

1 **Research Article:**

2 **Title: *ONC201 in combination with paxalisib is a therapeutic strategy for***
3 ***diffuse midline glioma***

4 **Authors:**

5 Evangeline R. Jackson^{1,2,#}, Ryan J. Duchatel^{1,2,#}, Dilana E. Staudt^{1,2,#}, Mika L. Persson^{1,2}, Abdul
6 Mannan^{1,2}, Sridevi Yadavilli^{3,4}, Sarah Parackal^{5,6}, Shaye Game^{5,6}, Wai Chin Chong^{5,6}, W. Samantha
7 N. Jayasekara^{5,6}, Marion Le Grand⁷, Padraic S. Kearney^{1,2}, Alicia M. Douglas^{1,2}, Izac J. Findlay^{1,2},
8 Zacary P. Germon^{1,2}, Holly P. McEwen^{1,2}, Tyrone S. Beitaki^{1,2}, Adjanie Patabendige^{8,9}, David A.
9 Skerrett-Byrne^{10,11}, Brett Nixon^{10,11}, Nathan D. Smith¹², Bryan Day¹³, Neevika Manoharan¹⁴,
10 Sumanth Nagabushan¹⁴, Jordan R. Hansford¹⁵, Dinisha Govender¹⁶, Geoff B. McCowage¹⁶, Ron
11 Firestein^{5,6}, Meegan Howlett¹⁷, Raelene Endersby¹⁷, Nicholas G. Gottardo^{17,18}, Frank Alvaro^{2,19},
12 Sebastian M. Waszak^{20,21}, Martin R. Larsen²², Yolanda Colino-Sanguino^{23,24}, Fatima Valdes-
13 Mora^{23,24}, Andria Rakotomalala^{25,26}, Samuel Meignan^{25,26}, Eddy Pasquier^{7,27}, Nicolas André^{7,27,28},
14 Esther Hulleman²⁹, David D. Eisenstat^{30,31}, Nicholas A. Vitanza^{32,33}, Javad Nazarian^{3,34,35}, Carl
15 Koschmann³⁶, Sabine Mueller^{34,37}, Jason E. Cain^{5,6}, Matthew D. Dun^{1,2,38,39}

16 **Affiliations:**

17 ¹ Cancer Signalling Research Group, School of Biomedical Sciences and Pharmacy, College of
18 Health, Medicine and Wellbeing, University of Newcastle, Callaghan, NSW, AUSTRALIA.

19 ² Precision Medicine Research Program, Hunter Medical Research Institute, New Lambton Heights,
20 NSW, AUSTRALIA.

21 ³ Center for Genetic Medicine Research, Children's National Hospital, Washington, DC, USA.

22 ⁴ Brain Tumor Institute, Children's National Hospital, Washington, DC, USA.

23 ⁵ Centre for Cancer Research, Hudson Institute of Medical Research, Clayton, VIC, AUSTRALIA.

24 ⁶ Department of Molecular and Translational Science, Monash University, Clayton, VIC,
25 AUSTRALIA.

26 ⁷ Centre de Recherche en Cancérologie de Marseille, Aix-Marseille Université, Inserm, CNRS,
27 Institut Paoli Calmettes, Marseille, FRANCE.

28 ⁸ Brain Barriers Group, School of Biomedical Sciences and Pharmacy, College of Health, Medicine
29 and Wellbeing, University of Newcastle, University Drive, Callaghan, NSW, AUSTRALIA.

30 ⁹ Department of Biology, Edge Hill University, Ormskirk, UK.

31 ¹⁰ School of Environmental and Life Sciences, College of Engineering, Science and Environment,
32 University of Newcastle, Callaghan, NSW, AUSTRALIA.

33 ¹¹ Infertility and Reproduction Research Program, Hunter Medical Research Institute, New Lambton
34 Heights, NSW, AUSTRALIA.

35 ¹² Analytical and Biomolecular Research Facility Advanced Mass Spectrometry Unit, University of
36 Newcastle, Callaghan, NSW, AUSTRALIA.

- 37 ¹³ QIMR Berghofer Medical Research Institute, Herston, QLD, AUSTRALIA.
- 38 ¹⁴ Department of Paediatric Oncology, Sydney Children's Hospital, Randwick, NSW, AUSTRALIA
- 39 ¹⁵ Michael Rice Cancer Centre, Women's and Children's Hospital, South Australia Health and
40 Medical Research Institute, South Australia ImmunoGenomics Cancer Institute, University of
41 Adelaide, Adelaide, SA, AUSTRALIA.
- 42 ¹⁶ Department of Oncology, The Children's Hospital at Westmead, Westmead, NSW, AUSTRALIA.
- 43 ¹⁷ Brain Tumor Research Program, Telethon Kids Cancer Centre, Telethon Kids Institute, University
44 of Western Australia, Perth, WA, AUSTRALIA.
- 45 ¹⁸ Department of Pediatric and Adolescent Oncology and Hematology, Perth Children's Hospital,
46 Perth, WA, AUSTRALIA.
- 47 ¹⁹ John Hunter Children's Hospital, New Lambton Heights, NSW, AUSTRALIA.
- 48 ²⁰ Centre for Molecular Medicine Norway (NCMM), Nordic EMBL Partnership, University of Oslo and
49 Oslo University Hospital, Oslo, NORWAY.
- 50 ²¹ Department of Neurology, University of California, San Francisco, San Francisco, CA, USA.
- 51 ²² Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense M,
52 DENMARK.
- 53 ²³ Cancer Epigenetics Biology and Therapeutics, Precision Medicine Theme, Children's Cancer
54 Institute, Sydney, NSW, AUSTRALIA
- 55 ²⁴ School of Women's and Children's Health, University of NSW, Sydney, NSW, AUSTRALIA
- 56 ²⁵ Tumorigenesis and Resistance to Treatment Unit, Centre Oscar Lambret, Lille, FRANCE
- 57 ²⁶ University of Lille, CNRS, Inserm, CHU Lille, UMR9020-U1277, CANTHER, Cancer
58 Heterogeneity Plasticity and Resistance to Therapies, Lille, FRANCE
- 59 ²⁷ Metronomics Global Health Initiative, Marseille, FRANCE.
- 60 ²⁸ Department of Pediatric Oncology, La Timone Children's Hospital, AP-HM, Marseille, FRANCE
- 61 ²⁹ Princess Máxima Center for Pediatric Oncology, Utrecht, THE NETHERLANDS.
- 62 ³⁰ Children's Cancer Centre, The Royal Children's Hospital Melbourne, Parkville, Victoria,
63 AUSTRALIA
- 64 ³¹ Neuro-Oncology Laboratory, Murdoch Children's Research Institute, Department of Paediatrics,
65 University of Melbourne, Parkville, Victoria, AUSTRALIA
- 66 ³² Ben Towne Center for Childhood Cancer Research, Seattle Children's Research Institute, Seattle,
67 WA, USA
- 68 ³³ Division of Pediatric Hematology/Oncology, Department of Pediatrics, Seattle Children's Hospital,
69 Seattle, WA, USA
- 70 ³⁴ Department of Pediatrics, University Children's Hospital Zurich, Zurich, SWITZERLAND.
- 71 ³⁵ The George Washington University, School of Medicine and Health Sciences, Washington, DC,
72 USA.
- 73 ³⁶ Division of Pediatric Hematology/Oncology, Department of Pediatrics, University of Michigan, Ann
74 Arbor, MI, USA.

75 ³⁷ Department of Neurology, Neurosurgery and Pediatric, University of California, San Francisco,
76 CA, USA.

77 ³⁸ Paediatric Program, Mark Hughes Foundation Centre for Brain Cancer Research, College of
78 Health, Medicine, and Wellbeing, Callaghan, NSW, AUSTRALIA

79 ³⁹ Lead contact

80 # These authors shared the first authorship for this work.

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93 **Corresponding authors:**

94 Matthew D. Dun Ph.D.

95 University Drive, Callaghan, NSW 2308, Australia

96 Phone: +61 2 4921 5693

97 Email: matt.dun@newcastle.edu.au, Twitter: @MattDun17

98

99 Sabine Mueller M.D., Ph.D., MAS.

100 Department of Neurology, Neurosurgery and Pediatrics, University of California, San Francisco, 675

101 Nelson Lane Ring, Room 402, San Francisco, CA 94158. U.S.A.

102 Phone: +1 415 476 3831

103 E-mail: sabine.mueller@ucsf.edu

104

105 Jason E. Cain Ph.D.

106 27–31 Wright Street, Clayton, VIC 3168, Australia

107 Phone: +61 3 8572 2720

108 Email: jason.cain@hudson.org.au

109

110 Carl Koschmann M.D.

111 3520D MSRB 1, 1150 W Medical Center Dr., Ann Arbor MI, 48109

112 Phone: +1 734 936 9814

113 Email: ckoschma@med.umich.edu

114

115 Javad Nazarian Ph.D.

116 Department of Oncology, Children's Research Center, University Children's Hospital

117 Zürich, Balgrist Campus, Lengghalde 5, 8008 Zürich, Switzerland

118 Phone: +41 44 266 3776

119 Email: Javad.Nazarian@kispi.uzh.ch

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121 **Conflict of interest:**

122 M.D. Dun is a director of the not-for-profit charity RUN DIPG Ltd.

123

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126 **Abstract**

127 Diffuse midline gliomas (DMG), including diffuse intrinsic pontine gliomas (DIPGs), are the most
128 lethal of childhood cancers. Palliative radiotherapy is the only established treatment, with median
129 patient survival of 9-11 months. ONC201 is a DRD2 antagonist and ClpP agonist that has shown
130 preclinical and emerging clinical efficacy in DMG. However, further work is needed to identify the
131 mechanisms of response of DIPGs to ONC201 treatment and to determine whether recurring
132 genomic features influence response. Using a systems-biological approach, we showed that
133 ONC201 elicits potent agonism of the mitochondrial protease ClpP to drive proteolysis of electron
134 transport chain and tricarboxylic acid cycle proteins. DIPGs harboring *PIK3CA*-mutations showed
135 increased sensitivity to ONC201, while those harboring *TP53*-mutations were more resistant.
136 Metabolic adaptation and reduced sensitivity to ONC201 was promoted by redox-activated PI3K/Akt
137 signaling, which could be counteracted using the brain penetrant PI3K/Akt inhibitor, paxalisib.
138 Together, these discoveries coupled with the powerful anti-DIPG/DMG pharmacokinetic and
139 pharmacodynamic properties of ONC201 and paxalisib have provided the rationale for the ongoing
140 DIPG/DMG phase II combination clinical trial NCT05009992.

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142 **50-word statement of significance**

143 PI3K/Akt signaling promotes metabolic adaptation to ONC201-mediated disruption of mitochondrial
144 energy homeostasis in diffuse intrinsic pontine glioma, highlighting the utility of a combination
145 treatment strategy employing ONC201 and the PI3K/Akt inhibitor paxalisib.

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149 **Introduction**

150 High-grade gliomas (HGGs) are responsible for 10-15% of all pediatric central nervous system
151 (CNS) cancers, but account for over 40% of deaths (1). Diffuse midline gliomas (DMG), including
152 those of the brainstem (diffuse intrinsic pontine glioma - DIPG) are universally fatal childhood
153 malignancies and responsible for half of all pediatric HGG diagnoses (2). Despite half a century of
154 clinical trials, radiotherapy (RT) remains the only life prolonging treatment for DIPG, with the median
155 overall survival (OS) remaining stagnant at 9-11 months post-diagnosis, and <10% of patients with
156 pontine tumors surviving more than two years post-diagnosis (3,4). The diffuse and infiltrative
157 growth characteristics of DIPG which enmesh the critical structures of the brainstem make surgical
158 resection extremely challenging. However, over the last 10 years image-guided stereotactic biopsy
159 at diagnosis has been shown to be safe and feasible (5), helping to isolate tumor tissue to identify
160 the recurring molecular (6) and immunological (7) features of the disease.

161 Global loss of trimethylation at lysine 27 (K27) of histone H3 drives epigenetic dysregulation in
162 primitive neuronal stem cells/oligodendrocyte precursor cells, caused by a methionine to lysine
163 substitution (H3K27M) in either *HIST1H3B* (H3.1) or *H3F3A* (H3.3) genes (8-10) or through the
164 overexpression of *EZH1* (EZH inhibitory protein) in patients harboring wildtype H3 (11). These H3-
165 alterations inhibit the catalysis of H3K27 trimethylation by the polycomb repressive complex 2
166 (PRC2) (12) and co-occur with mutations in tumor suppressor and signaling genes (13). Together,
167 these changes promote the activity of oncogenic signaling cascades that sustain mitogenesis,
168 immune system avoidance and drive cellular immortality (14).

169 Preliminary clinical efficacy for the oral, small molecule imipridone anticancer therapy, ONC201, has
170 been reported in patients diagnosed with DIPG (15) and recurrent H3K27M DMG (16). Previous
171 studies in hematological (17), colorectal (18), breast (19), uterine (20), and non-midline brain
172 cancers (such as glioblastoma) (21), showed ONC201 triggered p53-independent cancer cell
173 apoptosis driven in part by an atypical integrated stress response, initiating expression of the anti-
174 tumor protein TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) (22,23). The
175 identification of a durable objective response observed in a patient with a secondary glioblastoma
176 harboring a H3.3K27M mutation encouraged continued testing in patients with these mutations,
177 such as DIPG (21).

178 Described as a dopamine receptor D2 (DRD2) selective antagonist, corroborated by Bayesian
179 machine-learning approaches (24), more recent studies show ONC201 is also a potent agonist of
180 the ATP-dependent Clp protease proteolytic subunit (ClpP), a mitochondrial protein that degrades
181 mitochondrial respiratory chain proteins to disrupt energy homeostasis (23,25). Recently, mRNA
182 expression analysis correlated *CLPP* expression with tumor grade and overall survival in DMG (25).
183 These studies also demonstrated that DMG cell lines with sensitivity to ONC201 and ONC206 (a

184 fluorinated analog of ONC201 in a phase I pediatric clinical trial for DMG PNOC023,
185 NCT04732065), impaired tumor cell metabolism and caused mitochondrial damage, inducing
186 reactive oxygen species (ROS) production to activate an integrative stress response and apoptosis
187 *in vitro* and *in vivo*.

188 Metabolic effects highlight the potential of ONC201 for the treatment of DIPG, potentially
189 circumventing the inter- and intra-tumoral heterogeneity that has previously plagued the use of
190 precision-therapy based approaches (6). Indeed, ONC201 induces a state of energy depletion as
191 outlined by a significant decrease in ATP levels and a hypo-phosphorylated state in glioblastoma
192 (26). Potentially, ONC201 represents an important first step in the establishment of a recognized
193 targeted treatment strategy for some patients with H3K27-altered DMG; however, monotherapeutic
194 benefits are transient, whilst for other patients ONC201 offers no survival improvements and these
195 individuals succumb quickly (27).

196 Here we employ a systems-wide approach to identify combination strategies to increase the
197 therapeutic response to ONC201, thereby providing the preclinical and preliminary clinical evidence
198 for the commencement of the phase II clinical trial to test ONC201 in combination with the potent
199 brain-penetrant PI3K/Akt inhibitor, paxalisib (28,29), for the treatment of H3K27M DIPG and DMG
200 patients at diagnosis and disease progression (NCT05009992).

201 **Materials and Methods**

202 **Reagents**

203 Unless otherwise stated, all reagents were obtained from ThermoFisher Scientific, Waltham, MA,
204 USA.

205 **Drugs**

206 ONC201 (Chimerix, Philadelphia, PA, USA) and paxalisib (Kazia Therapeutics Limited, Sydney,
207 NSW, Australia) were obtained under a materials transfer agreement.

208 **Cell lines**

209 The use of patient derived DIPG neurosphere cell cultures in this study was approved by the
210 Human Ethics Research Committee, University of Newcastle (H-2018-0241). Cell lines (summarized
211 in Table S1) were cultured as previously described (30).

212 **Sensitivity**

213 Drug effect on cellular growth and proliferation was determined using the resazurin cell proliferation
214 assay as previously established (15). Briefly, DIPG cells were seeded at 2.5×10^4 cells/well in a 96-
215 well plate, incubated overnight at 37°C and treated with a 1:2 serial dilution of ONC201 from 150 μ M
216 for 96 h. Cells were treated as neurospheres without growth matrix. For low oxygen testing, DIPG

217 cells were grown in 5% O₂ conditions for at least 1 week before commencement of assays. Plates
218 were read using a Fluostar system at 544/590 nm and values graphed compared to the untreated
219 control.

220 **Annexin-V FITC assay**

221 Cell death was measured using an Annexin-V FITC apoptosis detection kit (BD Biosciences,
222 Sydney, NSW, Australia) as previously established (15). Cells were seeded at a density of 5 × 10⁴
223 per well in a 96-well plate and were incubated with ONC201, for 96 h before propidium iodide and
224 Annexin V staining as per manufacturer's recommendations. Stained cells were analyzed using a
225 FACS Canto II flow cytometer and data were processed using FlowJo software.

226 **Colony formation assay**

227 SU-DIPG-VI colony forming ability was assessed via soft agar growth matrix colony formation assay
228 as previously described (31). A total of 3,000 SU-DIPG-VI cells/well were plated into the top agar
229 layer of 24-well plates with indicated doses. MTT was used to count proliferative cells after 2 weeks
230 of growth (5% CO₂ conditions). These data were analyzed with ImageJ and are presented as colony
231 number compared to untreated wells, performed in biological triplicate.

232 **Western Blotting**

233 Protein was extracted from DIPG cells using RIPA buffer as per manufacture's recommendations
234 and previously described (32). BCA quantification was performed using a Pierce BCA Protein Assay
235 Kit (Catalogue No. 23227) according to the manufacturer's instructions. Primary antibodies were
236 incubated overnight at dilutions described in Table S2. Secondary horseradish peroxidase (HRP)
237 conjugated antibody (1662408) (Bio-Rad, Hercules, CA, USA) was used at a dilution of 1:5000.
238 Labeled protein bands were imaged using enhanced chemiluminescence (ECL – Classico,
239 Crescendo (Merck KGaA, Darmstadt, Germany)) in combination with a Chemidoc MP Imaging
240 System (Bio-Rad) and data were analyzed using ImageLab software.

241 **Res259 H3 mutation transfection**

242 The human pediatric glioma cell line Res259 (grade II, diffuse astrocytoma) was transfected to
243 express the wildtype or mutated histone H3 forms, using a Cell Line Nucleofector™ Kit V (Lonza,
244 Basel, Switzerland) with 1 µg of the plasmid containing K27M mutated *H3F3A* or *HIST1H3B* gene
245 fused with the *mCherry* gene, and bearing a resistance gene for Hygromycin B. As a control, cells
246 were transfected with a similar plasmid containing the wildtype *H3F3A* or *HIST1H3B* gene. Cells
247 were selected using Hygromycin B and sorted for mCherry expression. Histones PTMs were
248 collected using a histone extraction kit (Abcam ab113476, Cambridge, UK) and analyzed using
249 immunoblotting.

250 **CRIPS-Cas9**

251 A total of 2×10^5 cells were seeded in a 6-well plate and incubated overnight. Cells were then
252 replenished with fresh complete media containing 5 $\mu\text{g}/\text{mL}$ polybrene (ThermoFisher Scientific). A
253 250 μL aliquot of lentiviral cocktail containing either Lenti-Cas9-Blast plasmid (SU-DIPG 13;
254 Addgene, Watertown, MA, USA), Lenti-Cas9-2A-Blast (SU-DIPG 36; Addgene) or FUCas9Cherry
255 (DIPG-HSJD-007; Addgene) was supplemented into the cell media and incubated for 72 h.
256 Transduced cells were selectively maintained in complete media containing 10 $\mu\text{g}/\text{mL}$ blasticidin
257 (Jomar Life Research, Scoresby, Victoria, Australia) for at least 7 days, or sorted for mCherry
258 expression, prior to experiment. *CLPP*, *DRD2*, *TP53*, and non-targeting control (NTC) single guide
259 RNA (sgRNA), cloned into the U6-gRNA/hPGK-puro-2A-BFP vector, were obtained from the Human
260 Sanger Whole Genome Lentiviral CRISPR Library (ThermoFisher Scientific). The details of gRNA
261 sequence for the *CLPP*, *DRD2*, and *TP53* were as follows: *CLPP*: 5'-
262 GGTGTGGTGACCGCGGGCCTGG -3', *DRD2*: 5'- GGCAATGATGCACTCGTTCTGG -3', *TP53*: 5'-
263 CTCGAAGCGCTCACGCCACGG -3'

264 A total of 5×10^5 Lenti-X HEK29T were seeded in 6-well plates and the following day were
265 transfected with sgRNA plasmids along with the viral packaging plasmids, psPAX-D64V (Addgene)
266 and pMD2.G (Addgene) using Lipofectamine™ LTX Reagent with PLUS™ reagent as per the
267 manufacturer's recommendations. Transfection media was replaced with fresh media after 6 h and
268 incubated for a further 72 h prior to collection of virus-containing media. Viral media was added to 2
269 $\times 10^5$ Cas9-expressing DMG cells in a 6-well plate in the presence of 1 $\mu\text{g}/\text{mL}$ polybrene,
270 centrifuged at 800 x g for 30 min and then incubated for 72 h. Selection of transduced cells using 2
271 $\mu\text{g}/\text{mL}$ of puromycin in fresh media was performed until non-transduced control cells were dead.
272 Heterogenous cell lines were maintained in 2 $\mu\text{g}/\text{mL}$ puromycin. For the establishment of single cell
273 clones from the heterogenous population, single BFP positive cells were sorted in 96-well plates
274 containing a 1:1 mixture of conditioned media and fresh media. Single cell clones were expanded
275 and screened using immunoblotting to identify clones with reduced or absent target protein.

276 **Mass Spectrometry**

277 Proteomic analysis was conducted as previously reported (33). Briefly, protein was extracted from
278 DIPG cells using a Na_2CO_3 solubilization method capable of differentiating between soluble and
279 membrane bound proteins. Oasis solid-phase extraction (SPE) columns (Waters, Milford, MA, USA)
280 were blocked using a trypsin digest of bovine serum albumin (BSA) prior to being used to desalt
281 protein extracts. A total of 100 μg of each sample (as determined by Qubit 2.0 Fluorometer
282 quantification) was labelled with TMT 16 plex pro labelling tags (as per Table S3) according to the
283 manufacturer's instructions. Samples were fractionated by offline high-pH reverse phase
284 fractionation using a Dionex Ultra 3000 uHPLC system (Thermo Fisher Scientific) using nano Ease
285 M/Z Peptide CSH C18 column (130 A, 1.7 μm , 300 $\mu\text{m} \times 100 \text{ mm}$) (Waters). LC-MS/MS was
286 performed using EASY-nLC 1000 (Thermo Fisher Scientific) coupled online to an Orbitrap Exploris

287 480 mass spectrometer (Thermo Fisher Scientific). Raw files were processed via Proteome
288 Discoverer 2.5.

289 Hierarchical clustering was performed using Perseus. For our parameters, we used a Euclidean
290 distance, with average linkage and no constraint. A pre-process with k-means was performed, with
291 a maximum of 300 clusters, no more than 10 iterations and 1 restart. Due to the small size of some
292 clusters, we grouped like clusters where the same treatment had similar expression profiles. Cluster
293 1 was the combination of two clusters, while cluster 3 was the combination of 7 clusters, 4 of which
294 with clusters less than 5 genes. Cluster 2 and cluster 4 are standalone. Hierarchical clustering trees
295 have been highlighted. Ingenuity Pathway Analysis software (IPA; Qiagen) was used for
296 bioinformatic analysis of proteomic dataset. Canonical pathways, and upstream regulator analyses
297 were generated and assessed based on p-value and z-scores.

298 **DIPG xenograft modeling**

299 All *in vivo* experiments were conducted in compliance with the approved CNH Institutional Animal
300 Care and Use Committee protocol (#30425), the University of Newcastle Animal Care and Ethics
301 Committee (#A-2019-900) and the University of California San Francisco Institutional Animal Care
302 and Use Committee (IACUC). Five-week-old, male, NSG mice were implanted with 100,000 SU-
303 DIPG-VI/Luc and 300,000 HSJD-DIPG-007 tumor cells into the pontine region of the brainstem
304 using coordinates with Lambda as the reference point (Y: 1.5 mm, X: 0.8 mm, Z: 5 mm) at a rate of
305 1 μ L/min. Mice were allowed to recover for 4 and 3 weeks, respectively before commencement of
306 treatment.

307 For SU-DIPG-VI model, ONC201 and paxalisib were administered by oral gavage at 125 mg/kg
308 (PBS) and 10 mg/kg (0.5% methyl cellulose/0.2% Tween 80), respectively, at a frequency of 1
309 time/week and 3 times/week. Animals were monitored for weight loss (compared to base weight)
310 and clinical signs. Dose holidays were given at 10% weight loss and resumed at 5% weight
311 recovery. Mice were humanely sacrificed when neurological symptoms were observed, or with more
312 than 20% weight loss.

313 For HSJD-DIPG-007 xenograft model, ONC201 and paxalisib were administered as above, except
314 paxalisib was given twice daily at 5 mg/kg. Mice were treated for 5 weeks. Mice were sacrificed at
315 endpoints as described above.

316 For the SF8628 study, five to six week-old, female athymic (homozygous, nu/nu) mice were
317 obtained from Harlan-Envigo Laboratory (Livermore, CA, USA). For tumor inoculation 500,000
318 human SF8628 DIPG cells with the luciferase reporter gene were intracranially implanted into the
319 right pons as previously described (34). Briefly, anesthetized animals received 2 μ L of cell
320 suspensions into the right pontine area, with injection coordinates 1 mm to the right from the
321 lambda, top of lamboid suture and 4 mm depth. Treatment initiated at day 14, when

322 bioluminescence indicated log phase growth. Mice were euthanized when tumor burden reached
323 levels determined by IACUC guidelines.

324 Tumor size was monitored bi-weekly, using an IVIS-Lumina III imaging system (PerkinElmer,
325 Waltham, MA, USA) for SU-DIPG-VI-Luc. For SF8628, bioluminescence was measuring using an
326 IVIS Lumina imaging station (Caliper Life Sciences, Alameda, CA) (35). Mice were intraperitoneally
327 injected with 150 mg/kg of D-luciferin (Gold Biotechnology) and imaged 10 min following D-luciferin
328 injection. BLI signal intensities were quantified using the region of interest feature of Living Image
329 software. BLI signal at each time point was plotted as an average of total flux (photons/sec) for all
330 animals in each group.

331 In SU-DIPG-VI, following 2 *in vivo* treatments with ONC201, paxalisib or combination, brainstems
332 were resected lysed with RIPA buffer, immunoblotting was then performed as described above.
333 Samples for immunohistochemistry were collected in the middle of the fourth week of treatment in
334 HSJD-DIPG-007 xenograft model and staining was conducted as previously described for H3K27M,
335 Ki67 and SDHA (15). Images were visualized using ImageScope and pixel intensity was quantified
336 using Image J in technical triplicates across three biological replicates.

337 **Patient experience**

338 Written informed consent was obtained from each of the families whose child's data is included in
339 this study. Two children with biopsy/autopsy-confirmed H3K27M, PIK3CA or PIK3R1 mutant DIPG
340 were treated with ONC201 and paxalisib.

341 DIPG patient at 5 years old was diagnosed in March 2021 with *H3.1K27M*, *PIK3R1* and *ACVR1*
342 mutations. A biopsy was performed in the two weeks following this diagnosis, with RT started soon
343 after. The combination of ONC201 (15 mg/kg) and paxalisib (27 mg/m²) was started 3 months
344 following diagnosis and is ongoing.

345 DIPG patient at 16 years old, was diagnosed with *H3.3K27M*, *TP53* and *PIK3CA* mutations on the
346 19th of December 2018, without a biopsy. They began radiation and ONC201 (15 mg/kg) treatment
347 on the 9th of January 2019. ONC201 alone began February 2019. February 2020 saw further
348 progression, with ONC201 and panobinostat (45 mg daily three times per week), stopped 20th of
349 May 2020 at further progression. Re-irradiation and ONC201 began 29th May. ONC201 and
350 paxalisib (27 mg/m²) dual compassionate use began 22nd of June 2020.

351 **Statistical Analysis**

352 GraphPad Prism 9 software (La Jolla, CA, USA) was used for statistical analyses. Unless otherwise
353 stated, two sample unpaired Student's t-tests or one-way ANOVA was used to determine significant
354 differences between groups. Where samples sizes were smaller, comparing different biological
355 samples, non-parametric tests, one-way ANOVA, and t-tests were used. Survival analysis was

356 performed using the Log rank test. Values shown are the mean \pm SEM. Significance values:
357 $*=p<0.05$, $**=p<0.01$, $***=p<0.001$, $****=p<0.0001$ are used throughout.

358 **Data availability**

359 Data generated in this study have been included in the article and supplementary material. The
360 proteomics data is deposited to ProteomeXchange via the PRIDE with the dataset identifier
361 PXD036245 (36). All other raw data is available upon request from the corresponding author.

362 **Results**

363 **Comprehensive drug profiling predicts reduced sensitivity to ONC201 in *TP53*-mutant DIPG**

364 Using fourteen patient-derived neurosphere-cell culture models harboring DMG molecular subtypes
365 (H3-wt n=2, H3.1K27M n=4, and H3.3K27M n=7) and immortalized neural cell controls (HCMEC/D3
366 blood-brain barrier (BBB) endothelial cells, HMC3 microglial cells and ReN neural progenitor cells),
367 we assessed sensitivity to ONC201 via inhibition of proliferation, induction of apoptosis and cell
368 death. Overall, 43% of DIPG models, showed >50% reduction in proliferation following ONC201
369 exposure (Fig. 1A, Table S4). However, we identified a subpopulation of DIPG models, including
370 controls, which demonstrated <50% reduction in proliferation, even at very high concentrations of
371 ONC201 (>150 μ M) for up to 96 h. (Fig. 1A, Table S4).

372 Analysis of cell death markers via annexin V/PI cytotoxicity analysis corroborated proliferation data,
373 showing ONC201 is cytotoxic to SU-DIPG-XXXIII ($p=0.0043$) and HSJD-DIPG-007 ($p=0.0018$), with
374 UON-JUMP4 and SU-DIPG-XIII demonstrating decreased sensitivity (Fig. 1B), akin to previous
375 studies testing ONC201 in DIPG models (15,25) and at physiologically relevant doses (5 μ M) (37).
376 Neurosphere morphology was assessed following 6-day ONC201 treatment to account for
377 variations in doubling times (Fig. S1) across eleven DIPG cell line models. ONC201 sensitive cells
378 showed reduced cell number and less viability, while neurosphere models similarly featured less
379 robust sphere formation accompanied by more non-viable, singular cells (Figs. 1C upper panel, S2).
380 By contrast, models with decreased sensitivity retained cell number and neurosphere morphology
381 and presented with fewer differences in non-viable cells compared to untreated controls (Figs. 1C
382 lower panel, S2B).

383 To determine whether recurring mutations influenced the sensitivity of DIPG cell lines to ONC201,
384 we performed pharmacogenomic analysis using next generation sequencing (summarized in Fig.
385 1D). As a means of determining the comparative sensitivity across models we calculated the area
386 under the curve (AUC) of cell lines treated with ONC201 (Fig. 1A), and grouped DIPG models by
387 H3K27 status and assessed whether there were differences in sensitivity (Fig. 1E). No difference in
388 ONC201 sensitivity was seen in H3K27-altered subtypes (Fig. 1E, wt-H3 vs. H3.1K27M, $p=0.0696$;
389 wt-H3 vs. H3.3K27M, $p=0.09999$; H3.1K27M vs. H3.3K27M, $p=0.1711$). In line with previous studies
390 of ONC201 efficacy in glioblastoma models (21), and to confirm the role histone mutations may play

391 in response to ONC201, we knocked in H3.1K27M or H3.3K27M mutations into wt-H3 astrocytoma
392 models (Res259) (38). Res259 cells harbor overexpression of PDGFRA and KIT (39), which
393 represent a similar genetic architecture to DMGs without the H3-/EZHIP- alterations. In line with
394 previous studies Res259-H3.3K27M+ cells showed significantly increased sensitivity compared with
395 H3.1K27M+ cells (H3.3K27M vs. H3.1K27M $p=0.0139$) (Figs. 1F,G, S3A,B).

396 Besides H3K27M-alterations, *TP53* loss of function mutations (LoF) were the next most frequently
397 identified in our DIPG models ($n=9$), and included missense variants ($n=5$), stop gains ($n=3$) and a
398 splice donor variant ($n=1$), predominantly affecting H3.3K27M DIPG models (Fig. 1D). *TP53*-mutant
399 DIPG models were significantly less sensitive to ONC201 than wt-*TP53* DIPG models ($p = 0.014$)
400 (Fig. 1H). Receiver operating characteristic (ROC) curve analysis supported the pharmacogenomic
401 observation that *TP53*-mutant DIPGs possessed decreased sensitivity to ONC201
402 (AUROC=0.9722, $p=0.0087$) (Fig. S3C). To further explore the influence of *TP53* mutations, we
403 performed CRISPR-Cas9 mediated *TP53* knockdown (KD) and single cell knockout (KO) using the
404 ONC201 sensitive HSJD-DIPG-007 DIPG model, which harbors wt-*TP53*, H3.3K27M and mutant
405 *PPM1D* (Fig. 1A,B,D). Modulating expression of *TP53* did not influence proliferation rate (Fig. S3D),
406 however, in agreement with our pharmacogenomics studies, *TP53* KD/KO decreased sensitivity of
407 HSJD-DIPG-007 to ONC201 treatment compared to non-targeting gRNA controls (NTC) (ONC201
408 IC_{50} wt-*TP53*=2.202 μ M, *TP53*-KD=7.344 μ M $p=0.0117$, *TP53*-KO=NR $p=0.004$) (Figs. 1I,J, S3E).
409 The role LoF *TP53* mutations in response to ONC201 was further investigated using
410 immunoblotting, which demonstrated that 5 μ M ONC201 induced robust cleavage of PARP,
411 indicative of apoptosis in wt-*TP53* cells, moderate cleavage in *TP53*-KD cells, but no cleavage of
412 PARP in *TP53*-KO HSJD-DIPG-007 cells (Fig. 1K), corroborating the pharmacogenomic analysis
413 showing that DIPG cells harboring *TP53* mutations show decreased sensitivity to ONC201. Using
414 the small molecule MDM2 inhibitor of Nutlin-3, we show that knockdown/knockout of *TP53* mimics
415 LoF mutations, driving senescence only in non-transfected control (*TP53*-NTC $IC_{50} = 3.507$; *TP53*-
416 KD and *TP53*-KO $IC_{50} =$ NR) than *TP53*-KD and *TP53*-KO, which did not reach IC_{50} (Fig. 1L).
417 Further, even though HSJD-DIPG-007 cells harbor a *PPM1D* mutation, these cells are as sensitive
418 to MDM2 antagonism in line with other *PPM1D* mutant cells lines, (40) and conversely to HSJD-
419 DIPG-007 cells harboring *TP53* LoF, show reduced sensitivity to ONC201 (Figs. 1L, S3F,G). As
420 *TP53* and H3.3K27M mutations are known to associate with aneuploidy, and a chromosomal
421 instability signature, we examined if *TP53* mutations and ONC201 sensitivity correlated with
422 chromosomal instability (41), through the measurement of tumor mutational burden (TMB),
423 microsatellite instability (MSI) (Figs. 1D, S3H,I) and chromosomal gains and losses (Fig. S3J,K). No
424 difference between *TP53* status and MSI, TMB or chromosomal gains/losses was observed,
425 suggesting this may not be a feature of *TP53* mutant DMGs in our cohort. Furthermore, TS0-500
426 revealed high number of *ACVR1*-mutant DIPGs (38%, $n=5$), often co-occurring with H3.1K27M

427 (23%, n=3) (Fig. 1D). We next examined whether *ACVR1* promoted sensitivity to ONC201, however
428 showed no difference in ONC201 sensitivity between wt-*ACRV1* and mutant DIPGs ($p=0.1274$, Fig.
429 1M). Additionally, as *ACVR1* and *PIK3CA* regularly co-occur (23%, n=3), with recurrent *PIK3CA*
430 mutations seen in our DMG models (31%, n=4), we examined if *PIK3CA* mutations could predict
431 sensitivity and show here that they are more sensitive to ONC201 compared to wt-*PIK3CA* DIPGs
432 ($p=0.012$, Fig. 1N).

433 **Somatic pharmacogenomic analysis identified DRD2 and CLPP to be targets of ONC201 in** 434 **DIPG**

435 *In vitro* profiling of the G-protein coupled receptor (GPCR) superfamily has previously shown
436 ONC201 to be a dopamine receptor (DRD2/3/4) antagonist (24), as well as an agonist of the
437 mitochondrial protease ClpP (19,23). Recently we performed molecular modeling of both ClpP and
438 DRD2 to show that ONC201 binds to both targets with high affinity (15). Therefore, to identify
439 targets of, and hence pathways influenced by ONC201, we correlated ONC201 sensitivity (z-AUC)
440 with basal gene (Fig. S4A) and protein (Fig. 2A) expression profiles of known putative targets. High
441 DRD2 protein expression was significantly correlated with increased sensitivity to ONC201
442 ($R^2=0.2348$, $p=0.0027$) (Fig. 2B), and at the transcript level ($R^2=0.1382$, $p=0.0431$) (Fig. S4B). A
443 significant correlation was also identified for ClpP at the protein level ($R^2=0.1240$, $p=0.0352$) (Fig.
444 2B) however, not at the transcript level ($R^2=0.06571$, $p=0.1715$) (Fig. S4B). Pediatric HGG patients,
445 including DIPG patients, harbor ubiquitously high-*CLPP* expression, more so than any other
446 pediatric CNS tumor (Fig. 2C) (42). Agonism of ClpP by ONC201, increases its proteolytic activity to
447 drive degradation of respiratory chain complex subunits including Succinate dehydrogenase A and
448 B (SDHA and SDHB), among others (23) (Fig. S4C). Succinate dehydrogenase enzymes form
449 integral components of both the TCA cycle and mitochondrial respiratory ETC, and not only oxidize
450 succinate to fumarate to support energy production, but their loss promotes oxidative stress through
451 the production of ROS and release intermediates that control chromatin modifications and gene
452 expression (43). Our recent study of ONC201 used by DIPG patients, showed ONC201 elicited
453 potent degradation of SDHA in DIPG patient derived xenograft (PDX) tumor tissue *in vivo* (15).
454 Analysis shows that SDHA protein expression did non-significantly correlate with ONC201
455 sensitivity ($R^2=0.05555$, $p=0.1664$) (Fig. 2B), and this was also not at the transcript level ($R^2=0.118$,
456 $p=0.071$) (Fig. S4B). However, the ratio of SDHA (proteolytic target) to ClpP (protease) protein
457 expression profiles may influence DMG cell sensitivity to ONC201 ($R^2=0.1823$, $p=0.0094$) (Fig. 2B),
458 providing further evidence that ClpP is a target of ONC201 in DIPG. We further examined the role of
459 ClpP and DRD2 in mediating ONC201 sensitivity using CRISPR-Cas9 mediated knockdown (Fig.
460 2D). Indeed, loss of *CLPP* expression had no effect on the ONC201-cell line harboring reduced
461 sensitivity (SU-DIPG-XIII) yet abrogated the effects of ONC201 in the sensitive line (SU-DIPG-
462 XXXVI) (Fig. 2D,E). Interestingly, DRD2 was shown to be indispensable for DIPG cell line

463 proliferation *in vitro*, regardless of sensitivity (Fig. 2D,E), analogous to *in vitro* and *in vivo* studies
464 performed in patient derived glioblastoma models (44).

465 **Quantitative proteomic profiling confirms ONC201 drives mitochondrial degradation,**
466 **rescued by redox-regulated PI3K/Akt signaling**

467 Pharmacogenomics coupled with gene editing predicted *TP53* mutations/LoF to influence sensitivity
468 to ONC201 (Fig. 1), which is at odds with previous studies in non-DIPG cancers (17,22). However,
469 biochemical correlation of putative targets including *DRD2* and *CLPP* supports previously identified
470 mechanisms of the anti-cancer effects of ONC201 in non-DIPG cells.

471 Given the critical role *SDHA* plays in mitochondrial respiration, we performed high-resolution
472 quantitative proteomic profiling following ONC201 exposure (5 μ M, 24 h) in both normoxic and low
473 oxygen conditions to mimic the spatial heterogeneity of DIPG using SU-DIPG-VI cells (H3.3K27M,
474 *TP53*-mutant, *DRD2*-low, *SDHA*-high, ONC201 resistant) (Table S5). Hierarchical clustering
475 revealed subtle but significant changes in protein expression induced by ONC201 treatment in cells
476 grown under different oxygen tensions (Fig. S5A). By interrogating differentially and commonly
477 expressed clusters, assigned using the differences influenced by ONC201 or oxygen concentration
478 (Fig. S5B,C) using Ingenuity Pathway Analysis (IPA), we identified mitochondrial dysfunction as the
479 most significantly altered canonical process across these clusters and across both oxygen tensions
480 following ONC201 treatment ($p=1E-27$) (Figs. 3A, S5B,D) (Table S6-8), with oxidative
481 phosphorylation the most significantly downregulated cellular process ($p=1.58e-24$, z -score=-4.49)
482 (Figs. 3A, S5B,C). Activated upstream regulator analysis further revealed the role that ONC201
483 plays in promoting *ClpP* ($p=6.65E-09$, z -score=3.051) and *KDM5A* ($p=2.22E-15$, z -score=4.2)
484 activity, disrupting mitochondrial homeostasis ($p=6.65E-09$, z -score=3.051) (Fig. 3B) and degrading
485 mitochondrial and tricarboxylic acid cycle (TCA) proteins, (*SDHA*, $p=2.58E-04$ and *IDH3B*, $p=5.29E-$
486 03 , respectively), as well as additional enzymes of the mitochondrial energy production pathways
487 (Fig. 3C). Immunoblotting confirmed the changes in protein expression revealed by mass
488 spectrometry, here ONC201 elicited degradation of mitochondrial proteins *SDHA* and *IDH3A/B* and
489 increased phosphorylation of *H2AX* (Fig. 3D).

490 Protein expression profiles significantly regulated by ONC201 treatment across oxygen tensions
491 predicted the Akt serine/threonine kinase, the key effector of the PI3K pathway, to be up-regulated
492 following ONC201 treatment (*AKT1*, z -score=2.399; *Akt*, z -score=2.349), (Fig. 3B) (Table S9).
493 Additionally, IL-15 activity (z -score=2.416), which is known to stimulate the JAK-STAT pathway and
494 PI3K/Akt signaling was predicted to be increased (45). Taken together, the predicted increase of
495 PI3K/Akt signaling is potentially responsible for the significantly altered protein expression profiles
496 seen following ONC201 treatment (Fig. 3E). These include decreased expression of the
497 proapoptotic protein *BAD*, increased expression of the antiapoptotic protein *BCL2* as well as
498 increased expression of markers of quiescence and progenitor cell types such as *SOX2* and *EZH2*.

499 Predicted increased activity of activating transcription factor 4 (ATF4) (z-score=2.051, $p=0.0104$)
500 (Fig. 3B), leading to anti-apoptosis through unfolded protein response ($p=5.89E-04$) (Fig. S5B) (46)
501 is also driven by increased PI3K/Akt signaling, inferring a mechanism of avoiding cell death
502 processes following ONC201 treatment.

503 To further elucidate the role PI3K-Akt activation may be playing in resistance to ONC201 we
504 performed high-resolution comparative and quantitative proteomic profiling following ONC201
505 exposure (5 μ M, 24 h) across additional DIPG cell lines with varying sensitivity to ONC201; SU-
506 DIPG-XXXVI, SU-DIPG-XIII and compared to SU-DIPG-VI (Fig. 3F). SU-DIPG-VI and SU-DIPG-XIII
507 cells, less sensitive to ONC201 clustered together, away from SU-DIPG-XXXVI, which is more
508 sensitive to ONC201. Analysis using IPA revealed mitochondrial dysfunction and activation of
509 CLpP/KDM5A following treatment with ONC201 across all cell lines (Fig. 3G), further validating
510 ONC201 to be elucidating anti-DIPG effects through mitochondrial dysfunction. Treatment with
511 ONC201 induced activation of PI3K-Akt signaling proteins (Fig. 3G), including Akt, IGF1 and
512 downregulated PTEN signaling, in all cell lines, suggesting Akt activation is a reciprocal mechanism
513 associated with ONC201 treatment, however greater upregulation of Akt signaling was observed in
514 cell lines less sensitive to ONC201 (z-score: Akt SU-DIPG-XXXVI=1.067, SU-DIPG-VI=1.692, SU-
515 DIPG-XIII=2.039, mTOR: SU-DIPG-XXXVI=0.378, SU-DIPG-VI=1.134, SU-DIPG-XIII=1.890). Given
516 that unbiased global proteomic profiling results predicted increased PI3K/Akt activity following
517 ONC201 exposure, we orthogonally validated phosphorylation changes of proteins regulated by this
518 pathway, all of which showed increased phosphorylation in cells refractory to ONC201 following
519 treatment (Fig. 3H). Activated PI3K/Akt signaling potentiated phosphorylation of Akt at Thr308 and
520 Ser473 across DIPG lines regardless of ONC201 sensitivity, however activation of downstream
521 pathway proteins GSK3 α , GSK3 β , p70S6K was only present in cell lines showing reduced
522 sensitivity to ONC201 (SU-DIPG-VI, SU-DIPG-XIII and SU-DIPG-XVII) (Fig. S5E). In cell lines more
523 sensitive to ONC201, the increase in Akt phosphorylation occurred earlier (24 h for HSJD-DIPG-
524 007), however after 48 h, these cells became apoptotic as indicated by increased cleaved PARP
525 (Fig. 3H). Such data aligns with our recent demonstration that ONC201 drives mitochondrial ROS
526 production and mitochondrial structural abnormalities (25), and thereby links these responses with
527 the oxidative DNA damage seen in these cells (γ H2AX, Fig. 3D).

528 Together, the increased mitochondrial oxidative stress caused by ONC201's ClpP agonism and
529 electron leakage (25), commensurate with increased PI3K/Akt signaling activity, may be promoting
530 the activity of the stress sensing transcription factor nuclear factor erythroid 2-related factor 2
531 (NRF2) (Fig. 3G), as increased expression of its downstream target, the reductase NQO1 was
532 detected following ONC201 treatment (log2 fold-change = 0.28, $p=0.0017$ and orthogonally
533 validated) (Figs. 3C,E,H). NQO1 is responsible for promoting redox homeostasis and cell survival
534 (47). In this regard, KEAP1 is known to regulate the activity of NRF2, and is degraded with ONC201

535 treatment, leading to decreased abundance (Fig. 3H). These observations are in line with previous
536 studies that show loss of expression/degradation of KEAP1 promotes the transcriptional activity of
537 NRF2 resulting in partial epithelial to mesenchymal transition but only in tumors harboring *TP53* LoF
538 mutations (48). These observations potentially explain the persistent proliferation of *TP53*-mutant
539 DIPG cells even in the presence of high dose ONC201.

540 **ONC201 driven oxidative stress drives PI3K/Akt signaling highlighting the potential of** 541 **ONC201 combined with the PI3K/Akt inhibitor paxalisib**

542 Proteomic profiling predicted that increased PI3K/Akt signaling may be leading to decreased
543 sensitivity of DIPG cells to ONC201 (Fig. 3). Previously we showed ONC201 increased ROS
544 production (25) therefore, we used the potent ROS scavenger *N*-acetyl-L-cysteine (NAC) to
545 investigate whether there was a link between increased ROS and increased PI3K/Akt signaling.
546 NAC abrogated phosphorylation of Akt, while hydrogen peroxide (H₂O₂) increased phosphorylation
547 (Figs. 4A, S6A,B). As such, we hypothesized that inhibition of PI3K signaling may prevent the Akt-
548 mediated cell survival signaling induced following exposure to ONC201. To investigate this, we
549 tested whether the brain penetrant PI3K/Akt inhibitor paxalisib (previously GDC-0084) (28,29) could
550 also suppress PI3K/Akt signaling in response to ONC201. Paxalisib decreased phosphorylation of
551 Akt in the H3.3K27M, *TP53*-mutant and ONC201-refractory model SU-DIPG-XVII either alone or in
552 combination with ONC201 (Fig. 4B) (49). Again, ONC201 modulated the abundance of proteins
553 mapping to NRF2 regulated antioxidant response including the loss of KEAP1, increased NQO1,
554 both abrogated by the combination with paxalisib (Fig. 4B) to drive cell death.

555 To assess adhesion-independent cell proliferation and survival of DIPG cells treated with ONC201
556 we performed soft agar colony forming assays using SU-DIPG-VI that show decreased sensitivity to
557 ONC201 as a monotherapy. Encouragingly, at physiologically relevant dosing, single agents
558 decreased colony formation (ONC201 -0.43 log₂ fold, *p*=0.007; paxalisib -0.5 log₂ fold, *p*=0.0032),
559 with the combination of ONC201 and paxalisib significantly decreasing colony formation beyond that
560 achieved using either of the single agents (combination vs. UT, -1.4 log₂ fold, *p*<0.0001,
561 combination vs. ONC201, -1 log₂ fold, *p*=0.0007, combination vs. paxalisib, -0.93 log₂ fold,
562 *p*=0.0014) (Fig. 4C). Indeed, ONC201 in combination with paxalisib synergized, particularly in
563 H3.3K27M *TP53*-mutant DIPG models, regardless of whether the treatment was performed under
564 normoxic or low oxygen conditions (Figs. 4D,E, S7, S8A,B, Table S10), however, the combination
565 was additive in the UON-JUMP4 model, grown in low oxygen conditions (Fig. S8A,B). As an
566 additional control, we assessed the sensitivity of human peripheral blood mononuclear cells *in vitro*
567 donated from healthy volunteers to each drug individually and in combination, which revealed no
568 increase in cell death; however, a reduction in PI3K/Akt/mTOR signaling was observed (Fig.
569 S8C,D).

570 To determine if *TP53* status influenced ONC201 PI3K/Akt signaling we investigated the effect of
571 ONC201 in the *TP53*-KD and *TP53*-KO HSJD-DIPG007 models via immunoblotting. Here, ONC201
572 decreased SDHA abundance and ERK1/2 phosphorylation regardless of p53 status (Fig. 4F).
573 Again, ONC201 significantly increased phosphorylation of Akt at both T308 and S473 residues
574 (Figs. 4F, S9) across models, including cells harboring either *TP53*-KD or *TP53*-KO. Interestingly,
575 *TP53*-KD and *TP53*-KO HSJD-DIPG-007 cells harbored significantly increased basal levels of
576 phosphorylation of Akt at T308, a marker of active PI3K signaling compared to the NTCs, which was
577 further potentiated using ONC201 (Figs. 4F, S9). Therefore, to determine whether paxalisib could
578 rescue the decreased response promoted by KD and KO of *TP53* in HSJD-DIPG-007 cells we
579 tested ONC201 in combination with paxalisib and identified very high-level synergy in the *TP53*-KD /
580 *TP53*-KO cells, greater than three times that of parental cells and corresponding to the level of
581 increased PI3K signaling seen (Figs. 4G,H, S10). Together, these *in vitro* results highlight the
582 potential for the use of paxalisib in combination with ONC201 even in highly aggressive H3.3K27M
583 *TP53*-mutant DIPG models.

584 **Preclinical optimization of ONC201 combined with paxalisib**

585 Clinical trials testing ONC201 and paxalisib as monotherapies in DIPG/DMG have demonstrated
586 acceptable safety and toxicity profiles (NCT03416530 and NCT03696355, respectively). Therefore,
587 to test the preclinical utility of ONC201 combined with paxalisib we first examined their efficacy
588 using the SU-DIPG-VI-Luc (H3.3K27M, *TP53*-mutant) and HSJD-DIPG-007 (H3.3K27M, *TP53*-
589 wildtype) DIPG xenograft mouse models, using mouse equivalent MTDs (125 mg/kg q.w. ONC201,
590 in combination with paxalisib 10 mg/kg t.i.w., or 5 mg/kg/b.i.d., respectively) (42,43), engrafted into
591 the fourth ventricle/pons of NSG mice (Fig. 5A). SU-DIPG-VI-Luc mice were treated continuously
592 and HSJD-DIPG-007 mice were treated for five weeks from treatment start (Fig. 5A). *In vivo*
593 bioluminescence imaging (BLI) was performed immediately before drug or vehicle control
594 administration to assess baseline tumor burden (Fig. S11A,B). Using BLI as a surrogate for tumor
595 size in SU-DIPG-VI-Luc, ONC201 had no significant effect on tumor size, while paxalisib
596 significantly reduced tumor burden (paxalisib=404.84 p sec⁻¹ cm⁻² sr⁻¹, $p=0.0309$) (Fig. S11A,B).
597 ONC201, combined with paxalisib, decreased tumor burden throughout the treatment regimen
598 compared to vehicle control (4-week mean BLI ONC201+paxalisib=158.34 p sec⁻¹ cm⁻² sr⁻¹,
599 $p=0.0038$).

600 Both ONC201 and paxalisib as single agents significantly extended the survival of SU-DIPG-VI-Luc
601 xenograft models compared to vehicle controls, with the combination significantly extending the
602 survival compared to all treatments (vehicle=45 days, ONC201=56 $p=0.0082$, paxalisib=54 days
603 $p=0.0082$, ONC201+paxalisib=72 days, combination vs. vehicle $p=0.0027$, combination vs. paxalisib
604 $p=0.0198$, and combination vs. ONC201 $p=0.0044$, post treatment start) (Fig. 5B) (50). In the SU-
605 DIPG-VI model, we identified some early toxicity using 10 mg/kg/day (Fig. S11C), therefore, treated

606 HSJD-DIPG-007 mice with 5 mg/kg/b.i.d. to improve tolerability (Fig. S11D). In the ONC201
607 sensitive, HSJD-DIPG-007 model, ONC201 provided an increased survival (vehicle=43.5 vs.
608 ONC201=50 days, $p=0.0009$), and twice daily low dose paxalisib also provided an improved survival
609 advantage (vehicle vs. paxalisib=55 days, $p<0.0001$) (Fig. 5C). Together the combination both
610 significantly increased survival effect vs. controls (combination=61 days, $p<0.0001$) and was
611 synergistic compared to monotherapies (ONC201 vs. combination, $p=0.0003$; paxalisib vs.
612 combination, $p=0.0019$) (Fig. 5C). Analogous to *in vitro* studies (Fig. 4), tumors resected from SU-
613 DIPG-VI-Luc+ DIPG xenograft mice treated for two weeks, showed increased Akt phosphorylation
614 and expression of EZH2 following ONC201 treatment alone, consistent with our *in vitro* proteomic
615 profiling, with the former rescued using paxalisib (Fig. 5D). To determine whether systemic effects of
616 ONC201 treatment *in vivo*, we measured the expression of tyrosine hydroxylase (TH) (Fig. 5E).
617 ONC201 treatment decreased TH expression in the prefrontal cortex, but not in brainstem where
618 the SU-DIPG-VI cell line was engrafted (Fig. 5E). ONC201 decreased ERK phosphorylation in both
619 the prefrontal cortex and brainstem (Fig. 5E), commensurate with global effects on DRD2 inhibition,
620 suggesting that systemic effects of DRD2 inhibition and ERK phosphorylation may contribute to
621 efficacy observed in these models. To assess pharmacodynamic markers of treatment response,
622 we performed immunohistochemistry on fixed tumor tissue following 4 weeks of treatment. Tumor
623 was detected in the pons of all animals; however, compared to the controls, decreased H3K27M
624 staining was seen across biological replicates, including in the cerebellum of treated mice (Fig.
625 5F,G) ($p<0.05$). Compared to the controls, decreased staining of the proliferation marker Ki67 was
626 also seen across treatments (ONC201, $p=0.0114$; paxalisib, $p=0.0023$; combination $p=0.0002$), with
627 the combination also significantly decreased compared to ONC201 alone ($p=0.0275$) (Fig. 5F,G).
628 Significantly decreased staining for SDHA was seen in samples treated with ONC201 and the
629 combination (ONC201, $p=0.008$; ONC201+paxalisib, $p=0.0436$, respectively) (Fig. 5F,G).

630 Using the highly aggressive H3.3K27M+ SF8628 DIPG xenograft model (51), paxalisib alone and
631 the combination of ONC201 and paxalisib decreased tumor burden at early timepoints (Day 4;
632 vehicle vs. paxalisib, $p=0.0075$, vehicle vs. combination $p=0.0152$, Day 10; vehicle vs. paxalisib,
633 $p=0.0042$, vehicle vs. combination $p=0.0032$) (Fig. S11E), commensurate with survival analysis,
634 where paxalisib alone provided a significant survival benefit compared to the vehicle (vehicle=22.5
635 days, paxalisib=28 days, $p=0.0453$) as did the combination therapy (ONC201+paxalisib=28 days,
636 $p=0.0002$) (Fig. S11F). The combination of ONC201 and paxalisib also increased survival of
637 xenograft mice compared to ONC201 alone ($p=0.0024$), and provided a modest benefit compared to
638 paxalisib alone ($p=0.0442$) (Fig. S11F).

639 **Case reports of ONC201 combined with paxalisib in DIPG patients at diagnosis or disease**
640 **progression**

641 To demonstrate the potential utility of ONC201 in combination with paxalisib, we report two recent
642 DIPG case studies of patients that received both ONC201 and paxalisib through compassionate
643 access. These patients underwent radiographic analysis according to response assessment in
644 pediatric neuro-oncology (RAPNO). The first is a 6-year-old patient diagnosed in March 2021,
645 harboring H3.1K27M, *ACVR1* and *PIK3R1* mutant DIPG identified following biopsy (Fig. 6A). At
646 diagnosis, a diffuse pontine lesion was identified (Fig. 6B,C, tumor area 1554 mm²). The patient
647 received 54 Gy of RT delivered in 30 fractions of 1.8 Gy in the 1-3 months following diagnosis. Post-
648 RT magnetic resonance images (MRIs) indicated a tumor reduction of 38.1% compared to diagnosis
649 (Fig. 6B,D, tumor area 962 mm²). The patient began the combination treatment of ONC201 (15
650 mg/kg q.w.) and paxalisib (27 mg/m² daily) 7 weeks following the post-RT scan, corresponding to 5
651 months post-diagnosis. Tumor size remained relatively stable over the next consecutive MRIs (Fig.
652 6E,F, tumor area=1156 mm² and 1224 mm², respectively). Encouragingly, 9 months post-diagnosis,
653 a substantial 62.1% decrease in tumor area based on T2 weighted images was recorded from the
654 previous MRI, representing a 70.1% reduction compared to diagnosis and 51.8% reduction
655 compared to post-RT (Fig. 6B,G, tumor area=464 mm²), showing a partial response. Furthermore,
656 12 months post-diagnosis, 8 months into the ONC201 and paxalisib combination, the tumor had
657 reduced by 80.3% and 68.2%, compared to diagnosis and post-RT, respectively (Fig. 6B,H, tumor
658 area=306 mm²). Clinically, twenty-four months post-diagnosis, the tumor remains stable (Fig. 6I)
659 and the patient continues to do well, experiencing continued reduction in DIPG associated clinical
660 symptoms, and has returned to school. Intermittent toxicities during treatment included grade II
661 mucositis during the initial few months on the combination, which responded well to dexamethasone
662 mouthwash.

663 The second DIPG patient was a 16-year-old diagnosed with DIPG (tumor area=977.8 mm²) (Fig. 6J-
664 L). The patient did not have a biopsy and began radiotherapy in combination with ONC201 (625 mg,
665 q.w.) soon after diagnosis. The patient then continued to receive ONC201 as a maintenance
666 therapy. Clinical and radiological signs of first progression were detected 15 months post-diagnosis
667 (Fig. 6K,M, tumor area=1303.3 mm²). The patient then received the combination of ONC201 and
668 panobinostat (45 mg daily three times per week) but stopped after 3 months upon detection of
669 further signs of disease progression (Fig. 6K,N, tumor area=1814 mm²). The patient then
670 immediately underwent re-irradiation (20 Gy delivered in 10 x 2 Gy fractions). Paxalisib (45 mg, 27
671 mg/m²) was then combined with ONC201 (625 mg) 18 months post-diagnosis and continued until
672 the patient succumbed to *Pneumocystis pneumonia* (PCP), 24 months post-diagnosis. The acquired
673 PCP was attributed to concomitant steroid use and hence, the patient was unable to continue either
674 therapy, ultimately passing away 6 months after re-irradiation. T2 axial MR scans during ONC201
675 and paxalisib treatment showed partial response, with a 34% reduction in total tumor area
676 compared to regression (Fig. 6N to Fig. 6P) and 9% reduction during treatment with ONC201 and

677 paxalisib (Fig. 6O to Fig. 6P), a reduction not seen when ONC201 was combined with RT at
678 diagnosis (Fig. 6K,O,P). Autopsy analysis revealed viable tumor with no evidence of growth when
679 compared with the latest MRI 1 month earlier, with the family of the child also reporting no signs of
680 DIPG associated clinical symptoms prior to the infection. NGS of post-mortem tumor tissue
681 identified typical H3.3K27M, *TP53*, *PIK3CA* mutations (Fig. 6J), highlighting the potential of
682 combined use of ONC201 and paxalisib for the treatment of DIPG at diagnosis and disease
683 progression.

684 Discussion

685 The recent development and sharing of patient-derived models has helped to illustrate the high level
686 of inter- and intra-tumoral heterogeneity of DIPG and DMG, results that highlight the need for
687 combined therapies that target the metabolic rather than the genomic/epigenetic heterogeneity of
688 the disease (6,13). In this study, we have employed a pharmaco-proteogenomic approach to inform
689 a combination treatment regimen to improve response to the imipridone, ONC201, and build upon
690 the preliminary promising efficacy of the drug for the treatment of DIPG (15,21).

691 ONC201 is currently being assessed in 12 clinical trials worldwide, including in H3K27-altered
692 gliomas (NCT03295396, NCT03416530 and NCT02525692) which reveal a preliminary survival
693 benefit (27). ONC201 increased median OS for H3K27M brainstem tumor patients (DIPG) to 20
694 months ($p=0.0002$), from the historical 11.9 months. Patients who received ONC201 outside of trials
695 purchased by their families from a German oncologist survived 18 months, while those who also
696 underwent re-irradiation survived 22 months (15,52). Although these preliminary results are
697 favorable, patients still succumb within 18-20 months, with some patients failing upfront treatment,
698 indicating mechanisms of intrinsic resistance. Here, we present evidence that decreased response
699 is influenced by PI3K/Akt signaling; prompting us to test the clinically relevant PI3K/Akt inhibitor,
700 paxalisib (NCT03696355) (28,49), both in DIPG cell line models that were sensitive and resistant to
701 ONC201. Combined treatment with ONC201 and paxalisib rescued the therapeutic potential of
702 ONC201 in refractory models, independent of the availability of oxygen and independent of *TP53*
703 status, highlighting the potential for this drug combination therapy to combat the metabolic, spatial
704 and genetic heterogeneity of DIPG. Commensurate with these *in vitro* discoveries, combined
705 treatment tested in two out of three DIPG xenograft models significantly extended the survival of
706 mice compared to monotherapies, while the combination had an additive effect in the other.

707 We show ONC201 targets DRD2 and ClpP in DIPG models *in vitro* and xenograft models *in vivo*.
708 DIPG models harboring *TP53* mutations show decreased sensitivity to ONC201. This is distinct from
709 previous studies revealing ONC201 to be effective in *TP53*-mutant non-DIPG cancer models (17).
710 Irrespective of sensitivity, ONC201 elicits potent agonism of the mitochondrial protease, ClpP, which
711 drives mitochondrial degradation and ROS production. Previous studies showed ONC201 to be a

712 selective antagonist of DRD2 and DRD3, causing cell death through tumor necrosis factor (TNF)-
713 related apoptosis-inducing ligand (TRAIL) signaling (22). Overexpression of DRD2 has been
714 correlated with ONC201 sensitivity (53), with antagonism shown to decrease the pro-proliferative
715 effects of DRD2 signaling in glioblastoma, mediated, in part, by Ras/Erk, confirmed in DMG cell
716 lines *in vitro* (15). This mode of action is in agreement with the DRD2 antagonist activity of
717 haloperidol, an FDA approved antipsychotic, which also decreased Erk activity, analogous to the
718 response of DIPG cells that are refractory to ONC201 (15), while having no effect on Akt. The
719 importance of DRD2 antagonism was further highlighted following CRISPR-Cas9 mediated DRD2
720 knockdown, a strategy that proved lethal to DIPG cells *in vitro*. In several DIPG cell line models,
721 including SU-DIPG-VI, ONC201 showed limited cytostatic effects; however, when the same cell line
722 was implanted into the brainstem of mice, ONC201 provided a significant survival advantage
723 compared to controls. It is plausible that these *in vivo* results reflect ONC201's role in global DRD2
724 antagonism rather than in the tumor alone. DIPG synthesize and secrete dopamine, a characteristic
725 that is likely supportive of DIPG gliomagenesis (54). In glioblastoma, elevated DRD2 expression is
726 seen in glioma-initiating cell populations, with stimulation causing neuron-like hyperpolarization
727 exclusively driving sphere-formation and increasing tumor engraftment in PDX models (47). Here
728 we observe that treatment of mice with ONC201 decreased expression of tyrosine hydroxylase (TH)
729 in the prefrontal cortex suggestive of global antagonism of DRD2, however, further mechanistic
730 insights are needed to elucidate the anti-tumor benefit in DMGs at this time. It is highly probable that
731 paracrine dopamine signaling also occurs in DIPG as these cells express TH, analogous to
732 electrochemical communications between DIPG and neurons transmitted through synapses to drive
733 proliferation, differentiation, and survival (55). It was recently shown that DIPG patients with
734 increased ¹⁸F-DOPA uptake during MRI showed decreased sensitivity to radiotherapy ($p=0.001$)
735 and experienced worse outcomes independently correlating ¹⁸F-DOPA uptake with OS (54). These
736 studies highlight the potential benefit in assessing ¹⁸F-DOPA during routine MRI monitoring of
737 patients receiving ONC201 and may contribute to predicting response to ONC201.

738 Our studies support the findings that ClpP is an important target of ONC201 in DIPG, where
739 agonism caused mitochondrial dysfunction (22), and *CLPP* knockdown abrogated ONC201's anti-
740 DIPG effects *in vitro* (24). Regardless of sensitivity, ONC201 drives oxidative stress (24) (following
741 ClpP-mediated degradation of SDHA, IDH3B, CLS, COX4L1, COX5A, and COX10), however in
742 non-sensitive cells, promotes redox activation of PI3K/Akt, however the mechanism promoting
743 reduced sensitivity in *TP53*-mutant lines remains unknown. Akt inactivates GSK3 α/β a well-
744 characterized mechanism of metabolic rescue driven by increased glycogen and protein synthesis
745 to promote cell survival (49). GSK3 α/β also cooperates with Kelch-like ECH-associated protein 1
746 (KEAP1) (50) to repress the activity of the transcription factor, NRF2 (*NFE2L2*). Yet under
747 mitochondrial and oxidative stress, KEAP1 is degraded and NRF2 translocates to the nucleus,

748 binding to the antioxidant response elements at gene promoters to combat oxidative stress, by
749 promoting expression of the two-electron reductase NQO1 (38), this response could be inhibited
750 through the combination with paxalisib to drive cell death. Here we show that *TP53*-KO in HJSD-
751 DIPG-007 leads to increased phosphorylation of Akt at Thr308 and Ser473, further promoting
752 expression of NQO1 in line with this proposed mechanism of action. Additionally, treatment with
753 ONC201 increases phosphorylation of Akt at Thr308 and Ser473 further promoting expression of
754 NQO1. It is important to note that the DIPG xenograft mouse survival benefit provided by the
755 combination was modest, commensurate with the insidious clinical journey experienced by DIPG
756 patients. Early clinical experience from the two cases we report using ONC201 in combination with
757 paxalisib is promising. Both patients demonstrated resolution of clinical symptoms and radiographic
758 tumor regression. The first patient, who demonstrated the more dramatic response and continual
759 regression of the primary tumor extending >18 months post-diagnosis, remains on the combination
760 at the time of submission. Additionally, this strategy was also used in a H3.3K27M, *TP53*, *PIK3CA*
761 mutant DIPG patient enrolled on the phase I clinical trial (NCT03416530) testing oral ONC201 in
762 pediatric patients with newly diagnosed DIPG, experiencing an almost complete regression of the
763 progressive tumor to initial diagnosis size and a reversal of clinical symptoms, regression not seen
764 following upfront RT+ONC201, although the re-RT might have contributed to this response. Both
765 patients tolerated the treatments well by combining treatment with dexamethasone mouthwash. The
766 optimal dose and timing of the combination and whether these enhance the effects of standard of
767 care RT either in the upfront or relapse setting remain to be determined, given that both patients
768 also received either upfront radiotherapy or re-irradiation, respectively. However, in this study we
769 cannot explicitly rule out the contribution of paxalisib to patient response as both patients
770 commenced ONC201 before or at the same time as paxalisib, further, the contribution of paxalisib
771 for patients harboring PI3K mutations has not yet been determined; questions that will be elucidated
772 under clinical trial conditions. Additionally, the off-target effects of paxalisib are currently unknown.

773 We acknowledge that ONC201 in combination with paxalisib may not be solely responsible for the
774 almost complete resolution of the disease, particularly at advanced stages, given the modest
775 xenograft results using immune-compromised mouse models. Indeed, H3K27M DIPG are known to
776 reside in an immunologically cold tumor microenvironment (TME) devoid of inflammatory immune
777 cells (7). The global loss of the H3K27me3-mediated epigenetic landscape within DIPG cells is
778 similar to those seen in embryonic stem cells (ESCs) (56) characterized by little to no expression of
779 the major histocompatibility complex I (MHC I) proteins, making these primitive cells less visible to
780 the immune system (57). The observed change in the epigenetic landscape following ONC201
781 treatment and following modulation of oxidative stress may play a role in the immunogenicity of
782 DIPG, particularly in patients with an active immune system. The partial restoration in H3K27me3
783 following ONC201 treatment is consistent with recent data showing that H3K27M mutations drive

784 TCA cycle protein expression (58). Here we show that ONC201 drives potent degradation of
785 IDH3A/B and hence loss of mitochondrial TCA cycle function. This, in turn, may modulate the
786 production of epigenetic cofactors required to maintain hypomethylation of H3K27me3 (59). This
787 highlights the emerging link between H3K27M mutations and metabolic and epigenetic plasticity
788 (58), which may play a role in the immunogenicity of the tumor, driving an anticancer response from
789 the immune system.

790 The preclinical and clinical data provided here underpins the recently commenced phase II clinical
791 trial (NCT05009992), where we are seeking to determine if ONC201 in combination with paxalisib is
792 an effective regimen for treating DIPG and DMG patients at diagnosis, post-RT and at the time of
793 progression when patients are eligible for re-irradiation. This multimodal clinical trial will assess
794 safety of single agents in combination with upfront radiotherapy or re-irradiation for patients
795 commencing the trial at advanced stages that we hope will form the backbone of future combination
796 studies. We hypothesize that rationally designed combination trials, informed by rigorous preclinical
797 data, will improve outcomes for these poor prognosis cancers. Integration of correlative studies will
798 be critical for assessment of predictive biomarkers of response and refinement of inclusion and
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843 **Author contributions**

844 **E.R. Jackson:** Investigation, formal analysis, writing – original draft, writing – reviewing and editing,
845 visualization, **R.J. Duchatel:** Investigation, formal analysis, writing – original draft, writing –
846 reviewing and editing, visualization, supervision, **D. Staudt:** Investigation, formal analysis, writing –
847 reviewing and editing, **M.L. Persson:** Investigation, **A. Mannan:** Investigation, **S. Yadavilli:**
848 Investigation, **S. Parackal:** Investigation, **S. Game:** Investigation, **W. Chin Chong:** Investigation,
849 **W.S.N. Jayasekara:** Investigation, **M. Le Grand:** Investigation, writing – reviewing and editing, **P.S.**
850 **Kearney:** Investigation, **A.M. Douglas:** Project administration, **I.J. Findlay:** Investigation, formal
851 analysis, **Z.P. Germon:** Investigation, **H.P. McEwen:** Investigation, **T.S. Beitaki:** Investigation, **A.**
852 **Patabendige:** Resources, writing – reviewing and editing, **D.A. Skerrett-Byrne:** Investigation,
853 methodology, writing – reviewing and editing, **B. Nixon:** Methodology, writing – reviewing and
854 editing, **N.D. Smith:** Methodology, **B. Day:** Resources, **N. Manoharan:** Investigation, patient care,

855 **S. Nagabushan**: Investigation, patient care, **J.R. Hansford**: Writing – reviewing and editing, **D.**
856 **Govender**: Investigation, **G.B. McCowage**: Investigation, Writing – reviewing and editing, **R.**
857 **Firestein**: Writing – reviewing and editing, **M. Howlett**: Writing – reviewing and editing, **R.**
858 **Endersby**: Writing – reviewing and editing, **N.G. Gottardo**: Writing – reviewing and editing, **F.**
859 **Alvaro**: Investigation, writing – reviewing and editing, **S.M. Waszak**: Investigation, writing –
860 reviewing and editing, **M.R. Larsen**: Methodology, writing – reviewing and editing, **Y. Colino-**
861 **Sanguino**: Writing – reviewing and editing, **F. Valdes-Mora**: Writing – reviewing and editing, **A.**
862 **Rakotomalala**: Writing – reviewing and editing, **S. Meignan**: Investigation, writing – reviewing and
863 editing, **E. Pasquier**: Resources, investigation, writing – reviewing and editing, **N. André**: Writing –
864 reviewing and editing, **E. Hulleman**: Resources, Writing – reviewing and editing, **D.D. Eisenstat**:
865 Writing – reviewing and editing, **N.A. Vitanza**: Resources, Writing – reviewing and editing, **J.**
866 **Nazarian**: Conceptualization, resources, writing – reviewing and editing, supervision, **C.**
867 **Koschmann**: Conceptualization, resources, writing – reviewing and editing, supervision, patient
868 care, **S. Mueller**: Conceptualization, resources, writing – reviewing and editing, supervision, **J.E.**
869 **Cain**: Conceptualization, resources, writing – reviewing and editing, supervision, **M.D. Dun**:
870 Conceptualization, resources, writing – original draft, writing – reviewing, editing, resubmission,
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1027 Figure captions

1028 **Figure 1. DIPG patient derived cell lines show variable response to ONC201 treatment. A,**
1029 Resazurin proliferation (percentage compared to untreated) after 96 h ONC201 exposure in DIPG
1030 patient-derived cell lines; EZHIP+ (circles) = CNMC-XD-760, DIPG-VUMC10; H3.1K27M (squares)
1031 = UON-JUMP4, SU-DIPG-IV, SU-DIPG-XXXIII, SU-DIPG-XXXVI and H3.3K27M (triangles) =
1032 HSJD-DIPG-007, SU-DIPG-VI, SU-DIPG-XIII, SU-DIPG-XVII, SU-DIPG-XXIV, SU-DIPG-XXV, SU-
1033 DIPG-XXIX. Endothelial cell line, HCMEC/D3, SV-40 dependent human microglial line, HMC3 and
1034 neural progenitor cell line, ReN cells, were used as controls (diamonds). Values shown as mean +/-
1035 SEM (n=3). **B,** Annexin V apoptosis assay after 96 h exposure with 5 μ M ONC201 (dark grey)
1036 compared to untreated (light grey) in SU-DIPG-XXXVI, HSJD-DIPG-007, UON-JUMP4 and SU-
1037 DIPG-XIII. Unpaired t-test, values shown as mean +/- SEM (n=3), ** p <0.01. **C,** Representative
1038 phase contrast images of biological triplicates (n=3) of HSJD-DIPG-007 and SU-DIPG-XIII following
1039 6 days exposure to 1.25 μ M ONC201, scale bar = 0.2 mm. **D,** Oncoplot of somatic mutations
1040 determined using TSO500. Cell lines ordered from the least to most sensitive to ONC201 exposure
1041 (top to bottom). Larger values of microsatellite instability (MSI) and tumor mutational burden (TMB)

1042 are associated with increased pathogenicity. **E**, Proliferation data was grouped by H3 status; wt-H3
1043 (n=5), H3.1K27M (n=4) and H3.3K27M (n=7), and sensitivity to ONC201 was determined by the
1044 area under the curve (AUC), \pm SEM. Statistical analysis was performed via non-parametric unpaired
1045 one-way ANOVA. **F**, Resazurin proliferation, AUC, following ONC201 exposure for 96 h in Res259
1046 cells harboring knock-in of either H3.1K27M or H3.3K27M mutations. Statistical analysis performed
1047 via parametric unpaired t-test, with Welch's correction. **G**, Western blot validation of H3K27M knock-
1048 in in Res259 cells. **H**, *TP53* status; wt- and mutant-*TP53* (n=4 vs. n=9), and sensitivity to ONC201
1049 was determined by the AUC, with values shown as mean \pm SEM. Statistical analysis performed via
1050 non-parametric unpaired t-test. **I**, Resazurin proliferation, AUC, following ONC201 exposure in wt-
1051 *TP53* HSJD-DIPG-007 DIPG cell lines transduced with a non-targeting control (NTC) gRNA, *TP53*-
1052 KD (knockdown) and *TP53*-KO (knockout). Statistical analysis was performed via parametric
1053 unpaired one-way ANOVA with Welch's correction. **J**, Western Blot confirmation of *TP53* knockout
1054 (KO) and knockdown (KD) in HSJD-DIPG-007 cells. **K**, Validation of decreased response to
1055 ONC201 in *TP53*-KD or *TP53*-KO HSJD-DIPG-007 cell lines was performed by Western Blot
1056 analysis of PARP cleavage (cPARP). **L**, Resazurin proliferation, AUC, following Nutlin-3 exposure
1057 for 96 h in HSJD-DIPG-007 NTC, *TP53*-KD and *TP53*-KO. Statistical analysis was performed via
1058 parametric unpaired one-way ANOVA with Welch's correction. **M**, Proliferation data was grouped by
1059 *ACVR1* status; *ACVR1* wildtype (n=8) vs. *ACVR1* mutant (n=5) and **N**, *PIK3CA* status; *PIK3CA*
1060 wildtype (n=9) vs. *PIK3CA* mutant (n=4) and compared to AUC following ONC201 exposure.
1061 Statistical analysis performed via non-parametric unpaired t-test.

1062
1063 **Figure 2. Pharmacoproteogenomic analysis identifies DRD2 and ClpP as targets of ONC201**
1064 **in DIPG.** **A**, Western blot analysis of basal DRD2, SDHA and CLPP expression across DIPG
1065 models. **B**, Densitometry of protein expressions were normalized to DIPG-VUMC10 and compared
1066 with the z-AUC (median area under the curve (AUC) for the control cell lines (HMC3, HCMEC/D3,
1067 ReN) – AUC of DIPG cells after exposure to ONC201. Pearson's linear regression, accounting for
1068 replicates, was used to determine ONC201 sensitivity correlation for DRD2, CLPP, SDHA and the
1069 ratio of SDHA to CLPP (SDHA/CLPP). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns (not significant), (n=12). **C**,
1070 *CLPP* RNA expression from RNA-seq data publicly available through St Jude's PeCan database,
1071 normalized to FPKM (Fragments Per Kilobase of transcript per Million mapped reads). NBM (normal
1072 bone marrow CD34 positive hemopoietic stem cells/mononuclear cells), DIPG (diffuse intrinsic
1073 pontine glioma), non-BS-HGG (non-brainstem-high grade glioma, including not otherwise specified),
1074 LGG (low grade glioma), MB (medulloblastoma) and BT other (brain tumor other - ependymoma,
1075 atypical teratoid rhabdoid tumor, choroid plexus carcinoma, cranio and CNS tumor not specified).
1076 Statistical significance determined via one-way ANOVA. **D**, Resazurin proliferation following
1077 ONC201 exposure (compared to untreated, 96 h) of CRISPR-Cas9 mediated knockdown of *CLPP*
1078 and *DRD2* was performed in SU-DIPG-XIII (blue) and SU-DIPG-XXXVI (yellow). Values shown as

1079 mean +/- SEM (n=3). **E**, Western blot validation of successful knockdown of CLPP and DRD2 in SU-
1080 DIPG-XIII and SU-DIPG-XXXVI.

1081 **Figure 3. Quantitative proteomic profiling identifies increased PI3K/Akt signaling in resistant**
1082 **models.** High resolution quantitative proteomic profiling was conducted on SU-DIPG-VI, exposed to
1083 5 μ M ONC201 for 24 h. Cells were treated in low oxygen (5% O₂, 5% CO₂) and normoxic conditions
1084 (20% O₂, 5% CO₂) in biological triplicate. **A**, Major canonical pathways and **B**, activated upstream
1085 regulators (determined by Ingenuity Pathway Analysis – IPA) of proteins significantly altered
1086 following 5 μ M ONC201, regardless of oxygen tension (Student's t-test, $p < 0.05$, n=6). **C**, Expression
1087 changes of proteins were calculated as log₂ fold change and grouped by mitochondrial proteins,
1088 transcription factors and proteins markers of apoptosis. Student's t-test of average change, * $p < 0.05$,
1089 ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **D**, Orthogonal validation of mitochondrial changes, such as
1090 decreased SDHA were analyzed in DIPG cell lines (HSJD-DIPG-007, SU-DIPG-XXXVI, SU-DIPG-
1091 VI, SU-DIPG-XIII, SU-DIPG-XVII) via Western blot, exposed to 5 μ M ONC201 for up to 48 h. **E**,
1092 Network of proteins from upregulated PI3K/Akt signaling predicted by IPA were integrated in
1093 Cytoscape StringApp. Predicted increase (orange) and predicted decrease (blue) functional
1094 networks indicated with sharp and dark lines linking proteins to indicate a higher confidence interval.
1095 Protein expression changes mapped as log₂ fold change of ONC201/untreated calculated using the
1096 right-tailed Fisher Exact Test with the smaller the p -value, the more likely the association between
1097 proteins not to be a random event ($p < 0.05$). **F-G**, High resolution quantitative proteomic profiling
1098 was conducted on SU-DIPG-XXXVI, SU-DIPG-VI and SU-DIPG-XXXVI, exposed to 5 μ M ONC201
1099 for 24 h. **F**, Heatmap and unbiased hierarchical clustering of protein expression values normalized
1100 using z-score of abundances in Perseus. **G**, Canonical pathways and predicted upstream regulators
1101 determined by IPA analysis of proteins altered following ONC201 exposure. Positive z-score value
1102 is predictive of pathway activation, whereas a negative z-score is predictive of inhibition. **H**,
1103 Orthogonal validation of proteins associated PI3K/Akt/mTOR signaling and the antioxidant response
1104 element (ARE) axis were assessed in DIPG cell lines following ONC201 exposure.

1105 **Figure 4. ONC201 in combination with paxalisib is synergistic across DIPG models.** **A**, SU-
1106 DIPG-VI was treated with 5 μ M ONC201 for 48 h, 20 mM NAC for 24 h and 1 mM H₂O₂ for 1 h, and
1107 protein changes downstream PI3K/Akt and reductase signaling were validated by Western blot. **B**,
1108 Western blot analysis of PI3K/Akt, Erk and antioxidant response element (ARE) signaling in SU-
1109 DIPG-XVII treated with 5 μ M ONC201 (48 h) and 1 μ M paxalisib (24 h). **C**, SU-DIPG-VI was grown
1110 in soft agarose in colony formation for 2 weeks treated with 0.5 μ M ONC201, 100 nM paxalisib and
1111 the combination. The number of colonies were then quantified using ImageJ. Assay was performed
1112 in biological triplicate with representative images shown (One-way ANOVA, * $p < 0.05$, ** $p < 0.01$
1113 *** $p < 0.001$ and **** $p < 0.0001$, values shown as mean +/- SEM). **D-E**, DIPG cells SU-DIPG-VI, SU-

1114 DIPG-XIII, SU-DIPG-XVII, were passaged, grown in low oxygen (5% O₂, 5% CO₂) or atmospheric
1115 oxygen (20% O₂, 5% CO₂) conditions for a week and then proliferation assays were performed
1116 using ONC201, paxalisib or both for 96 h (n=3). Synergy was determined using **D**, Chou-Talalay via
1117 CompuSyn or **E**, Bliss synergy analysis where a combination index (CI) where <1 (dotted line)
1118 demonstrates a synergistic effect and Bliss score >10 represents a strong synergism. **F-H**, Parental
1119 wt-*TP53* HSJD-DIPG-007 and HSJD-DIPG-007 cell lines transduced with a non-targeting control
1120 (NTC) gRNA, harboring knockout (KO) or knockdown (KD) of *TP53* were subjected to analysis
1121 following ONC201 treatment alone or in combination with paxalisib. **F**, Western blot confirmation of
1122 mitochondrial marker, SDHA, PI3K/Akt, Erk and ARE signaling to ONC201 (5 μM, 48 h). **G-H**, Cells
1123 were treated with increasing concentrations of ONC201, paxalisib or both for 96 h, in biological
1124 triplicate. Synergy was determined using **G**, Chou-Talalay or **H**, Bliss synergy analysis. Chou-
1125 Talalay and Bliss synergy graphs are reported as mean +/- SD.

1126 **Figure 5. ONC201 in combination with paxalisib is a synergistic drug combination in DIPG**
1127 **xenograft models.** **A**, SU-DIPG-VI/Luc and HSJD-DIPG-007 cells were injected into the brainstem
1128 of NOD SCID Gamma (NSG) mice. Treatment was started at 4 or 3 weeks, respectively from
1129 xenograft date. ONC201 and paxalisib were administered by oral gavage. Xenografts were
1130 sacrificed for pharmacodynamics and survival was tracked where they were culled at ethical
1131 endpoints. **B-C**, Survival curve analysis of days post treatment start at animal sacrifice, with
1132 significance determined by survival curve comparison for **B**, SU-DIPG-VI and **C**, HSJD-DIPG-007.
1133 Shading indicates treatment duration. Log-rank (Mantel Cox) Test, **p*<0.05, ***p*<0.01, ****p*<0.001,
1134 *****p*<0.0001. **D**, Tumor tissue from SU-DIPG-VI xenografts sacrificed at 2 weeks following start of
1135 treatment analyzed by Western Blot. **E**, SU-DIPG-VI *in vitro* cells exposed to 5 μM ONC201 0, 24,
1136 48 and 72 hr compared to *in vivo* SU-DIPG-VI tissue collected from the prefrontal cortex (PFC) and
1137 brainstem (BS), treated with ONC201. **E**, Tumor tissue was resected from HSJD-DIPG-007
1138 xenografts following 4 weeks of treatment and analyzed by immunohistochemistry. **F**, Sections were
1139 stained for H3K27M, Ki67 and SDHA (representative images are presented, scale bars = 2 mm, 200
1140 μM or 50 μM). **G**, IHC images quantified via ImageJ (measured in technical triplicate, across
1141 biological replicates, n=3).

1142 **Figure 6. ONC201 in combination with paxalisib drives tumor regression and increased**
1143 **survival in DIPG case studies.** **A**, Six-year-old *H3.1K27M*, *PIK3R1*, *ACVR1* mutant DIPG patient
1144 underwent biopsy soon after diagnosis and received 54 Gy radiotherapy over 30 fractions. MRI was
1145 performed six weeks after the completion of radiotherapy, and compassionate access was granted
1146 for the use of paxalisib to target *PIK3R1* mutations. Family of the patient sourced German ONC201
1147 and started concurrently with paxalisib. **B**, Tumor size at diagnosis, following radiotherapy and
1148 throughout treatment. **C**, T2 and T1 post contrast MR axial scans at patient diagnosis, tumor area =
1149 1554 mm². **D**, Following radiotherapy, tumor area decreased by 38.1% to 962 mm² compared to

1150 diagnosis. **E**, MRI showed tumor area was stable following radiotherapy = 1156 mm², 20.2%
1151 progression. Following this scan, the ONC201 (15 mg/kg QW) and paxalisib (27 mg/m² daily)
1152 combination was started. **F**, MRI following 8 weeks on the combination tumor area was stable =
1153 1224 mm², 6% increase. **G**, Tumor regression was seen after 20 weeks on the drug combination.
1154 Tumor area = 464 mm², tumor reduction by 62% compared to the last scan. **H**, Most recent MRI,
1155 tumor area = 306 mm², total tumor area reduction compared to diagnosis = 80%. **I**, The patient
1156 continues to remain on the combination 22 months following diagnosis. **J-P**, Sixteen-year-old
1157 *H3.3K27M*, *TP53*, *PIK3CA*, mutant DIPG patient received 54 Gy over 30 fractions. Patient enrolled
1158 on the ONC201 monotherapy trial NCT03416530 and experienced stable disease for 2 months.
1159 Following radiological and clinical progression, the patient received panobinostat (45 mg daily three
1160 times a week) with ONC201 (625 mg, once a week). Further progression was seen in the
1161 subsequent MRI, where the patient then received reirradiation. The patient immediately commenced
1162 ONC201 and paxalisib, both on compassionate grounds. **K**, Tumor area measured throughout
1163 treatment. **L**, T2 and T1 post-contrast MR axial scans at patient diagnosis, tumor area = 977.8 mm².
1164 **M**. MRI following first progression tumor area = 1303.3 mm². **N**, Patient received panobinostat in
1165 combination with ONC201, MRI image following the combination tumor area = 1814 mm².
1166 Following, this patient received reirradiation and ONC201 (15 mg/kg QW) and paxalisib (27 mg/m²
1167 daily) **O**, Tumor regression was seen 8 weeks post re-RT, whilst receiving ONC201 in combination
1168 with paxalisib, tumor area = 1322.6 mm². **P**, Tumor regression was again seen after 20 weeks on
1169 the combination, tumor area = 1209 mm², 20 months post diagnosis. Patient continued to receive
1170 ONC201 and paxalisib for the next 3 months and then contracted pneumonia and passed away 24
1171 months from diagnosis.

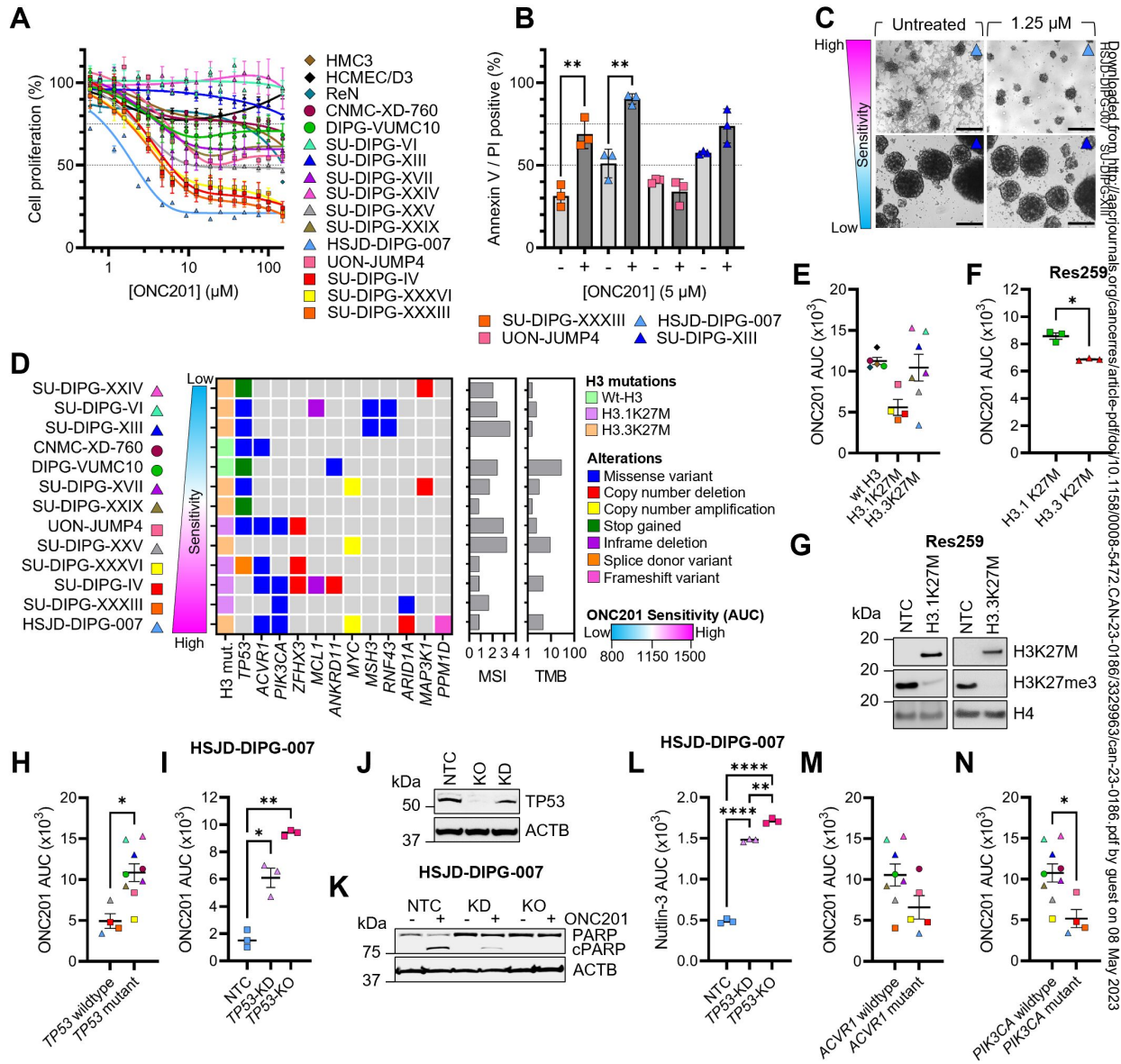
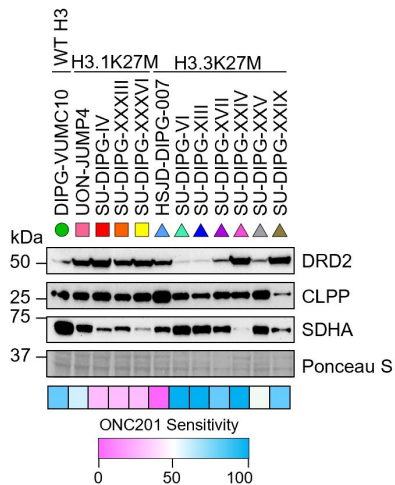
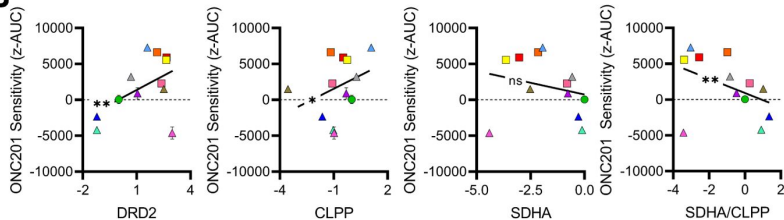
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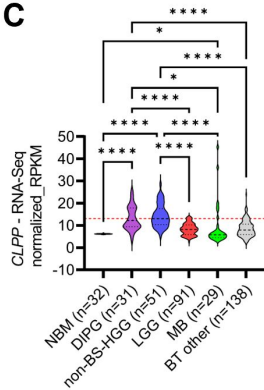
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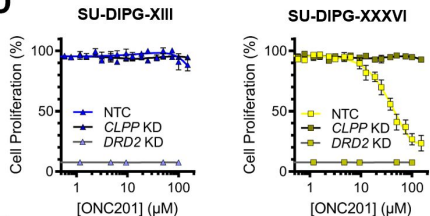
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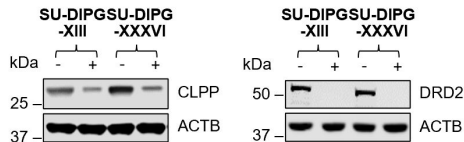


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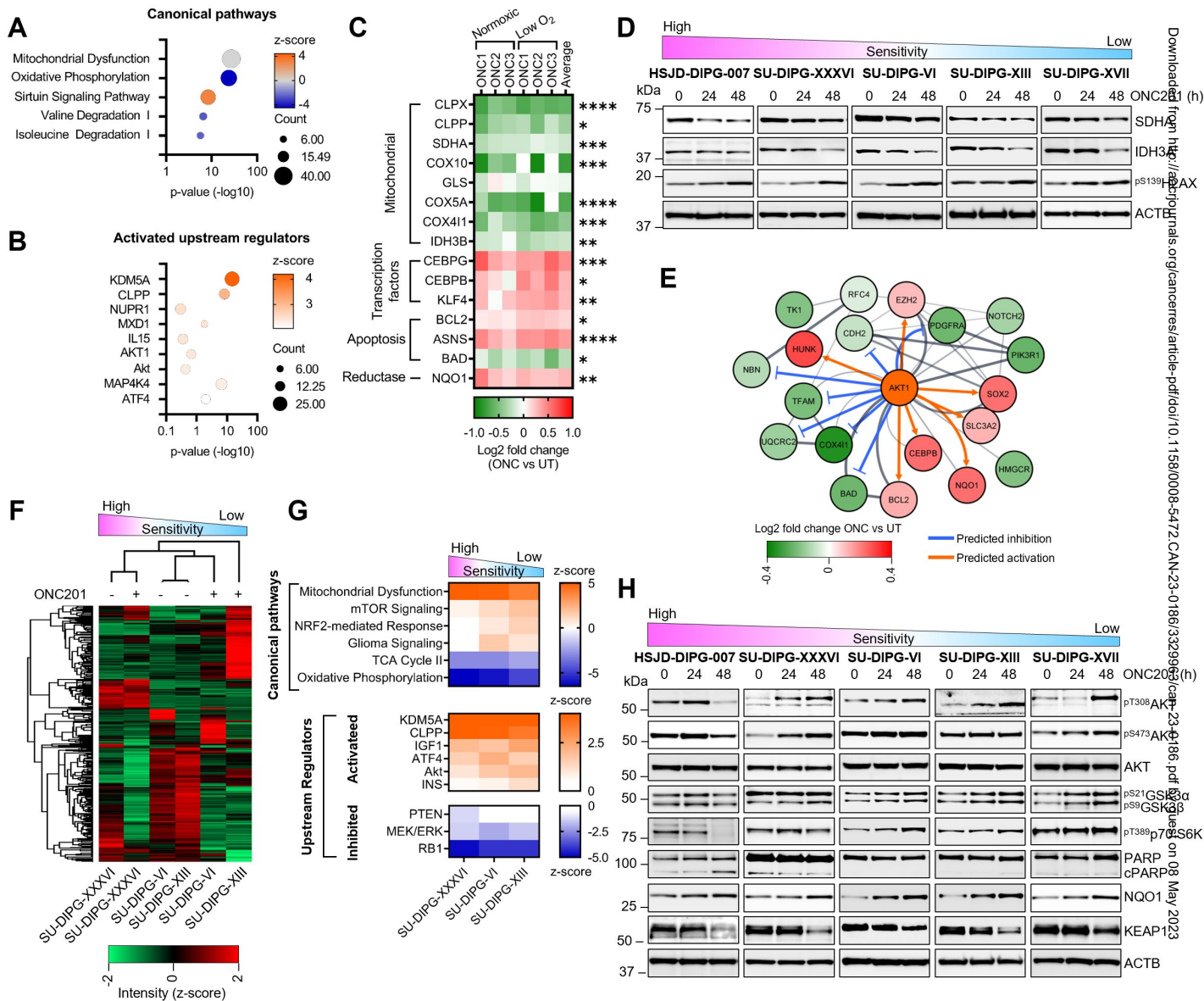


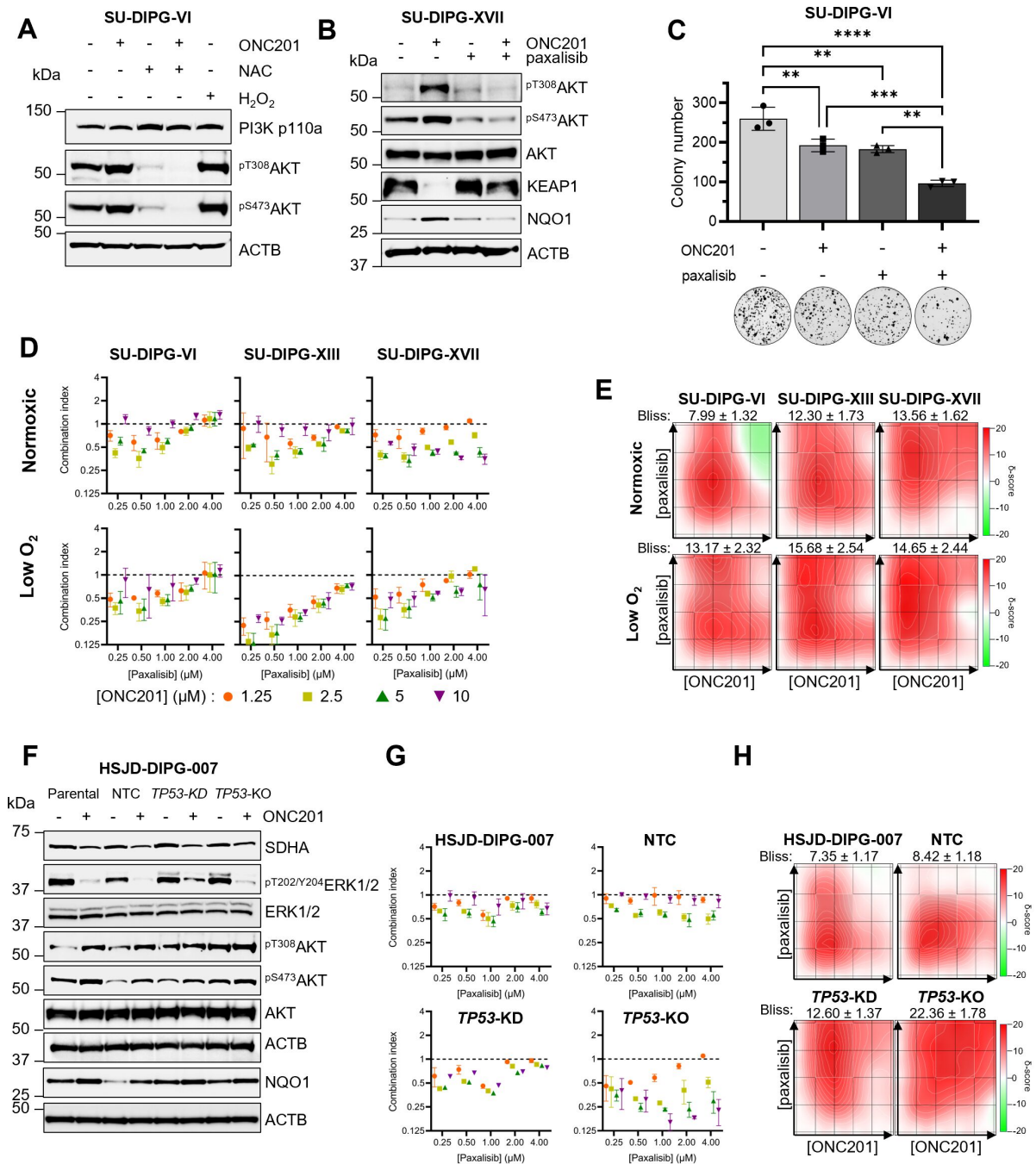
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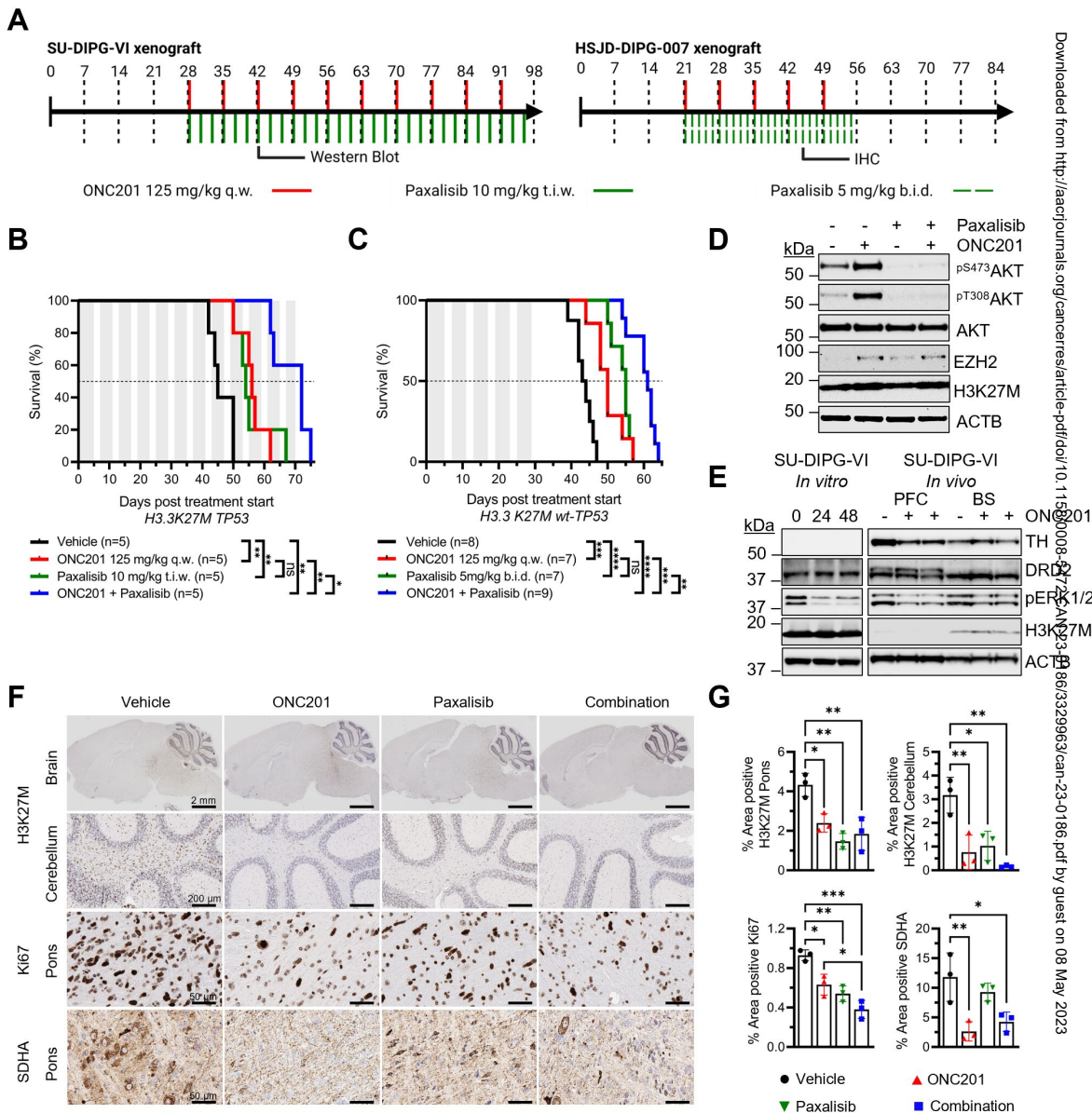
Figure 5:

Figure 6:

