nature portfolio

Peer Review File

Tumour-intrinsic endomembrane trafficking by ARF6 shapes an immunosuppressive microenvironment that drives melanomagenesis and response to checkpoint blockade therapy

Editorial note: Parts of this Peer Review File have been redacted as indicated to remove third-party material where no permission to publish could be obtained.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author): with expertise in ARF6, cancer

In this manuscript, the authors showed that Arf6 is involved in promoting immune evasion in melanoma and that melanoma patients with high Arf6 levels are sensitive to ICB treatment, while melanoma patients with low Arf6 levels do not respond well to ICB treatment. This reviewer felt that the manuscript was excellent in that it included many multifaceted analyses and provided many new insights into Arf6 function. On the other hand, however, it may be difficult to accept the main statements in this manuscript as they are, due to several concerns discribed below.

(1) Related to Figure 4B: Arf6 mRNA is known to have a G-quadruplex structure and its protein level is mainly regulated by mRNA translational control. Therefore, the protein level of Arf6 should be examined by immunostaining or other methods, and the mRNA level as shown in Fig. 4B may not be meaningful.

(2) The possibility that a loop structure exists in which IFNgR activates Arf6 and activated Arf6 promotes IFNgR recycling may be one of the key points in this manuscript. On the other hand, multiple GEFs may activate Arf6, and this manuscript showed that ACAP1 and ARAP2 are highly expressed in melanomas. However, it is not clarified whether these GEFs function downstream of IFNgR and activate Arf6 upon IFNg stimulation. Without such results, the logic of this paper may be very weak.

3) Fig. 7 states that Arf6 high is an "ICB responder." However, given the reality of ICB treatment, such a too simple statement is rather misleading.

4) With respect to immune evasion of cancer, it has already been shown that Arf6 promotes PD-L1 recycling. This is not mentioned at all in this manuscript.

5) Arf6 may be involved in the recycling of such as transferrin R and carbonic anhydrase 9. Thus, in addition to immune evasion, several basic cellular functions, as well as metabolism, may be impaired in Arf6 f/f cells. However, these points are not mentioned at all in this manuscript, and the logic described in this manuscript seems somewhat one-sided.

Reviewer #2 (Remarks to the Author): with expertise in cancer immunology, melanoma

Wee et. al. investigate the impact of ARF6, a target they have extensively studied previously in a different context, on tumor-immune interactions in the context of a genetically engineered Braf-activated, Cdkn2a-deleted murine model of melanoma. They identify that genetic loss of ARF6 reduced tumor incidence and size. In contrast, prior studies had suggested that expression of constitutively active ARF6 enhanced tumor metastasis without changing primary tumor growth. Characterizing the TME of ARF6-deleted and constitutively active tumors, the group found increased cytokine signaling in ARF6-null tumors and increased CD8 T cell clustering. Flow cytometry showed reduced immune infiltration in ARF-deleted tumors and no difference in CD8 T cell infiltration. However, ARF6 -null tumors had increased IFNg+ and GZMB+ CD8+ T cells and decreased FOXP3+ T regs. scRNAseq was performed which identified increased MDSC in ARF6 WT tumors compared with ARF6-null tumors. The group then tested ICB in mice with palpable and pre-palpable tumors and identified a relatively increased response in ARF6 WT tumors compared with ARF6-null tumors. They hypothesized that this is due to ARF6 control of IFNGR at the plasma membrane and show that, indeed, modulating ARF6 can affect IFNGR surface expression and signaling. They validated this finding across a range of human tumor cell lines. They also performed correlative analysis suggesting that GAP/GEF levels that are expected to correlate with ARF6 function suggest that a lower ARF6 level may correlate with reduced response to ICB.

Overall this is an intriguing story that adds to the complex literature surrounding the regulation and impact of IFNGR signaling in tumor immunity. Strengths include the use of genetically engineered mouse models to study tumorigenesis as well as ICB response, an extensive amount of data and an interesting mechanism that is at least partly corroborated in human tumor cells. Although IFNGR signaling is a longstanding and critical regulator of

anti-tumor immunity, as highlighted decades ago by Bob Schrieber (and noted by the authors), its role has since been complicated by studies of IFNg-mediated resistance to immunotherapy with the argument that chronic IFNg signaling may both sensitize and drive resistance to immunotherapy depending upon the context (e.g. Benci et al, Cell, 2016 and 2019 from Andy Minn's lab). Viewed in this context, a relative challenge in this study is the extensive reliance on a single mouse model and incomplete characterization of the mechanisms of purported IFNGR-mediated resistance beyond PD-L1. Notably the finding that ARF6 regulates IFNGR levels is recapitulated in human cell lines, which is helpful, and there is an effort made to identify potential correlative biology in human sequencing datasets. However, the cell line models fail to recapitulate the interactive complexity of the TME and the human models are correlative and limited by the degree to which the indirect inference of ARF6 activity can be relied upon. Overall, the conclusions about ARF6 regulation of IFNGR expression seem stronger than the conclusions about the role this has in ICB and anti-tumor immunity. In particular, I worry that the increased growth of ARF6-null tumors may be due to something other than IFNGR regulation and the 'adaptive resistance' mechanism proposed. Experiments in immunodeficient mice (IFNGR knockout, NSG, SCID or others) would help to address this concern. That said, in my opinion, the strengths of this work outweigh the weaknesses, which are, in any case, common to the field. I make a few suggestions of experiments that could potentially further elucidate the biology.

In particular, I wonder if the mechanistic depth and impact could be increased by:
- IFNGR blocking or knockout experiments. IFNGR expression is shown to be altered on ARF6-modulated tumors. However, The T cells in ARF6 knockout tumors were also making more IFNg. Furthermore, IFNg signatures tend to go up in successful anti-tumor immune responses, even if the mechanism is not direct modulation of IFNg signaling. Thus, it would be helpful in my mind to establish a direct role for IFNg signaling in dictating the tumor immune response rather than assuming that this is the driver of the phenotype.
- It could be useful to perform further immune inhibitory ligand profiling, focusing on those that have previously been identified as upregulated following chronic IFNg stimulation (e.g. Benci et al, Cell, 2016). PD-L1, CD80 and IDO1 are discussed, but are not the only IFNg-mediated regulators of resistance proposed (e.g. Gal9, CD155, etc).
- For the PD-1 treatment experiments: The relevance of PD-1 treatment experiments is

increased by treating after tumors become palpable instead of prior to tumor onset. I see this was done in Supplemental Figure 4 for the WT but is not compared to the ARF6-deleted setting. Also, why is the ARF6-deleted experiment carried out to 90 days, whereas the ARF6 WT is stopped at d75 (when mice appear to be actively continuing to reach endpoint)? These data seem somewhat less convincing to me than some of the prior points made.
- I think that I am less confident than the authors in some of the correlative inferences they make in the RNAseq data, particularly when concluding that gene expression values of particular GAPs or GEFs will reliably predict ARF6 function or immune infiltration. As an example, CYTH4 is discussed in several sentences and concluded to potentially reflect tumor infiltrating immune cells. In this case, is CYTH4 correlated with inferred immune infiltration (e.g. via CIBERSORT or another approach)? How specific is ACAP1 for ARF6? - A quick look suggests that it may act in cargo sorting in other contexts. I wonder if it possible to place a bit less weight on these inferences in the story.
- Given that IFNg-signaling has been proposed as a mechanism of resistance not only to endogenous antitumor immunity but to ICB, I wonder how the authors think about the situations in which targeting ARF6 would be desirable.
- For cell cluster identification in scRNAseq experiments it would be very helpful to include the gene lists / marker genes that help to identify 'naive-like' T cells or other types. The methods say that this was done using SingleR and ProjectTILs, but both of these algorithms are subject to error and should be cross-checked with intuitive markers that are included in publication, in my opinion.
- What were the sizes/weights of tumors at the time of TME analysis? Was there a significant size difference (which can contribute to changes in immune populations)?

Reviewer #3 (Remarks to the Author): with expertise in cancer immunology

This manuscript sought to examine how endomembrane trafficking machinery involving ARF6 affects TME. To address this question, the authors used a conditional knock-out mouse and human cell lines and concluded that the downregulation of ARF6 results in resistance to immune checkpoint blockade therapy, attributable to its involvement in modulating the expression of the IFNγ receptor. This study addresses interesting findings in immunotherapy. However, the manuscript seems to have several conflicting data, and the

authors should address the following concerns:

1. The authors are linking these results to antitumor immunity. However, the provided data are insufficient. The authors should compare in vitro cellular proliferation or tumor growth in immunodeficient mice between Arf6 wild-type (WT) and Arf6-knock-out (KO) cancer cells. In Figure 3H, the authors depleted CD8+ T cells in Arf6-KO models without tumor growth data. However, CD8+ T-cell depletion generally enhanced tumor growth and shorten survival. Therefore, the authors should provide tumor growth and survival in Arf6-WT models in addition to those in Arf6-KO models.

2. In Fig. 2C, the authors conclude that apoptosis is enhanced in ARF6-KO tumors. However, since the expression of MHC-I and IFNγR is reduced in ARF6-KO tumors, it is difficult to understand these data and I think that these data could be conflicting. The IFNɤ signaling pathways could be lost and CD8+ T cells difficulty recognizing cancer cells due to low expression of MHC.

3. In Fig. 3B, the authors show that the ratio of CD8+ IFNγ+ T cells and CD8+ GzmB+ T cells increases. However, similar to the second comment, it is questionable that CD8+ T cells are activated in tumors that hardly express MHC-I. Looking at the plot on the right side of Fig. S5B, there are many ARF6-KO tumor cells in which MHC-I expression does not increase even after IFNγ stimulation. In contrast, IFNγ stimulation is sufficient to increase MHC-I expression in ARF6-WT tumor cells. Despite this difference, can you experimentally explain the mechanism by which CD8+ T cells are activated in ARF-KO with low MHC-I expression? Particularly, several recent studies emphasized the importance of MHC expression among the IFNɤ signaling pathways in antitumor immunity (doi: 10.1158/2326-6066.CIR-22-0815.).

4. Also, regarding CD8+ T cell activation, such as the ratio of CD8+ IFNγ+ T cells and CD8+ GzmB+ T cells, it would be better to show whether the data evaluated in four groups, not only the treated with Isotype control group but also the treated with Anti-PD-1 group in ARF6-WT and ARF6-KO tumors, is consistent with the authors' theory.

5. The authors should analyze chemokine expression as IFNɤ signaling pathways. Single-cell

sequencing data could be helpful.

6. In Figures 2B and 5A, these photos are a little difficult to understand.

Reviewer #4 (Remarks to the Author): with expertise in skin cancer, IFNs

The paper presents some interesting and potentially impactful findings related to the role of ARF6 in remodeling the tumor microenvironment and regulating response to immunotherapy. This study reveals the importance of ARF6 mediated endomembrane trafficking to impose immunosuppressive tumor microenvironment (TME) to accelerate tumor development. This ARF6 dependent TME makes it vulnerable to immune checkpoint inhibitor blockade therapy.

The work is significant, original, and supports the conclusions. The methodology is sound and provides enough details. Here are some suggestions:

1. The introduction, though informative, is very lengthy. The background can be shortened to couple of paragraphs on what is known in the field and focus on the gaps in knowledge that need to be addressed.
2. Figure 3 and supplement can be split into two for clarity of multilineage immune cell data.
3. The authors have looked at ARF6 mediated mechanisms in this study. Did they look at other ARFs that have been reported in cancer? If so, what was their expression?
4. There was low expression of ARF6 expression in ARF6fl/fl tumors but the expression of ARF1 remained intact. The effect of ARF1 in ARF6fl/fl mice is not clear.
5. The authors found that immune checkpoint blockage was effective in tumors that expressed PD-L1 by ARF6 dependent mechanism. Can this mechanism be applied to “cold tumors” that do not express PD-L1?
6. The limitation and future directions section can be further elaborated.

REVIEWER COMMENTS and AUTHOR RESPONSES

Reviewer #1 (Remarks to the Author): with expertise in ARF6, cancer

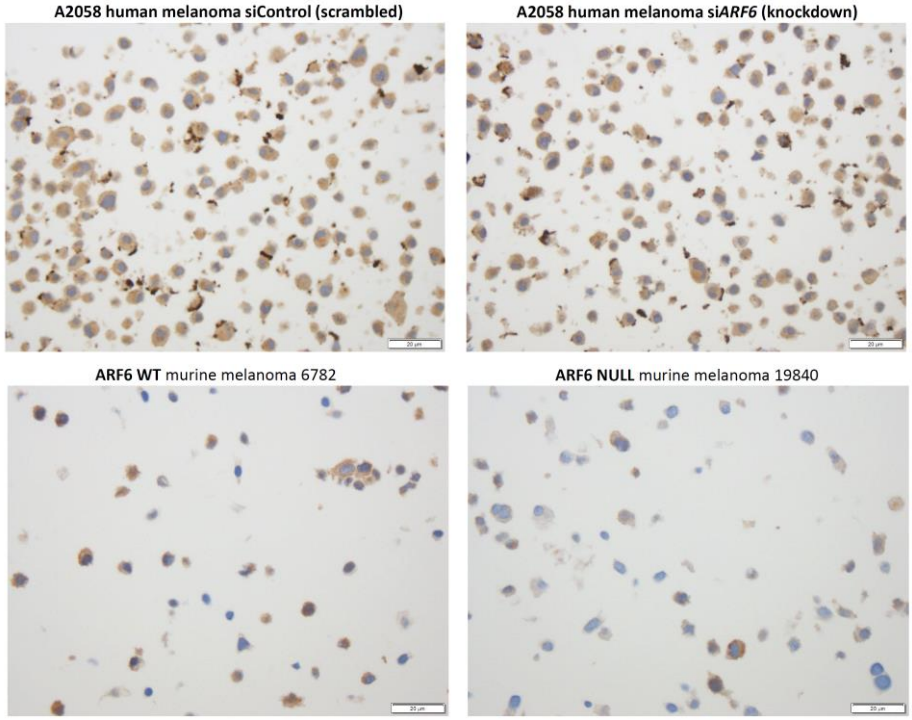
In this manuscript, the authors showed that Arf6 is involved in promoting immune evasion in melanoma and that melanoma patients with high Arf6 levels are sensitive to ICB treatment, while melanoma patients with low Arf6 levels do not respond well to ICB treatment. This reviewer felt that the manuscript was excellent in that it included many multifaceted analyses and provided many new insights into Arf6 function. On the other hand, however, it may be difficult to accept the main statements in this manuscript as they are, due to several concerns described below.

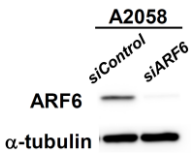
(1) Related to Figure 4B: Arf6 mRNA is known to have a G-quadruplex structure and its protein level is mainly regulated by mRNA translational control. Therefore, the protein level of Arf6 should be examined by immunostaining or other methods, and the mRNA level as shown in Fig. 4B may not be meaningful.

Author response: We agree, and we would prefer to perform ARF6 immunohistochemistry (IHC) on the clinically archived melanoma specimens, however ARF6 IHC is not feasible because 1) all available antibodies tested are nonspecific and 2) lack of access to the clinically archived specimens:

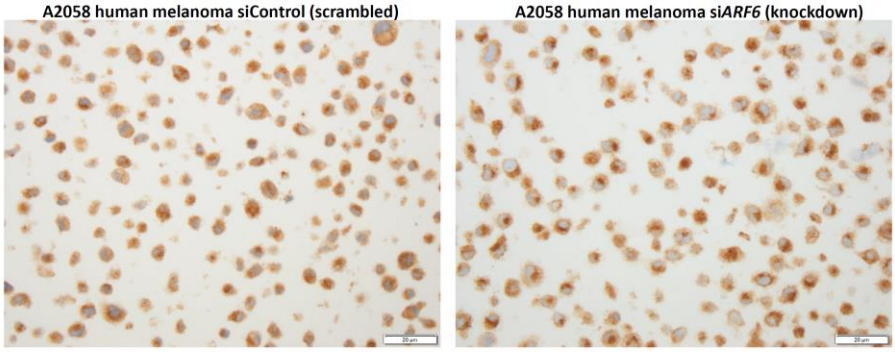
1) The available ARF6 antibodies for IHC (from vendors Santa Cruz and Aviva) show nonspecific staining. See images below and summary table below of all conditions we tested with both available antibodies. All other antibodies reported to be validated for IHC have been discontinued and are no longer available.

Aviva antibody (ARP72395_P050, rabbit polyclonal, anti-human raised against a synthetic peptide of the C-terminus of human ARF6, which is 100% homologous to murine ARF6 amino acid sequence). Nonspecific staining was seen for both human and murine samples.





Santa Cruz antibody (sc-7971, murine monoclonal, raised against amino acids 1-174 representing full length of ARF6 of human origin). Nonspecific staining seen.



ARF6 IHC Antibodies Tested and Conditions Used					
Vendor	**Catalogue No.**	**Tissue**	**Antigen Retrieval Condition**	**Antibody Dilution**	**Primary Antibody Incubation (mm:ss)**
Santa Cruz Biotechnology	sc-7971	Human Melanoma Cell Line, A2058 *siCtrl* and A2058 *siArf6* Cell Pellets	pH 6.0	1:50 1:100 1:150 1:500	15:00
			pH 9.0	1:50 1:100 1:150 1:500	
Aviva Systems Biology	ARP72395_P050	Human Melanoma Cell Line, A2058 *siCtrl* and A2058 *siArf6* Cell Pellets	pH 6.0	1:50 1:100 1:200 1:500	15:00
				1:100	60:00
			pH 9.0	1:50 1:100 1:200 1:500	15:00
				1:100	60:00
		1° Murine Melanoma Cell Lines, $ARF6^{WT}$ and $ARF6^{NULL}$ Cell Pellets	pH 6.0	1:50 1:100 1:500	15:00
				1:100	60:00
			pH 9.0	1:50 1:100 1:500	15:00
				1:100	60:00

2) Even if there was a sensitive and specific ARF6 antibody for *quantitative* IHC, neither the primary melanoma samples from the Leeds Melanoma Cohort (Figure 1 in our manuscript), nor the melanoma samples queried via Cancer-Immu analysis (Figure 4 in our manuscript) are available for testing. While primary melanomas are routine samples in clinical archives, those with annotated overall survival outcome data are extremely limited and challenging to access. It took months for the Leeds group to respond and to execute a data access and usage agreement for their unique sample set, and they have not made the FFPE blocks available for other testing. The melanoma transcriptomes queried with Cancer-Immu represent non-standard of care biopsies, procured under IRBs specifically for unique clinical trials (> 10 clinical trials). These tissues were likely depleted for the transcriptomes and even if they were not, it is not realistic to expect the multiple investigators involved in those studies to yield unstained slides for one IHC stain by one investigator.

Please note that the corresponding author, Dr. Allie Grossmann, is expertly positioned to evaluate the feasibility and quality of the IHC testing requested. Dr. Grossmann is a licensed and board-certified Anatomic and Molecular pathologist (American Board of Pathology). As a medical director for 11 years at a national reference clinical laboratory owned by the University of Utah (ARUP Laboratories), Dr. Grossmann has been involved in numerous IHC validations and has extensive experience interpreting IHC stains, including quantitative, US FDA-approved, companion diagnostic assays. In addition, she routinely evaluates proposed clinical specimen use protocols at the University of Utah for the Clinical Trials Feasibility Administrative Review Committee, Huntsman Cancer Institute. Nationally, she is a member of the Immuno-MATCH Biomarkers and Specimen Management Working Group, who oversees the use of IHC and molecular biomarkers in a new precision medicine clinical trial platform being piloted by the US National Cancer Institute.

(2) The possibility that a loop structure exists in which IFNgR activates Arf6 and activated Arf6 promotes IFNgR recycling may be one of the key points in this manuscript. On the other hand, multiple GEFs may activate Arf6, and this manuscript showed that ACAP1 and ARAP2 are highly expressed in melanomas. However, it is not clarified whether these GEFs function downstream of IFNgR and activate Arf6 upon IFNg stimulation. Without such results, the logic of this paper may be very weak.

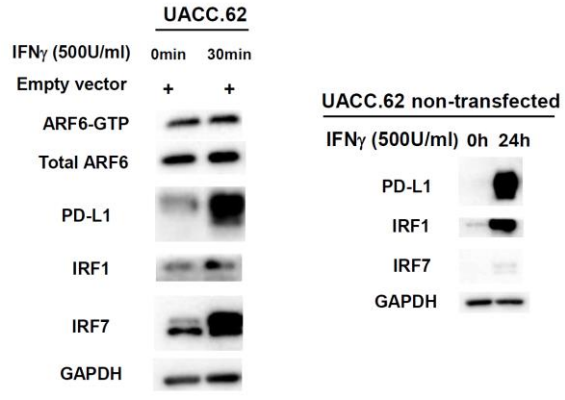
Author response: Thank you for the suggestion and we agree that the ARF6 GEF data could be of key interest to the readership. Importantly, the foundation of our story rests with ARF6 and the ARF6 logic is based on extensive data, both human and murine. Our main conclusion – that ARF6 exerts rheostatic control over IFNγ-driven AIR (based on testing ARF6 directly) - *is independent of ARF6 GEFs (and GAPs)*. Evidence for an IFNγR-ARF6 positive feedback loop is derived from orthogonal, concordant experiments, irrespective of identifying a specific GEF involved – and – ARF6 expression correlated with immunotherapy outcome irrespective of identifying the GEF that mediates ARF6 activation downstream of IFNγ stimulation.

To clarify our specific GEF findings relevant to IFNγ-signaling, we identified the GEF, cytohesin-1 (*CYTH1*), in the human data sets pertaining to IFNγ-driven adaptive immune resistance/response to immunotherapy (Figure 4 of our manuscript). The *CYTH1* data was analyzed independent of ARF6 and was included as a positive finding that enriches the story by raising the possibility of dysregulation of small GTPase activity in cancer by aberrant expression of GEFs. Importantly, knockdown of *CYTH1* reduced ARF6 activation in human melanoma cells cultured in full serum (see new Figure S1D). To the best of our knowledge, control of ARF6 activation by cytohesin-1, in cells, has never been shown previously. Due to technical limitations (described below), we are unable to test if knockdown or overexpression of *CYTH1* impacts IFNγ-induced ARF6 activation. Thus, we have moved all the *CYTH1* data to supplemental figure section (Figures S1C-D).

In contrast to cytohesin-1, ACAP1 is an ARF6 GAP. ACAP1 expression is *variable* in untreated primary melanomas and correlates with overall survival. We did not observe any correlations for the GAP *ARAP2* (although we certainly looked, Tables S1 and S3 of our manuscript). While *ACAP1* expression correlated with survival after immunotherapy (Figure S4), as a prognostic marker (independent of treatment) in primary and metastatic melanomas, we did not claim that ACAP1 expression was linked to ARF6 function in the IFNγ pathway. Rather, the point of the *ACAP1* data is that it implicates ARF6 in primary melanoma progression, and we followed this by testing ARF6 directly (Figure 1 in the manuscript). There are numerous ligand-receptor pathways reported to contribute to melanoma progression. These include pathways that activate ARF6, such as HGF-MET (Tague, SE et al. 2004 Jun 29; 101(26): 9671–9676) and WNT5A-FZD4 (Grossmann AH et al. 2013 Science Signaling). Hence, ACAP1 may play a functional role in regulating ARF6 in one or more of these pathways during melanoma progression. In our revised submission, we added "Purified ACAP1 has been reported to have selective GAP activity for ARF6 over ARF1 or ARF5. Interestingly, *ACAP1* is prognostic in both primary (Leeds cohort, Figure 1B) and metastatic (TCGA cohort) tumours, and ectopic expression of ACAP1 inactivated ARF6 (reduced ARF6-GTP level) in human cutaneous melanoma cells (Figure 1C), consistent with previous studies in other cell lineages. Overall, these data suggest that variable expression of ACAP1 in primary melanoma (Figure 1B) impacts the activation level of ARF6 and can influence both primary and metastatic disease progression. "

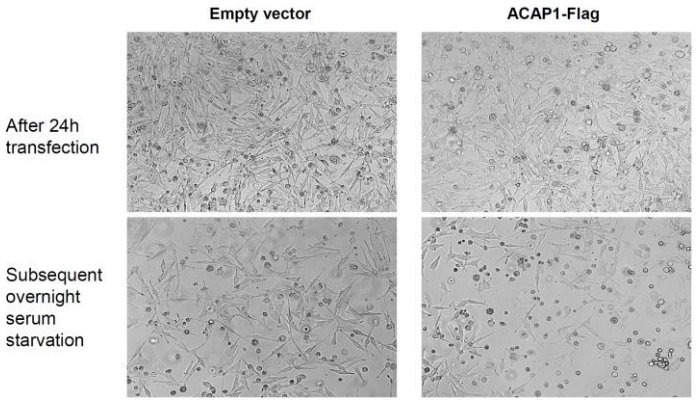
In response to the suggestion to evaluate ARF6 GEFs downstream of IFNγ-IFNγR, we are limited in our ability to test cytohesin-1 (*CYTH1*) and the GAP, ACAP1, in IFNγ-mediated activation of ARF6 due to the following **technical limitations**:

I. In attempting to express ectopic cytohesin-1 or ACAP1, we found that unlike non-transfected cells (blots on the right), transfection of the empty vector plasmid (blots on the left) activates Interferon signaling (PD-L1 and IRF1 expression was induced without IFNγ ligand; IRF7 expression was induced without IFNα ligand). More importantly, unlike non-transfected cells (Figure 6I of our manuscript), transfection with nucleic acid (empty vector) activates ARF6 in the absence of ligand (left blot, left column, high basal ARF6-GTP). Interferon activation in response to foreign nucleic acid is, of course, a well-known biologic response (to pathogens), and we now know this foreign-DNA induced IFN signaling is associated with ARF6

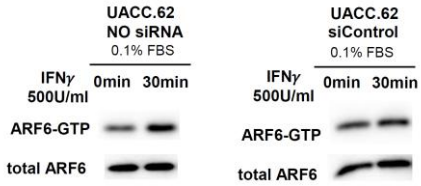


activation. While this result is interesting and consistent with our observations that IFNγ activates ARF6 (Figure 6I), specific to this suggestion, we are not able to ascertain if ectopic expression of cytohesin-1 or ACAP1 is sufficient to alter IFNγ-induced ARF6-GTP levels because ligand-independent ARF6-GTP levels are artifactually high after plasmid transfection.

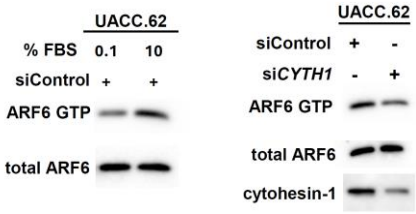
II. Unlike experiments conducted in serum or with purified growth factor ligands, acute IFNγ stimulation *in vitro* has cytostatic and cytotoxic effects on mammalian cells. The serum starvation required to detect IFNγ signaling (change in phospho-JAK level) is an additional insult to the cells. Adding a third insult, plasmid transfection, causes cells to detach and die. While we are able to generate lysates from the residual attached cells, there is significant artifact in the system from the serial experimental manipulations (nucleic acid transfection → serum starvation → IFNγ).



Lastly, serum starvation followed by IFNγ stimulation is also problematic for cells transfected with siRNAs. As shown in Figure 6I and below (left blots), IFNγ robustly and reliably activates ARF6 after serum starvation. In contrast, liposomal-based transfection of control (scrambled) siRNA introduces artifact in the form of marginal increases in ARF6-GTP (activation) after IFNγ (right blots). Thus, we are unable to test IFNγ-induced ARF6 activation with siRNAs in the system.



Note that testing siRNAs in full serum is not limited by this artifact – the ARF6-GTP level markedly rises with serum after siRNA transfection (below, left blot). Unlike the IFNγ treatment after serum starvation, there are no issues testing a role for cytohesin-1 in ARF6 activation when the cells are incubated in full serum (below, right blot).



3) Fig. 7 states that Arf6 high is an "ICB responder." However, given the reality of ICB treatment, such a too simple statement is rather misleading.

Author response: Thank you for the feedback. To address this, we have added the adjective "relatively" in the description of ARF6 high and low in the Figure 7 legend, which is accurate based on the murine and human data and avoids over-simplification. The ARF6 high and low status is illustrated in a way that directly links it to tumor-intrinsic IFNγ AIR – the target of immunotherapy. Note that the schematic does portrays ARF6 high/low status as specifically related IFNγ -AIR, as our data reflects.

4) With respect to immune evasion of cancer, it has already been shown that Arf6 promotes PD-L1 recycling. This is not mentioned at all in this manuscript.
Author response: In our original submission we did include this specific published observation from the Sabe Lab. We cited Hashimoto, S. *et al.* 2019 *PNAS* **116**, 17450-17459, in the results section, where we stated "Although ARF6 could potentially control the trafficking of PD-L1, *Cd274* expression after IFNγ exposure was ARF6-dependent (Figure 5D)." We are unable to confirm a role for ARF6 in PD-L1 trafficking in melanoma, NSCLC, CRC and TNBC, because any manipulation of ARF6 affected IFNγR levels in these cancer cells, which impacts IFNγ-induction of *CD274* mRNA. It is interesting that Hashimoto S. *et al*. did not observe any change in total, IFNγ-induced PD-L1 protein (by Western blot) after *ARF6* knockdown (Figure S5A in Hashimoto et al. 2019) in the pancreatic cancer cell line tested (MIAPaCa-2). We are curious whether this is true in other pancreatic adenocarcinoma cell lines.

5) Arf6 may be involved in the recycling of such as transferrin R and carbonic anhydrase 9. Thus, in addition to immune evasion, several basic cellular functions, as well as metabolism, may be impaired in Arf6 f/f cells. However, these points are not mentioned at all in this manuscript, and the logic described in this manuscript seems somewhat one-sided.
Author response: We agree that there may be other mechanisms of ARF6-mediated melanoma progression. Please note, in our original submission, we stated in the abstract "Mechanistically, these phenotypes are at least partially explained by ARF6-dependent recycling, which controls plasma membrane density of the Interferon-gamma receptor ". Also, we noted in the discussion, "Although our data support that ARF6 recycles constitutively internalized IFNγR back to the surface, other ARF6 mechanisms may be at play". In our revised discussion, we have added additional citations and text, "…other mechanisms may be at play, including ARF6-dependent intracellular trafficking and subcellular localization of other integral membrane proteins and of mitochondria. Hence, future research into ARF6-dependent basic cellular functions and endocytic transport of immune-modulating cargo may yield important insights that advance the development of immuno-therapeutics."

Reviewer #2 (Remarks to the Author): with expertise in cancer immunology, melanoma

Wee et. al. investigate the impact of ARF6, a target they have extensively studied previously in a different context, on tumor-immune interactions in the context of a genetically engineered Braf-activated, Cdkn2a-deleted murine model of melanoma. They identify that genetic loss of ARF6 reduced tumor incidence and size. In contrast, prior studies had suggested that expression of constitutively active ARF6 enhanced tumor metastasis without changing primary tumor growth. Characterizing the TME of ARF6-deleted and constitutively active tumors, the group found increased cytokine signaling in ARF6-null tumors and increased CD8 T cell clustering. Flow cytometry showed reduced immune infiltration in ARF-deleted tumors and no difference in CD8 T cell infiltration. However, ARF6 -null tumors had increased IFNg+ and GZMB+ CD8+ T cells and decreased FOXP3+ T regs. scRNAseq was performed which identified increased MDSC in ARF6 WT tumors compared with ARF6-null tumors. The group then tested ICB in mice with palpable and pre-palpable tumors and identified a relatively increased response in ARF6 WT tumors compared with ARF6-null tumors. They hypothesized that this is due to ARF6 control of IFNGR at the plasma membrane and show that, indeed, modulating ARF6 can affect IFNGR surface expression and signaling. They validated this finding across a range of human tumor cell lines. They also performed correlative analysis suggesting that GAP/GEF levels that are expected to correlate with ARF6 function suggest that a lower ARF6 level may correlate with reduced response to ICB.

Overall this is an intriguing story that adds to the complex literature surrounding the regulation and impact of IFNGR signaling in tumor immunity. Strengths include the use of genetically engineered mouse models to study tumorigenesis as well as ICB response, an extensive amount of data and an interesting mechanism that is at least partly corroborated in human tumor cells. Although IFNGR signaling is a longstanding and critical regulator of anti-tumor immunity, as highlighted decades ago by Bob Schrieber (and noted by the authors), its role has since been complicated by studies of IFNg-mediated resistance to immunotherapy with the argument that chronic IFNg signaling may both sensitize and drive resistance to immunotherapy depending upon the context (e.g. Benci et al, Cell, 2016 and 2019 from Andy Minn's lab). Viewed in this context, a relative challenge in this study is the extensive reliance on a single mouse model and incomplete characterization of the mechanisms of purported IFNGR-mediated resistance beyond PD-L1. Notably the finding that ARF6 regulates IFNGR levels is recapitulated in human cell lines, which is helpful, and there is an effort made to identify potential correlative biology in human sequencing datasets. However, the cell line models fail to recapitulate the interactive complexity of the TME and the human models are correlative and limited by the degree to which the indirect inference of ARF6 activity can be relied upon. Overall, the conclusions about ARF6 regulation of IFNGR expression seem stronger than the conclusions about the role this has in ICB and anti-tumor immunity. In particular, I worry that the increased growth of ARF6-null tumors may be due to something other than IFNGR regulation and the 'adaptive resistance' mechanism proposed. Experiments in immunodeficient mice (IFNGR knockout, NSG, SCID or others) would help to address this concern. That said, in my opinion, the strengths of this work outweigh the weaknesses, which are, in any case, common to the field. I make a few suggestions of experiments that could potentially further elucidate the biology. In particular, I wonder if the mechanistic depth and impact could be increased by:
- IFNGR blocking or knockout experiments. IFNGR expression is shown to be altered on ARF6-modulated tumors. However, The T cells in ARF6 knockout tumors were also making more IFNg. Furthermore, IFNg signatures tend to go up in successful anti-tumor immune responses, even if the mechanism is not direct modulation of IFNg signaling. Thus, it would be helpful in my mind to establish a direct role for IFNg signaling in dictating the tumor immune response rather than assuming that this is the driver of the phenotype.

Author response: Thank you for your thoughtful and insightful input. We appreciate your point that the growth of "ARF6-null tumors may be due to something other than IFNGR regulation". Note that we have previously interrogated ARF6 in an immunodeficient melanoma model (Grossmann A et al. 2013 Science Signaling) and added the following information to our introduction "pharmacologic inhibition of ARF6 reduced spontaneous metastasis without altering primary tumour growth in an immunodeficient model of cutaneous melanoma [13]". See below from that published study, tumor growth (LOX IMVI BRAF-mutant human melanoma cells injected into NOD-SCID mice).

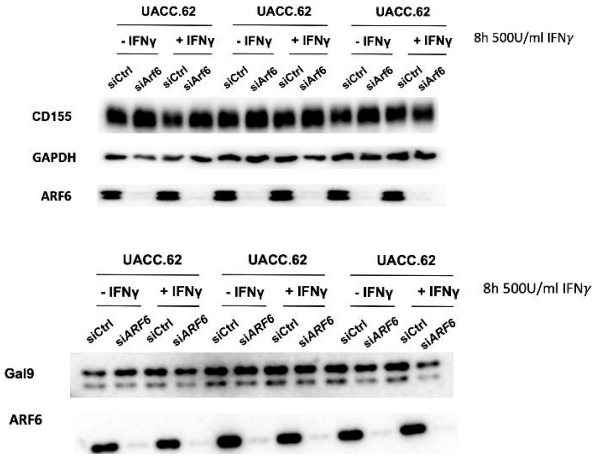
[figure redacted]

Furthermore, we have added supplemental data (Figure S1D) showing there is no difference in growth between ARF6 WT and NULL murine melanoma cell lines *in vitro*. Please also note, in our original submission we acknowledged that "other mechanisms of ARF6-mediated tumor progression may be at play" and have elaborated on this further in our revised discussion. Lastly, note that in contrast to cutaneous melanoma, pharmacologic inhibition of ARF6 in *uveal* melanoma (UM) *does* inhibit tumor growth (ARF6 controls downstream signaling from the oncogenic driver, mutant GNAQ, in UM (Yoo, JH et al 2016 Cancer Cell). Thus, it appears that ARF6 can have context-specific roles in distinct cancer types.

We agree that directly testing tumor IFNγR in our murine model could be informative, however, unlike syngeneic models, the *in vivo* approaches you suggest would likely take up to 3 years to complete in our genetic model and are beyond the scope of this study. Please note that ARF6 loss does not completely ablate IFNγR, as would an IFNγR blocking antibody or tumor specific *Ifngr* homozygous knockout. Rather, ARF6 loss *reduces* total and surface IFNγR. How to simulate this *in vivo* is unclear. Perhaps an *Ifngr* heterozygous state (in the melanocytic lineage) could accomplish this through haploinsufficiency protein levels, but this would need to be confirmed before crossing an *IfngR* floxed locus into our model. For now, we have included definitive evidence that blocking a downstream effector of IFNγ, the PD-1 – PD-L1 pathway, significantly controls both tumor development and progression in our model, as long as ARF6 is present in the tumor cells (Figures 4A and S4A).

- It could be useful to perform further immune inhibitory ligand profiling, focusing on those that have previously been identified as upregulated following chronic IFNg stimulation (e.g. Benci et al, Cell, 2016). PD-L1, CD80 and IDO1 are discussed, but are not the only IFNg-mediated regulators of resistance proposed (e.g. Gal9, CD155, etc).

Author response: Great suggestion, however, we found that unlike PD-L1 (and CD80 and IDO1), Gal9 and CD155 are constitutively expressed proteins in human melanoma cells (see below). Perhaps there are cell lines where Gal9 and CD155 are inducible with IFNγ, but chasing these down is beyond the scope of our current study.



- For the PD-1 treatment experiments: The relevance of PD-1 treatment experiments is increased by treating after tumors become palpable instead of prior to tumor onset. I see this was done in Supplemental Figure 4 for the WT but is not compared to the ARF6-deleted setting. Also, why is the ARF6-deleted experiment carried out to 90 days, whereas the

ARF6 WT is stopped at d75 (when mice appear to be actively continuing to reach endpoint)? These data seem somewhat less convincing to me than some of the prior points made.

Author response: Great suggestion and this experiment was ongoing and incomplete during our original submission. The results of anti-PD-1 treatment of established tumors in *Arf6* $^{f/f}$ mice has been added to Figure S4A alongside updated results for *Arf* ^{WT} mice. As with treatment of nonpalpable, microscopic tumors, anti-PD-1 is ineffective against established tumors in the *Arf6*$^{f/f}$ mice. Mice from each genotype were each treated for 5 weeks total. Because the onset of ARF6$^{f/f}$ tumors is later than ARF6^{WT} tumors, the end dates are distinct. The goal of the experiment was to treat each tumor genotype with identical protocols, including length of treatment (same 5 week endpoint).

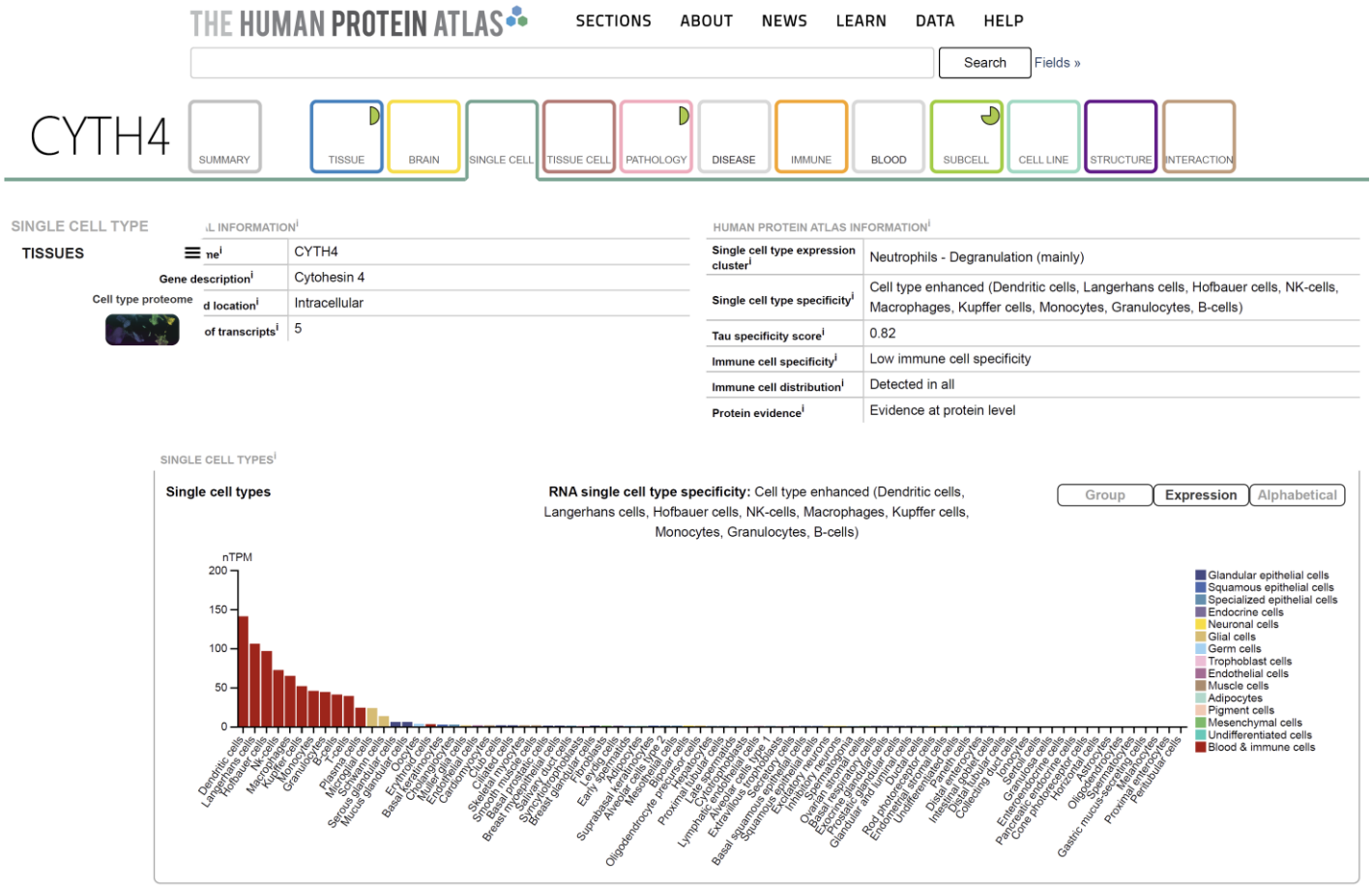
- I think that I am less confident than the authors in some of the correlative inferences they make in the RNAseq data, particularly when concluding that gene expression values of particular GAPs or GEFs will reliably predict ARF6 function or immune infiltration. As an example, CYTH4 is discussed in several sentences and concluded to potentially reflect tumor infiltrating immune cells. In this case, is CYTH4 correlated with inferred immune infiltration (e.g. via CIBERSORT or another approach)? How specific is ACAP1 for ARF6? - A quick look suggests that it may act in cargo sorting in other contexts. I wonder if it possible to place a bit less weight on these inferences in the story.

Author response: We appreciate your thoughts about the GEF and GAP data. These data are included for a broad audience that includes investigators who study small GTPases. While the ARF6 data stands alone, we think the GEF and GAP data enrich the story because it raises awareness about potential regulation of small GTPase activity via aberrant expression of GEFs and GAPs. In the small GTPase research community, these are provocative data because ARF6 is in the RAS superfamily, which includes hundreds of small GTPases, and their GEFs and GAPs are understudied. Nevertheless, to de-emphasize, we have moved the *CYTH1* human correlates to the supplemental figures (Figure S4C) and now show that cytohesin-1 is an ARF6 GEF in melanoma cells (*CYTH1* knockdown reduces ARF6-GTP, Figure S4D.

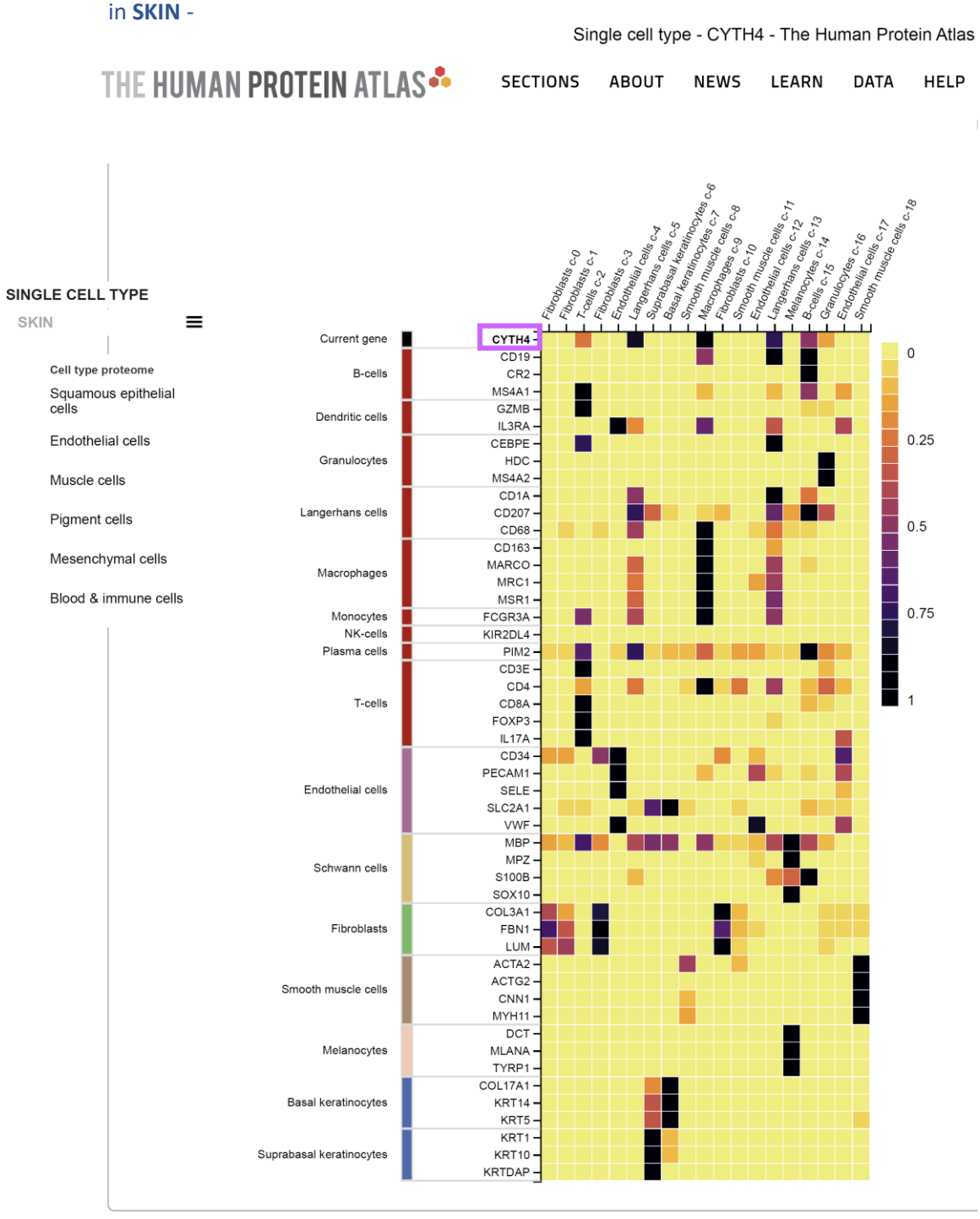
We have also added new results and text with references to strengthen the link between ACAP1 and ARF6 in melanoma: "Purified ACAP1 has been reported to have selective GAP activity for ARF6 over ARF1 or ARF5. Interestingly, *ACAP1* is prognostic in both primary (Leeds cohort, Figure 1B) and metastatic (TCGA cohort) tumours, and ectopic expression of ACAP1 inactivated ARF6 (reduced ARF6-GTP level) in human cutaneous melanoma cells (Figure 1C), consistent with previous studies in other cell lineages. Overall, these data suggest that variable expression of ACAP1 in primary melanoma (Figure 1B) impacts the activation level of ARF6 and can influence both primary and metastatic disease progression."

Regarding *CYTH4*, in addition to the prior work by others cited in our original manuscript showing that *CYTH4* expression is limited to immune cells, we now also cite the interactive Human Protein Atlas (https://www.proteinatlas.org/, Karlsson M et al., **A single-cell type transcriptomics map of human tissues.** *Sci Adv.* (2021), which provides extensive data from multiple cell lines and single cell transcriptomes of tissues showing that *CYTH4* expression is limited to immune cells. Examples procured from the Human Protein Atlas are shown below.

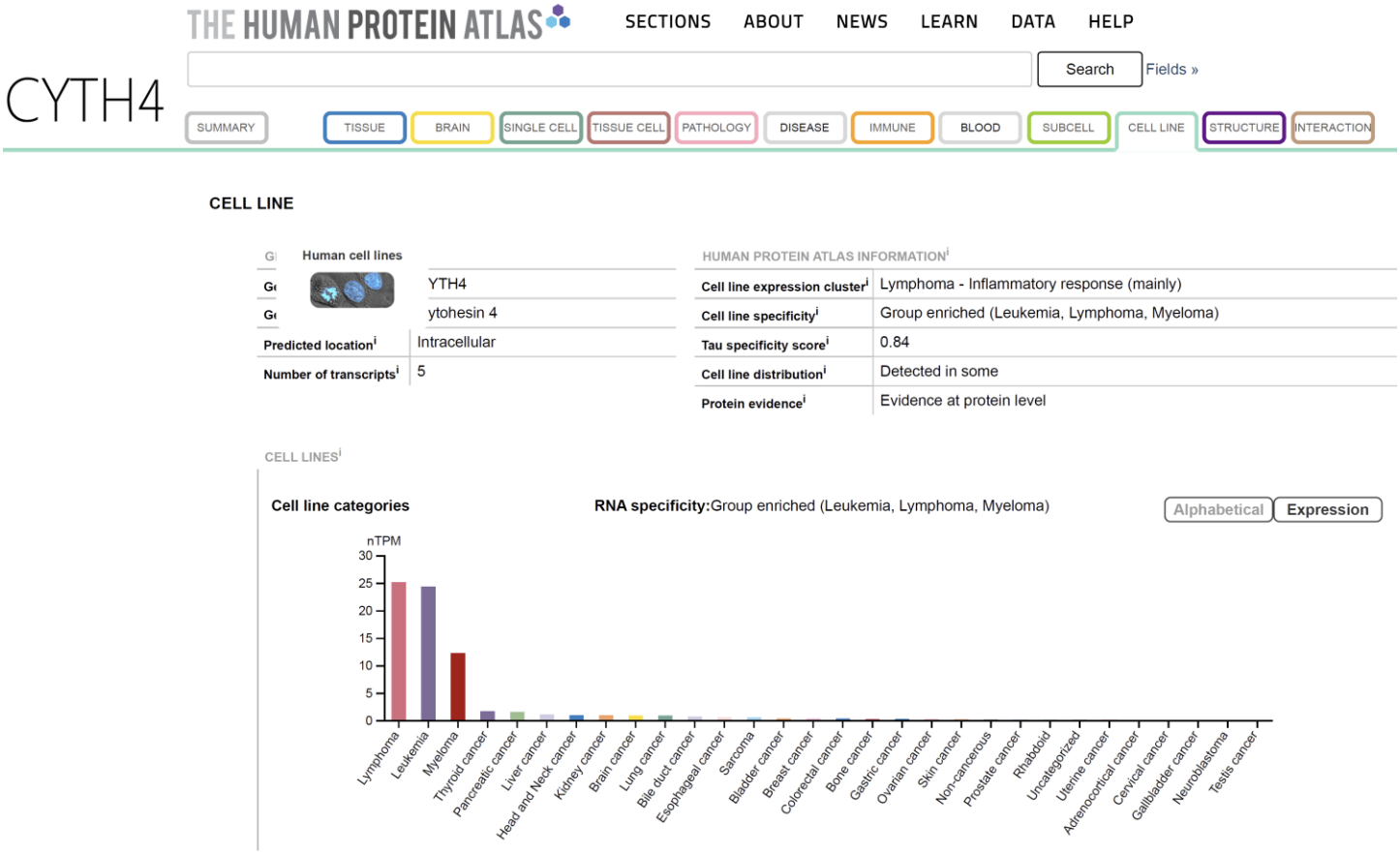
I. Detection of expression specifically in dendritic cells, Langerhans cells, Hofbauer, cells, NK cells, macrophages, Kupfer cells, monocytes, granulocytes and B cells.



II. Detection of *CYTH4* expression specifically in T cells, Langerhans cells, macrophages, B cells, granulocytes of in **SKIN** -



III. Detection of *CYTH4* expression specifically in hematopoietic (vs. other) **cells lines**:



Regarding *CYTH4* expression as a surrogate marker of tumor immune infiltrates in patient tumor samples - we queried the melanoma clinical trial data sets in Cancer-Immu for evidence of immune cell infiltrates in the pre-treatment melanoma biopsies. We found that like *CYTH4*, *CD8A* (T cells), *CD4* (T cells), *ITGAM+CD14+FCGR3A+CD68* (CD11b+ macrophages), and *ITGAX* (CD11c+ dendritic cells) expression correlate with overall survival after ICB therapy (Figures S4F, G, H, I). In other words, tumors with evidence of immune infiltration have superior outcomes.

- Given that IFNg-signaling has been proposed as a mechanism of resistance not only to endogenous antitumor immunity but to ICB, I wonder how the authors think about the situations in which targeting ARF6 would be desirable.
Author Response: We have thought about this at length and appreciate the question. While ARF6 mRNA may have clinical utility as a biomarker in the ICB-naïve setting, it is too early to speculate much about pharmacologic targeting of ARF6. In theory, an ARF6 *agonist*, in conjunction with ICB, might improve ICB sensitivity in ICB treatment naïve patients by acutely increasing IFNγR density in tumor cell plasma membranes, as long as the down-stream signaling components of IFNγ signaling are intact. Note in Figure 4D and E that despite differences in *ARF6* knockdown efficiencies, all the human cancer cell lines tested show a drop in IFNγR1 protein. Whether an ARF6 agonist (like QS11) could reliably boost IFNγR levels in tumors remains to be explored. Importantly, we would want to understand if and how ARF6 impacts immune cell function before considering ARF6 as a pharmacologic target in cancer. If ARF6 regulates surface density of immune or cytokine/chemokine receptors in immune cells, there could be unanticipated consequences (helpful or unhelpful) from treating with a systemic agonist of ARF6 with ICB.

- For cell cluster identification in scRNAseq experiments it would be very helpful to include the gene lists / marker genes that help to identify 'naive-like' T cells or other types. The methods say that this was done using SingleR and ProjectTILs, but both of these algorithms are subject to error and should be cross-checked with intuitive markers that are included in publication, in my opinion.
Author Response: Thank you for noticing this inadvertent omission. All cell clusters were manually reviewed to confirm or clarify cell lineage identities. These details have been added to the Supplemental Information section (see table titled Gene Expression Markers for Distinguishing Immune Cell Lineages).

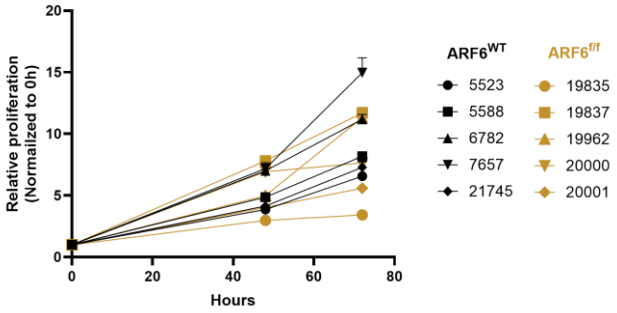
- What were the sizes/weights of tumors at the time of TME analysis? Was there a significant size difference (which can contribute to changes in immune populations)?
Author Response: For TME analysis (flow cytometry and scRNAseq), all tumors were harvested when the largest dimension reached 2cm. This has now been clarified in the revised Methods section.

Reviewer #3 (Remarks to the Author): with expertise in cancer immunology

This manuscript sought to examine how endomembrane trafficking machinery involving ARF6 affects TME. To address this question, the authors used a conditional knock-out mouse and human cell lines and concluded that the downregulation of ARF6 results in resistance to immune checkpoint blockade therapy, attributable to its involvement in modulating the expression of the IFNγ receptor. This study addresses interesting findings in immunotherapy. However, the manuscript seems to have several conflicting data, and the authors should address the following concerns:

1. The authors are linking these results to antitumor immunity. However, the provided data are insufficient. The authors should compare in vitro cellular proliferation or tumor growth in immunodeficient mice between Arf6 wild-type (WT) and Arf6-knock-out (KO) cancer cells. In Figure 3H, the authors depleted CD8+ T cells in Arf6-KO models without tumor growth data. However, CD8+ T-cell depletion generally enhanced tumor growth and shorten survival. Therefore, the authors should provide tumor growth and survival in Arf6-WT models in addition to those in Arf6-KO models.

Author Response: Points well taken.
Tumour Proliferation: *In vitro*, $ARF6^{NULL}$ murine melanoma cell lines show a similar range of growth kinetics as $ARF6^{WT}$ cell lines in CyQuant assay. See results below and the newly added Figure S1D showing that the means of each genotype are not significantly different.



These *in vitro* data are consistent with our prior *in vivo* studies in both immunocompetent and immunodeficient hosts: 1) constitutively active ARF6 ($ARF6^{Q67L}$) accelerated metastases but did not alter primary tumor growth, nor mitotic frequency (Ki67), in our immunocompetent genetic model (Yoo, JH et al. 2019 Cancer Research 79(11):2892).

[figure redacted]

[figure redacted]

2) pharmacologic inhibition of ARF6 with SecinH3 reduced spontaneous metastasis but did not alter primary tumor growth in a xenograft (**immunodeficient**) cutaneous melanoma model (Grossmann, AH et al. 2013 Science Signaling).

Both of these prior publications are cited in our current manuscript under review. Thus, we do not have any evidence, in immunocompetent nor immunodeficient mice, to support that ARF6 controls cutaneous melanoma proliferation *in vivo*. In clarification, in our revised manuscript, we added a sentence to the introduction, "Specifically, ARF6-GTP in primary tumours promoted metastasis without increasing primary tumour growth. Likewise, pharmacologic inhibition of ARF6 reduced spontaneous metastasis without decreasing primary tumour growth in an immunodeficient model of cutaneous melanoma."

CD8 depletion: The CD8 depletion experiment in *Arf6*^{WT} mice was ongoing during our original submission and is now complete (Figure S3C). Tumour growth rates have now been included, as requested, for both *Arf6*^{WT} and *Arf6*$^{f/f}$ mice (Figure 3I, S3C-D). Unlike the *Arf6*$^{f/f}$ mice, CD8 depletion does not accelerate tumor development or progression because the adaptive immune response is not controlling tumor growth in *Arf6*^{WT}mice. Our data shows that CD8 T cell effector function is suppressed in *Arf6*^{WT}mice (Figure 3B,F) and as such, removing these cells has no effect.

2. In Fig. 2C, the authors conclude that apoptosis is enhanced in ARF6-KO tumors. However, since the expression of MHC-I and IFNγR is reduced in ARF6-KO tumors, it is difficult to understand these data and I think that these data could be conflicting. The IFNɣ signaling pathways could be lost and CD8+ T cells difficulty recognizing cancer cells due to low expression of MHC.
Author Response: Thank you for raising this interesting topic. We have addressed your concern below in response to #3.

3. In Fig. 3B, the authors show that the ratio of CD8+ IFNγ+ T cells and CD8+ GzmB+ T cells increases. However, similar to the second comment, it is questionable that CD8+ T cells are activated in tumors that hardly express MHC-I. Looking at the plot on the right side of Fig. S5B, there are many ARF6-KO tumor cells in which MHC-I expression does not increase even after IFNγ stimulation. In contrast, IFNγ stimulation is sufficient to increase MHC-I expression in ARF6-WT tumor cells. Despite this difference, can you experimentally explain the mechanism by which CD8+ T cells are activated in ARF-KO with low MHC-I expression? Particularly, several recent studies emphasized the importance of MHC expression among the IFNɣ signaling pathways in antitumor immunity (doi: 10.1158/2326-6066.CIR-22-0815.).
Author Response: Thank you for the question. To avoid confusion, we will reserve the term "activation" to describe the priming of T cells to proliferate after initial antigen presentation (by antigen presenting cells, which are ARF6^{WT} in our model). As you know, this was not measured in our study. Rather, we performed the gold-standard assay for cytotoxic T cell effector function (measured IFNγ and granzyme B production by CD8+ T cells) from tumors and spleen, and then confirmed these findings with an orthogonal method (scRNAseq of CD45 sorted tumor infiltrating immune cells). Note in the new Figure S4B that in ARF6^{WT} mice, whose tumors respond to anti PD-1 therapy, T cell effector function is significantly increased with therapy compared to untreated mice, as expected, and these data provide an independent positive control for the gold-standard, T cell effector function assay.

Regarding your request "can you experimentally explain the mechanism by which CD8+ T cells are activated in ARF-KO with low MHC-I expression?" While MHC-I presentation of antigen by tumours has certainly been shown to be important, we understand from the large published body of work that regulation of T cell effector function (after activation) is not likely to be solely dependent on the number of tumor MHC-I synapses with T cells, especially since T Cell Receptor (TCR) affinity for antigens presented by MHC-I can potentially influence the number of synapses needed to stimulate effector function. There may be several potential tumor antigens that elicit CD8+ T cell response in our model. Identifying those antigens and understanding the TCR-antigen affinity in our model is for future studies, nevertheless, there are other evident clues from our data that can mechanistically explain how CD8+ T cell effector function is increased in the ARF6 knockout tumors. The following paragraph, with citations, has been added to the discussion to address your astute questions about MHC-I:

Our study reveals a provocative finding in that CD8+ T cells produced more effector molecules (Figure 3B) in ARF6$^{f/f}$ tumors expressing relatively low MHC-I compared to ARF6^{WT} tumors (Figure S5B). Despite the low tumour MHC-I, more effector CD8+ T cells were detected in ARF6$^{f/f}$ tumours (Figure 3F) and CD8+ T cells were necessary to limit tumor development in *Arf6*$^{f/f}$ mice (Figure 3H and S3D). This may be explained by cumulative changes in the ARF6$^{f/f}$ TME that alleviated suppression of CTLs and compensated for the diminished MHC-I, including loss of immune checkpoint ligands PD-L1 and CD80 (Figure 5), as well as the reduction in Tregs (Figure 3D) and MDSCs (Figure 3E). Our data are consistent with findings reported by Benci, J.L. et al. [62], who described that elimination of tumour IFNγ signaling increased IFNγ produced by CTLs.

In this study, CTLs functioned in a supportive role in tumours with low or absent MHC-I expression. Specifically, IFNγ produced by exhausted T cells induced maturation of innate immune cells, including NK cells, to kill tumor. In a more recent study by Lerner, E.C. et al.[63], MHC-I independent, CTL killing of tumor was revealed. The authors showed that CD8+ T cells maintain the ability to eliminate tumor cells that completely lack MHC-I expression. T cells engaged nonclassical MHC class-I like, NKG2D ligands on tumor cells to release granzyme and perforin. The contribution of these MHC-I independent mechanisms of CTL-dependent tumour elimination, and how they might coordinate in a tumor with variably low MHC-I expression as seen in our model, remains to be investigated.

Lastly, note that CD8+ T cell depletion completely restores tumor development and progression in *Arf6*$^{f/f}$ mice (see new Figure S3D) – note that the tumor growth rate in CD8-depleted *Arf6*$^{f/f}$ mice is equivalent to the tumor growth rate in untreated *Arf6*^{WT} mice. To what extent the CD8+ T cells rely on MHC-I antigen presentation or other mechanisms in our model is unknown and beyond the scope of this study, nevertheless it is clear that CD8+ T cells are essential for the anti-tumor immune response and that tumor intrinsic ARF6 facilitates suppression of CD8+ T cell effector function.

4. Also, regarding CD8+ T cell activation, such as the ratio of CD8+ IFNγ+ T cells and CD8+ GzmB+ T cells, it would be better to show whether the data evaluated in four groups, not only the treated with Isotype control group but also the treated with Anti-PD-1 group in ARF6-WT and ARF6-KO tumors, is consistent with the authors' theory.
Author Response: Please see new Figure S4B, CD8+ T Cell Effector Function in tumors from anti-PD-1 treated mice. Treatment was initiated after tumors were established. As expected, tumor infiltrating CD8+ T cells from ARF6^{WT} tumors showed significantly increased IFNγ and GzmB production, reflecting the efficacy of anti-PD-1 therapy in stimulating CD8+ T cell effector function and in limiting tumor progression (Figures 4A, S4A). In contrast and also as expected, anti-PD-1 therapy failed to stimulate CD8+ T cell effector function in tumors from *Arf6*$^{f/f}$ mice. This makes sense because the PD-L1/PD-1 pathway is inactive in ARF6$^{f/f}$ tumors, i.e. targeting a nonexistent pathway is futile (Figures 4A, S4A).

5. The authors should analyze chemokine expression as IFNγ signaling pathways. Single-cell sequencing data could be helpful.
Author response: We appreciate the suggestion to analyze chemokine expression within the context of IFNγ signaling pathways using scRNAseq data from tumor infiltrating immune cells in our model. Conceptually this is a great idea, however the reality is that interpreting chemokine production by immune cells is quite challenging without geospatial data to overlay and guide interpretation. Our scRNAseq data showed complex patterns of differential expression of chemokines in the various immune cell lineages, suggesting intricate communication between immune cells and between immune and tumor cells, and that multiple factors contribute to chemokine regulation. This complexity presents limits to interpreting TME chemokine expression patterns solely within the framework of IFNγ signaling. While we acknowledge the importance of understanding these complexities, unraveling the role of tumor intrinsic ARF6 in chemokine regulation extends beyond the scope of our current study.

6. In Figures 2B and 5A, these photos are a little difficult to understand.
Author response: We apologize and have made adjustments that we hope will resolve confusion (not knowing exactly what was challenging to interpret). In the legend of Figure 2B – we added as description for the H&E images that identify tumor infiltrating immune cells morphologically as “small round blue cells”, which is a standard morphologic descriptor given by pathologists (the corresponding author, Dr. A. Grossmann, is a pathologist). For Figure 5A we have converted the original table into a histogram.

Reviewer #4 (Remarks to the Author): with expertise in skin cancer, IFNs

The paper presents some interesting and potentially impactful findings related to the role of ARF6 in remodeling the tumor microenvironment and regulating response to immunotherapy. This study reveals the importance of ARF6 mediated endomembrane trafficking to impose immunosuppressive tumor microenvironment (TME) to accelerate tumor development. This ARF6 dependent TME makes it vulnerable to immune checkpoint inhibitor blockade therapy. The work is significant, original, and supports the conclusions. The methodology is sound and provides enough details. Here are some suggestions:

1. The introduction, though informative, is very lengthy. The background can be shortened to couple of paragraphs on what is known in the field and focus on the gaps in knowledge that need to be addressed.

Author Response: Thank you for the suggestion and we are happy to shorten the introduction with guidance. We defer to the editorial team to advise if and how to shorten. The current version includes content that, based on our experience, audiences need to comprehend the story.

2. Figure 3 and supplement can be split into two for clarity of multilineage immune cell data.

Author Response: Thank you for the suggestion. The purpose of grouping multilineage immune data together is to 1) emphasize the more global impact of tumor intrinsic ARF6 on the TME and 2) to avoid considering CD8 effector function independent of other key immune subsets, such as Tregs and MDSCs, which regulate CD8 T cell function. While our grouping was intentional, we defer to the editor to advise if and how to split these groupings.

3. The authors have looked at ARF6 mediated mechanisms in this study. Did they look at other ARFs that have been reported in cancer? If so, what was their expression?

Author Response: Yes, and we're glad you asked because it validates the logic behind our analysis, which we included in the original manuscript. Please see Table S1 and Table S3. There are five members in the *ARF* gene family and among these, only ARF6 functions at the plasma membrane. Expression of each of the *ARF* genes, along with ARF6 GEFs and GAPs, was queried in the human data sets (Figures 1, 4, S4). Among the *ARF* genes, only expression of *ARF6* correlated with outcome.

4. There was low expression of ARF6 expression in ARF6fl/fl tumors but the expression of ARF1 remained intact. The effect of ARF1 in ARF6fl/fl mice is not clear.

Author response: Thank you for the feedback. ARF1 was evaluated by Western blot as an internal negative control (not deleted) because it functions in similar pathways as ARF6, even though it localizes to the Golgi rather than the plasma membrane. With the exception of ARF6, all ARFs, including ARF1, localize to the Golgi. In the revised submission we have added more citations and explained in the results, "Among the ARF family of proteins, ARF6 uniquely localizes and functions at the cell periphery but has overlapping and synergistic roles with ARF1. Notably, in ARF6 knockout cells, expression of ARF1 remained intact (Figure S1A)."

5. The authors found that immune checkpoint blockage was effective in tumors that expressed PD-L1 by ARF6 dependent mechanism. Can this mechanism be applied to "cold tumors" that do not express PD-L1?

Author response: We would not expect cold tumors, i.e. those with absent or low immune infiltration, to respond, and there are numerous publications by other groups to support this. Note that consistent with this expected result, we added additional supplemental data showing that melanoma patients with tumors with low expression of immune lineage markers for T cells, dendritic cells and macrophages have inferior survival outcomes (Figures S4 F, G H, I). In our murine model, $ARF6^{f/f}$ tumors fail to express PD-L1 (Figure 5A-E) and fail to respond to anti-PD-1 therapy (Figure 4A and newly added Figure S4A). In human tumors, absent or low PD-L1 expression in either tumor or tumor infiltrating immune cells, detected with the 22C3 antibody, is predictive for a lack of response to pembrolizumab (anti-PD-1 therapy) and this is the basis of the US FDA-approval of the 22C3 IHC stain as a companion diagnostic test for NSCLC, Head & Neck SCC, cervical cancers, triple negative breast cancer, and gastric/GEJ cancers. Nevertheless, as a stand-alone marker, the field of oncology recognizes that PD-L1, like most other biomarkers, is imperfect in predicting response and outcome.

6. The limitation and future directions section can be further elaborated.

Author response: Indeed, thank you for the suggestion. Based on common questions we field from various audiences we have added a paragraph to the discussion that addresses unanswered questions about MHC Class I. Please see paragraph beginning and ending with, "Our study reveals a provocative finding… remains to be investigated."

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The basic finding of this manuscript is that Arf6 may regulate intracellular dynamics and cell surface expression INFγR in melanoma cells. Thus, the authors described when Arf6 expression in melanoma cells is low, Arf6GAP expression is high, or Arf6GEF expression is low, melanomas may be less responsive to INFγ produced within the TME and may hence also be less responsive to ICB therapy. For example, as the authors also demonstrated, such unresponsiveness to INFγ may impair INFγ-induced IDO1 expression which is known to produce kynurenine and shape the immune evasion properties of TME.

In contrast, however, silencing of Arf6 and related factors in pancreatic cancer MIAPaCa-2 cells has been demonstrated not to substantially impair their INFγ responsiveness (PNAS 2019, 116:17450). Therefore, as the authors state in their answer letter, the Arf6-INFγR relationship may be diverse across cancer types and cells. Therefore, as this reviewer previously suggested, the molecular details linking Arf6 activity and INFγR in melanoma cells need to be clarified, which was still not done in the revised manuscript. Without such data, the Arf6-INFγR relationship may not be clearly applicable to clinical settings.

Furthermore, the authors have not even performed a cell biological assay of the INFγR recycling. Hence, while Arf6 can be closely linked to some process(es) of the intracellular dynamics and cell surface expression of INFγR in melanoma cells, it is not clear which process(es) Arf6 may primarily regulate.

Overall, the manuscript describes a variety of important findings, but the most basic statements at the core of the manuscript are still descriptive. It is not clear whether Arf6 directly regulates INFγR dynamics, or may affect INFγR indirect manner.

Reviewer #2 (Remarks to the Author):

Overall the revised manuscript presented is, in my view, functionally similar to the original presentation.

In many cases, the authors raised a number of arguments as to why the reviewer-recommended experiments are either unnecessary or infeasible. I find some of these arguments compelling and others less so. Among the more compelling include that there is a lot of orthogonal circumstantial data to support the plausibility of their conclusions.

An example of this approach: in response to the suggestion that they block or knockout IFNGR to prove that the anti-tumor effects are actually attributable to altered IFNGR expression, they respond both that this is unnecessary because they see a response in a different, human, immunodeficient model and in vitro data and that it is infeasible either because the genetic manipulations would be too difficult or because the blocking-antibody experiment would not fully reflect the biology. I am sympathetic to the idea that the genetic manipulations would be onerous in this model. However, I find it somewhat puzzling that the argument is made that the mechanism / reasoning should be acccepted as likely based on a lack of effect immunodeficient murine studies (which could reflect a host of non-IFNGR issues), but that blocking IFNGR is not sufficiently relevant to have any bearing on whether the mechanism is IFNGR-related. While this experiment wouldn't be a perfect phenocopy of the partial modulation of IFNGR, a lack of phenotype in this setting would narrow the mechanism to one that is, at the least, IFNGR-dependent. I dont think I would put as fine a point upon it except that it does seem fairly central to their anti-tumor immunity argument / mechanism.

A small point: I had asked about the timing of flow experiments- whether there was a difference in the size/weights of the groups at the time of TME analysis. The response was that the tumors were harvested 'when the largest dimension reached 2 cm'. To clarify, I think it is important to report the indivudal sizes / weights of tumors at the time of takedown and between group differences, not just the size criterion that triggered takedown of the entire group. It seems unlikely that all tumors in all groups reached the same size endpoint on the precise day of takedown.

Ultimately, however, while I think there was probably more space to address the concerns raised (including the issues raised by other reviewers—e.g. transfection certainly alters IFN

signaling and consequently limits evaluation of the effect of expressing cytohesin-1, ACAP1… but just as clearly transfection isn't the only way to achieve expression), and there remain some gaps in connecting the proposed mechanisms and the biology, I think that the paper remains sufficiently interesting to be considered for publication.

Reviewer #3 (Remarks to the Author):

Related to my query #5, IFNɣ-related chemokines such as CXCL9, 10, and 11 play important roles in CD8+ T-cell recruitment. Thus, the expression in cancer cells, immune cells, and tumor tissues should be analyzed.

Reviewer #4 (Remarks to the Author):

The paper presents some interesting and potentially impactful findings related to the role of ARF6 in remodeling the
tumor microenvironment and regulating response to immunotherapy. This study reveals the importance of ARF6
mediated endomembrane trafficking to impose immunosuppressive tumor microenvironment (TME) to accelerate
tumor development. This ARF6 dependent TME makes it vulnerable to immune checkpoint inhibitor blockade therapy.
The work is significant, original, and supports the conclusions.
The authors have addressed all the comments suggested by the reviewer.

Reviewer #1 (Remarks to the Author)

The basic finding of this manuscript is that Arf6 may regulate intracellular dynamics and cell surface expression INFγR in melanoma cells. Thus, the authors described when Arf6 expression in melanoma cells is low, Arf6GAP expression is high, or Arf6GEF expression is low, melanomas may be less responsive to INFγ produced within the TME and may hence also be less responsive to ICB therapy. For example, as the authors also demonstrated, such unresponsiveness to INFγ may impair INFγ-induced IDO1 expression which is known to produce kynurenine and shape the immune evasion properties of TME.

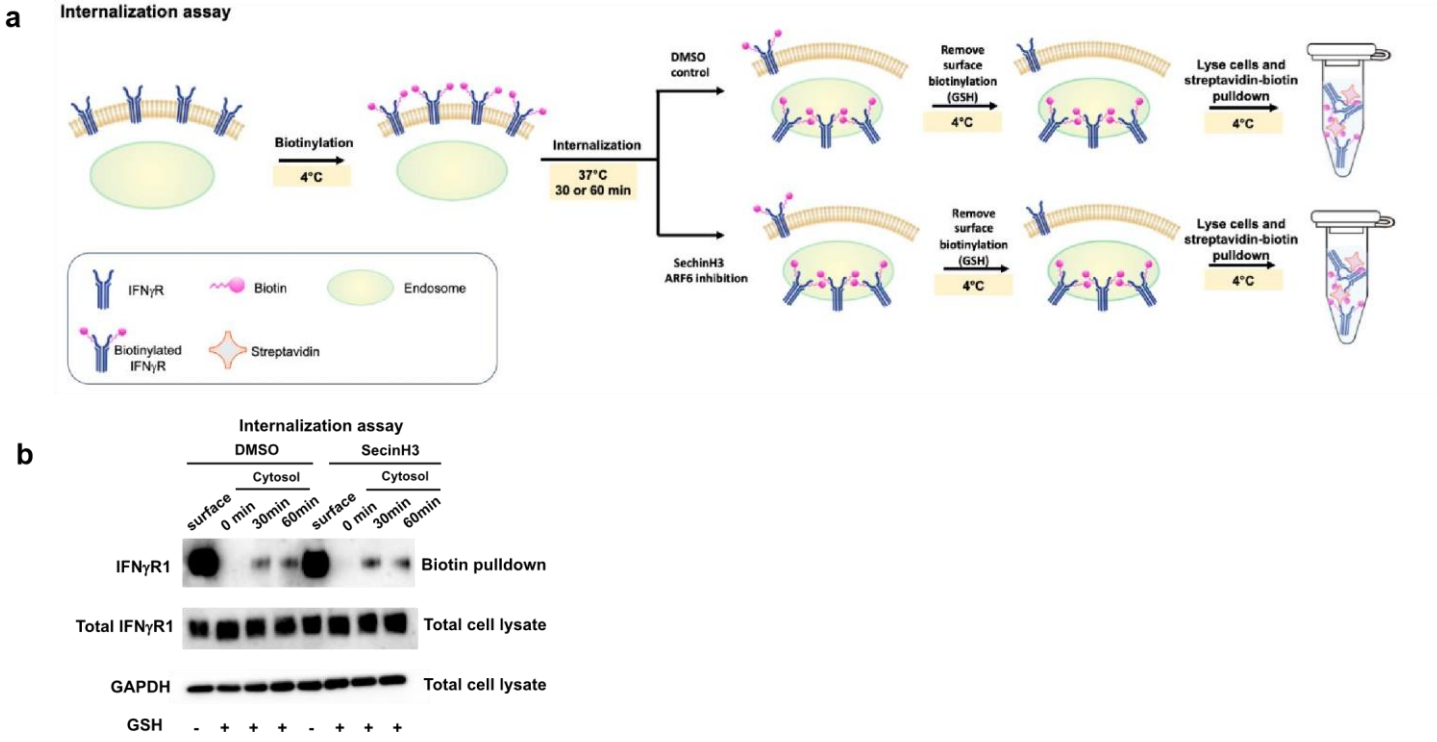
In contrast, however, silencing of Arf6 and related factors in pancreatic cancer MIAPaCa-2 cells has been demonstrated not to substantially impair their INFγ responsiveness (PNAS 2019, 116:17450). Therefore, as the authors state in their answer letter, the Arf6-INFγR relationship may be diverse across cancer types and cells. Therefore, as this reviewer previously suggested, the molecular details linking Arf6 activity and INFγR in melanoma cells need to be clarified, which was still not done in the revised manuscript. Without such data, the Arf6-INFγR relationship may not be clearly applicable to clinical settings.

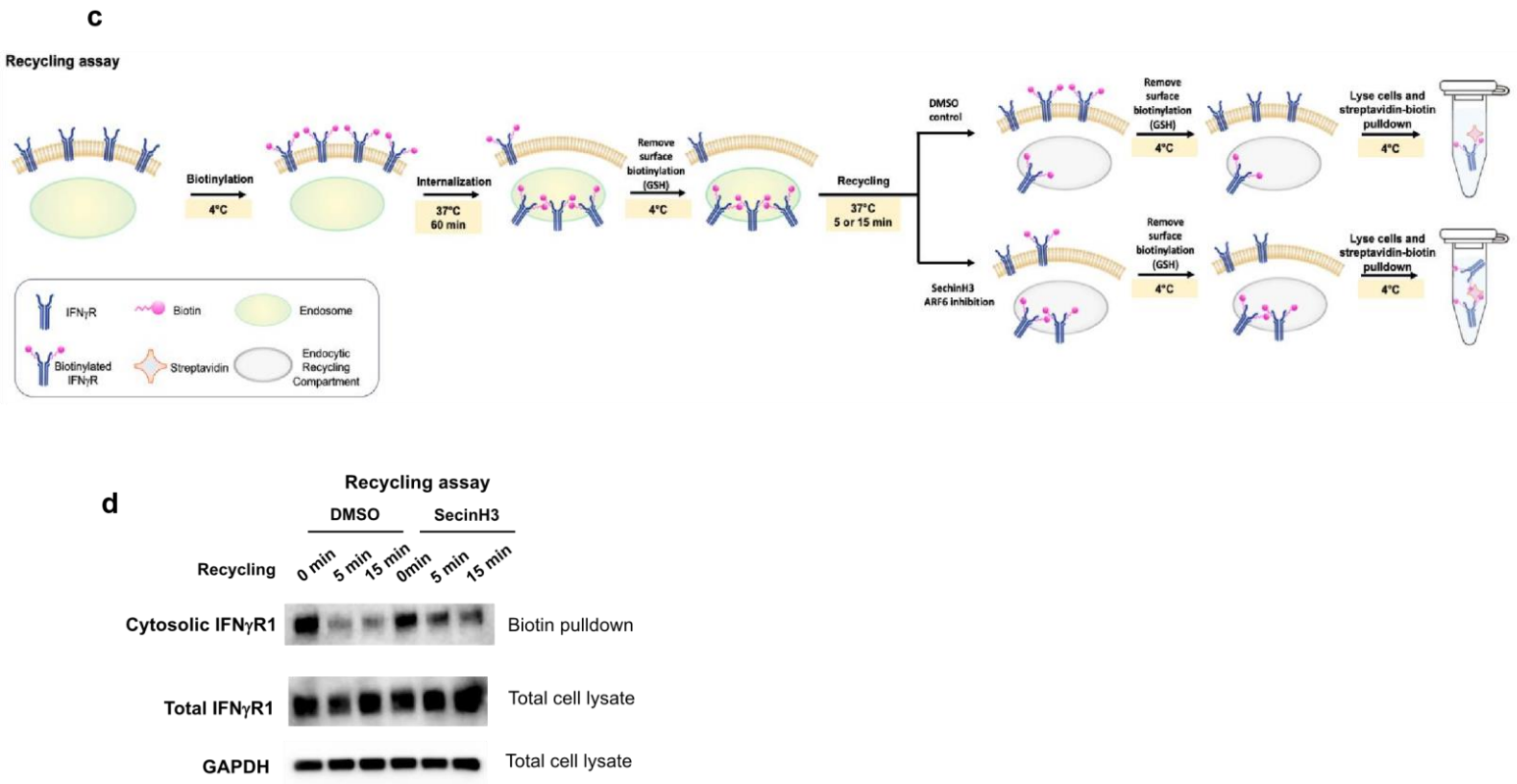
Furthermore, the authors have not even performed a cell biological assay of the INFγR recycling. Hence, while Arf6 can be closely linked to some process(es) of the intracellular dynamics and cell surface expression of INFγR in melanoma cells, it is not clear which process(es) Arf6 may primarily regulate.

Overall, the manuscript describes a variety of important findings, but the most basic statements at the core of the manuscript are still descriptive. It is not clear whether Arf6 directly regulates INFγR dynamics, or may affect INFγR indirect manner.

Author response:

We performed IFNγR1 internalization and recycling assays shortly after our first submission. The figures below show that pharmacologic inhibition of ARF6 has no effect on IFNγR1 constitutive internalization (a,b). In contrast, recycling of the receptor is compromised by inhibition of ARF6 (c,d), The results are consistent with our flow cytometry results (Figure 6a, 6g, Supplementary 6d). We added the following results to the manuscript as supplementary 6f-g.





ARF6 controls IFNγR1 recycling in human melanoma (UACC.62). (a) Schematic representation of the internalization assay of IFN□R1 in human melanoma (UACC.62) cells treated with DMSO (control) or SechinH3. After cell surface biotinylation, the cells were incubated for 30 or 60 minutes at 37°C to allow endocytosis. Glutatione (GSH) was applied to remove biotin remaining at the cell surface, followed by cell lysis. Biotin in cell lystate were precipitated with streptavidin beads, and the expression of IFN□R1 was analyzed by Western blot **(b)**. **(c)** Schematic representation of the recycling assay of IFN□R1 in human melanoma (UACC.62) cells treated with DMSO (control) or SechinH3. After cell surface biotinylation, the cells were incubated for 60 minutes at 37°C to allow internalization of cell surface proteins. An initial GSH treatment removed any remaining biotin from the cell surface, after which the cells were incubated again at 37°C for 5 or 15 minutes to enable the recycling of biotinylated IFNγR1. A subsequent GSH treatment then cleaved all biotinylated proteins that had returned to the cell surface, leaving the biotinylated proteins within the cytosol unaffected. Biotin in cell lysates were precipitated with streptavidin beads, and the expression of IFNγR1 was analyzed by western blot **(d)**.

While these data are novel because the machinery that trafficks the IFNγR was previously unknown, it is not surprising that ARF6 mediates recycling as this is a known function for ARF6 with other receptors. Rather, the unanticipated and unique aspects of our story are the *in vivo* outcomes, the human correlates that are consistent with the murine phenotypes, and the fact that ARF6 controls IFNγR1 protein levels in at least four common cancer types that rely on IFNγ-mediated adaptive immune resistance and are sensitive to ICB therapy.

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Author response:

Regarding the 'small point,' we added more details to the methods to answer the weight question (rather than a size question): 'When the tumors reached 2 cm in greatest dimension, both the tumors and spleens were harvested after euthanasia. Portions of the tumors and the whole spleen were then taken, and their weights were recorded for flow cytometry analysis.' This is included in the flow cytometry analysis methods section. Please note that weight was used to normalize the data when comparing the amount of infiltrating immune cells between genotypes, (see Y axis of Figure S2A, CD45+ cells per gram of tumor).

Regarding the two potential *in vivo* experiments. The approach and timeline for completion are detailed below with highlighted caveats. Note that the Cre-inducible *Dct::TVA; Braf*^{V600E}*;Cdkn2a*$^{f/f}$ model is distinct from syngeneic models where *Ifnγr1* knockouts have previously been tested and reported. Our model uniquely interrogates the entire tumor initiation to progression sequence, including the elimination, equilibrium and

escape phases of tumor-host immune interactions. Hence, it allows for dynamic co-evolution of the cancer and immune system and more closely resembles human disease. While this is a strength and may yield new information that syngeneic models cannot, it is a much more labor intensive, costly and time-consuming system to deploy.

IFNγR1 Genetically Engineered, Conditional Mouse Melanoma Model

To interrogate the functions of tumour-specific Interferon-gamma receptor (IFNγR), we would compare three *Ifnγr1* genotypes in our Cre-inducible *Dct::TVA; Braf*^{V600E}*;Cdkn2a*$^{f/f}$ model: homozygous (floxed) knockout (*Ifnγr1*$^{f/f}$) vs. heterozygous (*Ifnγr1*$^{f/+}$) vs. wild type (*Ifnγr1*$^{+/+}$) cohorts. The *Ifnγr1*$^{f/f}$ mice are available through The Jackson Laboratory. Tumour-intrinsic IFNγ signaling has both acute, anti-tumour (cytotoxic) and chronic, protumor (immunosuppressive) effects. In addition, IFNγ induces expression of "classical", MHC I genes involved in antigen presentation and immune vulnerability, as well as "non-classical" MHC I genes that mediate immune evasion and escape (reviewed by Zaidi, MR 2019 JICR 39:30-8 PMID: 30388040). Homozygous deletion of the receptor would completely abolish both pro- and anti-tumour intrinsic IFNγ pathway functions during malignant transformation and progression and, as a result, may mask phenotypes and obscure interpretation, leading to incomplete conclusions. While this approach has been published in multiple syngeneic models, it cannot recapitulate regulation of the receptor by endocytic trafficking, which (we have shown) alters IFNγR protein levels and surface localization without ablating the receptor. In order to evaluate the consequences of a reduction in total and surface IFNγR1 protein , heterozygous (*Ifnγr1*$^{f/+}$) mice would be included. **If** IFNγR1$^{+/-}$ tumours from the initial crosses showed hemizygous expression levels of IFNγR1, then we would proceed with the following breeding and experimental scheme:

Based on the anti-tumour effect of anti-PD-1 therapy in our *Arf6*^{WT} mice (Figure 4A, S4A), we estimate we will need at least 25 tumor-bearing mice for each of the three *Ifnγr1* cohorts. In our model, homozygous *Dct::TVA* is embryonically lethal, so only half of the mice generated are TVA+. Hence, we have to generate twice as many mice for every generation and at every stage of the project. We expect an average of 4 mice born per litter, and after Cre injection/tumour induction, we would plan to observe for at least 100 days for tumor generation and end-point growth, similar to our *Arf6* knockout tumor model.

Since our mouse colony has been truncated to a bare minimum (4 breeding pairs) in advance of our move to a new institution July 1, 2024, generating enough mating mice from our existing colony to cross in the *Ifn□r1*$^{f/f}$ locus would take at least 4 months after the mice are transferred. Mouse transfer will occur in Sept/October of 2024 once the weather cools (the climate in Salt Lake City, Utah is high mountain desert, i.e too hot to ship mice in the summer). We estimate that a total of 4 generational crosses would be sufficient to achieve the correct genotype and strain background mix. The projected percentage of mice with a genotype usable for each generation of crossing is as follows: F1 = 50%, F2 = 14.9%, F3 = 15.7%, F4 = 12.5%. Criteria for determining the percentage of usable mice from each generation are as follows: F1 - TVA+ and all other alleles are heterozygous, F2 – TVA+ and at least 1 other allele is homozygous, F3 – TVA+ and at least 2 other alleles are homozygous, F4 – TVA+ and all other alleles are homozygous. For generations F2-F4, the expected percentage of mice with a usable genotype is low, which may require extra time and mice at each generation to have enough breeders to set up for the next cross. As such, we expect that producing each generation of mice would take ~4 months. Therefore, an estimated total of 22 months would be required to create these new genetic combinations. Once the goal genotypes are achieved, expanding our breeder population for *Ifn□r1*$^{+/+}$, *Ifn□r1*$^{f/+}$ and *Ifn□r1*$^{f/f}$ mice to approximately 15 breeding pairs each would take ~9 months. (This is, indeed, what we did to complete the each anti-CD8 and anti-PD-1 experiment reported in our manuscript). As females mature and produce litters, we would then begin inducing tumors for the ensuing ~ 12 months as litters are continuously born and prospectively enrolled (in series) in the study. After tumor inductions begin, we estimate

that all tumors will have reached the end point and the experiment would be complete within ~ 24 months. **In summary, we estimate overall project completion 3-4 years from now, after generating >500 mice**.

IFNγR1 blocking antibody treatment in Cre-inducible *Dct::TVA; Braf*^{V600E}*;Cdkn2a*$^{f/f}$ model

Note that treating mice systemically with IFNγR1 blocking antibody **will disrupt IFNγ signaling in both tumour and immune cells.** IFNγ, secreted by CD8+ T cells, impacts the differentiation and function of other immune cells. For example, we observed IFNγ gene expression signatures (antigen presentation and phagocytosis) in tumour infiltrating macrophages (Figure S2e in our manuscript). Because this approach fails to specifically target the tumour, this experiment is truly a "scorched earth" approach, ablating the IFNγ pathway in the whole organism. It is the furthest possible, opposite extreme from our ARF6 studies. Also, while this is a much faster experiment that the genetic interrogation of *Ifnγr1* described above, there is still significant lead time to generate enough breeding pairs and tumour bearing mice.

Based on our experience treating *Dct::TVA; Braf*^{V600E};*Cdkn2a*$^{f/f}$ mice with anti-PD-1 antibody, we would design the experiment similarly. We would enroll 25 mice into the two treatment arms (isotype control vs. anti-IFNγR1) for a total of 50 mice. As described above, only heterozygous mice transmit the TVA transgene, therefore about 50% of the offspring would be TVA positive. Hence, we would need to breed a total of 100 mice, 50 of which would be TVA positive and appropriate for the experiment.

To generate 100 experimental mice, we would generate TVA positive mice for 15 breeding pairs. Due to our current, limited number of maintenance breeders, establishing the necessary breeding pairs would take approximately 4 months. Breeding pairs mature and litters are born in series. After enrollment by tumor induction (Cre-injection) and then treatment, the experiment would be complete in ~9 months, considering gestation periods (3 weeks per litter), the gradual generation of litters for enrollment and the treatment timeline (10 weeks). Overall, we estimate the experiment would take >12 months to complete from now.

Reviewer #3 (Remarks to the Author)

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We did not observe, nor report, any significant differences in the amount of CD8+ T cell recruitment (infiltration) to ARF6^{WT} vs. ARF6$^{f/f}$ tumours (Figure S2b). As such, it is not surprising that within our single-cell RNA-seq data of CD45+ tumour infiltrating immune cells, expression levels of CXCL9 and CXCL10 were either undetectable or inconclusive across various immune cell types when comparing the ARF6^{WT} and ARF6$^{f/f}$.

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