Ion channel dysregulation and ferroptosis susceptibility in a heterogeneous DTP state

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***Short Abstract* — Recently, we reported that BRAF-mutant melanoma cells under prolonged BRAF inhibition enter a "drug tolerant persister" (DTP) state with balanced rates of division and death, termed "idling." Here, we use single-cell barcoding, single-cell transcriptomics, and bulk epigenomics to show that idling DTP populations, while more homogeneous than untreated populations, still comprise multiple epigenetic states. Furthermore, gene ontology analyses point to ion channel dysregulation in idling DTPs, confirmed by calcium flux experiments. We also show that idling DTPs are more prone to ferroptotic cell death than untreated cells, suggesting that secondary treatments targeting idling cells could increase the efficacy of targeted drug treatments.**

***Keywords* — Cancer, melanoma, drug tolerant persisters, targeted therapies, tumor heterogeneity, single-cell RNA-seq, ATAC-seq, gene ontology, ion channels, ferroptosis.**

Cancer is a complex, dynamic disease characterized by intratumoral heterogeneity, which has been implicated in treatment evasion and acquired resistance [1]. Historically, tumor heterogeneity has been viewed through the lens of genetics, i.e., in terms of pre-existing or acquired genetic resistance mutations. Recently, however, non-genetic sources of tumor heterogeneity have received increased attention [2]. Multiple investigators have reported cancer cell subpopulations, broadly termed "drug-tolerant persisters" (DTPs), capable of withstanding drug treatments via non-genetic mechanisms. DTPs have been variously described as quiescent [3] or slow cycling [4] and potential reservoirs from which genetic resistance mutations may emerge [5]. It has been suggested that a window of opportunity may exist before the acquisition of drug resistance to deploy a secondary treatment targeting DTPs [5]. Doing so will require detailed knowledge about the molecular drivers underlying DTPs, which are poorly understood.

Recently, we described a DTP state, termed "idling," in BRAF-mutant melanoma cell populations under prolonged BRAF inhibition [5]. Idling DTPs differ from previously reported DTPs in that idling is a *population state*. Idling cells are distributed across multiple phenotypic states with varying proliferation rates and cell transitions allow the population to achieve a net proliferation rate near zero. Here, we demonstrate using single-cell RNA sequencing that idling DTPs are less heterogeneous than untreated populations but still composed of multiple transcriptomic states. DNA barcoding shows that idling DTPs are composed of cells from across the phenotypic states of the untreated population,

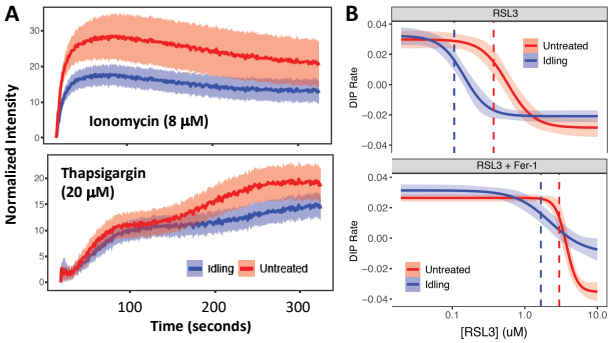

Fig. 1. Calcium flux and dose-response assays for untreated and idling melanoma cells. (A) Calcium flux assays for the agonists Ionomycin and Thapsigargin show reduced Ca^{2+} flux in idling vs. untreated cells, indicating possible impairment of store operated calcium entry (SOCE). (B) Dose responses for untreated and idling cell populations treated with RSL3 and RSL3+ferrostatin-1. Ferrostatin-1 restores insensitivity to ferroptosis induction to idling cells. DIP: drug-induced proliferation.

indicating that idling is not the result of competitive selection of pre-existing drug-tolerant clones. A subsequent gene ontology analysis, based on data from both transcriptomics and epigenomics, indicates that ion channel activity is significantly altered in idling cells, pointing to a role for mitochondrial metabolism. This is supported by calcium flux assays that show store-operated calcium entry (SOCE) is significantly altered in idling cells (**Fig. 1A**). We also show that idling DTPs have increased susceptibility to ferroptosis (**Fig. 1B**), an alternative form of programmed cell death, supporting the notion that the reduced heterogeneity of DTPs due to drug pressure could be leveraged clinically via a targeted secondary treatment.

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