Peroxisome disruption alters lipid metabolism and potentiates anti-tumor response with MAPK-targeted therapy in melanoma

Fan Huang, … , Wilson H. Miller Jr., Sonia V. del Rincón


Graphical abstract

Find the latest version:

https://jci.me/166644/pdf
Peroxisome disruption alters lipid metabolism and potentiates anti-tumor response with MAPK-targeted therapy in melanoma

Authors:
Fan Huang\textsuperscript{1,2}, Feiyang Cai\textsuperscript{1,2}, Michael S. Dahabieh\textsuperscript{1,2}, Kshemaka Gunawardena\textsuperscript{1}, Ali Talebi\textsuperscript{4}, Jonas Dehairs\textsuperscript{4}, Farah El-Turk\textsuperscript{5,6}, Jae Yeon Park\textsuperscript{5}, Mengqi Li\textsuperscript{1,2}, Christophe Goncalves\textsuperscript{1}, Natascha Gagnon\textsuperscript{1}, Jie Su\textsuperscript{1}, Judith H LaPierre\textsuperscript{1,2}, Perrine Gaub\textsuperscript{7}, Jean-Sébastien Joyal\textsuperscript{7}, John J Mitchell\textsuperscript{5}, Johannes V Swinnen\textsuperscript{4}, Wilson H. Miller Jr.\textsuperscript{1,2,3}, Sonia V. del Rincón\textsuperscript{1,2,3}\textsuperscript{*}

Affiliations:
\textsuperscript{1} Lady Davis Institute, McGill University, Montreal, QC, Canada
\textsuperscript{2} Department of Experimental Medicine, McGill University, Montreal, QC, Canada
\textsuperscript{3} Department of Oncology, McGill University, Montreal, QC, Canada
\textsuperscript{4} Laboratory of Lipid Metabolism and Cancer, Department of Oncology, KU Leuven and Leuven Cancer Institute (LKI), Belgium
\textsuperscript{5} McGill University Health Centre, Montreal, QC, Canada
\textsuperscript{6} Centre Hospitalier Universitaire Sainte Justine, Montreal, QC, Canada.
\textsuperscript{7} Centre de Recherche, CHU St. Justine, Montréal, QC, Canada.

Correspondence: Sonia V. del Rincón
Address: E-537, Lady Davis Institute for Medical Research, 3755 Chemin de la Côte-Ste-Catherine, Montréal, Québe, Canada, H3T 1E2.
Phone: (514) 340-8222, ext. 22246
E-mail: soniavictoria.delrincon@mcgill.ca

Conflicts of interest: The authors have declared that no conflict of interest exists.
Abstract:

Melanomas reprogram their metabolism to rapidly adapt to therapy-induced stress conditions, allowing them to persist and ultimately develop resistance. We report that a subpopulation of melanoma cells tolerate MAPK-pathway inhibitors (MAPKi) through a concerted metabolic reprogramming mediated by peroxisomes and UDP-glucose ceramide glycosyltransferase (UGCG). Compromising peroxisome biogenesis, by repressing PEX3 expression, potentiated the pro-apoptotic effects of MAPKi via an induction of ceramides, an effect limited by UGCG-mediated ceramide metabolism. Co-targeting PEX3 and UGCG selectively eliminated a subset of metabolically active, drug-tolerant CD36+ melanoma persister cells, thereby sensitizing melanoma to MAPKi and delaying resistance. Increased levels of peroxisomal genes and UGCG were found in patient-derived MAPKi-relapsed melanomas, and simultaneously inhibiting PEX3 and UGCG restored MAPKi sensitivity in multiple models of therapy resistance. Finally, combination therapy comprised of a newly identified inhibitor of the PEX3-PEX19 interaction, a UGCG inhibitor and MAPKi demonstrated potent anti-tumor activity in pre-clinical melanoma models, thus representing a promising approach for melanoma treatment.

Keywords: Peroxisome; melanoma; targeted therapy; resistance; lipid metabolism; CD36
**Introduction:**

Melanoma is the deadliest form of skin cancer. Current treatments for patients with metastatic melanoma include therapies that target the MAPK pathway (BRAF and MEK inhibitors) and immunotherapies targeting immune checkpoints. However, as with all therapies, MAPK- and immune-targeted treatments have their limitations, often with patients being intrinsically resistant or developing resistance (1). Expanding the toolbox of treatments available for patients with melanoma requires a thorough understanding of the underlying biology of this disease.

Melanomas typically exhibit high intratumoral heterogeneity and phenotype plasticity, which ultimately promote MAPKi-resistance, as a combined result of clonal evolution (MAPKi-mediated cell selection) and the emergence of phenotypically and metabolically distinct cell states (2, 3). Metabolic reprogramming is a mechanism through which subsets of melanoma cells adapt to microenvironmental cues and MAPK-targeted therapy (4-8). PGC1α-mediated mitochondrial oxidative phosphorylation (OXPHOS) and PPARα-mediated fatty acid oxidation (FAO) are two metabolic pathways that have emerged as promising targets to overcome therapy resistance in melanoma (5-8).

Peroxisomes are highly specialized membrane-bound organelles with vital metabolic functions including beta-oxidation of very-long chain fatty acids (VLCFAs), biosynthesis of C24-bile acids and ether-phospholipids (EPLs), and reactive oxygen species (ROS) metabolism. In mammalian cells, the peroxins PEX3, PEX16, and PEX19 are required for de novo biogenesis of peroxisomes (9, 10). Patients with peroxisomal disorders often exhibit impaired lipid metabolism (11). Moreover, recent studies suggest that dysregulated sphingolipid metabolism, marked by increased levels of ceramide and altered sphingomyelin abundance, might be a potential biomarker of peroxisomal disorders (12-15), indicating a previously underappreciated role of peroxisomes in sphingolipid metabolism. Moreover, emerging evidence highlights the peroxisome as playing significant roles in maintaining human health, with altered peroxisome functions impacting an expanding list of diseases including cancer (16). Although there has been a growing appreciation for the roles of EPLs and ceramides in melanoma progression, stress response, and drug sensitivity (17, 18), whether peroxisomes play an essential role in these processes remains largely underexplored.
Herein, we sought to investigate the role of peroxisome-mediated lipid metabolism in melanoma response and resistance to MAPK inhibition. We chose genetic inhibition of PEX3 as a mode to disrupt peroxisome biogenesis in a panel of melanoma cells harbouring different genetic driver mutations. Reduced PEX3 expression in melanoma cells potentiated their response to MAPK inhibition in vitro and in vivo. Using mouse models and lipidomic analysis, we showed that peroxisome-deficient melanoma cells relied on the UDP-Glucose Ceramide Glucosyltransferase (UGCG)-mediated ceramide metabolism for survival. Interrogating a previously published single-cell RNA-sequencing (scRNA-seq) dataset (19), we identified a CD36\(^+\) cell state population with dependence on peroxisome/UGCG-mediated metabolic rewiring for MAPKi tolerance. Finally, we identified an inhibitor of PEX3-PEX19 binding and showed that it worked in concert with a UGCG inhibitor to potentiate melanoma response to MAPK-targeted therapy.

**Results:**

**Compromising peroxisome biogenesis potentiates melanoma cell response to MAPK-targeted therapies.**

Continuous exposure to MAPK-targeted therapies, such as BRAF and MEK inhibitors, can trigger metabolic reprogramming to promote cell state transitions, known as phenotype plasticity, enabling melanoma cells to persist, ultimately contributing to disease relapse. Given the emerging role of peroxisomes in cancer, we posited that peroxisome biogenesis and lipid metabolism might change in melanoma cells in response to MAPKi. We re-analyzed publicly available RNA-seq data from four studies assessing transcriptomic alterations in patient samples collected before and after MAPK-targeted therapy (20-23). Gene set enrichment analysis (GSEA) revealed that approximately 70% of patients showed an overall induction of peroxisome-associated genes after treatment with BRAF inhibitors (BRAFi) alone or when combined with a MEK inhibitor (MEKi) (Figure 1A, S1A). Conversely, interrogating the same data, less than 50% of patients showed an enriched gene signature involved in OXPHOS (Figure S1A), previously shown to promote MAPKi tolerance in a subset of melanoma cells (5, 24). These in silico-derived data support a potential role of peroxisomes in MAPKi-driven metabolic rewiring, which allows melanoma cells to escape therapeutic pressure.
To formally test the impact of disrupting peroxisome biogenesis on response to MAPKi, we knocked down PEX3 with siRNAs in four human melanoma cell lines harbouring different genetic mutations (Figure 1B) to assess whether this would alter their response to MAPK inhibition. Knockdown of PEX3 significantly decreased the number of peroxisomes in all four melanoma cell lines (Figure S1B), with minimal impact on cell viability (Figure 1B, S1C). We treated BRAFV600E-mutant melanoma cell lines with the BRAFi vemurafenib (vemu) alone or in combination with the MEKi cobimetinib (cobi). The non-BRAF-mutant WM3406 and MeWo cells were treated with cobi alone, since the BRAFi would be ineffective in these melanoma subtypes. PEX3 knockdown sensitized all melanoma subtypes to MAPKi-induced apoptosis, compared with the same cells transfected with scrambled siRNA-control (siCtrl) (Figure 1B, S1C). Similar phenotypes were observed with PEX19 knockdown (Figure S1D). Knockdown of PEX3 alone, or treatment of PEX3 knockdowns with vemu+cobi did not induce apoptosis in the non-malignant melanocyte line termed MelST (Figure 1B) (25).

To model our in vitro results in mice, we sought to knock out Pex3 in the BRAFV600E-mutant melanoma cell line D4M.3a (26). Although none of the 80 clones that we screened showed completed loss of PEX3 expression, we successfully isolated two clones (6D and 9G) with single-allele Pex3 knockout. As expected, characterization of these Pex3−/− cell lines showed decreased levels of PEX3, PEX16, PEX19, and reduced number of peroxisomes (Figure 1C). Similar to our human melanoma cell data (Figure 1B), the D4M.3a Pex3−/− clones were more susceptible to MAPKi-mediated cell death than their Cas9-Ctrl counterparts (Figure 1D). To test the sensitivity of Pex3−/− melanomas to BRAF inhibition in vivo, we next injected D4M.3a Cas9-Ctrl, Pex3−/− clones 6D or 9G cells into syngeneic mice. A significant delay of tumor onset was observed in mice injected with clone 6D or 9G, compared with mice injected with the Cas9-Ctrl cells (Figure 1E), indicating that compromised peroxisome biogenesis impairs the tumor-initiating ability of melanoma cells. Once initiated, the Pex3−/− melanomas grew at a similar rate as the Cas9-Ctrl group (Figure S1E). When melanomas of different genotypes reached a volume of approximately 200mm³, mice were administered a diet containing the vemurafenib analog PLX4720 (27). Mice from the 6D and 9G arms showed a rapid decrease in tumor volume within 48 hours of PLX4720 administration (short-term response, STR), while the Cas9-Ctrl tumors continued increasing in size during this timeframe (Figure 1F). As the study continued, 6D and 9G treatment groups showed a significantly
improved best response (BR) in tumor shrinkage and improved progression-free survival (PFS), compared with the Cas9-Ctrl treatment group (Figure 1G-1H). The increased treatment benefit in mice bearing Pex3+/− melanomas was recapitulated in a separate cohort of mice that were administered PLX4720 chow when their tumors reached 800mm³ in size (Figure S1F-S1H).

**Lipidomic analyses of Pex3+/− D4M.3a melanoma cells reveal altered levels of ceramide-derived lipid species and increased metabolic vulnerability.**

Next, we investigated potential mechanisms through which inhibiting peroxisomes improves MAPKi response in melanoma. Our data suggested that compromised peroxisome biogenesis potentiates MAPKi-induced apoptosis through a mechanism largely independent of alterations in ROS homeostasis and mitochondrial respiration (Figure S2A-S2G).

Given the essential role of peroxisomes in cellular lipid metabolism (Figure 2A), we performed lipidomic analyses using our stable Pex3+/− D4M.3a (6D and 9G) models to assess whether alterations in lipid species underpin the anti-tumor phenotypes observed when peroxisome biogenesis is repressed. Our data revealed that Pex3+/− D4M.3a cells have an increased abundance of phospholipids (PLs), dramatically decreased levels of ether-phospholipids (EPLs), and a slight increase in lysophospholipids (Lyso-PLs) (Figure 2B). We next compared the abundance of each fatty acid-containing species in Pex3+/− cells versus Cas9-Ctrl cells. We found 29 commonly upregulated and 69 commonly downregulated species in Pex3+/− D4M.3a cells (Figure 2D-2E). Similar to the observed changes in their relative concentrations (Figure 2B), 11 PL species were increased and 57 EPL species were decreased in the Pex3+/− cells compared with the Cas9-Ctrl cells (Figure 2E). The increased PL and decreased EPL levels in Pex3+/− D4M.3a cells are consistent with clinical phenotypes detected in patients with peroxisomal disorders and corresponding PEX3 biallelic-mutant cell lines in vitro (28-30). The latter support the robustness of our lipidomic analyses and the Pex3+/− D4M.3a cells as a reliable model to understand the biological implications of altering peroxisome biogenesis.

Interestingly, our lipidomic analysis revealed changes in several sphingolipid species, centering on the synthesis and metabolism of ceramides (Figure 2E-2F, S3A-S3B). We observed increased levels of ceramides
and hexosylceramides (HexCer) (Figure 2E-2F), and decreased levels of dihydroceramides (DCER) and lactosylceramides (LacCer) (Figure S3A). Notably, although several sphingomyelin species (SM) were altered in Pex3<sup>−/−</sup> cells (Figure 2E), no consistent changes were observed in total SM level (Figure S3A). Our data are consistent with previous studies showing elevated ceramide levels and altered composition of SM species in patients with peroxisomal disorders (13, 14), further supporting a role of peroxisomes as regulators of sphingolipid metabolism (15). Intriguingly, myriocin, an inhibitor of de novo sphingolipid synthesis, blocked the sensitization of Pex3<sup>−/−</sup> cells to vemu-induced apoptosis (Figure S3B-S3C). This data indicates that the hypersensitivity of Pex3<sup>−/−</sup> cells to vemu is likely attributed to increased levels of certain sphingolipid species, namely ceramides and HexCer, depletion of both were confirmed after myriocin treatment (Figure S3C, bottom). Knockdown of PEX3 or PEX19 in A375M cells also increased the levels of ceramides and HexCer (Figure S3D).

Ceramides are critical mediators of cell fate and lie at the nexus of sphingolipid metabolism (Figure S3B). While increased ceramides can compensate the loss of EPLs due to disrupted peroxisomal function (15), several cell stressors stimulate the production of ceramides to promote apoptosis (31, 32). We hypothesized that the increased susceptibility of peroxisome-deficient melanoma cells to MAPKi-induced apoptosis is mediated via a mechanism involving ceramide-dependent cell death. To test this, we used C2-Ceramide (C2-Cer), a cell-permeable ceramide analog. While C2-Cer demonstrated limited cytotoxicity in Cas9-Ctrl D4M.3a cells, it potentiated vemu-induced apoptosis in a dose-dependent manner (Figure 2G, left). In Pex3<sup>−/−</sup> (9G) cells, which are characterized by increased ceramide levels (Figure 2F), C2-Cer alone was able to induce cell death, albeit at a higher dose (Figure 2G, right), suggesting a potential metabolic vulnerability in peroxisome-compromised cells. When combined with vemu, C2-Cer further induced apoptosis in Pex3<sup>−/−</sup> 9G cells (Figure 2G, right). Next, we used the HDAC inhibitor vorinostat (Vor), known to induce peroxisomes (33, 34), to test whether increased peroxisomes could protect cells from ceramide/vemu-induced apoptosis. As expected, Vor increased peroxisome numbers in both Cas9-Ctrl and Pex3<sup>−/−</sup> 9G cells (Figure S3E) and significantly decreased apoptosis induced by C2-Cer and vemu (Figure 2H). Notably, induction of peroxisomes protected Pex3<sup>−/−</sup> 9G cells against high-dose C2-Cer (Figure 2H, right), further supporting a role of peroxisomes in ceramide metabolism. Similar
results were observed in A375M cells (Figure 2I, S3F). Together, our data suggest that reducing peroxisomes in melanoma cells potentiates their response to MAPKi through increased ceramides.

**Dual inhibition of peroxisome biogenesis and UGCG potentiates melanoma response to MAPKi.**

Having shown that Pex3<sup>+/−</sup> cells have a high level of ceramides and undergo apoptosis in response to further ceramide increases (Figure 2F-2G), we next sought to exploit this metabolic vulnerability for therapeutic intervention. Melanoma cells with reduced peroxisomes are characterized by increased levels of HexCer (Figure 2F, S3D), which include glucosylceramides (GluCer) and galactosylceramides (GalCer) (Figure S3B). Given that GluCer can be pro-survival in melanoma cells (35), we posited that melanoma cells with compromised peroxisomes rely on the UGCG-catalyzed ceramide-to-GluCer metabolism as a pro-survival mechanism. Dual blockade of PEX3 and UGCG would thus be anticipated to result in a greater clinical benefit. Indeed, knockdown of Ugcg significantly increased apoptosis in treatment-naïve Pex3<sup>−/−</sup> 9G cells (Figure 3A), indicating that these cells are reliant on UGCG-mediated ceramide clearance for survival. Additionally, knockdown of Ugcg further potentiated the response of the Pex3<sup>−/−</sup> 9G cells to vemu, resulting in ~80% cell death within 24 hours of MAPK inhibition (Figure 3A). Consistent with ceramide being pro-apoptotic (Figure 2G), Ugcg-silenced Cas9-Ctrl D4M.3a cells were also sensitized to vemu-induced apoptosis (Figure 3A). Conversely, silencing Gba, which encodes the enzyme catalyzing the reverse GluCer-to-ceramide metabolism (Figure S3B), protected Pex3<sup>−/−</sup> 9G cells, but not Cas9-Ctrl cells, from vemu-induced apoptosis (Figure 3B). To verify these results in human melanoma cells, we generated PEX3 knockout (PEX3-KO) A375M cells and similar data were obtained as in our murine models (Figure S4A-S4C).

We next tested the impact of blocking UGCG enzymatic activity on MAPK-targeted therapy response using the inhibitor D,L-threo-PPMP (PPMP), which induced endogenous ceramide level, decreased HexCer (Figure S4D) (36, 37), and increased peroxisome abundance (Figure S4E). PEX3 knockdown combined with PPMP potentiated MAPKi-induced apoptosis in our melanoma cell lines (Figure 3C). Similarly, PPMP+vemu led to the highest level of cell death in the PEX3-KO A375M cells (Figure S4F).
We next evaluated the impact of reducing peroxisomes in melanoma cells on their response to combined UGCG inhibition and MAPKi in vivo. Murine and human peroxisome-deficient cells showed delayed tumor initiation compared with their control counterparts (Figure S4G-S4H, S4J-S4K). When melanomas reached a volume of 200mm³, mice were administered a PLX4720 diet, and PPMP therapy was initiated (Figure S4G, S4J). While both the D4M.3a Cas9-Ctrl- and A375M-Ctrl-derived melanomas continued increasing in size 2 days following PLX4720 monotherapy, the addition of PPMP rapidly potentiated their short-term response to PLX4720 (Figure 3D, 3G). The long-term treatment with combined PPMP and PLX4720 significantly improved the BR and PFS in the D4M.3a Cas9-Ctrl group, a phenotype that was much stronger in mice bearing Pex3+/- 9G-derived tumors (Figure 3E-3F). Similarly, the most improved BR and PFS were observed in the A375M PEX3-KO group when mice were treated with PPMP+PLX4720, compared with all other arms (Figure 3H-3I). PPMP treatment, alone or in combination with PLX4720, did not cause overt toxicity (Figure S4I, S4L). Together, our mouse modelling supports the clinical potential of blocking UGCG and PEX3 to enhance anti-tumor responses to MAPK-targeted therapy.

**Peroxisome and UGCG functions are required for MAPKi-tolerant CD36⁺ persister melanoma cells.**

Co-targeting PEX3 and UGCG not only potentiated melanoma response to MAPK inhibition but also delayed the onset of resistance (i.e., prolonged PFS) (Figure 3). We posit that this strategy may eliminate specific populations of MAPKi-persister melanoma cells present in minimal residual disease (MRD), which ultimately lead to the development of resistance (19, 38). Rambow et al. identified four MAPKi-tolerant melanoma states in a patient-derived xenograft (PDX) model, termed pigmented, starved-like melanoma cells (SMC), dedifferentiated (invasive), and neural crest stem cells (NCSC) (19). Re-analysis of the Rambow scRNA-seq dataset revealed that, among these drug-tolerant states present during the early MAPKi-adapting phase, a peroxisomal gene signature was specifically enriched in the SMC state which is marked by the expression of the fatty acid transporter CD36 (Figure 4A). Our analysis is consistent with prior work wherein the transcript levels of FAO, which encompassed both mitochondrial and peroxisomal enzymes, were found to characterize the SMC population (8). Importantly, in our analysis, the expression of peroxisome signature genes and UGCG were positively correlated with CD36 expression in the human melanoma dataset of The Cancer Genome Atlas.
activity with cobi their CD36 following melanoma (i.e. combined of expression of those genes gene compared with activity metabolism abundance throughout the drug response in the SUCell score originally defined from the mitochondrial melanoma cells revealed that PPARGC1A, critical for mitochondrial metabolism-mediated MAPKi-resistance in a subset of melanoma cells (5, 24), was predominantly expressed in the pigmented MITF\textsuperscript{high} cell state and showed no correlation with CD36 in the TCGA dataset (Figure 4A-4B). The SMC and pigmented melanoma cell states are reliant on altered metabolism for their drug tolerance (7, 24). We thus hypothesized that the CD36\textsuperscript{+} SMC state is distinguished from the mitochondrial-dependent pigmented state by their reliance on a peroxisome/UGCG-dependent mechanism.

We next confirmed that CD36 marker expression was sufficient to identify the SMC population, which was originally defined by the high expression of a subset of SMC signature genes including CD36 (termed “SMC AUCCell score”) (19). By setting a normalized CD36 expression ≥2.2 as a cutoff (considered as “CD36\textsuperscript{+}”) (Figure S5A), we were able to highlight almost identical cell populations that were defined by SMC AUCCell score (≥0.05) in the Rambow 2018 dataset (Figure S5B). These CD36\textsuperscript{+} cells and SMC populations also showed similar abundance throughout the drug response (Figure S5C). Although dramatic decreases in the cancer cell metabolism gene signature ensue with combined BRAF/MEK inhibition, indicative of an overall low metabolic activity in MRD (19), this metabolic signature was decreased to a significantly lesser extent in the CD36\textsuperscript{+} cells, compared with the CD36\textsuperscript{-} subpopulation (Figure 4C). Consistent with these results, while peroxisome signature genes (AGPS, SCP2, PEX1) and UGCG expression were significantly decreased in the CD36\textsuperscript{-} cells, the expression of those genes was retained in the CD36\textsuperscript{+} population (Figure 4D). We further verified the robustness of these data using the A375M xenograft model. Mice bearing A375M-derived tumors were administered combined BRAFi/MEKi therapy (PLX4720 diet+Cobi) for 8 days, a regimen to which melanomas fully respond (i.e., tumor shrinkage) (Figure 4E, S5D). Combined BRAFi/MEKi treatment significantly increased the CD36\textsuperscript{+} melanoma population (Figure S5E). Despite an overall decrease of AGPS (a peroxisomal enzyme) and UGCG following MAPK inhibition, the CD36\textsuperscript{+} cells had significantly higher levels of AGPS and UGCG compared with their CD36\textsuperscript{-} counterpart (Figure 4F-4G). Similar results were observed in vitro using vemurafenib alone or combined with cobi (Figure 4H-4I). Together, these data suggest a potential mechanistic link between the higher metabolic activity of MAPKi-tolerant CD36\textsuperscript{+} SMCs and a role of the peroxisome and UGCG therein. We next formally tested
whether peroxisomes and UGCG are required for MAPKi-tolerant CD36+ persister cells. Consistent with a previous study (7), treatment with vemu, cobi, or vemu+cobi increased the percentage of CD36+ A375M cells to a similar extent (Figure S5F). We thus used vemu to model the dynamics and drug response/tolerance of these MAPKi-induced CD36+ cells. The abundant CD36+ population was observed from Day 1 to Day 10 post-vemu treatment (Figure S5G), recapitulating an overall trend observed in the PDX model MEL006 (Figure S5C). After a 48-hour vemu treatment, A375M cells staining negative for both Annexin V and PI were sorted into CD36- and CD36+ populations (Figure 5A). As expected, both populations showed lower sensitivity to vemu than the parental A375M cells (Figure S5H). Moreover, CD36+ cells developed resistance within the shortest time, compared with either CD36- cells or parental cells (Figure S5H).

In agreement with our previous data (Figure 4D-4I), vemu-induced CD36+ A375M cells showed higher expression of peroxisomal genes (AGPS, SCP2, PEX1) and had more peroxisomes compared with CD36- cells (Figure 5B-5C). UGCG expression was also higher in the CD36+ population (Figure 5B, 5D). Consistently, these CD36+ cells exhibited increased ceramide tolerance, compared with their CD36- counterparts (Figure S5I). Supporting the essential role of peroxisomes in CD36+ drug-tolerant cells, the CD36+ population was more susceptible to apoptosis when PEX3 was knocked down, compared to the CD36- population. Moreover, this effect was potentiated when UGCG activity was simultaneously blocked using PPMP (Figure 5E). Using both genetic and pharmacological approaches, we further showed that co-targeting PEX3 and UGCG most efficiently eliminated the CD36+ population (Figure 5F-5H). Similar results were also observed in the NRAS-driven WM3406 cells upon MEKi treatment (Figure S5J). Together, these data revealed a distinct peroxisome/UGCG-dependent mechanism of tolerance in the MAPKi-induced CD36+ melanoma persister cells. Dual blockade of PEX3 and UGCG, therefore, enhances efficacy of MAPKi by selectively eliminating these CD36+ persister cells.

**Increased peroxisomal activity and UGCG marks MAPKi-resistance and poor outcome in melanoma.**

Having shown that inhibiting peroxisomes and UGCG can induce death of CD36+ MAPKi-tolerant cells, we next interrogated the status of CD36, peroxisomes and UGCG in melanomas from patients treated with
MAPKi. We re-analyzed a publicly available RNA-seq dataset (20), which included patient melanoma samples collected before, during, and after relapse of MAPKi therapy. In 77% patients (17/22), we observed an overall induction of CD36 expression following MAPKi treatment (Figure 6A, left). In this patient cohort, expressions of the peroxisomal gene signature and UGCG were most dramatically increased in the relapsed samples (Figure 6A-6B). Notably, a small group of patients (5/22) showed decreased levels of CD36 during treatment. No significant pattern of change in peroxisomal genes or UGCG was observed in this group of samples, suggesting alternative CD36+/peroxisome-independent mechanisms through which drug tolerance and resistance can occur (Figure S6A). Similar trends were detected in a separate melanoma patient-derived RNA-seq dataset (21, 22). Approximately 76% (19/25) patients showed increased expression of CD36 following MAPK-targeted therapy (Figure S6B). Both peroxisomal genes (AGPS, SCP2, PEX1) and UGCG were significantly increased after therapy resistance (Figure S6C). In this cohort, a slight decrease in PEX1 and UGCG expression was observed in samples collected during therapy, consistent with the scRNA-seq data (Figure 4D).

Our data support that peroxisomes and UGCG are essential for the survival of MAPKi-tolerant melanomas and changes in their expression can alter lipid profiles (Figure 2-5). We next interrogated lipidomic data from a human melanoma PDX (MEL006) and cell line model (451Lu) of acquired MAPKi-resistance (19, 39) to understand whether they showed lipid profiles indicative of heightened peroxisome and/or UGCG activity. Several ether-phosphatidylethanolamine (PE-P) species were increased in the MEL006 PDX model after tumors relapsed (Figure 6C), strongly indicative of increased peroxisomal/AGPS activity. Similarly, we observed significantly increased abundance of EPL species, including PC-P, PE-O and PE-P, in the vemu-resistant 451Lu-R cells, compared with the parental 451Lu cells (Figure 6D). In addition, HexCer was significantly increased in relapsed MEL006 tumors (Figure 6C) and in the 451Lu-R cells (Figure 6D). These data are consistent with our results showing that the expression of AGPS and UGCG are upregulated in a panel of MAPKi-resistant melanoma cell lines (Figure 6E-6F, S6D).

Finally, we assessed potential value of CD36, AGPS and UGCG as biomarkers in predicting patients' response to MAPK-targeted therapy. We collected data from seven independent studies assessing transcriptomic changes in matched treatment-naïve versus MAPKi-treated melanomas from a total of 80 patients
(20-23, 40-42). Using baseline and/or treatment-induced expression of CD36, AGPS, UGCG as three individual risk factors, patients were categorized into high- versus low-risk groups (see Methods and Table S5). Indeed, the high-risk group of patients, marked by high or increased expression of CD36, AGPS and UGCG, showed poorer clinical response to MAPK-targeted therapy and reduced PFS compared to the low-risk group (Figure 6G-6H).

To summarize, increased peroxisomal/AGPS activity and UGCG occur in a significant proportion of relapsed melanomas, which are likely driven through the CD36+ drug-tolerant SMC state. This subset of melanoma patients may have limited clinical response to MAPK-targeted therapy, which can potentially be improved by combined inhibition of PEX3 and UGCG.

**Overcoming MAPKi-resistance by co-targeting peroxisomes and UGCG**

We next evaluated the efficacy of dual inhibition of PEX3 and UGCG in a panel of melanoma cell lines with acquired MAPKi-resistance. These cells include previously characterized BRAFV600E-mutant A375, WM164 and SK-Mel-28 cells that are resistant to vemurafenib single agent (VSR) (43, 44); newly generated BRAFV600E-mutant 1205Lu and SK-Mel-28 cells that are vemurafenib+cobimetinib dual-resistant (VCDR); and NF1-mutant MeWo cells that are resistant to cobimetinib single agent (CSR) (See Table S1). Knockdown of either PEX3 or UGCG induced apoptosis in all drug-resistant cells that were cultured in the presence of MAPKi. The UGCG inhibitor PPMP significantly increased apoptosis in PEX3-silenced drug-resistant cells, without any such effect in UGCG-silenced cells (Figure 7A, S7A), indicating that UGCG inhibition cooperates with peroxisome reduction to overcome MAPKi-resistance. Similar results were observed in PEX19-silenced A375-VSR cells upon PPMP treatment (Figure S7B).

Next, we modeled the in vivo efficacy of PPMP therapy in PLX4720-resistant melanoma models that have, or not, altered peroxisomes. Mice bearing size-matched D4M.3a Cas9-Ctrl- versus 9G-derived melanomas were kept on PLX4720 chow and PPMP treatment was initiated at the time of monitored tumor relapse (Figure 7B, S7C). While peroxisome-deficient melanomas (9G) had a delayed onset of PLX4720-resistance compared with Cas9-Ctrl-derived tumors, both groups showed similar growth rates once tumor relapse was detected (Figure S7C, top). No significant difference in OS was observed between vehicle-treated Cas9-Ctrl versus 9G groups
after acquired resistance to PLX4720 (Figure 7C, S7C). PPMP treatment led to prolonged tumor control in 43% (3/7) of mice in the PLX4720-resistant Cas9-Ctrl group (Figure S7C). However, 100% (7/7) of mice harboring PLX4720-resistant Pex3<sup>−/−</sup> melanomas had improved tumor control and significantly increased OS on the PPMP therapy (Figure 7C, S7C). Similar results were observed in another cohort of mice where PPMP was used to treat late-stage therapy-resistant melanomas (Figure 7D-7E, S7D). Without PPMP treatment, all mice needed to be euthanized within 48-72 hours regardless of their Pex3 status due to their large tumor size (Figure 7E, S7D). Intriguingly, PPMP treatment improved the OS of mice bearing Cas9-Ctrl- and 9G-derived late-stage melanomas by approximately 4 and 8 days, respectively (Figure 7E, S7D). We here conclude that dual inhibition of PEX3 and UGCG has potential clinical benefit in MAPK-targeted therapy-resistant melanomas.

The PEX3-PEX19 interaction is druggable with NNC 55-0396 and has therapeutic efficacy in melanoma.

Protein-protein interactions are potential vulnerabilities for therapeutic intervention in human disease, including cancer (45). The PEX3-PEX19 interaction is crucial for peroxisome biogenesis (46, 47), thus provides a potential intervention target with a small molecule inhibitor. NNC, a T-type calcium channel (TTCC) inhibitor (48), amongst others, was identified from a recent screen for drugs that potentially inhibit the PEX3-PEX19 interaction (Figure S8A-S8B) (49). We showed NNC decreased peroxisome numbers in A375M cells (Figure S8C) and dramatically reduced the PEX3-PEX19 interaction (Figure 8A). Structural analysis revealed that the predicted binding site of NNC on PEX3 is in close proximity to PEX19-interacting residues (Figure S8D) (50). Notably, overexpressing GFP-PEX3 alone resulted in degradation of PEX3, which is likely due to proteasomal degradation (Figure S8E). However, co-overexpressing PEX3 and PEX19 prevented PEX3 degradation (Figure 8A, S8E), suggesting that the PEX3-PEX19 interaction is crucial for PEX3 stability. In agreement with these data, we showed that NNC promoted PEX3 degradation within 48-72 hours (Figure S8E-S8F). Prolonged NNC treatment subsequently led to decreased expression of PEX16, PEX19, and AGPS (Figure S8F), and increased levels of ceramides and HexCer (Figure S8G), characteristic of cells with compromised peroxisomes (Figure 2F, S3D).
Similar to the data obtained in PEX3-silenced or -knockout melanoma cells, NNC treatment potentiated vemurafenib-induced apoptosis in A375M-Ctrl cells (Figure 8B). The induction of apoptosis was significantly more robust when A375M-Ctrl cells were treated with the triple-therapy of vemurafenib and NNC in combination with PPMP (Figure 8B). Furthermore, NNC was sufficient to counteract the vemurafenib-mediated increase in CD36-expressing cells (Figure 8C). Combined treatment of NNC and PPMP efficiently and preferentially eliminated the vemurafenib-induced CD36+ population (Figure 8C-8D). Similar effects were not observed in NNC-treated A375M PEX3-KO (AG3) cells, supporting that the observed biological effects of NNC is through a mechanism involving PEX3 (Figure 8B-8C). TTCCs, such as CaV3.1 and CaV3.2, can promote melanoma cell proliferation and protect against apoptosis (51). Thus, we tested the effect of NNC in melanoma cells expressing or not TTCCs. Knocking down CaV3.1 or CaV3.2 did not impede the ability of NNC to potentiate vemurafenib-induced apoptosis or the NNC-mediated decrease in the CD36+ melanoma cell population (Figure S8H-S8I), suggesting that the effects that we observe with NNC are largely attributed to its inhibitory effect on PEX3-PEX19 binding rather than on TTCCs. In addition, NNC combined with PPMP efficiently killed a panel of drug-resistant cells cultured in the presence of MAPKi (Figure 8E), recapitulating a phenotype we observed via genetic inhibition of PEX3 or PEX19 (Figure 7A, S7A-S7B).

Next, we tested in vivo efficacy of NNC combined with PPMP and PLX4720 in the A375M xenograft model. In the first cohort, when tumors reached approximately 200mm³ in size, mice were switched to the PLX4720 diet and simultaneously treated with NNC, PPMP or NNC+PPMP (Figure S9A). NNC+PPMP significantly potentiated the PLX4720 anti-tumor response in A375M-derived melanomas, compared with any other groups, a phenotype observed as early as 48 hours following the initiation of therapy (Figure 8F-8G, S9B). Moreover, long-term administration of the triple-therapy (PLX4720+NNC+PPMP) resulted in significantly improved BR and PFS, without causing any overt toxicity (Figure 8G-8H, S9B). In a separate cohort to model therapy resistance, mice were switched to the PLX4720 diet when tumors reached approximately 200mm³ in size. NNC, PPMP or NNC+PPMP treatments were subsequently initiated when relapsed tumors reached a size of 400mm³ (Figure S9C). NNC treatment significantly prolonged tumor control and improved OS, an effect that was far more pronounced when NNC was combined with PPMP (Figure 8I, S9D). Based on these results, we next tested
whether addition of a MEKi (cobi) to the triple-therapy (PLX4720+NNC+PPMP) would yield similar tumor control (Figure 9A, S9E). Indeed, NNC+PPMP rapidly improved the response of A375M-derived melanomas to PLX4720+cobi treatment (Figure 9B-9C). After a 10-day course of treatment, melanomas were collected and analyzed by flow cytometry to confirm that the NNC+PPMP therapy decreased the abundance of CD36+ drug-tolerant cells and reduced AGPS expression (Figure 9D-9E). Finally, to test whether we have an exploitable therapy for BRAFi/MEKi dual-resistant melanomas, we injected mice with 1205Lu-VCDR cells and treated them with PLX4720+cobi when their tumors were palpable. When these drug-resistant tumors reached a size of approximately 250mm³, mice were administered either NNC+PPMP therapy or vehicle control (Figure S9E). Similar to the results obtained in the PLX4720 monotherapy-resistant cohort (Figure 8I, S9D), NNC+PPMP continuously inhibited the growth of BRAFi/MEKi dual-resistant melanomas for at least 4 weeks and dramatically improved OS (Figure 9F, S9F). Together, these data provide a proof-of-concept that combining NNC, a drug that disrupts the PEX3-PEX19 interaction, with a UGCG inhibitor demonstrates efficacy in sensitizing melanoma to MAPK-targeted therapy.
Discussion:

In this present study, we showed that compromising peroxisome biogenesis by targeting PEX3 (or PEX19) sensitizes melanoma to MAPK pathway inhibition and overcomes MAPKi-resistance, a phenotype that is more robust when UGCG is simultaneously inhibited. Mechanistically, we identified two lipid pathways, the peroxisome/AGPS-driven EPL biosynthesis pathway, and the UGCG-catalyzed ceramide-to-GluCer pathway, that collaboratively regulate ceramide homeostasis and mediate drug tolerance in a subset of CD36+ persister cells and melanoma cells with acquired MAPKi-resistance. Furthermore, we identified NNC 55-0396 as an inhibitor of the PEX3-PEX19 interaction, with its potent anti-tumor effects demonstrated in preclinical melanoma models.

Melanomas typically exhibit high intratumoral heterogeneity and cancer cell plasticity, ultimately favoring metastasis and therapeutic resistance (2, 3, 19, 38). Metabolic reprogramming represents an important mechanism by which melanoma cells switch between different states and quickly adapt to therapy-induced stress conditions (5, 24). For example, the PAX3/MITF-PGC1α axis promotes MAPKi-tolerance in a subset of melanoma cells through a mitochondrial-mediated metabolic shift (6, 19, 52, 53). Blockade of mitochondrial biogenesis combined with MAPKi induced apoptosis in these MITFhigh/pigmented melanoma cells (5). These studies provide evidence that targeting cell state-specific metabolism is a promising strategy to selectively kill drug-tolerant melanoma cell subpopulations. Importantly, our work revealed a distinct metabolic rewiring mechanism harnessed by a different subset of melanoma cells, namely the CD36+ SMCs, to mediate their tolerance to MAPK-targeted therapies (Figure 4 and 5). The CD36+ SMC state is an attractive therapeutic target in melanoma, as it represents a significant subset of drug-tolerant melanoma cells, accounting for approximately 20-80% of the population persisting from the early MAPKi-response phase to MRD (7, 19). Both CD36+ SMC and pigmented melanoma cell states can rely on altered metabolism for their drug tolerance (7, 24). Previous studies suggest that SMCs rely on increased FAO, mediated by PPARα, for their survival during MAPK inhibition (7). Another study identified the peroxisomal enzyme ACOX1, a downstream target of PPARα, as a mediator of drug tolerance generally in melanoma persister cells (8). Although the latter study revealed an enrichment of FAO-related genes in SMCs, including a subset of peroxisomal enzymes, the authors did not explore the role of
the PPARα-ACOX1 axis specifically in the CD36⁺ SMC population (8). Notably, the FAO-related genes that were enriched in their persister cells (total of 26 genes) also included 11 mitochondrial-specific enzymes and PPARGC1A (encoding PGC1α) (8), a key regulator of mitochondria-dependent metabolic rewiring that functions predominately in a distinct MITF<sup>high</sup>/pigmented melanoma persister cell state (Figure 4A) (6, 19, 52, 53). Using CIBERSORTx (54), we showed that the enrichment of peroxisomal genes in MAPKi-treated melanoma samples (Figure 1A) is indeed associated with SMC induction (Figure S10A). Approximately 41% (19/46) of patients showed an increase of SMC (>1.5-fold increase) following MAPKi treatment (Figure S10A, left). Interestingly, an enrichment of the KEGG_Peroxisome gene signature was observed in ~95% (18/19) of patients in this group. While within patients with no change or decrease in SMC abundance after MAPK-targeted therapies, only 57% (8/14) and 46% (6/13) showed enriched KEGG_Peroxisome gene signature, respectively (Figure S10A, right).

Our data further demonstrated that the CD36⁺ SMCs utilize a peroxisome/UGCG-dependent mechanism for their lipid metabolic rewiring and drug tolerance, distinguishing them from the aforementioned mitochondrial/PGC1α-dependent MITF<sup>high</sup>/pigmented state. Dual blockade of PEX3 and UGCG effectively eliminated CD36⁺ cells, leading to a dramatic induction of apoptosis in MAPKi-treated melanoma cells and improved response to MAPKi and PFS in our preclinical models. Conversely, blocking mitochondrial OXPHOS by oligomycin potentiated melanoma cell response to vemurafenib without affecting CD36⁺ cell abundance (Figure S10B), further indicating that the peroxisome/UGCG-mediated lipid metabolism and the mitochondria-mediated OXPHOS independently promote drug tolerance in different melanoma cell subpopulations. Therefore, combined inhibition of both metabolic pathways can potentially work in concert to achieve better clinical responses to MAPKi (Figure S10C). These data further support that targeting cell state-specific metabolism is an effective way to potentiate drug response. It is also worth mentioning that, while CD36 is a hallmark of the SMC state, it is not functionally associated with the increased FAO that allows SMCs to survive under the MAPKi-induced metabolic stress (7). For example, knocking out CD36 did not alter fatty acid uptake or FAO rate in MAPKi-treated A375 cells. Using a fatty acid transport protein inhibitor, Aloia et al. further showed that CD36 does not function as a fatty acid transporter in MAPKi-induced FAO (7). Therefore, existing inhibitors or neutralizing antibodies against CD36 might not be sufficient to eliminate the SMC population. Together, these
data highlight the significance of combining small molecules that disrupt PEX3-PEX19 binding and UGCG inhibitors to target CD36+ persister melanoma cells. Finally, interrogation of TCGA datasets revealed that the expression of the peroxisome marker ABCD3 and UGCG were also positively correlated with CD36 expression in other tumor types (Figure S10D), suggesting that combined inhibition of peroxisomes and UGCG may have broader clinical utility.

Notably, the use of dual BRAF and MEK inhibition still comes with some clinical challenges. For example, only patients with class 1 BRAF-mutant melanomas respond to current targeted therapy agents, and acquired resistance invariably occurs, which may also confer cross-resistance to immunotherapy through MAPKi-driven phenotype switching/dedifferentiation (2, 3). Our data showed that co-targeting PEX3 and UGCG could (a) sensitize RAS-mutant and NF1-mutant melanoma cells to MEKi-induced apoptosis, (b) delay the onset of acquired resistance in vivo, and (c) restore drug sensitivity in resistant/relapsed melanomas. We further demonstrated that dual blockade of PEX3 and UGCG eliminated MAPKi-induced CD36+ persister cells. Together, these data are consistent with previous observations that not only BRAFi but also MEKi induce CD36 expression in melanoma cells (7) and that the CD36+ SMC state is an important transitory state leading to the emergence of other MAPKi-tolerant states, which promote resistance (19). Importantly, as dedifferentiated and NCSC states are also responsible for MAPKi-induced cross-resistance to immunotherapy (2, 3), targeting these CD36+ cells by dual PEX3+UGCG inhibition could also potentially sensitize melanoma to combined immunotherapy and MAPKi and prevent cross-resistance.

Finally, peroxisomes have only recently been identified as a potential target for cancer management (9). There are no effective and bioavailable inhibitors of peroxisomes currently available for clinical use. Drug repurposing represents an attractive strategy for identifying new uses (i.e., inhibition of peroxisomes) for existing drugs (55). A recent drug screen aiming to discover inhibitors that disrupt trypanosomatid PEX3-PEX19 binding allowed us to identify NNC 55-0396 as an inhibitor of human PEX3-PEX19 interaction (49). NNC was originally developed and is now commonly used as a selective TTCC blocker, with an IC50 of 7μM for inhibition of CaV3.1 T-type channels (48). We showed that NNC effectively disrupted PEX3-PEX19 interaction at 4μM concentration and that the anti-tumoral effect of NNC is dependent on peroxisomal functions, but not on TTCCs. Moreover,
NNC is bioavailable and demonstrates potent anti-melanoma activity in combination with PPMP and PLX4720 (or PLX4720+cobi) in vivo. These data support the use of NNC as an PEX3-PEX19 binding inhibitor and provide a proof-of-concept that pharmacological blockade of peroxisomes and UGCG represents a promising strategy in melanoma.
Methods

Mice

Male C57BL/6N mice (6-8 weeks old) were purchased from Charles River Laboratories. Male and female nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice (6-10 weeks old) were kindly gifted by Dr. Moulay Alaoui-Jamali. All mice were randomized before injection. All melanoma cell lines were freshly prepared in PBS and subcutaneously injected to the right flank of mice. Tumor initiation was determined once palpable tumors were formed. Tumors were then measured in length (L) and width (W). Tumor volumes (V) were calculated based on the formula $V = \frac{3.1416}{6} \times L \times W^2$. The short-term response (STR) rate was calculated based on the initial tumor volume ($V_0$) and the tumor volume measured at 48-hour timepoint post treatment ($V_{48}$), using the formula $\text{STR} = \frac{(V_{48} - V_0)}{V_0} \times 100\%$. The best response (BR) rate was calculated based on the initial tumor volume ($V_0$) and the smallest three consecutive tumor volumes ($V_{\text{min}1}$, $V_{\text{min}2}$, $V_{\text{min}3}$) measured during the course of treatment, using the formula $\text{BR} = \frac{[(V_{\text{min}1} + V_{\text{min}2} + V_{\text{min}3})/3 - V_0]}{V_0} \times 100\%$.

Detailed descriptions of experiments involving subcutaneous injection of D4M.3a, A375M, and 1205Lu-VCDR cells, drug preparation, and treatment timeline can be found in Supplemental Methods.

Cells and reagents

Sources, culture conditions and treatment timelines of human melanoma cell lines are listed in Table S1. The SK-Mel-28-VSR and WM164-VSR cells were previously generated and described as SK-Mel-28R and WM164, respectively (44). The vemu/cobi dual-resistant 1205Lu-VCDR cells were generated by culturing of the parental 1205Lu cells in elevated doses of vemu+cobi simultaneously. SK-Mel-28-VCDR cells were generated by exposing the vemu-resistant SK-Mel-28-VSR cells sequentially to elevated doses of cobi (with the presence of vemu). The cobi-resistant MeWo-CSR cells were generated by culturing of the parental MeWo cells in elevated doses of cobi. The murine melanoma D4M.3a cell line was a kind gift from Dr. C.E. Brinckerhoff, and was cultured in Advanced DMEM/F12 media supplemented with 5% FBS, 1x GlutaMax and 1x Pen/Strep.

Generation of CRISPR cell lines

CRISPR/Cas9-mediated knockout of $PEX3/Pex3$ in A375M and D4M.3a cells was accomplished using commercially available plasmids. Pre-designed sgRNAs targeting $PEX3$ or $Pex3$ were constructed into the
pSpCas9 BB-2A-GFP (PX458) vector by the manufacturer (GenScript). A375M and D4M.3a cells were transfected with either control pSpCas9 BB-2A-GFP plasmid (Cas9-Ctrl) or PEX3/Pex3-targeting sgRNA/pSpCas9 BB-2A-GFP plasmids. Individual GFP-positive clones were sorted into single cells in 96-well plates 48h after transfection. Single-cell clones were expanded and subsequently validated for PEX3 status by western blot and sequencing.

**RNA interference**

siRNAs were transfected into cells using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, 13778) following the manufacturer’s instructions, and 18 hours later, the media was changed. All cells were harvested between 48h-96h after siRNA transfection. All siRNA sequences are listed in Table S2.

**Immunofluorescence**

One coverslip/well was placed in a 24-well plate before cells were seeded. At the time of harvesting, media was aspirated, and cells were washed with PBS. Cells were fixed in 4% formaldehyde/PBS for 20 min at room temperature (RT) and washed with PBS. Cell membranes were permeabilized using 0.2% Triton X-100/PBS for 10min. Cells were washed and blocked with 10% BSA/PBS for 1h at RT, and then incubated with indicated primary antibodies diluted in 2% BSA/PBS overnight at 4 °C in a humid chamber. Detailed antibody information is listed in Table S3. Next day, cells were washed and incubated with secondary antibodies for 1h at RT in a humid, dark chamber. Cells were then washed with PBS, followed by incubation with 1:1000 DAPI/PBS nuclear stain for 15min. Cells were washed and mounted to a glass slide using ProLong gold mounting media (Invitrogen, P36930). Slides were stored in the dark until confocal fluorescence images were taken. Stacking and coloring of images were performed using FIJI software. ABCD3 puncta were manually counted on FIJI software using stacked images.

**Flow cytometry-based assays**

Following indicated treatments, cells were trypsinized, centrifuged at 240g for 5min, and washed in PBS. For apoptosis detection of non-fixed cells, Alexa Fluor™ 647-Annexin V (Invitrogen™, A23204) and Propidium Iodide (PI) Staining Solution (BD Biosciences, 556463) were used following the manufacturer’s instructions. For DCFDA staining, cells were stained with 5μM H2-DCFDA (ThermoFisher Scientific, D399) for 30min at 37 °C,
followed by Annexin V/PI staining. For CD36 staining, cells were stained with Brilliant Violet 421™ anti-human CD36 (Clone 5-271, BioLegend, 336229) antibody diluted at 1:400 in PBS containing 2% FBS for 30 mins on ice. Cells were then washed and sorted into CD36+ and CD36− populations, or fixed for subsequent intracellular staining. For tumor samples, freshly resected tumors were minced and digested in 4mg/mL collagenase A (Sigma Aldrich) at 37°C for 1 hour to obtain single cell suspension and were stained with indicated antibodies as well as a live/dead discrimination dye (see Figure S11 and Table S3). For all other panels, representative images showing gating strategy and antibody information can be found in Figure S11. All flow cytometry experiments were conducted on the LSRFortessa (BD Biosciences).

**Western blotting and Quantitative real-time PCR**

Immunoblots and qPCR were performed as previously described (44, 56). Detailed descriptions can be found in Supplemental Methods. Antibody information and primers used for qPCR are listed in Table S3 and S4, respectively.

**Lipidomic analyses**

From three separate passages per cell line, D4M.3a Cas9-Ctrl, Pex3+/− Clone 6D and 9G cells were cultured to 70-80% confluence, trypsinized, washed twice in PBS, pelleted in a 15mL conical tube, and flash frozen in liquid nitrogen. The samples were homogenized and mixed with 0.9mL MeOH:HCl(1 N) (8:1), 0.8mL CHCl₃ and 200μg/mL of 2,6-di-tert-butyl-4-methylphenol (Sigma, B1378). The organic fractions were evaporated, reconstituted in MeOH/CHCl₃/NH₄OH (90:10:1.25) and lipid standards were added (Avanti Polar Lipids). Phospholipids were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a hybrid quadrupole linear ion trap mass spectrometer (4000 QTRAP system, AB SCIEX) equipped with a TriVersa NanoMate robotic nanosource (Advion Biosciences) as described (39). Concentrations of lipid species were normalized to DNA quantity, and relative lipid concentrations were presented as nmol lipid per mg DNA. To obtain statistical information corresponding to fold-changes in lipids between cell lines, data were uploaded onto Perseus (MaxQuant). Values and names of lipid species were numerically and textually characterized. Numerical values were then Log₂-transformed to obtain a normal numerical distribution pattern within sample types. Zeroes were then imputed using standard settings to fall within the normal curve distribution. Triplicates
were grouped according to each cell line, and a multiple-sample one-way ANOVA statistical test was performed to determine whether lipid values differed between cell lines. A post-hoc test was then conducted to determine which cell lines exhibited statistically significant (p ≤ 0.05) differences in Log₂-transformed lipid abundances. To determine enrichment of species between cell lines, a ≥1.5 fold-change cut-off and p-value ≤ 0.05 was set.

**Co-immunoprecipitation**

HEK-293T cells were transfected with plasmids overexpressing TurboGFP-tagged PEX3 (OriGene Technologies, RG202031) and Myc-DDK-tagged PEX19 (OriGene Technologies, RC201756) using Lipofectamine 2000 (Invitrogen, 11668-019). The next day, media was changed, and cells were treated with 4μM NNC 55-0396 or DMSO control for 24 hours. Cells were then scraped and lysed with cell lysis buffer (HEPES 25mM pH7.5, potassium acetate 115mM, EDTA 1mM, 1% NP40) supplemented with protease and phosphatase inhibitors (Roche). Equal amounts of cell lysates were incubated with anti-myc (Cell Signaling Technology, 2276S) antibody for 1.5h and immunoprecipitated with 25μl of Protein G Dynabeads (ThermoFisher Scientific, 10004D) for 1h at 4℃. After washing with cell lysis buffer, the immunocomplexes were analysed by western blotting using anti-PEX3, anti-PEX19, and anti-α-Actinin antibodies (See Table S3).

**Access and re-analysis of previously published datasets**

Normalized scRNA-seq data from the MEL006 PDX model were downloaded from the Gene-Expression Omnibus public database, accession number GSE116237 (19). A total of 674 cells were projected into a two-dimensional space using t-distributed stochastic neighbor embedding (t-SNE). To determine the four established drug-tolerant states and cell metabolic activity, we used the AUCell algorithm based on their characteristic gene signatures as previously described (19). CD36 expression was further examined and cells with a CD36 expression ≥2.2 were considered as CD36+ and were highlighted in the t-SNE map.

Normalized RNA-seq data from patient samples (Kwong 2015 dataset) were kindly shared by Dr. Genevieve M. Boland and can be found in the European Genome-phenome Archive (EGA S00001000992) (20). Separate RNA-seq datasets (Hugo 2015 and Song 2017) were downloaded from the Gene-Expression Omnibus public database, accession numbers GSE65186 and GSE75313 (21, 22). A final RNA-seq dataset from a total of 6 patients (Tirosh 2016) was directly accessed from the original publication Supplementary Table 9 (23).
Normalized and background-corrected microarray data (Kakavand 2017, Long 2014, and Rizos 2014) were downloaded from the Gene-Expression Omnibus public database, accession numbers GSE99898, GSE61992 and GSE50509 (40-42).

For GSEA, RNA-seq data from matched pre- and post-treatment samples were analyzed through https://www.gsea-msigdb.org/gsea/index.jsp using pre-defined gene sets (see Table S6). For patient response, PFS, and risk analysis, only patients with gene expression (CD36, AGPS, UGCG) data from matched tumor samples that were collected before, on-treatment and/or after relapse were further analyzed and categorized into high- versus low-risk groups using 3 risk factors: RF1, RF2 (a or b), and RF3 (a or b). Briefly, baseline expression of AGPS (RF2a) and UGCG (RF3a) before treatment and fold change of CD36 (RF1), AGPS (RF2b), UGCG (RF3b) expression after treatment (compared to before) were normalized into Z-scores within each dataset. High Z-score of each risk factor values 1 risk score. High- and low-risk groups are defined by a risk score ≥2 and ≤1, respectively. Detailed risk scores and patient information are listed in Table S5.

Processed quantitative lipidomic data from the MEL006 PDX model and lipidomic data from 451Lu and 451Lu-R cells (39) were kindly shared by Drs. Ali Talebi, Jonas Dehairs and Johannes V Swinnen.

Statistics

In vitro data are presented as mean ± SD. In vivo data are presented as mean ± SEM. Prism software (GraphPad) was used to determine statistical significance of differences. Figure legends specify the statistical analysis used. P values are indicated in the figures and p <0.05 were considered significant.

Study approval

Animal experiments were conducted according to the regulations established by the Canadian Council of Animal Care, and protocols approved by McGill University Animal Care and Use Committee (#2015-7672).

Data availability

Data are available in the “Supporting data values” XLS file. Supporting analytic code for re-analysis of scRNA-seq data can be accessed at https://github.com/MMdR-lab/Huang-peroxisome-melanoma.
Author contributions

FH, SVDR designed research studies. FH, FC, MSD, KG, AT, JD, FET, JYP, ML, JHL, and PG conducted experiments. FH, FC, MSD, KG, AT, JD, FET, JYP, CG, NG, JS, and PG acquired data. FH, FC, MSD, KG, AT, JD, FET, JYP, ML, CG, NG, JS, JHL, and PG analyzed and interpreted data. FH, MSD, AT, JJM, WHM and SVDR wrote, reviewed, and/or revised the manuscript. JSJ and JVS provided administrative, technical, or material support. WHM and SVDR supervised the study.

Acknowledgments

This research was funded by the Canadian Institutes of Health Research (CIHR) (grant PJT-162260 to SVDR). FH was endowed by McGill Integrated Cancer Research Training Program graduate studentships. We thank Dr. Genevieve M. Boland (Massachusetts General Hospital, Boston, USA) for sharing RNA-seq data from patient samples (Kwong 2015 dataset). We thank Christian Young, Mathew Duguay, and Darleen Element for experimental advice and technical support.
References:


Figure 1. Compromising PEX3 sensitizes melanoma to MAPK inhibition.

(A) Left: Pie chart showing percentage of patients (n=46) with increased or decreased transcript levels of peroxisome-related genes (KEGG_Peroxisome) after treatment with MAPK-targeted therapies. Right: Normalized enrichment scores (NES) assessing increase (positive) or decrease (negative) of KEGG_Peroxisome gene set in samples from each patient collected post- versus pre-treatment with indicated MAPK inhibitors. 

(B) Top: Percent apoptotic cells as measured by the sum of PI/Annexin V double-positive and Annexin V-positive staining. Bottom: Western blot analysis of the indicated proteins in human melanoma cells or immortalized melanocytes (MeISt) following PEX3 knockdown (or siCtrl transfection) and treatment of indicated MAPK inhibitors (n=4). Equal volumes of DMSO were added to the control groups. Detailed treatment and timeline are presented in Table S1. Two-way ANOVA.

(C) Western blot analysis of the indicated proteins (left) and relative number of ABCD3 puncta (right) in D4M.3a Cas9-Ctrl, Pex3^+/− Clone 6D and 9G cells. Representative immunofluorescence (IF) staining for ABCD3 (green) and DAPI nuclear stain (blue) are presented (n=3). One-way ANOVA. 

(D) Percent apoptosis detected in D4M.3a Cas9-Ctrl, 6D and 9G cells following vemurafenib (vemu) or DMSO treatment for 24 hours (n=3). Two-way ANOVA. 


(F-G) Waterfall plots showing (F) the short-term response (STR, 48h-treatment) and (G) the best response (BR) of D4M.3a Cas9-Ctrl-, 6D- and 9G-derived melanomas to PLX4720. Values represent % volume change of each tumor from baseline. One-way ANOVA. 

(H) Kaplan-Meier curves showing progression-free survival (PFS) of mice bearing D4M.3a Cas9-Ctrl-, 6D- and 9G-derived melanomas, fed with PLX4720 chow. Log-rank test. 

(B-D) Data represent mean ± SD. Number of biological replicates is indicated in each graph.
Figure 2. Pex3<sup>−/−</sup> D4M.3a melanoma cells have altered lipidomes.

(A) Schematic of peroxisome-mediated lipid metabolism. (B) Pie charts showing lipid composition (relative abundance of each lipid family in % total) in D4M.3a Cas9-Ctrl, Clone 6D and 9G cells. Concentrations of each lipid family (normalized to per mg of DNA) are indicated. (n=3). (C) Volcano plots comparing abundance of lipid species in Clone 6D vs. Cas9-Ctrl (left) and Clone 9G vs. Cas9-Ctrl (right). Yellow and blue shades highlight respective increased (≥1.5-fold) and decreased (≤1.5-fold) lipid species in Pex3<sup>−/−</sup> cells relative to Cas9-Ctrl D4M.3a cells. (D) Venn diagrams showing lipid species that were significantly increased (top) or decreased (bottom) in D4M.3a Clone 6D and Clone 9G cells, comparing with Cas9-Ctrl cells. (E) Top: Heatmap showing lipid species that were commonly altered in D4M.3a Pex3<sup>−/−</sup> (6D and 9G) cells comparing with Cas9-Ctrl cells. Bottom: number of lipid species, categorized by family, enriched in D4M.3a Cas9-Ctrl or Pex3<sup>−/−</sup> (6D and 9G) cells. (F) Concentrations of ceramides (left) and hexosylceramides (HexCer, right) detected in D4M.3a Cas9-Ctrl, 6D and 9G cells (n=3). Two-way ANOVA. (G-I) Percent apoptosis (PI<sup>−</sup>/Annexin V<sup>+</sup>, PI<sup>−</sup>/Annexin V<sup>+</sup>) detected in DMSO- or vorinostat-treated (G-H) D4M.3a Cas9-Ctrl (left) or Pex3<sup>−/−</sup> Clone 9G (right) or (I) A375M cells. Cells were pretreated with (G, I) C2-ceramide (C2-Cer) at escalated doses or with (H-I) C2-Cer (10μM) and vorinostat (Vor, 1μM) 24 hours prior to vemu treatment (n=4 for H, n=3 for G, I). Two-way ANOVA. All data represent mean ± SD.
Figure 3. Dual blockade of PEX3 and UGCG sensitized melanoma to MAPK inhibition.

(A-B) Percent apoptosis detected in D4M.3a Cas9-Ctrl (left) or Pex3−/− Clone 9G (right) cells, following (A) Ugcg or (B) Gba knockdown and treatment with vemu or DMSO control (n=4 for A, n=3 for B). (C) Percent apoptosis detected in human melanoma cells following PEX3 knockdown and treatment with indicated MAPK inhibitors and/or D,L-threo-PPMP (PPMP). Equal volumes of DMSO were added to the control groups (n=4). Detailed treatment and timeline are presented in Table S1. (A-C) Data represent mean ± SD. (D-E) Waterfall plots showing (D) the STR and (E) the BR of D4M.3a Cas9-Ctrl- or 9G-derived melanomas to PLX4720 alone or PLX4720 combined with PPMP. (F) Kaplan-Meier curves showing PFS of mice bearing D4M.3a Cas9-Ctrl- or 9G-derived melanomas, treated with PLX4720 alone or PLX4720 combined with PPMP. (G-H) Waterfall plots showing (G)
the STR and (H) the BR of A375M-Ctrl- or PEX3-KO AG3-derived melanomas to PLX4720 alone or in combination with PPMP. (I) Kaplan-Meier curves showing PFS of mice bearing A375M-Ctrl- or PEX3-KO AG3-derived melanomas, treated with PLX4720 alone or PLX4720 combined with PPMP. (D-I) Number of biological replicates (mice) is indicated in each graph. Detailed experimental timelines are shown in Figure S4G and S4J. (D-E, G-H) Values represent % volume change of each tumor from baseline. (A-E, G-H) Two-way ANOVA. (F, I) Log-rank test.
Figure 4. CD36+ MAPKi-tolerant melanoma cells have retained peroxisome levels and UGCG. 
(A) Heatmap showing relative expression of indicated melanoma cell state-specific markers, a panel of peroxisomal genes, and PPARGC1A in 4 drug-tolerant melanoma populations in the MEL006 PDX model during early dabrafenib+trametinib treatment (Day 4). (B) Expression of CD36 and a peroxisomal gene signature, UGCG, GBA, and PPARGC1A (HTSeq-FPKM) in the GDC TCGA Melanoma dataset (SKCM, n=472). Spearman rank-order. (C-D) Violin plots of scRNA-seq data highlighting the distribution of (C) a gene signature indicating cancer cell metabolic activity and (D) indicated peroxisomal genes and UGCG in CD36+ (<2.2) versus CD36+ (≥2.2) cells, before (Pre) or after dabrafenib+trametinib treatment for 28 days (MRD). (E) Schematic of
experimental design for panel (F-G). (F) Relative expressions of AGPS (left) and UGCG (right) in CD36⁻ and CD36⁺ cell populations isolated from A375M melanoma xenografts following vehicle (n=4) or PLX4720+cobi (n=7) treatment for 8 days. (G) Fold change of AGPS (left) and UGCG (right) transcripts in CD36⁺ cells relative to CD36⁻ cells isolated from A375M melanoma xenografts following PLX4720+cobi treatment for 8 days. RPLP0 was used as a reference gene. Cells from a total n=7 tumors were pooled before sorting. Two-sided unpaired t-test. (H) Schematic of experimental design for (I). (I) Relative expressions of AGPS (left) and UGCG (right) in CD36⁻ versus CD36⁺ A375M cells following treatment with DMSO, vemu (2.5μM), or vemu (2.5μM) combined with cobi (100nM) for 96 hours. (C-D, F, I) Two-way ANOVA. (F-I) Data represent mean ± SD.
Figure 5. Peroxisomes and UGCG are required for survival of CD36+ MAPKi-tolerant melanoma cells. 
(A) Schematic of experimental design for (B-E). A375M cells were treated with vemu (2.5μM) for 48 hours before CD36 staining and subsequent sorting. (B) Fold change of the indicated mRNAs in vemu-exposed CD36+ A375M cells relative to CD36- A375M cells, normalized to RPLP0 as a reference gene (n=4). The SCP2 primers amplify the N terminus of the transcript initiated from the proximal promoter, encoding the peroxisome-specific protein SCPx. (C) Number of ABCD3 puncta and (D) relative UGCG expression in vemu-exposed CD36+ versus CD36- A375M cells. Representative IF staining for (C) CD36 (red), ABCD3 (green) and DAPI (blue) or (D) CD36 (red), UGCG (green) and DAPI (blue) are presented. Red arrows highlight cells stained positive for CD36. (E) Percent apoptosis (top) and representative images (bottom) of vemu-exposed CD36+ versus CD36- A375M cells following PEX3 knockdown and/or PPMP treatment (n=5). (F-H) Percentage of CD36+ populations in (F) A375M cells following PEX3 knockdown and the indicated treatment (n=3), (G) A375M-Ctrl versus PEX3-KO AG3 cells upon indicated treatment (n=3), or (H) A375M-Ctrl versus PEX3-KO AG3 cells following UGCG knockdown and subsequently treated with vemu (n=4). (B-D) Two-sided unpaired t-test. (E-H) Two-way ANOVA. Data represent mean ± SD.
Figure 6. Increased peroxisomal and UGCG activity commonly occurs in melanomas that rapidly acquire resistance to MAPK-targeted therapy.

(A) Normalized gene expression of CD36 (left), relative expression of a peroxisomal gene signature (right), and (B) expressions of AGPS, SCP2, PEX1 and UGCG, in a cohort of melanoma samples (n=17 out of a total of 22 patients, Kwong 2015 dataset) collected pre-, on-, or relapsed on MAPK-targeted therapy showing an overall trend of CD36 induction upon MAPKi. Data represent Mean + SEM. One-way ANOVA. (C-D) Normalized abundance of indicated lipid species grouped by carbon chain length detected in (C) MEL006 PDX tumor.
samples collected before (Pre, n=6) or relapsed (Res, n=5) on dabrafenib+trametinib treatment, or (D) 451Lu parental cells versus vemu-resistant 451Lu-R cells (n=3). Two-way ANOVA. (E) Fold change of AGPS and UGCG mRNA levels in a panel of MAPKi-resistant melanoma cells relative to their corresponding parental cells, normalized to ACTB as a reference gene (n=4). (C-E) Data represent mean ± SD. (F) Western blot analysis of the indicated proteins in a panel of parental versus MAPKi-resistant melanoma cells. (G) Waterfall plots showing the best overall response and (H) Kaplan-Meier curves showing PFS of melanoma patients treated with MAPK-targeted therapy. Risk score ranging between 0-3 was calculated based on expressions of CD36, AGPS, and UGCG before and after treatment (See Table S5 for detailed information). Patients were subsequently grouped into high-risk (risk score ≥2) versus low-risk (risk score ≤1). (G) Values represent % response of individual patient from baseline. (E, G) Two-sided unpaired t-test. (H) Log-rank test.
Figure 7. Dual inhibition of PEX3 and UGCG overcomes MAPKi-resistance in melanoma.

(A) Percent apoptosis detected in MAPKi-resistant cells following PEX3 or UGCG knockdown and treatment with PPMP. Equal volumes of DMSO were added to the control groups. Cells were maintained in the presence of indicated MAPK inhibitors (Table S1). Detailed treatment and timeline are presented in Table S1. Data represent mean ± SD (n=3). Two-way ANOVA. (B, D) Schematic of detailed experimental design. (C, E) Kaplan-Meier curves showing overall survival (OS) of Cas9-Ctrl- or 9G-tumor-bearing mice treated with PPMP or vehicle (C) after tumors relapsed on PLX4720, or (E) after relapsed tumor reached a volume of 1,300mm$^3$. All mice were kept on PLX4720 chow after PLX4720 treatment initiated when individual tumor first reached a volume of 200mm$^3$. Number of biological replicates (mice) is indicated in each graph. Individual tumor growth curves are shown in Figure S7C and S7D, respectively. Log-rank test.
Figure 8. NNC 55-0396 disrupts PEX3-PEX19 interaction and cooperates with PPMP to sensitize melanoma to BRAF inhibition.

(A) Western blot analysis of PEX3 and PEX19 from Myc co-immunoprecipitation (co-IP) of HEK-293T cells co-transfected with GFP-PEX3- and Myc-PEX19-expressing plasmids, treated with DMSO or NNC 55-0396 (NC, 4 μM, 24h). Left portion displays Myc co-IP of HEK-293T cells transfected with empty vector (EV), Myc-PEX19-, or GFP-PEX3-expressing plasmid alone. Immunoblots for inputs (10% of protein from IP) are shown below (representative of n=3).

(B) Percent apoptosis or (C) percentage of CD36+ populations detected A375M-Ctrl cells (left) or PEX3-KO AG3 cells (right) following indicated treatment with NNC (4 μM), vemu, and/or PPMP.

(D)
Percent apoptosis detected in vemu-exposed CD36+ versus CD36- A375M cells following NNC and/or PPMP treatment (n=3). (B-D) Data represent mean ± SD. (E) Colony formation assays of a panel of MAPKi-resistant melanoma cells cultured in the presence of indicated MAPK inhibitors and treated with NNC and/or PPMP for 5 days (representative of n=3). (F-G) Waterfall plots showing (F) the STR and (G) the BR of A375M-derived melanomas to PLX4720 alone or in combination with NNC, PPMP, or NNC+PPMP. Values represent % volume change of each tumor from baseline. (H) Kaplan-Meier curves showing PFS of mice bearing A375M-derived melanomas, fed with PLX4720 chow and simultaneously treated with vehicle, NNC, PPMP, or NNC+PPMP. See detailed experimental design in Figure S9A. (I) Kaplan-Meier curves showing OS A375M-tumor-bearing mice treated with vehicle, NNC, PPMP, or NNC+PPMP after relapsed (PLX4720-resistant) tumor reached a volume of 400mm³. Detailed experimental design and individual tumor growth curves are shown in Figure S9C and S9D, respectively. (F-I) Number of biological replicates (mice) is indicated in each graph. All mice were kept on PLX4720 chow when individual tumor first reached a volume of 200mm³. (B-D, F-G) Two-way ANOVA. (H-I) Log-rank test.
Figure 9. NNC 55-0396 and PPMP potentiates melanoma response to combined BRAF/MEK inhibition. 

(A) Schematic of experimental design for (B-E). (B) Tumor growth curve and (C) waterfall plots showing the STR of A375M-derived melanomas to PLX4720+cobi treatment. Tumors were allowed to grow to a volume of 750mm$^3$ before indicated treatments started. (D) Percentage of CD36$^+$ cells and (E) relative AGPS expression in total (CD45$^-$) tumor cells, isolated from A375M-derived melanomas following indicated treatment for 10 days. (F) Kaplan-Meier curves showing OS of mice bearing 1205Lu-VCDR-derived melanomas treated with NNC+PPMP or vehicle. All mice were kept on PLX4720 chow and simultaneously treated with cobi. NNC+PPMP treatment started when PLX4720/cobi dual-resistant tumors first reached a volume of 250mm$^3$. Detailed experimental design and individual tumor growth curves are shown in Figure S9E and S9F, respectively. (B-F) Number of biological replicates (mice) is indicated in each graph. (B, D-E) Data represent mean ± SEM. (C) Values represent % volume change of each tumor from baseline. (B) Two-way ANOVA. (C-E) Two-sided unpaired t-test. (F) Log-rank test.