Tumor-intrinsic SIRPA promotes sensitivity to checkpoint inhibition immunotherapy in melanoma

Graphical abstract

Highlights

- High SIRPA expression correlates with response to anti-PD-1 treatment in melanoma
- The loss of SIRPA expression is a key marker of melanoma dedifferentiation
- Tumor-specific SIRPA overexpression enhances anti-PD-L1 response
- Multiple mechanisms affect SIRPA heterogeneity, including SIRPAP1 co-regulation

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In brief

Zhou et al. reveal that tumor-intrinsic SIRPA can enhance the sensitivity to anti-PD-1 treatment in melanoma patients, whereas macrophage SIRPA has a well-established role as a major inhibitory modulator in antitumor immunity. This study highlights that the same target in different cell types can have antagonistic effects on immunotherapy.
Tumor-intrinsic SIRPA promotes sensitivity to checkpoint inhibition immunotherapy in melanoma

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SUMMARY

Checkpoint inhibition immunotherapy has revolutionized cancer treatment, but many patients show resistance. Here we perform integrative transcriptomic and proteomic analyses on emerging immuno-oncology targets across multiple clinical cohorts of melanoma under anti-PD-1 treatment, on both bulk and single-cell levels. We reveal a surprising role of tumor-intrinsic SIRPA in enhancing antitumor immunity, in contrast to its well-established role as a major inhibitory immune modulator in macrophages. The loss of SIRPA expression is a marker of melanoma dedifferentiation, a key phenotype linked to immunotherapy efficacy. Inhibition of SIRPA in melanoma cells abrogates tumor killing by activated CD8+ T cells in a co-culture system. Mice bearing SIRPA-deficient melanoma tumors show no response to anti-PD-L1 treatment, whereas melanoma-specific SIRPA overexpression significantly enhances immunotherapy response. Mechanistically, SIRPA is regulated by its pseudogene, SIRPAP1. Our results suggest a complicated role of SIRPA in the tumor ecosystem, highlighting cell-type-dependent antagonistic effects of the same target on immunotherapy.

INTRODUCTION

Checkpoint inhibition immunotherapy has become one of the most successful strategies for cancer treatment and functions through the stimulation of the patient’s immune system (Hamberbacher and Snyder, 2017; Liu and Mardis, 2017). Many cancer patients, even those with advanced refractory cancers, show beneficial clinical responses, sometimes long-lasting ones, to checkpoint inhibitors targeting programmed death 1 (PD-1), programmed death-ligand 1 (PD-L1), and cytotoxic T lymphocyte antigen 4 (CTLA-4) (Hodi et al., 2010; Postow et al., 2015; Topalian et al., 2012). In particular, PD-1/PD-L1 blockades have achieved the most success in clinical development (Tan et al., 2020). However, the response rate varies by cancer type, ranging from ~10% to ~60% (Ansell et al., 2015; Yarchoan et al., 2017), and the average objective response rate is only ~26% across all cancer types (Shen et al., 2020). Combination therapy is a promising approach to overcoming PD-1/PD-L1 resistance and increasing the response rate (Zhang et al., 2020a). A recent study shows that ~80% of active trials in PD-1/PD-L1 blockades are testing combination regimens with other cancer therapies, such as immuno-oncology agents (Upadhaya et al., 2021). The daunting complexity of the human immune system and increased toxicity associated with combination therapy, however, necessitates the identification of key factors affecting the immunotherapy response and elucidating synergistic/antagonistic effects of different agents in the context of the whole tumor ecosystem (Bagaev et al., 2021; Ho et al., 2022; Newell et al., 2022).

In recent years, several studies have generated transcriptomic or proteomic profiles of clinical patient cohorts under anti-PD-1 treatment, which provide rich resources to characterize key regulators affecting immunotherapy response. However, it remains challenging to digest these data to make translational impacts for several reasons. First, owing to limited sample sizes of clinical cohorts, the datasets obtained are often underpowered to detect key changes, especially given the multiple-testing burden in a genome-wide survey. To overcome this, it would be more powerful to focus on a subset of clinically actionable targets and assess the signal robustness across multiple cohorts. Second, clinical samples have highly heterogeneous cell compositions that confer additional complexity in analysis (Zaitsev...
B

Anti-PD-1 treated patient cohorts

Differential expression analysis

Survival analysis

* Concordance between DEG and survival analysis

- High expression in responding group (Strong significance with FDR ≤ 0.15)
- High expression in responding group (Significance with p' ≤ 0.05)
- High expression in non-responding group (Strong significance with FDR ≤ 0.15)
et al., 2022): bulk samples reflect the average of mixed cell types, while single-cell data usually have very limited patient representativeness. Therefore, it is essential to perform complementary bulk sample and single-cell analyses and borrow the information from each other (Davis-Marcisak et al., 2021). Last but not least, pure clinical phenotype-driven association analyses often generate statistically significant but biologically trivial hits. It is also important to consider the pattern of potential targets in disease progression or lineage plasticity. The convergent hits of the above two analyses would give more credible hypotheses for subsequent investigation. With these considerations in mind, we focused on the anti-PD-1 treated patient cohorts of melanoma, the frontier of checkpoint inhibition immunotherapy, and performed an integrative analysis of bulk and single-cell data across multiple clinical cohorts to obtain insights that can maximize the benefits of immunotherapy.

RESULTS

High SIRPA expression correlates with response to PD-1 blockade in bulk samples

To identify effective therapies that can potentially overcome the resistance to checkpoint inhibitors, we focused on a set of emerging immuno-oncology target genes and performed an integrative analysis across five melanoma patient cohorts under anti-PD-1 treatment (four with available transcriptomic data and one with proteomic data) (Figure 1A). We collected 60 immuno-oncology target genes that have active agents tested in ≥10 active clinical trials (Table S1). Given these highly actionable targets, for each of the five patient cohorts we performed two parallel analyses: (1) differential expression analysis between responding and non-responding groups; and (2) patient survival analysis between high-expression and low-expression groups of each gene (Figure 1A). Through the differential expression analysis, seven genes showed significant expression-response correlations in multiple cohorts (p ≤ 0.05, false discovery rate [FDR] ≤ 0.15, Figure 1B). Among these genes, SIRPA showed the most consistent pattern in the differential expression analysis across different patient cohorts (Figure 1C).

SIRPA is an emerging target in cancer immunotherapy (Uger and Johnson, 2020; Xiang et al., 2021). The protein product of SIRPA, signal regulatory protein α1 (SIRPα), also known as CD172a or SHPS-1, is a multifunctional transmembrane glycoprotein (Barclay and Brown, 2006). SIRPα was thought to be selectively expressed on myeloid cells (e.g., macrophages, dendritic cells, and neutrophils) and neurons (Adams et al., 1998). Recently, however, many tumor cell lines have been shown to express SIRPα on their surface (Yamasaki et al., 2007; Yanagita et al., 2017). SIRPα exerts its effects through interaction with its ligand CD47, a transmembrane glycoprotein, ubiquitously expressed in different cell types and often overexpressed in solid and hematologic tumors. Our results revealed that tumor samples with high SIRPA expression were significantly more sensitive to PD-1 blockade not only at the RNA level (p = 0.01 and FDR < 0.15 in the Hugo cohort [Hugo et al., 2016], and p = 0.05 in the Gide and Liu cohorts [Gide et al., 2019; Liu et al., 2019], Figure 1D) but also at the protein level (p = 0.01 and FDR < 0.15 in the Harel cohort [Harel et al., 2019], Figure 1E). Furthermore, patient survival analyses in multiple cohorts showed that the patient group with high SIRPA expression had a better prognosis than that with low SIRPA expression (log-rank test, p = 0.016 in the Hugo cohort, Figure 1D; p = 0.026 in the Harel cohort, Figure 1E). Among the five cohorts assessed, the Riaz cohort (Riaz et al., 2017) is the only one that did not show any significant patterns in tumor response or patient survival analyses, likely because (1) this cohort has a small sample size and (2) the majority of the patients underwent complicated treatments. To further evaluate its potential as an immunotherapy biomarker, we found that SIRPA expression showed no correlation with response to other therapies such as BRAF inhibitors (Figures S1A and S1B), established immunotherapy biomarkers such as tumor mutation burden (Figure S1C), or prognosis in the general patient population (Figure S1D). These results suggest a positive and unique role of SIRPA in tumors responding to anti-PD-1 immunotherapy.

High SIRPA expression in anti-PD-1-responding tumors comes from tumor cells

SIRPα on macrophages, interacting with CD47 on tumor cells or T cells, was recently established as the first macrophage checkpoint (Chao et al., 2019; Jalil et al., 2020; Uger and Johnson, 2020). Since the interaction between SIRPα-bearing macrophages and CD47-positive tumor cells triggers a “do not eat me” signaling cascade to inhibit the phagocytosis of tumor cells, the blockade of such an interaction would lead to efficient phagocytosis of tumor cells by macrophages. In light of this mechanism, several clinical trials were launched to target the SIRPα-CD47 interaction, alone or in combination with the anti-PD-1 treatment (Jalil et al., 2020), for a number of cancer types,
**Figure 2. SIRPA expression in tumor cells and macrophages in melanoma patient samples**

(A and B) Uniform manifold approximation and projection (UMAP) plot visualizing cell-type annotations (A) and SIRPA expression (B) in single cells of a melanoma patient cohort from Jerby-Arnon et al. (2018).

(C) Top: heatmap showing the proportion of cells positive for four melanoma gene markers in all melanoma cells of each patient. Middle: heatmap showing the proportion of SIRPA+ cells in different cell types of each patient. Right: bar plot showing the proportion of SIRPA+ cells in different cell types where cells from all patients are combined.
including melanoma. However, this specific paradigm seemingly contradicts the SIRPA-related favorable response to the anti-PD-1 treatment we observed here.

To resolve this paradox, one key question is which cells contribute to the high expression of SIRPA in anti-PD-1-responding tumors, since all the above analyses were based on bulk samples consisting of a mixture of tumor cells and various non-tumor cells. Since tumor cells constitute a substantial proportion of a bulk tissue sample, as demonstrated by tumor purity often >70% in both the surveyed melanoma dataset (Hugo et al., 2016) and a larger pan-cancer cohort (Aran et al., 2015), we hypothesized that the difference in SIRPA expression level between patients with distinct clinical benefits could mainly reflect a tumor-intrinsic SIRPA pattern. To validate this, we collected multiple single-cell RNA sequencing (scRNA-seq) datasets from melanoma patients with diverse clinical backgrounds, where the expression patterns of SIRPA can be investigated in individual cell populations. First, we performed the analysis based on two published scRNA-seq datasets of melanoma patients (Jerby-Arnon et al., 2018; Smalley et al., 2021).

In the Jerby-Arnon dataset, we found that SIRPA mRNA expression was comparably enriched in melanoma, macrophages, and monocyte cells while completely depleted in stromal, endothelial, and non-monocyte immune cells (Figures 2A–2C). We observed the same phenomenon in another scRNA-seq dataset of distant melanoma metastases (Smalley et al., 2021), suggesting a stable and abundant expression of SIRPA in melanoma cells even after developing a secondary malignant growth, regardless of the metastatic site (Figures 2D–2F). Importantly, although tumor cells were primarily clustered by patients, indicating significant inter-patient transcriptomic heterogeneity, considerable SIRPA expression was detected in most of the patients, demonstrating a ubiquitous presence of SIRPA in melanoma cells. Consistent with the findings in human patient samples, melanoma cell lines are among those with the highest SIRPA expression at both RNA and protein levels across >50 lineages (Figures S2A and S2B). Finally, we analyzed a single-cell proteomics dataset of a melanoma cell line (WM989) and a monocyte cell line (U937) (Leduc et al., 2022) and found that SIRPA was expressed at a ubiquitously high level in both cell types, indicating that SIRPA expression is constitutive of the melanoma cell state (Figures 2G and 2H).

We next aimed to elucidate the exact contribution of tumor cells and macrophages to high SIRPA signals in tumors responding to anti-PD-1 treatment. As there is no single-cell profile of both cell types in the bulk sample cohorts surveyed, we first applied a computational deconvolution algorithm, CIBERSORTx (Newman et al., 2019) to the bulk RNA-seq dataset of Hugo et al. (2016) to infer tumor- and macrophage-specific gene expression profiles for each sample, using the Tirosi scRNA-seq dataset (Tirosi et al., 2016) as a cell signature reference (Figure 2I). We found that SIRPA was significantly upregulated in melanoma cells of patients responding to anti-PD-1 treatment (p = 0.022, Figure 2I) but significantly downregulated in macrophages for the responding group (p = 0.033, Figure 2I). To further confirm this pattern, we obtained another two scRNA-seq datasets that respectively surveyed tumor and macrophage transcriptomes in melanoma patients receiving anti-PD-1 treatment. With the first dataset (Jerby-Arnon et al., 2018), we showed that SIRPA was expressed less abundantly in the melanoma cells of the post-treatment-resistant group compared with the treatment-naive group (p = 8.5 × 10^{-4}, Figure 2J). In the second dataset (Sade-Feldman et al., 2018), we found that SIRPA was significantly downregulated in macrophages responding to anti-PD-1 therapy (p = 1.1 × 10^{-7}, Figure 2K). Collectively, these results from both deconvoluted bulk samples and single-cell profiling data suggest that the high expression of SIRPA correlated with anti-PD-1 treatment response is due to melanoma cells rather than macrophages.

SIRPA is a melanocytic marker that decreases during melanoma progression

Having established the clinically relevant high expression of SIRPA in melanoma cells, we sought to further explore its role in melanoma biology. Because cellular dedifferentiation is a key axis of the melanoma phenotype (Agaimy et al., 2016; Kohler et al., 2017; Riesenberg et al., 2015), we asked whether the SIRPA expression dynamics reside in a meaningful topology along the melanoma dedifferentiation trajectory. To address this question, we analyzed an RNA-seq dataset comprising a panel of human melanoma cell lines spanning four consecutive differentiation stages, namely, undifferentiated, neural crest-like, transitory, and melanocytic (Tsoi et al., 2018). Intriguingly, SIRPA expression appears to be a monotonically increasing function of the melanoma differentiation status, as readily visualized in the embedded principal component analysis (PCA) space, with the most differentiated melanoma cells showing the strongest expression of SIRPA (Figures 3A and 3B).
Figure 3. SIRPA expression dynamics in melanocyte maturation and melanoma dedifferentiation
(A and B) PCA projection of human melanoma cell lines from Tsoi et al. (2018), based on gene expression profiles and colored by dedifferentiation stages (A) or normalized SIRPA expression level (B).
(C–E) PCA projection of human melanoma cell lines from CCLE, based on gene expression profiles and colored by differentiation score (C), SIRPα protein expression by RPPAs (D), or SIRPα protein expression by quantitative proteomics (E).
(F and G) PCA projection of in vitro differentiating human melanocytes from Mica et al. (2013), based on gene expression profiles and colored by differentiation time (F) or normalized SIRPA expression level (G).
further measured the SIRPα protein expression using reverse-phase protein arrays (RPPAs) in an independent collection of 48 melanoma cell lines with parallel publicly available RNA-seq and quantitative proteomics data. After scoring each cell line for differentiation status using a signature derived from a previous study (Tsai et al., 2018), we found a very strong correlation between SIRPα protein abundance and melanoma differentiation (Figures 3C–3E). Melanocytes are known to be the cell of origin of melanoma tumors (Gupta et al., 2005; Kohler et al., 2017; Moon et al., 2017). Thus, we hypothesized that SIRPα would show a similar pattern of expression on a melanocyte maturation trajectory. Indeed, based on an RNA-seq dataset derived from cultured human melanocytes and their progenitors (Mica et al., 2013), we showed that the high-expression status of SIRPα is gradually institutional as melanocytes reach maturity (Figures 3F and 3G).

Bridging the tumorigenic melanoma dedifferentiation process and the physiological melanocytic differentiation process, we next aimed to monitor SIRPα expression in a setting where melanocyte-to-melanoma transformation is captured. A recent study on the stepwise introduction of oncogenic mutations into primary human melanocytes generated scRNA-seq data on genetically distinct melanoma cellular models (Hodis et al., 2022) (Figure S3A). This allowed us to query SIRPα expression along a phylogenetically related trajectory of melanoma from its normal cell of origin and associate SIRPα expression shifts with key oncogenic events. Using well-established melanocytic markers (e.g., PMEL, MLANA, MITF, and TYR) to form a reference for the differentiation program, we observed that this highly matched SIRPα expression changes between the populations, including a drop when cells obtained replicative immortality through a TERT promoter mutation and an increase when a PTEN exon mutation led to MITF duplication (Figure S3B).

Finally, to validate our findings on the in vitro SIRPα expression dynamics in melanocytes and melanoma cells in real-world physiological and pathological contexts, we further performed two analyses on data from human samples. First, we analyzed an scRNA-seq dataset of human skin samples across three developmental stages (Belote et al., 2021): fetal, neonatal, and adult. Consistent with the in vitro data, we found a ubiquitous expression pattern of SIRPα in melanocytes of all developmental stages (Figures 3H–3J). Interestingly, SIRPα expression was exclusive to melanocytes, emphasizing its important role in establishing the identity of this cell type. A pseudo-time trajectory-based analysis of fetal-stage melanocytes during the establishment of their identity further characterized SIRPα as a marker gene of melanocytic maturation (Figure S3C). Second, we reanalyzed The Cancer Genome Atlas (TCGA) cutaneous melanoma cohort (n = 472) with a specific focus on the association between SIRPα expression and well-established pathological/clinical features. We found that the expression level of SIRPα was highly negatively correlated with melanoma tumor dedifferentiation (Figure 3K), consistent with our observation in the cell line data. Together, these results support that SIRPα is a melanocytic marker whose loss is a hallmark of melanoma progression.

**SIRPα loss confers anti-PD-L1 resistance through the interaction of tumor cells and CD8+ T cells**

Melanoma dedifferentiation is a known mechanism of resistance to T cell-mediated immunotherapy through loss of melanocytic antigens, which has been shown in both mice (Landsberg et al., 2012) and humans (Mehta et al., 2018). Based on our observations that SIRPα, a previously understudied melanocytic antigen gene, showed strong positive correlations with both anti-PD-1 immunotherapy response and melanoma differentiation status, we hypothesized that SIRPα is directly involved in enhancing T cell-mediated immunotherapy, and its loss then serves as a mediator of immunotherapy resistance that accompanies the attenuation of melanocytic identity.

To identify a possible molecular mechanism for this phenotype, we first focused on the canonical SIRPα–CD47 interaction model. According to the current research paradigm, this interaction provides an antiphagocytic signal that modulates the crosstalk between macrophages and tumor cells (Morrissey et al., 2020; Takizawa and Manz, 2007; Willingham et al., 2012). However, given our findings on SIRPα expression prevailing in tumor cells and a widespread presence of CD47 in all major immune cell populations (Figures S4A–S4D), SIRPα–CD47 interaction may contribute to the communication among alternative cell-type combinations. To identify the donor and receptor cell populations that host SIRPα and CD47 in the melanoma ecosystem, we analyzed a melanoma spatial transcriptome dataset (Thrane et al., 2018), where the localization of the tumor, stromal, and immune cells was determined using hematoxylin and eosin (H&E) staining — and was consistent with our gene-expression-based deconvolution results (Figure 4A). The juxtaposition of slide-wide cell population distributions and the expression patterns of SIRPα and CD47 showed enrichment of SIRPα in melanoma cells and its co-localization with CD47 in T cells (Figure 4A). Given that tumor-intrinsic SIRPα overexpression in patient samples correlates with a favorable response to PD-1 blockade therapy, we hypothesized that such an effect is caused by an enhanced SIRPα–CD47 communication between melanoma cells and T cells. To test this hypothesis, we inferred a SIRPα–CD47 interaction scoring using CellPhoneDB (Efremova et al., 2020) based on the co-expression patterns of SIRPα in melanoma cells and CD47 in CD8+ T cells in the Tirosh scRNA-seq dataset.
(Tirosh et al., 2016). Indeed, we observed a significant SIRPα-CD47 interaction score between melanoma cells and CD8+ T cells (p = 0.008, permutation test, Figure 4B).

To directly examine the effect of SIRPα on T cell-dependent tumor immune responses, we designed a co-culture system and performed cytotoxic T cell killing assays (Figure 4C). We established two stable cell lines with verified SIRPα knockdown (KD) and overexpression (OE) (Figure 4D) and confirmed that SIRPα perturbation in tumor cells had no impact on in vitro tumor growth during the co-culture experiment (Figure S4E). We then co-cultured these cells with activated cytotoxic CD8+ T cells for 24 h. We found that cytotoxic T cells killed tumor cells with SIRPα overexpression more efficiently compared with the non-targeting controls (NTCs), whereas tumor cells with the loss of SIRPα were more resistant to T cell-mediated killing (Figure 4E). This suggests a positive role of SIRPα on the tumor cell surface in T cell-mediated tumor cytotoxicity.

To further confirm the role of SIRPα in the context of anti-PD-1/PD-L1 treatment, we added a mouse PD-L1 (mPD-L1) antibody into the co-culture system and observed that the difference in T cell killing effect was even more striking (Figure 4F) than in the setting without the antibody (Figure 4E). The same results were observed when adopting an additional SIRPα KD cell line with an extended co-culture time of 96 h (Figures S4F and S4G). To rule out the possibility that SIRPα perturbation leads to expression changes of melanoma differentiation antigens (MDAs), which directly mediate the immunogenicity of melanoma cells (Pitcovski et al., 2017), we profiled the transcriptomes of SIRPα-KD and SIRPα-OE B16F10 cells and found no significant expression changes for six well-established MDA-encoding genes, PMEL, TYR, TYRP1, DCT, MLANA, and MITF (Figure 4G). To provide more direct support for the proposed immunostimulatory activity of SIRPα via CD47-mediated interactions with CD8+ T cells, we utilized two antibodies capable of blocking the SIRPα-CD47 interaction, MIA410 and MIA430 (Han et al., 2000; Willingham et al., 2012), to pre-treat the T cells before the co-culture and tested whether this blockade would affect T cell-mediated cytotoxicity. The results showed that the tumor-killing effect was hampered in T cells with CD47 blocked, and the effect was enhanced in the culture with SIRPα-overexpressing melanoma cells (Figure 4H). Taken together, these results suggest that melanoma-intrinsic SIRPα can effectively trigger T cell immunogenicity through CD47 interaction in PD-1/PD-L1 blockade immunotherapy.

**Tumor-intrinsic SIRPα enhances the antitumor response of checkpoint blockade in mice**

To investigate whether melanoma-intrinsic SIRPα affects the efficacy of checkpoint inhibition immunotherapy in vivo, we utilized the well-established B16F10 murine melanoma model. We subcutaneously inoculated B16F10 cells with different SIRPα perturbations—overexpression (mSIRPA-OE), knockdown (mSIRPA-KD), and NTCs—into C57BL/6J mice. We implemented a two-step randomization strategy to ensure homogeneous tumor volumes before treatment with anti-mPD-L1 or isotype control (Figure 5A). Compared with tumors treated with isotype control, tumors overexpressing SIRPα showed the most remarkable response to anti-mPD-L1 treatment, both at specific time points and across time points (p < 10⁻³); NTC tumors showed a moderate but significant response to the treatment (p < 10⁻²); whereas tumors bearing SIRPα KD essentially exhibited no response (Figures 5B, 5C, and 5S). We next examined the tumor volume changes from baseline (anti-mPD-L1 versus isotype control) at a single-mouse scale. At different time points (days 10, 12, 14, and 16), the three mouse groups showed distinct tumor volume change rates from each other: all the mice in the mSIRPA-OE group consistently benefited from anti-mPD-L1 treatment and showed considerable tumor shrinkage, and in some cases tumors even disappeared completely; most mice in the NTC group showed response to the treatment, but in a small proportion of mice, the tumor size increased; in sharp contrast to mSIRPA-OE, approximately half of the mice in the mSIRPA-KD group suffered from tumor expansion (Figures 5D and 5E). Finally, we examined the impact of different SIRPα perturbations on animal survival. The mSIRPA-OE mice treated with anti-mPD-L1 showed a much better prognosis than those treated with isotype control (log-rank test, p < 10⁻³), and the median survival time increased from 14 days to 20 days; NTC mice showed a marginally significant survival benefit (log-rank test, p < 0.08); mSIRPA-KD mice showed no difference at all (log-rank test, p < 0.8) (Figure 5F). Collectively, these results provide strong evidence supporting...
**A**

Randomization before treatment  
Treatment of Anti-mPD-L1 and Isotype con.  
Measurement of tumor growth

**B**

Mean of tumor volume (mm³)

**C**

Tumor volume (mm³)

**D**

Change from baseline

**E**

Averaged change from baseline

**F**

Survival probability

(legend on next page)
a positive role of tumor-intrinsic SIRPA in PD-1/PD-L1 induced, T cell-mediated antitumor immunity.

**SIRPα expression is positively regulated by SIRPAP1 in melanoma**

To identify potential regulators that affect the heterogeneity of SIRPα protein expression in melanoma patients, we quantified SIRPα protein expression in 349 TCGA melanoma samples using RPPAs and then performed an association analysis with other TCGA molecular profiling data, including DNA methylation, somatic mutation, somatic copy-number alteration, and the expression of its endogenous pseudogene, SIRPAP1. Among the three cis-regulatory features, SIRPA gene amplification showed a significant positive correlation, but the effect size was limited (Figures 6A and 6B). In the Hugo cohort (Hugo et al., 2016), SIRPA gene amplification showed no relation to SIRPα expression (Figure S6A). Intriguingly, SIRPAP1 RNA expression was strongly correlated with SIRPα expression (Figures 6A and 6C). We further confirmed this pattern in CCLE melanoma cancer cell lines (Figure S6B) based on quantitative proteomics data. These results suggest that SIRPAP1 is a key non-coding regulator of SIRPα.

To dissect the competitive relationship between SIRPA and SIRPAP1, we conducted a microRNA (miRNA)-centered analysis (Figure S6C). We adopted three common miRNA target prediction tools, RNAhybrid (Kruger and Rehmsmeier, 2006), miRDB (Wong and Wang, 2015), and TarPmiR (Ding et al., 2016), to identify potential miRNAs and their corresponding target sites shared by SIRPA and SIRPAP1. We then performed an association analysis on the expression levels between the miRNA candidates and SIRPA or SIRPAP1 using TCGA melanoma data. We identified three miRNAs, let-7a-2-3p, miR-149-3p, and miR-3154, that targeted the homologous regions of SIRPA and SIRPAP1 and exhibited a significantly negative correlation with both of their expression levels (Figures 6D and S6D). In particular, let-7a-2-3p showed the strongest anticorrelation with SIRPA/SIRPAP1 expression (Figures 6E and 6F). These results suggest that the co-regulation of SIRPA and SIRPAP1 in melanoma is mediated by a group of miRNAs.

To test whether SIRPAP1 can causally regulate SIRPα expression in melanoma cells and whether such regulation can affect the protein abundance on the cell surface, we overexpressed SIRPAP1 in A375 melanoma cells (Figure 6G) and measured the total and membrane-anchored SIRPα level by western blot and flow cytometry, respectively (Figures 6H and 6I). Indeed, we found that the SIRPAP1 overexpression greatly increased the total and cell surface SIRPα level. To further confirm this observation, we utilized the CRISPR-Cas9 synergistic activation mediator (SAM) system to transcriptionally activate SIRPAP1 in A375 cells (Figure 6J). We observed the same increased protein expression after induced SIRPAP1 overexpression (Figures 6K and 6L). Thus, we established SIRPAP1 as a positive regulator of the SIRPα expression on the surface of melanoma cells.

Taken together, we propose a model of tumor-intrinsic SIRPα-mediated immunotherapy response (Figure 7). Specifically, multiple mechanisms contribute to SIRPα expression heterogeneity, including SIRPAP1 as a competing endogenous RNA to upregulate SIRPA. The tumor cells with high SIRPα expression on the surface then interact with CD47 on CD8+ T cells. Such interaction may enhance cell-cell adhesion between tumor cells and CD8+ T cells, thereby facilitating T cell killing activity. As a result, patients whose tumors carry high expression of SIRPA show favorable responses to anti-PD-1 immunotherapy.

**DISCUSSION**

Checkpoint inhibition immunotherapy has revolutionized cancer treatment by leveraging the cytotoxic potential of the human immune cells, especially cytotoxic T cells, yet we still have a very limited ability to predict patients’ responses to immunotherapy. In this study, focusing on emerging immune-oncology targets, we developed an integrative analysis strategy to prioritize actionable targets in combination with anti-PD-1/PD-L1 therapy. Combining single-cell bulk and RNA-seq datasets from melanoma patients, we showed that high SIRPA expression correlated with a favorable response to anti-PD-1 treatment and that it is melanoma cells, rather than macrophages, that contribute to the observed pattern. Through both in vivo and in vitro experiments, we further demonstrated that tumor-intrinsic SIRPA promotes T cell-mediated immunotherapy response. This is in sharp contrast to the well-established role of SIRPA as a major inhibitory immune modulator in macrophages.

Although the CD47-SIRPα signaling axis is an innate immune checkpoint in cancer, durable antitumor responses require adaptive immune cell stimulation (Sockolosky et al., 2016).
Figure 6. Regulation of SIRPα protein expression in melanoma patients
(A–C) Summarized plot showing possible regulations of SIRPα protein expression across TCGA-SKCM patients assayed by RPPAs in this study, including SIRPA DNA methylation, somatic mutation, somatic copy-number alteration (SCNA), and SIRPAP1 expression.
(A) Top: bar plot of TCGA-SKCM samples ranked by SIRPα protein expression. Color keys indicate normalized levels for SIRPα protein expression, SIRPA DNA methylation, and SIRPAP1 expression, respectively. Bottom: heatmap showing different regulatory features. (B) Boxplots showing the association between gene
Several studies suggest the synergistic effect of the anti-CD47-SIRPα signaling axis and anti-PD-1/PD-L1 therapy in syngeneic mouse models (Kuo et al., 2020; Sockolosky et al., 2016; Yanagita et al., 2017). Our results from the analysis of clinical patient cohorts and functional assays reveal a positive role of tumor-intrinsic SIRPA in the activated T cell-mediated cytotoxicity. Interestingly, a recent study identified a functional subpopulation of SIRPA+ CD8+ T cells in humans and mice during chronic immune exhaustion, and showed that these T cells kill the CD47+ target more efficiently compared with the SIRPA− CD8+ T cells both in vivo and in vitro (Myers et al., 2019). Similarly, our data showed that tumor cells with SIRPA overexpression were sensitive to cytotoxic T cell-mediated killing. One possible mechanism is that CD47-SIRPα interaction stabilizes cell-to-cell contacts and cytolytic synapses. The strength of cell-to-cell interaction is determined not only by the affinity between the receptor and the ligand but also by their avidity. As the spanning distance of the end-to-end bound CD47-SIRPα complex (~14 nm) is very similar to TCR-MHC, CD28+ CD86, and CD40+ CD40L, tumor cells or CD8+ T cells bearing more SIRPA could have stronger and longer interactions with cells expressing CD47, leading to a greater cytotoxicity effect (Myers et al., 2019). Furthermore, another study shows that the interaction of SIRPα on dendritic cells and CD47 on T cells is important for T cell activation (Seiffert et al., 2001). Thus, these studies and ours collectively suggest that any disruption to the interaction between SIRPα and CD47 may affect T cell activation and the related antitumor activity.

The CD47-SIRPα signaling axis is a hot topic in the field of immunotherapy and has been under intensive clinical investigation. Results from multiple clinical trials show that monoclonal antibodies against CD47 exhibited varied efficacy between different cancer types (Huang et al., 2017; Zhang et al., 2020b). The ubiquitous expression of CD47 on all cell types may contribute to the low efficacy and side effects of the monotherapy. Given the notion that SIRPA is predeterminately expressed in neurons, dendritic cells, and macrophages, some clinical trials were initiated to evaluate the possibility of SIRPA as an alternative target. However, we found that many different types of cancer cells expressed SIRPA. Some cancer cells, for example, melanoma cells, express as much SIRPA as tumor-associated macrophages. Given the dual role of SIRPA in immunotherapy response, we would like to emphasize that it is the overall net effect that determines the clinical benefits.

Specifically, our study may have significant clinical implications for SIRPα-related therapies (Figure 7). First, for indication selection, the effect of SIRPα blockade may depend on the relative abundance of tumor-intrinsic SIRPα in a given cancer lineage. In cancer types such as melanoma, where tumor-intrinsic SIRPα expression is high, the blockade may significantly dampen tumor killing mediated by CD8+ T cells, whereas in other cancer types such as breast cancer and lymphoma, where SIRPA expression is much lower, the efficacy of SIRPα blockade in pre-clinical models (Gauttier et al., 2020; Ring et al., 2017) likely reflects a dominant role of macrophage-mediated phagocytosis of tumor cells. Therefore, the relative abundance of SIRPA in tumor cells must be considered when choosing suitable cancer types for anti-SIRPα treatment. Second, to stratify patients for better immunotherapy response within a cancer type, tumor-specific SIRPA expression may be a more effective biomarker than the bulk-level SIRPA expression, as the latter would be confounded by signals from different cell components within a tumor. Currently, biomarker identification is largely based on the analysis of bulk-level expression data, and more efforts should be made to assess cell-type-specific gene expression signatures as potential biomarkers. Finally, for drug development, we propose that antibodies that specifically bind to SIRPα on the surface of macrophages would be more effective. For that purpose, it would be of particular interest to design bispecific antibodies that can simultaneously bind to two different types of antigens, one to target macrophage-specific antigens and the other to target SIRPα.

Our study highlights cell-type-dependent antagonistic effects of the same target on immunotherapy, an issue largely ignored in the field. Besides SIRPA, tumor-intrinsic PD-1 and CTLA-4 have been reported (Wang et al., 2020; Zhang et al., 2019). The unexpected expression of these immune checkpoints on the surface of tumor cells may antagonize or agonize the immune cells’ anti-tumor activity (Kleffel et al., 2015; Zhang et al., 2019). Considering the high fraction of tumor cells in the tumor ecosystem, it would be critical to systematically elucidate the effects of immunotherapeutic targets in different cell types and take this into account in the design of clinical studies.

### STAR+ METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Material availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Cell lines

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amplification and SIRPα protein expression. Patient samples with somatic copy number with log2 transformation >1 are denoted as amplified and the others as non-amplified. The middle line in the box is the median, the bottom and top of the box are the first and third quartiles, and the whiskers extend to the 1.5× interquartile range of the lower and the upper quartiles, respectively. (C) Scatterplot showing the correlation between SIRPA and SIRPAP1 RNA expression. The correlation coefficient and p value are based on Spearman’s rank correlation.

(D) Correlation plot showing the miRNAs which are negatively correlated with SIRPA expression in TCGA-SKCM samples. The color bar represents Spearman’s rank correlation coefficient.

(E and F) Scatterplots showing the correlations between let-7a-2-3p and SIRPA (E) or SIRPAP1 (F) expression.

(G) Cartoon summary of SIRPAP1 overexpression by lentivirus transduction.

(H and I) Western blot (H) and flow cytometry (I) of SIRPα protein expression upon vector-based SIRPAP1 overexpression.

(J) Cartoon summary of SIRPAP1 overexpression by CRISPR-Cas9 SAM system.

(K and L) Western blot (K) and flow cytometry (L) of SIRPα protein expression upon CRISPR-Cas9-based SIRPAP1 overexpression.

See also Figure S6.
**METHOD DETAILS**

- Mice
- Expression plasmids
- Generation of stable cell lines
- Generation of CRISPR gene activation system
- Curation of immuno-oncology targets
- Integrative analysis of bulk transcriptomic and proteomic data from anti-PD-1 treatment patient cohorts
- Analysis of single-cell RNA-seq data of melanoma and normal skin samples
- Pseudo-time trajectory analysis of melanocyte scRNA-seq data
- Analysis of single-cell proteomics data of human melanoma and monocyte cell lines
- In-silico inference of cell-type-specific gene expression from bulk samples
- Analysis of melanoma spatial transcriptomic data
- Estimation of cell-cell communication using single-cell RNA-seq data
- Generation and analysis of SIRPA-perturbed B16F10 RNA-seq data
- Profiling of SIRPα protein expression in TCGA melanoma and CCLE samples
- Analysis of potential regulators of SIRPα protein expression
- MicroRNA analysis
- Flow cytometry analysis

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**Figure 7. The proposed model of SIRPα-mediated T cell-centric immunotherapy response**

The proposed model consists of three consecutive layers, from the molecular mechanism to impacts on clinical outcomes. The left and right sides show the contrasting situations of SIRPA high expression versus SIRPA low expression in tumor cells. On the left side, within tumor cells, SIRPA1 upregulates SIRPA by functioning as a competing endogenous RNA; within the tumor microenvironment (TME), SIRPα on the surface of tumor cells interacts with CD47 on CD8+ T cells to enhance cell-cell adhesion between these two cell types; and consequently, the enhanced cell-cell interaction increases the T cell killing effect, leading to a better response to anti-PD-1/PD-L1 therapy. In contrast, on the right side, tumor cells with low SIRPA expression have moderate cell-cell adhesion with CD8+ T cells, thereby rendering resistance to anti-PD-1/PD-L1 therapy. The bottom panels show clinical implications of the proposed model in terms of indication for treatment, immunotherapy biomarker, and therapeutic development.
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ccell.2022.10.012.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

M.-J.M.C. and H.L. conceived the project. M.-J.M.C. and Y.L. led the data analysis. Z.Z. led the experiments and contributed to the data analysis. K.M. and X.P. contributed to the experiments, H.C. contributed to the data analysis. S.V.K., R.A., and Y.L. contributed to RPPA experiments. Z.Z., M.-J.M.C., Y.L., and H.L. wrote the manuscript. H.L. supervised the project.

DECLARATION OF INTERESTS

H.L. is a shareholder and scientific adviser of Precision Scientific Ltd.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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Published: November 3, 2022

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myeloid checkpoint receptor SIRPalpha potentiates innate and adaptive im-


Science 376, eabi8175.

Huang, Y., Ma, Y., Gao, P., and Yao, Z. (2017). Targeting CD47: the achieve-


Kuo, T.C., Chen, A., Harabi, O., Sockolosky, J.T., Zhang, A., Sangalang, E., Doyle, L.V., Kauder, S.E., Fontaine, D., Bollini, S., et al. (2020). Targeting the myeloid checkpoint receptor SIRPαβα potentiates innate and adaptive imm-


### STAR★METHODS

#### KEY RESOURCES TABLE

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<td>Miltenyi Biotec Inc</td>
<td>130-099-896</td>
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<td>REA Control-FITC</td>
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<td>Anti-SIRPα antibody</td>
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<td>Endura™ Chemically Competent Cells</td>
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<td>jetPRIME® Versatile DNA/siRNA transfection reagent(0.75 mL)</td>
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<td>Bulk proteomics data from anti-PD-1-treatment-responding and non-responding melanoma patients (Figures 1A–1C and E)</td>
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<td>Tumor mutation burden in melanoma patients (Figure S1C)</td>
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<td>Bulk RNA-seq data of in vitro differentiating melanocytes derived from ESC/iPSC (Figures 3F and 3G)</td>
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<td>Whole-exome sequencing data from anti-PD-1-treatment-responsive and non-responding melanoma patients (Figure S6A)</td>
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Experimental models: cell lines

| HEK293T | MD Anderson Characterized Cell Line Core Facility HEK293T |
| A375 | MD Anderson Characterized Cell Line Core Facility A375M |
| A375-dCas9-SAM | This study N/A |
| B16F10 | ATCC CRL-6475 |

Experimental models: organisms/strains

| Mouse:B6.Cg-Thy1a/Cy Tg(TcraTcrb)8Rest/J | The Jackson Laboratory JAX:005023; RRID:IMSR_JAX:005023 |
| Mouse: C57BL/6J | The Jackson Laboratory JAX:000664; RRID:IMSR_JAX:005023 |

Oligonucleotides

| Scramble gRNA-F: 5’-CACCGGTATTACTGATATTGGTG-3’ | This study N/A |
| Scramble gRNA-R: 5’-AACCACCCACCAATATCTCAATACC-3’ | This study N/A |
| SIRPAP1-1-F: 5’-CACCGGTAGGGTCGCGAGACGGATG-3’ | This study N/A |
| SIRPAP1-1-R: 5’-AAACCATTCCGTCGACCGAGCCCTACC-3’ | This study N/A |

Recombinant DNA

| pCMV- SV-G | Addgene 8454; RRID:Addgene_8454 |
| pCMV-dR8.2 dvpr | Addgene 8455; RRID:Addgene_8455 |
| lentiviral sgRNA (MS2), zeo backbone | Addgene 61427; RRID:Addgene_61427 |
| lentiviral dCAS-VP64, Blast | Addgene 61425; RRID:Addgene_61425 |
| lentiviral MPH | Addgene 89308; RRID:Addgene_89308 |
| plenti-CMV-Puro-Dest | Addgene 17452; RRID:Addgene_17452 |
| pDONR221-Human SIRPAP1 | Epoch Life Science GS65919 |
| pDONR221-Mouse SIRPA | Epoch Life Science GS68006 |
| plenti-CMV-Puro-Human SIRPAP1 | This study N/A |
| plenti-CMV-Puro-Mouse SIRPA | This study N/A |
|MISSION® plKO.1-puro Non-Target shRNA Control Plasmid DNA | Sigma-Aldrich SHC016-1EA |
| SHCLNG MISSION shRNA-1 | Sigma-Aldrich TRCN0000029914 |
| SHCLNG MISSION shRNA-2 | Sigma-Aldrich TRCN0000055053 |
| SHCLNG MISSION shRNA-3 | Sigma-Aldrich TRCN0000029915 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Han Liang (hliang1@mdanderson.org).

Material availability
This study did not generate new unique reagents.

Data and code availability
The newly generated bulk RNA-seq data of B16F10 cells with SIRPA perturbations are available at Gene Expression Omnibus (GEO) with an accession number GSE211226; and SIRPα RPPA data of TCGA-SKCM and CCLE samples are available at TCPA data portal (https://tcpaportal.org). The source and accession numbers for previously published datasets used in this study are as follows: bulk RNA-seq data from melanoma patients under anti-PD-1 treatment: GEO, GSE78220 and GSE91061, ENA, PRJEB23709, and dbGaP, phs000452.v3.p1; whole-exome sequencing data of melanoma patients under anti-PD-1 treatment, SRA: SRP090294 and SRP067938; bulk proteomics data from melanoma patients under anti-PD-1 treatment, Harel et al.; bulk RNA-seq data from melanoma patients under BRAFi treatment, GEO, GSE50509 and GSE99898; TCGA genomic data and clinical data, NCI Genome Data Commons; single-cell RNA-seq data of melanoma patients, GEO, GSE115978, GSE70630, GSE174401, GSE120575; single-cell proteomics data from human melanoma and monocyte cell lines, Leduc et al. (2022); CCLE quantitative mass spectrometry data, -Nusinow et al., 2020; CCLE gene expression data, CCLE data portal (https://portals.broadinstitute.org/ccle); bulk RNA-seq data of patient-derived melanoma cell lines, GEO, GSE80829; bulk RNA-seq data of in vitro differentiating melanocytes derived from ESC/iPSC, GEO: GSE45227; scRNA-seq data from human normal skin samples of different developmental stages, GEO: GSE151091; scRNA-seq data from step-wise-edited melanoma cell lines, Single Cell Portal: SCP1334; spatial transcriptomics data from treatment-naïve melanoma patients, http://www.spatialomics.org/SpatialDB; DICE immune cell type gene expression data,
https://dice-database.org; bulk proteomics data from human hematopoietic cell populations sorted from peripheral blood, http://www.improt.org. Code used for all processing and analysis is available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
B16F10, A375, and HEK293T cells were cultured in complete DMEM media (10% FBS and 50 U/mL Penicillin-Streptomycin). B16F10 cells with SIRPA perturbation and A375 cells stably expressing SIRPAP1 were cultured in complete DMEM media supplemented with 2 ug/mL puromycin. A375 cells stably integrated with synergistic activation mediator (SAM) dCas9 and effector components were cultured as described previously (Konermann et al., 2015). CD8+ T cells isolated from Pmel-1 mice were cultured in complete RPMI-164 media supplemented with 10% FBS, 20 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 2 mM L-glutamine. All cell lines prepared at MD Anderson Cancer Center were confirmed by short tandem repeat analysis and were periodically tested for mycobacterium contamination at the MD Anderson Characterized Cell Line Core.

Mice
We purchased 6-8 weeks old C57BL/6J (#000664) and B6.Cg-Thy1a/Cy Tg(TcraTcrb)8Rest/J (#005023) female mice from the Jackson Laboratory. All animal experiments and procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. Our sample size predetermination experiments indicated that \( n \geq 15 \) mice were needed in each group to detect the expected effect. In the experiment, all the animals were randomized before tumor inoculation; and three days after inoculation, mice with established tumors were randomized again before the treatment.

METHOD DETAILS

Expression plasmids
The full-length human SIRPAP1 and mouse Sirpa were synthesized by Epoch Life Science and cloned into pDONR221. The sequences of the two genes were verified by Sanger Sequencing. The expression clones were generated via the LR Clonase Reaction between the Entry clone and the pLenti CMV Puro DEST (w118-1). The pLenti CMV Puro DEST was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid # 17452; http://n2t.net/addgene:17452; RRID: Addgene_17452) (Campeau et al., 2009).

Generation of stable cell lines
HEK293T cells were co-transfected with lentiviral vectors encoding the gene of interest or shRNAs together with packaging vectors pCMV-dR8.2 dvpr and pCMV-VSV-G using JetPRIME. At 48 h post-transfection, the supernatant was collected and filtered using a sterile syringe filter with a 0.45 \( \mu \)m pore size hydrophilic PVDF membrane. The filtered supernatant was applied to infect the target cells for 18 h. The infected cells were selected using the appropriate antibiotics for a week before the conduction of the experiments.

Generation of CRISPR gene activation system
Lentivectors encoding dCAS9-VP64, MS2-P65-HSF1, and lenti-sgRNA(MS2) zeo backbone were gifts from Feng Zhang (Konermann et al., 2015). Human A375 cells stably expressing dCAS9-VP64, MS2-P65-HSF1 were infected with lentivirus expressing gRNA. We designed gRNAs to target 700 bp upstream of SIRPAP1’s transcription start site.

Curation of immuno-oncology targets
We collected the immuno-oncology (IO) targets listed in Global Immuno-Ontology Drug Development Pipeline 2020, a pdf file downloaded by selecting “Specific sheets from this dashboard” and “2020”). Then, we mapped the collected 508 IO targets (504 non-redundant names) to HGNC-approved symbols by HGNC multi-symbol checker (https://www.genenames.org/tools/multi-symbol-checker/). We manually reviewed the mapped gene symbols, filled blanks if a proper gene symbol could be found in GeneCards (https://genecards.org), and then excluded IO targets without gene symbols. Next, we merged redundant target names by summing the numbers of clinical trials from each record. Lastly, we focused on those IO targets with at least 10 active agents/clinical trials and obtained 60 IO targets for the subsequent analyses.

Integrative analysis of bulk transcriptomic and proteomic data from anti-PD-1 treatment patient cohorts
To collect suitable datasets, we first reviewed all available anti-PD-1 trial studies in the literature and at the website of Tumor Immune Dysfunction and Exclusion (TIDE; http://tide.dfci.harvard.edu). We collected the anti-PD1 treated melanoma patient cohorts with available RNA-seq or proteomic data, leading to four transcriptomic datasets (Gide et al., 2019; Hugo et al., 2016; Liu et al., 2019; Riaz et al., 2017) as well as one proteomic dataset (Harel et al., 2019) (see key resources table). We followed quality control of the samples from each study and then removed samples collected from sites other than skin to exclude potential confounders in survival analysis. In particular, seven non-skin samples were removed from the 41 anti-PD1 treated samples from the Gide dataset; two
unqualified and 33 non-skin samples were removed from the 121 samples of the Liu dataset; 7 unqualified samples were removed from the 74 samples of the Harel dataset. Next, to analyze RNA-seq data, we used gene annotation (version 37) from GENCODE. Then, we adopted Salmon v1.4.0 to obtain read counts and TPMs for each dataset using the default parameter setting. We developed an integrative analytic procedure combining proteomic and transcriptomic data with the treatment response and survival information. Specifically, we computed expression-response and expression-survival associations in anti-PD-1-treated melanoma patients and then examined the concordance of the two associations to evaluate the combinational potential of an active IO target for anti-PD-1 therapy in melanoma. First, we performed differential expression analysis. For transcriptomic data, we adopted three well-established methods (DE-seq2/edgeR/limma) to detect differentially expressed (DE) genes between the responding and non-responding patients given anti-PD-1 treatment. To identify the most robust differentially expressed IO targets, we then constructed a consensus set of DE features by overlapping the three DE lists (P ≤ 0.05 and FDR ≤ 0.15 among all three tests with two-sided testing). To detect differential signals more sensitively, we also computed p values with one-sided testing (P') to identify significant associations. For proteomic data, we used the Mann-Whitney U test (MW test), incorporating a permutation procedure to identify DE proteins. For each protein, we randomly shuffled the sample labels and calculated U scores of the MW test 10,000 times to obtain the background distribution and re-computed a p-value. Proteins with a p-value < 0.05 were defined as DE proteins. Second, we retained the IO targets that were differentially expressed in at least two patient cohorts. Third, we adopted a log-rank test (by ggpermplot with the option log.rank.weights = “n” to identify early survival differences) for survival analysis. For each candidate IO target, the patients in a cohort were divided into high- and low-expression groups based on the median expression. We then computed the association between the overall survival rate and the expression groups. Lastly, we examined the concordance between DE and survival analyses to identify the IO targets showing a consistent pattern in terms of tumor response and patient survival rate.

Analysis of single-cell RNA-seq data of melanoma and normal skin samples
We downloaded gene expression profiles of single cells in raw counts (UMIs for data generated with droplet-based platforms) along with clustering annotations and clinical metadata from the Gene Expression Omnibus (GEO) and Single Cell Portal (SCP; see key resources table). No quality control was further applied beyond what was already conducted by the original studies. We followed the Scanpy workflow (Wolf et al., 2018) for downstream analyses. Specifically, we 1) applied the log1pCP10K normalization to the raw counts, 2) selected highly variable genes, 3) regressed out the effects of the total count per cell and the percentage of mitochondrial gene count, 4) calculated the first 50 principal components, 5) applied Harmony (Korsunsky et al., 2019) to remove sample-level batch effects, 6) reduced the data dimension through Uniform Manifold Approximation and Projection (UMAP), 7) clustered the single cells using an unsupervised graph-based clustering algorithm (Leiden), 8) identified cluster-specific marker genes using Student’s t-test, and 9) annotated the clusters based on the expression patterns of literature-derived marker genes along with referring to the annotations from the original studies.

Pseudo-time trajectory analysis of melanocyte scRNA-seq data
We extracted the single-cell gene expression profiles of the human melanocytes at the fetal stage from the original dataset to build a pseudo-time trajectory. The data were normalized and processed following the same scRNA-seq data analysis procedure described above. Force Atlas was applied to generate a graph layout of the cells, upon which a differentiation progression was inferred by Diffusion Pseudo-time with the root set to be the cells with the lowest expression levels of canonical melanocytic lineage gene markers, including PMEL, MITF, and TYR. SIRPA expression was then visualized along the same trajectory to be compared against the differentiation progression.

Analysis of single-cell proteomics data of human melanoma and monocyte cell lines
We downloaded protein expression profiles of single cells in log-normalized and batch-corrected units from the Single-cell Proteomics Data Repository (see key resources table). Data processing and analysis were conducted using the same procedure as in scRNA-seq analysis.

In-silico inference of cell-type-specific gene expression from bulk samples
To computationally enumerate seven cell types, namely, tumor cells, T cells, B cells, macrophages, natural killer cells, fibroblasts, and endothelial cells, from bulk melanoma RNA-seq samples from Hugo et al., we used CIBERSORTx to estimate their relative fractions. Following estimations by the CIBERSORTx algorithm, we constructed gene expression signature matrices for the seven cell types based on the scRNA-seq from Tiros et al. Briefly, for each of the desired cell types, half of all the single cells were randomly selected without replacement and merged into a mega cell with average TPM values. Such random combinations were conducted 10 times to generate replicates for each cell type and were used as an input for a DE gene analysis to identify cell-type signature genes. Specifically, aggregated gene expression replicates of each cell type were compared against replicates of all other cell types using a Mann-Whitney U test, and the top 200 genes with an adjusted p-value < 0.01 and the highest log2FC were defined as signature genes. The average expression levels of these genes across all single cells were pooled into a final signature matrix for that cell type. We excluded 671 genes involved in the cell cycle (GO:0007049), 108 genes involved in ribosome biogenesis (GO:0042254), 21 genes involved in cell apoptosis (GO:0008637), and 37 genes mapped to the mitochondrial genome. With the signature matrices as a
reference, we first ran the “Impute Cell Fractions” task in CIBERSORTx to estimate the relative fractions of the cell types within each bulk RNA-seq sample and then the “Impute Cell Expression” task with the “High-Resolution” mode to obtain sample-specific expression profiles of all cell types.

Analysis of melanoma spatial transcriptomic data
Spatial transcriptomic data of a stage III cutaneous malignant melanoma sample was downloaded from SpatialDB (http://www.spatialomics.org/SpatialDB/). To decompose the spatial distribution of tumor cells and immune cells from the expression data, we first queried the top 200 marker genes of each of the seven cell types (see above) surveyed in the scRNA-seq study by Tirosh et al. We then used GSVA to compute cell-type signature scores for all the dots, which quantified the enrichment or depletion of each of the cell populations at each position. Based on the concordance between transcriptome-deconvoluted and H&E staining-informed cell type distributions, the cell type-specific expression patterns of SIRPA and CD47 were visualized across the slide.

Estimation of cell-cell communication using single-cell RNA-seq data
The melanoma patients’ scRNA-seq data from Tirosh et al. were used to evaluate the confidence in SIRPα-CD47-mediated cell-cell communication between melanoma cells and CD8⁺ T cells based on their co-expression patterns. We ranked all T cells by the expression level of CD4 and CD8 and extracted the gene expression profiles of the top 1,044 CD8⁺ T cells that were completely depleted of CD4. These cells were then combined with all melanoma cells in the TPM scale as input to CellPhoneDB.

Generation and analysis of SIRPA-perturbed B16F10 RNA-seq data
For RNA-seq experiments, total RNA was extracted from mSIRPA KD, OE, or control B16F10 cells using the Qiagen RNeasy Mini kit according to the manufacturer’s instructions and were subjected to mRNA paired-end sequencing at Novogene Co., LTD. Each cell line had three biological repeats. We employed a similar processing pipeline to the integrative analysis of public datasets to analyze the B16F10 data. Briefly, we (i) performed quality control of RNA-seq raw reads using MultiQC, (ii) pseudo-aligned reads to GENCODE mm39 mouse reference genome and obtained read counts and TPM using Salmon, (iii) identified differentially expressed genes in each condition through log₂ fold changes and adjusted p-values using DESeq2, and (iv) focused on the changes of six well-established MDA-encoding genes, namely PMEL, TYR, TYRP1, DCT, MLANA, and MITF.

Profiling of SIRPα protein expression in TCGA melanoma and CCLE samples
We quantified SIRPα protein expression in 349 TCGA melanoma samples and 48 CCLE melanoma samples using a reverse phase protein array at the RPPA core facility at MD Anderson. The SIRPα antibodies (Abcam, Cat # ab8120) were validated by comparison with immunoblotting, as previously described (Hennessy et al., 2010; Li et al., 2017). Briefly, lysates were manually serial-diluted in 5 two-fold dilutions with lysis buffer and printed on nitrocellulose-coated slides using an Aushon Biosystems 2470 arrayer. Slides were probed with validated primary antibodies. Signals were captured by Dako GenePoint Tyramide Signal Amplification System (Agilent, Cat. # K0620). Stained RPPA slides were first quantified using ArrayPro (Media Cybernetics) to generate signal intensities. The raw data were further normalized by SuperCurve, median polish, and replicate-based normalization (Akbari et al., 2014; Ju et al., 2015) for downstream analyses.

Analysis of potential regulators of SIRPα protein expression
To identify potential regulators of SIRPα expression, we integrated the SIRPα protein expression of TCGA-SKCM samples with other TCGA-SKCM molecular profiles (DNA methylation, mutation, somatic copy-number alteration, and pseudogene expression) that were obtained from the TCGA Pan-Cancer Atlas website (https://gdc.cancer.gov/about-data/publications/pancanatlas), except for pseudogene expression. For the pseudogene SIRPA1, we downloaded TCGA-SKCM bam files from the GDC data portal (https://portal.gdc.cancer.gov/), extracted uniquely mapped reads, and computed RPM by using featureCounts. Then, we used multiple statistical methods to examine associations between SIRPα protein expression and other types of molecular data. For categorical variables such as wild-type versus mutant, we performed two-sample Wilcoxon tests (Mann-Whitney tests). For continuous variables, including DNA methylation and SIRPA1, we computed Spearman’s rank correlation. To understand whether copy-number alteration of SIRPA affects the gene expression in the Hugo anti-PD1-treated patient cohort, we downloaded the whole-exome sequencing data of Hugo et al. and mapped them to human reference genome GRCh37 with the BWA-MEM algorithm (version 0.7.17). Picard (version 2.23.8) was used to mark duplications. The R package PureCN was used to estimate sample purity and ploidy. All normal samples were combined to build a reference panel, against which each tumor sample was compared to infer integer and categorical copy number using the cnvkit package (version 0.9.6). We categorized “Amp” for integer somatic copy number > 3, “Del” for integer somatic copy number < 1, and all others are considered somatic copy number neutral (“N”).

MicroRNA analysis
We employed three miRNA-mRNA interaction prediction tools, RNAhybrid, miRDB, and TarPmiR, which use different methodologies to infer miRNA targets, to search for miRNAs that potentially bind to the shared regions of SIRPA and SIRPA1 transcripts. We first built a union set of miRNAs combining the results from all three tools and then used an additional filter that relied on the co-expression between SIRPA/SIRPA1 and the miRNAs across patient samples of the TCGA melanoma cohort. With these two filters, we identified the miRNAs as potential mediators of the competitive endogenous mechanism that governed SIRPA-SIRPA1 coordination (Figure S6C).
Flow cytometry analysis
The surface expression of SIRPα on B16F10 and A375 cells was examined by flow cytometry as described previously (Motegi et al., 2003). Briefly, cells were washed by PBS twice and then detached from the culture dishes by treatment with 0.05% Trypsin-EDTA. 1×10^6 cells were resuspended in 100 μl staining buffer (pH 7.2 PBS, 0.5% bovine serum albumin (BSA), and 2 mM EDTA). 10 μl FITC-conjugated SIRPα antibody was added to the cell suspension. Cells were incubated with the antibody for 10 min in the dark in the refrigerator (4°C), washed twice with PBS, and then analyzed by a BD FACSComp™ cytometer. Data were analyzed using the FlowJo software (version 10.7 Flowjo).

T cell killing assay based on co-cultures of B16F10 and T cells
We performed T cell killing assays as described previously (Pan et al., 2018). Briefly, CD8+ T cells were isolated from the spleen of Pmel-1 transgenic mice using the EasySep mouse CD8+ T cell isolation kit according to the manufacturer’s protocol. Freshly isolated CD8+ T cells were then activated with anti-CD3/CD28 beads according to the manufacturer’s protocol. 20 ng/mL mouse IL-2 was added to the culture medium. T cells were in vitro stimulated for at least 6 days before the co-culture with B16F10 cells. B16F10 cells (1×10^5) were plated into a well of 6-well plate. The next day, cells were pulsed with 1 μM gp100<sup>25–33</sup> for 2 h. After that, the in vitro activated Pmel-1 T cells were added into co-culture with tumor cells at 0, 50%, or 100% of the number of tumor cells. We added 10μg/mL anti-mPD-L1 to the co-culture system together with T cells. For CD47 blockade treatments, the indicated antibodies (10μg/mL) were pre-incubated with T cells for 2 h. Then, the T cells were washed with PBS twice and added to the B16F10 cells. Each condition had 3 replicates. At 24 h after co-culture, the tumor cells were washed with PBS and then detached from the culture dishes by treatment with 0.05% Trypsin-EDTA. Before flow cytometry analysis, 10 μl of CountBright™ absolute counting beads were added to each sample. The samples were run through the flow cytometer and set to stop after 500 beads were acquired. The number of tumor cells in each sample was computed based on the reference beads. The average cell number in each condition was normalized to its corresponding no-T-cell control to get the relative cell viability proportion. The experiments were repeated three times using T cells from three separate mice. In each experiment, each condition was measured in triplicate. The relative cell survival in each condition was normalized to its corresponding no-T-cell control of each cell type within the experiment. The controls’ relative cell survival was set as 1. The error bar represents the standard error. For 96 h co-culture experiments, the B16F10 cells were not pulsed with 1μM gp100<sup>25–33</sup>. The in vitro activated Pmel-1 T cells were added into co-culture with tumor cells at 0, 50%, or 100% of the number of tumor cells. At 96 h after co-culture, the tumor cells were counted by flow cytometer as described previously.

Cell growth assay
On day 0, B16F10 cells (1×10<sup>5</sup>) with different SIRPA perturbations were seeded into one well of 6 well plates. At different time points, cancer cells were detached from the plates by the treatment with 0.05% Trypsin-EDTA and counted using Cellometer Auto T4. Each condition has 3 repeats. The error bar represents the standard deviation.

Immunoblotting
These experiments were performed as described previously (Xu et al., 2019). The cells were lysed in RIPA buffer. Protein concentrations were measured using the Pierce BCA protein assay kit. Cell lysates were boiled and separated on a 10% SDS-PAGE gel. The proteins were transferred to a PVDF membrane, which was then incubated with specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The protein expression was detected with an ECL western blot detection kit. The membranes were imaged with the ChemiDoc MP Imaging System.

In vivo experiments using B16F10 cells
B16F10 syngeneic mouse melanoma models were performed as described previously (Overwijk and Restifo, 2001; Pan et al., 2018). Briefly, all the animals were randomized before tumor inoculation. Then 0.4×10<sup>6</sup> control (non-targeting knockdown RNA), mSIRPA-KD, or mSIRPA-OE B16F10 cells were subcutaneously injected into 7-8 weeks old female C57BL/6 mice (The Jackson Laboratory #000664). Three days after inoculation, mice in each condition with established tumors were randomized again before the treatment. For each condition of mice implanted with control, mSIRPA-KD or mSIRPA-OE B16F10 tumor cells, two treatments were performed: anti-mPD-L1 and isotype control antibody. Specifically, αPD-L1 (clone B7-H1, #BP0101, 200 μg/mice) or isotype control antibodies (clone LTF-2; #BP0090, 200 μg/mouse) mAbs were administered on days 3, 7, 10, 14, and 17. Accordingly, there were six groups: control tumors treated with isotype antibody (n = 17); mSIRPA-KD tumors treated with isotype antibody (n = 17); mSIRPA-OE tumors treated with isotype antibody (n = 15); mSIRPA-OE tumors treated with anti-mPD-L1 (n = 17); and mSIRPA-OE tumors treated with anti-mPD-L1 (n = 17). Tumors were measured 3-6 times a week, beginning on day 10 after inoculation, until either the survival endpoint (37 days) was reached or no palpable tumor remained. Tumor volumes were calculated using the formula for a hemiellipsoid (volume = length×width×height/2). Mice were sacrificed when tumors reached 20 mm in diameter or 1500 mm<sup>3</sup> in volume.

QUANTIFICATION AND STATISTICAL ANALYSIS
We performed quantification and statistical analysis using GraphPad Prism 6, R (version 3.4) and python (version 3.6). Detailed descriptions of statistical tests are provided in the method details section and the respective figure legends.