Supplemental Methods

Passaging of organoids

Media was gently removed using a 1 mL pipet tip and transferred to a 15 mL conical tube. RPMI supplemented with 10% FBS and a final concentration of 5 mg/mL Collagenase/Dispase (Millipore 10269638001) was added to the well(s) to just cover the Cultrex. The Cultrex was then loosened by running a pipet tip around the edge of the well. Organoids were incubated at 37˚C 5% CO_2 in a humidified incubator. Digestion was monitored microscopically every 15 minutes. Digestion was deemed complete when the majority of the Cultrex was digested and organoids were just starting to dissociate. The slurry was transferred to a 15 mL centrifuge tube and any remaining Cultrex was scraped into the 15 mL tube. The well(s) were washed with 1 mL of Dulbecco's Phosphate Buffered Saline (DPBS) (Thermo Fisher Scientific 14190-144) and added to the 15 mL centrifuge tube. Dissociated organoids were spun at 500 x *g* for 5 minutes. The supernatant was aspirated, leaving behind any undigested Cultrex. The organoid pellet was resuspended in 10 mL of DPBS and centrifuged again as above. The supernatant was aspirated, and the organoids were either frozen in 90% FBS, 10% Dimethyl sulfoxide (DMSO) (Sigma D2650) or replated in RPMI supplemented with 10% FBS, 1X Anti/Anti, 1X Glutamax, 0.01% rock inhibitor and 5% Cultrex on Cultrex-coated wells. If necessary, a portion of the dissociated cells was used for nucleic acid isolation.

To understand how to best generate and maintain the biobank, PDO growth patterns were monitored. PDOs were slow growing in culture, consistent with observed slow clinical progression of this tumor type intraocularly. While some tumors could be passaged in as little as 1-2 weeks, while others required 2-3 months of slow growth prior to initial passaging, especially when the amount of material collected from the primary tumor was small or consisted of a larger fraction of necrotic pigment compared to live tumor. Smaller samples required smaller starting well size, and care was necessary to avoid suctioning off early non-adherent PDOs when feeding.

Immunohistochemistry

For PDOs, approximately 50 µL of washed organoid pellet was dispensed into molten HistoGel (Epredia HG-4000-012) according to manufacturer's instructions. Mouse tissue was embedded in formalin. The following primary antibodies were evaluated: Melan-A 1:200 (Abcam ab785), SOX10 1:200 (Abcam ab227680), BAP1 and hematoxylin and eosin (H&E) staining were done by the Mayo Clinic Rochester Clinical Pathology Lab. The following secondary antibodies were used: Alexa Fluor 647 (Abcam ab150083) and Alexa Fluor 488 (Abcam ab150113). Vectashield Plus containing DAPI (Vector Laboratories H-2000) was used for nuclear staining. Images were analyzed with a confocal laser microscope (Zeiss LSM 780).

Exome-sequencing

Previously frozen organoids were thawed on ice. The organoids were briefly spun to collect contents to the bottom of the tube. TE buffer (10 mM Tris pH8, 1mM EDTA) was added to bring the volume to 500 µL. The samples were digested in the presence of 1% SDS and 40 units of proteinase K (New England Biolabs P8107). Digestion proceeded at 55°C for one hour. The samples were transferred to 37°C for 15 minutes. A visual inspection was done to ensure the samples were homogeneous. Recovery of nucleic acids was accomplished by adding sodium acetate to a final concentration of 0.3M after which the samples were phenol (ThermoFisher Scientific 15513039) extracted one time followed by a phenol:chloroform:isoamyl alcohol extraction (ThermoFisher Scientific 15593031). DNA was precipitated by adding 15 mg of glycoblue (ThermoFisher Scientific AM9515) and an equal sample volume of isopropanol. The precipitation was carried out overnight at room temperature. The DNA was recovered from the isopropanol by spinning at >20,000 x *g* at room temperature for thirty minutes. Pellets were washed twice with 70% ethanol and resuspended in TE buffer (10 mM Tris pH8, 1mM EDTA). Quantification was carried out using the Qubit DNA HS kit (ThermoFisher Scientific Q33230). The manufacturer's protocol was used. Data were collected using a SpectraMax i3 multi-mode

plate reader (Molecular Devices, CA). The excitation/emission wavelengths were 510/530 respectively.

For samples that had small quantities of DNA (<200 ng total), pHI29 amplification was employed to generate sufficient material for whole exome sequencing using a modified version of the protocol described by Yooseph et al.[46] The reaction was comprised of 1X pHI29 reaction buffer (New England Biolabs M0269), 1 mM each dNTP, 0.05 mM random heptamers with 3'phosphorothioate modification (Integrated DNA Technologies), 500 pg of input template, 1.0 unit of pHI29 DNA polymerase (New England Biolabs M0269), 0.1 units inorganic yeast pyrophosphatase (New England Biolabs M2043), and 1 µg of recombinant albumin (New England Biolabs M0269). One to three reactions were set up for each sample. The amplification protocol was performed in a thermocycler as follows: samples were heated to 70°C for 5 minutes in the presence of reaction buffer, dNTPs, heptamers, then quickly cooled to 4°C. Samples were removed to ice and the enzyme master mix comprised of pHI29, yeast inorganic pyrophosphatase, and recombinant albumin was added. The samples were briefly mixed and returned to the thermocycler. Samples were incubated at 4°C for 10 minutes. A temperature ramp of 0.1°C/sec was applied to the samples until 10°C was reached; incubation at 10°C was for 10 minutes. This ramp and incubation was repeated at 15°C, 20°C, and 25°C with a final 0.1°C/sec ramp to 30°C. Samples were then incubated at 30°C for 4 hours, followed by incubation at 65°C for 10 minutes. Amplified DNA from the same sample was pooled, phenol:chloroform:isoamyl alcohol (ThermoFisher Scientific 15593031) extracted, and ethanol precipitated overnight at room temperature in the presence of 0.3 M sodium acetate and 15 mg glycoblue (ThermoFisher Scientific AM9515). Amplified DNA was recovered by centrifugation at 14,000 x *g* for 30 minutes at 4°C. Samples were washed with 70% ethanol, centrifuged, and supernate was removed. DNA was resuspended in TE buffer (10 mM Tris pH8, 1mM EDTA).

Residual heptamers in the sample were removed with approximately 30 units of RecJf (New England Biolabs M0264). Incubation was for one hour at 37°C followed by phenol:chloroform:isoamyl alcohol (ThermoFisher Scientific 15593031) extraction, ethanol precipitation and centrifugation as described above. The amplified DNA was incubated in a 50 µL reaction comprised of 1X pHI29 reaction buffer (New England Biolabs M0269), 1 mM each dNTP, amplified template, 10 units of pHI29 DNA polymerase (New England Biolabs M0269), 0.1 units inorganic yeast pyrophosphatase (New England Biolabs M2043), and 1 µg of recombinant albumin (New England Biolabs M0269). Samples were incubated for two hours at to 30°C. Forty units of Bst DNA polymerase large fragment (New England Biolabs M0275) was added to the reaction and incubation proceeded for an additional 2 hours at 65°C. Samples were extracted with phenol:chloroform:isoamyl alcohol (ThermoFisher Scientific 15593031) and ethanol precipitated in the presence of 0.3 M sodium acetate. To remove any remaining branched structures, the samples were incubated in a reaction containing 1X NEB buffer 2 (supplied with the enzyme) and 10 units of T7 endonuclease I (New England Biolabs M0302S) for 30 minutes at 37°C. Samples were extracted with phenol:chloroform:isoamyl alcohol (ThermoFisher Scientific 15593031) and ethanol precipitated overnight at room temperature in the presence of 0.3 M sodium acetate. Following centrifugation to recover the DNA, the samples were resuspended in TE buffer and yield was determined using the Qubit DNA HS kit (ThermoFisher Scientific Q33230). Data were collected using a SpectraMax i3 multi-mode plate reader (Molecular Devices, CA). The excitation/emission wavelengths were 510/530 respectively. Whole exome sequencing was performed by Azenta Life Sciences.

After library preparation and sequencing, raw BCL files were converted to FASTQ for each sample, and data QC and analysis was performed by using DNA-seq Analysis Pipeline. Clean reads were aligned to the GRCh38 reference genome using Sentieon 202112.01. Somatic SNVs and small INDELs were called by using Sentieon 202112.01 (TNseq algorithm). The VCF files generated by the pipeline were then normalized (left alignment of INDELs and splitting multiallelic sites into multiple sites) using bcftools 1.13. Overlapped transcripts were identified

for each variant and the effects of the variants on the transcripts were predicted by Ensembl VEP 104. Impact of the variants were classified using the R package for MAFtools 2.14.0 to visualize the landscape of critical mutations. Known pathogenic mutations in PDOs were compared to clinical sequencing test results from corresponding primary tumors. The Exome-seq data are available in Sequence Read Archive (BioProject accession number PRJNA1068520).

PCR Resequencing

When exome-sequencing failed to detect an expected mutation that had been seen on clinical testing of the primary tumor, PCR resequencing was done to look for the expected mutation. Primers were designed (**Supplemental Table 2)** to span the region of interest using PrimerBlast3.[47] Amplification was performed in a reaction containing 100 ng of genomic DNA, 1X HF Phusion Buffer (New England Biolabs M0530), 0.2 mM each dNTP, 2.5 µM forward primer, 2.5 µM reverse primer and 0.2 units of Phusion DNA polymerase (New England Biolabs M0530). The following program was used for amplification: 98°C for 30 seconds followed by thirty cycles of 98°C for 5 seconds, 55°C for 15 seconds, and 72°C for 15 seconds. A final extension step of 72°C for 5 minutes completed the PCR. Amplified products were gel-purified by low melting point agarose gel electrophoresis. PCR products were recovered from the gel by beta-agarose (New England Biolabs M0329) treatment in the presence of 0.3 M sodium acetate for 2 hours at 37°C followed by one phenol extraction (ThermoFisher Scientific 15513039) and phenol:chloroform:isoamyl (ThermoFisher Scientific 15593031) extraction. Samples were isopropanol precipitated overnight at room temperature in the presence of 15 µg of glycoblue. Samples were resuspended in TE buffer (10 mM Tris pH8, 1 mM EDTA) and yield was determined by nanodrop spectrophotometry. Sanger sequencing was performed by Azenta Life Sciences.

RNA-sequencing

During PDO passaging, approximately one-third of the cell slurry was removed to a multiwell plate prior to centrifugation. The collagenase/dispase digestion of the Cultrex was allowed to continue until the organoids were completely dissociated and the Cultrex was largely digested. Dissociated organoids were stored at -70°C in the presence of TRIzol LS (Thermo Fisher Scientific 10296010). RNA was isolated using a modified version of the RNA clean and concentrator kit from Zymo Research (Zymo Research R2062). Briefly samples were thawed at room temperature and further incubated at room temperature for 15 minutes to ensure that any nucleoprotein complexes were fully dissociated. The cell slurry was briefly centrifuged to pellet the contents to the bottom of the tube. A one tenth volume of 1-Bromo-3-Chloropropane (BCP) (Sigma B9673) was added to the samples, and the samples were subjected to constant vortexing for 15 minutes at room temperature. Samples were centrifuged at 12000 x *g* for 15 minutes at 4°C. The top layer was removed to a fresh RNAase free tube. The interface was back extracted with half of the original TRIzol volume of nuclease free water, spun as above and the top layer was pooled with the initial top layer. If necessary, a third water back extraction was done. Total RNA was applied to the column and centrifuged at 1500 x *g* for 2 minutes. To increase RNA recovery, the flow through was recovered and reapplied to the column and centrifuged at 10000 x *g* for 30 sec. The remainder of the total RNA isolation followed the manufacturer's recommendations. The yield of total RNA was determined using a Nanodrop spectrophotometer. Samples sent for RNA-Seq were quantified using the Qubit HS RNA quantification kit (Thermo Fisher Scientific Q32852). Data were collected using a SpectraMax i3 multi-mode plate reader (Molecular Devices, CA). The excitation/emission wavelengths were 644/673. RNA sequencing was performed by Azenta Life Sciences using the Illumina Ultra-Low Input protocol for library preparation and Illumina 2x150 bp PE reads. RNA-seq FASTQ files were aligned to HG38 Reference Genome using STAR 2.7.8a.

Gene-level counts were obtained using HTseq-count v.0.9.1 (Galaxy), and differential gene expression analysis was done with R package DESeq2 version 2.11.40.7 (RStudio). Genes with

a base mean ≥1, $\log_2$(fold change) > 1 or < –1, and an adjusted P value <0.05 were considered significantly differentially expressed, but genes with adjusted P value <0.1 were included in the global gene expression heatmap. Heatmaps were created using the R package ggplot2 version 3.3.3. The RNA-seq data are available in Sequence Read Archive (BioProject accession number PRJNA1068520).

ATAC-sequencing

Media was gently aspirated from each well of a 6-well plate containing PDOs using a 1 mL pipet tip. RPMI supplemented with 10% FBS and a final concentration of 5 mg/mL Collagenase/ Dispase was added to the well (100µL/well) to cover the Cultrex. Organoids were incubated at 37˚C 5% CO2 in a humidified incubator. Digestion was monitored microscopically every 15 minutes. For ATAC-seq, 2 hours of enzymatic digestion was found to be sufficient for lifting Cultrex off the plate and dissociating organoids. The slurry was transferred to a 15 mL centrifuge tube. The well(s) were washed with 1 mL of ice-cold PBS to collect any remaining Cultrex stuck to the wells, added to the 15 mL centrifuge tube, and spun at 500 x *g* for 5 minutes at 4˚C. The supernatant was removed and 1 mL of Gentle Cell Dissociation solution was added to the organoid pellet and incubated at room temperature for 20 mins on an orbital shaker. After incubation, samples were centrifuged at 500 x *g* for 5 minutes at 4˚C and supernatant was carefully removed. The organoid pellet was washed with 1 mL of ice-cold 1x PBS with gentle mixing and then centrifuged at 500 x *g* for 10 minutes at 4˚C. Supernatant was gently removed with a 200 µL pipet tip, and 100 µL of ice-cold anti-freezing buffer (10% DMSO, 10% FCS in RPMI-1640) was added immediately to re-suspend the pellet. The samples were then transferred on dry ice to ship to the Genomics Core facility, Mayo Clinic, for further processing.

ATAC-sequencing was done as previously reported.[48] Briefly, DNA libraries were prepared and cleaned using the Zymo DNA clean/concentrator kit followed with AMPure XP bead cleanup to remove primer dimers and under-digested chromatin. Sequencing was done on an Illumina HiSeq 4000. ATAC data were analyzed via the HiChIP pipeline developed by the Mayo Bioinformatics Core.[49] Paired-end reads were mapped to the HG38 reference genome using Burrows-Wheeler Alignment tool (BWA).[50] Read pairs with one or both reads uniquely mapped were retained and duplicates removed using Picard MarkDuplicates command (http://broadinstitute.github.io/picard/). Peaks were identified with the Model-based Analysis of ChIP-Seq (MACS2) software package using FDR ≤0.01.[51] BEDTools[52] combined with in-house scripts were used to generate normalized tag density profiles at a window size of 200 bp and step size of 20 bp. Differential analysis of chromatin accessibility was done with DiffBind version 3.8.4.[53] Raw read counts were normalized using trimmed mean of M values (TMM) method, and sites with fold change ≥2 and FDR ≤0.05 in DiffBind were extracted to represent differential chromatin accessibility sites. The ATAC-seq data are available in Sequence Read Archive (BioProject accession number PRJNA1068520). GREAT was used to identify genes associated with differentially accessible chromatin peaks, with FDR ≤0.05 for global analysis and FDR ≤0.1 for pathway-specific analysis. The Integrative Genomics Viewer (IGV) was used to visualize tracks.

Drug studies

Approximately 4.5×10^5 cells in 5 mL Cultrex were dispensed in 50 µL aliquots in each well of a black, clear bottom 96-well plate (Greiner 655090). Following 30 minutes incubation at 37°C and 5% CO_2, complete RPMI containing 10 µM Y-27632 dihydrochloride was added. Media was changed bi-weekly, and media containing drug or vehicle control was added after an initial 2-week incubation period. Drug doses included 5 nM, 50 nM, or 500 nM FR900359 (Cayman Chemical 33666); 5 nM, 50 nM, or 500 nM quisinostat (Selleckchem S1096); 0.18 nM, 1.8 nM, 18 nM trametinib (Selleckchem S267309); 100 nM, 1000 nM, 5000 nM buparlisib (Selleckchem S2247); or 100 nM, 1000 nM, 5000 nM copanlisib (Selleckchem S2802). PDOs were incubated with drug for 72-hours using 8 technical replicates per condition. Viability was assessed by adding 4 µL PrestoBlue HS Cell Viability Reagent (Invitrogen P50201) to each well, with

fluorescence read (SpectraMax i3) after 30 minutes with excitation and emission wavelengths of 560 nm and 590 nm, respectively. Fold-change was established by averaging technical replicates of each condition and comparing them to their respective vehicle control. Data were analyzed using a 2-tailed, paired t-test and $p \leq 0.05$ was considered statistically significant. Relative area under the curve (AUC) was calculated as previously described.[15] Drug study experiments had n=3 biological replicates for FR900259, quisinostat, and trametinib and n=5 for buparlisib and copanlisib.