CDKN2A^{Low} cancer cells outcompete macrophages for microenvironmental zinc to drive immunotherapy resistance

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Running title: Zinc modulates immunotherapy response in *CDKN2A*^{Low} tumors

Keywords: SLC39A9, ZIP9, tumor microenvironment (TME), phagocytosis, p16, anti-PD1, anti-PD-L1, immune checkpoint blockade (ICB), tumor interstitial fluid (TIF), dietary intervention, zinc, metabolism, metals

ABSTRACT

Approximately 50% of cancers exhibit decreased *CDKN2A* expression (*CDKN2A*^{Low}), which is linked to immune checkpoint blockade (ICB) resistance. While *CDKN2A* is traditionally recognized as a tumor suppressor and cell cycle regulator, we have previously put forth a new paradigm demonstrating its role in intracellular metabolic reprogramming. Whether the metabolic derangement due to *CDKN2A* loss alters metabolites within the tumor microenvironment (TME) and how that affects the immune compartment and ICB response has never been investigated. Here we found that *CDKN2A*^{Low} cancer cells reorganize zinc compartmentalization by upregulating the zinc importer SLC39A9 in the plasma membrane, leading to intracellular zinc accumulation in cancer cells and concurrent zinc depletion in the TME. This competition for zinc results in zinc-starved tumor-associated macrophages (TAMs), leading to reduced phagocytic activity. Increasing zinc in TAMs through multiple approaches, including a dietary intervention that increases availability of TME zinc, re-educates these TAMs to a pro-phagocytic phenotype. Remarkably, both knockdown of Slc39a9 in cancer cells or providing a high zinc diet sensitizes *Cdkn2a*^{Low} tumors to ICB. TAMs, not T cells, are indispensable for ICB response. Clinically, TAMs from *CDKN2A*^{Low} cancer patients have decreased zinc signatures, corresponding to reduced phagocytosis signatures. Moreover, patients with low circulating zinc levels have reduced time-to-event outcomes compared to those with higher zinc levels. Our work reveals a previously unrecognized mechanism through which *CDKN2A*^{Low} cancer cells outcompete TAMs for zinc, directly disrupting their function and ICB efficacy.

INTRODUCTION

The Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*) gene, located on chromosome 9p21, is a critical tumor suppressor and one of the most frequently altered loci in human cancers, with disruptive alterations observed in approximately 50% of all cases[1,2]. *CDKN2A* encodes two distinct proteins through alternative reading frames: $p14^{ARF}$ ($p19^{ARF}$ in mice) and $p16^{INK4A}$ (hereafter p16), both of which are essential regulators of the cell cycle[3]. While canonical roles of *CDKN2A* in cell cycle regulation and senescence induction is well established, evidence from our laboratory and others suggests that its functions extend beyond this paradigm[4-15]. We have demonstrated that loss of *CDKN2A*/p16 expression reprograms intracellular metabolism, enhancing key biosynthetic metabolites that contribute to cellular proliferation and tumorigenesis, including nucleotides and cholesterol[4,7]. However, whether this metabolic disruption downstream of *CDKN2A* loss affects the metabolic landscape of the tumor microenvironment (TME) remains unexplored.

A number of data mining studies have highlighted a significant correlation between *CDKN2A* genetic alterations and a pro-tumorigenic TME, as well as poor response to immune checkpoint blockade (ICB) therapies[5,16-32]. ICB therapies, particularly monoclonal antibodies targeting the PD-1/PD-L1 axis, have become a cornerstone in cancer treatment, demonstrating significant clinical success across several cancers[33-37]. Their effectiveness is primarily attributed to enhancing T cell activity and T cell-mediated tumor killing[38]. Interestingly, recent evidence suggests that at least a subset of tumor-associated macrophages (TAMs) play a role in the response to ICB[39-45]. Anti-PD-1/PD-L1 therapies

directly target monocytes and macrophages to improve therapeutic responses at least in part via increasing their phagocytic activity[39,46,47]. Indeed, studies have shown that increased TAM phagocytic activity inhibits tumor growth in mouse models[48-50]. While metabolic reprogramming is known to influence macrophage phagocytic activity[48,51,52], the role of cancer-TAM nutrient competition in driving this process remains poorly understood.

Zinc is an essential trace element and the second most abundant metal in the human body[53]. Zinc is a micronutrient that is obtained through the diet and absorbed in the intestine from where it is distributed across various organs[54]. At the tissue microenvironment and cellular level, zinc homeostasis is tightly regulated by solute carrier transporters. SLC39A family members, also known as ZRT- and IRT-like proteins (ZIP), increase cytosolic zinc by importing it from the extracellular environment or releasing it from intracellular organelles[55]. In contrast, SLC30A family members, or zinc transporter (ZNT) proteins, reduce cytosolic zinc by transporting it into organelles or exporting it to the extracellular space[56]. Additionally, metallothioneins (MTs), small cysteine-rich proteins that bind and store zinc, provide an additional layer of regulation[57]. It is estimated that over 2,500 human proteins rely on zinc for structural integrity or biological function (e.g., zinc fingers and cofactors)[53]. As a result, zinc plays a critical role in multiple cellular processes, including immune responses[58-65]. Thus, it is not surprising that dysregulated zinc homeostasis via altered transport and storage or systemic zinc deficiencies have been observed in multiple malignancies[66-78]. Indeed, *in vivo* studies show that zinc supplementation can reduce tumor burden, enhance resistance to tumor challenges, and decrease the

incidence of spontaneous tumors in mice[79-82], suggesting a potential therapeutic role for zinc in cancer treatment. Additionally, a recent report found that patients who respond to ICB have TAMs with metal/zinc signatures[40]. While some studies have explored the link between zinc and immunotherapy response[83-87], the results remain contradictory, and the precise molecular mechanisms underlying zinc in the TME are unclear.

Here we found that *CDKN2A*^{Low} tumor cells disrupt zinc homeostasis in the TME, driving resistance to ICB. By cross-comparing differentially regulated metabolites and metals in the tumor interstitial fluid (TIF) and negatively enriched plasma membrane solute carrier transporter (SLCs) from a metabolism-focused *in vivo* CRISPR knockout screen, we identified zinc and the zinc importer Slc39a9 as critical regulators of ICB resistance in *Cdkn2a*^{Low} tumors. *CDKN2A*^{Low} cells have increased plasma membrane (PM) localization of SLC39A9, leading to elevated intracellular zinc and a zinc-depleted TME. This cancer cell outcompetition for zinc leads to decreased zinc in TAMs, reducing their phagocytic activity and impairing ICB response. Restoring TME zinc levels through Slc39a9 knockdown or dietary zinc supplementation rescues TAM function and enhances ICB response. Using publicly available scRNA-Seq, we also found that zinc and phagocytosis signatures were impaired in TAMs from patients with *CDKN2A*^{Low} tumors. These findings highlight zinc homeostasis as essential for ICB efficacy in *CDKN2A*^{Low} tumors and have implications for the use of zinc supplements to improve patient outcomes.

RESULTS

Zinc and the zinc importer SLC39A9 are critical regulators of ICB resistance in *CDKN2A*^{Low} tumors

Published studies show that tumors with loss of *CDKN2A* function correlate with decreased response to anti-PD1 or anti-PD-L1 ICB[16,17,20,27,29-32]. Using publicly available data, we found that patients with low *CDKN2A* mRNA expression (*CDKN2A*^{Low}) have shorter overall survival compared to those with high *CDKN2A* mRNA expression (*CDKN2A*^{High}) when treated with anti-PD1 or anti-PD-L1 (**Extended Data Fig. 1A-B**). *MTAP* and the *IFN* cluster are frequently co-deleted with *CDKN2A* and have been previously linked to ICB resistance[17,88]. Notably, *CDKN2A*^{Low} patients also have shorter overall survival on anti-PD1/anti-PD-L1 regardless of *MTAP* or *IFN* gene expression (**Extended Data Fig. 1C-D**). To investigate the mechanism whereby loss of *CDKN2A* alone, and more specifically p16 alone, in cancer cells drives ICB resistance, we developed two complementary syngeneic models. In the Yumm5.2 (*Cdkn2a*^{High}) model, we knocked down *Cdkn2a*, while in the Yumm1.7 (*Cdkn2a*^{Null}) model, we overexpressed p16 (**Fig. 1A and Extended Data Fig. 2A-F, see Methods for syngeneic mouse model details**). Both syngeneic tumors mimic the ICB response seen in *CDKN2A*^{Low} cancer patients (**Extended Data Fig. 2G-J**), providing us models to explore the mechanism of *de novo* ICB resistance in the context of low *Cdkn2a*/p16 expression.

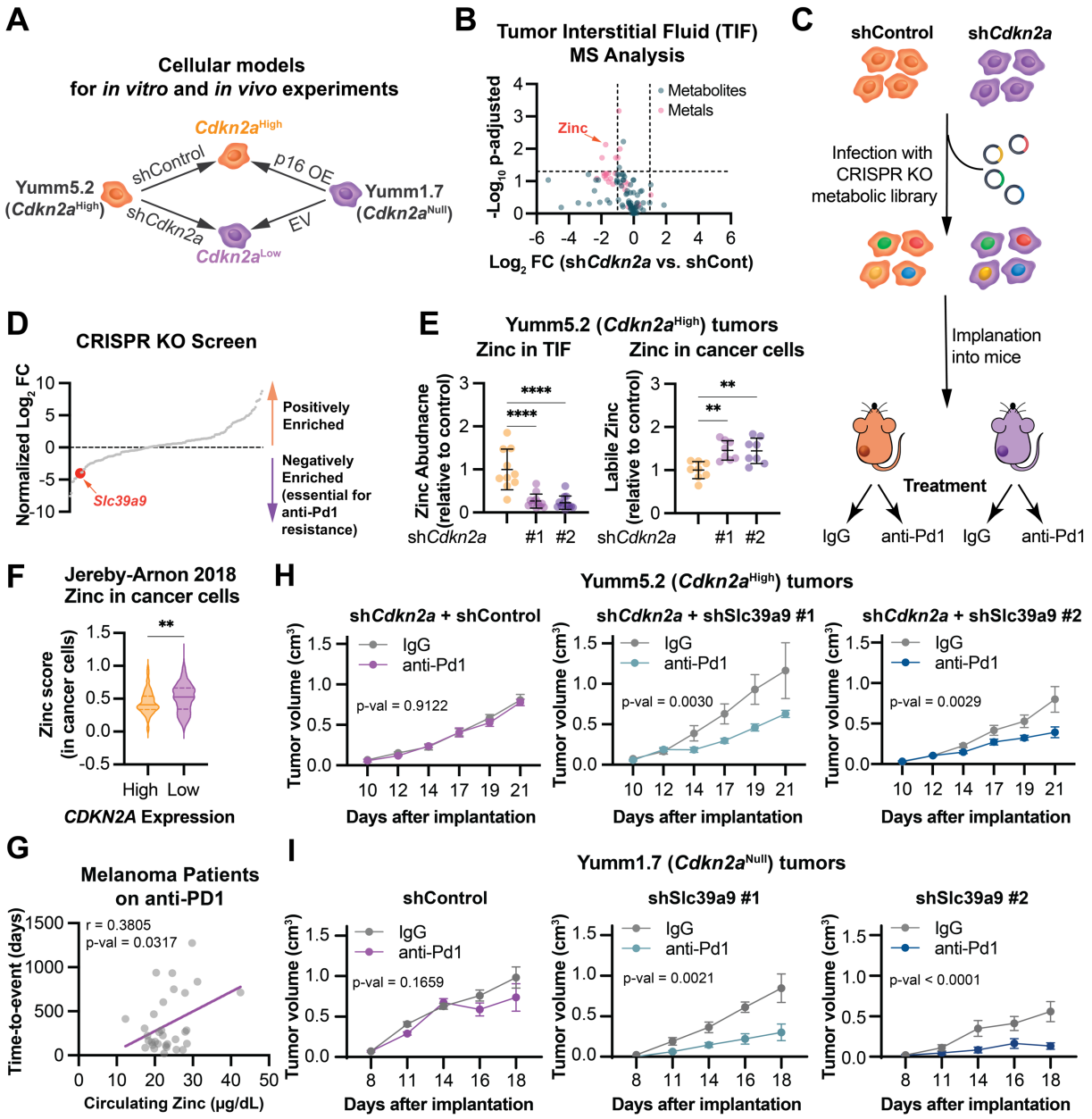


Figure 1. Zinc and Slc39a9 are key determinants of ICB response in *Cdkn2a*^{Low} tumors. (A) Schematic of cellular models. **(B)** Volcano plot of metabolites and metals in the TIF from Yumm5.2 (*Cdkn2a*^{High}) with knockdown of *Cdkn2a* (sh*Cdkn2a*) or control (shCont) tumors. **(C)** Schematic of the *in vivo* metabolic CRISPR knockout (KO) screen design. **(D)** Results from the CRISPR KO screen in mice bearing Yumm5.2 (*Cdkn2a*^{High}) shControl and sh*Cdkn2a* tumors treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Data show Log_2 fold change (FC) of CRISPR negative score of the plasma membrane solute carrier transporters in sh*Cdkn2a* tumors treated with anti-Pd1 vs. IgG vs. shControl tumors treated with anti-Pd1 vs. IgG. See Table S1. **(E)** ICP-MS quantification of zinc levels in the TIF (left) or labile zinc quantification in cancer cells (right) from Yumm5.2 (*Cdkn2a*^{High}) shControl and sh*Cdkn2a* (hairpins #1 and #2). Data represent mean ± SD (n=12/group right, n=8/group left). One-way ANOVA. **(F)** Zinc scores calculated in the cancer cells of patients harboring *CDKN2A*^{High} or *CDKN2A*^{Low} melanoma in the Jerby-Arnon 2018 data set. Violin plot showing median and quartiles. Student's t-test. **(G)** Correlation between the amount of zinc in plasma

quantified by ICP-MS and the time-to-event outcome in patients treated with anti-PD1, alone or combined with additional immunotherapy from the melanoma cohort. See Table S6. Pearson Correlation. **(H)** Yumm5.2 (*Cdkn2a*^{High}) cells with sh*Cdkn2a* alone or combined with knockdown of Slc39a9 (shSlc39a9 hairpins #1 and #2) were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Longitudinal tumor growth as mean ± SEM (n=5/group). Mixed-effects model. **(I)** Same as (H) but mice were implanted with Yumm1.7 (*Cdkn2a*^{Null}) cells with Slc39a9 knockdown (shSlc39a9, hairpins #1 and #2). Longitudinal tumor growth as mean ± SEM (n=6/group). Mixed-effects model. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

We and others have published that suppression of *CDKN2A*/p16 alters cell-intrinsic metabolism[4,6,7,9,10,89,90]. Excitingly, using unbiased metabolomics and metallomics in the tumor interstitial fluid (TIF), we found that multiple analytes are also differentially regulated in the TME of Yumm5.2 sh*Cdkn2a* tumors compared to controls (**Fig. 1B**). Previous studies have shown that altered cancer cell metabolism contributes to the composition and activity of immune cells within the TME, thereby affecting tumor responses to ICB[91-94]. Thus, we reasoned that *CDKN2A*^{Low}-mediated metabolic changes in the TME may contribute to the observed ICB resistance in these tumors (**Extended Data Fig. 2G-J**). To identify drivers of this phenotype, we employed a metabolism-focused sgRNA knockout library in our Yumm5.2 isogenic cell model (**Fig. 1C**). We identified 137 genes with significant negative enrichment in sh*Cdkn2a* tumors treated with anti-Pd1 vs. IgG compared to shControl tumors treated with anti-Pd1 vs. IgG, indicating their requirement for inducing ICB resistance in the context of low *Cdkn2a* expression (**Table S1**). Given the role of plasma membrane solute carrier transporters in metabolite compartmentalization between the intracellular and extracellular space, we focused on these genes (**Fig. 1D and Table S2**) for cross-comparison with depleted analytes in sh*Cdkn2a* TIF (**Fig. 1B**). Using this comparison, we identified zinc and the plasma membrane zinc importer *Slc39a9* as potential drivers of ICB resistance in sh*Cdkn2a* tumors (**Fig. 1B and Fig. 1D**). Notably, we found that the decrease of zinc in the TIF in sh*Cdkn2a* tumors correspond to increased

zinc in cancer cells (**Fig. 1E**). Consistently, data mining of single-cell RNA sequencing (scRNA-Seq) from melanoma patients showed that *CDKN2A*^{Low} cancer cells have higher zinc scores compared to *CDKN2A*^{High} cancer cells (**Fig. 1F**). Altogether these data demonstrate that loss of *CDKN2A* drives a differential zinc compartmentalization within the tumor, leading to zinc accumulation in cancer cells and concurrent depletion of zinc in the TME. Interestingly, ICB-treated patients with low circulating zinc levels have reduced time-to-event outcomes compared to those with higher zinc levels (**Fig. 1G**), providing support for the role of zinc in ICB response. Finally, we validated that knockdown of Slc39a9 sensitizes *Cdkn2a*^{Low} tumors to anti-Pd1 treatment (**Fig. 1H-I and Extended Data Fig. 3A-C**). Altogether, these data demonstrate that Slc39a9 is essential for ICB resistance in *Cdkn2a*^{Low} tumors and indicate that zinc compartmentalization may play an important role in this process.

CDKN2A^{Low} cancer cells reprogram zinc compartmentalization via plasma membrane SLC39A9 in a mechanism mediated by cholesterol

Next, we sought to determine how *CDKN2A* regulates zinc homeostasis via SLC39A9. Prior work found that SLC39A9 is localized in the plasma membrane and imports zinc ions from the extracellular space into the cytosol[95-99]. We found that multiple human and mouse *CDKN2A*^{Low} cancer cell models have increased plasma membrane SLC39A9 (**Fig. 2A-B and Extended Data Fig. 4A-C**). Increased plasma membrane Slc39a9 correlated with elevated intracellular zinc levels and reduced extracellular zinc levels (both labile and

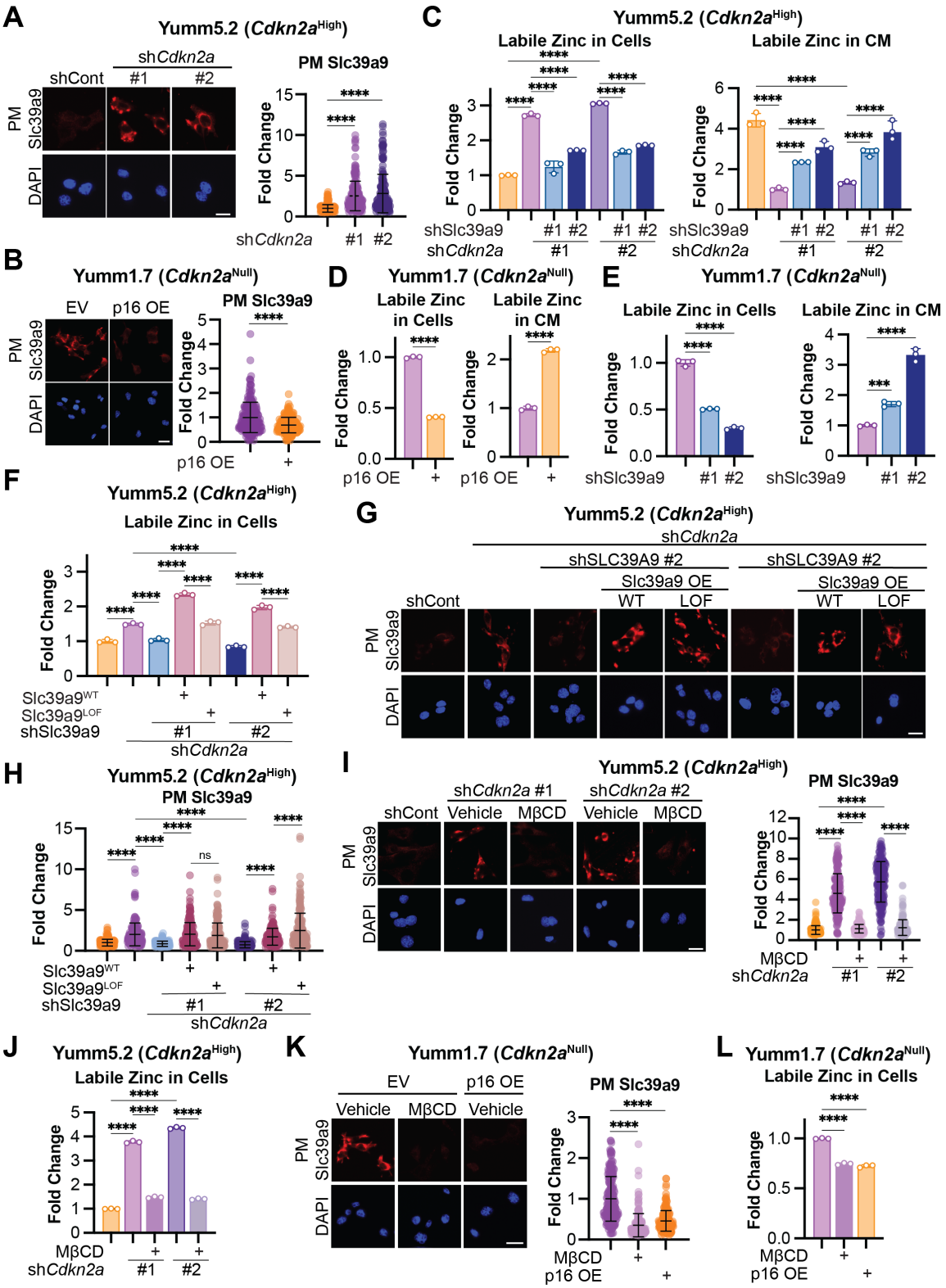


Figure 2. Increased plasma membrane Slc39a9 via cholesterol drives zinc uptake in *Cdkn2a*^{Low} cancer cells. (A-B) Representative images (left) and quantification (right) of Slc39a9 IF on non-permeabilized cells to visualize plasma membrane (PM) Slc39a9 in **(A)** Yumm5.2 (*Cdkn2a*^{High}) with *Cdkn2a* knockdown (sh*Cdkn2a* hairpins #1 and #2) or shControl, and **(B)** Yumm1.7 (*Cdkn2a*^{Null}) with p16 overexpression (p16 OE) or empty vector control. Data represent mean ± SD (n=200/group). **(C-**

E) Labile zinc quantification in cells (left) and conditioned media (CM; right) in **(C)** Yumm5.2 (*Cdkn2a*^{High}) with shControl or sh*Cdkn2a* (hairpins #1 and #2) alone or combined with knockdown of Slc39a9 (shSlc39a9 hairpins #1 and #2), **(D)** Yumm1.7 (*Cdkn2a*^{Null}) with empty vector control or p16 overexpression (p16 OE), and **(E)** Yumm1.7 (*Cdkn2a*^{Null}) with shControl or shSlc39a9 (hairpins #1 and #2). Data represent mean ± SD (n=3/group). **(F-H)** Yumm5.2 (*Cdkn2a*^{High}) with shControl or with sh*Cdkn2a* alone or combined with shSlc39a9 (hairpins #1 and #2) and empty vector (EV) control, wildtype Slc39a9 (WT), or loss-of-function Slc39a9 mutant (LOF). **(F)** Labile zinc quantification. Data represent mean ± SD (n=3/group). **(G)** Representative images of PM Slc39a9 IF on non-permeabilized cells. **(H)** Quantification of (G). Data represent mean ± SD (n=200/group). **(I-J)** Yumm5.2 (*Cdkn2a*^{High}) with shControl or sh*Cdkn2*a (hairpins #1 and #2) were treated with 10mM Methyl-β-cyclodextrin (MβCD) or vehicle for 1h. **(I)** Representative images (left) and quantification (right) of PM Slc39a9 IF on non-permeabilized cells. Data represent mean ± SD (n=200/group). **(J)** Labile zinc quantification. Data represent mean ± SD (n=3/group). **(K-L)** Yumm1.7 (*Cdkn2a*^{Null}) with p16 overexpression (p16 OE) or empty vector (EV) control were treated with 10mM Methyl-β-cyclodextrin (MβCD) or vehicle for 1h. **(K)** Representative images (left) and quantification (right) of PM Slc39a9 IF on non-permeabilized cells. Data represent mean ± SD (n=200/group). **(L)** Labile zinc quantification. Data represent mean ± SD (n=3/group). Scale bars = 25μm. Shown are representative data from 3 independent experiments. One-way ANOVA or student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

total), an effect that was reversed by knockdown of Slc39a9 in *Cdkn2a*^{Low} cancer cells (**Fig. 2C-E** and **Extended Data Fig. 4D-G**). Overexpression of wildtype Slc39a9 rescued the intracellular labile zinc defect in Slc39a9 knockdown cells, whereas the putative loss-of-function Slc39a9^{H156R} mutant[100-102] did not (**Fig. 2F and Extended Data Fig. 5A**). The lack of zinc uptake in cells expressing the Slc39a9^{H156R} mutant was not due to the inability of the mutant to localize to the plasma membrane (**Fig. 2G-H and Extended Data Fig. 5B-C**). We did not observe a compensatory upregulation of other *Slc39* or *Slc30* genes known to transport zinc upon knockdown of Slc39a9 (**Extended Data Fig. 5D and Table S3**), further confirming Slc39a9 as the key zinc transporter in these cells. Additionally, none of the metal ions commonly transported by SLC39 family members were altered upon knockdown of Slc39a9 (**Extended Data Fig. 5E**), indicating the observed changes are not due to differential uptake of other metals. These findings demonstrate that SLC39A9 is a major and specific regulator of zinc homeostasis in *CDKN2A*^{Low} cancer cells.

Finally, we sought to determine the mechanism whereby *CDKN2A*^{Low} cancer cells have increased plasma membrane SLC39A9. We did not observe differential mRNA expression of *Slc39a9* upon *Cdkn2a* knockdown or p16 overexpression (**Extended Data Fig. 5F**). SLC39A9 is a transmembrane protein, which are often anchored to the plasma membrane through cholesterol[103]. Our prior study found that human cells with knockdown of *CDKN2A*/p16 have increased cholesterol[7], a finding we validated in the complementary mouse cell line models used in this study (**Extended Data Fig. 5G-H**). Thus, we hypothesized that increased cholesterol levels in *CDKN2A*^{Low} cancer cells may enhance plasma membrane SLC39A9 expression. Indeed, depletion of plasma membrane cholesterol using methyl-β-cyclodextrin (MβCD) decreased plasma membrane Slc39a9 and reduced intracellular labile zinc in *Cdkn2a*^{Low} cancer cells (**Fig. 2I-L**). Altogether, these data demonstrate that *CDKN2A*^{Low} cancer cells increase intracellular zinc via increased plasma membrane SLC39A9, which is regulated through cholesterol.

Macrophages, not T cells, are critical for ICB response in *Cdkn2a*^{Low} shSlc39a9 tumors

We found that *Cdkn2a*^{Low} tumors, which are ICB resistant (**Extended Data Fig. 2**), have depleted microenvironmental zinc due to enhanced cancer cell zinc uptake via Slc39a9 (**Fig. 2**). Next, we aimed to determine the effects of depleted TME zinc on the immune compartment. Given the well-established role of T cells in ICB response[104,105], we first assessed whether *Cdkn2a*^{Low} tumors with Slc39a9 knockdown remain responsive to anti-

Pd1 in RAG-1 deficient mice, which lack mature functional T and B cells. Unexpectedly, we found that *Cdkn2a*^{Low} tumors with Slc39a9 knockdown responded to anti-Pd1 in RAG-1 deficient mice to a similar extent as in wildtype C57BL/6J mice (**Fig. 3A**), indicating that T and B cells are not essential for the ICB response in this context. To identify the immune cell populations that are altered in *Cdkn2a*^{Low} tumors, we conducted scRNA-Seq on Cd45^{+} cells in our Yumm5.2 isogenic model with and without anti-Pd1 treatment. Interestingly, we observed an increase in the percentage of macrophages in *Cdkn2a*^{Low} tumors regardless of treatment (**Fig. 3B**). This is consistent with prior observation in human

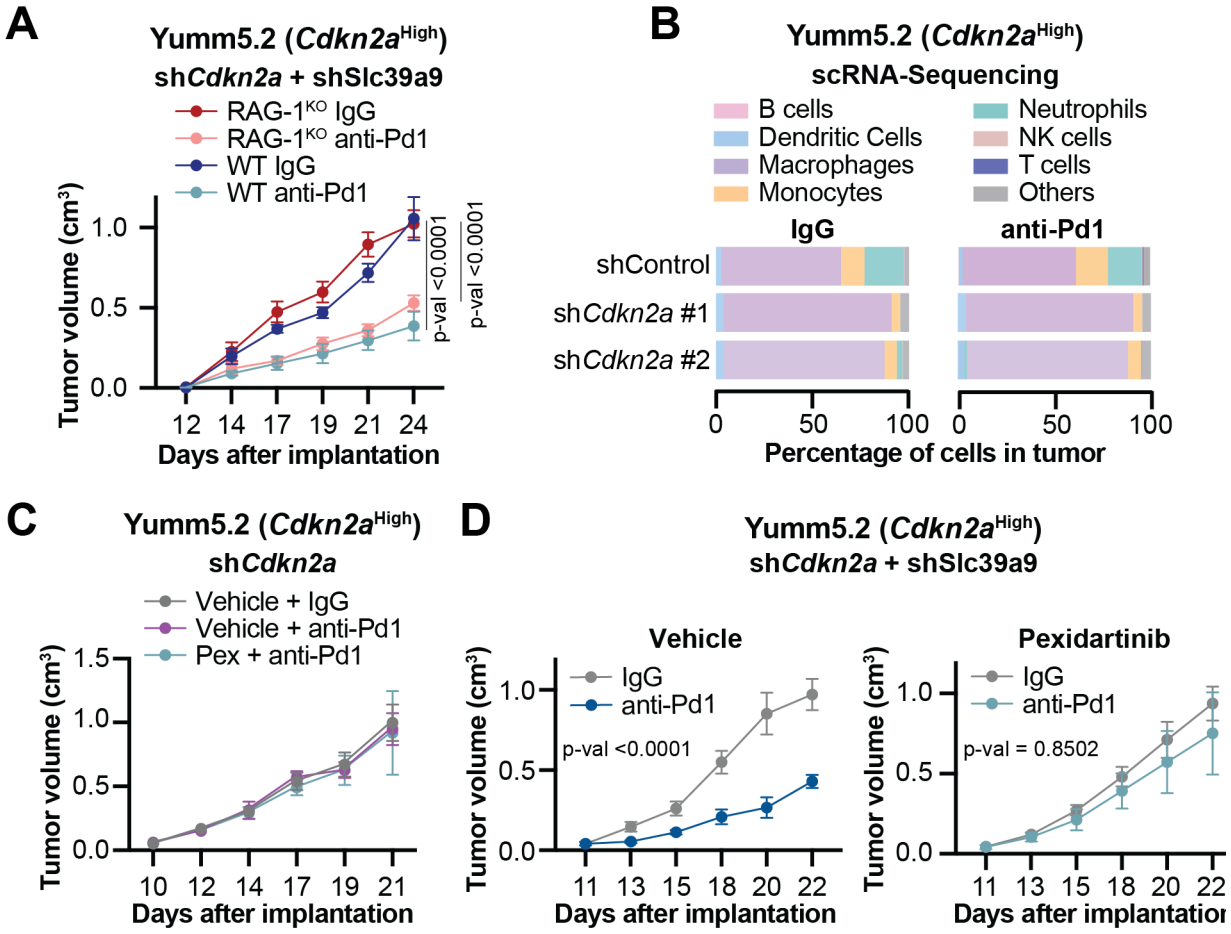


Figure 3. TAMs are necessary for the anti-Pd1 response in *Cdkn2a*^{Low} tumors upon knockdown of Slc39a9. (A) Yumm5.2 (*Cdkn2a*^{High}) cells with *Cdkn2a* knockdown (sh*Cdkn2a*) were implanted into wildtype (WT) and B6.129s7-*Rag1*^{tm1Mom}/J (RAG-1^{KO}) C57BL/6J mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Longitudinal tumor growth as mean ± SEM

(n=5/group). Mixed-effects model. **(B)** scRNA-Seq of Cd45^{+} cells sorted from Yumm5.2 (*Cdkn2a*^{High}) with knockdown of *Cdkn2a* (sh*Cdkn2a* hairpins #1 and #2) or shControl tumor-bearing mice treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Data represent the percentage of the indicated populations. **(C)** Yumm5.2 (*Cdkn2a*^{High}) cells with sh*Cdkn2a* were implanted into mice. Mice were treated with 45 mg/kg of Pexidartinib (Pex) or vehicle (o.g., 3x/week) and 200 µg IgG or anti-Pd1 (i.p., 3x/week). Longitudinal tumor growth as mean ± SEM (n=6/group). Mixed-effects model. **(D)** Yumm5.2 (*Cdkn2a*^{High}) sh*Cdkn2a* cells with knockdown of Slc39a9a (sh*Cdkn2a* + shSlc39a9) were implanted into mice. Mice were treated with 45 mg/kg of Pex or vehicle (o.g., 3x/week) and 200 µg IgG or anti-Pd1 (i.p., 3x/week). Longitudinal tumor growth as mean ± SEM (n=6/group). Mixed-effects model.

tumors with loss of *CDKN2A*[17,25]. Tumor-associated macrophages (TAMs) have context-dependent roles in ICB response and are often thought to be immunosuppressive[106,107]. Thus, we reasoned that depleting TAMs would restore ICB response in *Cdkn2a*^{Low} tumors. However, depletion of TAMs with the CSF1R inhibitor pexidartinib did not enhance the anti-Pd1 response in *Cdkn2a*^{Low} tumors (**Fig. 3C and Extended Data Fig. 6A**). Consistently, we did not observe a significant increase in the prototypical immunosuppressive genes *Mrc1* (encoding Cd206) or *Arg1*[108] in the TAMs from sh*Cdkn2a* tumors (**Extended Data Fig. 6B**). TAMs also have anti-tumor functions and have been shown to be important for improved ICB response in some cancers[39,40,42,43,109-111]. Thus, we explored whether TAMs in *Cdkn2a*^{Low} tumors with Slc39a9 knockdown are required to promote an ICB response. Indeed, depletion of TAMs reversed the ICB response in *Cdkn2a*^{Low} tumors with Slc39a9 knockdown (**Fig. 3D**). These data together indicate that the TAMs in *Cdkn2a*^{Low} tumors may not be immunosuppressive and demonstrate the requirement of TAMs for ICB response in this context.

Competition for zinc between *Cdkn2a*^{Low} cancer cells and TAMs regulates phagocytosis and ICB response

Next, we aimed to determine the mechanism whereby TAMs are essential for ICB response in *Cdkn2a*^{Low} tumors with Slc39a9 knockdown. A recent publication found that increased metal signatures in TAMs, mainly genes related to zinc function, correlate with improved ICB responses in patients[40]. Moreover, new evidence demonstrates the important role of TAMs in ICB responses at least in part via increasing phagocytosis[39,45,112]. Given the data that T cells are dispensable for ICB response of *Cdkn2a*^{Low} tumors with Slc39a9 knockdown (**Fig. 3A**), we reasoned that lack of TAM phagocytosis of cancer cells may be contributing to the observed ICB resistance of *Cdkn2a*^{Low} tumors. Indeed, we observed decreased zinc and phagocytosis signatures in TAMs from *CDKN2A*^{low} patient samples, including melanoma and head and neck squamous cell carcinoma (**Fig. 4A-B and Extended Data Fig. 7A**), cancers where decreased *CDKN2A* is both common and clinically significant[1], highlighting the relevance of these pathways in humans. In line with this, we found that RAW264.7 macrophages with higher intracellular zinc have greater phagocytic activity *in vitro* (**Extended Data Fig. 7B**). Further analysis of our scRNA-Seq data demonstrated that the TAMs in *Cdkn2a*^{Low} tumors have decreased zinc signature score (**Extended Data Fig. 7B and Table S4**). A number of phagocytosis signatures were modestly reduced in sh*Cdkn2a* tumors compared to controls (**Extended Data Fig. 7C-D and Table S4**). Excitingly, while anti-Pd1 treatment increased phagocytosis signatures in TAMs from shControl tumors, it failed to do so in TAMs from sh*Cdkn2a* tumors (**Extended Data Fig. 7D-E and Table S4**). Indeed, we found that anti-Pd1 treatment increased the expression of phagocytosis-related genes in RAW264.7 macrophages *in vitro*, which was further enhanced by increased microenvironmental zinc but suppressed by extracellular zinc chelation (**Extended Data Fig. 7G**). This demonstrates that zinc plays an important

role in the transcriptional regulation of phagocytosis-related genes. To confirm the connection between phagocytosis and zinc *in vivo*, we implanted dTOMATO-labeled cancer

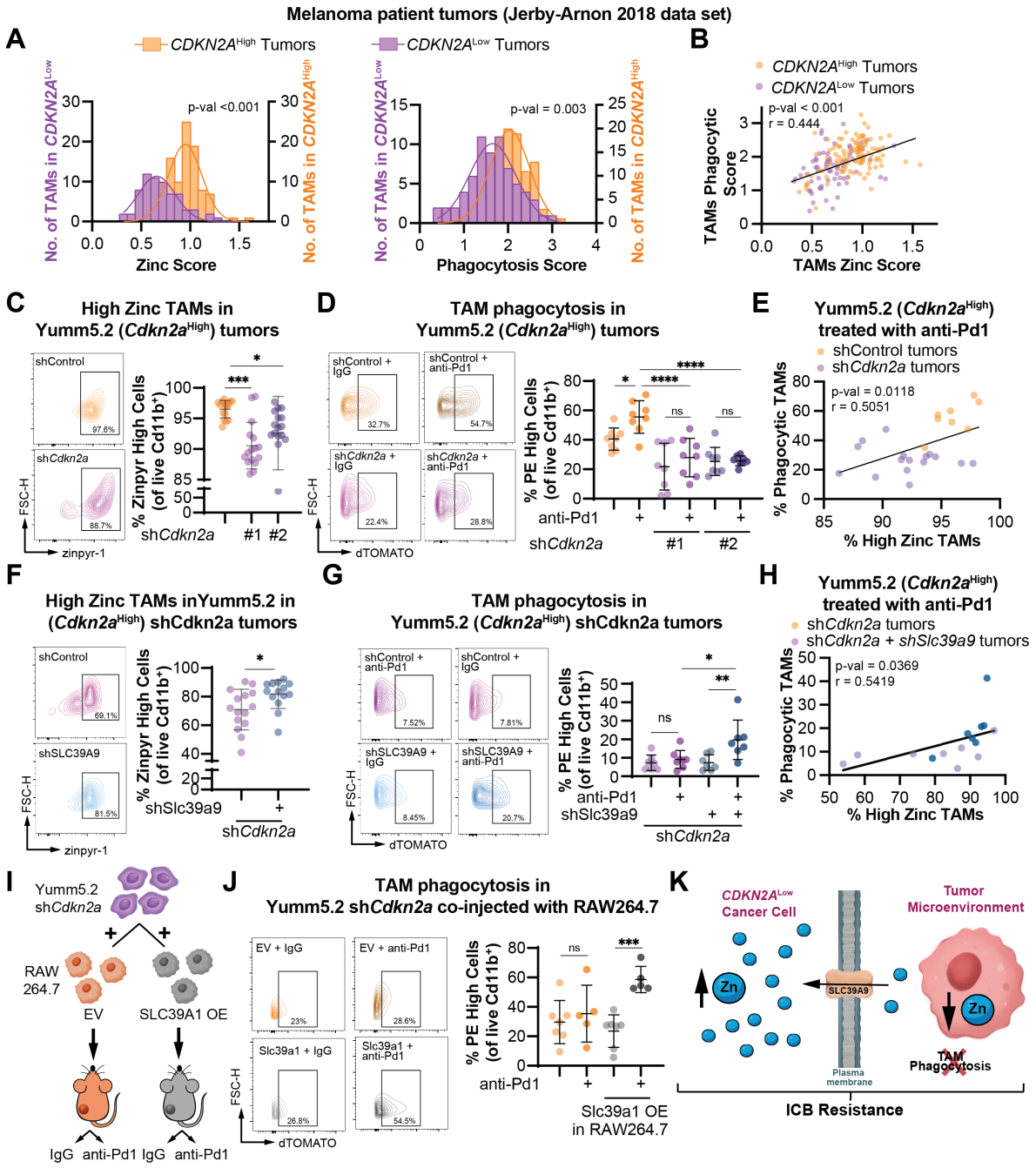


Figure 4. TAMs from *Cdkn2a*^{Low} tumors have less zinc and are less phagocytic. (A) Zinc and phagocytic scores calculated in the TAM population of melanomas harboring *CDKN2A*^{High} or

CDKN2A^{Low} tumors in the Jerby-Arnon 2018 data set. Histogram of number of macrophages per tumor group with indicated zinc (left) or phagocytic (right) score. Linear Mixed Model. **(B)** Same as (A) but correlation of zinc and phagocytic scores per each TAM. Pearson Correlation. **(C-E)** dTOMATO-expressing Yumm5.2 (*Cdkn2a*^{High}) cells with *Cdkn2a* knockdown (sh*Cdkn2a*) or control were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). **(C)** Representative flow cytometry plots (left) and quantification (right) showing the percentage of TAMs with high Zinpyr-1 (labile zinc) staining. **(D)** Representative flow cytometry plots (left) and quantification (right) showing the percentage of TAMs with dTOMATO signal (phagocytic TAMs). **(E)** Correlation between the percentage of high-zinc TAMs and phagocytic TAMs per mouse. **(F-H)** dTOMATO-expressing Yumm5.2 (*Cdkn2a*^{High}) sh*Cdkn2a* cells alone or with knockdown of Slc39a9a (sh*Cdkn2a* + shSlc39a9) were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). **(F)** Representative flow cytometry plots (left) and quantification (right) showing the percentage of TAMs with high Zinpyr-1 (labile zinc) staining. **(G)** Representative flow cytometry plots (left) and quantification (right) showing the percentage of TAMs with dTOMATO signal (phagocytic TAMs). **(H)** Correlation between the percentage of high-zinc TAMs and phagocytic TAMs per mouse. **(I)** Schematic of macrophage co-injection model. **(J)** RAW264.7 macrophages with Slc39a1 overexpression (Slc39a1 OE) or empty vector control were co-injected with Yumm5.2 (*Cdkn2a*^{High}) sh*Cdkn2a* cancer cells (ratio 1:10). Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Representative flow cytometry plots (left) and quantification (right) showing the percentage of TAMs with dTOMATO signal (phagocytic TAMs). **(K)** Schematic of zinc competition between *Cdkn2a*^{Low} cells and TAMs leading to decreased phagocytosis and ICB response. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

cells in mice and analyzed the PE and zinpyr-1 (labile zinc) signals in the TAM compartment (**Extended Data Fig. 7H**). We found that sh*Cdkn2a* tumors had a decreased proportion of high zinc TAMs, corresponding to decreased phagocytic activity upon anti-Pd1 treatment (**Fig. 4C-E**). Consistent with our scRNA-Seq data, we found that TAMs in shControl (*Cdkn2a*^{High}) tumors, which have high zinc (**Fig. 4C and Extended Data Fig. 7B**), are intrinsically more phagocytic than those in sh*Cdkn2a* tumors, and anti-Pd1 treatment further enhanced this phenotype (**Fig. 4D-E**). Knockdown of Slc39a9 in cancer cells increased the high zinc TAM population, corresponding to increased phagocytic activity upon anti-Pd1 treatment (**Fig. 4F-G**). Importantly, we did not observe consistent significant differences in “eat me” or “don’t eat me” signals in sh*Cdkn2a* cells alone or in combination with shSlc39a9 (**Extended Data Fig. 8A and Table S5**). Additionally, we did not observe changes in Pd-l1 expression on cancer cells (**Extended Data Fig. 8B-C**), suggesting that this effect is macrophage-intrinsic. These data indicate that TAMs are

dysfunctional due to zinc outcompetition with *Cdkn2a*^{Low} cancer cells in these tumors, and elevating TME zinc re-educates TAMs to a pro-phagocytic phenotype, thereby improving ICB responses in the context of low *Cdkn2a*. Finally, to demonstrate that zinc replete macrophages increase phagocytosis *in vivo*, we engineered zinc-replete RAW264.7 macrophages by overexpressing Slc39a1 and co-implanted them with Yumm5.2 sh*Cdkn2a* cells (**Fig. 4I and Extended Data Fig. 8D-E**). Consistent with our hypothesis, zinc-replete TAMs were more phagocytic when mice were treated with anti-Pd1 than control TAMs (**Fig. 4J**). Together, our data demonstrate that zinc is critical for TAM phagocytosis, and zinc-replete TAMs respond to anti-Pd1 by increasing their phagocytic gene expression and activity. Thus, increased zinc uptake by *Cdkn2a*^{Low} cancer cells impairs TAM phagocytosis by outcompeting macrophages for zinc (**Fig. 4K**).

High zinc diet combined with ICB decreases tumor burden and enhances TAM phagocytosis in *CDKN2A*^{Low} tumors

We found that *Cdkn2a*^{Low} cancer cells deplete the TME of zinc and outcompete TAMs for zinc by upregulating plasma membrane Slc39a9, and that zinc-replete TAMs have increased phagocytosis (**Fig. 2 and Fig. 4**). Since no SLC39A9 inhibitors are currently available, we hypothesized that replenishing the TME with zinc via the diet would increase zinc availability for TAMs to promote an anti-Pd1 response. Indeed, a high zinc diet (~3X higher than normal chow) significantly elevated TME zinc levels (**Fig. 5A-B**). This corresponded to sensitization of *Cdkn2a*^{Low} tumors to ICB (**Fig. 5C-D and Extended Data Fig. 9A**). The dietary intervention did not significantly affect food intake or body weight

(**Extended Data Fig. 9B-E**) nor did it markedly alter tumor growth in the IgG control treatment group (**Extended Data Fig. 9F-G**). Similar to *Cdkn2a*^{Low} tumors with Slc39a9 knockdown (**Fig. 3D**), TAM depletion abrogated this anti-Pd1 response (**Fig. 5E**), indicating that

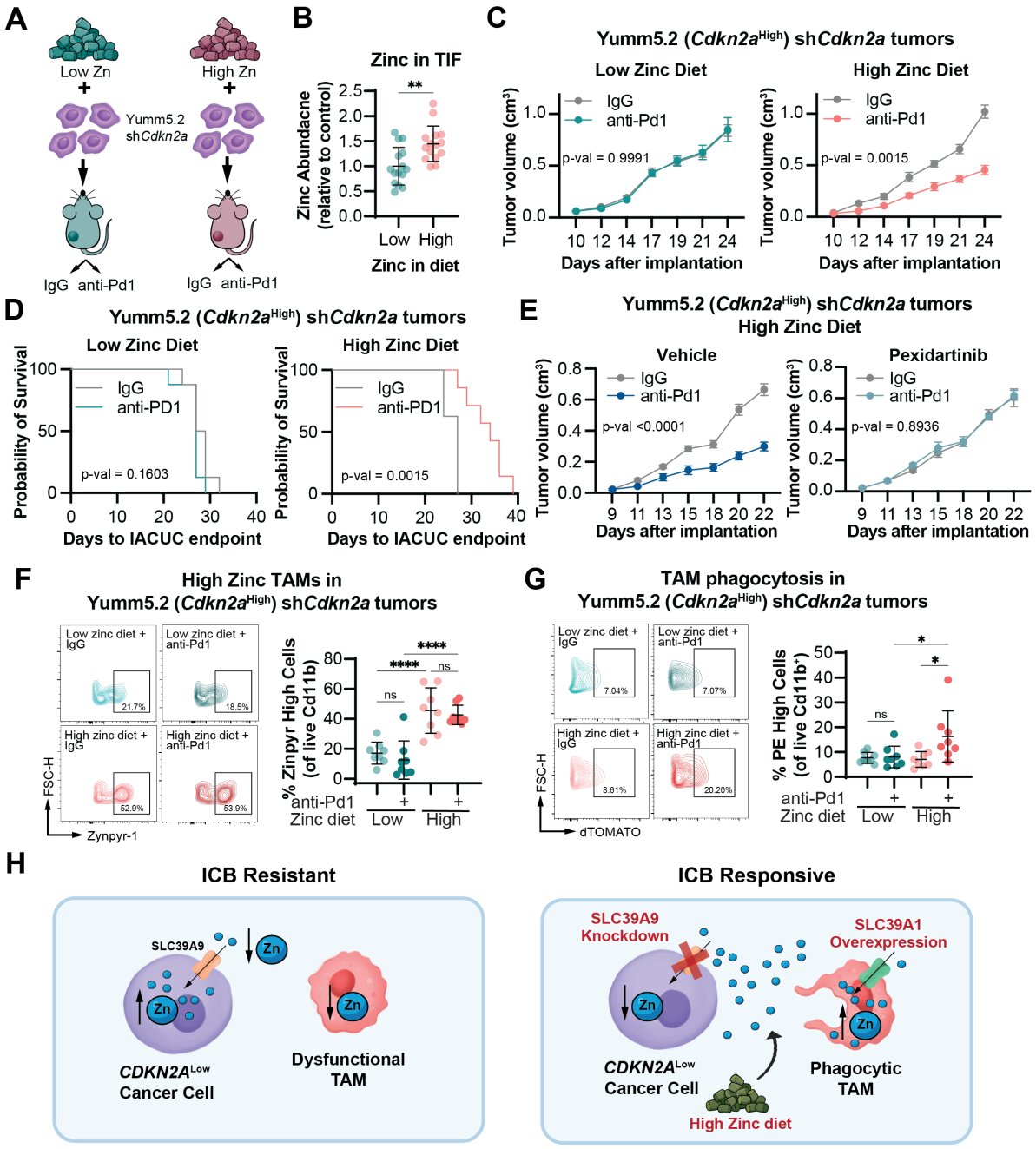


Figure 5. Replenishing TME zinc via a dietary intervention in *Cdkn2a*^{Low} tumors re-educates TAMs and primes phagocytosis activity for ICB response. (A) Schematic of dietary intervention. **(B-D)** Mice were assigned to low (55 mg/kg) or high (300 mg/kg) zinc diets. Yumm5.2

(*Cdkn2a*^{High}) cells with *Cdkn2a* knockdown (sh*Cdkn2a*) were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). **(B)** ICP-MS of zinc in the tumor interstitial fluid (TIF) of indicated groups. **(C)** Longitudinal tumor growth as mean ± SEM (n=8/group). Mixed-effects model. **(D)** Survival analysis Logrank test. **(E)** Mice were assigned to low (55 mg/kg) or high (300 mg/kg) zinc diets. Yumm5.2 (*Cdkn2a*^{High}) with sh*Cdkn2a* were implanted into mice. Mice were treated with 45 mg/kg of Pexidartinib or vehicle (o.g., 3x/week) and 200 µg IgG or anti-Pd1 (i.p., 3x/week). Longitudinal tumor growth as mean ± SEM (n=8/group). Mixed-effects model. **(F-G)** Mice were assigned to low (55 mg/kg) or high (300 mg/kg) zinc diets. dTOMATO-expressing Yumm5.2 (*Cdkn2a*^{High}) sh*Cdkn2a* cells with knockdown of Slc39a9a (sh*Cdkn2a* + shSlc39a9) were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). **(F)** Representative flow cytometry plots (left) and quantification (right) showing the percentage of TAMs with high Zinpyr-1 (labile zinc) staining. **(G)** Representative flow cytometry plots (left) and quantification (right) showing the percentage of TAMs with dTOMATO signal (phagocytic TAMs). **(H)** Schematic of proposed mechanism. *CDKN2A*^{Low} cancer cells sequester TME zinc via increased plasma membrane SLC39A9 leading to macrophage dysfunction and ICB resistance. Replenishment of TME zinc via knockdown of SLC39A9 in cancer cells, overexpression of Slc39a1 in co-implanted macrophages, or high zinc diet increases intracellular zinc in macrophages priming their phagocytic activity to respond to ICB. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

TAMs are essential for the anti-Pd1 response of *Cdkn2a*^{Low} cancer cells on a high zinc diet. Excitingly, we confirmed that the high zinc diet increased TAM zinc levels (**Fig. 5F**), and these zinc-replete TAMs had enhanced phagocytic activity following anti-Pd1 treatment (**Fig. 5G**). Similar to our findings with knockdown of Slc39a9, the observed increase in TAM phagocytosis was independent of changes in Pd-l1 expression on cancer cells (**Extended Data Fig. 9H**), further confirming that this effect is macrophage-intrinsic. Taken together, these findings support the notion that replenishing the TME with zinc via a dietary intervention can reverse ICB resistance in *Cdkn2a*^{Low} tumors, which is associated with an enhanced macrophage phagocytotic response (**Fig. 5H**).

DISCUSSION

The role of *CDKN2A* as a critical regulator of the cell cycle has been well established for >30 years[113,114]. However, accumulating evidence from our lab and others has revealed that *CDKN2A*/p16 loss has non-canonical pro-tumorigenic functions that extend beyond its role in cell cycle control, including in metabolism[4-14,89,90]. Moreover, there is recent evidence that nutrient competition within the TME can affect immune cell activity and function[91-94], although how the micronutrient zinc is compartmentalized within cancer and immune cells has never been explored. Here, we demonstrate that loss of *CDKN2A* in cancer cells enhances zinc uptake through upregulation of the SLC39A9 transporter at the plasma membrane, a process that is mediated by cholesterol. The increased zinc uptake by *Cdkn2a*^{Low} cancer cells leads to a zinc-depleted TME, impairing TAM phagocytic activity and diminishing the efficacy of anti-Pd1 treatment. Our findings underscore the critical role of *CDKN2A*/p16 expression in modulating the immune response through zinc, demonstrate an anti-tumorigenic role for zinc-replete TAMs, and open up new therapeutic possibilities through dietary interventions.

Our study demonstrates that *CDKN2A*/p16 itself drives inherent resistance to ICB. There are a handful of prior reports linking loss of *CDKN2A* expression, or 9p21 deletion, with changes in anti-tumor immunity and ICB response[5,16-32]. Here, we found that *CDKN2A*, and more specifically p16 alone, can drive differential ICB responses (**Extended Data Fig. 2**). Consistent with our findings, an integrated analysis of immunogenomic and clinical data from over 1000 patients across 8 solid tumors demonstrated that 9p21 deletions, specifically encompassing *CDKN2A* and *MTAP*, were associated with decreased tumor

immune responses and resistance to anti-PD1 or anti-PD-L1 monotherapy[17]. In contrast, a prior study using cells with different 9p21 deletions showed deletions involving only the *CDKN2A* gene did not affect the response to anti-CTLA4[88]. This discrepancy may arise from differences in the models and immunotherapies used. Despite these insights, routine assessment of *CDKN2A*/p16 expression remains uncommon in clinical practice, except in specific cases such as HPV-positive tumors or when germline alterations are suspected, particularly in individuals with a family history of melanoma and/or pancreatic cancer[115-117]. Together with the prior research, our work provides compelling evidence for the need to routinely assess *CDKN2A* status in cancer diagnostics, particularly as our research suggests that treatment outcomes of patients harboring *CDKN2A*^{Low} tumors may be improved by combining ICB therapy with dietary zinc supplementation.

CDKN2A has non-canonical roles in intracellular metabolism[4,6,7,9,10,89,90], and we emphasize here a role for *CKDN2A* in influencing metabolites within the TME. Specifically, we found that *CDKN2A*^{Low} cancer cells sequester zinc from the TME (**Fig. 2**), reducing macrophage phagocytic activity during ICB treatment, thereby diminishing ICB response (**Fig. 4**). Zinc is an essential micronutrient critical for the proper functioning of cells. For example, zinc deficiency impairs immune cell activity, while zinc supplementation can either restore normal function in deficient cells or enhance immune responses in healthy cells[118]. Despite decades of research on zinc's role in cancer, its biological effects remain poorly understood and are often contradictory[119]. These inconsistencies stem in part from a historical focus on systemic zinc levels, which overlook the intricate regulation and compartmentalized distribution of zinc within the TME. In this study, we found that increased

intracellular zinc in *CDKN2A*^{Low} cancer cells was mediated by a significant upregulation of the zinc transporter SLC39A9 at the plasma membrane (**Fig. 2**). Upregulation of plasma membrane SLC39A9 was regulated by cholesterol (**Fig. 2**), which we have previously found to be elevated in sh*CDKN2A*/shp16 cells[7] (**Extended Fig. 5G-H**). Interestingly, several epidemiological studies have shown a strong correlation between statin use—drugs that lower cholesterol—and improved response to ICB in cancer patients[120-125]. Although outside of the scope of the present study, it is intriguing to speculate that this effect is mediated, at least in part, by decreased plasma membrane SLC39A9 in cancer cells, leading to increased zinc availability for immune cells. Moreover, these results contribute to the growing body of research highlighting the critical role of metabolite compartmentalization within the TME[91,93,126,127] and underscores the limitations of systemic zinc-level assessments in evaluating zinc's role in cancer.

Zinc can be modulated by the diet, and prior work has associated high dietary zinc intake with improved cancer outcomes or decreased risk[128-133]. However, others report adverse[134-137] or neutral effects[138-141]. Here, we found that high dietary zinc effectively increases the zinc content in the TME and improves ICB response of *Cdkn2a*^{Low} tumors (**Fig. 5**). This increase in zinc in the diet is ~3X more than the normal chow. The recommended daily allowance (RDA) of zinc is 8 mg for adult women and 11 mg for adult men[142]. Assuming similar zinc metabolism between humans and mice, this increase in dietary zinc is equivalent to a 30 mg zinc supplement, which is available over-the-counter. Future studies are required to explore the potential benefits of combining ICB with this highly accessible and cost-effective zinc supplementation for patients with *CDKN2A*^{Low}

tumors. In contrast to our study, a recent article found that high dietary zinc promoted tumor progression in a B16F10 melanoma model by increasing regulatory T cells[85]. These differences are likely due to the amount of zinc (300mg/kg in our study vs. 80mg/kg in[85]) or delivery method. Indeed, 80 mg/kg is below our normal chow levels, suggesting that this study did not achieve high enough intratumoral zinc levels. Nonetheless, these discrepancies underscore the importance of considering tumor-specific factors, zinc delivery methods, and bioavailability when exploring zinc-based therapies. In this regard, our data show that the high zinc diet used in this study effectively increases zinc levels not only in the TME (**Fig. 5B**), but also in intratumoral macrophages (**Fig. 5F**). Although we found that replenishing the TME with zinc enhances TAM zinc content and phagocytic activity in C*DKN2A*^{Low} tumors, the role of increased intracellular zinc in *CDKN2A*^{Low} cancer cells remains unclear. The literature reports both pro-tumorigenic[143-145] and cytotoxic[146-149] effects of high intracellular zinc in cancer cells. Thus, it is interesting to speculate that zinc may have pleiotropic roles within cancer cells similar to that observed for ROS[150], where excessively high levels are detrimental, but moderate to high levels promote tumorigenesis. Further research is needed to determine the intracellular mechanisms of zinc in C*DKN2A*^{Low} cancer cells.

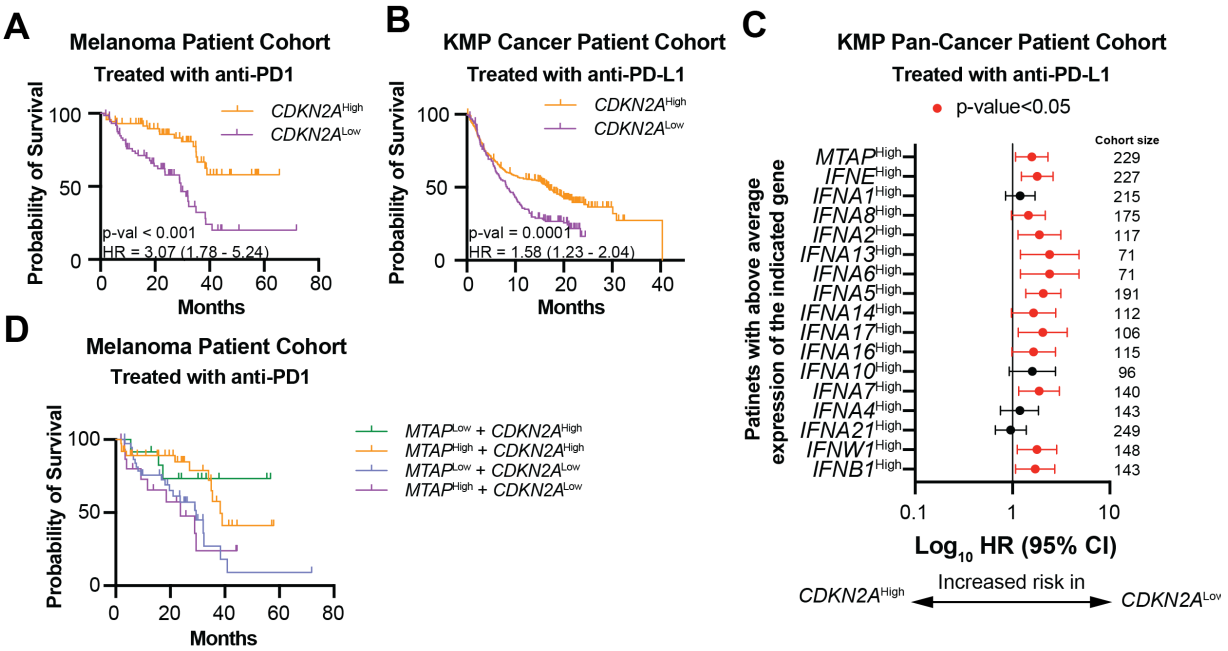
Our studies and others have shown that suppression of *CDKN2A*/p16 correlates with reduced expression of pro-inflammatory factors across various diseases, including cancer[5,15,151-155]. Consistently, *CDKN2A*^{Low} tumors exhibit 'cold' immune phenotypes with reduced immune infiltration, which has been linked to the observed resistance to ICB[17,18,22,23,25,26,156-159]. Excitingly, our findings reveal that TAMs, rather than T or B cells,

are the critical immune cell impairing the response to ICB in *CDKN2A*^{Low} (**Fig. 3**). We found that *Cdckn2a*^{Low} murine tumors have increased TAMs (**Fig. 3B**), consistent with prior reports in human patients[17,25]. However, while other research focused on the macrophage polarization status[17,21,22], here we found that *CDKN2A*^{Low} cancer cells modulate TAM phagocytic activity via rearrangement of zinc compartmentalization in the TME. In this regard, recent studies have unveiled the impact of myeloid cells—including macrophages—in orchestrating both direct and indirect responses to ICB[39-45]. This paradigm shift underscores the critical importance of studying TAM activity, not simply polarization status or macrophage markers, in the context of ICB treatment to improve anti-tumor efficacy. We observed that TAMs are depleted of zinc in *Cdkn2a*^{Low} tumors, which was reversed by Slc39a9 knockdown in cancer cells or high zinc diet (**Fig. 4 and 5**). Zinc-replete TAMs exhibited elevated intracellular zinc levels and increased phagocytic activity. These findings align with previous research indicating that TAMs can directly respond to ICB in part by enhancing phagocytosis[39,45,112]. Additionally, a recent pan-cancer study in patient samples identified a subgroup of TAMs that upregulate zinc-binding metallothioneins (named *metalloMac*) that are enriched in ICB-responsive patients[40]. This is consistent with our data demonstrating that zinc metabolism in TAMs boosts the response to ICB. Indeed, zinc has been previously shown to enhance phagocytosis capacity and/or pathogen clearance[58-60,160-163], although none of this work is in cancer and the mechanism is not well understood. Here we found that there is a correlation between the amount of intracellular zinc in TAMs and their cancer-phagocytic activity *in vivo*, both in human and mouse (**Fig. 4-5 and Extended Data Fig. 7**). Moreover, zinc supplementation enhances the expression of phagocytosis-related genes in macrophages treated with anti-Pd1 *in*

vitro, an effect that is abolished by zinc chelation (**Extended Data Fig. 7G**). The human genome encodes over 700 zinc-finger proteins, the largest class of putative transcription factors[164], and prior research has explored whether intracellular zinc levels directly influence gene transcription[165]. Future studies will investigate the specific transcription factors that drive macrophage phagocytic activity in response to zinc replenishment. Collectively, our findings underscore the critical role of TAM zinc metabolism in modulating the efficacy of ICB therapies, particularly in the context of *CDKN2A*^{Low} tumors, where zinc homeostasis within the TME is disrupted. This study also highlights the potential for boosting zinc metabolism in TAMs as a strategy to enhance the therapeutic response to ICB in cancer patients.

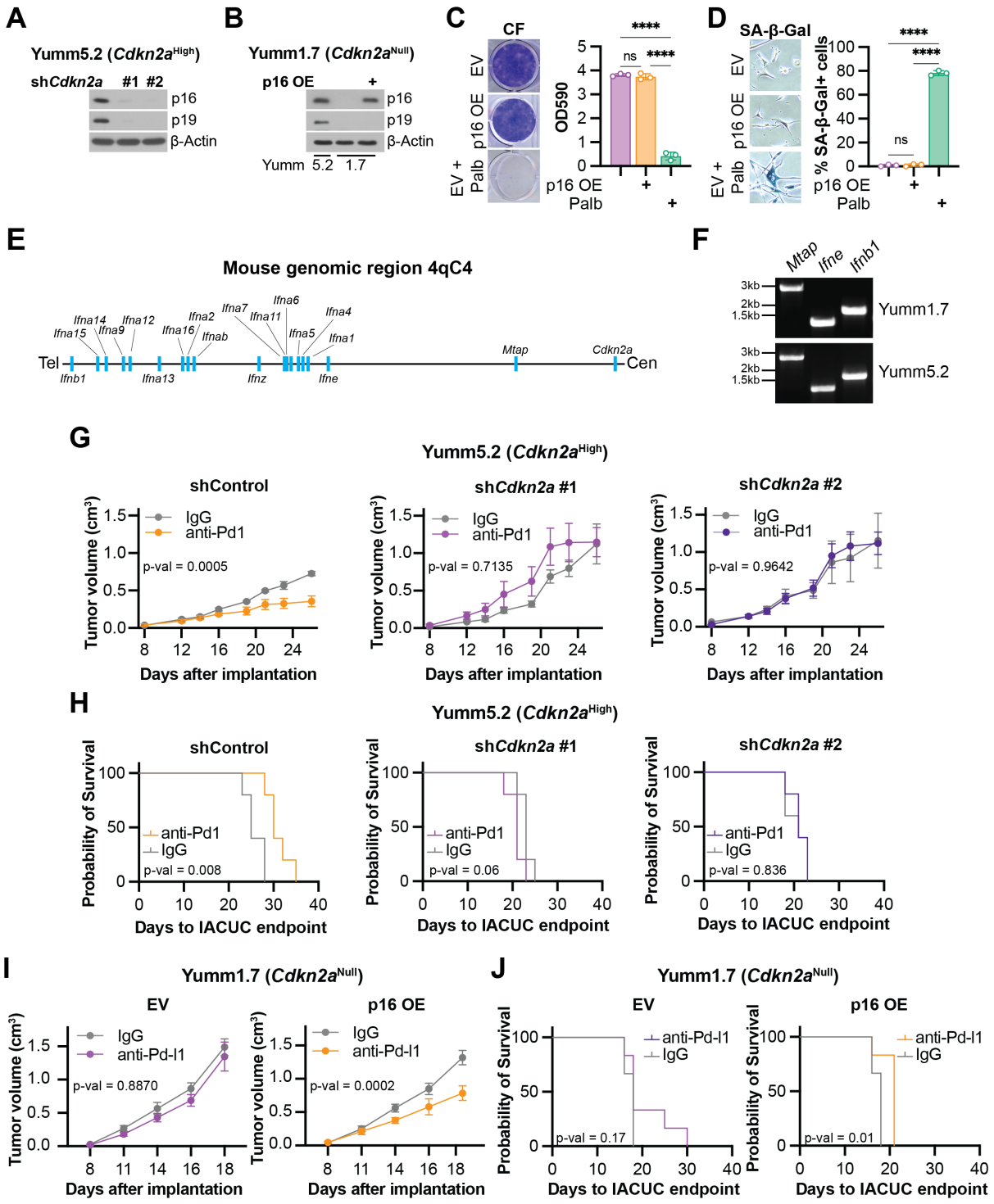
In conclusion, the TME represents a competitive environment where access to nutrients, such as zinc, critically shapes the dynamics between tumor and immune cells. Our findings demonstrate that suppression of *CDKN2A* confers the ability to deplete zinc from the TME by upregulating the SLC39A9 transporter, impairing TAM function, and reducing the efficacy of ICB therapy. This study highlights a previously unrecognized role of *CDKN2A* in maintaining a balanced TME metabolism that supports immune activity and therapeutic responses. In summary, our results identify an unexpected role for *CDKN2A* as a master regulator of zinc homeostasis, which impacts the TME and ICB therapy. These results have major implications not only for how *CDKN2A*^{Low} tumors are treated, but also in underscoring novel roles for *CDKN2A* outside of the cell cycle.

EXTENDED DATA FIGURES



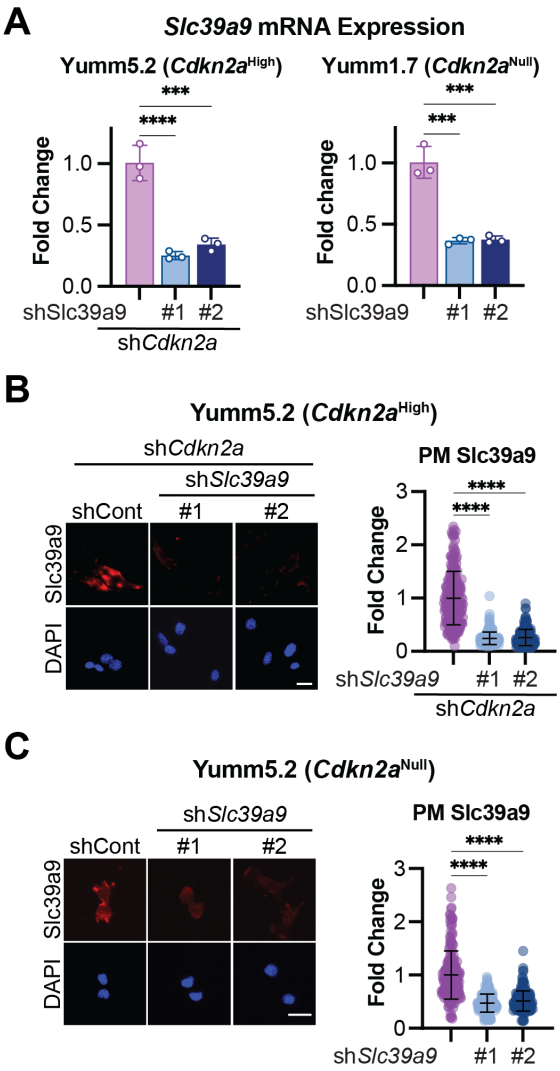
Comparison	HR (95% CI)	p-value
MTAP^{High} + *CDKN2A*^{Low} Vs. *MTAP*^{High} + *CDKN2A*^{High}	2.43 (0.89 - 6.68)	0.04
MTAP^{Low} + *CDKN2A*^{Low} Vs. *MTAP*^{Low} + *CDKN2A*^{High}	3.08 (1.28 - 7.42)	0.05
MTAP^{Low} + *CDKN2A*^{Low} Vs. *MTAP*^{High} + *CDKN2A*^{High}	2.43 (1.19 - 4.95)	0.01

Extended Data Figure 1: Patients harboring *CDKN2A*^{Low} tumors have shorter overall survival on ICB. Related to Figure 1. (A) Overall survival of melanoma patients treated with anti-PD1 stratified based on *CDKN2A* mRNA expression level. Melanoma Patient Cohort. **(B)** Overall survival of cancer patients treated with anti-PD-L1 stratified based on *CDKN2A* mRNA expression level. KMP Cancer Patient Cohort. **(C)** Forest plot of hazard ratios (HR) and 95% Confidence intervals (CI) of cancer patients treated with anti-PD-L1 stratified based on *CDKN2A* mRNA expression levels but only on patients with high expression of the indicated gene. KMP Cancer Patient Cohort. **(D)** Overall survival of melanoma patients treated with anti-PD1 stratified based on *CDKN2A* and *MTAP* mRNA expression. Melanoma Patient Cohort. Log-rank (Mantel-Cox) test.

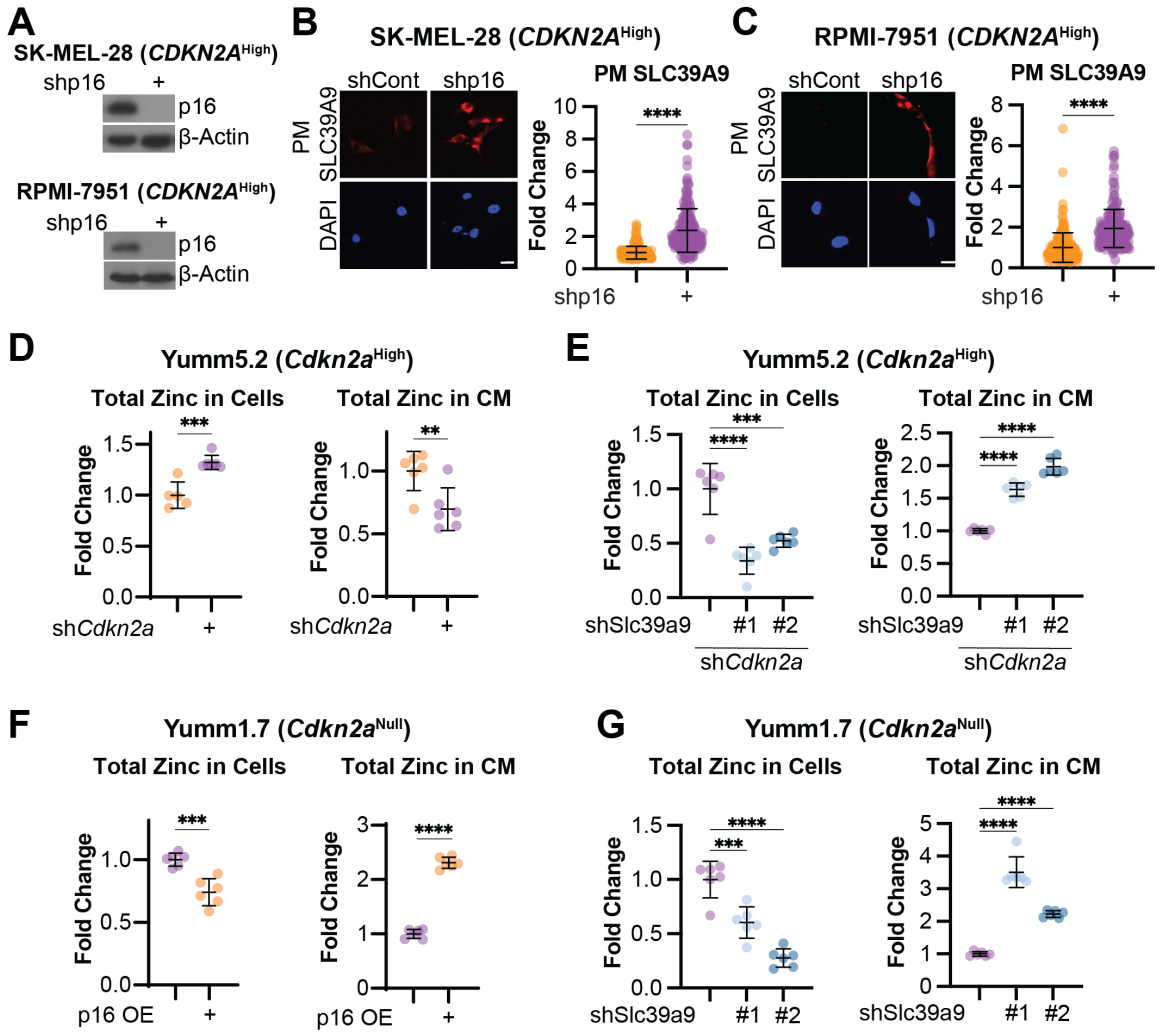


Extended Data Figure 2: Cellular models used in this study. Related to Figure 1. (A-B) Immunoblot analysis of p16 and p19. β-Actin was used as loading control. **(A)** Yumm5.2 (*Cdkn2a*^{High}) cells. **(B)** Yumm1.7 (*Cdkn2a*^{Null}) cells. **(C-D)** Yumm1.7 (*Cdkn2a*^{Null}) cells expressing empty vector control or overexpression of p16 (p16 OE). Induction of senescence with

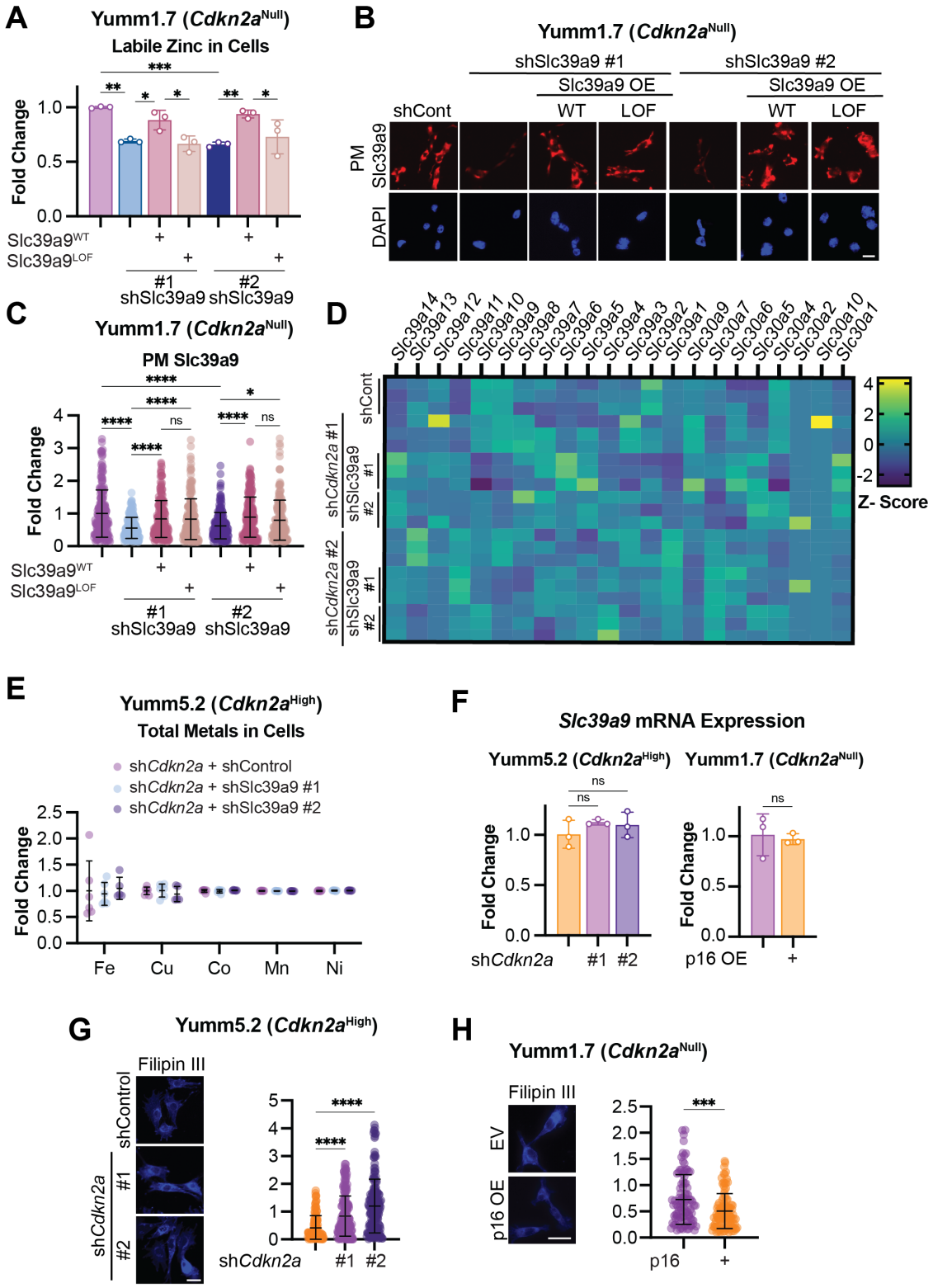
palbociclib (palb; 3 μM, 10 days) was used as positive control. **(C)** Colony formation (CF) Assay (left) and quantification (right). One-way ANOVA. **(D)** Representative images of Senescence-Associated β-Galactosidase (SA-β-Gal) activity (left) and quantification (right). One-way ANOVA. **(E)** Schematic representation of the murine 4qC4 genomic region containing *Cdkn2a* gene and gene neighbors. **(F)** Genomic DNA PCR amplification of indicated genes in Yumm1.7 and Yumm5.2 cell lines. **(G-H)** Yumm5.2 (*Cdkn2a*^{High}) cells with knockdown of Cdkn2a (sh*Cdkn2a* hairpins #1 and #2) or shControl were implanted in mice. Mice were treated with 200 μg IgG or anti-Pd1 (i.p., 3x/week). **(G)** Longitudinal tumor growth as mean ± SEM (n=4/group). Mixed-effects model. **(H)** Kaplan-Meier curves (n=5/group). Log-rank (Mantel-Cox) test. **(I-J)** Yumm1.7 (*Cdkn2a*^{Null}) cells expressing empty vector (EV) control or overexpression of p16 (p16 OE) were implanted in mice. Mice were treated with 200 μg IgG or anti-Pd1 (i.p., 3x/week). **(I)** Data shows longitudinal tumor growth as mean ± SEM (n=6/group). Mixed-effects model **(J)** Data shows Kaplan-Meier curves (n=6/group). Log-rank (Mantel-Cox) test. Mixed-effects model and Log-rank (Mantel-Cox) test.



Extended Data Figure 3: Knockdown of Slc39a9 in cancer cells. Related to Figure 1. (A) *Slc39a9* mRNA expression in the indicated cell lines. Fold change to shControl group (mean ± SD). **(B-C)** Representative images (left) and quantification (right) of Slc39a9 IF on non-permeabilized cells to visualize plasma membrane (PM) Slc39a9 in **(B)** Yumm5.2 (*Cdkn2a*^{High}) with *Cdkn2a* knockdown (sh*Cdkn2a* hairpins #1 and #2) alone or combined with knockdown of Slc39a9 (hairpins #1 and #2), and **(C)** Yumm1.7 (*Cdkn2a*^{Null}) with knockdown of Slc39a9 (hairpins #1 and #2) or shcontrol. Data represent mean ± SD (n=200/group). Representative images (left) and quantification (right) of Slc39a9 IF on non-permeabilized cells. Data represent mean ± SD (n=200/group). Shown are representative data from at least 3 independent experiments. Scale bars = 25μm. One-way. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

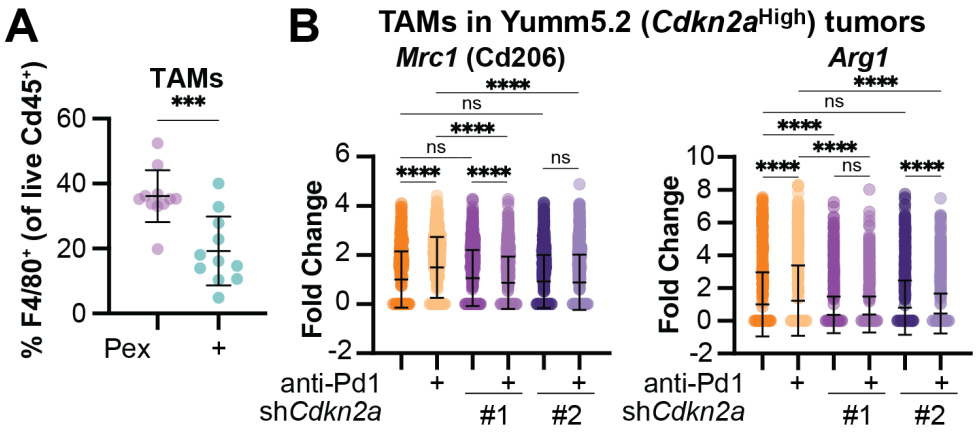


Extended Data Figure 4: Knockdown of p16 in human melanoma cells increases plasma membrane SLC39A9; Slc39a9 regulates zinc uptake in *Cdkn2a*^{Low} cells. Related to Figure 2. (A) Immunoblot analysis of p16 in the indicated human melanoma cell lines. β-Actin was used as loading control. **(B)** Representative images (left) and quantification (right) of plasma membrane (PM) SLC39A9 IF on non-permeabilized SK-MEL-28 (*Cdkn2a*^{High}) cells with p16 knockdown (shp16) or control (shCont). Data represent mean ± SD (n=200/group). **(C)** Same as (A), but in RPMI-7951 (*Cdkn2a*^{High}) cells. **(D)** Total zinc quantification by ICP-MS in cells (left) and conditioned media (CM, right) in Yumm5.2 (*Cdkn2a*^{High}) cells with knockdown of *Cdkn2a* (sh*Cdkn2a*) or shcontrol. Data represent mean ± SD. **(E)** Same as (D) but in Yumm5.2 (*Cdkn2a*^{High}) with sh*Cdkn2a* alone or in combination with knockdown of Slc39a9 (shSlc39a9, hairpins #1 and #2). **(F)** Same as (D), but in Yumm1.7 (*Cdkn2a*^{Null}) cells overexpressing p16 (p16 OE) or empty vector control. **(G)** Same as (E) but in Yumm1.7 (*Cdkn2a*^{Null}) cells with shSlc39a9 (hairpin #1 and #2) or shControl. Scale bars = 25μm. Shown are representative data from 2 independent experiments. Student's t-test and one-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

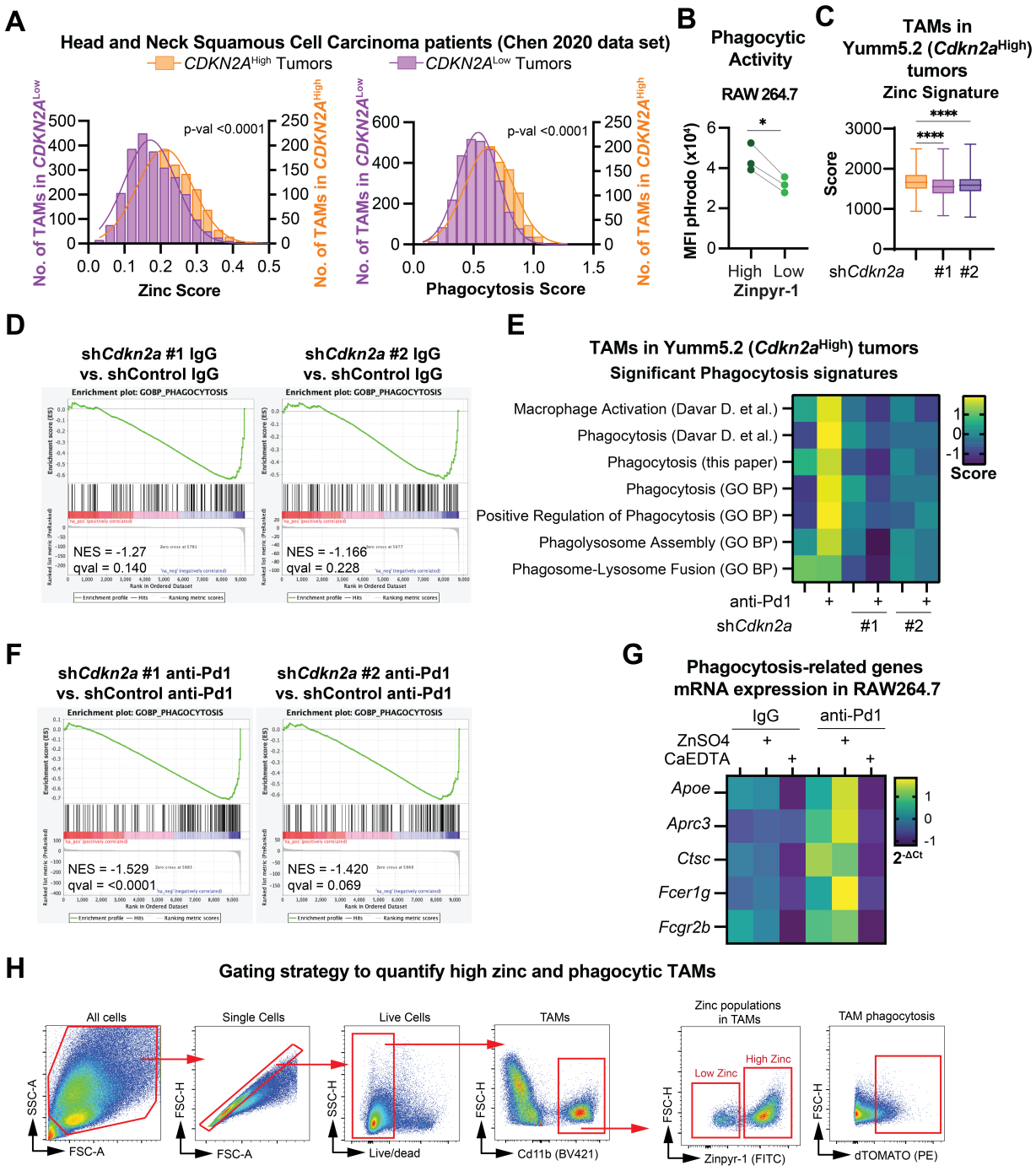


Extended Data Figure 5: Slc39a9 loss-of-function mutant does not increase intracellular zinc; Knockdown of Slc39a9 does not affect other metals nor induce compensatory expression of other metal transporters; *Cdkn2a*^{Low} cancer cells have decreased cholesterol. Related to Figure 2. (A-C) Yumm1.7 (*Cdkn2a*^{Null}) cells with knockdown of Slc39a9 (shSlc39a9, hairpins #1 and #2) alone or in combination with empty vector (EV) control,

wildtype Slc39a9 overexpression (WT), or loss-of-function Slc39a9 mutant overexpression (LOF). **(A)** Labile zinc quantification. Data represent mean ± SD. **(B)** Representative images of plasma membrane (PM) Slc39a9 IF on non-permeabilized cells. Scale bars = 25μm. **(C)** Quantification of (B). Data represent mean ± SD (n=200/group). **(D)** Heatmap of expression values for the indicated genes in the indicated Yumm5.2 isogenic cells. **(E)** Quantification of indicated metals by ICP-MS in Yumm5.2 (*Cdkn2a*^{High}) cells with knockdown of *Cdkn2a* (sh*Cdkn2a*) alone or in combination with Slc39a9 shSlc39a9 (hairpins #1 and #2). Data represent mean ± SD. **(F)** *Slc39a9* mRNA expression in the indicated cell lines. **(G-H)** Representative images of Filipin III staining to detect cholesterol (left) and quantification (right) in **(G)** Yumm5.2 (*Cdkn2a*^{High}) cells with knockdown of *Cdkn2a* (sh*Cdkn2a* hairpins #1 and #2) or shControl and **(H)** Yumm1.7 (*Cdkn2a*^{Null}) cells with p16 overexpression (OE) or empty vector control. Scale bars = 50μm. Data represent fold change to control group as mean ± SD. One-way ANOVA and student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

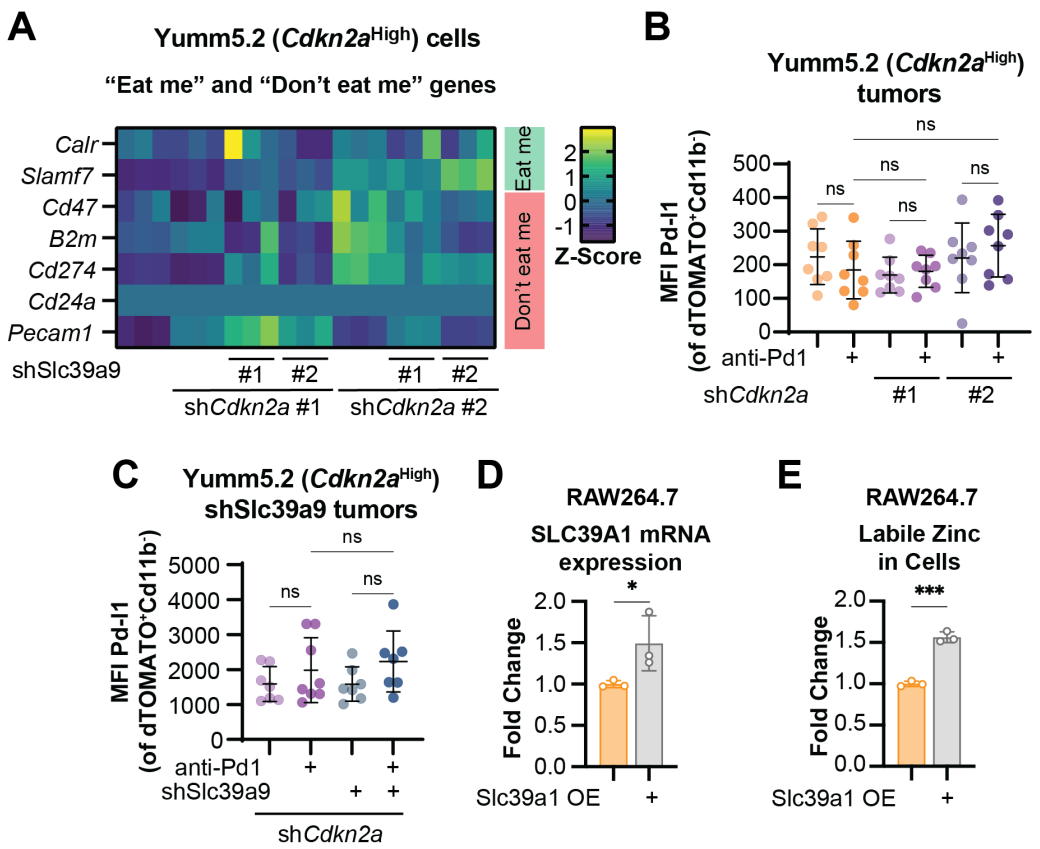


Extended Data Figure 6: Pexidartinib reduces TAMs; TAMs in *Cdkn2a*^{Low} tumors do not have increased mRNA expression of immunosuppressive markers. Related to Fig. 3. (A) Percent F4/80^{+} cells in the live Cd45^{+} cell gate in Yumm5.2 sh*Cdkn2a* tumors treated with or without Pexidartinib (Pex). Student's t-test. **(C)** mRNA expression of the prototypical immuno-suppressive markers *Mrc1* (Cd206) and *Agr1* in the TAM compartment of indicated Yumm5.2 tumors. Data from scRNA-Seq represent mean ± SD of expression fold change relative to shControl. One-way ANOVA. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

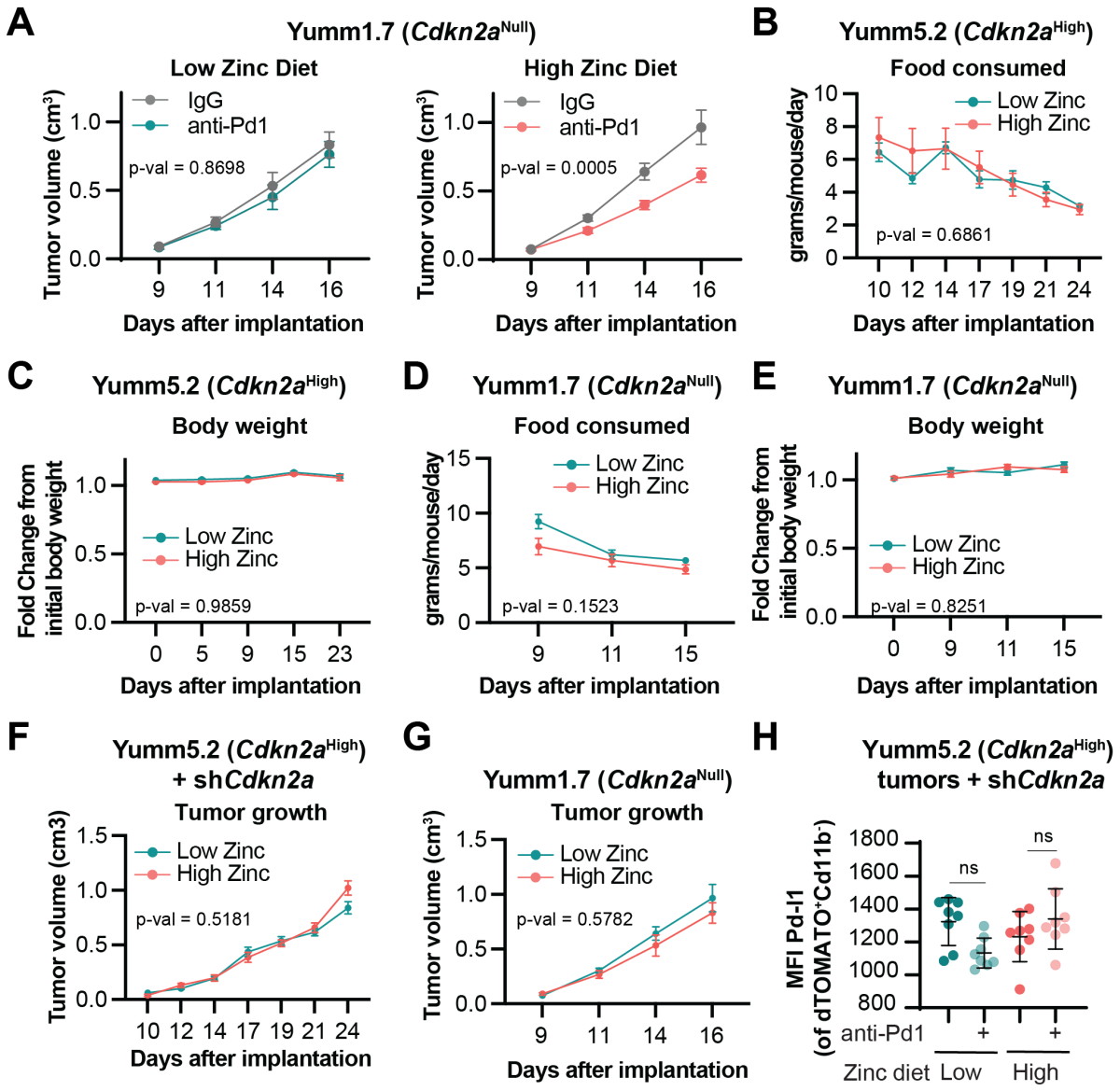


Extended Data Figure 7: TAMs in *CDKN2A*^{Low} tumors have decreased zinc and phagocytosis signatures; Zinc regulates the expression of phagocytic-related genes; TAM gating strategy. Related to Fig. 4. (A) Zinc and phagocytic scores calculated in the TAM population of head and neck squamous cell carcinoma patients harboring *CDKN2A*^{High} or *CDKN2A*^{Low} tumors in the Chen 2020 data set. Histogram of number of macrophages per tumor group with indicated zinc (left) or phagocytic (right) score. Linear Mixed Model. **(B)** Mean fluorescence intensity (MFI) of pHrodo in RAW 264.7 macrophages stratified based on high or low Zinpyr-1 staining of intracellular labile zinc **(C)** Zinc signature scores in the TAM compartment of

Yumm5.2 tumors with knockdown of *Cdkn2a* (sh*Cdkn2a* hairpins #1 and #2) or shControl. Data from scRNA-Seq represent mean ± SD. **(D)** Gene Set Enrichment Analysis (GSEA) of the indicated comparisons using the GOBP_PHAGOCYTOSIS mouse gene set. **(E)** Heatmap of scores for the indicated phagocytosis signatures in the TAM compartment of Yumm5.2 tumors with shControl and sh*Cdkn2a* (hairpins #1 and #2) treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Data from scRNA-Seq. **(F)** Gene Set Enrichment Analysis (GSEA) of the indicated comparisons using the GOBP_PHAGOCYTOSIS mouse gene set. **(G)** RAW264.7 macrophages were treated with 30µg of $ZnSO_4$ or 3mM of CaEDTA in combination with 1 µg/mL of IgG or anti-Pd1 for 16h in FBS-free media. Heatmap of phagocytic-related genes. **(H)** TAM gating strategy. One-way ANOVA and student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.



Extended Data Figure 8: Knockdown of *Cdkn2a* alone or in combination with Slc39a9 knockdown does not markedly affect the mRNA expression of 'eat me' and 'don't eat me' signals. *Cdkn2a*^{Low} cancer cells alone or with knockdown of Slc39a9 do not have altered Pd-l1 expression *in vivo*; Overexpression of Slc39a1 in RAW264.7 increases intracellular labile zinc. Related to Fig. 4. (A) Heatmap of expression values for the indicated genes associated to the 'eat me' and 'don't eat me' signaling in Yumm5.2 cells with shControl and sh*Cdkn2a* (hairpins #1 and #2) alone or in combination with shSlc39a9 (hairpins #1 and #2). **(B)** dTOMATO-expressing Yumm5.2 (*Cdkn2a*^{High}) cells with *Cdkn2a* knockdown (sh*Cdkn2a*) or control were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Mean fluorescence intensity (MFI) of Pd-l1 in cancer cells (dTOMATO^{+} Cd11b^{-}). **(C)** dTOMATO-expressing Yumm5.2 (*Cdkn2a*^{High}) sh*Cdkn2a* cells alone or with knockdown of Slc39a9a (sh*Cdkn2a* + shSlc39a9) were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Mean fluorescence intensity (MFI) of Pd-l1 in cancer cells (dTOMATO^{+} Cd11b^{-}). **(D)** *Slc39a1* mRNA expression in RAW264.7 cells with Slc39a1 overexpression (Slc39a1 OE) or empty vector control. Fold change to shControl group (mean ± SD). **(E)** Fold change relative to shControl of Mean fluorescence intensity (MFI) of zinpyr-1 (labile zinc) in RAW264.7 cells with Slc39a1 overexpression (Slc39a1 OE) or empty vector control. One-way ANOVA and student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.



Extended Data Figure 9: High zinc diet overcomes anti-Pd1 resistance in the Yumm1.7 model; Dietary intervention does not alter body weight or food consumed; zinc dietary intervention does not alter cancer cell Pd-l1 expression *in vivo.* Related to Fig. 5. (A) Mice were assigned to low (55 mg/kg) or high (300 mg/kg) zinc diets. Yumm1.7 (*Cdkn2a*^{Null}) cells were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Longitudinal tumor growth as mean ± SEM (n=12/group). Mixed-effects model. **(B-C)** Mice were assigned to low (55 mg/kg) or high (300 mg/kg) zinc diets. Yumm5.2 (*Cdkn2a*^{High}) cells with *Cdkn2a* knockdown (sh*Cdkn2a*) were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). **(B)** Food consumed. **(C)** Body weight. **(D-E)** Mice were assigned to low (55 mg/kg) or high (300 mg/kg) zinc diets. Yumm1.7 (*Cdkn2a*^{Null}) cells were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). **(D)** Food consumed**. (E)** Body weight. **(F)** Same as (B-C) but longitudinal tumor growth in IgG treated groups. **(G)** Same as (D-E) but longitudinal tumor growth in IgG treated groups. Data in (F) and (G) are the same show in Fig. 5C and Extended data S9A but plotted together for better assessment of the growth curves of IgG treated mice. **(H)** Mice were assigned to low (55 mg/kg) or high (300 mg/kg) zinc

diets. dTOMATO-expressing Yumm5.2 (*Cdkn2a*^{High}) sh*Cdkn2a* cells with knockdown of Slc39a9a (sh*Cdkn2a* + shSlc39a9) were implanted into mice. Mice were treated with 200 µg IgG or anti-Pd1 (i.p., 3x/week). Mean fluorescence intensity (MFI) of Pd-l1 in cancer cells (dTOMATO^{+} Cd11b^{-}). Mixed Models and One-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Acknowledgements

This work was supported by grants from the National Institutes of Health (R37CA240625 to KM. Aird, R01CA259111 to KM. Aird and NW. Snyder, R01DE031729 and P50CA254865 to R. Bao, and T32 CA082084 to RE. Dadey), the Congressionally Directed Medical Research Program (HT9425-23-1-0436 to KM. Aird and OC230324 to NK. Tangudu), the Melanoma Research Foundation (to R. Buj), the HERA Ovarian Cancer Foundation (to A. Amalric, A. Uboveja, and NK. Tangudu), the University of Chicago Cancer Center Support Grant (to A. Muir), the Ludwig Center for Metastasis Research (to A. Muir), the MaryEllen Connelan Award (to JJ. Apiz Saab), the Robert C and Mary Jane Gallo Scholarship Fund (to JJ. Apiz Saab) and the Harper Fellowship at the University of Chicago (to JJ. Apiz Saab). We utilized resources from the Hillman Cancer Center shared facilities, partially supported by award P30CA047904 including the Animal Facility, the Cytometry Facility (RRID:SCR_025361), the Biostatistics Facility (RRID:SCR_025355), and the Cancer Bioinformatics Services (RRID:SCR_025355). The Cancer Bioinformatics Services were additionally supported by the University of Pittsburgh Center for Research Computing, specifically the HTC high-performance computing cluster, which is supported by NIH (S10OD028483). We also used the University of Pittsburgh Single Cell Core Facility (RRID:SCR_025110), whose services and instruments were in part graciously supported by the University of Pittsburgh and the Department of Medicine. Similarly, we also used the Health Sciences Sequencing Core at UPMC Children's Hospital of Pittsburgh (RRID:SCR_023116), which resources were generously supported by the University of Pittsburgh, the Office of the Senior Vice Chancellor for Health Sciences, the Department of Pediatrics, the Institute for Precision Medicine, and the Richard K. Mellon Foundation

for Pediatric Research. Finally, we extend our gratitude to Francisco J. Vazquez Moreno for his exceptional contribution to the graphic illustrations included in this paper.

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Declaration of Interests

The authors declare no competing interests.

MATERIAL AND METHODS

Melanoma Patient Cohort

This cohort comprises 248 cutaneous melanoma patients, pooled from five independent, publicly available studies[45,166-169], with publicly available pre-treatment RNA-Seq and overall survival data. Details are provided in **Table S6**. Briefly, eligible patients had resectable stage III melanoma treated with neoadjuvant immunotherapy or unresectable stage III/IV treated with systemic immunotherapy. Treatment regimens included anti-PD1 monotherapy or combination therapy with anti-CTLA-4 or TLR9 agonists. Patients were stratified according to *CDKN2A* expression, either alone or in combination with *MTAP* (**Extended Data Fig. 1**). Expression levels of the target genes were categorized as follows: patients in or above the third tertile were considered to have high expression, while those in or below the first tertile were considered to have low expression.

Kaplan-Meier plotter (KMP) Cancer Patient Cohort:

Survival data from cancer patients was downloaded from Kaplan-Meier (KM) plotter 'Start KM Plotter for immunotherapy' (kmplot.com)[170-172] in January 2025. The analysis was performed using the following parameters: the gene symbol was set to *CDKN2A* alone (**Extended Data Fig. 1B**) or in combination with *Filter by median expression* of patients with high expression of *MTAP* and *IFN* genes (**Extended Data Fig. 1D**). Patients were split based on *lower tertile*. *Overall survival* was selected as the survival metric, with no restrictions on follow-up thresholds. The analysis was restricted to patients who had received *all anti*-PD-L1 therapy and included *all* tumor types and sex. The resulting data was exported as text for subsequent analysis in Graph Prism 10.

Cell Lines and culture conditions

All cell lines used in this study were purchased from ATCC and were used within 50 passages. ATCC performs cell line authentication by short tandem repeat (STR) profiling. Cells were cultured within 3 months of receipt or resuscitation. The murine melanoma cell lines Yumm5.2 (ATCC, catalog no. CRL-3367, RRID: CVCL_JK43) and Yumm1.7 (ATCC, catalog no. CRL-3362, RRID: CVCL_JK16) were cultured in DMEM and Ham's F-12 50/50 media (Corning, catalog no. 10-092-CV), supplemented with nonessential amino acids (Corning, catalog no. 25-025-CI). The murine macrophage cell line RAW 264.7 (ATCC, catalog no. TIB-71 ™, RRID: CVCL_0493), the human melanoma cell line SK-MEL-28 (ATCC, catalog no. HTB-72, RRID: CVCL_0526), and the human embryonic kidney cell line HEK293-FT (ATCC, catalog no. CRL-3216, RRID: CVCL_6911) were cultured in DMEM (Corning, catalog no. 10-013-CV), while the human melanoma cell line RPMI-7951 (ATCC, catalog no. HTB-66, RRID: CVCL_1666) was cultured in MEM (Corning, catalog no. 10-009-CV). All cell lines were supplemented with 10% FBS (BioWest, catalog no. S1620) and 1% Penicillin/Streptomycin (Gibco, catalog no. 15-140-122) and maintained at 37 °C with 5% CO_2. To ensure the absence of Mycoplasma contamination, all cell lines were tested monthly as described in[173].

Plasmid information and cloning:

To perform knockdown experiments, short hairpin RNAs (shRNAs) targeting specific genes were used. The shRNA sequences used in this study included: human shp16 (TRCN0000010482), mouse sh*Cdkn2a* #1 (TRCN0000077816) and #2 (TRCN0000362595), and mouse shSlc39a9 #1 (TRCN0000346746) and #2

(TRCN0000346750). The hairpins for shp16 and shSlc39a9 #1 and #2 were purchased pre-cloned into the lentiviral vectors expressing puromycin resistance (Sigma-Aldrich). The sh*Cdkn2a* #1 and #2 hairpins sequences (TRCN0000077816 and TRCN0000362595, respectively) were obtained from Integrated DNA Technologies (IDT) as desalted oligos and subsequently cloned into the lentiviral pLKO.1 vector expressing blasticidin resistance (Addgene, catalog no. 26655). Briefly, 2 µg of pLKO.1 vector was digested with 1 µL of EcoRI-HF (New England Biolabs, catalog no. R3101S), 1 µL of AgeI-HF (New England Biolabs, catalog no. R3552S), and 1 µL of rCutsmart 10X Buffer (New England Biolabs, catalog no. B6004S) in a final reaction volume of 10 µL. The digestion was incubated for 1 hour at 37°C. The linearized vector was separated by electrophoresis on a 1% agarose (IBI Scientific, catalog no. IB70070) gel and purified using the Qiagen Gel Extraction Kit (Qiagen, catalog no. 28704), following the manufacturer's instructions. Simultaneously, shRNA oligos were reconstituted to a final concentration of 100 µM in molecular-grade water (Corning, catalog no. 46-000-CM) and annealed by incubating 11.25 µL of each oligo with 2.5 µL of 10X annealing buffer (1M NaCl, 100 mM Tris, pH 7.4). The reaction was performed in a thermal cycler (Bio-Rad, T100™ Thermal Cycler, serial no. 621BR59583) starting at 95°C for 5 minutes, followed by a ramp-down to 25°C at 5°C/min. The ligation reaction was prepared by combining 50 ng of purified pLKO.1 vector with 1 µL of annealed shRNA oligos (diluted 1:400 in 0.5X annealing buffer), 1 µL of T4 ligase (New England Biolabs, catalog no. M0202T), and 1 µL of T4 ligase buffer (New England Biolabs, catalog no. B0202S) in a final volume of 10 µL. The reaction was incubated at room temperature for 3 hours, after which 5 µL of the ligation mixture was used to transform One Shot™ Stbl3™ Chemically Competent *E. coli*

(Thermo Fisher Scientific, catalog no. C737303) following the manufacturer's instructions. Control lentiviral vectors, (Addgene, catalog no. 110419, RRID: Addgene_110419 and Addgene, catalog no. 30323, RRID: Addgene_30323), were used for both human and mouse experiments. For overexpression experiments, lentiviral plasmids were synthesized by Twist Bioscience. The Twist Bioscience empty vector (EV) was used as the control in overexpressing experiments. The pLX304-dTOMATO lentiviral plasmid was generously provided by the Gomes Lab at the H. Lee Moffitt Cancer Center.

Lentiviral Packaging and Infection conditions

Lentiviral vectors were packaged as follows: 10 µg of the corresponding lentiviral vector was mixed with 7.5 µg of psPAX2 (Addgene, catalog no. 12260, RRID: Addgene_12260) and 7.5 µg of pMD2.G (Addgene, catalog no. 12259, RRID: Addgene_12259) in 1.5 mL of Opti-MEM™ media (Gibco, catalog no. 31985070) and incubated for 5 minutes at room temperature. In parallel, 50 µL of Lipofectamine™ 2000 Transfection Reagent (Invitrogen, catalog no. 11668019) was diluted in 1.5 mL of Opti-MEM™ media and incubated for 5 minutes at room temperature. The Lipofectamine mixture was added dropwise to the plasmid mixture, gently swirled to mix, and incubated for 20 minutes at room temperature. The Lipofectamine-lentiviral complexes were then added dropwise to a 60-70% confluent 10 cm dish of HEK293-FT cells, seeded 24 hours in advance and containing 7 mL of regular media, and incubated at 37°C with 5% CO2. Six to eight hours later, the media was replaced with 9 mL of fresh regular media and incubated at 37°C with 5% CO_2. Seventy-two hours later, the viral supernatants were collected, filtered through a 45 µm filter (Fisherbrand™, catalog no. 09-720-005), and used to infect target cells for 16 hours.

Infected cells were selected for 3 days with 1 µg/mL puromycin (Thermo Fisher Scientific, catalog no. A1113802) for SK-MEL-28 and RPMI-7951, 2 µg/mL puromycin for RAW 264.7 cells, and 3 µg/mL puromycin and/or 3 µg/mL blasticidin (InvivoGen, catalog no. ant-bl-05) for Yumm5.2 and Yumm1.7 cells.

Mouse Models

All mice were housed in a HEPA-filtered ventilated rack system at the Animal Facility of the Assembly Building of The Hillman Cancer Center, University of Pittsburgh School of Medicine. Mice were maintained on a 12-hour light/dark cycle at 21 ± 2 °C and were group-housed up to 5 mice per cage. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh.

To investigate the role of *CDKN2A* in the response to immunotherapy, we developed two complementary subcutaneous syngeneic melanoma models. In the first model, *Cdkn2a* expression was knocked down in the *Cdkn2a*^{High} murine melanoma cell line Yumm5.2 (**Extended Data Fig. 2A**). In the second model, p16 was overexpressed in the *Cdkn2a*^{Null} melanoma cell line Yumm1.7 to physiological levels that did not induce a senescence-associated phenotype (**Extended Data Fig. 2B-D**). Note that Yumm1.7 cells are p19^{Null} (**Extended Data Fig. 2B**). Also note that both cell lines have intact *MTAP* and *IFN* gene cluster (**Extended Data Fig. 2E-F**). Thus, results with this model specifically relate to p16 function. For simplicity, Yumm1.7 with p16 overexpression and Yumm5.2 cells are referred as *Cdkn2a*^{high} while Yumm5.2 with knockdown of *Cdkn2a* and Yumm1.7 cells are referred as *Cdkn2a*^{Low} cancer cells **(Fig. 1A)**. To investigate the role of SLC39A9 in

resistance to anti-Pd1 treatment in *Cdkn2a*^{Low} cancer cells, we used Yumm5.2 cells with dual knockdown of *Cdkn2a* and *Slc39a9* (sh*Cdkn2a*n + sh*Slc39a9*), as well as Yumm1.7 cells with *Slc39a9* knockdown (sh*Slc39a9*). In all models described above, 350,000 cells were subcutaneously implanted into the right flank of 6–8 weeks old male C57BL/6J mice (The Jackson Laboratory, strain #000664, RRID: IMSR_JAX:000664). To assess the role of T and B cells in anti-PD1 response, 350,000 Yumm5.2 sh*Cdkn2a*/sh*Slc39a9* double knockdown cells were implanted into B6.129s7-*Rag1*^{tm1Mom}/J mice (The Jackson Laboratory, strain #002216, RRID: IMSR_JAX:002216). For experiments where TMA phagocytosis was quantified, dTOMATO-expressing cancer cells were implanted. For co-injection experiments, 350,000 Yumm5.2 sh*Cdkn2a* cells expressing dTOMATO were mixed with 35,000 RAW264.7 cells overexpressing Slc39a1 and subcutaneously implanted into the right flank of 6-8 weeks old male C57BL/6J mice (The Jackson Laboratory, strain #000664, RRID: IMSR_JAX:000664). In all cases, control groups received cells overexpressing corresponding shControl (shGFP) or empty vector (see *Plasmid Information and cloning* section).

In all cases described above, cells were resuspended in 200 µL DPBS (Corning, catalog no. 21-031-CV) and subcutaneously implanted using a 1 mL tuberculin syringe and a 25G needle (Becton Dickinson, catalog no. 309626). Tumor formation was monitored, and all models developed palpable tumors within 8–14 days of implantation. Tumor length (L) and width (W) were measured three times a week once palpable tumors appeared using an electronic caliper set in mm (Adoric, model number# KC24, resolution 0.1mm ± 0.2mm). Tumor volume was calculated using the formula: $\frac{1}{2}(L \times W^2)$ where L > W. Mouse

body weight was measured weekly using an electronic scale. The experimental endpoint for longitudinal tumor growth studies was defined as the point when the first mouse reached IACUC-approved criteria: either a tumor volume exceeding 1 cm^3 or a 20% loss of body weight, whichever occurred first. For survival studies, the endpoint for each animal was similarly determined based on IACUC guidelines.

Mice treatments

For anti-Pd-1 and anti-Pd-l1 experiments, mice were administered 200 µg of the respective antibody or placebo, diluted in DPBS to a final volume of 200 µL, by intraperitoneal injection three times per week using insulin syringes (Excel International, catalog no. 26028). The antibodies used were: anti-mouse Pd-1 (Leinco Technologies, catalog no. P362) or placebo IgG2a (Leinco Technologies, catalog no. I-1177) and anti-mouse Pd-l1 (InVivoPlus™, catalog no. BP0101) or placebo IgG2b (InVivoPlus™, catalog no. BP0090). For macrophage depletion, mice were treated with 45 mg/kg Pexiclartinib (Selleck Chemicals, catalog no. S7818), diluted in a vehicle solution containing 45% PE300 (Sigma-Aldrich, catalog no. 202371), 45% ddH_2O, 5% Tween-80 (Sigma-Aldrich, catalog no. P4780), and 5% DMSO (Fisher BioReagents, catalog no. BP231-100), administered via oral gavage at a final volume of 150 µL, three times per week. Treatment was delivered using a 1 mL syringe (Becton Dickinson, catalog no. 309659) and a reusable animal feeding needle (Cadence Science, catalog no. 7910; 2.5 mm ball diameter, 3.81 cm length, 20G, curved), which were autoclaved between uses. Control mice received the same volume of vehicle solution following the same dosing schedule. All treatments described above were initiated upon detection of palpable tumors. For dietary zinc

experiments, standard rodent chow (LabDiet, catalog no. 5P76) containing 110 ppm (110 mg/Kg) zinc was replaced with custom-made diets from LabDiet. Mice were fed either a high-zinc diet (LabDiet, catalog no. 5SNB) containing 300 ppm (300 mg/Kg) zinc or a low-zinc diet (LabDiet, catalog no. 5SNA) containing 55 ppm (55 mg/Kg) zinc. Both experimental diets were identical in composition to the standard chow, except for the zinc content. Mice were acclimated to the new diet for 10 to 15 days prior to tumor cell implantation and continued on the assigned diet throughout the experiment. The amount of food per cage was measured before and after replenishing the feeder one to two times per week to estimate food intake per mouse and day on each diet.

CRISPR Drop out Screen

The pooled mouse metabolic-focused CRISPR KO library was a gift from Dr. Kivanc Birsoy (Addgene, catalog no. 160129). Fifteen million Yumm5.2 cells expressing sh*Cdkn2a* or control shGFP were infected with pooled CRISPR KO library to an MOI <0.25 to achieve ~500-fold library coverage after selection with 3 µg/mL puromycin. The cells were passaged every 2–3 days for two weeks, maintaining the full population, before implantation into mice. A total of 350,000 cells per mouse were injected into 36 mice per group, achieving a 550-fold library coverage. Mice were treated with either anti-Pd1 or IgG as described in the *Mice Treatments* section above. Once tumors reached 0.5 cm^3, they were harvested, and DNA was isolated using the Quick-DNA Miniprep Plus kit (Zymo Research, catalog no. D4069) following the manufacturer's instructions. DNA from each mouse in the same group was pooled and used for gRNA amplification. To ensure adequate gRNA representation, calculations from Wohlhieter et al.[174] were used to achieve

>450-fold coverage. In brief, 64 µg of genomic DNA per condition was divided into 16 PCR reactions, with 4 µg per reaction. The gRNA region was amplified using primers listed in **Table S7** and Ex Taq DNA polymerase (Takara, catalog no. RR001A) following the manufacturer's protocol. The PCR conditions were as follows: one cycle at 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 20 seconds, 63 °C for 30 seconds, and 72 °C for 45 seconds, concluding with a final extension step at 72 °C for 1 minute. The reaction was performed in a thermal cycler (Bio-Rad, T100™ Thermal Cycler, serial no. 621BR59583). The resulting PCR amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, catalog no. A9282) following the manufacturer's instructions and pooled. The cleaned amplicons were sequenced using a NextSeq high output 75 cycle kit 1x50 in an Illumina NextSeq 500 (Serial no. NB: 501446). Data was analyzed using the MAGeCK bioinformatics pipeline to identify negatively enriched genes (Li et al., 2014). **Fig. 1D** shows the Log_2 fold change of the negative CRISPR score of solute carrier transporter in YUMM5.2 sh*Cdkn2a* tumors in mice treated with anti-Pd-1 vs. IgG vs. shControl tumors in mice treated with anti-Pd-1 vs. IgG.

Isolation of plasma and tumor interstitial fluid (TIF)

Blood from melanoma patients treated with anti-PD1 immunotherapy was collected by venipuncture into ACD-A tubes (Becton-Dickinson, catalog no. 364606) as part of an IRB-approved biorepository protocol (HCC 20-2019, MOD20010266-016). Mouse blood was collected via lancet check bleed (Braintree Scientific, catalog no. GR 4MM) into EDTA tubes (SAI Infusion Technologies, catalog no. K3EDTA). Plasma was separated by centrifugation at 1500g for 10 minutes at 4°C and stored at -80°C for later analysis. After

bleeding, animals were euthanized by cervical dislocation, and tumors were harvested, weighed, rinsed briefly in saline (150 mM NaCl), and excess saline removed using filter paper (Cytiva, catalog no. 1001-813). TIF was isolated using a previously described centrifugal method[175]. Briefly, tumors were placed on a 20 µm strainer (pluriSelect, catalog no. 43-50020-03) within a sterile 50 mL conical tube (FisherbrandTM, catalog no. 06-443-19) and centrifuged at 11,000g for 10 minutes at 4°C. TIF was collected from the conical tube, rapidly frozen in liquid nitrogen, and stored at -80°C. TIF volumes ranged from 5 to 100 µL, <20% of the tumors did not yield any fluid. The interval from euthanasia to centrifugation was <2 minutes per mouse.

Quantification of metabolite levels

LC/MS was performed using a QExactive orbitrap mass spectrometer using an Ion Max source and heated electrospray ionization (HESI) probe coupled with a Dionex Ultimate 3000 UPLC system (Thermo Fisher). External mass calibration was set every 7 days and internal mass calibration (lock masses) was not used. The mass spectrometer was operated in full-scan, polarity switching mode with the spray voltage set to 3.0 kV, the heated capillary held at 275 °C, and the HESI probe held at 350 °C. Seath gas flow rate was set to 40 units, the auxiliary gas flow at 15 units, and the sweep gas flow at 1 unit. Data acquisition was performed in a range of 70 to1000 *m/z*, with resolution set to 70,000, the AGC target at 1e6, and the maximum injection time at 20 milliseconds.

A library of 149 chemical standards of plasma polar metabolites was built as we have previously described (see first paragraph of *Quantification of metabolite levels in TIF and plasma* from the methods section in Sullivan MR et al. article[175]). Polar metabolites were

extracted from 5 μL of each sample using 45 μL of a solvent mixture consisting of acetonitrile, methanol, and formic acid (75:25:0.1), which included isotopically labeled internal standards (ISTDs) listed in **Table S8**. Samples were vortexed at 4°C for 10 minutes and centrifuged at 15,000g for 10 minutes to separate the supernatant containing soluble polar metabolites. Aliquots of 2 μL of sample were injected onto a ZIC-pHILIC (2.1x150 mm) analytical column equipped with a guard column (2.1 x 20mm), both of 5 μm particle size (EMD Milipore, catalog no. 150454). Column was maintained at 25°C and the autosampler was set at 4°C. The mobile phase consisted of Buffer A (20 mM ammonium carbonate, 0.1% ammonium hydroxide), and Buffer B (acetonitrile). The chromatographic gradient was run at a flow rate of 0.150 mL/minute as follows: 0–20 min: linear gradient from 80% to 20% B; 20–20.5 min: linear gradient from 20% to 80% B; 20.5–28 min: hold at 80%. All solvents used in this protocol were LC/MS gradient.

Metabolite identification was performed using XCalibur 2.2 software (Thermo Fisher Scientific), applying a 5 ppm mass accuracy window and a 0.5-minute retention time window. External standard pools were used for metabolite peak identification based on *m/z* values and retention times. The limit of detection ranged from 100 nM to 3 μM, depending on the metabolite. For metabolite quantification, two methods were used: stable isotope dilution for metabolites with available ISTDs, and external standard calibration for those without Briefly, for stable isotope dilution quantification, peak areas of labeled ISTDs were compared to external standard pools to determine their concentration. The peak area of each unlabeled metabolite in the TIF and plasma samples was then compared to the quantified ISTDs to calculate metabolite concentrations. For metabolites without ISTDs,

quantification by external calibration involved normalizing the peak area of each analyte to that of a labeled ISTD to account for sample recovery differences. This normalization was applied to both biological samples and external standard pool dilutions. A standard curve was generated from the normalized peak areas, with linear fits typically above $r^2 = 0.95$. These curves were used to convert peak areas in TIF or plasma samples into metabolite concentrations. These results are considered semiquantitative due to matrix differences between external standards (prepared in water) and the samples. Please, refer to *Quantification of metabolite levels in TIF and plasma* from the methods section in Sullivan MR et al. article[175] for more details.

Harvest cells and conditioned media for ICP-MS and labile zinc quantification

For ICP-MS and labile zinc quantification, cells were seeded in 10 cm tissue culture dishes (Cellstar, catalog no. 82050-916) at densities of 500,000 and 300,000 cells per dish, respectively. Cells were seeded in complete media and incubated for 48 hours (refer to the *Cell Lines and Culture Conditions* section for details). After this initial incubation, cells were washed twice with DPBS, and the media was replaced with 6 mL of FBS-free DMEM and Ham's F-12 50/50 media (Corning, catalog no. 10-092-CV) and incubated for an additional 24 hours. Conditioned media was filtered through a 0.45 µm filter (Fisherbrand™, catalog no. 09-720-005), aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

For ICP-MS, attached cells were washed twice with ice-cold DPBS, scraped into 1 mL of 80% methanol (Fisher Scientific, catalog no. A456-4) over dry ice, snap-frozen in liquid

nitrogen, and stored at -80°C until analysis. For Labile Zinc Quantification, attached cells were washed twice with room-temperature DPBS, scraped into 1 mL of room-temperature DPBS, and immediately processed for staining. Six and three replicates per condition (for both cells and conditioned media) were harvested for ICP-MS and labile zinc quantification, respectively. In both cases, two additional plates were cultured in parallel to estimate the cell number at the end of the experiment, which was used to normalize zinc levels per cell. Cell counts were determined using a hemocytometer, by counting four squares in two independent aliquots taken from the same sample and averaging the results.

Quantification of metals by mass spectrometry

Human and Mouse Plasma and Mouse TIF were collected as described in the *Isolation of Plasma and Tumor Interstitial Fluid (TIF)* section. *In vitro* cells and conditioned media were collected as outlined in the *Harvesting Cells and Conditioned Media for ICP-MS and Labile Zinc Quantification* section. For cells collected in 80% methanol, the methanol was evaporated to dryness under nitrogen gas prior to further processing. All samples including methanol-evaporated cells, conditioned media, TIF, and plasma, were spiked with 159Tb and 89Y (High-Purity Standards, catalog nos. 1057-1 and 1067-1, respectively) as ISTDs to final concentrations of 0.2 ppb and 1 ppb, respectively. ISTDs were used to correct variations in sample preparation and to monitor fluctuations during the ICP-MS acquisition. Samples were digested with 250 µL of concentrated nitric acid (Fisher Chemical™, catalog no. A509P500) at 50°C for 16 hours. Following digestion, samples were diluted to a final volume of 5 mL with metal-free water (produced using a Milli-Q system, Millipore Sigma, catalog no. IQ7003). Multi-element metal standard calibration curves

were generated at the same time with each batch of samples, using a premixed metal ion stock solution (High-Purity Standards, catalog no. QCS-27-100), using the exact sample procedure as for the samples. The calibration curves used ½ serial dilutions ranging from 250 ppb to 0.12 ppb, and were spiked with same amount of ISTD as the samples. Quality controls were prepared by exactly same protocol as the samples, and were ran at the beginning and the end of the analysis of each batch, to verify measurement accuracy thorough the batch. The limit of quantification for each metal was determined for each batch, and was usually 0.12 ppb or 0.24 ppb, depending on the batch. All solvents used in the protocol were of LC/MS or TraceMetal grade. All samples, including controls, were prepared in 15 mL polypropylene tubes (Fisher Scientific; catalog no. 14-955-238).

Metal quantification was performed using an iCAP RQ ICP-MS (Thermo Scientific, serial no. ICAPRQ01408) equipped with a Cetac ASX-560 autosampler (Teledyne Cetac Technologies). The ICP-MS system was operated under constant argon plasma gas flow at 110 psi. Elemental detection was performed in kinetic energy discrimination (KED) mode using helium gas at 17 psi. Data for each sample were acquired in triplicate, and averaged values were reported.

In vitro labile Zinc quantification

Cells and conditioned media were collected as outlined in the *Harvesting Cells and Conditioned Media for ICP-MS and Labile Zinc Quantification* section. One million scraped cells were pelleted in a round-bottom 96-well plate (Corning, catalog no. 3366) by centrifugation at 350 g for 5 minutes at 4°C. Cells were then stained with 50 μM Zinpyr-1

(Cayman Chemical, catalog no. 15122) diluted in DPBS, containing a 1:1000 dilution of Live/Dead Fixable Near-IR Dead Cell Stain kit (Invitrogen, catalog no. L34976), in a final volume of 50 µL for 20 minutes at 4°C, protected from light. Following staining, cells were fixed in 4% paraformaldehyde (Sigma Aldrich, catalog no. 158127-3Kg) for 15 minutes at 4°C, shielded from light, and resuspended in FACS buffer (1x PBS, 10 mM HEPES [Gibco, catalog no. 15630080], 1% heat-inactivated FBS [Sigma Aldrich, catalog no. F4135-100ML], 0.05% sodium azide [Sigma Aldrich, catalog no. S2002-25G]). Stained cells were immediately analyzed on a CytoFlex Flow Cytometer (Beckman Coulter, serial no. 822338373). Flow cytometry data were analyzed with FlowJo version 10. The median fluorescence intensity (MFI) of single live cells was measured to assess intracellular zinc levels per sample.

Labile zinc concentration was measured as previously described[176]. Briefly, 50 µL of conditioned media was mixed with 50 µL of 50 µM Zinpyr-1 (Cayman Chemical, catalog no. 15122) in a 96-well polystyrene microplate (Greiner Bio-One, catalog no. 655101). The mixture was incubated for 20 minutes at 37°C in a CO_2 incubator. Fluorescence was then measured in a fluorescent plate reader (Promega GloMax explorer, serial no. 9720100141) with excitation at 475 nm and emission at 500-550 nm. This first fluorescence measurement reflects the signal of Zinpyr-1 bound to zinc in the sample (F). To determine the baseline fluorescence, 50 µL of 600 µM of the zinc chelator N,N,N',N'-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN, MilliporeSigma, catalog no. 6163941100MG), was added to each well. After another 20-minute incubation at 37°C in a CO_2 incubator, fluorescence was measured again, representing the minimum

fluorescence (F_{min}) due to the chelation of zinc. Finally, to obtain the maximum fluorescence (F_{max}), 50 µL of 2 mM $ZnSO_4$ (Sigma Aldrich, catalog no. Z2876-500ML) was added to each well. After a final 20-minute incubation at 37°C in a CO_2 incubator, the fluorescence was measured one last time. The zinc concentration in each sample was then calculated using the formula: Zn^{2+} concentration = $K_D \times ((F - Fmin) / (Fmax - F))$, where K_D is the dissociation constant of the zinc:Zinpyr-1 complex (0.7 nM)[176]. Finally, zinc concentrations were normalized to the number of cells in each sample at the time of harvesting.

Immunofluorescence (IF)

Approximately 30,000 to 50,000 cells were seeded onto coverslips in 12-well plates (Corning, catalog no. 3513) and incubated at 37°C with 5% CO_2. Becauwe Yumm1.7 cells have low attachment properties, these cells were seeded onto poly-L-ornithine-coated coverslips. Briefly, coverslips were first washed with 2 mL of 200 proof ethanol (Decon Labs) for 5 minutes, followed by two washes with molecular-grade water (Corning, catalog no. 46000CM). Then, 500 µL of poly-L-ornithine (Sigma Aldrich, catalog no. P4957-50mL) was added to each well containing a coverslip. The plates were sealed with parafilm (VWR, catalog no. 52858-032) to prevent evaporation, and incubated overnight at 37°C. Before seeding the cells, the coverslips were washed three times with 1 mL of molecular-grade water and air-dried for 10 minutes. Approximately 16 hours after seeding, the cells were fixed with 4% paraformaldehyde (Sigma Aldrich, catalog no. 158127-3Kg) for 10 minutes followed by permeabilized with 0.2% Triton X-100 (VWR, catalog no. 97062-208) for 5 minutes. For plasma membrane staining of SLC39A9, however, permeabilization

was omitted. The cells were then blocked with 5 µg/mL of Cd16/Cd32 (Invitrogen, catalog no. 14016185) diluted in 3% BSA/PBS (Fisher BioReagents, catalog no. BP1600) for 10 minutes. Both human and mouse cells were subsequently incubated with 10 µg/mL of anti-Slc39a9 (Thermo Scientific, catalog no. PA521074), diluted in 3% BSA/PBS for 1 hour. After incubation, the cells were washed three times with PBS and then incubated with 0.75 µg/mL of secondary anti-rabbit Cy3-conjugated antibody (Jackson ImmunoResearch, catalog no. 111-165-003), diluted in 3% BSA/PBS for 1 hour in the dark. Cells were then washed 10 times with PBS and permeabilized with 0.2% Triton X-100 for 5 minutes. Cells were stained with 0.15 µg/mL DAPI (diluted in PBS) for 1 minute. Finally, coverslips were mounted (Sigma Aldrich, catalog no. F6182-20ML) and sealed with transparent nail polish. Controls included staining with 10 µg/mL normal rabbit IgG (Cell Signaling, catalog no. 2729) and secondary-only staining.

For filipin staining cells were cultured on coverslips and fixed with 4% paraformaldehyde for 10 minutes. Cells were incubated with 0.1 mg/mL filipin III (Cayman Chemicals, catalog no. 70440) for 1h at room temperature. Coverslips were mounted and sealed with transparent nail polish. Of note that Yumm1.7 cells were seeded in media containing lipid-depleted FBS (Omega Scientific, catalog no. FB-50).

Five to ten images were captured per condition using an inverted microscope (Nikon ECLIPSE Ti2, serial no. 542740) equipped with a 20x/0.17 objective (Nikon DIC N2 Plan Apo), a digital camera (ORCA-Fusion, catalog no. C1440-20UP, serial no. 000670) and the NIS-Elements AR software. Image analysis was conducted using Python (v. 3.10).

SLC39A9 quantification was performed as follows: Nuclear staining images (DAPI) were normalized to the 3rd and 97th percentiles before segmentation, which was carried out using a pre-trained CellPose model ("cyto")[177]. Nuclei touching the image borders were excluded from further analysis. The mean Cy3 fluorescence signal (anti-SLC39A9) was quantified within the nuclear regions. Background signal was corrected by subtracting the median intensity of the entire image. Finally, the number of cells per condition was randomized, and 200 cells per condition were plotted as fold change relative to the control condition. Filipin quantification was performed as follows: Individual cells were defined as regions of interest (ROIs) and captured using image thresholding. Analysis was conducted using the 'Analyze Particles' tool in ImageJ (Fiji), excluding cells at the image periphery. Integrated Density values were determined for each ROI. A total of at least 100 cells per condition were analyzed, and data are presented as fold change relative to the control condition.

Methyl-β-cyclodextrin (MβCD) treatment

Cells seeded in 12-well plates (Corning, catalog no. 3513) for immunofluorescence (IF) or in 6 cm plates (Falcon, catalog no. 353002) for flow cytometry were treated with 10 mM MβCD (Sigma Aldrich, catalog no. C4555-10G) diluted in FBS-free DMEM and Ham's F-12 50/50 media (Corning, catalog no. 10-092-CV), supplemented with 10 mM HEPES (Gibco, catalog no. 15630080). The treatment was carried out for 1 hour at 37°C with 5% CO_2. After treatment, cells were collected for downstream analysis (i.e. IF or labile zinc quantification). Note that MβCD-treated cells are extremely loosely adhered, so washes

must be done gently. To prevent cell loss during the immunofluorescence staining procedure, fixation time was increased to 20 minutes.

Tumor disassociation

To obtain a single-cell suspension, dissected tumors were enzymatically and mechanically disrupted. First, the tumors were mechanically minced using surgical disposable scalpels (Surgical Design, catalog no. SCALPEL 12B) into pieces smaller than 5 mm in diameter on a 6 cm plate (Falcon, catalog no. 353002). Next, 2 mL of enzymatic cocktail [8 mg of collagenase IV (Gibco, catalog no. 17104-019), 800 µL of dispase (Stem Cell Technologies, catalog no. 07913), and 25 µg of DNase (Sigma Aldrich, catalog no. D4263-5VL) diluted in FBS-free MEM (Corning, catalog no. 10-009-CV)] was added to the minced tumors. The mixture was incubated for 20 minutes at 37°C in a non-CO_2 incubator. Minced tumor pieces were then further disrupted by smashing them between two fully frosted cytology slides (Fisherbrand, catalog no. 1255015). The resulting single-cell suspension was filtered through a 70 µm strainer (Fisherbrand, catalog no. 22-363-548) and resuspended in 5 mL of FBS-free media. The cells were centrifuged at 350 g for 5 minutes at 4°C and resuspended in 1x RBC lysis buffer (BD, catalog no. 555899). The RBC lysis reaction was incubated for 2–5 minutes at room temperature, after which the reaction was stopped by adding 5 mL of FBS-free media. The cells were then centrifuged at 350 g for 5 minutes at 4°C, resuspended in DPBS, and were ready for subsequent procedures.

Single-cell RNA-Sequencing

Yumm5.2 sh*Cdkn2a* hairpins #1 and #2, and control shGFP cells were subcutaneously implanted into five mice per condition, as outlined in the *Mice Models* section. Once reached a volume of 0.5 cm^3, tumors were harvested and processed into a single-cell suspension as described in the *Tumor cell disassociation* section. Single-cell suspensions were first stained with a 1:4000 dilution of viability dye (ThermoFisher Scientific, catalog no. 65-0863-14) in DPBS for 20 minutes on ice and in the dark. Then, cells were centrifuged at 350 g for 5 minutes at 4°C and stained with 2 µg/mL of anti-Cd45 PE-conjugated antibody (ThermoFisher Scientific, catalog no. 12-0451-83), diluted in EasySep™ buffer (Stemcell Technologies, catalog no. 20144) for 20 minutes on ice and protected from light. After centrifugation at 350 g for 5 minutes at 4°C, cells were resuspended and pooled by sample group in 5 mL of FBS-free MEM media (Corning, catalog no. 10-009-CV). Single live PE-positive (Cd45+) cells were sorted using a Sony MA900 cell sorter (serial no. 0714313) and collected in 0.04% BSA/PBS for downstream processing. cDNA Libraries were prepared at the University of Pittsburgh Single Cell Core Facility. Briefly, viability was confirmed with greater than 80% live cells using a Nexcelom automated cell counter (serial no. Auto2000-201-1019). Samples were then labeled with TotalSeq-C antibody hashtags (Biolgened, catalog no. 155881, 155883, 155885, 155887, 155889, 155891) following manufacturer's instructions. Equal numbers of cells from each sample were then pooled together and loaded onto the Chromium controller (serial no. CXVG11091) for single-cell capture and barcoding. cDNA library preparation was carried out using the Chromium Next GEM Single Cell 5' Reagent Kits v2 (10X Genomics, catalog no. PN-1000265) following the manufacturer's protocol. The sizes of the library pools were analyzed using a Standard Sensitivity NGS Fragment Analyzer Kit (Agilent) followed

by fluorescent quantification using the Promega QuantiFluor ONE dsDNA kit on the Infinite F Nano+ (Tecan). The library pools were normalized per manufacturer protocol (Illumina). Sequencing was performed on the NovaSeq 6000 platform (serial no. A00524), with 28bp/90bp paired-end reads. The sequencing data was demultiplexed with 10X Genomics Cell Ranger mkfastq version v6.0.2. The resulting sequence data was then aligned to the mouse reference genome (mm10) using cellranger multi (v.7.1.0) to generate count matrices for each condition. Analysis was conducted using R (v.4.3.0). Seurat's was used for quality control, data normalization, and data analysis. The data was normalized using the NormalizeData function, following which the top 2000 variable genes were chosen using the 'vst' parameter setting of the FindVariableFeatures function. The 6 data objects were merged and used for further analysis. Celltypes were annotated using ImmGen data and the SingleR package. Gene Set Enrichment Analysis (GSEA) was performed using ranked gene lists (log2foldchange * log10pval) using the GOBP_PHAGOCYTOSIS mouse gene set from MSigDB. Pathway signature scores for each cell were computed using the ssGSEA framework as implemented in the escape package. Raw and processed RNA-seq data can be found on Gene Expression Omnibus (GSE291530).

Immunostaining and flow cytometry

Tumors were harvested and processed into a single-cell suspension as described in the *Tumor disassociation* section. Note that for experiments where phagocytosis was assessed, dTOMATO-expressing cancer cells were implanted. Single-cell suspensions were first blocked with 5 µg/mL of Cd16/Cd32 (Invitrogen, catalog no. 14016185) diluted

in DPBS for 10 minutes on ice. Cells were stained for 20 minutes at 4°C protected from the light after which cells were fixed in 4% paraformaldehyde (Sigma Aldrich, catalog no. 158127-3Kg) for 15 minutes at 4°C, shielded from light, and resuspended in FACS buffer (1x PBS, 10 mM HEPES [Gibco, catalog no. 15630080], 1% heat-inactivated FBS [Sigma Aldrich, catalog no. F4135-100ML], 0.05% sodium azide [Sigma Aldrich, catalog no. S2002-25G]). Stained cells were immediately analyzed on a Fortessa (Beckman Coulter, serial no. H64717700020). FlowJo version 10 was used for further analysis. Antibodies and dyes used in this study include: 0.5 µg/mL of anti-Cd11b Brilliant Violet 421-conjugated antibody (Biolegend, catalog no. 101236), 4 µg/mL of anti-F4/80 APC-conjugated antibody (Biolegend, catalog no. 123116), 2 µg/mL of anti-Pd-l1 PE/Cyanine5-conjugated antibody (Biolgened, catalog no. 124344), 50 µM Zinpyr-1 (Cayman Chemical, catalog no. 15122). Antibodies were diluted in DPBS, containing a 1:1000 dilution of Live/Dead Fixable Near-IR Dead Cell Stain kit (Invitrogen, catalog no. L34976).

In vitro phagocytosis activity

Seventy-five thousand RAW 264.7 cells were seeded in 12-well plates (Corning, catalog no. 3513) containing 1.5 mL of regular media and incubated at 37°C and 5% CO_2. Seventy-two hours later cells were washed with PBS and media replaced for 1.5 mL of 200 µg/mL of pHrodo red E.coli particles (Invitrogen, catalog no. P35361) resuspended in media without FBS and incubated 1h at 37°C and 5% CO_2. Cells were scrapped out and stained with zinpry-1 and Live/Dead Fixable Near-IR Dead Cell Stain as described in *In vitro labile Zinc quantification* section above. Stained cells were immediately analyzed on a Fortessa (Beckman Coulter, serial no. H64717700020). Flow cytometry data were

analyzed with FlowJo version 10. The median fluorescence intensity (MFI) of single live cells was measured to assess the labile zinc content and the phagocytic activity per sample.

Patient scRNA-Seq data sets and processing

The Jereby-Arnon 2018 melanoma dataset[178] and the Chen 2020 Head and Neck dataset[179] were downloaded from the Gene Expression Omnibus (GSE115978 and GSE150430, respectively). For sample processing, genes were retained if expressed in at least three cells, cells were kept if they had more than 200 features, and cells with >20% mitochondrial reads were removed. Library size bias was corrected using Seurat's NormalizeData, scaling expression values to 10,000 counts per cell and log-transforming the data. Data was then scaled using Seurat's ScaleData with all genes as features. Cancer cells and macrophages were annotated based on publicly available cell type labels. Tumor samples were then stratified into *CDKN2A*^{High} and *CDKN2A*^{Low} groups based on *CDKN2A* median expression in the cancer cell compartment. Signature scores were calculated as the mean expression of genes in the zinc signature and phagocytosis signature annotated in this paper (**Table S4**) for each cell in the macrophage or cancer cell compartment.

Western Blotting

Cell lysates were collected in 1X sample buffer [2% SDS (Millipore Sigma, catalog no. 75746-1Kg), 10% glycerol (Sigma Aldrich, catalog no. G5516), 0.01% bromophenol blue (Millipore Sigma, catalog no. BX1410), 62.5 mM Tris (Fisher Scientific, catalog no.

AAJ65594A1), pH=6.8 and 0.1 M DTT (Thermo Fisher, catalog no. 15508-013)] and boiled at 95° C for 10 minutes. Protein concentration was determined using Bradford assay (Bio-Rad, catalog no. 5000006) following manufacturer's instructions. Proteins were resolved using SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, catalog no. 10600001) as previously described[4]. Antibodies used include: anti human p16 (Abcam, catalog no. ab108349, working concentration 1.5 µg/mL), mouse anti-p16 (Cell Signaling Technology, catalog no. 29271S, working concentration 1 µg/mL), anti-p19 (Abcam, catalog no. ab80, working concentration 1 µg/mL), anti-β-Actin (Sigma-Aldrich, catalog no. A1978, working concentration 1 µg/mL), anti-mouse HRP (Cell Signaling Technology, catalog no. 7076, working concentration 0.1 µg/mL), and anti-rabbit HRP (Cell Signaling Technology, catalog no. 7074, , working concentration 0.2 µg/mL).

Bulk RNA-Seq and RT-qPCR

Total RNA was extracted from cells with Trizol (Ambion, catalog no. 15596018) and DNase treated, cleaned, and concentrated using Zymo columns (Zymo Research, catalog no. R1013) following the manufacturer's instructions. Optical density values for RNA were measured using NanoDrop One (Thermo Scientific) to confirm an A260 and A280 ratios above 1.9. For bulk RNA-Seq, RNA integrity number (RIN) was measured using BioAnalyzer (Agilent Technologies; RRID:SCR_019715) RNA 6000 Nano Kit to confirm RIN above 7 for each sample. The cDNA libraries, next-generation sequencing, and bioinformatics analysis was performed by Novogene. Differential expression analysis was done using DESeq (v2_1.6.3).

For RT-qPCR, a total of 2 µg of RNA was reverse-transcribed to cDNA (Applied Biosystems, catalog no. A25742) following manufacturer's instructions in a thermal cycler (Bio-Rad, T100™ Thermal Cycler, serial no. 621BR59583). Relative expression of target genes was analyzed using the Real-Time PCR System (Bio-Rad, CFX Connect, serial no. 788BR09362) with clear 96-well plates (Greiner Bio-One, catalog no. 652240). Primers were designed using the Integrated DNA Technologies (IDT) web tool (**Table S7**). Conditions for amplification were: 5 min at 95° C, 40 cycles of 10 sec at 95° C and 7 sec at 62° C. The assay ended with a melting curve program: 15 sec at 95° C, 1 min at 70° C, then ramping to 95° C while continuously monitoring fluorescence. Each sample was assessed in triplicate. Relative quantification was determined to multiple reference genes (Rplp0, Gusb, and Rp132) to ensure reproducibility using the delta-delta CT method.

Senescence and proliferation assays

SA-β-Gal staining was performed as previously described[180]. Cells were fixed in a mixture of 2% formaldehyde (Fisher Scientific, catalog no. BP531-500) and 0.2% glutaraldehyde (Fisher Scientific, catalog no. 01909-100) diluted in PBS for 5 minutes, followed by staining with a staining cocktail containing: 40 mM Na_2HPO_4 (Sigma Aldrich, catalog no. 567547), 150 mM NaCl (Sigma Aldrich, catalog no. S5886), 2 mM $MgCl_2$ (Sigma Aldrich, catalog no. M1028), 5Mm $K_3Fe(CN)_6$ (Sigma Aldrich, catalog no. 244023), 5 mM $K_4Fe(CN)_6$ (Sigma Aldrich, catalog no. 455989), and 1 mg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma Aldrich, catalog no. B4252). Staining was incubated overnight at 37° C in a non-CO2 incubator after which reaction was stopped with tap water.

Images were acquired immediately upon reaction stopped using an inverted microscope (Nikon Eclipse Ts2, serial no. 139045) with a 20X/0.40 objective (Nikon LWD) equipped with a camera (Nikon DS-Fi3). Each sample was assessed in triplicate and at least 100 cells per well were counted (>300 cells per experiment). For colony formation, an equal number of cells were seeded in 12-well plates and cultured for an additional 1-2 weeks. Colony formation was visualized by fixing cells in 1% paraformaldehyde for 5 minutes followed by staining with 0.05% crystal violet for 20 minutes on rocking. Wells were de-stained in 500mL 10% acetic acid for 10 minutes on rocking and absorbance (590 nm) measured using a spectrophotometer (Promega GloMax explorer, serial no. 9720100141). Each sample was assessed in triplicate.

Qualification and Statistical analysis

GraphPad Prism version 10.0 was used to perform statistical analysis. The level of significance between two groups was assessed with pared or unpaired Student's *t* test as necessary and indicated in figure caption. For data sets with more than two groups, one-way ANOVA followed by Šidák correction for multiple comparisons was applied. For longitudinal tumor growth studies mixed-effects model was used and Šidák correction for multiple comparisons was applied. For survival analysis log-rank (Mantel-Cox) test was used. Pearson correlation was used to assess correlation. P-values <0.05 were considered significant. Specific statistical details used in scRNA-Seq and bulk RNA-Seq data are included in the corresponding section.

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