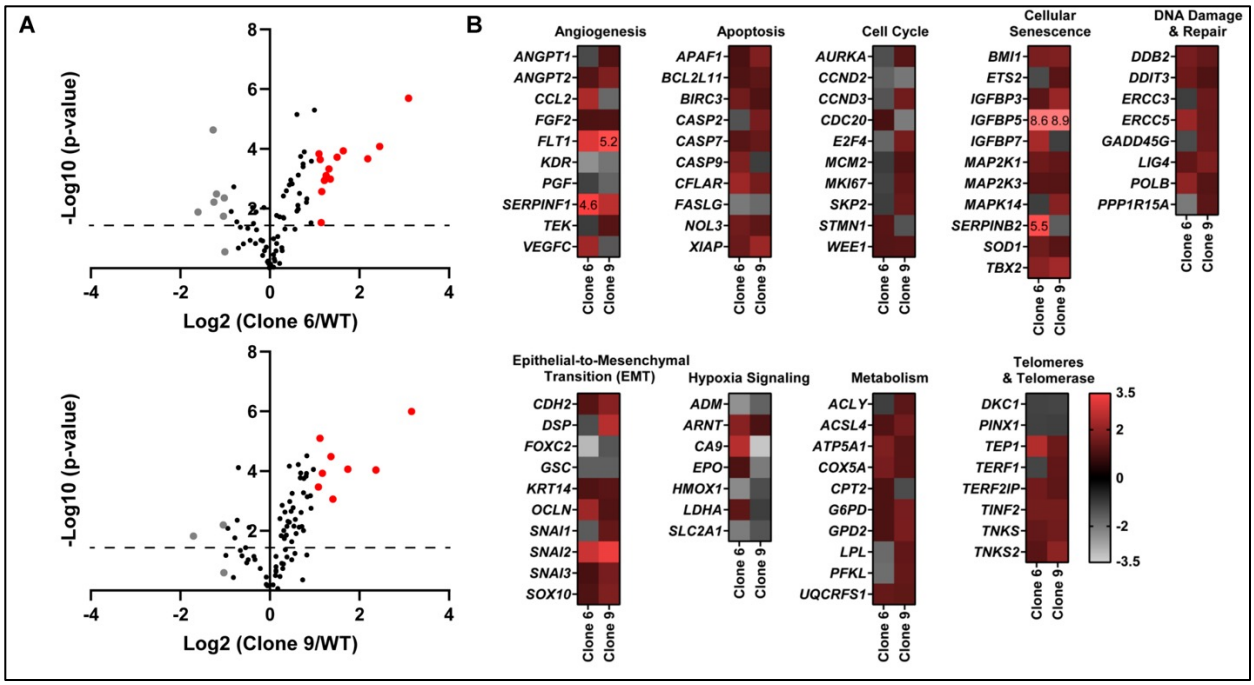

Figure 2. CRISPR/Cas9-mediated SIRT6 knockout causes alterations in multiple cancer-associated pathways. Qiagen Human Cancer PathwayFinder RT² Profiler PCR Array was performed using A375 WT and SIRT6 KO cells (clones #6 and 9). (A) Volcano plots outlining the distribution of 84 tested genes within the Qiagen Human Cancer PathwayFinder RT² Profiler PCR Array are displayed. Data was graphed log2 (ratio) of SIRT6 KO clone 6 (left) or SIRT6 KO clone 9 (right) vs WT against the -Log10 (p-value). Upregulated genes that reached the 2-fold cut-off are shown in red whereas the downregulated are depicted in grey, and black if no/negligible fold change was observed. The dashed line indicates the 0.05 p-value cut off. (B) Heat maps of each SIRT6 KO clone vs WT were generated to display gene fold changes classified by the cancer pathway identified in the array. Upregulated genes are shown in red whereas the downregulated are depicted in grey. To view clear differences in gene expression, the scale covers fold

regulation of -3.5 (grey) to 3.5 (red). Gene expression values falling outside of this scale are indicated by the indicated fold change value in the relevant box.

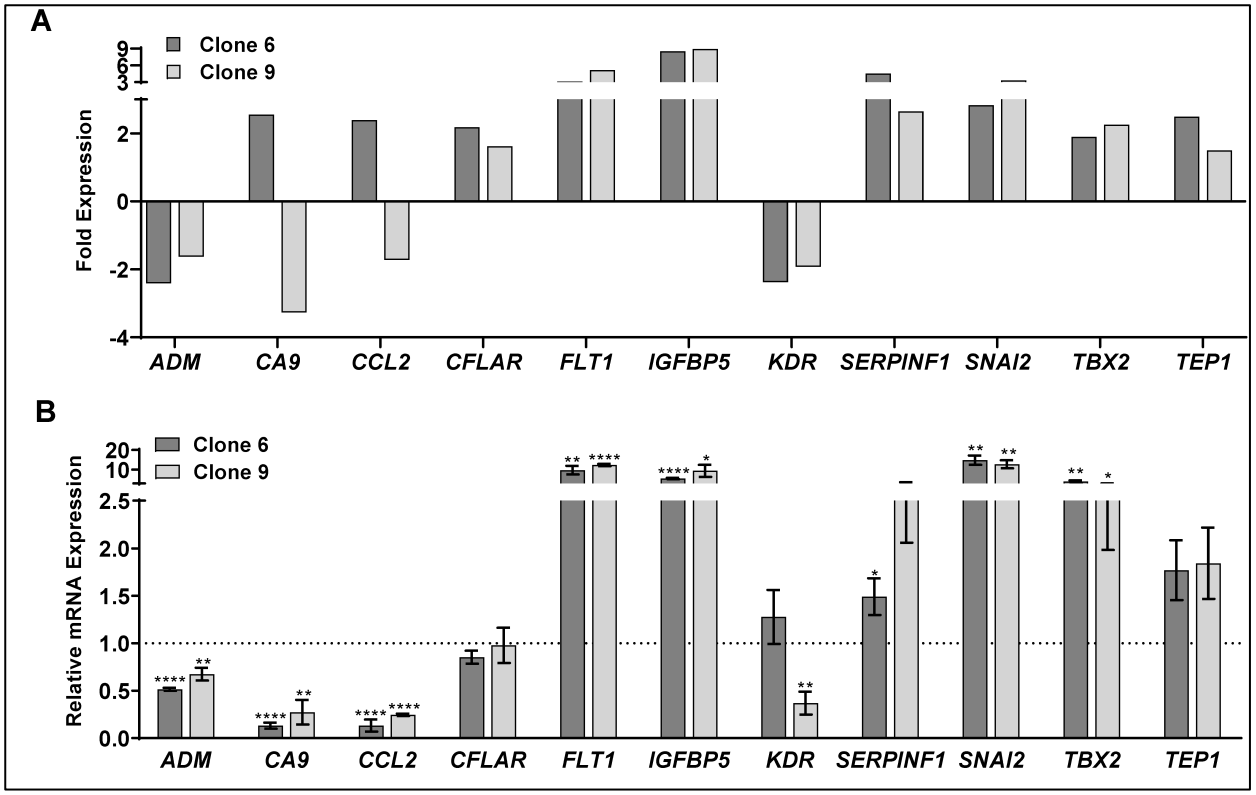

Figure 3. SIRT6 knockout by CRISPR/Cas9 results in alteration of key cancer-related genes. Key genes were found to be significantly modulated (>2-fold change in one clone and >1.5-fold change in the other, with statistical significance for both) after SIRT6 knockout. (A) Graphical representation of the 11 genes that were found differentially expressed upon SIRT6 KO. The data shown represent three biological replicates. (B) RT-qPCR analysis was performed to validate the key altered genes at mRNA levels in WT and SIRT6 KO cells as detailed in Supplementary Materials. Data are represented as mean ± SEM of minimum three technical and two biological replicates, and were analyzed via GraphPad Prism software using multiple t-tests and the Holm-Sidak method for multiple comparisons, with significance indicated by * P≤0.05, **P<0.01, ***P<0.001, ****P<0.0001.

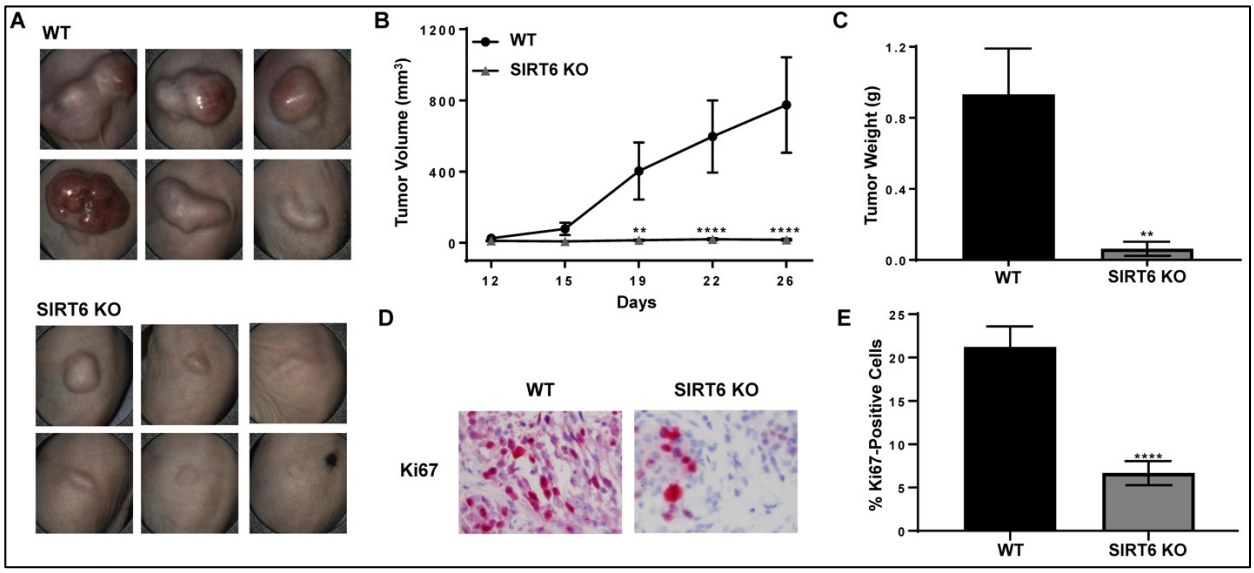

Figure 4. CRISPR/Cas9-mediated SIRT6 knockout results in marked decrease in melanoma cells-implanted tumors in athymic nude mice. A375 SIRT6 KO and WT cells were subcutaneously implanted and allowed to grow in athymic nude mice and tumor growth was assessed as described in Materials and Methods. (A) Pictures of tumors before resection were captured using the Biopticon TumorImager. (B) Tumor volume as calculated by the Biopticon TumorManager software and (C) final tumor weight after resection are shown. After excision, tumors were subjected to immunohistochemistry for Ki67. (D) Representative images and (E) percent positive cells are shown. Data is shown as mean ±SEM. Statistical analysis was performed using two-way ANOVA (B) or t-test (C and E) using GraphPad Prism Software, with significance denoted as **P<0.01, ****P<0.0001.

SUPPLEMENTARY MATERIALS

Supplementary Figures

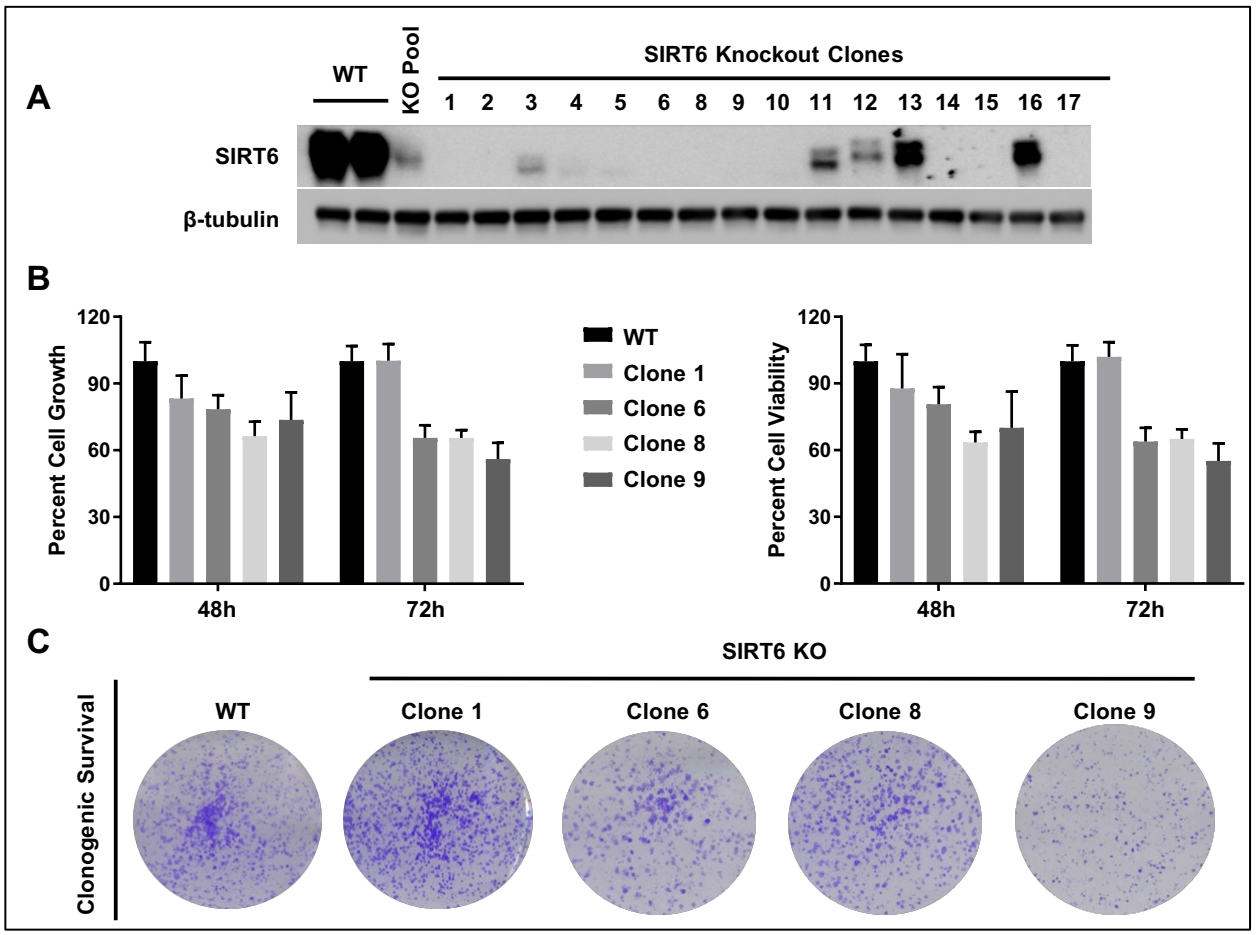

Figure S1. CRISPR/Cas9-mediated SIRT6 knockout results in reduced A375 cell growth *in vitro*. A375 SIRT6 Knockout (KO) cell pool was plated for isolation of individual colonies, and clones were tested for SIRT6 protein KO and growth effects. (A) Cell lysates from each clone (with equal amounts of protein) were subjected to immunoblot analysis to confirm SIRT6 KO. β-tubulin was used as a loading control. (B) Cell growth and viability of the 4 clones with the lowest SIRT6 expression was determined by trypan blue exclusion assay. Results are expressed as relative growth from 24h and percentage of viable SIRT6

KO cells compared to WT. (C) To determine the clonogenic survival, an equal number of viable cells were plated in triplicate into 6-well plates at low density. After ~10 days, cells were fixed with methanol and stained with crystal violet. The images shown are representative of three experiments with similar results. Statistical analysis was done by two-way ANOVA analyzed via GraphPad Prism Software. * $P \leq 0.05$.

Supplementary Tables

Table S1. Antibodies used for immunoblot (IB) and immunohistochemistry (IHC) analyses.

Antibodies	Company	Catalog Number	RRID*	IB Dilution	IHC Dilution
SIRT6	Cell Signaling	12486	AB_2636969	1:1000	n/a
β-Tubulin	Cell Signaling	86298	AB_2715541	1:1000	n/a
Ki67	Cell Signaling	12202	AB_2620142	n/a	1:350

*RRID is ID number found at https://scicrunch.org/resources

Table S2. Primers used in this study.

Gene	Amplicon size (bp)	Primer	Primer Sequence (5' - > 3')	Length	Location	Primer Bank ID[1]
ADM	146	F	ATGAAGCTGGTTTCCGTCG	19	1 - 19	4501944c1
		R	GACATCCGCAGTTCCCTCTT	20	146-127	
CA9	97	F	TTTGCCAGAGTTGACGAGGC	20	775-794	169636419c2
		R	GCTCATAGGCACTGTTTTCTTCC	23	871-849	
CCL2	190	F	CAGCCAGATGCAATCAATGCC	21	70-90	4506841a1
		R	TGGAATCCTGAACCCACTTCT	21	259-239	
CFLAR	107	F	TCAAGGAGCAGGGACAAGTTA	21	525-545	2653418a1
		R	GACAATGGGCATAGGGTGTTATC	23	631-609	
FLT1	117	F	TTTGCCTGAAATGGTGAGTAAGG	23	186-208	229892219c1
		R	TGGTTTGCTTGAGCTGTGTTC	21	302-282	
IGFBP5	136	F	ACCTGAGATGAGACAGGAGTC	21	540-560	171460920c1
		R	GTAGAATCCTTTGCGGTCACAA	22	675-654	
KDR	124	F	GTGATCGGAAATGACACTGGAG	22	277-298	195546779c2
		R	CATGTTGGTCACTAACAGAAGCA	23	400-378	
SERPINF1	87	F	TCATTCACCGGGCTCTCTACT	21	308-328	318037587c2
		R	GGGCAGTGACCGTGTCAAG	19	394-376	
SNAI2	87	F	CGAACTGGACACACATACAGTG	22	60-81	324072669c1
		R	CTGAGGATCTCTGGTTGTGGT	21	146-126	
TBX2	100	F	GCTGACGATTGCCGCTATAAG	21	478-498	44921604c2
		R	GGCTGTCTGGGTGGATGTA	19	577-559	
TEP1	81	F	CCACCCTCTCTAGTCTAAAGAGC	23	338-360	225735570c1
		R	CAGCTTGCGTCATGTGAGATA	21	418-398	

1. Wang X, Spandidos A, Wang H, Seed B. PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. Nucleic Acids Res. 2012;40:D1144-1149.

Table S3. Significantly modulated genes.

Gene Symbol	Name	Category	Fold Change (Clone 6/ WT)	T-TEST p value	Fold Change (Clone 9/ WT)	T-TEST p value
ADM	Adrenomedullin	Hypoxia Signaling	-2.41	0.000023	-1.63	0.000077
CA9	Carbonic anhydrase 9	Hypoxia Signaling	2.55	0.00102	-3.27	0.015027
CCL2	Chemokine (C-C motif) ligand 2	Angiogenesis	2.39	0.000779	-1.72	0.017164
CFLAR	CASP8 and FADD-like apoptosis regulator	Apoptosis	2.18	0.000229	1.62	0.002401
FLT1	FMS-like tyrosine kinase 1	Angiogenesis	3.11	0.000115	5.15	0.000092
IGFBP5	Insulin-like growth factor binding protein 5	Cellular Senescence	8.55	0.000002	8.96	0.000001
KDR	Kinase insert domain protein receptor	Angiogenesis	-2.38	0.006097	-1.92	0.008281
SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	Angiogenesis	4.55	0.000214	2.65	0.000868
SNAI2	Snail homolog 2 (Drosophila)	Epithelial-to-Mesenchymal Transition (EMT)	2.83	0.000188	3.34	0.000086
TBX2	T-box 2	Cellular Senescence	1.9	0.000257	2.25	0.000118
TEP1	Telomerase associated protein 1	Telomeres & Telomerase	2.49	0.000466	1.5	0.012255

Statistically significant genes with >2-fold change in one clone and ≥1.5-fold change in the other clone as found via Qiagen Human Cancer PathwayFinder RT² Profiler PCR Array.

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Appendix 3: Mechanisms of Immunotherapy Resistance in Cutaneous Melanoma: Recognizing a Shapeshifter

Status: Published

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ABSTRACT

Melanoma is one of the seven most common cancers in the United States, and its incidence is still increasing. Since 2011, developments in targeted therapies and immunotherapies have been essential for significantly improving overall survival rates. Prior to the advent of targeted and immunotherapies, metastatic melanoma was considered a death sentence, with less than 5% of patients surviving more than 5 years. With the implementation of immunotherapies, approximately half of patients with metastatic melanoma now survive more than 5 years. Unfortunately, this also means that half of the patients with melanoma do not respond to current therapies and live less than 5 years after diagnosis. One major factor that contributes to lower response in this population is acquired or primary resistance to immunotherapies via tumor immune evasion. To improve the overall survival of melanoma patients new treatment strategies must be designed to minimize the risk of acquired resistance and overcome existing primary resistance. In recent years, many advances have been made in identifying and understanding the pathways that contribute to tumor immune evasion throughout the course of immunotherapy treatment. In addition, results from clinical trials focusing on treating patients with immunotherapy-resistant melanoma have reported some initial findings. In this review, we summarize important mechanisms that drive resistance to immunotherapies in patients with cutaneous melanoma. We have focused on tumor intrinsic characteristics of resistance, altered immune function, and systemic factors that contribute to immunotherapy resistance in melanoma. Exploring these pathways will hopefully yield novel strategies to prevent acquired resistance and overcome existing resistance to immunotherapy treatment in patients with cutaneous melanoma.

INTRODUCTION

Melanoma is one of the deadliest cancers of skin and its incidence has increased significantly at a constant pace in the past few decades in the United States. Historically, the diagnosis of distant metastatic melanoma has been associated with significant mortality. Before the invention of immunotherapies, less than 10% of patients who received such a diagnosis survived more than 5 years. From the 1990's through 2010's novel treatments were lacking for melanoma, until the approval of the first immune checkpoint inhibitor (ICI) in 2011 (reviewed in [1, 2]). This discovery launched a revolution that resulted in this once death sentence into a better manageable disease with a 5-year survival rate as high as 44% (reviewed in [3]). Melanocytes have complex interplay with the immune system, as spontaneous regression of nevi is common in early life, suggesting an active immune surveillance system that eliminates transformed melanocytes to prevent tumor formation [4, 5]. However, neoplastic cells skew the immune system to an immunosuppressed state and acquire mechanisms to escape immune responses [6]. This is reflected by a long lag between enhanced melanocytic proliferation to neoplastic progression [7].

Immune checkpoint inhibitors are drugs that block proteins known as immune checkpoints, which monitor the immune system (e.g. programmed cell death protein 1/programmed death ligand 1 (PD-1/PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)) (4). These checkpoints negatively regulate the immune response that normally protects the individual from severe reactions [8]. In cancer patients, this prevents immune cells from targeting tumor cells, permitting malignant cells to evade the immune

response, which is considered a hallmark of cancer [3]. Presently, immune checkpoint inhibitors are some of the standard treatments used for melanoma patients.

The clinical trial data showing superior anti-melanoma effects of ICIs such as pembrolizumab (anti-PD-1), nivolumab (anti-PD-L1), and ipilimumab (anti-CTLA-4), over traditional therapies, has led to their rapid FDA approval [9, 10]. However, approximately 66% of melanoma patients still experience disease progression while on immunotherapy and approximately 50% still die of melanoma [3]. Thus, despite the promising clinical outcomes, immune checkpoint inhibitors have been associated with lower than desirable responses due to resistance leading to disease progression or relapse. The etiology of the resistance to immunotherapies that often underlies these deaths is often multifactorial. Mechanisms of immunotherapy resistance are poorly understood across all cancer types, and melanoma is no exception. Since the driving cause of resistance is not fully understood, it is currently not possible to identify which patients are likely to respond and which patients will develop resistance to a chosen immunotherapy before initiating treatment.

Although, there are various reviews available focusing on melanoma and immunotherapies, the goal of this review is to discuss the recent advancements towards elucidating the mechanisms of melanoma resistance to immunotherapies. As this is a pressing area of research, the focus will be on tumor intrinsic characteristics of resistance, epigenetic mechanisms of resistance, altered immune function, and systemic factors that contribute to immunotherapy resistance. Due to the complexity of these pathways, there is some overlap between the groups. We have also discussed the possible ways to overcome the resistance against immunotherapies in melanoma.

CURRENT IMMUNOTHERAPIES FOR MELANOMA

Immune Checkpoint Inhibitors (ICIs)

A number of ICIs are being currently used for melanoma treatment. Ipilimumab is an anti-CTLA-4 monoclonal antibody, meaning that it binds to CTLA-4 protein, blocking the interaction between CTLA-4 and its ligands (CD80 and CD86), and consequently inhibiting the activity of this protein [11, 12]. This negative regulation activates the proliferation of T cells and promotes the attack of cancerous cells, including melanoma [13]. Ipilimumab was one of the first treatments to prolong the survival of metastatic melanoma patients and indeed, after FDA approval in the year 2011, National Comprehensive Cancer Network recommended it as category 1 for patients with late-stage melanoma [12, 14]. Ipilimumab is recommended for unresectable or metastatic melanoma cases, and as an adjuvant therapy. This marked a milestone in immunotherapy, as selected patients reaped long-term responses after treatment. Survival increased in some for up to 10 years [15]. On the other hand, at the beginning of treatment, some patients experience atypical responses or pseudo-progression — meaning that they experience an increase of tumor size and/or new lesions present due to a weak antitumor immune response, or even immune effector cells aiding tumor growth [16]. However, continued treatment results in an anti-melanoma response [16]. Moreover, since ipilimumab stimulates T-cell proliferation, it can lead to immune-related adverse effects (irAEs) like dermatitis, endocrinopathy, and hepatitis, and other side effects including pruritus, fatigue, and colitis, the latter being the most prevalent [12, 14, 15]. Fortunately, most of these reactions can be treated and even reversed. Even though a higher percentage of patients (20-25%) experience long-term beneficial effects including

increased survival rates, overall response rates range on the lower end (12-19%) [13]. Therefore, researchers have been searching for prognostic and predictive biomarkers that could help determine which patients would benefit from this treatment the most [12]. Interestingly, patients who experience changes in blood markers during treatments, like increased counts of eosinophil and lymphocytes, have better outcomes [17]. Nonetheless, more biomarkers are being explored including peripheral blood cell markers, molecular markers, and even markers found in the gut microbiome [17, 18]. Overall, due to the low efficacy and high cost (approximately $80,000-$120,000 per patient) other treatments are now preferred.

In 2014, pembrolizumab (anti-PD-1) and nivolumab (anti-PD-L1) were approved as melanoma treatments and are currently used as first-line therapy for patients with advanced melanoma [1, 8, 19]. Both of these drugs work by inhibiting PD-1, an immune checkpoint that, unlike CTLA-4, is activated later in the immune response since it is expressed after continuous exposure to antigens [1]. PD-1 is expressed in T cells and after biding to its ligands (PD-L1 and PD-L2) decreases the proliferation, activity, and survival of T cells [11, 20]. Interestingly, cancer cells often increase the expression of PD-L1, which allows them to evade being targeted by immune cells [11, 20]. Therefore, inhibiting PD-1 stimulates immune cells to attack tumor cells [20]. Nivolumab is used as monotherapy, combination therapy, or adjuvant therapy to treat patients with unresectable or metastatic melanoma [20]. Monotherapy with this agent was shown to increase survival and response rate (up to 44%) in advanced melanoma patients [15]. Some patients experience minor and manageable side effects (e.g. diarrhea, nausea, pruritus) and/or irAEs, but nivolumab is considered safe and effective [21]. Comparatively,

pembrolizumab imparts long-term antitumor effects (more than 5 years), increases survival in advanced melanoma patients, and decreases the risk of disease progression and death in more than one-third of the patients [22-24]. Interestingly, treatment regimens are adaptable, but the efficacy remains constant and depending on the patient, treatment can go on for up to two years [16, 24]. Likewise, this immunotherapy agent is well-tolerated by melanoma patients since the adverse effects are less frequent, thyroid disorders being one of the most frequent effects observed [22, 23]. Treatments with nivolumab and pembrolizumab have been more effective than ipilimumab monotherapy, yielding increased survival rates in 35%-50% of patients as well as having a favorable toxicity profile and lower rates of irAEs [3, 25]. Overall, checkpoint inhibitors are modern immunotherapeutics that have been shown to improve melanoma patient prognosis in resectable tumors as well as metastatic melanoma [13, 19].

Oncolytic Enhanced Immunotherapy

In 2015, FDA approved the use of talimogene laherparepvec (T-VEC or Imlygic) for advanced melanoma patients, becoming the first oncolytic virus therapy (OVT) in the United States (reviewed in [26]). In fact, OVT leads to oncolysis, apoptosis, necrosis, autophagic cell death, and stimulates an overall anti-tumor immune response [27]. T-VEC is an oncolytic herpes simplex virus type 1 (HSV-1) that has mutations in several genes including c34.5, a47, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [27]. Particularly, the first two genes are deleted while the latter is a transgene derived from humans that are inserted into the deleted c34.5 loci of the virus (reviewed in [28]). Deletion of c34.5 is crucial for the virus to selectively replicate in cancer cells without

infecting normal cells, while a47 usually negatively regulates antigen presentation, hence its deletion promotes antitumor immune response [29, 30]. On the other hand, the reasoning behind adding GM-CSF relies on its potential of enhancing antitumor activity, nevertheless, researchers have suggested that other human genes might be preferred (e.g. interleukins 12 and 18) due to their key immune-related roles (reviewed in [28]). The immunosuppressive TME favoring melanoma ICI resistance can be taken advantage of as a target for viral attack; dysfunctional immune signaling allows genetically engineered non-pathogenic viruses to selectively target cancer cells and consequently replicate in them to a greater extent than in normal cells [28, 31]. OVTs have been associated with activation of T and NK cells, release of immunogenicity stimulating agents, release of tumor-specific antigens for APC uptake, type I IFN signaling, and major histocompatibility complex (MHC) upregulation [32, 33]. The overall response rate to T-VEC monotherapy is only around 25% [34], but the true promise of OVTs arguably lies in their immunomodulatory properties.

T-VEC and Anti-PD-1 Therapy

Tumor adenovirus OVT injection drastically alters the immune landscape with increased NK, T cell and APC migration occurring. Interestingly, initial trials observed that OVTs were associated with an increase of PD-1 and PD-L1 expression [32]. In response to these findings, recent reports reveal PD-L1 expression is an adaptive mechanism used by melanoma to generate OVT resistance [33, 35]. The exact relationship between PD-L1 expression and anti-PD1 efficacy remains cloudy, but multiple studies associate high PD-L1 with improved therapeutic outcomes [36, 37]. This suggests OVTs may prevent

anti-PD-1 resistance acquisition through PD-L1 regulation, or even reverse acquired resistance. This hypothesis is supported by a study that found Newcastle Disease Virus (NDV) sensitized B16-F10 tumors to anti-PD-1 therapy [33]. T-VEC was also found to enhance anti-PD-1 therapy responses [38]. Another recent study taking advantage of PD-L1 OVT response generated a novel OVT that expressed a PD-L1 inhibitor. This “double-armed” OVT reactivated T cell responses and specifically targeted B16-F10 melanoma alone, or even more effectively when also paired with PD-L1 antibodies [39]. The re-activation of tumor immune pathways in response to viral infection already gives a basis for sensitization to ICIs, but the possibility to overcome resistance problems through OVT mediated PD-L1 control provides an appealing prospect for further research.

T-VEC and Anti-CTLA-4 Therapy

T-VEC also positively augments anti-CTLA-4 therapies in melanoma, as do other OVTs [38, 40]. A measles OVT was developed to express CTLA-4 antibodies and showed promising results in human melanoma xenografts [41]. A recent study using an anti-CTLA-4 expressing NDV OVT found the treatment was similar in effectiveness to traditional anti-CTLA-4 therapy in combination with radiation, in B16-F10 melanoma [42]. Zamarin *et al* have discussed how NDV treatment re-actives the melanoma immune landscape through T and NK cells, MCH I and II, and interferon signaling to provide a synergistic effect when combined with anti-CTLA-4 therapy in B16-F10. They also found NDV therapy increased the expression of CTLA-4 ligands (CD80 and CD86), thus providing a potential mechanism for direct modulation of CTLA4 resistance [43]. The interplay between OVTs and anti-CTLA-4 resistance is poorly researched, but initial

studies show promising results for both combined therapies and insight into potential mechanisms for ICI therapy resistance deterrence via OVTs.

T-VEC and STING Signaling

As a master regulator of innate immunity pathways, it is unsurprising that STING signaling inhibits OVT efficacy [31, 44, 45]. A recent study used T-VEC to effectively target STING deficient melanoma [31]. OVTs have good potential for targeting ICI-resistant tumors as the same pathways developing resistance also develop viral susceptibility. Furthermore, if STING reactivation occurs as a result of OVT, it may re-sensitize melanoma to ICIs. This remains untested but should be investigated as it may provide a basis for therapies alternating between OVT and ICI therapy admission. Overall, OVTs directly augment ICI therapy efficacy through their viral-induced immune responses. Many OVTs have poor lytic capabilities, but recent combinations with ICI therapies have revealed potential possibilities for avoiding secondary resistance or overcoming it in primary instances. The relationship between OVTs and known resistance-pathways desperately needs to be studied so that novel, exploitive, and highly effective OVT/ICI combinations can be employed.

KEY PLAYERS IN RESISTANCE AGAINST IMMUNE CHECKPOINT INHIBITORS (ICI)

Despite promising clinical results, immune checkpoint inhibitors have been associated with sub-optimal responses to single-agent therapies due to resistance or relapse. Around half of advanced melanoma patients develop resistance to PD-1 treatments (reviewed in [25]). Even though antitumor effects from anti-PD-1 and anti-

CTLA-4 therapies are durable, they are associated with low response rates likely due to intrinsic and acquired resistance to treatments (reviewed in [8, 46]). For these reasons, researchers have been exploring the potential of combining treatments (multiple immunotherapies as well as other types of treatments together with immunotherapies). This pertains especially to nivolumab and pembrolizumab since they are well tolerated by patients and have the most promising outcomes.

Beyond honing the current therapeutic arsenal, melanoma targets governing resistance are also under investigation. T cell immunoglobulin and mucin domain 3 (TIM-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) are two extensively studied immune checkpoints responsible for fortifying melanoma against current immunotherapies, especially anti-PD-1 treatments [47] and (reviewed in [48-51]. The human leukocyte antigen (HLA) complexes I and II are responsible for antigen presentation, a crucial factor in lymphocyte function. HLA malfunction in melanoma is well documented and responsible for deterring anti-PD-1 and anti-CLTA-4 consequences (reviewed in [52, 53]. The tumor microenvironment (TME) is another well-reviewed emerging area of interest in immunotherapy resistance research (reviewed in [54, 55]. Indoleamine 2,3 dioxygenase-1 (IDO) is another target shown to manifest immunotherapy resistance through the accumulation of TME immunosuppressants [56] and reviewed in [50]. Past these well-documented resistance factors, newer reports detail many other mechanisms utilized by melanoma to impede immunotherapies. Below, we summarize the most recent advancements towards elucidating the mechanisms of melanoma resistance to immunotherapies.

Epigenetic Mechanisms of Resistance

Epigenetics is a rapidly evolving field that studies changes in gene expression that occur without modification of the underlying DNA sequence. Major epigenetic processes often involve the addition or removal of compounds to either DNA sequence itself, or the histones DNA is wrapped around. Histone acetylation, deacetylation, and methylation are often carried out by histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone methyltransferases (HMTs), respectively. Similar impermanent modifications can also be made to regulatory elements in DNA by a variety of enzymes, including DNA methyltransferases (DNMT). Epigenetics is intertwined with melanoma resistance to immunotherapies through the modulation of proteins that promote resistance. Aleotti *et al* authored a review article that covers many of the key roles of global loss of DNA methylation with promoter CpG island hypermethylation for tumor promotion and lists many hypermethylated and hypomethylated genes that are associated with melanoma [57].

A review by Gracia-Hernandez *et al* has described many of the epigenetic modifiers involved in melanoma pathogenesis, resistance to targeted and immunotherapies, and potential epigenetic targets for therapeutic adjuvants. They discuss the role of reduced 4-1BB (CD137) expression by DNA hypermethylation, HDAC6 promotion of IL-10 and PD-L1 expression, and mutations in the histone methyltransferase EZH2, which reduces expression of proteins involved in the antitumor immune response, such as RASSF5 and ITGB2 (reviewed in [58]). Recent research has expanded the association of EZH2 with immune evasion. Xu *et al* revealed that EZH2 inhibition enhances STING expression and signaling. Combining EZH2 inhibition with STING

agonist successfully suppressed melanoma in a mouse model (B16-F10) with poor immunogenicity. These mice displayed increased MHC I expression and antigen presentation, increased $CD8^+$ T cell infiltration of tumors, and improved survival [59]. This finding is promising for the development of a therapy to combat immune evasion in melanoma that has demonstrated resistance to immunotherapies.

Recent research has identified several epigenetic regulators that are altered in melanoma and may contribute to immunotherapy resistance. KDM5B is a H3K4 demethylase that has been implicated in melanoma resistance to targeted BRAF inhibitor therapies [60]. However, recent studies have shown that KDM5B also promotes immune evasion in mouse models of melanoma by recruiting a H3K9 methyltransferase, SETDB1 [61]. SETDB1 is enriched in many human cancers and its overexpression is associated with suppressed anti-tumor immune activity and resistance to immune checkpoint blockade [62-64]. SETDB1 suppresses the anti-tumor immune response by blocking the expression of immune-related gene clusters and transposable elements that encode MHC I antigens recognized by T cells [62]. The use of mithramycin A and mithramycin analog EC-8042 in melanoma cells enhanced the efficacy of MAPK inhibitor therapies [63, 65]. However, despite the clear impact of KDM5B and SETDB1 on immune evasion, there have not been any studies on the effects of their inhibition in melanoma resistance to immunotherapies. The results of the above studies on KDM5B and SETDB1 indicate that an epigenetic axis is likely involved in tumor immune evasion, as discussed by Galassi *et al* [66]. Further research into this epigenetic regulator axis may help elucidate therapeutic targets for melanoma that have successfully evaded immune surveillance with current immunotherapies.

Fat mass- and obesity-associated (FTO) protein is a m6A RNA demethylase that is strongly linked to obesity and is highly expressed in certain types of acute myeloid leukemia [67]. In melanoma, overexpression of FTO is associated with increased growth, proliferation, cell migration, and invasion [68]. It is upregulated when melanoma cells are placed under conditions of metabolic stress, through activation of the autophagy pathway that also increases PD-1 expression [68]. In a study using immunocompetent C57BL/6 mice with B10-F10 melanoma tumors as a model for melanoma resistance to immunotherapies, FTO knockdown restored the efficacy of anti-PD-1 blockade and significantly inhibited tumor growth [68]. Another recent study showed that Dac51-mediated inhibition of FTO reduced the growth of injected B16-OVA melanoma tumors [69]. A combination of Dac51 and anti-PD-1 checkpoint blockade demonstrated even slower tumor growth and prolonged survival of mice without evidence of significant general toxicity [69]. The results are promising for the use of FTO inhibition as an adjuvant to current ICI approaches. Further research to identify selective FTO inhibitors and more research on their effects in melanoma models are necessary before these results can be adapted to human trials.

Tumor Mutational Burden (TMB) and Neoantigens

A major factor that impacts the immunogenicity of cancers is tumor mutational burden (TMB), or the number of nonsynonymous mutations present in a tumor. Higher TMB indicates a greater number of mutations, which in turn increases the likelihood that an abnormal protein may be identified by the immune system. These proteins are termed neoantigens. Consistent with this theory, high TMB is generally associated with improved

overall survival and immunotherapy response in melanoma patients [8, 70-73]. However, there is still some debate about a good way to make high and low TMB definitions more consistent across studies to improve its use as a biomarker for immunotherapy response (reviewed in [74, 75]). Recent research has shown that TMB and neoantigen burden scores can predict response and likelihood of resistance to immunotherapies [76]. However, these scores are limited when mutations that affect antigen presentation are present, which is true in approximately 50% of melanoma tumors [8]. Many of the mechanisms underlying this loss are discussed in a recent review by Olbryt, Rajczykowski, and Widlak [8]. In addition, immunotherapy creates positive selective pressure for low TMB cells. Patients demonstrating melanoma progression post-ipilimumab treatment subsequently treated with nivolumab saw a decreased TMB when this course of action was effective [77]. If a given tumor has subclones with different levels of neoantigen burden, the ICI therapy may eliminate all high TMB clones but leaving low TMB clones behind, resulting in a newly resistant tumor [77]. Research to develop combination therapy that targets both hot and cold immunogenic subclones may decrease rates of acquired resistance by preventing this selection.

Altered Immune Signaling

Lymphocyte-activation gene 3 (LAG3)

Lymphocyte-activation gene 3 (LAG3) is both an activator of antigen-presenting cells when bound to MHC II and a negative regulator of T cell activation when found on T cells. Because of this mechanism, LAG3 has both been associated with non-response to therapy and therapeutics mimicking it has been used to induce antitumor immunity [78-

81]. Elevated serum concentrations of soluble LAG3 are seen in non-responders to anti-PD1 therapy and increased tumor infiltration with T cells positive for LAG3 and TIM3 was also correlated with shorter progression-free survival in patients receiving anti-PD1 immunotherapy [79, 80]. To corroborate these findings, preliminary results from a recent clinical trial combining a monoclonal antibody against LAG3 (relatlimab) with nivolumab showed improved progression-free survival in patients treated with both relatlimab and nivolumab compared to only nivolumab [82]. To note, the cohort treated with the combination relatlimab and nivolumab treatment did have a higher rate of treatment-related adverse events than the cohort only treated with nivolumab [82]. More analyses regarding overall survival and objective response are forthcoming, but the initial evidence supports the theory that LAG3 may play a role in impairing response to immunotherapies. Moreover, a clinical trial combining eftilagimod alpha, a soluble LAG3 protein that activates antigen-presenting cells, with pembrolizumab, also shows promising activation of antitumor immune activity [78]. Further research focusing on the many roles of LAG3 in driving antitumor immunity is necessary to determine the optimum therapy that can hijack this pathway to overcome and prevent resistance to immunotherapies.

Sphingosine Kinase 1

Sphingosine kinase 1 (SK1) is a kinase that catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P), an important regulatory protein for lymphocyte trafficking and differentiation [83, 84]. Past research has shown that SKI is overexpressed in melanoma and causes elevated levels of S1P, though the mechanism through which this impacts immunotherapy response is still unknown [84]. However, high

expression of SKI in melanoma cells has been shown to be associated with resistance to anti-PD-1 immunotherapies in patients [84]. In accordance with this, inhibition of SKI has been associated with the enhancement of the effect of anti-CTLA-4 and anti-PD-1 immunotherapies on melanoma cells *in vitro* [84]. This is promising as several therapies that target the S1P axis have been approved by the food and drug administration for use in conditions like multiple sclerosis, and several S1P modulators are under investigation for use in various human cancers [83]. Further preclinical and clinical research is necessary before these agents are ready for adaptation to melanoma treatment, but the initial preclinical data appears promising.

Stimulator of Interferon Genes Protein (STING)

Stimulator of interferon genes (STING) is an endoplasmic reticulum (ER) protein that regulates the immune response through interferon (IFN) and chemokine signaling [45, 85-89]. STING signaling is often suppressed or even absent in melanoma [45]. Activation of STING signaling was recently shown to increase MHC I expression and promote the T cell response against melanoma through increased type I IFN, and chemokine CXCL10 activity [85]. In B16-F10 mouse models, STING stimulation improved anti-CTLA-4 and anti-PD-1 therapy outcomes through IFN signaling [90]. The small molecule agonist of STING, diABZI, was shown to prevent Nuclear Factor Erythroid 2–Related Factor 2 (NRF2) activation in melanoma [86]. NRF2 is relevant to therapy resistance as it regulates PD-L1 expression and drives oxidative metabolic adaptation [86, 91]. Another recent study modified DNA methylation to enhance STING expression; this approach vastly increased TIL-mediated killing in melanoma [85]. As discussed

previously, EZH2 inhibitor mediated acetylation enhanced STING expression to improve therapeutic effects. STING agonist-loaded lipid nanoparticles (STING-LNPs) are a third method currently under investigation to enhance STING signaling. STING-LNPs diminished anti-PD-1 resistance in B16-F10 metastatic melanoma via IFN signaling, and synergistically provided an antitumor effect [88]. Countering the suppression of STING signaling is an emerging method to improve the innate anti-tumor response and immunotherapy effectiveness while deterring therapy resistance. Clinical trials promoting STING signaling should be developed, and their results studied to further understand how these potent effects are elicited.

Nucleotide-binding domain, leucine-rich containing family, pyrin domain-containing-3 (NLRP3)

NLRP3 regulates the caspase 1-dependent release of proinflammatory interleukins IL-1β and IL-18 through the formation of the NLRP3 inflammasome [92, 93]. IL-1β is a known pro-tumorigenic and immunosuppressive agent in melanoma [94, 95]. The orally active inhibitor, OLT1177, was recently used to target NLRP3 in a B16F10 mouse model. This approach resulted in decreased progression, inflammation, and normalization of Myeloid-Derived Suppressor Cell (MDSC) levels. OLT1177 combined with anti-PD-1 therapy further decreased MDSC levels and promoted T Cell tumor infiltration for an enhanced antitumor effect [95]. These results were consistent with another recent study that both silenced NLRP3, or inhibited it with the small molecule MCC950, to report a greatly reduced MDSC presence and increased anti-PD-1 therapy response. NLRP3 activity is especially pertinent in the scope of immunotherapy, as

MDSC recruitment was recently identified as a driver of resistance acquisition. Furthermore, this recruitment is unavoidable as PD-L1 was found to inhibit STAT3, which in turn activates the NLRP3 inflammasome [96]. However, the role of NLRP3 in melanoma is unclear as another recent report found its activity induces cell death in BRAF inhibitor-resistant melanomas and improves prognosis [97]. The results of NLRP3 manipulation may therefore depend on unknown genetic primings previously introduced through therapy. NLRP3 is also active in the TME; NLRP3 inhibition in macrophages with Celastrol resulted in decreased B16-F10 migration [94]. The role of NLRP3 in negating immunotherapy resistance should be further explored, especially as a key component in adaptive anti-PD-1 resistance.

Plasminogen Activator Inhibitor-1 (PAI-1)

Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor family, specifically responsible for inhibition of tissue plasminogen activator and urokinase [98-101]. PAI-1 was previously reported to promote macrophage M2 polarization and tumor infiltration through an IL-6/STAT3 pathway [100, 102]. PAI-1 inhibition with tiplaxtinin in a B16-F10 mouse model decreased M2 macrophage and TREG tumor infiltration [101]. Exogeneous PAI-1 was recently shown to also internalize PD-L1 in B16-F10 melanoma through endocytosis mediation. In a B16-F10 mouse model, PAI-I inhibition with tiplaxtinin prevented PD-L1 surface loss and provided a synergistic anti-tumor effect when combined with anti-PD-L1 therapy [98]. Interestingly, a separate study also verified authophagically derived exogenous PAI-1 contributes to an immunosuppressive TME in melanoma mouse models. Melanoma cells challenged with

mitoxantrone used autophagic PAI-1 release to gain resistance, suggesting a similar mechanism may be employed in immunotherapy cases [100]. PAI-1 is also a known regulator of autophagy and its ability to create a paracrine positive feedback loop fostering further secretion has been verified, although not in melanoma [100, 101]. Targeting PAI-1 may prove useful to deter treatment resistance through negation of autophagic signaling, macrophage polarization and halting of PD-L1 loss. The exogenous nature of PAI-I also merits further investigation in the scope of novel treatment development focused on TME modification.

OVERCOMING IMMUNOTHERAPY RESISTANCE

The immune system plays a key role in cancer pathogenesis, prognosis and therapy responses. The use of ICI in cancer immunotherapy aims to target the interaction between immune cells and cancer cells, enhancing the immune system's capabilities against tumors. Melanoma has been characterized as one of the most immunogenic tumors due to the existence of tumor-infiltrating lymphocytes (TILs) in resected melanoma and positive clinical responses to immune stimulation [103]. Due to high immunogenicity, melanomas have had widespread success in being treated using ICI. The major advantage to immunotherapy over targeted therapy is the more durable response on cancer growth that can be present even after the drugs have been discontinued. However, a large percentage of partial responders (primary resistance) and high rates of resistance acquisition remain the greatest obstacles to the optimal success of these therapies [104, 105]. As discussed above, immunotherapy resistance is the result of developing multiple interactions between cancer cells and the immune system. Thus, it

appears that the optimal treatment of melanoma is likely to involve therapeutic regimens that include multiple agents, given together or in sequence, with molecularly defined targets. Below, we have addressed some of the strategies that may synergize with ICIs for maximal anti-melanoma responses.

Use of molecularly targeted therapy in combination with immunotherapy

This approach relies on the combinations of drugs targeting two different signaling pathways to induce apoptosis leading to the release of tumor-associated antigens, and/or modulating key cellular pathways that allow cancer cells to maintain an adaptive resistance. BRAF inhibitors, such as dabrafenib and vemurafenib, have demonstrated a survival advantage as both monotherapy and in combination with MEK inhibitor trametinib in both resectable and unresectable or metastatic melanomas [106-108]. However, most of the patients ultimately acquire resistance, thereby failing to achieve durable tumor regression [109]. Interestingly, preclinical and clinical studies combining anti–PD-1/PD-L1 with BRAF/MEK inhibitors have demonstrated enhanced anti-melanoma responses and tolerability [110]. Similarly, bevacizumab, an anti-vascular endothelial growth factor (VEGF) monoclonal antibody, has been found to possess immunomodulatory properties, as VEGF exerts immunosuppressive functions via inhibiting dendritic cell maturation and T-cell tumor infiltration. With this rationale, several clinical trials are evaluating the combination of immune checkpoint inhibitors with anti-VEGF therapies across multiple tumor types including melanoma [111, 112].

Concurrent inhibition of two or more immune checkpoints

Combination therapy with anti–CTLA-4 and anti–PD-1 has been approved or in clinical trials for certain cancers including melanoma. In a phase I clinical study, involving 142 patients with metastatic melanoma, the objective-response rate and progression-free survival have been found significantly higher with ipilimumab (CTLA-4 inhibitor) and nivolumab (PD-1 inhibitor) combined therapy than ipilimumab monotherapy [113]. Three years post-trial, the average survival rate of the nivolumab-plus-ipilimumab group was 58%, compared to 52% for nivolumab alone [114]. At five years, the survival rate was 52% for nivolumab-plus-ipilimumab and 44% for nivolumab alone [115]. At six and a half years, the survival rate was 49% for nivolumab-plus-ipilimumab and 42% for nivolumab alone [116]. These results demonstrate the durability of the response achieved through utilizing nivolumab alongside ipilimumab [116]. The clinical benefits with this combination therapy may have been due to complementary mechanisms as ipilimumab is known to prime T cells, whereas nivolumab reactivates effector responses. Similarly, anti-PD-L1 along with anti-CTLA-4 and radiotherapy has been demonstrated to promote a better response in a subset of patients with metastatic melanoma [117].

Influence of the microbiome

Several studies have noted the connection between gut microbiome and melanoma response to immunotherapy, and postulated some theories for how microbiome influences immunotherapy responses [118-121]. In patients with microbiota that favors immunotherapy response, microbiota-derived STING agonists induce IFN1 signaling and spur anti-tumor immune response [118, 119]. It is theorized that gut

microbiome may influence response to immunotherapy through the production of short-chain fatty acids and their subsequent influence on the epigenome of melanoma cells. A recent study found that pentanoate induced epigenetic reprogramming of T cells by inhibiting class 1 histone deacetylases, increasing mTOR activity in $CD8^+$ T cells, and enhancing expression of CD25 and IL-2, which empirically increased anti-tumor activity of $CD8^+$ T cells treated with these short-chain fatty acids [120]. In one study, oral administration of probiotic *Bifidobacterium* in combination with PD-L1 has been found to almost abolish melanoma tumor growth in a mouse model [122]. The molecular analyses suggest that the effects were mediated by improved dendritic cell function resulting in enhanced $CD8^+$ T cell priming and accumulation in the TME [122]. In an analysis of fecal microbiome samples of melanoma patients undergoing anti-PD-1 immunotherapy, diversity and composition of gut microbiome of responders showed significantly higher alpha diversity and relative abundance of bacteria of *Ruminococcaceae* family compared to nonresponders [123]. Moreover, fecal microbiota transplantation has been found to overcome resistance to PD-1 blockade in germ-free mice [123]. Based on these promising preclinical data, a small phase 1 clinical trial of fecal microbiota transplant (FMT) and reinduction of anti-PD-1 therapy has been conducted in patients with melanoma refractory to initial anti-PD-1 immunotherapy [121]. The FMT was sourced from patients with melanoma who responded to anti-PD-1 treatment [121]. The study found that FMT was associated with favorable changes in immune cell infiltrates and deemed FMT safe to pursue in larger phase clinical trials [121]. If replicated in these larger trials, FMT could be a viable option for patients who experience melanoma progression while on anti-PD-1 immunotherapy.

Other combination strategies with immunotherapy

Similarly, several other combination therapeutic strategies to overcome immunotherapy resistance are being investigated (reviewed in [124]). For example, combining cancer vaccines with ICI has been found to be beneficial in multiple preclinical studies as it increases antigen presentation and prime T cells. Enhanced survival has been noticed with multi-peptide vaccine and nivolumab adjuvant therapy in melanoma patients [125]. In another study, a personalized neoantigen vaccine (that targets up to 20 predicted tumor neoantigens) paired with anti-PD-1 therapy showed complete tumor regression in melanoma patients [126].

Further, a small molecule IDO inhibitor Epacadostat was tested in combination with pembrolizumab in a metastatic melanoma trial with 928 patients, however, progression-free survival was not significantly affected [127]. Other Phase 1 and 2 trials combined the nuclear factor erythroid 2-related factor 2 (NRF2) agonist, Omaveloxolone, with Ipilimumab or Nivolumab in hopes of abrogating MDSC-driven immunosuppression (ClinicalTrials.gov Identifier: NCT02259231). Omaveloxolone was associated with decreases in tumor iNOS, PD-L1, and IDO-1 expression without any dose-limiting toxicities and thus, it may overcome ICI resistance [128]. Furthermore, Ipilimumab was also combined with oncolytic virus (T-VEC) therapy, yielding, a 50% progression-free survival and 67% overall survival at 18 months demonstrating superiority over T-VEC monotherapy. (ClinicalTrials.gov Identifier: NCT01740297) [129]. Given these promising findings, several new clinical trials have been initiated to devise the strategies to overcome immunotherapy resistance in melanoma. Concurrently, research investigations

are also underway to identify biomarkers associated with ICI resistance and treatment responses.

CONCLUSION

Melanoma has been characterized as one of the most immunogenic tumors due to the existence of TILs in resected melanoma, occasional spontaneous regressions, and clinical responses to immune stimulation. The immunogenicity of melanoma has led researchers to identify novel immune strategies to overcome tumor immune evasion. Nevertheless, high rates of resistance acquisition, lack of long-lasting anti-melanoma responses and higher percentages of limited responders remain key obstacles to the realization of immunotherapies. The mechanisms of immunotherapy resistance need to be identified for successful future drugs targeting those mechanisms. There are definite advancements in current research exploring novel mechanisms of resistance against immunotherapy for melanoma. Since melanoma is notoriously resistant to treatment and current therapeutic approaches have not been able to effectively manage this neoplasm, there is no doubt that the future treatment of melanoma will involve therapeutic regimens that include multiple agents, given together or in sequence, with wide varieties of molecularly defined and immunologic targets.

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FIGURES

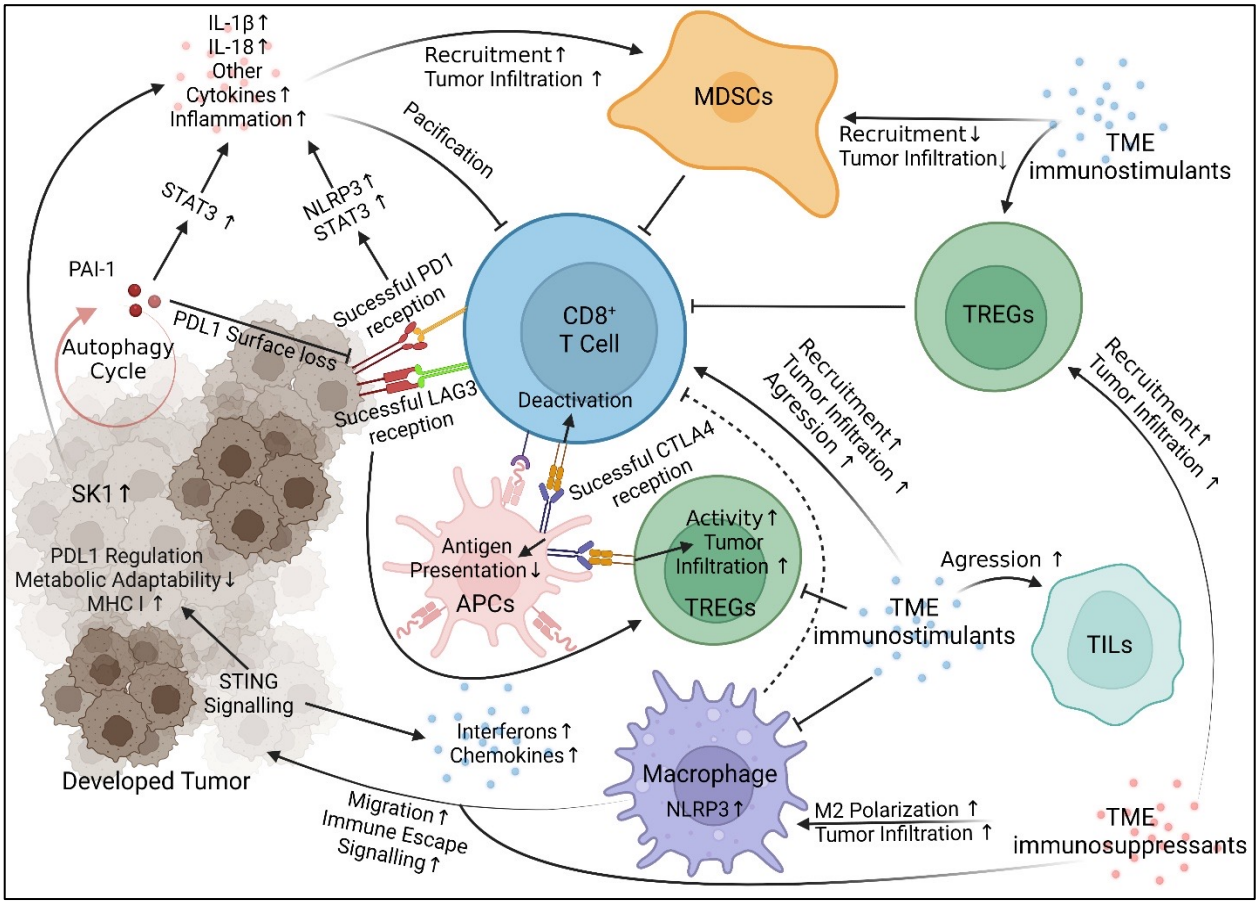

Figure 1. Immunotherapy Resistance Signaling. Key targets of interest negate therapeutic responses through internal melanoma pathways, acquisition of TME immunosuppressants (red) and loss of TME immunostimulants (blue). STING, Stimulator of Interferon Genes; NLRP3, Nucleotide-binding domain, leucine-rich containing family, pyrin domain-containing-3; PAI-1, Plasminogen Activator Inhibitor-1; LAG3 (Green on T Cell), Lymphocyte-activation gene 3; SK-1, Sphingosine kinase 1; PD1, Programmed cell death protein 1 (yellow on T Cell); CLTA4, Cytotoxic T-Lymphocyte Associated Protein 4 (Blue on APC); STAT3, Signal transducer and activator of transcription 3; IL, interleukin,

MDSCs, Myeloid-derived suppressor cells; TREGs, Regulatory T cells; TILs, Tumor-infiltrating lymphocytes; APCs, Antigen Presenting Cells [45, 59, 85-102, 130-133].

TABLES

Table 1. Recently revealed drivers of immunotherapy resistance and their influences.

Drives of Immunotherapy Resistance	Immune Influence(s)	References
CpG promoter	Methylation of melanoma genes of interest	[57]
EZH2	MHC I expression, antigen presentation, $CD8^+$ T cell infiltration, STING regulator	[59]
HDAC6	IL-10 and PD-L1 expression	[134]
RASSF5 and ITGB2	Immunogenicity Generation	[134]
KDM5B	Recruits the H3K9 methyltransferase SETDB1	[61]
SETDB1	Regulates expression of immune-related gene clusters, MHC I expression, antigen presentation	[62-64]
FTO	Increased PD-1 expression through autophagy	[68]
TMB	Generates neoantigens to promote successful immunosurveillance	[8, 70-72]
LAG3	APC activator when bound to MHC II and negative regulation of T cells	[80, 135, 136]
SK1	Lymphocyte trafficking and differentiation	[83, 137]
STING	MHC I expression, metabolic regulation, PD-L1 expression, immunostimulating interferon and chemokine signals	[45, 85-89]
NLRP3	Inflammatory interleukin signals, MDSC control, macrophage polarization	[92-95, 138, 139]
PAI-1	Macrophage polarization, PD-L1 surface levels, autophagy cycle, TREG control	[99-101, 140]
Microbiome	Activation of various pathways (including STING) involved in T cell response and interleukin signaling	[120-123, 141, 142]
VEGF	Inhibition of dendritic cell maturation and T cell tumor infiltration	[111, 112]

AUTHOR CONTRIBUTIONS

Conceptualization, JT, GC, and NA. Writing—original draft preparation, JT, GC, CKS, GGP and CAS. Writing—review and editing, JT, GC, CKS, GGP, CAS and NA. Tables and figures, CAS, GC and CKS. Supervision, NA. Project administration, NA. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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