TITLE

Purinergic calcium signaling drives drug tolerance through ERK reactivation in BRAF-mutant melanoma

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SUMMARY

We report a previously unrecognized signaling mechanism underlying drug tolerance in BRAF-mutant melanoma treated with BRAF inhibitors. Its key feature is sustained intracellular Ca^{2+} signaling initiated by purinergic ligand-gated cation channels, P2X receptors. Src family kinases act as mediators for cytoplasmic Ca^{2+} spikes to activate ERK1/2, well-known to support cell survival and proliferation. An intriguing feature of this network is that extracellular ATP, virtually ubiquitous in living systems, is the ligand that can initiate Ca^{2+} spikes via P2X channels. ATP is abundant in the tumor microenvironment and is released by dying cells, thereby implicating the death of drug-sensitive cells as a source of trophic stimuli that leads to ERK reactivation and drug tolerance. Such a mechanism immediately offers an explanation of the inevitable relapse after BRAFi treatment in BRAF-mutant melanoma.

INTRODUCTION

Drug tolerance, originally described in bacteria treated with antibiotics [1,2], is recognized as a major challenge to cancer treatment, particularly with molecularly targeted agents. It is considered a major cause of residual disease, tumor relapse and treatment failure [3–8]. Drug-tolerant cells survive treatment by non-genetic means, presumably via phenotypic adaptation [9–13]. Therefore, tolerance is distinct from resistance, which is based on pre-existing or acquired genetic mutations. The drug-tolerant state is thought to be reversible [4,14,15], and it has been proposed that redirecting drug-tolerant cells into non-tolerant states across the phenotypic landscape should be possible [10,16], such as by epigenetic modulators [14,17]. In spite of intensive efforts [6,7,15,18–23], the molecular basis for drug tolerance remains poorly understood, hampering actionable intervention.

It is widely recognized that drug tolerance to targeted therapies, such as BRAF inhibitors (BRAFi), requires time for non-genetic adaptation to occur [4,8,14,24]. However, major gaps in knowledge remain, pertaining to the dynamics and consequences of adaptive processes (see Discussion). To complicate matters, it has been convincingly shown that drug tolerance is a heterogeneous collection of distinct cell states [8,25–27].

We previously described a distinct form of drug tolerance in BRAFi-treated BRAF-mutant melanoma, termed idling [28,29]. After surviving initial drug exposure, these drug-tolerant melanoma cell populations enter an idling state characterized by low, approximately equal rates of stochastic division and death events, resulting in approximately zero net growth of the population. In these idling cell populations, oncogenic BRAF stays inhibited [28,30], but since cell division still occurs sporadically the cell population as a whole can be considered drug-tolerant. We proposed that these idling melanoma cell populations may be responsible for “residual disease” after BRAFi targeted therapy, which is thought to be a prelude to the acquisition of resistance-conferring mutations that ultimately defy treatment [8,31,32].

Here, we report that, unexpectedly, purinergic signaling underlies drug tolerance in idling melanoma cell populations. Our data indicate that extracellular ATP (eATP), known to be abundant in the tumor microenvironment, trigger cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) spikes in BRAFi treated melanoma cells, through ATP-gated cation channels. We demonstrate that purinergic signaling is then responsible for promoting viability and cell division by a series of steps that

lead to re-activation of ERK, at least in part, via Src-family kinases (SFK). To the best of our knowledge, this mechanism has not been described before and could produce actionable insights into drug tolerance.

RESULTS

Purinergic Ca^{2+} signaling is upregulated in idling drug tolerant cells.

Previous findings by our lab implicated changes in ion channel expression and Ca^{2+} signaling as a mechanism of adaptation and drug tolerance to BRAFi in BRAF-mutant melanoma [30]. While ion channels and Ca^{2+} signaling have been studied in the context of cellular physiology and to a lesser extent in cancer [33–38], no studies have directly explored their potential involvement in drug tolerance. To investigate this possibility, we first measured gene expression changes in a panel of BRAF-mutant melanoma cell lines in the idling drug-tolerant state (8-days under BRAFi conditions). To confirm the previously described effects of continuous BRAFi, we assured expected reduction in MEK-dependent genes associated with oncogenic BRAF activity [39] and increased expression of genes responsible for melanocyte differentiation [40,41]. The surviving cell population exhibited significant upregulation of numerous genes involved in Ca^{2+} signaling (Fig. 1A and Supplementary Figs. S1–3). Notably, the ATP-gated, monoatomic cation conducting channel Purinergic Receptor P2X7 (gene name P2RX7) was one of the highest upregulated genes, with a >250 fold increase at the mRNA level in drug-tolerant A375 cells and >6 fold across all drug-tolerant cell lines combined (Figs. 1A, 1B, and Supplementary Figs. S1–3), which was confirmed at the protein level by flow cytometry (not shown).

P2X7 belongs to a family of ionotropic (P2X) and metabotropic (P2Y) purinergic receptors that have been studied extensively in the context of the nervous system and the immune system, largely with respect to cellular signaling, inflammation, and inflammatory pathologies [42–44]. Purinergic signaling, however, has been broadly overlooked in the context of oncogenic BRAF activity. Nonetheless, the Human Protein Atlas and The Cancer Genome Atlas report a trend for P2RX7 enrichment at both protein and transcript levels in skin and melanoma tissues (Supplementary Fig. S4) [45,46], suggesting this signaling network is of general interest to the field. Indeed, a potential role for purinergic signaling in cancer is immediately obvious due to elevated ATP content in the tumor microenvironment. While the topic has gained traction in recent years [36], it is still understudied, and the role purinergic signaling may play in the drug-tolerant phenotype has never been explored. This, along with our previous observations of dysregulated Ca^{2+} handling [30], prompted us to further explore the contribution of Ca^{2+} signaling to drug tolerance. Measuring changes in $[Ca^{2+}]_{cyt}$ using the Ca^{2+} dye, Fura-2-AM, revealed abundant spontaneous spikes of $[Ca^{2+}]_{cyt}$ within a 60 min observation time in approximately 45% of the idling population (Fig. 1C). No such spikes were detected in any of the drug sensitive cells in the absence of BRAFi or after 60 minutes BRAFi, indicating that this is a time-dependent adaptive response that coincides with the acquisition of drug tolerance.

Broadly, these $[Ca^{2+}]_{cyt}$ spikes may be from intracellular or extracellular Ca^{2+} sources, or a combination of both. Ligand activation of plasma membrane receptors may lead to $[Ca^{2+}]_{cyt}$ release from intracellular Ca^{2+} stores in the endoplasmic reticulum (ER) [47]. Alternatively, $[Ca^{2+}]_{cyt}$ spikes could be a result of influx of extracellular Ca^{2+} ($e[Ca^{2+}]$) through plasma membrane ion channels, such as P2X receptors. To specifically explore whether these $[Ca^{2+}]_{cyt}$ spikes originate from P2X receptors we imaged idling cells as before with the addition of a P2X inhibitor (iso-PPADS) and found that approximately 50% fewer idling cells experienced $[Ca^{2+}]_{cyt}$ spikes (not shown), demonstrating that P2X signaling is a major mechanism of these BRAFi induced $[Ca^{2+}]_{cyt}$ spikes. Importantly, removal of $e[Ca^{2+}]$ diminished spontaneous $[Ca^{2+}]_{cyt}$ spikes to approximately 5% of the idling population (not shown), providing additional support for the importance of plasmalemma Ca^{2+} flux. Evidence against a role for release of intracellular stores of Ca^{2+} include that inositol trisphosphate (IP_3) receptor expression levels are significantly reduced in idling cells (Supplementary Fig. S5), and that drug-tolerant cells have reduced intracellular Ca^{2+} stores and greater store-operated Ca^{2+} entry (SOCE) than drug sensitive cells (Supplementary Figs. S6–8). Taken together, these results indicate that entry of $e[Ca^{2+}]$ rather than release of intracellular Ca^{2+} stores is responsible for generating these $[Ca^{2+}]_{cyt}$ spikes.

Our studies have thus far indicated that adaptation to BRAFi treatment alters multiple axes of Ca^{2+} signaling, which could be relevant to the drug-tolerant state. Moreover, this effect is not limited specifically to BRAFi since the $[Ca^{2+}]_{cyt}$ spikes persisted (39% of idling population) despite co-treatment with a MEK inhibitor (BRAFi + MEKi), a clinically relevant combination treatment (not shown). Altogether, these findings demonstrate drug-tolerant melanoma cells

overexpress purinergic receptor P2X7 by non-genetic adaptation and that We should P2X7 receptors are functionally active in inducing $[Ca^{2+}]_{cyt}$ spikes, likely due to greater ATP concentration in the microenvironment of BRAFi treated cells.

Purinergic Ca^{2+} signaling is a major mechanism of ERK reactivation in drug-tolerant cells

We then directed our attention to possible molecular mechanisms that may link these P2X-initiated $[Ca^{2+}]_{cyt}$ spikes to drug tolerance. ERK (re)activation is thought to be essential for drug tolerance because it is well known to promote cell survival and proliferation [48,49]. Ca^{2+} signals have been described to activate the MAPK pathway in many cell types [17,50–60], though it has never been considered in the context of drug tolerance. By performing single-cell Ca^{2+} measurements on idling cells, followed by fixation and staining for phospho-ERK T202/Y204 (ppERK), we found a positive correlation between $[Ca^{2+}]_{cyt}$ spikes and ppERK staining (not shown). Co-treatment with the P2X inhibitor iso-PPADS reduced the proportion of cells staining for ppERK by nearly 66% and reduced the intensity of ppERK staining in individual cells (Fig. 1D and not shown). Notably, approximately the same proportion of cells that lost $[Ca^{2+}]_{cyt}$ spikes also lost ppERK staining (at 2 hours), as would be expected from a causal relationship.

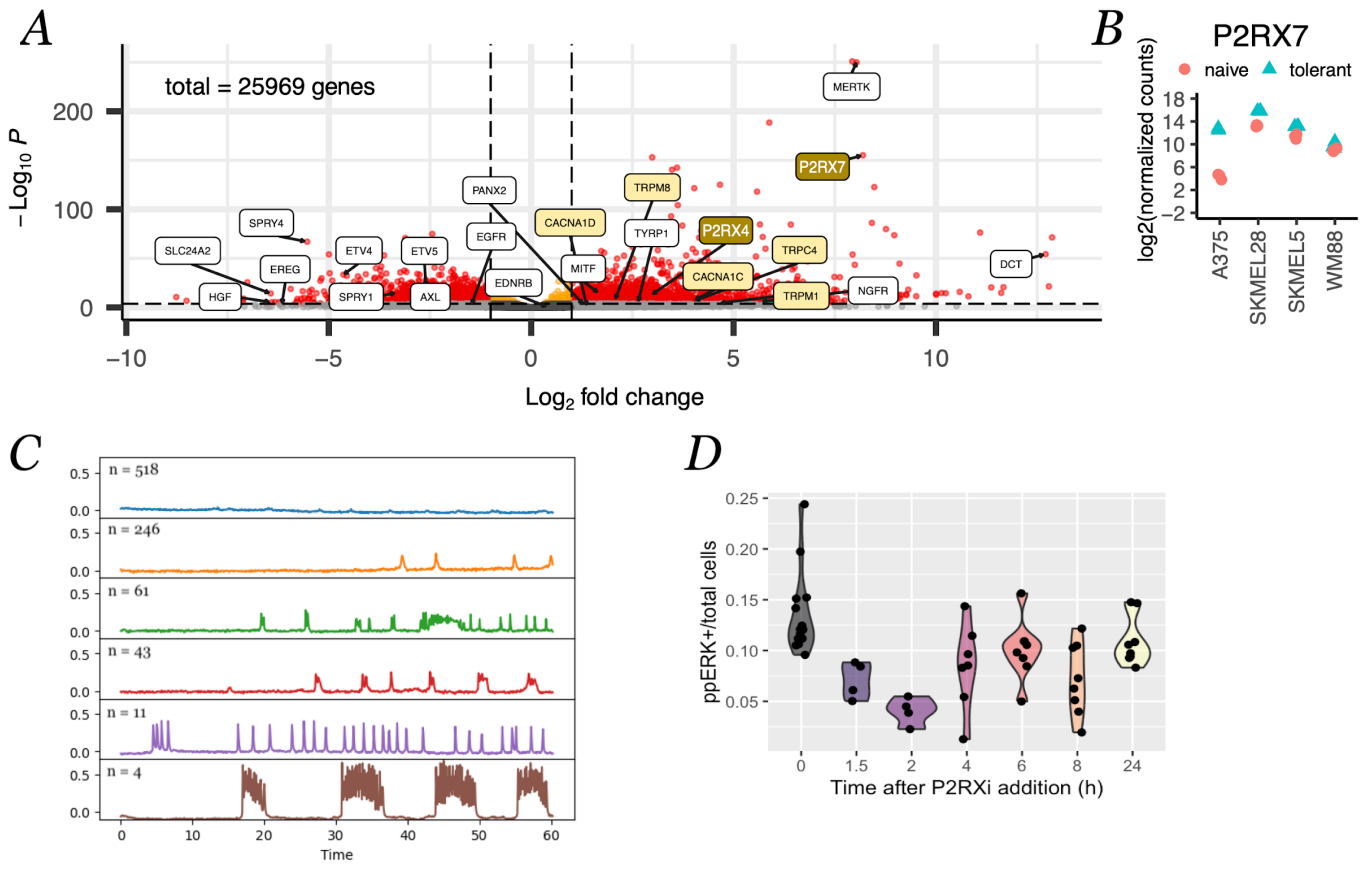

Figure 1. Purinergic signaling activity and ERK reactivation in drug-tolerant melanoma cells in the presence of BRAFi. *A*) Volcano plot of up- and down-regulated genes in melanoma cells under BRAFi conditions for 8 days, a treatment previously shown to induce an idling population of drug-tolerant cells [28,30]. Data are from bulk RNAseq of a representative cell line, A375, out of four that were tested (all data combined are shown in Supplementary Figures S1–3). Transcripts for the Ca2+ channel P2RX7 (upper left) are enriched by 250 fold, with a p value of $< 10^{-150}$. *B*) Log2 fold change in expression of P2RX7 transcripts before and after BRAFi treatment (8 days) in four BRAF-mutant melanoma cell lines. *C*) Representative whole cell Ca2+ traces recorded in drug tolerant cells (8 day BRAFi) over a 45 minute time period. Cells are grouped by an unsupervised classification algorithm and the number of cells that fall into each group is indicated for the representative traces of each group. *D*) phospho-ERK staining intensity in drug tolerant cells after treatment with the P2RX inhibitor, iso-PPADS. P2RXi was applied at indicated time points before fixation with PFA; that is all conditions were fixed at the same time. Time 0 represents vehicle control with no P2RXi added. Cells were fixed after 5 days of BRAFi.

Ca^{2+} mediated activation of ERK is potentiated in drug-tolerant cells

Previous work has demonstrated an increased ability of receptor tyrosine kinases to potentiate ERK activation in the presence of BRAFi due to the release of negative feedback [39]. This is an accepted mechanism of BRAFi bypass that underscores the adaptability of melanoma cells to treatment, presumably in a non-genetic fashion. Similarly, purinergic Ca^{2+} signaling in BRAFi tolerant cells may be a crucial and parallel component of this mechanism and likely reflects changes in cell states that lead to efficient Ca^{2+}-mediated reactivation of ERK. To directly test this possibility, we compared short- (60 minutes) versus long-term (3 days) BRAFi treatment, followed by a time-course exposure to the Ca^{2+} ionophore ionomycin, which drives e[Ca^{2+}] into the cytoplasm. Cells were then immunostained for ppERK to quantify Ca^{2+}-mediated activation of ERK. We found that in drug-tolerant cells, ERK phosphorylation began climbing at lower concentrations of ionomycin, reached much higher levels at a higher rate, and stayed elevated longer than drug sensitive cells (Fig. 2A and Supplementary Fig. S8). Furthermore, the maximum percentage of cells staining for ppERK in the long-term treated population (>40%) was higher than the short-term treated population (<25%) (Fig. 2A). Taken together, these data support the conclusion that cells that acquire tolerance over a period of days have an enhanced ability to activate the MAPK pathway in the presence of Ca^{2+} signaling.

We then asked if ATP, a physiological agonist of P2X receptors, could elicit similar results as ionomycin. Even after prolonged treatment with BRAFi that resulted in greater levels of basal ppERK levels, drug-tolerant cells respond to ATP with increased activation of ERK, whereas drug-sensitive cells do not (Fig. 2B). In addition, drug-tolerant cells experience greater Ca^{2+} flux and exhibit sustained high cytoplasmic Ca^{2+} levels in response to ATP stimulation, as would be expected in cells with increased expression of P2X receptors (Fig. 2C), although other possible explanations include differential channel gating (i.e., increased open probability) by post-transcriptional (e.g., alternative splicing) or post-translational mechanisms [61]. In addition, sustained $[Ca^{2+}]_{cyt}$ levels may partially be caused by reduced Ca^{2+} clearance in drug-tolerant cells (Supplementary Fig. S9), which is consistent with reduced expression of plasma membrane Ca^{2+} pumps (which pump $[Ca^{2+}]_{cyt}$ out of the cell) (Supplementary Fig. S10).

Taken together, these results indicate that drug-tolerant cells acquire enhanced ability to reactivate ERK via purinergic Ca^{2+} signaling. Furthermore, this endogenous mechanism of ERK reactivation may be particularly relevant in the tumor microenvironment, which is known to be ATP-rich [36]. In direct support of this, we found that cell lysate stimulated an increase in ppERK, similar to ATP and ionomycin (Supplementary Fig. S11).

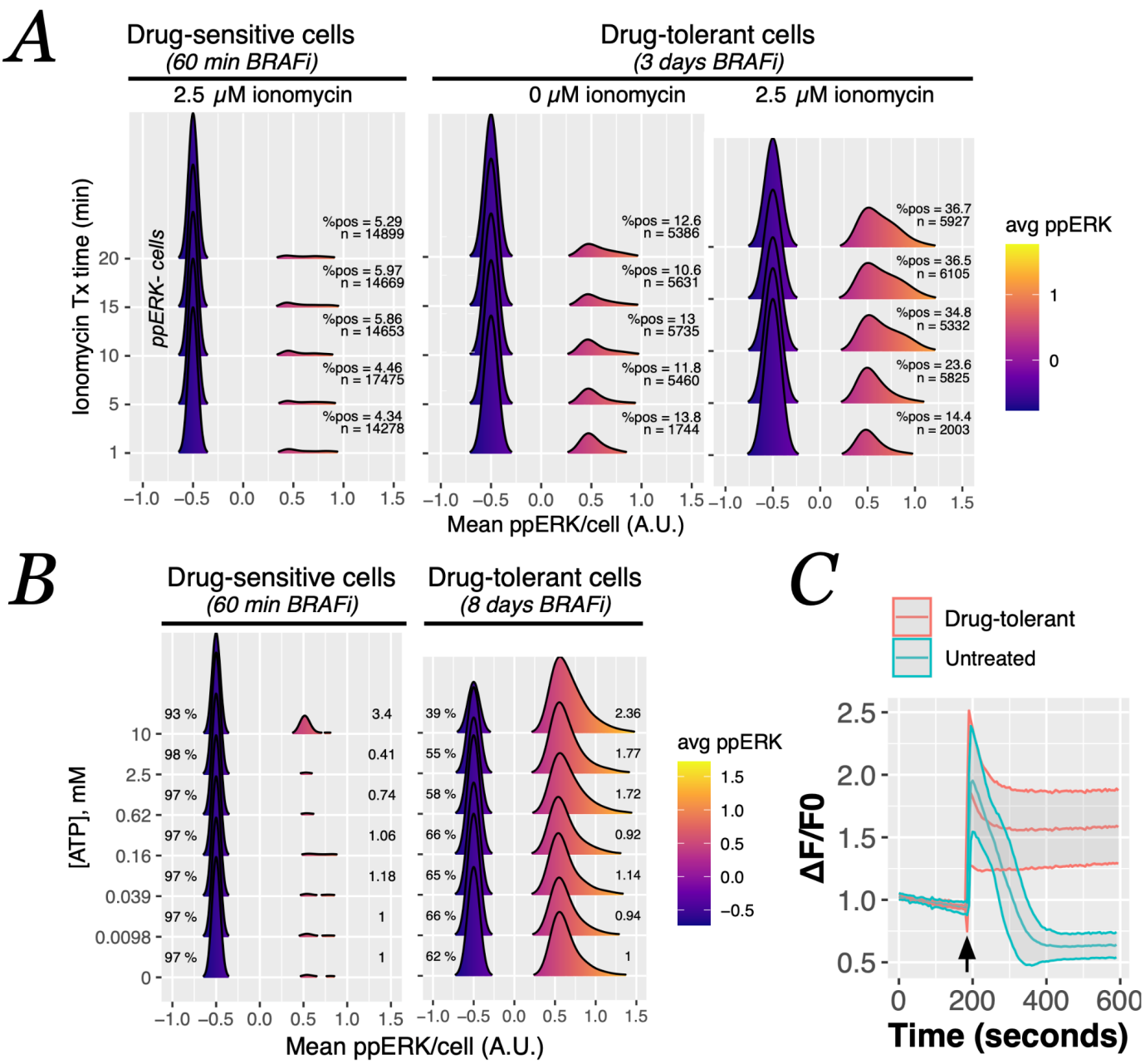

Figure 2. Increased $[Ca^{2+}]_{cyt}$ induced by eATP rapidly activates ERK. Immunofluorescence detection of dual-phosphorylated ERK (ppERK) was performed on drug-sensitive (60 min treatment with BRAFi) and drug-tolerant (3 day or 8 day treatment with BRAFi) cells in the presence of the calcium ionophore ionomycin (iono) (*A*) and eATP (*B*). Cells without detectable ppERK staining were given a value of –0.5 and the minimum ppERK level in the positive cells was set to 0. The percentage of positive cells and mean ppERK intensity per cell increased in a time-dependent manner after the addition of ionomycin and ATP in drug-tolerant cells but not in drug-sensitive cells. (*C*) 10 mM ATP was added (indicated by the black arrow) to drug-tolerant (8 days BRAFi) or untreated cells and $[Ca^{2+}]_{cyt}$ was measured using the ratio of fura-2 fluorescence at 340 nm over 380 nm and normalized to the baseline measurement (ΔF/F0).

Ca^{2+} signaling activates ERK by signaling through SFK

To better understand mechanisms behind the increased sensitivity to Ca^{2+} signaling in drug-tolerant cells, we asked which kinases are involved in the transduction of $[Ca^{2+}]_{cyt}$ spikes to ppERK and what differences may exist between drug-tolerant and sensitive states. Ca^{2+}-dependent activation of ERK has been shown to be mediated by various classes of kinases, including SFK, PKC, PI3K, CaMK2, and RAF, each of which can lead to the activation of MEK1/2, the dual specificity kinase responsible for phosphorylating ERK at T202/Y204 [50–60,62–69]. We reasoned that if a kinase is involved, inhibiting that kinase would reduce ionomycin-induced phosphorylation of ERK. Therefore, we applied inhibitors

targeting classes of kinases known to transduce Ca^{2+} signals to the MAPK pathway before applying conditions of ionomycin treatment that strongly activate ppERK. If a kinase is not involved in the transduction, then inhibitors should not reduce ppERK staining compared to vehicle control, and vice versa.

As expected due to its role as the immediate upstream kinase of ERK, inhibition of MEK1/2 reduced ppERK staining to near background levels (Fig. 3A). A well-studied mechanism of Ca^{2+}-mediated activation of ERK is through CaMK2 activation of CRAF dimers that transduce the signal through MEK and ERK[55,64]. However, targeting these kinases did not reduce ppERK staining (not shown), suggesting this pathway is not involved in drug tolerance. Other findings [50,59,69,70] suggest kinases such as PI3K, PKC, or SFKs may be responsible for the transduction of Ca^{2+} to ERK, but inhibitors to PI3K and PKC were unable to prevent Ca2+ mediated activation of ERK (Fig. 3A and not shown). SFK inhibitors, on the other hand, were able to reduce Ca^{2+}-mediated phosphorylation of ERK in drug-tolerant but not sensitive cells (Fig. 3B and Supplementary Fig. S12), implicating one or more SFKs. Therefore, our data indicate Ca^{2+} mediated activation of ERK in the drug-tolerant state is dependent on SRC activity.

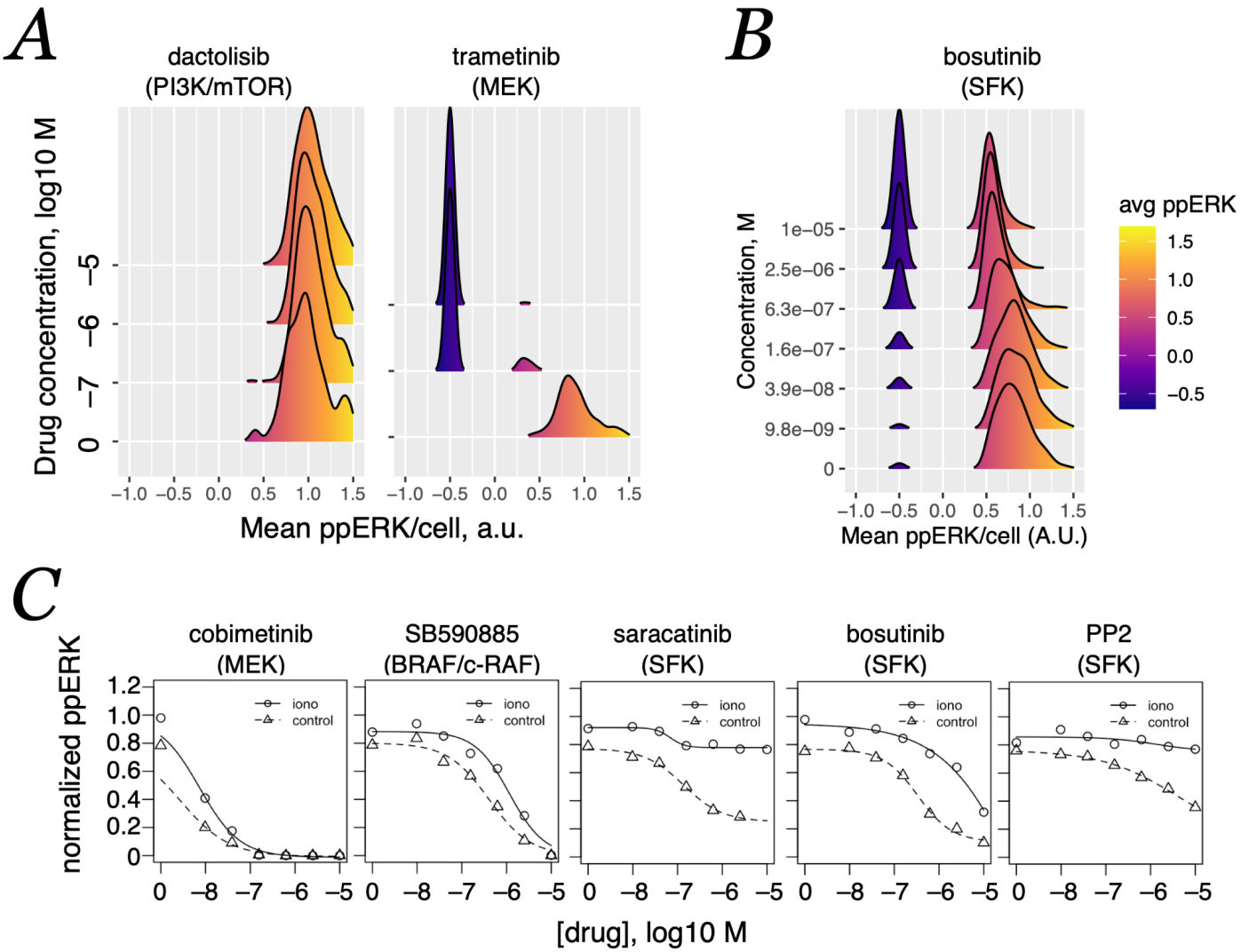

Figure 3. Drug-tolerant cells more efficiently transduce Ca2+ signaling to ERK activation. A and B) Cells were pre-incubated with indicated kinase inhibitors (30 minutes) prior to Ca^{2+} mobilization with ionomycin (10 uM, 10 minutes), in order to maximize Ca^{2+} mediated activation of ppERK. ppERK staining represented as in Figure 2. Average ppERK/cell is visualized by the heatmap on the right, on an arbitrary scale. No ppERK negative cells are detectable with inhibitors of pathways not involved in Ca^{2+}-mediated ERK phosphorylation. Dactolisib is a representative one, see Supplementary Information for additional pathway inhibitors. In contrast, significant amounts of ppERK negative appear in several SFK inhibitors (bosutinib is shown here, see Supplemental for others), and as expected with an inhibitor of the MAPK pathway.

DISCUSSION

In summary, our data identify a previously unrecognized signaling network responsible for ERK reactivation in drug-tolerant cells. The key feature of the network is increased output of Ca^{2+} signaling initiated by Ca^{2+} flux through ionotropic purinergic P2X receptors, which are utilized by the drug-adapted MAPK signaling network, with SFKs as mediators to ppERK. An intriguing feature of this network is that ATP, which is virtually ubiquitous in living systems, is the ligand that can initiate $[Ca^{2+}]_{cyt}$ spikes via P2X receptors. ATP is abundant in the tumor microenvironment and is released by dying cells, thereby implicating the death of drug sensitive cells as a source of trophic stimuli that leads to ERK reactivation and drug-tolerant behavior. Such a mechanism immediately offers an explanation of the inherent efficacy issues of BRAFi treatment in BRAF-mutant melanoma. Moreover, Pannexin-2, also enriched in drug-tolerant cells (>2 fold) may form a cell-to-cell positive feedback loop amongst adjacent drug-tolerant cells, since it reinforces purinergic receptor activation by ATP release. It is tempting to speculate ATP may be the diffusible ligand responsible for ppERK pulses in drug-tolerant melanoma cell clusters, as described by Gerosa et. al. [71]

Previous reports have linked melanoma tolerance to BRAFi with RTK activity (e.g., AXL, FGFR, NGFR, EGFR [23,72]) and ERK reactivation [71,73]. However, no clinically effective RTK combination in BRAF-mutant melanoma has been identified (although in BRAF-mutant colorectal cancer [74,75] that combination is an option, underscoring the possibility of a variety of context-dependent mechanisms of MAPK reactivation). On the whole, these RTK-based mechanisms of ERK reactivation are ligand dependent, raising the question of possible exogenous sources for these ligands *in vitro* and *in vivo*. In contrast, in the ppERK reactivation pathway we describe here, the ATP ligand may be released by cells dying from BRAFi, which can then be used for survival by neighboring cells to reactivate ERK via P2X receptor $[Ca^{2+}]_{cyt}$ spikes through SFKs. Additionally, it is possible that P2X7 signaling activity could also underlie RTK signaling activity that may occur in the drug-tolerant state [50,58,68,76–83].

It has been convincingly shown that drug tolerance is a heterogeneous collection of distinct cell states [8,25–27,84]. For instance, Raj and co-workers described a jackpot effect [23,73], whereby a stochastic combination of non-mutational (i.e., epigenetic) events generates rare cells in BRAF-mutant melanoma cultures under BRAFi conditions. These jackpot cells are implicated in ERK reactivation-dependent escape from BRAFi and appear in culture within a time frame similar to the idling tolerant cell state we describe. However, by definition the jackpot cells are a rare occurrence. In contrast, the drug tolerance we describe here is a cell population phenomenon. That is, the population as a whole adapts to the BRAFi stress, and no selection of rare clones takes place[30]. Thus, idling and jackpot effects are distinct, although possibly related. Spencer and co-workers described drug-tolerant cells that divide in the presence of BRAFi tend to have increased ER stress response signaling in comparison to non-dividing cells. In addition, ER stress-driven autophagy has previously been shown to be a survival mechanism utilized by melanoma cells under therapeutic treatment [85,86]. ER stress signaling can be induced by insufficient ER Ca^{2+} stores, and our data indicate that drug-tolerant cells have diminished ER Ca^{2+} content. While it remains to be explored, one can postulate that these signaling pathways are related.

In normal skin, UV light-induced ATP release from keratinocytes activates P2X7 receptors on melanocytes to induce melanogenesis and melanosome transfer to keratinocytes [87]. It remains to be determined whether the increased expression of P2RX7 in melanoma under BRAFi may be related to this physiological function, but BRAFi is known to induce melanocytic differentiation and a range of melanocytic differentiation states have been described across different melanomas by Heath and coworkers [15]. Moreover, this possible relationship may be reflected in the relatively high but wide range of P2RX7 transcript abundance in melanoma compared to other tumor types (Supplementary Fig. S4).

Purinergic signaling is an expansive area of active research. It is especially studied in the nervous system, where it can participate in synapse formation, action potentials, and neuropathologies. In the immune system, purinergic receptors and associated surface molecules modulate T cell activation and are a key player in inflammation[36]. In comparison, purinergic signaling has been less studied in cancer, although it has gained attention in recent years. Di Virgilio and co-workers reviewed immunosuppressive effects and modulation of host–tumor cell interactions mediated via purinergic receptors by ATP in the tumor microenvironment [36]. Here, we put forward the novel perspective that

ATP-driven activation of P2X receptors may be an adaptive mechanism of escape from targeted therapy (e.g., BRAFi), by promoting the rise of drug-tolerant cells.

Recently, P2X7 activation was associated with improved therapy responses in NRAS-mutant melanoma [88], presumably by reducing the rise of resistant cells. This is contrary to the role of purinergic signaling in BRAF-mutant melanoma that enhances drug tolerance and cell survivability to BRAFi therapy. It is nonetheless possible that these discordant findings point to fundamental distinct biology of mutations occurring at different steps in the MAPK pathway. In any case, more work is needed to reconcile these apparently conflicting observations. For instance, it is worth determining whether in one case there is much higher P2X7 activation than the other, such that the level of P2X7 activation and thus Ca^{2+} flux could cause opposite outcomes. Indeed, our findings indicated that excessive ATP stimulation in drug tolerant cells reduces viability (not shown), which is in agreement with prior studies showing prolonged exposure of cells to high concentrations of ATP are required to induce cell death [89].

Although our results implicate P2X7 as a primary driver of the increased Ca^{2+} signaling in drug tolerance, other Ca^{2+} regulatory genes are likely to contribute to the cellular responses. For example, P2X-induced $[Ca^{2+}]_{cyt}$ spikes may initiate Ca^{2+}-induced Ca^{2+} release through ryanodine receptors on the ER, which are facilitated by low-voltage-gated calcium channels (CACNA1C and CACNA1D) that are known to activate RYR in heart muscle. The increased release of ER Ca^{2+} in response to $[Ca^{2+}]_{cyt}$ spikes could explain the reduced ER Ca^{2+} content and be a contextual difference that explains the apparently discordant findings in NRAS and BRAF mutant melanoma.

A limitation of our studies is the exclusive focus on ERK reactivation as a consequence of purinergic-induced $[Ca^{2+}]_{cyt}$ spikes. We recognize the plethora of cellular functions in which Ca^{2+} plays a role, including bioenergetics, metabolism, stress response, and cell cycle progression [47]. These areas and others may be the subject of future work, since they may also have an impact on the generation of tolerant cells. Further, it will be interesting to see if Ca^{2+} signaling has similar influences in other oncogene addicted cancers where targeted inhibitors have proven disappointing. Indeed, such a role in SCLC has already been explored[76].

The mitogenic signaling architecture of drug-tolerant cells appears to be uniquely adapted to efficiently use Ca^{2+} signaling. This property suggests that targeting Ca^{2+} signaling may be key to increasing durability of treatment while maintaining enough target specificity for treatment tolerability. In preliminary experiments, pharmacologically inhibiting SOCE (Store Operated Calcium Entry) activity resulted in increased efficacy of BRAFi. Similar approaches are desirable because they could reduce patient suffering in comparison to the standard-of-care combinations with MEK inhibitors, which come with significant side effects. Indeed, clinical trials demonstrate P2X receptor inhibitors are well tolerated in patients, providing confidence that targeting P2X signaling in combination with BRAFi could be a promising strategy to combat drug tolerance.

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