

1 **From the clinic to the bench and back again in one dog year: identifying new treatments**
2 **for sarcoma using a cross-species personalized medicine pipeline**

3

4 Sneha Rao^{1*}, Jason A. Somarelli^{1,2*}, Erdem Altunel¹, Laura E. Selmic³, Mark Byrum³, Maya U.
5 Sheth⁴, Serene Cheng¹, Kathryn E. Ware¹, So Young Kim⁵, Joseph A. Prinz⁶, Nicolas Devos⁶,
6 David L. Corcoran,⁶ Arthur Moseley⁶, Erik Soderblom⁶, S. David Hsu^{1,2#}, and William C.
7 Eward^{2,7#}

8 ¹Department of Medicine, Duke University Medical Center, Durham, NC, USA, ²Duke Cancer Institute, Durham,
9 NC, USA, ³Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University,
10 Columbus, OH, USA, ⁴Pratt School of Engineering, Duke University, ⁵Department of Molecular Genetics and
11 Microbiology, ⁶Duke Center for Genomic and Computational Biology, ⁷Department of Orthopaedic Surgery,

12

13 ***These authors contributed equally.**

14 **#Address correspondence to: shiaowen.hsu@duke.edu or william.eward@duke.edu**

15

16 **Keywords:** precision medicine, cancer therapy, leiomyosarcoma, drug discovery, comparative
17 oncology

18

19 **Abstract.** Cancer drug discovery is an inefficient process, with more than 90% of newly-
20 discovered therapies failing to gain regulatory approval. Patient-derived models of cancer offer a
21 promising new approach to identifying personalized treatments; however, for rare cancers, such
22 as sarcomas, access to patient samples can be extremely limited, which precludes development
23 of patient-derived models. To address the limited access to patient samples, we have turned to
24 pet dogs with naturally-occurring sarcomas. Although sarcomas make up less than 1% of all
25 cancers in humans, sarcomas represent at least 15% of all cancers in dogs. Dogs with naturally-
26 occurring sarcomas also have intact immune systems, an accelerated pace of cancer progression,
27 and share the same environment as humans, making them ideal models that bridge key gaps
28 between mouse models and human sarcomas.

29 Here, we develop a framework for a personalized medicine pipeline that integrates drug
30 screening, validation, and genomics to identify new therapies. We tested this paradigm through
31 the study of a pet dog, Teddy, who presented with six synchronous leiomyosarcomas. By
32 integrating patient-derived cancer models, in vitro drug screens, and in vivo validation we
33 identified proteasome inhibitors as a potential therapy for Teddy. After showing an initial
34 response to the proteasome inhibitor, bortezomib, Teddy developed rapid resistance, and tumor
35 growth resumed. Whole exome sequencing revealed substantial genetic heterogeneity across
36 Teddy's multiple recurrent tumors and metastases, suggesting that intra-patient heterogeneity
37 was responsible for the heterogeneous clinical response. Ubiquitin proteomics coupled with
38 exome sequencing revealed multiple candidate driver mutations in proteins related to the
39 proteasome pathway. Together, our results demonstrate how the comparative study of canine
40 sarcomas can offer rapid insights into the process of developing personalized medicine
41 approaches that can lead to new treatments for sarcomas in both humans and canines.

42 **Introduction**

43 Despite spending billions of dollars on the preclinical development of new anti-cancer
44 drugs, fewer than 1 in 10 new therapies make it from the bench to the bedside and gain FDA
45 approval (1). These sobering statistics clearly demonstrate that the preclinical models and
46 paradigms currently being used to discover new cancer treatments require improvement. This
47 need for improvement is exemplified by the slow progress in finding new therapies for sarcoma.
48 Sarcomas are rare, but highly aggressive cancers that are prevalent in children and young adults.
49 While sarcomas make up less than 1% of adult solid tumors, they account for nearly 15% of
50 pediatric solid tumors(2). For patients who present with metastatic disease, the 5-year survival is
51 just 16% (3). Few new therapies have emerged in recent decades, underscoring the need for
52 creative new approaches in drug discovery.

53 One approach that has increasingly become a part of the discovery pipeline is the use of
54 patient-derived models of cancer, including low-passage cell lines and patient-derived xenografts
55 (PDXs). To create these patient-derived models, individual patient tumors are grown directly in
56 culture or in immunocompromised mice. Each type of patient-derived model has unique
57 advantages: For example, patient-derived cell lines enable large-scale drug screens to take place
58 quickly and at low cost. On the other hand, the use of PDXs reduces the selective bottleneck of
59 cell line generation and maintains the stromal components of the original tumor, which are
60 increasingly recognized as critical components of a tumor's relative therapeutic sensitivity(4, 5).
61 These patient-derived models are also being used to develop personalized treatments and guide
62 development of novel targeted agents (6, 7). One study in colorectal cancers showed a
63 correlation between transplanted xenograft tumors and clinical response to cytotoxic therapy (8).
64 Another pilot clinical trial of patients with advanced solid tumors received systemic cytotoxic

65 therapies based on *in vivo* validation in PDXs (9). This study showed that 11 out of 17 treatment
66 regimens identified in PDX were clinically efficacious (10). Drug screening in this study was
67 done *in vivo* rather than *in vitro* and used over 200 treatment regimens, including both targeted
68 and non-targeted agents (10). A similar study in advanced sarcoma patients with a variety of
69 histologic subtypes also yielded concordant results between PDX and patient responses, with 13
70 out of 16 patients showing a correlation between efficacy of the top drug identified through PDX
71 drug trials and clinical outcomes (11). Yet despite these exciting results, there remains a
72 disconnect between drug testing in mice and performance in human patients.

73 Another approach for cancer drug discovery that is rapidly gaining attention is the study
74 of pet dogs with spontaneously-occurring sarcomas and the inclusion of these patients in
75 therapeutic trials. Canine sarcomas are far more prevalent than their human counterparts,
76 representing approximately 15% of all canine malignancies (12) and rendering them an
77 underutilized “model” of human disease (13, 14). Unlike mouse models – which often fail to
78 recapitulate key conditions of spontaneous human disease - dogs share an environment with
79 humans, have an intact immune system, and have nearly identical treatment options. While there
80 are some differences in the histopathologic grading of soft tissue sarcomas between humans and
81 dogs, a study using canine soft tissue sarcomas to compare pathologic diagnoses between
82 veterinary and medical pathologists showed that the majority of canine tumors were given
83 diagnoses congruent with the human counterpart (15). Coupled with patient-derived models and
84 precision medicine strategies, a cross-species approach could illuminate new therapeutic options
85 for sarcoma patients with greater fidelity than the traditional “cells, then mice, then humans”
86 pathway. Most importantly, because the lifespan of dogs is much shorter than that of humans,

87 discoveries in canine clinical trials can be made more quickly in canine patients given the rapid
88 progression of their lives relative to humans.

89 In the present work, we report the development and testing of a personalized medicine
90 pipeline that combines patient-derived models, personalized genomics, and drug screening
91 strategies to identify new potential therapies for a young dog who presented with seven
92 synchronous, spontaneous leiomyosarcomas. Using this pipeline, we first developed an early
93 passaged cell line and PDX for our patient. Using high throughput drug screen on the cell line,
94 we identified proteasome inhibitors as a candidate therapy for this patient, then validated the
95 tumor response to proteasome inhibition *in vivo* using the patient's PDX, and finally treated the
96 patient's recurrent tumor in the clinic with the proteasome inhibitor, bortezomib. Our work
97 provides a generalizable framework for personalized medicine strategies and highlights key
98 challenges in the development of such approaches.

99

100 **Materials and Methods**

101 Generation of patient-derived xenograft models

102 Tumor samples were collected from a three-year-old male golden retriever following
103 surgical resection of the tumors at University of Illinois at Urbana-Champaign, College of
104 Veterinary Medicine (Urbana, IL, USA) with the informed consent of the owner. PDX models of
105 the patient's sarcoma were generated as described previously, and all *in vivo* mouse experiments
106 were performed in accordance with the animal guidelines and with the approval of the
107 Institutional Animal Care and Use committee (IACUC) at the Duke University Medical Center
108 (16). To develop PDXs, the tumor sample was washed in phosphate buffered saline (PBS),
109 dissected into small pieces (<2 mm), and injected into the flanks of 8-10-week-old JAX

110 NOD.CB17-PrkdcSCID-J mice obtained from the Duke University Rodent Genetic and Breeding
111 Core. Tumors were passaged into successive mice once the tumor size reached between 500 to
112 1,500 mm³. Resected PDX tumors were homogenized in a PBS suspension and 150 μ l of PDX
113 tissue-PBS suspensions at 150 mg/ml concentration were injected subcutaneously into the right
114 flanks of the 8 weeks old JAX NOD.CB17- PrkdcSCID-J mice. To maintain integrity of the
115 PDX tumor, passages were limited to the 3rd generation.

116

117 Low-passage cell line generation and characterization

118 Low passage cell lines were generated from the patient's PDX during passage one of the
119 PDX as follows. PDX tumor was surgically removed with a sterile blade, washed in PBS, and
120 small pieces (< 2mm) of tumor tissue were mechanically homogenized and then suspended in
121 cell growth media and cultured in 12-well plates with DMEM + 10% FBS + 1%
122 Penicillin/Streptomycin. To isolate tumor cells, growing colonies of cells were isolated by
123 trypsinization using O rings and cultured in fresh 12-well plates. This process was repeated until
124 a colony of cells was established that resembled pure tumor cells in morphology. Contamination
125 of the PDX cell line with mouse fibroblasts was detected by polymerase chain reaction (PCR)
126 using canine-specific and mouse-specific primers. The following primers were used: canine
127 reverse (5'-GTA AAG GCT GCC TGA GGA TAA G-3'), canine forward (5'-GGT CCA GGG
128 AAG ATC AGA AAT G-3'), mouse reverse (5'-AGG TGT CAC CAG GAC AAA TG-3'), and
129 mouse forward (5'-CTG CTT CGA GCC ATA GAA CTA A-3') (17).

130

131 High-throughput drug screening

132 Canine leiomyosarcoma low-passage cell line was cultured in DMEM + 10% FBS + 1%
133 Penicillin/Streptomycin. Automated systems were used for a 119- and 2,100- compound high-
134 throughput drug screens. The 119-drug screen library (Approved Oncology Set VI) was provided
135 by the NCI Developmental Therapeutics Program (<https://dtp.cancer.gov/>). Automated liquid
136 handling was provided by the Echo Acoustic Dispenser (Labcyte) for drug addition or Well mate
137 (Thermo Fisher) for cell plating, and assays were performed using a Clarioscan plate reader
138 (BMG Labtech). The BioActive compound library includes 2,100 small molecules that are
139 annotated for pathway and drug target (Selleckchem) and was screened in triplicate. Compounds
140 were stamped into 384 well plates for a final concentration of 1 μ M using an Echo Acoustic
141 Dispenser (Labcyte). Cells were then plated at a density of 2,000 cells/well using a WellMate
142 (ThermoFisher) and incubated in the presence of drug for 72 hours. After 72 hours of incubation,
143 Cell Titer Glo was added to each well and luminescence was measured using a Clariostar Plate
144 Reader (BMG Labtech). Percent killing was quantified using the formula $100 * (1 - (\text{average}$
145 $\text{CellTiterGlo}^{\text{drug}} / \text{average CellTiterGlo}^{\text{DMSO}}))$ where the value average $\text{CellTiterGlo}^{\text{DMSO}}$ was the
146 average DMSO CellTiterGlo value across each plate.

147 Validation of top drug candidates *in vivo*

148 To validate top candidates from the *in vitro* drug screens 150 μ l of homogenized PDX
149 tissue-PBS suspensions were injected at a concentration of 150 mg/ml of tumor tissue
150 subcutaneously into the right flanks of the 8-10 weeks old JAX NOD.CB17- PrkdcSCID-J mice.
151 Top drug targets identified by the high-throughput drug screens for *in vivo* validation,
152 bortezomib (PS-341) and 17-DMAG (alvespimycin) HCl were purchased from Selleck
153 Chemicals (Houston, TX). Drug were first solubilized in DMSO and then diluted in PBS for
154 intraperitoneal injections. When the tumor volumes reached 100-150 mm^3 , mice were

155 randomized (n = 5 mice for each treatment group) and 1 mg/kg bortezomib and 25 mg/kg
156 alvespimycin intraperitoneal injections were initiated three times a week (18, 19). Control tumors
157 were treated with 100 μ l of 5% DMSO diluted in PBS. Tumor volumes were measured three
158 times a week using calipers, and (length x (width)²)/2 was used to calculate the tumor size. Mice
159 were sacrificed on day 18 or if the tumor volume reached 1,500 mm³.

160

161 Whole exome sequencing

162 Genomic DNA from seven primary tumors, one recurrent tumor, a patient-derived
163 xenograft, and the cell line were isolated using the QIAGEN DNeasy Blood and Tissue kit. DNA
164 quality analysis, exome capture, and sequencing were performed at the Duke University
165 Sequencing and Genomics Technologies Shared Resource. Genomic DNA samples were
166 quantified using fluorometric quantitation on the Qubit 2.0 (ThermoFisher Scientific). For each
167 sample, 1 μ g of DNA was sheared using a Covaris to generate DNA fragments of about 300bp in
168 length. Sequencing libraries were prepared using the Roche Kapa HyperPrep Library prep Kit.
169 During adapter ligation, unique indexes were added to each sample. Resulting libraries were
170 cleaned using SPRI beads and quantified on the Qubit 2.0. Size distributions were checked on an
171 Agilent Bioanalyzer. Libraries were pooled into equimolar concentration (8 libraries per pool)
172 and library pools were finally enriched using the Roche SeqCap® EZ Dog Exome panel (design
173 1000003560). Each pool of enriched libraries was sequenced on one lane of a HiSeq 4000 flow
174 cell at 150bp PE, generating about 41 Million clusters per sample or ~12Gb of data. Sequence
175 data was demultiplexed and Fastq files generated using Bcl2Fastq2 conversion software
176 provided by Illumina.

177

178 Initial data analysis and variant calling were performed by the Duke University Genomic
179 Analysis and Bioinformatics Resource. Exome sequencing data was processed using the
180 TrimGalore toolkit (20), which employs Cutadapt(21) to trim low-quality bases and Illumina
181 sequencing adapters from the 3' end of the reads. Reads were aligned to the CanFam3.1 version
182 of the dog genome with the BWA algorithm (22, 23). PCR duplicates were flagged using the
183 PICARD Tools software suite (24). Alignment processing and variant calling were performed
184 using the MuTect2 (25) algorithm that is part of the GATK(22) following the Broad Institute's
185 Best Practices Workflow for identifying somatic variants(22). Variants for each sample were
186 called relative to the normal sample. Variant call files for each sample were filtered for single
187 nucleotide polymorphisms using the Genome Analysis Toolkit and converted to PHYLIP format
188 using the vcf2phylip package (27). Phylogenetic trees were generated using PHYLIP with 1,000
189 bootstrap replicates per tree (28) and visualized using the ape package in R (29). The number of
190 shared mutations was calculated pairwise between the matched tumor-normal variants of each
191 sample using VCFtools (30). Genes with deleterious mutations in each sample were identified
192 using Ensembl's Variant Effect Predictor tool (30). These results were analyzed and visualized
193 using BioVenn and the UpSetR package in R (31, 32).

194

195 Ubiquitin-tagged proteomics analysis of PDX tumors treated with bortezomib

196 *Sample Preparation.* Flash frozen vehicle- and bortezomib-treated PDX tumors (n = 3
197 per treatment) were provided to The Duke Proteomics and Metabolomics Shared Resource for
198 processing and analysis. Samples were normalized to 3.3 μ L of 8 M urea per mg of wet weight
199 and homogenized using a bead beater at 10,000 rpm. Protein concentration was determined via
200 Bradford assay and was normalized to 5,000 μ g of protein in 1.6 M of urea using 50 mM

201 ammonium bicarbonate. Samples were then reduced with 10 mM dithiothreitol for 45 minutes at
202 32°C and alkylated with 25 mM iodoacetamide for 45 minutes at room temperature. Trypsin was
203 added to a 1:25 ratio (enzyme to total protein) and allowed to proceed for 18 hours at 37°C. After
204 digestion, peptides were acidified to pH 2.5 with trifluoroacetic acid (TFA) and subjected to C18
205 SPE cleanup (Sep-Pak, 50 mg bed).

206 For ubiquitin antibody enrichment, samples were resuspended in 750 μ L 1X IAP Buffer
207 (50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl from Cell Signaling
208 Technology) using vortex and brief bath sonication. Pre-aliquoted PTMScan® Pilot Ubiquitin
209 Remant Motif (K- \square -GG) beads (Cell Signaling Technology) were thawed for each sample,
210 storage buffer was removed following slow centrifugation, and beads were pre-washed with 4 x
211 1 mL of 1X PBS buffer. Resuspended peptides were then transferred in IAP buffer directly onto
212 beads. Immunoprecipitation was performed for 2 hours at 4C using end-over-end mixing. After
213 spinning gently to settle the beads (VWR microfuge) the supernatants were removed. The IAP
214 resins containing the enriched ubiquitinated peptides were then washed with 1mL of IAP buffer
215 three times, and one time with 0.1X IAP buffer. After removing the supernatants, the antibody-
216 bound ubiquitinated peptides were eluted with a 50 μ L aliquot of 0.15% TFA in water for
217 approximately 10 minutes at room temperature, tapping gently on the bottom of the tube a few
218 times during elution to ensure mixing. Beads were eluted a second time with 45 μ L of 0.15%
219 TFA in water and added to the first elution. Combined eluents were lyophilized to dryness.
220 Samples were resuspended in 35 μ L 0.1% formic acid for a final cleanup on a C18 Stage Tip. All
221 samples were then lyophilized to dryness and resuspended in 12 μ L 1% TFA/2% acetonitrile
222 containing 12.5 fmol/ μ L yeast alcohol dehydrogenase. From each sample, 3 μ L was removed to
223 create a QC Pool sample that was run periodically throughout the acquisition period.

224 Quantitative LC/MS/MS was performed on 4 μ L of each sample, using a nanoAcquity
225 UPLC system (Waters Corp) coupled to a Thermo QExactive HF-X high resolution accurate
226 mass tandem mass spectrometer (Thermo) via a nanoelectrospray ionization source. Briefly, the
227 sample was first trapped on a Symmetry C18 20 mm \times 180 μ m trapping column (5 μ L/minute at
228 99.9/0.1 v/v water/acetonitrile), after which the analytical separation was performed using a 1.8
229 μ m Acquity HSS T3 C18 75 μ m \times 250 mm column (Waters Corp.) with a 90-minute linear
230 gradient of 5 to 30% acetonitrile with 0.1% formic acid at a flow rate of 400 nanoliters/minute
231 (nL/min) with a column temperature of 55°C. Data collection on the QExactive HF mass
232 spectrometer was performed in a data-dependent acquisition (DDA) mode of acquisition with a
233 $r=120,000$ (@ m/z 200) full MS scan from m/z 375 – 1600 with a target AGC value of $3e6$ ions
234 followed by 30 MS/MS scans at $r=15,000$ (@ m/z 200) at a target AGC value of 5×10^4 ions and
235 45 ms. A 20 second dynamic exclusion was employed to increase depth of coverage. The total
236 analysis cycle time for each sample injection was approximately 2 hours.

237 Data was imported into Proteome Discoverer 2.2 (Thermo Scientific Inc.), and analyses were
238 aligned based on the accurate mass and retention time of detected ions using Minora Feature
239 Detector algorithm in Proteome Discoverer. Relative peptide abundance was calculated based on
240 area-under-the-curve of the selected ion chromatograms of the aligned features across all runs.

241 The MS/MS data was searched against the TrEMBL *C. familiaris* database (downloaded in Nov
242 2017) with additional proteins, including yeast ADH1, bovine serum albumin, as well as an
243 equal number of reversed-sequence “decoys”) false discovery rate determination. Mascot
244 Distiller and Mascot Server (v 2.5, Matrix Sciences) were utilized to produce fragment ion
245 spectra and to perform the database searches. Database search parameters included fixed
246 modification on Cys (carbamidomethyl) and variable modifications on Lysine (Gly-Gly), and

247 Meth (oxidation). Peptide Validator and Protein FDR Validator nodes in Proteome Discoverer
248 were used to annotate the data at a maximum 1% protein false discovery rate.

249

250 Data analysis and statistics

251 JMP from SAS software (Cary, NC, USA) was used for the high-throughput drug screen
252 data analysis. Hierarchical clustering of data was used to identify the top drug candidates from
253 the 119-compound drug screen and the 2,100-compound screen. Tumor volumes were recorded
254 in GraphPad Prism 6 software (La Jolla, CA, USA). Two-way ANOVA analysis was used to
255 compare differences in tumor volumes between the control and treatment groups.

256

257 **Results**

258 Applying a personalized medicine pipeline to an unusual case of leiomyosarcoma

259 We enrolled a three-year-old Golden Retriever (Teddy) for this study who presented to a
260 veterinary primary care hospital with six synchronous leiomyosarcomas that underwent
261 excisional biopsy (**Figure 1**). Teddy was then referred to the Small Animal Oncology team at the
262 University of Illinois at Urbana-Champaign for treatment of a mass near the stifle. This tumor
263 was excised and scars of the resected tumors were excised. During clipping and preparation for
264 these surgeries, the treating surgeon noted two new masses in addition to previous surgical scars
265 that were also resected and also determined to be high grade leiomyosarcoma (**Figure 1**).

266 Pathology reports from the time of tumor excision noted an “ulcerated, inflamed, highly cellular,
267 invasive mass composed of neoplastic spindlyoid cells arranged in short interlacing streams and
268 bundles with many neutrophils throughout the neoplasm with clusters of lymphocytes and
269 plasma cells at the periphery”, which was consistent with high grade leiomyosarcoma. Following

270 surgery, Teddy was started on empirical treatment with toceranib, a multi-receptor tyrosine
271 kinase inhibitor and the only FDA-approved targeted cancer therapeutic for dogs, given the high
272 risk for recurrent disease.

273

274 Generation of patient-derived models of LMS-D48X

275 Using one of the excised recurrent tumors from this patient, we applied a personalized
276 medicine pipeline to identify new potential therapies in the event that Teddy's disease would
277 eventually recur (**Figure 2A**). The pipeline included successful development of a matching PDX
278 (designated "LMS-D48X") and low-passage cell line, a high throughput drug screen on the cell
279 line, genomic profiling of mutations in the original tumors, PDX, and cell line, and *in vivo*
280 validation of top drug candidates (**Figure 2A**). Hematoxylin and eosin staining of the canine
281 PDX revealed sheets of highly proliferative, spindle-like cells (**Figure 2B**). Similarly, the
282 matched cell line was also highly proliferative, with an estimated doubling time of 26-36 hours
283 and the presence of spindle-shaped, mesenchymal-like cells (**Figure 2C**). PCR using canine- and
284 mouse-specific primers demonstrated that the LMS-D48X cell line is made up of purely canine
285 tumor cells (**Figure 2D**).

286

287 High-throughput drug screens identify proteasome inhibitors as a potential candidate therapy

288 To identify potential candidate therapies to treat Teddy, we performed two high-
289 throughput drug screens. First, we used a panel of 119 FDA-approved anti-cancer drugs.
290 Importantly, this screen identified multiple standard-of-care therapies for soft tissue sarcomas,
291 such as doxorubicin and danurubicin (**Figure 2E**). Interestingly, however, in addition to
292 standard-of-care therapies, the drug screen also identified several novel candidate drugs, such as

293 proteasome inhibitors, HDAC inhibitors (i.e. romidepsin), and MEK inhibitors, as candidate
294 agents (**Figure 2E**). Analysis of drug hits grouped by pathway revealed sensitivity to protein and
295 nucleic acid synthesis pathways, autophagy, topoisomerases, HDACs, and c-kit/BCR/ABL
296 (**Figure 2F**).

297 To further identify and validate additional novel therapeutic targets, we next performed a
298 second-high throughput drug screen, this time using a larger panel of 2,100 bioactive
299 compounds. The BioActives compound library (Selleckchem) contains a mixture of FDA-
300 approved and non-FDA approved small molecules with confirmed bioactivity against known
301 protein or pathway targets. The Bioactives collection is structurally diverse and is designed to
302 target many key pathways regulating cellular processes including proliferation, apoptosis and
303 signal transduction. Using the targeted pathway annotation for each compound, we were able to
304 select targets and pathways for which multiple drugs had significant inhibitory effects. We
305 hypothesized that this strategy would increase the likelihood of identifying the candidate
306 targets/pathways for which a given tumor is most vulnerable. Our initial analysis of the screen
307 revealed that a large portion (>90%) of compounds had little to no inhibitory effect, with only
308 6.6% of compounds showing >50% inhibition and 4.2% of drugs showing >75% inhibition
309 (**Figure 3A**). Analysis of top hits by cellular target demonstrated vulnerability for this cell line to
310 some targets already identified from the 119-drug screen, such as proteasome inhibitors and
311 MEK inhibitors, as well as novel drug classes, such as HSP, PLK, CRM1, NAMPT, Kinesin, and
312 p53 inhibitors (**Figure 3B**). Analysis of the top inhibitors by pathway revealed enrichment in
313 drugs targeting cytoskeletal signaling, the proteasome, apoptosis, cell cycle, and NF- κ B (**Figure**
314 **3C**).

315 We further explored the potential therapeutic efficacy of top pathways by analyzing the
316 number of inhibitors for each pathway that had >50% cell growth inhibition. Notably, both the
317 HSP and proteasome pathways had multiple drugs with >50% inhibition (15/19 and 5/11,
318 respectively) (**Figure 3D and E**). In the proteasome inhibitor class, 4/11 drugs conferred >90%
319 cell growth inhibition. Likewise, in the HSP inhibitor drug class, 13 out of 19 drugs caused >90
320 % cell growth inhibition (**Figure 3D and E**). From these two drug classes, we selected
321 alvespimycin (HSP inhibitor) and bortezomib (proteasome inhibitor) for further study. Both of
322 these drugs have known toxicity profiles, with bortezomib being FDA approved for the treatment
323 of multiple myeloma. *In vitro* validation of alvespimycin and bortezomib showed sub-
324 micromolar IC₅₀ values of 345 nM and 6nM, respectively (**Figure 3D and E**).

325

326 *In vivo* validation of alvespimycin and bortezomib in PDX models of LMS-D48X

327 We next used the LMS-D48X PDX to assess whether the top candidate therapies we
328 identified *in vitro* would be therapeutically active in the patient's matched PDX *in vivo*.
329 Interestingly, while alvespimycin showed >95% growth inhibition *in vitro*, the PDX was
330 unresponsive to this HSP inhibitor, with no difference in growth rate between vehicle-treated and
331 alvespimycin-treated tumors (**Figure 4A**). On the other hand, tumors treated with bortezomib
332 showed significant tumor growth inhibition, consistent with the *in vitro* drug screen (**Figure 4B,**
333 **C**). Animal weights in LMS-D48 PDX mice did not change significantly from the vehicle-treated
334 tumors in either of the drug treatment groups (**Figure 4D**).

335

336 From bench to bedside: Applying preclinical modeling to clinical practice

337 For any personalized medicine approach to be clinically useful, it must provide insight
338 into the patient's disease within the time scale of clinical decision making. With an aggressive
339 disease course and high likelihood for recurrence, Teddy presented a unique opportunity to
340 assess the ability of our personalized medicine pipeline to meet the clinical demand for rapidly
341 providing data on potential therapies to treating clinicians. Teddy presented at a six month follow
342 up visit with lesions in the mediastinal and right iliac lymph nodes, nasal mucosa, and local
343 recurrence in the right pelvic limb (**Figure 1 and Supplementary Figure 1**). Using the *in vitro*
344 screening and *in vivo* validations data from our pipeline, a decision was made to treat the patient
345 with systemic bortezomib. The patient was treated with intravenous bortezomib infusions at
346 $1.3\text{mg}/\text{m}^2$ twice weekly for four weeks and also received local palliative radiation therapy to the
347 right pelvic limb to alleviate pain associated with the limb lesion. Measurements of the right
348 pelvic limb lesion showed an initial decrease in tumor size during the first three weeks of
349 treatment; however, tumor growth resumed by the sixth week of treatment (**Figure 5A**).
350 Metastatic lesions in other locations also increased in size on CT imaging at the conclusion of
351 bortezomib treatment (**Figure 5B**). Representative images of the tumors before and after
352 bortezomib demonstrated the increase in tumor size and aggressive disease, especially in the
353 infiltrative nature of the nasal mucosal lesion eroding into the maxilla (**Supplementary Figure**
354 **1**).

355

356 Whole exome sequencing reveals extensive inter-tumoral heterogeneity

357 Our analysis of patient-derived models of cancer identified bortezomib as a promising
358 treatment for Teddy. Consistent with these preclinical observations, Teddy showed an initial
359 response to bortezomib in the first three weeks of treatment. However, this response was short

360 lived and tumor growth resumed however, Teddy also developed rapid resistance to systemic
361 bortezomib by day 36 of treatment (**Figure 5A**). Given the substantial differences in response
362 between tumor sites, we sought to better understand the underlying genetic landscape of the
363 patient's tumors and the relationship between these tumors and our patient-derived models. To
364 do this, we performed whole exome sequencing and phylogenetic reconstructions on 11 samples
365 from Teddy, including seven primary tumors, one recurrent tumor, one PDX and matched cell
366 line, and normal tissue. Phylogenetic analysis of the tumors and patient-derived models grouped
367 the PDX and cell line with the recurrent tumor with strong bootstrap support (**Figure 6A and**
368 **Supplementary Figure 2**). With the exception of the distance trees, the grouping of the PDX
369 and cell line with the recurrent tumor was consistent for all other methods of phylogenetic
370 inference, including DNA compatibility, maximum parsimony, and maximum likelihood (**Figure**
371 **6B**). We also counted the number of shared somatic mutations across all samples and found the
372 greatest similarity between the PDX, cell line, the recurrent tumor, and tumor 1 (**Figure 6C**).
373 Together, these results suggest that the PDX and cell line most closely resemble the recurrent
374 tumor. All other tumor samples shared little genetic overlap (3% to 16%). Tumor 7 was
375 particularly distinct from the other tumors, sharing just 3.5% of somatic mutations with all other
376 tumors (**Figure 6C**). Analysis of unique and shared somatic mutations revealed that unique
377 mutations dominate the genetic landscape of each tumor (**Figure 6D**).

378

379 Integration of whole exome sequencing and ubiquitin proteomics identifies potential mechanisms 380 of action of bortezomib

381 To further understand the underlying molecular mechanisms of sensitivity and resistance
382 to bortezomib for this patient, we performed mass spectrometry proteomics analysis of ubiquitin-

383 tagged proteins in PDX tumors treated with vehicle or bortezomib. Since bortezomib is a
384 proteasome inhibitor, we analyzed proteins that were differentially ubiquitinated in the PDX
385 treated with bortezomib as compared to vehicle-treated tumors. We identified a total of 290
386 differentially ubiquitinated proteins in vehicle- vs. bortezomib-treated PDX tumors (adjusted p-
387 value <0.05), 160 of which showed increased ubiquitination and 130 of which showed decreased
388 ubiquitination (**Figure 7A**). Analysis of differentially ubiquitinated targets revealed enrichment
389 for myosins and HSPs as the proteins with the greatest increase in ubiquitination in bortezomib-
390 treated tumors as compared to vehicle-treated tumors (**Figure 7A and Supplementary File 1**). It
391 is worth noting that the top hits were unique to this PDX, as additional proteomics analysis of
392 bortezomib-treated osteosarcoma PDXs yielded a different suite of ubiquitinated proteins
393 (Altunel et al., in preparation). Pathway analysis of proteins with increased ubiquitination
394 revealed enrichments in pathways related to actin, contractile filament movement, and the
395 proteasome (**Figure 7B**) and pathways related to proteins with decreased ubiquitination were
396 enriched for adherens junctions, focal adhesions, and extracellular vesicles (**Figure 7C**).

397 We next cross-referenced the proteomics analysis with our whole exome sequencing data
398 to better understand the varied clinical response and rapid progression on bortezomib. We
399 identified 10 proteins that contained identical somatic mutations across multiple samples
400 predicted to alter protein function that were also differentially-ubiquitinated in the PDX and cell-
401 line (**Figure 7D**). Interestingly, two of these 10 proteins are involved in pathways relevant to
402 proteasome inhibition and HSPs, respectively (**Figure 7E**). Defective In Cullin Neddylation 1
403 Domain Containing 1 (DCUN1D1) is part of an E3 ubiquitin ligase complex for neddylation, and
404 heat shock protein 70 kDa member 8 (HSPA8) is integral to the HSP70 pathway and cellular
405 protein quality control systems (33, 34). Notably, the DCUN1D1 mutation was unique to the

406 PDX and cell line (**Figure 7E**), suggesting the tumor from which this PDX was derived may
407 have harbored unique genetics that could contribute to increased bortezomib sensitivity. Overall,
408 the presence of somatic mutations affecting genes related to the proteasome and the heat shock
409 protein pathway may explain the sensitivity to small molecule inhibitors targeting these
410 pathways. The extensive heterogeneity in somatic mutations across multiple tumors and the
411 patient-derived models may also help explain the rapid progression of the patient treated with the
412 proteasome inhibitor, bortezomib.

413

414 **Discussion**

415 A comparative oncology approach enables rapid testing of a drug discovery pipeline in the clinic

416 Our canine leiomyosarcoma patient provided an invaluable opportunity to test, in real
417 time, a personalized approach to cancer therapy. To do this, we generated patient-derived cancer
418 models, both *in vitro* and *in vivo*, that helped identify novel therapeutic options, including
419 proteasome inhibitors and HSP inhibitors. After identifying bortezomib as a potential drug for
420 clinical application, we provided the preclinical data to the veterinary oncology team who
421 initiated personalized therapy with bortezomib for local recurrence and metastatic disease.
422 Though there was initial response to bortezomib in the setting of adjuvant palliative radiation
423 therapy for the local recurrence, additional metastatic sites showed either stable disease or
424 progression on bortezomib. While the outcome for this patient was only a slight delay in disease
425 progression, the entire process of evaluating a personalized therapy – from presentation to death
426 – was able to be carried out in approximately one year.

427

428 The impact of genetic heterogeneity on treatment response

429 There are a number of possibilities to explain the rapid progression on bortezomib for this
430 patient. One possible cause is the potential genetic drift that could be associated with generation
431 and passage of the PDX and cell line. Indeed, recent studies have shown that PDXs are subject to
432 mouse-specific selective pressures beyond a few passages (9). While we strive to keep our
433 passage numbers low for this reason, it is possible that even the first implantation of a tumor into
434 mice leads to selection of a specific sub-clone that has different properties from the original
435 tumor. Interestingly, phylogenetic reconstruction of all seven tumors, a recurrent tumor and the
436 PDX/cell line supports the grouping of the PDX/cell line with the recurrent tumor in a distinct
437 clade. Consistent with this grouping, a recurrent tumor, like the PDX and cell line, had an initial
438 response to bortezomib (**Figure 5**).

439 One additional possibility for the rapid clinical progression on bortezomib could be that
440 there is not an established dosage or dosing schedule for treating canine cancer with bortezomib.
441 Bortezomib has been used in veterinary medicine as a therapy for golden retriever muscular
442 dystrophy and our therapeutic regimen was extrapolated from this (35). However, it is possible
443 our dosing regimen was incorrect in the context of leiomyosarcoma treatment.

444 A third possibility is that the recurrent and metastatic lesions acquired unique mutations
445 in key cellular pathways that conferred bortezomib resistance. Tumors are heterogeneous on the
446 individual level and within the population, greatly contributing to the challenge of discovering
447 novel universal drugs (37-39). Numerous studies across multiple cancer types have revealed
448 significant genotypic variability even within a single tumor (40, 41) (42-44). This is the case for
449 metastatic progression as well. For example, Wu et al. have shown that genetic signatures of
450 metastatic lesions are similar to each other, but distinct from primary tumors, suggesting key
451 genomic differences that could impact therapeutic response (45).

452 Driven by selective pressure from the tumor microenvironment , the inter-tumoral
453 heterogeneity exhibited by these tumors could explain the difference between the *in vivo*
454 response to bortezomib and the lack of response in the recurrent and metastatic lesions (46, 47).
455 Consistent with this hypothesis, our analysis of whole exome sequencing data revealed
456 substantial tumor heterogeneity across the multiple tumors from this patient, as well as between
457 the group of samples including the recurrent tumor, PDX, and cell line.

458 It is possible that heterogeneity-mediated differences in response to therapy could be
459 addressed with combination targeted therapy or with therapies that target multiple oncogenic
460 pathways simultaneously (39, 48, 49). Multiple studies in mouse models of EGFR mutant lung
461 cancer have shown the utility of combination therapies in overcoming treatment resistance (50-
462 52). Our 2,100-compound drug screen identified multiple candidate drugs with both single
463 cellular targets and those that target multiple pathways. In future iterations of this personalized
464 pipeline, using combination therapy of top drugs identified from the drug screen could yield
465 promising results.

466

467 A multi-omics analysis identifies mechanisms of sensitivity and resistance to bortezomib

468 Using whole exome sequencing we were able to characterize the genomic differences
469 between the tumor used for preclinical modeling and the recurrent tumors treated with
470 bortezomib. In the context of multiple myeloma, for which bortezomib is a standard therapy,
471 multiple cellular pathways have been associated with bortezomib resistance, including mutations
472 in genes regulating the active site for bortezomib (53-56). Our proteomics analysis identified
473 pathways related to actin-myosin filaments, HSPs, and the proteasome as downregulated by
474 bortezomib (**Figure 7** and **Supplementary File 1**). The downregulation of skeletal myosins

475 (MYH1, MYH2, MYH4) by bortezomib is not easily explained, since skeletal myosins are
476 typically markers of rhabdomyosarcoma rather than leiomyosarcoma (58). However, inhibition
477 of pathways related to HSPs and the proteasome further validates the target specificity and
478 mechanism of action for bortezomib. Our integrated comparison of the ubiquitin proteomics data
479 with the exome sequencing data identified 10 key genes that were both differentially
480 ubiquitinated and mutated. Remarkably, two of these genes are members of the HSP and
481 proteasome pathways. This integrated multi-omics analysis suggests that mutations within these
482 two genes may explain, in part, the response to bortezomib. Likewise, the lack of mutation in
483 these two genes within other tumors in this patient may also explain the differential response to
484 bortezomib in different metastatic lesions of this patient.

485

486 **Conclusions**

487 We have developed a translational drug discovery pipeline that integrates patient-derived
488 models of cancer, drug screening, genomics, and proteomics to provide a comprehensive view of
489 how to integrate translational preclinical research in the clinic. The unique biology of Teddy,
490 with multiple, synchronous leiomyosarcoma tumors and an aggressive clinical course, enabled us
491 to study the relationships between the molecular/genomic landscape and *in vitro*, *in vivo*, and
492 clinical response to therapy. This provided both the patient and the clinician with unique
493 information about tumor biology and response to novel therapeutics occurring in a very short
494 period of time. This suggests that utilizing pet dogs with cancer to model personalized medicine
495 approaches can facilitate rapid investigations of therapeutic successes and failures.

496

497 **Acknowledgments.** JAS acknowledges support from Meg and Bill Lindenberger, the Paul and
498 Shirley Friedland Fund, the Triangle Center for Evolutionary Medicine, and funds raised in
499 memory of Muriel E. Rudershausen (riding4research.org). We would like to thank Teddy and his
500 family for their participation in this study and the veterinary team at the University of Illinois at
501 Urbana–Champaign for contributing to Teddy’s clinical care. We acknowledge Wayne Glover
502 for contributing to *in vivo* PDX tumor propagation, the Duke Functional Genomics Shared
503 Resource, the Duke Proteomics and Metabolomics Shared Resource, the Duke Sequencing and
504 Genomics Shared Resource, and the Duke Genomic Analysis and Bioinformatics Core Facility.

505

506 **Figure Legends**

507

508 **Figure 1. An integrated preclinical drug discovery and validation pipeline.** A three year old
509 canine patient with synchronous leiomyosarcomas (LMS) was identified and recruited based on
510 high risk of disease recurrence. Using both *in vitro* and *in vivo* patient-derived models, we
511 identified proteasome inhibitors as candidates for validation in clinic. Clinicians applied the
512 information from this preclinical pipeline for the treatment of the patient’s recurrent and
513 metastatic disease.

514

515 **Figure 2. Patient-derived models of cancer enable seamless integration of high throughput**
516 **drug screening with *in vivo* validations.** **A.** Schematic of the personalized medicine pipeline
517 integrating *in vitro* and *in vivo* drug discovery and validation. **B.** Hematoxylin and eosin stain of
518 the patient derived xenograft model of the canine patient (LMS-D48) showing highly
519 proliferative spindle-like cells. **C.** The patient-derived cell line also displays a high proliferation

520 rate, with an estimated doubling time of 26-36 hours, and spindle-like mesenchymal
521 morphology. **D.** A species-specific PCR using mouse- and canine-specific primers confirms that
522 the patient-derived cell line is of canine origin. **E.** A preliminary drug screen of 119 FDA-
523 approved compounds in the LMS-D48 cell line identified single standard-of-care agents and
524 novel drug candidates. **F.** Analysis of drug screen data at the pathway level showed sensitivity to
525 protein synthesis, DNA/RNA synthesis, autophagy, and HDAC inhibitors. Novel agents,
526 including HDAC inhibitors and proteasome inhibitors were identified as top candidates for
527 validation.

528
529 **Figure 3. High-throughput drug screens identify HSP inhibitors and proteasome inhibitors**
530 **as promising therapies for personalized treatment. A.** LMS-D48 cells were plated at a density
531 of 2,000 cells/well on plates prestamped with 2,100 drug compounds and DMSO. Cell titer glow
532 assays were performed 72 hrs after cell plating to determine cell percent killing based on
533 luminosity values. **B.** Analysis of drug targets from the 2,100 screen with multiple drugs shows
534 HSP inhibitors and proteasome inhibitors among the top pathways for which this cell line
535 displays significant sensitivity. **C.** Analysis of cellular pathways targeted by all drugs in the
536 2,100 drug screen shows that the cytoskeletal signaling pathway has the highest cell percent
537 killing. **D.** LMS-D48 cells were sensitive to 15 out of 19 HSP inhibitors. Among these,
538 alvespimycin was the top candidate, with an estimated IC_{50} of 345nM. **E.** Bortezomib was
539 among the top drugs in the proteasome inhibitor class that killed LMS-D48 cells, with an
540 estimated IC_{50} value of 6 nM.

541

542

543 **Figure 4. *In vivo* validation of top drug candidates reveals sensitivity of the LMS-D48 PDX**
544 **to proteasome inhibition. A.** Alvepimycin (25 mg/kg) was administered intraperitoneally (i.p.)
545 *in vivo* to SCID beige mice harboring LMS-D48 PDX tumors (n = 5 mice per treatment group)
546 each in control and treatment groups. There was no statistical difference between control and
547 treatment groups as measured by analysis of variance. **B.** Bortezomib (1 mg/kg) was
548 administered i.p. as described for alvepimycin above. Bortezomib significantly inhibited tumor
549 growth of the PDX (p<0.0001). **C.** Representative images of resected tumors at treatment
550 endpoint from the treatment and control groups show that control tumors are approximately
551 twice the size of bortezomib-treated tumors (scale bar = 0.5 cm). **D.** Animal weights were not
552 significantly changed during treatment with either alvepimycin or bortezomib during the
553 treatment course.

554
555 **Figure 5. Translation of bortezomib into clinic. A.** At the time of metastatic spread of disease,
556 the patient had lesions in the mediastinal and right iliac lymph nodes, the nasal mucosa, and local
557 recurrence at the right pelvic limb. The patient was started on systemic bortezomib therapy at a
558 dose of 1.3mg/m² twice weekly and palliative radiation therapy of 8 Gy by four fractions, once
559 weekly, for pain from the right pelvic limb lesion. Measurement of the pelvic limb lesion during
560 therapy showed decrease in maximal tumor dimension throughout 3 weeks of radiation therapy
561 and systemic bortezomib; though, there was an increase in size two weeks after both therapies
562 were stopped. **B.** CT staging studies and physical exam demonstrated an interval increase in
563 tumor size at all sites of disease and after discontinuation of bortezomib therapy. The canine
564 patient was then transitioned to palliative care.

565

566 **Figure 6. Whole exome sequencing reveals inter-tumoral heterogeneity across the patient's**
567 **tumors. A.** Phylogenetic reconstruction using the DNA compatibility algorithm supports a clade
568 that includes the PDX, cell line, and the recurrent tumor with bootstrap support greater than
569 900/1000. **B.** With the exception of the distance tree (Fitch), trees based on maximum parsimony
570 and maximum likelihood also grouped the cancer models with the recurrent tumor. **C.** A
571 similarity matrix comparing all somatic variants from each sample shows the percentage of
572 shared mutations across all samples. **D.** Individual samples had higher numbers of mutated genes
573 that were unique to each sample. Common shared mutations were relatively rare, reflecting the
574 heterogeneity of the samples.

575
576 **Figure 7. Ubiquitin proteomics of PDX tumors treated with bortezomib show differential**
577 **ubiquitination in key pathways related to cytoskeletal dynamics and the proteasome. A.**
578 Mass spectroscopy proteomics of ubiquitin tagged proteins identified increased ubiquitination of
579 160 proteins and decreased ubiquitination of 130 proteins. Multiple myosins displayed increased
580 ubiquitination in bortezomib-treated tumors. **B.** Pathway analysis of proteins with increased
581 ubiquitination showed enrichment in pathways related to actin and proteasome subunits. **C.**
582 Pathway analysis of proteins with decreased ubiquitination showed enrichment in pathways
583 related to adherens junctions, focal adhesions, and extracellular vesicles. **D.** Genes affected by
584 deleterious mutations in each sample were determined by analyzing the whole exome sequencing
585 data with the Ensembl Variant Effect Predictor. Affected genes in the PDX were filtered by those
586 in the cell line to eliminate potential contamination by mouse tissue (left). Comparison of this
587 subset of genes with the proteins identified by proteomics analysis with increased or decreased
588 ubiquitination in the PDXs treated with bortezomib identified an overlap of only ten affected

589 proteins. **E.** The ten proteins identified in **D.** are shown and were affected in the tumors with high
590 variability.

591

592 **References**

- 593 1. J. A. DiMasi, R. W. Hansen, H. G. Grabowski, The price of innovation: new estimates of
594 drug development costs. *J Health Econ* **22**, 151-185 (2003).
- 595 2. L. Mirabello, R. J. Troisi, S. A. Savage, Osteosarcoma incidence and survival rates from
596 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program.
597 *Cancer* **115**, 1531-1543 (2009).
- 598 3. T. A. C. S. m. a. e. c. team. (2018), vol. 2018.
- 599 4. D. F. Quail, J. A. Joyce, Microenvironmental regulation of tumor progression and
600 metastasis. *Nat Med* **19**, 1423-1437 (2013).
- 601 5. A. Blomme *et al.*, Murine stroma adopts a human-like metabolic phenotype in the PDX
602 model of colorectal cancer and liver metastases. *Oncogene* **37**, 1237-1250 (2018).
- 603 6. J. J. Tentler *et al.*, Patient-derived tumour xenografts as models for oncology drug
604 development. *Nature Reviews Clinical Oncology* **9**, 338-350 (2012).
- 605 7. Z. Chen *et al.*, A murine lung cancer co-clinical trial identifies genetic modifiers of
606 therapeutic response. *Nature* **483**, 613-617 (2012).
- 607 8. I. Fichtner *et al.*, Anticancer drug response and expression of molecular markers in early-
608 passage xenotransplanted colon carcinomas. *Eur J Cancer* **40**, 298-307 (2004).
- 609 9. M. Hidalgo *et al.*, Patient-Derived Xenograft Models: An Emerging Platform for
610 Translational Cancer Research. *Cancer Discovery* **4**, 998 (2014).
- 611 10. M. Hidalgo *et al.*, A pilot clinical study of treatment guided by personalized tumorgrafts
612 in patients with advanced cancer. *Mol Cancer Ther* **10**, 1311-1316 (2011).
- 613 11. J. Stebbing *et al.*, Patient-derived xenografts for individualized care in advanced sarcoma.
614 *Cancer* **120**, 2006-2015 (2014).
- 615 12. N. Ehrhart, Soft-tissue sarcomas in dogs: a review. *J Am Anim Hosp Assoc* **41**, 241-246
616 (2005).
- 617 13. C. A. Stiller *et al.*, Descriptive epidemiology of sarcomas in Europe: report from the
618 RARECARE project. *Eur J Cancer* **49**, 684-695 (2013).
- 619 14. J. M. Dobson, S. Samuel, H. Milstein, K. Rogers, J. L. Wood, Canine neoplasia in the
620 UK: estimates of incidence rates from a population of insured dogs. *J Small Anim Pract*
621 **43**, 240-246 (2002).
- 622 15. M. Milovancev *et al.*, Comparative pathology of canine soft tissue sarcomas: possible
623 models of human non-rhabdomyosarcoma soft tissue sarcomas. *J Comp Pathol* **152**, 22-
624 27 (2015).
- 625 16. J. M. Uronis *et al.*, Histological and molecular evaluation of patient-derived colorectal
626 cancer explants. *PLoS One* **7**, e38422 (2012).
- 627 17. J. K. Cooper *et al.*, Species identification in cell culture: a two-pronged molecular
628 approach. *In Vitro Cellular & Developmental Biology - Animal* **43**, 344-351 (2007).

- 629 18. Y. Shapovalov, D. Benavidez, D. Zuch, R. A. Eliseev, Proteasome inhibition with
630 bortezomib suppresses growth and induces apoptosis in osteosarcoma. *Int J Cancer* **127**,
631 67-76 (2010).
- 632 19. Y. Hu, D. Bobb, J. He, D. A. Hill, J. S. Dome, The HSP90 inhibitor alvespimycin
633 enhances the potency of telomerase inhibition by imetelstat in human osteosarcoma.
634 *Cancer Biol Ther* **16**, 949-957 (2015).
- 635 20. B. Bioinformatics. (2018), vol. 2018.
- 636 21. M. Martin, Cutadapt Removes Adapter Sequences From High-Throughput Sequencing
637 Reads. *EMBnet.journal* **17**, (2011).
- 638 22. G. A. Van der Auwera *et al.*, From FastQ data to high confidence variant calls: the
639 Genome Analysis Toolkit best practices pipeline. *Curr Protoc Bioinformatics* **43**, 11 10
640 11-33 (2013).
- 641 23. H. Li, R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler
642 transform. *Bioinformatics* **25**, 1754-1760 (2009).
- 643 24. B. Institute. (Github), vol. 2018.
- 644 25. P. J. Kersey *et al.*, Ensembl Genomes: an integrative resource for genome-scale data from
645 non-vertebrate species. *Nucleic Acids Res* **40**, D91-97 (2012).
- 646 26. A. McKenna *et al.*, The Genome Analysis Toolkit: a MapReduce framework for
647 analyzing next-generation DNA sequencing data. *Genome Res* **20**, 1297-1303 (2010).
- 648 27. J. Felsenstein, Using the quantitative genetic threshold model for inferences between and
649 within species. *Philos Trans R Soc Lond B Biol Sci* **360**, 1427-1434 (2005).
- 650 28. E. Paradis, K. Schliep, ape 5.0: an environment for modern phylogenetics and
651 evolutionary analyses in R. *Bioinformatics*, (2018).
- 652 29. P. Danecek *et al.*, The variant call format and VCFtools. *Bioinformatics* **27**, 2156-2158
653 (2011).
- 654 30. D. R. Zerbino *et al.*, Ensembl 2018. *Nucleic Acids Res* **46**, D754-D761 (2018).
- 655 31. T. Hulsen, J. de Vlieg, W. Alkema, BioVenn - a web application for the comparison and
656 visualization of biological lists using area-proportional Venn diagrams. *BMC Genomics*
657 **9**, 488 (2008).
- 658 32. J. R. Conway, A. Lex, N. Gehlenborg, UpSetR: an R package for the visualization of
659 intersecting sets and their properties. *Bioinformatics* **33**, 2938-2940 (2017).
- 660 33. F. Wang *et al.*, Blocking nuclear export of HSPA8 after heat shock stress severely alters
661 cell survival. *Sci Rep* **8**, 16820 (2018).
- 662 34. D. C. Scott *et al.*, Blocking an N-terminal acetylation-dependent protein interaction
663 inhibits an E3 ligase. *Nat Chem Biol* **13**, 850-857 (2017).
- 664 35. K. P. Araujo *et al.*, Bortezomib (PS-341) treatment decreases inflammation and partially
665 rescues the expression of the dystrophin-glycoprotein complex in GRMD dogs. *PLoS*
666 *One* **8**, e61367 (2013).
- 667 36. R. Fisher, L. Pusztai, C. Swanton, Cancer heterogeneity: implications for targeted
668 therapeutics. *Br J Cancer* **108**, 479-485 (2013).
- 669 37. N. McGranahan, C. Swanton, Biological and therapeutic impact of intratumor
670 heterogeneity in cancer evolution. *Cancer Cell* **27**, 15-26 (2015).
- 671 38. J. Liu, H. Dang, X. W. Wang, The significance of intertumor and intratumor
672 heterogeneity in liver cancer. *Exp Mol Med* **50**, e416 (2018).
- 673 39. I. Dagogo-Jack, A. T. Shaw, Tumour heterogeneity and resistance to cancer therapies.
674 *Nat Rev Clin Oncol* **15**, 81-94 (2018).

- 675 40. B. A. Walker *et al.*, Intracлонаl heterogeneity and distinct molecular mechanisms
676 characterize the development of t(4;14) and t(11;14) myeloma. *Blood* **120**, 1077-1086
677 (2012).
- 678 41. M. Gerlinger *et al.*, Intratumor heterogeneity and branched evolution revealed by
679 multiregion sequencing. *N Engl J Med* **366**, 883-892 (2012).
- 680 42. A. Sottoriva *et al.*, Intratumor heterogeneity in human glioblastoma reflects cancer
681 evolutionary dynamics. *Proc Natl Acad Sci U S A* **110**, 4009-4014 (2013).
- 682 43. S. Bea *et al.*, Landscape of somatic mutations and clonal evolution in mantle cell
683 lymphoma. *Proc Natl Acad Sci U S A* **110**, 18250-18255 (2013).
- 684 44. A. Kogita *et al.*, Inter- and intra-tumor profiling of multi-regional colon cancer and
685 metastasis. *Biochem Biophys Res Commun* **458**, 52-56 (2015).
- 686 45. X. Wu *et al.*, Clonal selection drives genetic divergence of metastatic medulloblastoma.
687 *Nature* **482**, 529-533 (2012).
- 688 46. M. R. Junttila, F. J. de Sauvage, Influence of tumour micro-environment heterogeneity on
689 therapeutic response. *Nature* **501**, 346-354 (2013).
- 690 47. M. S. Lawrence *et al.*, Mutational heterogeneity in cancer and the search for new cancer-
691 associated genes. *Nature* **499**, 214-218 (2013).
- 692 48. A. A. Alizadeh *et al.*, Toward understanding and exploiting tumor heterogeneity. *Nat*
693 *Med* **21**, 846-853 (2015).
- 694 49. Z. Liao *et al.*, The Anthelmintic Drug Niclosamide Inhibits the Proliferative Activity of
695 Human Osteosarcoma Cells by Targeting Multiple Signal Pathways. *Curr Cancer Drug*
696 *Targets* **15**, 726-738 (2015).
- 697 50. V. Pirazzoli *et al.*, Afatinib plus Cetuximab Delays Resistance Compared to Single-Agent
698 Erlotinib or Afatinib in Mouse Models of TKI-Naive EGFR L858R-Induced Lung
699 Adenocarcinoma. *Clin Cancer Res* **22**, 426-435 (2016).
- 700 51. Y. Y. Janjigian *et al.*, Dual Inhibition of EGFR with Afatinib and Cetuximab in Kinase
701 Inhibitor-Resistant EGFR-Mutant Lung Cancer with and without T790M Mutations.
702 *Cancer Discovery* **4**, 1036-1045 (2014).
- 703 52. E. M. Tricker *et al.*, Combined EGFR/MEK Inhibition Prevents the Emergence of
704 Resistance in EGFR-Mutant Lung Cancer. *Cancer Discov* **5**, 960-971 (2015).
- 705 53. R. Oerlemans *et al.*, Molecular basis of bortezomib resistance: proteasome subunit beta5
706 (PSMB5) gene mutation and overexpression of PSMB5 protein. *Blood* **112**, 2489-2499
707 (2008).
- 708 54. D. Chauhan *et al.*, Blockade of Hsp27 overcomes Bortezomib/proteasome inhibitor PS-
709 341 resistance in lymphoma cells. *Cancer Res* **63**, 6174-6177 (2003).
- 710 55. D. J. Kuhn *et al.*, Targeting the insulin-like growth factor-1 receptor to overcome
711 bortezomib resistance in preclinical models of multiple myeloma. *Blood* **120**, 3260-3270
712 (2012).
- 713 56. W. Que, J. Chen, M. Chuang, D. Jiang, Knockdown of c-Met enhances sensitivity to
714 bortezomib in human multiple myeloma U266 cells via inhibiting Akt/mTOR activity.
715 *APMIS* **120**, 195-203 (2012).
- 716 57. T. Saku, N. Tsuda, M. Anami, H. Okabe, Smooth and skeletal muscle myosins in spindle
717 cell tumors of soft tissue. An immunohistochemical study. *Acta Pathol Jpn* **35**, 125-136
718 (1985).
- 719

Figure 1

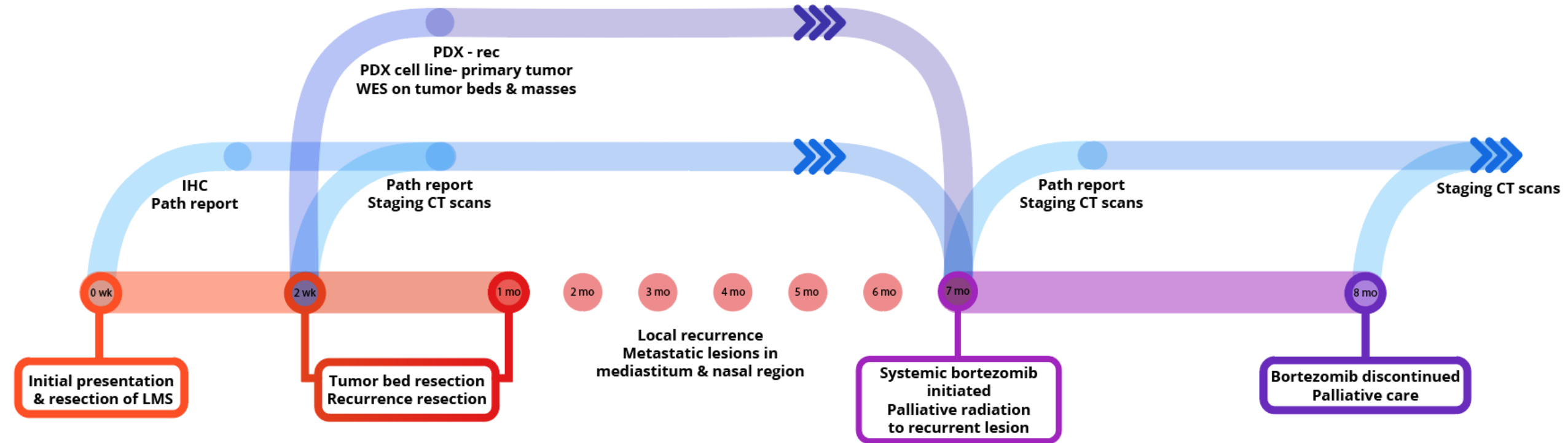
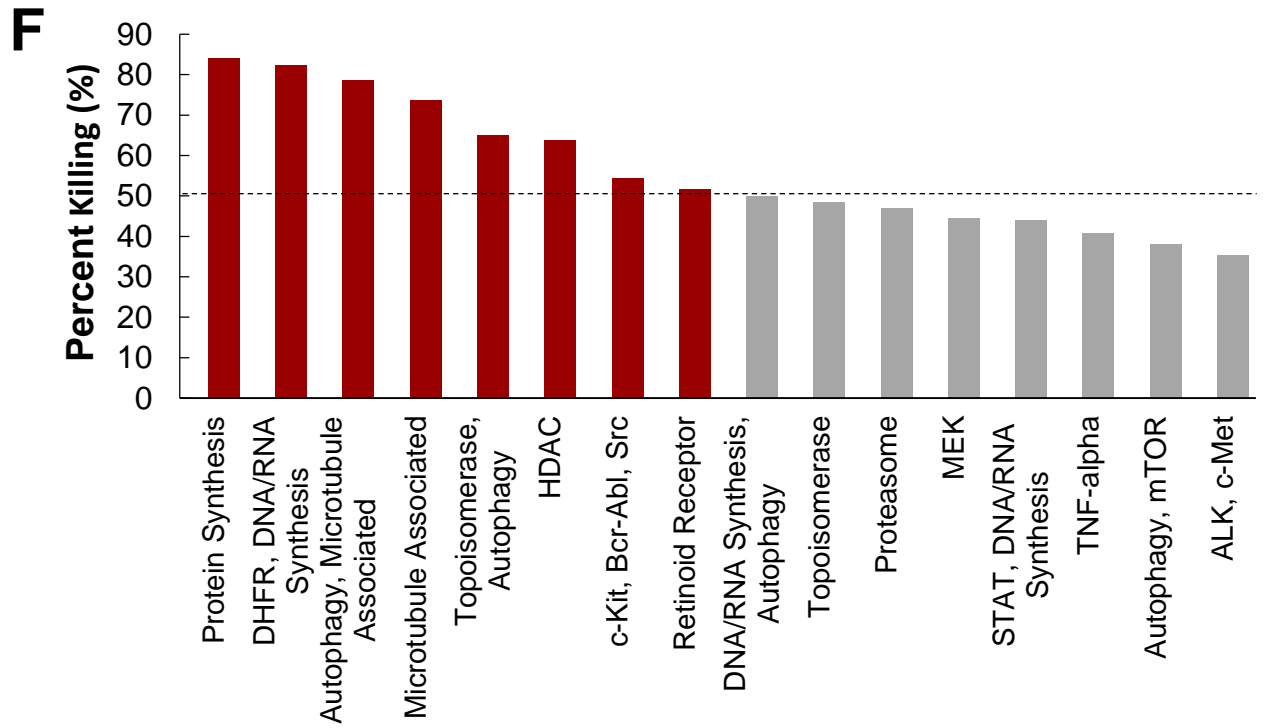
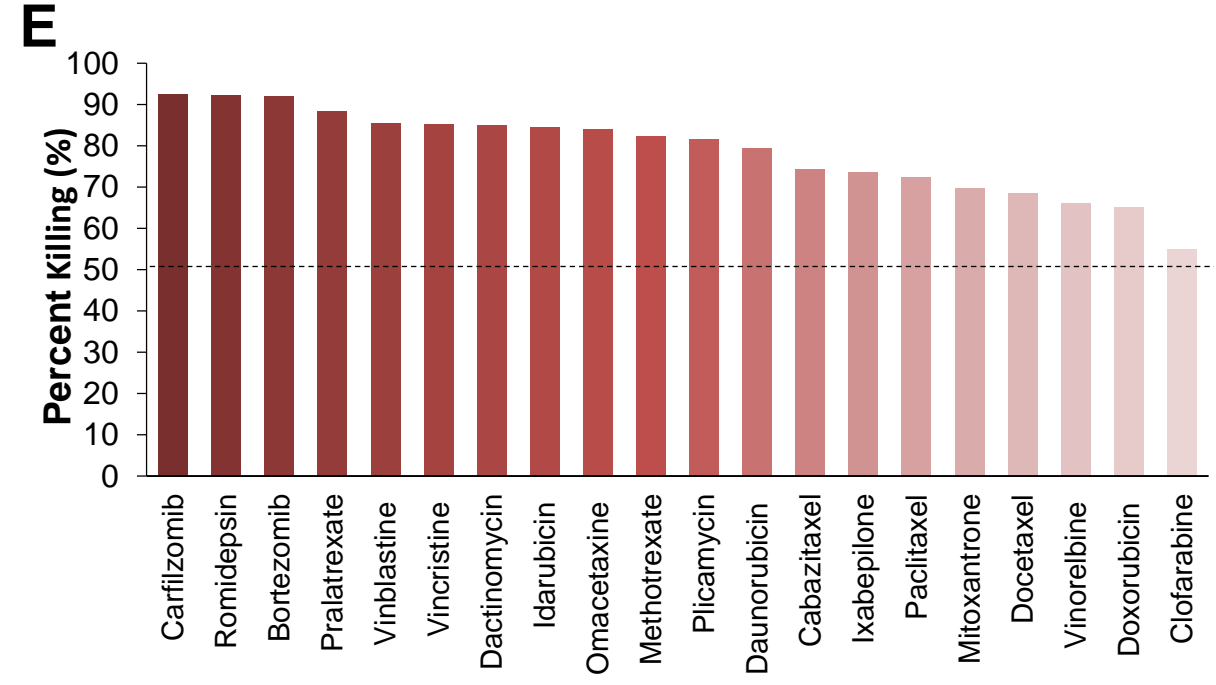
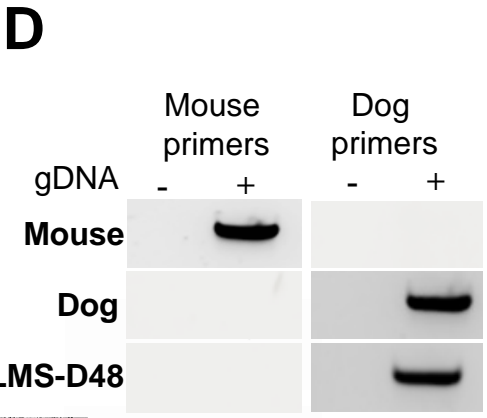
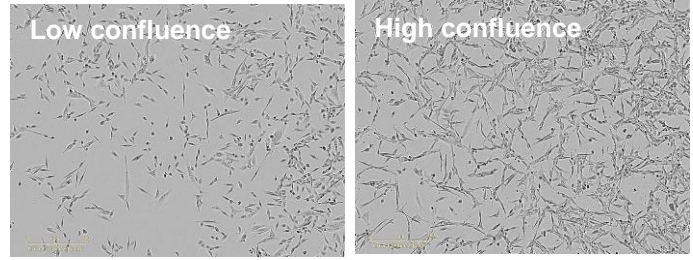
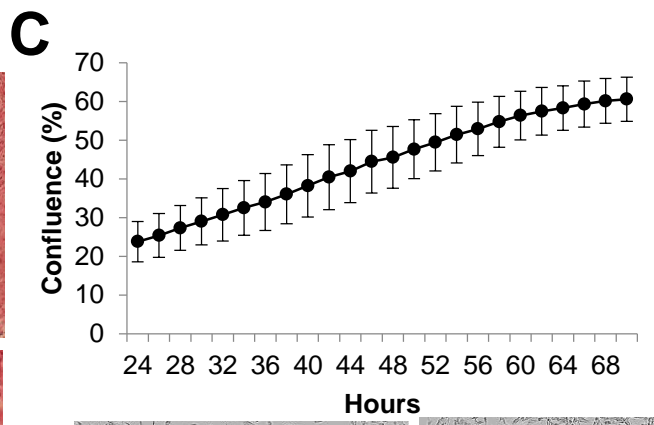
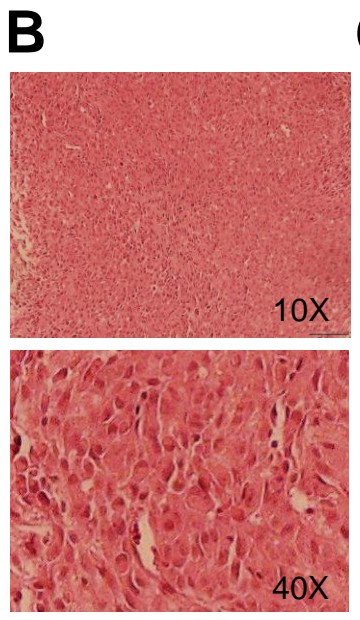
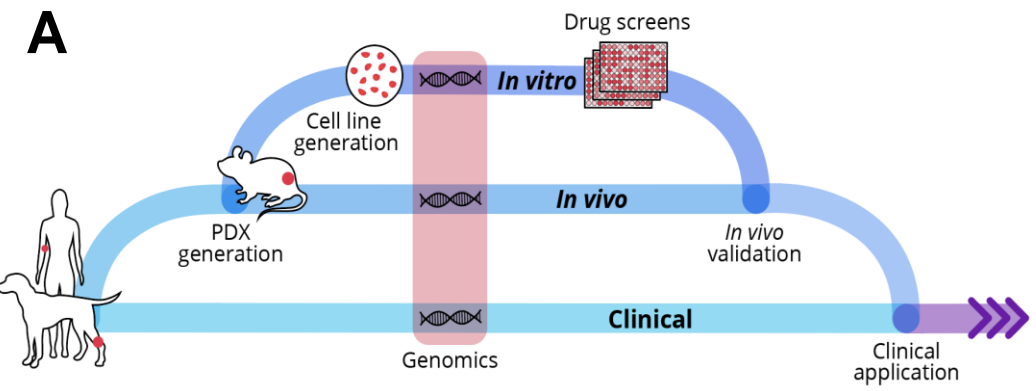


Figure 2



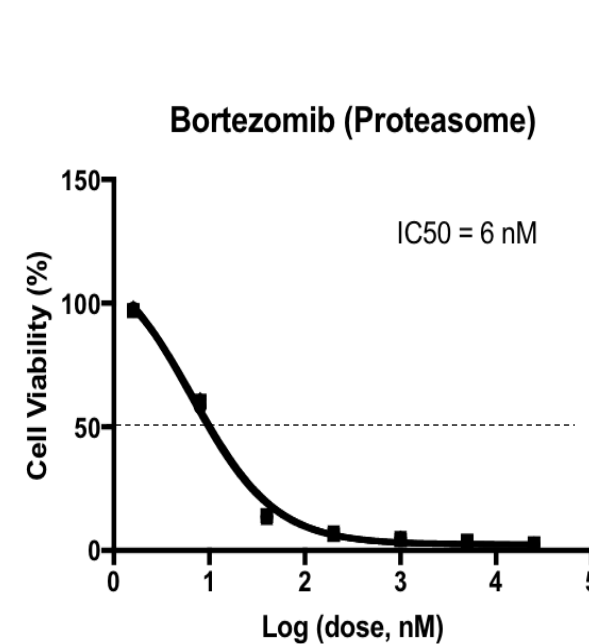
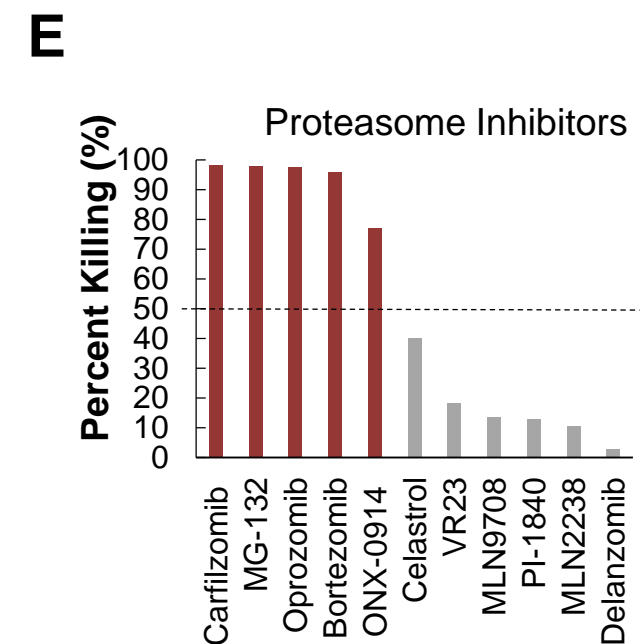
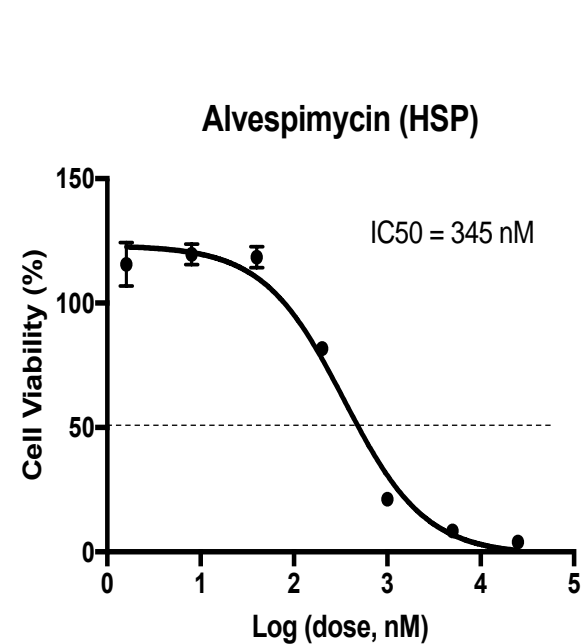
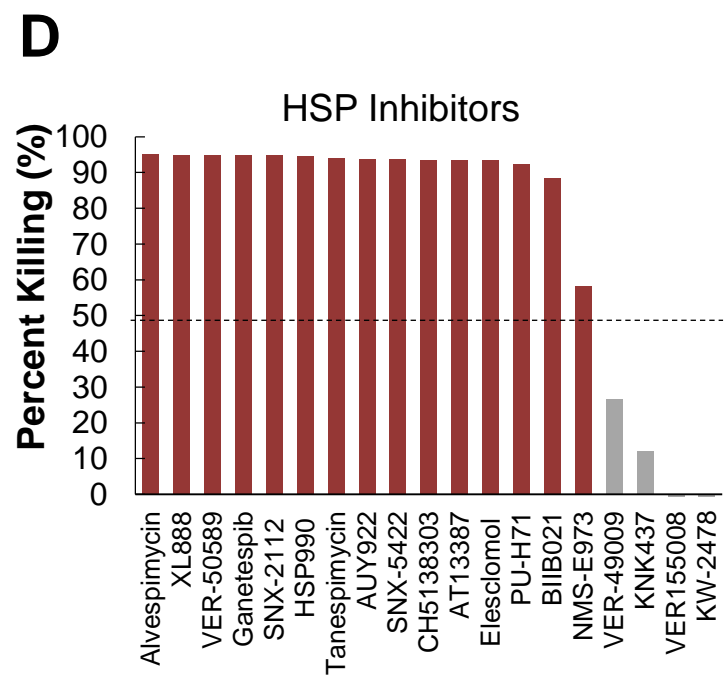
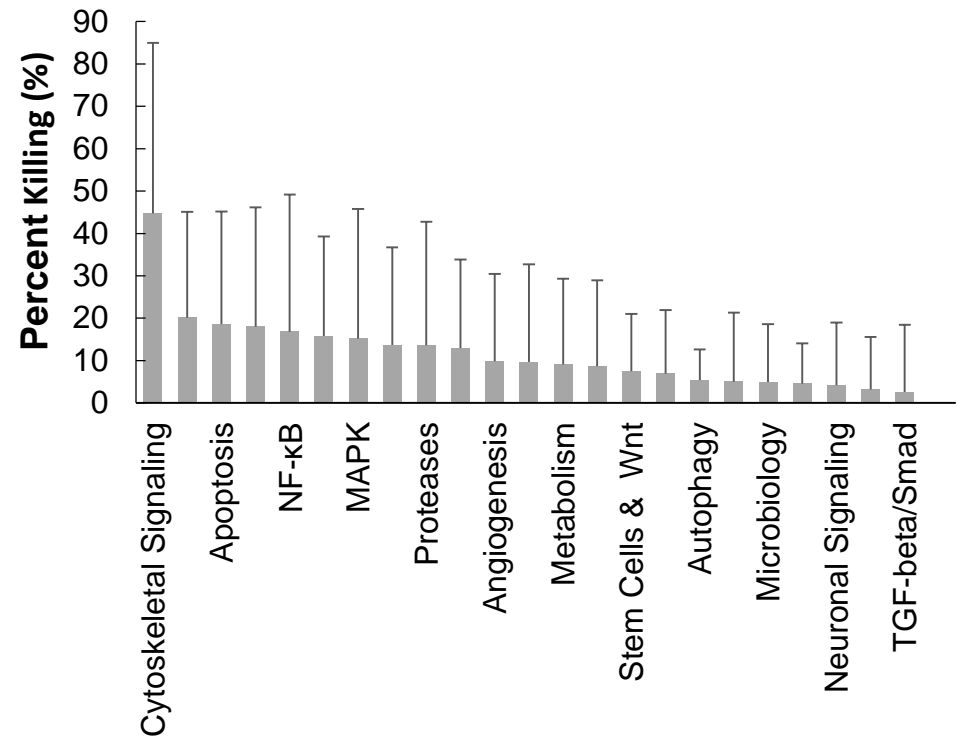
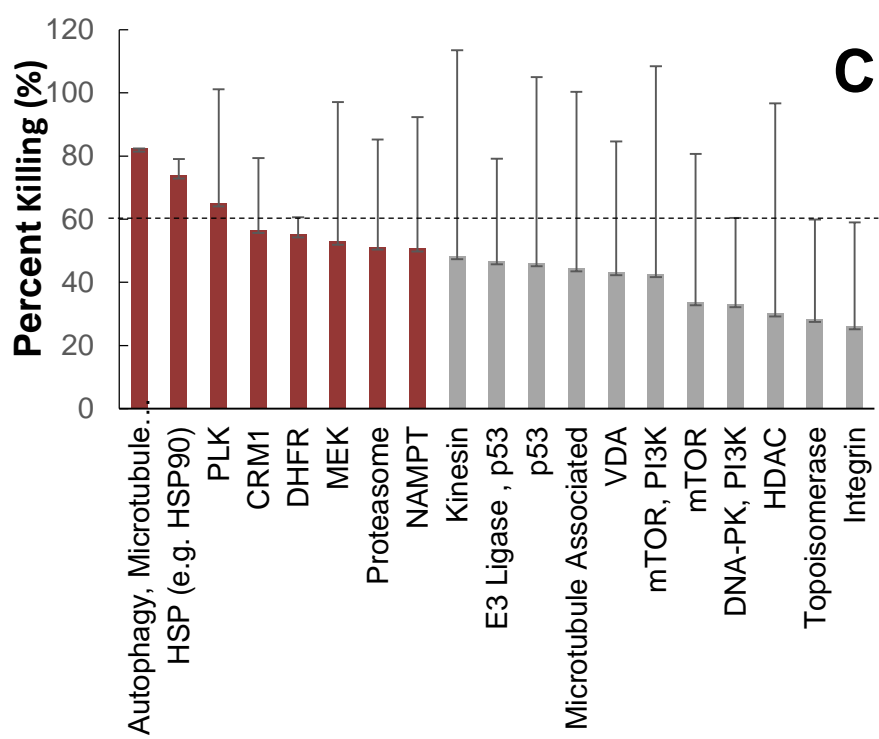
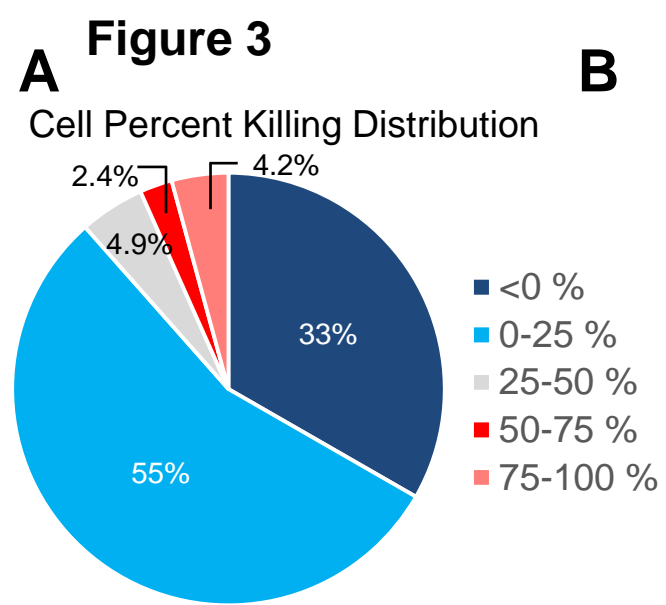
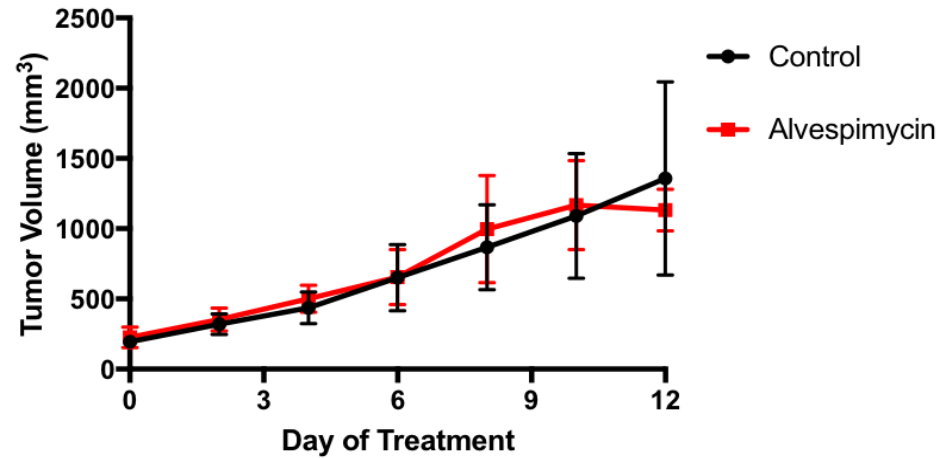
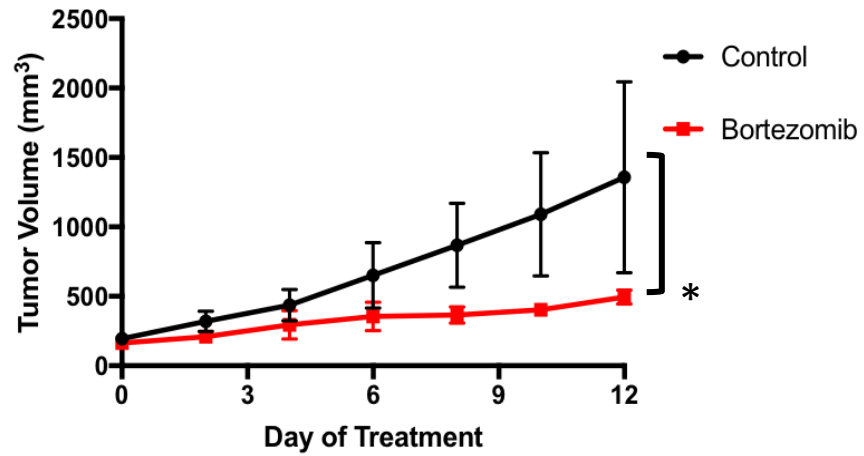


Figure 4

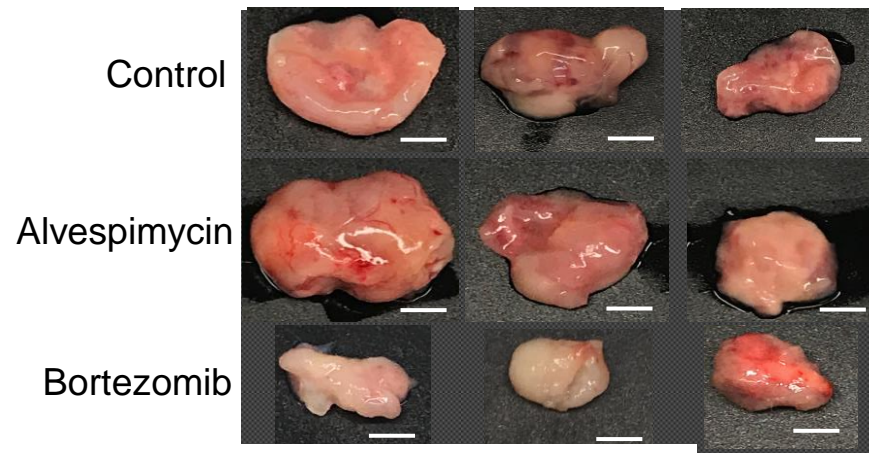
A



B



C



D

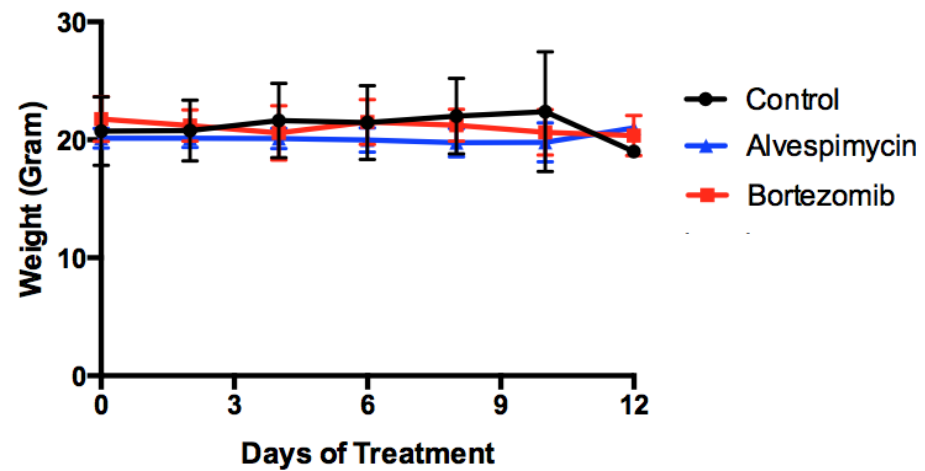
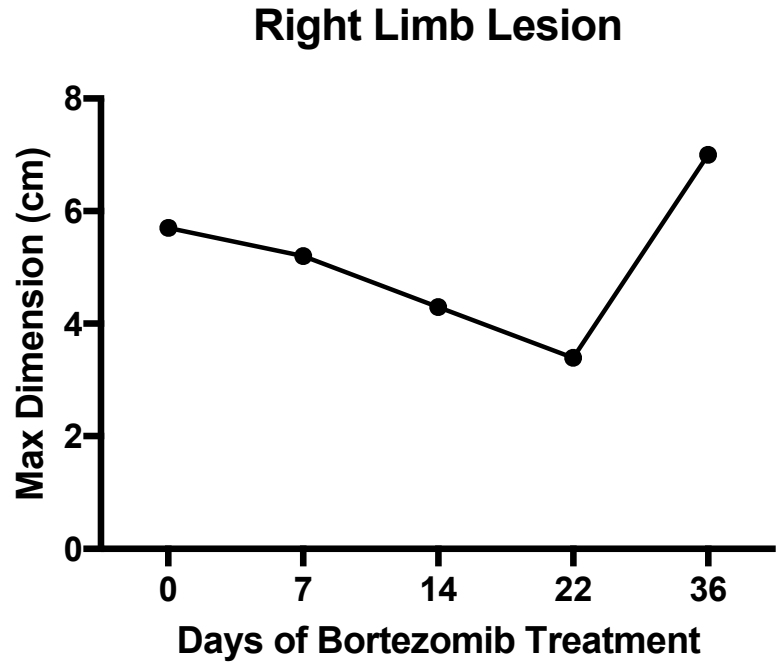


Figure 5

A



B

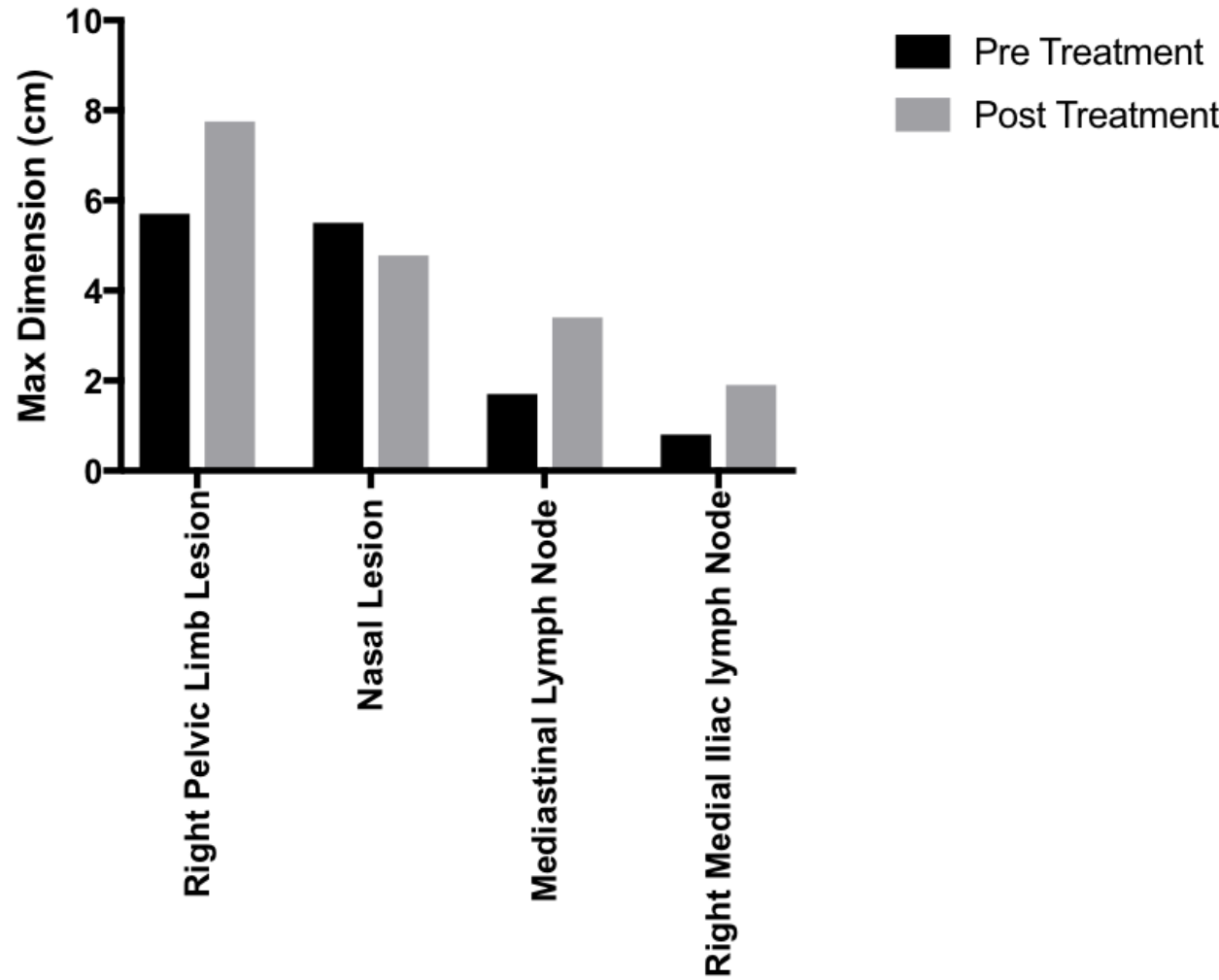


Figure 6

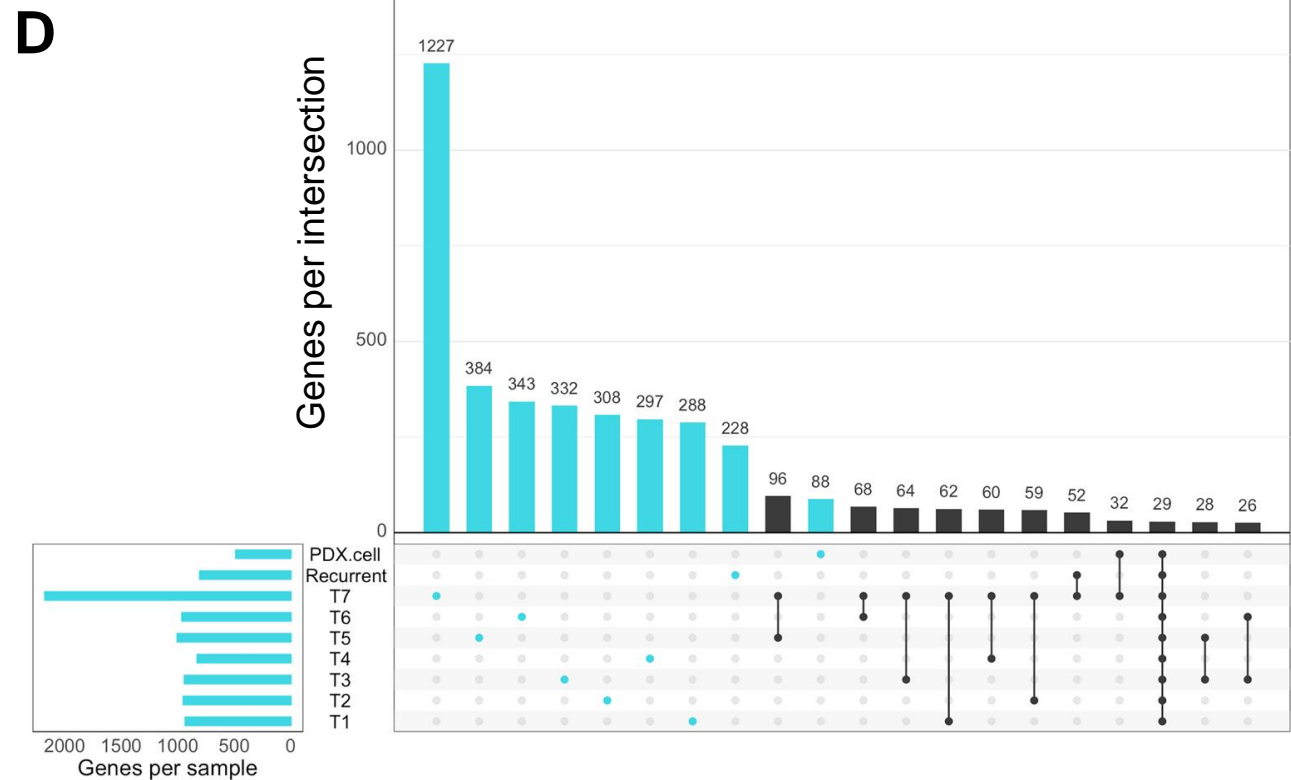
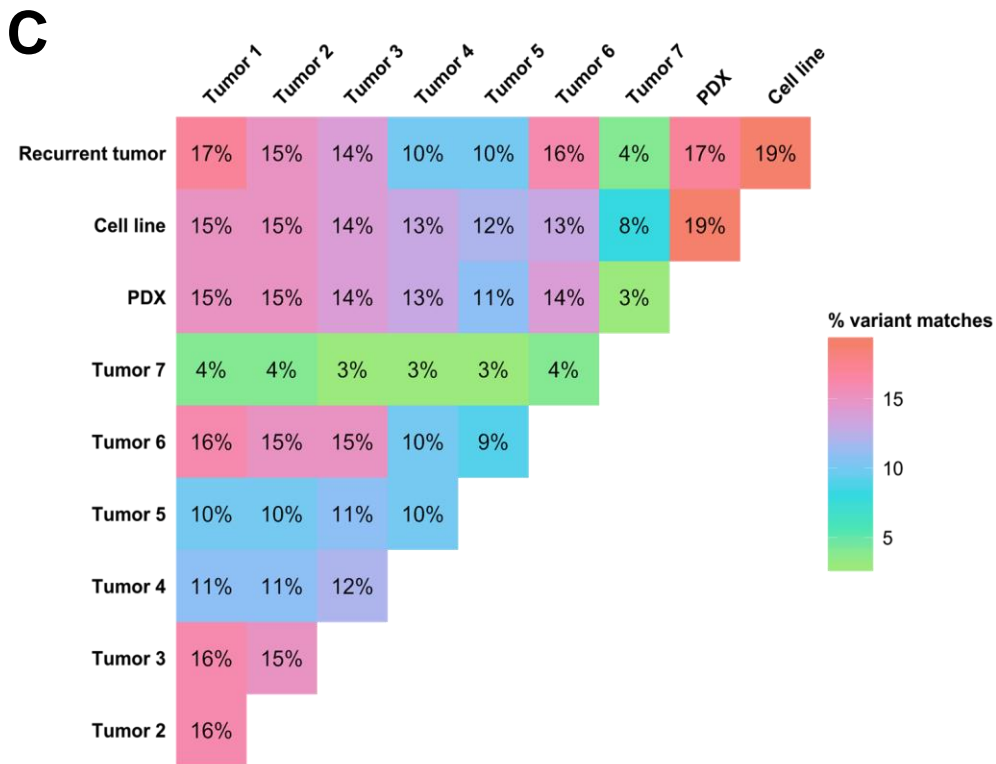
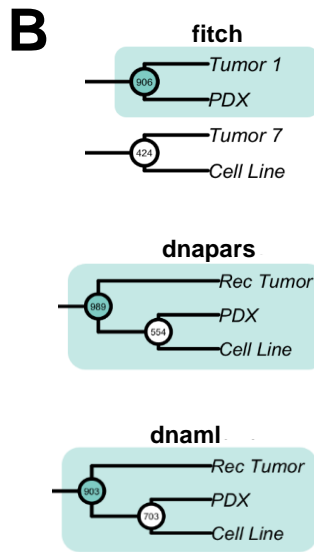
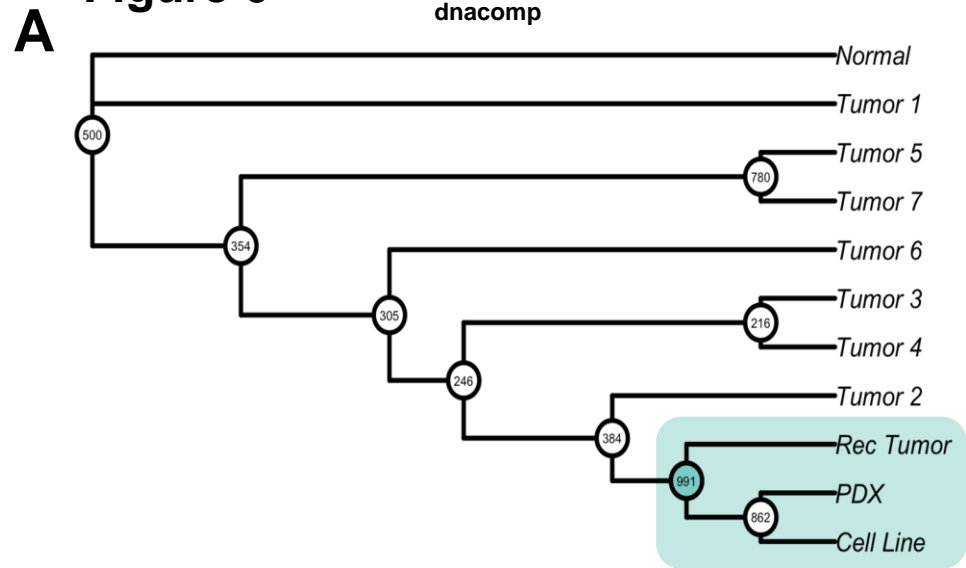


Figure 7

