

1 Efficient Development of Platform Cell Lines Using CRISPR-Cas9 2 and Transcriptomics Analysis

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10

11 Abstract

12

13 Antibody-drug conjugates offers many advantages as a drug delivery platform that allows
14 for highly specific targeting of cell types and genes. Ideally, testing the efficacy of these
15 systems requires two cell types to be different only in the gene targeted by the drug, with
16 the rest of the cellular machinery unchanged, in order to minimize other potential
17 differences from obscuring the effects of the drug. In this study, we created multiple
18 variants of U87MG cells with targeted mutation in the *TP53* gene using the CRISPR-Cas9
19 system, and determined that their major transcriptional differences stem from the loss of
20 p53 function. Using the transcriptome data, we predicted which mutant clones would have
21 less divergent phenotypes from the wild type and thereby serve as the best candidates to be
22 used as drug delivery testing platforms. Further *in vitro* and *in vivo* assays of cell
23 morphology, proliferation rate and target antigen-mediated uptake supported our
24 predictions. Based on the combined analysis results, we successfully selected the best
25 qualifying mutant clone. This study serves as proof-of-principle of the approach and paves
26 the way for extending to additional cell types and target genes.

27

28 **Keywords:** CRISPR-Cas9, TP53, CAGE, transcriptomics

29

30

31 1 Introduction

32

33 Targeted drug delivery through the use of monoclonal antibodies has been a prominent
34 therapeutic research area due to its promise of avoiding negative drug side effects by
35 targeting diseased cells expressing specific antigens (Scott et al., 2012). This can be
36 especially beneficial in cancer treatment, as the cytotoxic compounds currently used in
37 chemotherapy also bring about serious negative side effects in the patients (Firer and
38 Gellerman, 2012). While monoclonal antibodies by themselves often have low therapeutic
39 effects, they can be conjugated to highly potent drugs and act as their delivery vehicles to

40 their target cells marked by specific surface antigens, preventing the drugs from adversely
41 affecting healthy cells (Panowski et al., 2014). Especially promising are peptidic inhibitors
42 that can target specific protein-protein interactions and bring about desired cellular changes,
43 including apoptosis (Sorolla et al., 2020). Antibody drug conjugates (ADCs), as these
44 pairings are known, have been lauded as a revolutionary step in anti-cancer drug
45 development, but successful clinical application has proven challenging and the number of
46 clinically approved systems remains low due to the complicated requirement of optimizing
47 (Peters and Brown, 2015; Tang et al., 2019). Successful development of an ADC is a
48 complicated process that requires the simultaneous optimization of tumour-specific antigen
49 and its counterpart antibody, the stable linker between the antibody and the therapeutic
50 agent, and finally, the agent itself. Current ADC development strategies often employ a
51 limited number of existing microtubule inhibitors and their derivatives as the therapeutic
52 agent payload, whose performances are measured with both *in vitro* and *in vivo* cytotoxicity
53 tests (Lambert and Chari, 2014; Mullard, 2013). Efforts to expand the list of therapeutic
54 agents that can be used as ADC payload to other classes of compounds are ongoing, but the
55 choices still remain limited (Stein et al., 2018).

56

57 Given the requirements, novel ADC development would benefit from the availability of *in*
58 *vitro* testing platforms through which the specificity and the efficacy of both the antibody
59 and the drug can be evaluated. A number of human cell line models exists for testing
60 cellular response to anticancer drugs, but ADC development entails some additional
61 requirements to what is already available (Niu and Wang, 2015). Core requirements of a
62 good testing platform for ADCs are that both the test and control cell types express the
63 common antigens targeted by the antibodies at similar levels, and that any differences in the
64 cellular changes induced by the drug stem mainly from the disruption of its target. If the
65 first condition is not met, drugs would not be absorbed into the test and control cells at the
66 same levels, complicating downstream efficacy evaluations. As for the second condition, it
67 is necessary to limit the extent of off-target effects of the administered drugs so that they
68 would not add layers of complication in interpreting the cellular changes introduced by the
69 drugs. Unfortunately, because of the general lack of cell line pairings that differ in the drug
70 target genes only, researchers have to contend with using two distinct cell types with
71 differing origins to evaluate drug efficacy, despite the inherent risks of doing so. For
72 example, there are numerous ongoing studies on p53, which is the most well-known and
73 researched tumour suppressor gene, and a large portion of these studies focus on
74 manipulating its expression in cancer cells through various therapeutic means including
75 ADCs (Dangelmaier et al., 2019; Hafner et al., 2019; Kamp et al., 2016; Leroy et al., 2017;
76 Mantovani et al., 2019). Another advantage afforded by well researched genes such as p53
77 is that one can utilize the already existing drugs and focus on the development of novel
78 delivery mechanisms only. A popular testing platform for this purpose consists of the
79 glioblastoma cell lines U87MG and U251MG, with the former having wild type p53 and
80 the latter with an inactivated version that can no longer interact with MDM2, the main
81 regulator of p53 expression (Cobbs et al., 2008; Leroy et al., 2017; Qin et al., 2019; Yount

82 et al., 2001). The testing platform based on these two cell line pairs is particularly pertinent
83 to ADC research, as they both express integrin $\alpha\beta 3$ that can be targeted by the same
84 antibody and drugs that disrupt p53-MDM2 interaction are under intense research focus.
85 Integrin $\alpha\beta 3$ is a frequently targeted cancer cell surface marker, as it is well known that its
86 expression changes with tumour growth and metastasis (Sloan et al., 2006; Weigelt et al.,
87 2005). However, although both U87MG and U251MG are glioblastoma cell lines, they are
88 actually distinct cell types with completely different sources of origin, such that their
89 genetic differences extend far beyond just p53 (Camphausen et al., 2005). Thus, even
90 though many studies on p53 are performed on these two cell lines due to the known
91 mutation in U251MG, the interpretation of the obtained results can be obfuscated by other
92 inherent cellular differences.

93
94 Ideally, the best way to overcome this hurdle is to create targeted mutants of specific cell
95 lines using genome engineering. One of the major developments in the field of genome
96 engineering during the last decade has been the utilization of the CRISPR/Cas9 system for
97 targeted gene editing, bringing unprecedented precision and ease of use (Hsu et al., 2014;
98 Jinek et al., 2012; Ran et al., 2013). The system and its derivatives have gone through
99 intense development and refinement, and they are now employed for a multitude of
100 selective gene editing and regulation modifications. Wanzel *et al.* have employed this
101 system to create p53 knockout mutants in the human colorectal cancer cell line HCT116 to
102 investigate the mechanisms behind the disruption of p53-MDM2 interaction by the known
103 inhibitor RITA (Wanzel et al., 2015). They targeted multiple intronic and exonic regions,
104 resulting in mutants with both small indel mutations and large deletions and experimentally
105 validated p53 inactivation in the resulting p53 knockout mutants. However, there was no
106 comprehensive follow-up analysis of the p53 KOs to examine the extent of the
107 transcriptomic changes induced by these mutations, both from the p53 modification or
108 other potential off target effects.

109
110 In this study, we employed CRISPR-Cas9 to make targeted mutations of the *TP53* gene in
111 U87MG, with the goal of creating a mutant cell line that has otherwise minimal differences
112 from the wild type. We performed transcriptome analysis of the mutant clones created to
113 examine the genetic differences using Cap Analysis of Gene Expression (CAGE), which
114 can identify the location of transcription start sites and their expression levels (Carninci et
115 al., 2006; Kanamori-Katayama et al., 2011). We confirmed that the extent of genetic
116 differences between the wild type U87MG cells and the mutant clones were not extensive,
117 and that these changes were mostly attributable to the inactivation of p53. We down-
118 selected from the clones based on further analysis of their transcriptomic profiles compared
119 to the wild type, and subjected them to further experimental validation to ascertain that
120 there are no major changes to their *in vivo* phenotypes compared to the wild type U87MG
121 cells and that other attributes necessary for their utility as testing platform for ADC
122 development remain intact.

123

124 **2 Materials and Methods**

125

126 **2.1 CRISPR-Cas9**

127 *Cell culture:* U87MG cells (ATCC HTB-14) were grown in high glucose DMEM (Gibco)
128 supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco) and 100
129 µg/mL streptomycin (Gibco) at 37°C with 5% CO₂.

130 *Lentiviruses:* To prepare the TP53 knockout cells by CRISPR-Cas9 gene editing, sgRNA
131 targeting TP53 gene exon 5 (5'-GTTGATTCCACACCCCC.GCCcgg-3') was cloned into
132 lentiGuide-Puro vector (Addgene, #52963), named lentiGuide-Puro_e5.2. Lentivirus for
133 lentiCas9-Blast (Addgene, #52962) and lentiGuide-e5.2-Puro were prepared in a mixture of
134 the following packaging constructs: pCMV-VSV-G-RSV-Rev and pCAG-HIVgp (provided
135 by RIKEN BRC, Japan) according to the protocol (Suzuki et al., 2012).

136 *Mutation:* LentiCas9-Blast lentivirus transduction was performed with 8 µg/mL polybrane
137 supplemented DMEM at 2 MOI for 24 h. Fresh DMEM without polybrane was used 24 h
138 after transduction to avoid cell damage. After 48 hours, transduced cells were selected onto
139 medium containing 15 µg/mL blasticidin, and incubated for 7 days. LentiGuide-Puro_e5.2
140 lentivirus was transduced into Cas9-expressing cells at 2 MOI, and selected by 1 µg/mL
141 puromycin and incubated for 4 days. Cleavage efficiency was estimated through indel
142 detection performed using the Guide-it Mutation Detection Kit from Clontech.

143 *Clone selection:* Individual clones were scaled up in a 10 cm dish, and screened with
144 western blot using anti-human p53 IgG (CST, rabbit polyclonal). Sanger sequencing of the
145 TP53 cleavage target region in the selected clones was performed using Applied
146 Biosystems 3730xl DNA Analyzer with BigDye Terminator v3.1 Cycle Sequencing Kit
147 (ThermoFisher Scientific), and aligned to the wild type region using CLUSTAL version 2.1
148 (Larkin et al., 2007).

149 *Western blotting:* Cells were lysed in RIPA buffer (Pierce) containing proteinase inhibitors
150 (Halt Proteinase Inhibitor Cocktail, Thermo Scientific). Protein concentration was
151 determined with BCA Protein Assay Kit (Pierce). Protein lysates (20 µg) were separated on
152 NuPAGE Tris-Acetate Gels (Invitrogen) and transferred onto PVDF membranes.

153 Membranes were blocked with 5% skim milk in TBST for 1 h at room temperature and
154 incubated with anti-human p53 antibody (Cell Signaling Technologies, #9282, rabbit
155 polyclonal, 1:1000) in TBST containing 5% skim milk (4°C, overnight). Membranes were
156 incubated with anti-rabbit IgG-HRP (GE Healthcare, NA934V, 1:2000) in TBST for 2 h at
157 room temperature. Detection was performed with ECL Prime (GE Healthcare) and imaged
158 by chemiluminescence imaging system FUSION SOLO S (VILBER LOURMAT).

159 *Immunofluorescence:* Cells were seeded with 80,000 cells per well on a coverslip coated
160 with L-polylysine into 24-well plates. After overnight incubation, cells were fixed with 4%
161 paraformaldehyde in PBS for 10 min at room temperature. Cells were blocked with 1%
162 BSA-0.3 M glycine in PBST and incubated with mouse anti-human integrin $\alpha\beta3$ IgG
163 (1:200, monoclonal, LM609, Abcam, ab190147) in 1% BSA-PBST overnight at 4°C. For
164 isotype control, mouse (G3A1) IgG1 Isotype Control (Cell Signaling Technologies, #5451)

165 was used. Cells were incubated with donkey anti-mouse IgG-Alexa 488 conjugate (1:200,
166 Jackson ImmunoResearch Laboratories, code: 715-545-150) in 1% BSA-PBST for 1 h at
167 room temperature. The samples were embedded in VECTASHEELD mounting medium
168 (Vector Laboratories), and imaged by OLYMPUS IX71.

169 *Reverse transcriptase polymerase chain reaction*: With starting RNA amount of 100 ng per
170 sample, reverse transcription of *ITGAV*, *ITGB3*, and *GAPDH* (internal control) was
171 performed using SuperScript III and oligo-dT primers on GeneAmp PCR System 9700.
172 Quantitative PCR was performed with Hotstar Taq, customized primers on the ABI
173 7900HT Fast Real-Time PCR System. Experiments were done in triplicates per cell type.
174

175 **2.2 CAGE data generation and analysis**

176 *Data generation*: Total RNA was extracted from samples using RNeasy Mini Kit (Qiagen).
177 The CAGE libraries were constructed using the published nAnT-iCAGE protocol (Murata
178 et al., 2014): 5 µg of total RNA per sample from the mutant clones and the wild type cells
179 was reverse transcribed with SuperScript III reverse transcriptase to produce cDNAs, which
180 were then biotinylated and cap trapped to capture their 5' ends. After barcoding and
181 purification, they were sequenced on illumina HiSeq 2500 (50 nt single read). The
182 sequenced reads were processed using the MOIRAI pipeline (Hasegawa et al., 2014). After
183 filtering for rDNA and low quality reads, they were mapped to the human genome (hg38)
184 using BWA version bwa version 0.5.9 (r16), with default parameters except: (1) for
185 `bwa_aln`, maximum long deletion (-d) = 10, best hit limit (-R) = 30, seed length (-l) = 32,
186 and (2) for `bwa_samse`, maximum number of alignments (-n) = 100 (Li and Durbin, 2009).
187 The reads were overlapped with the FANTOM5 robust promoter set
188 (http://fantom.gsc.riken.jp/5/datafiles/latest/extra/CAGE_peaks/) and mapped to the nearest
189 GENCODE v27 annotations within 500 bp (FANTOM Consortium, 2014; Frankish et al.,
190 2019). CAGE clusters within 20 bp of each other were merged together, and enhancer
191 clusters overlapping existing GENCODE annotations were removed. For each processed
192 CAGE cluster, the number of overlapping 5' ends of the sequencing reads were summed to
193 arrive at the raw expression counts.

194 *Data analysis*: The expression counts table was first filtered for lowly expressed CAGE
195 clusters by first converting the values to counts per million (CPM), then removing those
196 expressed in less than 3 samples with at least 1 CPM and greater than 1 sample with at least
197 3 CPM. Differential expression analysis of the expression table was performed with edgeR
198 (version 3.28.0) in R 3.6.1 with TMM normalization and TREAT function with log fold
199 change threshold of 1 and false discovery rate threshold of 0.05 (R Development Core
200 Team, 2008; Robinson et al., 2010). KEGG pathway enrichment was performed using
201 edgeR's built-in `kegga` function and filtered for p-value threshold of 0.05. Motif Activity
202 Response Analysis (MARA) was performed with the implementation used in Alam *et al.*,
203 2020 (Alam et al., 2020; Balwierz et al., 2014). Briefly, transcription factor binding sites
204 were predicted using the SwissRegulon set of motifs in 300 bp downstream and 100 bp
205 upstream regions around each CAGE cluster, and motif activities calculated with the

206 normalized CAGE expression table (Pachkov et al., 2007). The motif activities were
207 converted to Z scores, and their differences between each mutant clone and the wild type
208 were taken and visualized. Motifs with Z scores greater than 2 were marked as the
209 significant set of motifs with notable regulatory effects on expression. TP53 ChipSeq peaks
210 data sets were downloaded from Chip-Atlas (<https://chip-atlas.org/>) and overlapped with
211 FANTOM5 promoter regions with +/-500 window. The TF activities were then calculated
212 as mean log₂ fold change of expression for genes with TP53 binding at the promoters. Off
213 target regions for the sgRNAs used were predicted using CasOFFinder (Bae et al., 2014),
214 and the GENCODE v27 annotations within 1 kb in either direction were retrieved and
215 compared against the list of differentially expressed genes. The scripts used to perform
216 these analyses are available at (https://github.com/tjkwon/CRISPR_p53).

217

218 **2.3 Cell proliferation assay**

219 U87MG (p53 wild-type) or the p53-mutant cells were seeded into 96-well plates at a
220 density of 2,000 cells/well, and incubated for 24 h before treatment. p53-MDM2 interaction
221 inhibitor, or nutlin-3a was added to cells at different concentrations, and the cells were
222 incubated for 5 days at 37 °C. Then, 10 µL of a WST-8 reagent (Nacalai Tesque, inc.) was
223 added to each well, and the cells were incubated for 2 h at 37 °C. The absorbance was
224 measured using a iMark™ Microplate Reader (Bio-Rad Laboratories, Inc.) at 450 nm to
225 count the living cells. IC₅₀ values for nutlin-3a were calculated from nonlinear regression
226 analysis (four parameters) using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA,
227 USA).

228

229 **2.4 Cellular uptake and intracellular localization**

230 FITC-labeled RGD peptide (Gly-Arg-Gly-Asp-Ser-Pro-OH), or FITC-RGD peptide was
231 purchased from AnaSpec (Fremont, CA, USA). To investigate cellular uptake of FITC-
232 RGD peptide, U87MG or the p53-mutant cells were seeded into 24-well plates at a density
233 of 2.5×10^5 cells/well, and incubated for 24 h before treatment. The cells were incubated
234 with 20 µM FITC-RGD peptide for 4 h at 37 °C. After the cells were washed twice with
235 PBS and harvested, the fluorescent intensity of cells was determined by Cell Sorter SH800
236 (Sony Corp., Tokyo, Japan) using SH800 software (Sony Corp.). To investigate
237 intracellular localization of FITC-RGD peptide, U87MG or the p53-mutant cells were
238 seeded into 3.5 cm glass bottom dishes at a density of 9×10^5 cells/dish, and incubated for
239 24 h before treatment. Cells were incubated with 20 µM FITC-RGD peptide for 4 h at 37
240 °C. The cells were washed twice with PBS, and incubated with 100 nM LysoTracker Red
241 DND-99 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 30 min at 37
242 °C. After washing twice with PBS, the cells were fixed in 4% paraformaldehyde for 20 min.
243 After washing twice with PBS again, the coverslips were mounted on glass slides with
244 ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen, Thermo Fisher Scientific,
245 Inc.), and observed using a confocal microscope (LSM710, Carl Zeiss GmbH, Oberkochen,
246 Germany).

247

248 **2.5 Tumour growth of rates in subcutaneously xenografted mice**

249 Male BALB/c nu/nu mice (5-week old) were purchased from Japan SLC, Inc. (Hamamatsu,
250 Japan). Mice were subcutaneously inoculated with U87MG or the p53-mutant cells ($3.0 \times$
251 10^6 cells per mouse) in 100 μ L of Hank's balanced salt solution (HBSS, Nacalai Tesque,
252 Inc., Kyoto, Japan). The tumour size was measured with a slide caliper, and tumour
253 volume was calculated using the following formula: tumour volume (mm^3) = $0.5 \times$ length
254 (mm) \times [wide (mm)]². All animal experimental protocols were approved by the Ethics
255 Committee on Animal Care and Use of the RIKEN Kobe Institute.

256

257 For the above 4 assays, statistical differences were analyzed using one-way analysis of
258 variance (ANOVA) followed by the Dunnett tests for multiple comparisons. Kaplan–Meier
259 curves were generated, and log-rank tests were performed. $P < 0.05$ was considered to be
260 statically significant.

261

262 **3 Results**

263

264 **3.1 3.1. Generation of p53-mutated U87MG cells and initial comparisons to wild type** 265 **cells**

266

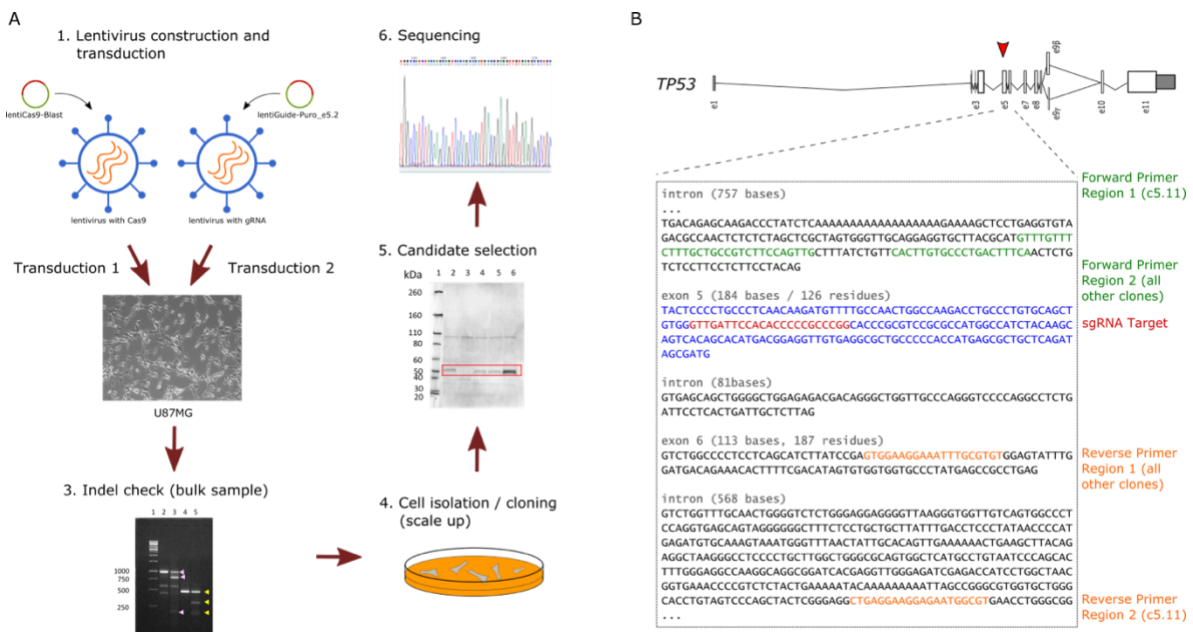
267 We decided on the CRISPR-Cas9 system based on guide RNA-containing plasmid
268 transfection and NHEJ pathway, as the goal of this study was to simply mutate p53 leading
269 to disruption of known downstream interactions, and as such did not require the specificity
270 offered by other methods. To increase our chance of success, we initially attempted the
271 mutation of the p53 gene body in U87MG cells with different sets of single guide RNAs
272 (sgRNAs), targeting introns 1, 2 and 9 for large deletions and exons 3 and 5 for indels.
273 When screened with Guide-it mutation detection kit and western blot, the sgRNA targeting
274 exon 5 was found to have achieved successful indel mutations in the target region (Figure
275 1, Supplementary Figure 1). These indels were confirmed with Sanger sequencing, and 7
276 unique p53 mutant clones were identified; their lengths suggest that they likely introduced
277 nonsense mutations (Supplementary Table 1). After culturing and scaling up the mutant
278 clones, their morphology was visually compared against the wild type U87MG cells, and in
279 general, they maintained similar shapes and growth rates as their wild type counterparts
280 (Supplementary Figure 2). The only exception was clone c4.2, which exhibited a higher
281 growth rate than the wild type cells. When immunostained for integrin $\alpha\beta 3$, all of the
282 mutant clones exhibited fluorescence levels at similar levels to the wild type, suggesting
283 that the expression of integrin $\alpha\beta 3$ was not impacted significantly. All of the mutant
284 clones exhibited similar levels of the integrin expression, with no discernible differences in
285 their fluorescence. We also carried out reverse transcriptase polymerase chain reaction (RT-
286 PCR) of *ITGAV* and *ITGB3*, the two component genes of integrin $\alpha\beta 3$ (Supplementary
287 Figure 3). All the mutant clones expressed *ITGAV* and *ITGB3* at levels similar or greater

288 than that of the wild type except for clone c4.2, which showed slightly lower *ITGB3*
 289 expression, and similar trends could be observed when the expression levels were compared
 290 against *GAPDH* for internal control. These observations demonstrate that the unwanted
 291 negative regulation of the integrin genes from the CRISPR-Cas9 process was minimal. As
 292 we envision utilizing the created mutant clones as an ADC testing platform with integrin
 293 $\alpha\beta3$ as the antigen, it is vital that the integrin expression is maintained in both the mutant
 294 clones and the wild type. Together, these results confirmed that the CRISPR-Cas9 process
 295 successfully disrupted p53 protein level abundance with minimal effects on cell
 296 morphology and integrin levels.

297

298 **Figure 1.** Targeted mutation of p53 gene at exon 5 in U87MG cells using CRISPR-Cas9
 299 system.

300 (a) Flowchart of the p53 mutation process. Virus particles containing the lentiCas9-Blast
 301 and lentiGuide-Puro_e5.2 vectors are constructed and transfected into U87MG cells in
 302 sequence. Those cells with indels are isolated and cloned for scale up, then candidates are
 303 selected based on western blot results, which are then subjected to Sanger sequencing for
 304 final confirmation of successful mutation. (b) The sgRNA target (red) in exon 5 (blue)
 305 and the forward and reverse primer regions for sub-cloning (green and orange, respectively) are
 306 shown. Clone 5.11 used different forward and reverse primer regions from the other clones.
 307



308

309

310 3.2 Transcriptome comparison of mutants using CAGE analysis

311

312 p53 is involved in numerous cellular processes, and as such it regulates and is regulated by
 313 numerous interacting partners, both upstream and downstream. Thus, any mutation in p53
 314 is bound to have both direct and indirect regulatory effects on many other genes.

315 Furthermore, although the CRISPR-Cas9 system allows for highly specific targeted

316 mutation, off-target effects resulting in unintended genomic changes still frequently occur
317 and must be checked for. We wanted to evaluate the extent of these gene expression
318 changes incurred by the p53 disruption using transcriptome profiling in each of the mutant
319 clones and ascertain that the changes were acceptable for our purpose. We used cap
320 analysis of gene expression (CAGE), which captures the 5' ends of transcripts to identify
321 transcription start sites (TSS) down to single nucleotide resolution, not only for promoters
322 of protein coding genes but also noncoding RNAs and enhancers. Compared to RNA-seq,
323 CAGE also has the advantage of detecting and analyzing alternative promoter usage at each
324 gene. Comparing the transcriptome profiles of each mutant clone to that of the wild type
325 using CAGE, enabled us to confirm the disruption of the p53-mediated pathways and use
326 the measured expression changes to calculate their distances from the wild type cells.

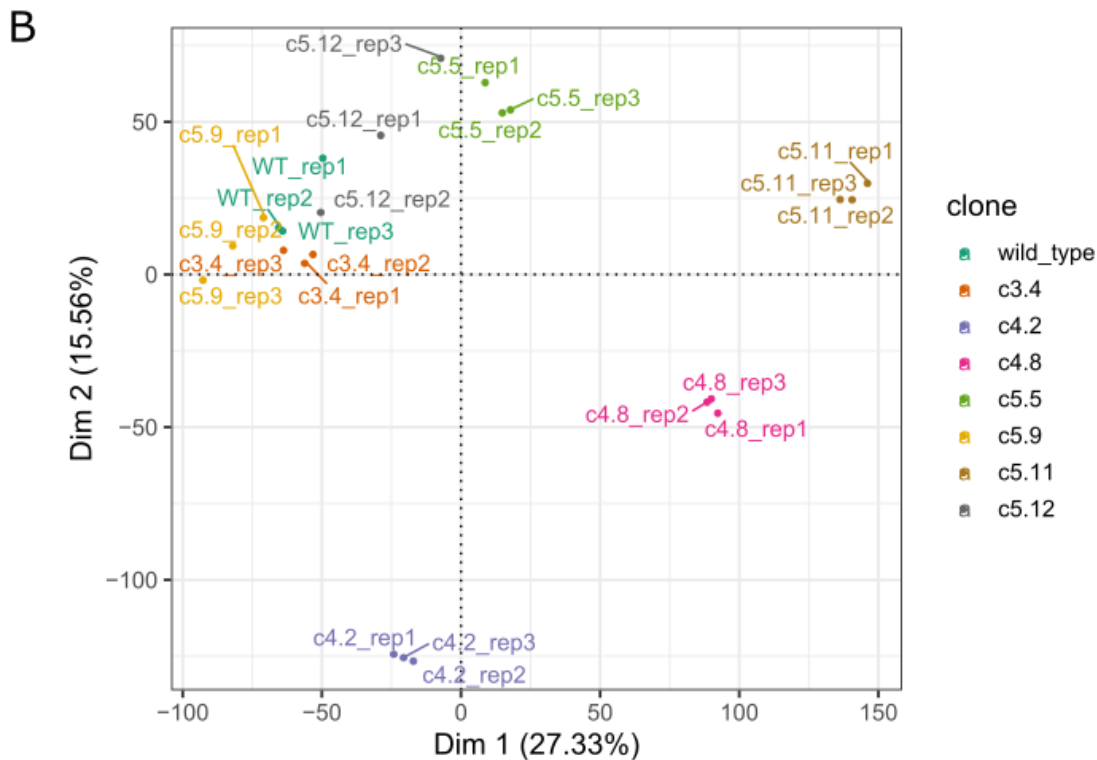
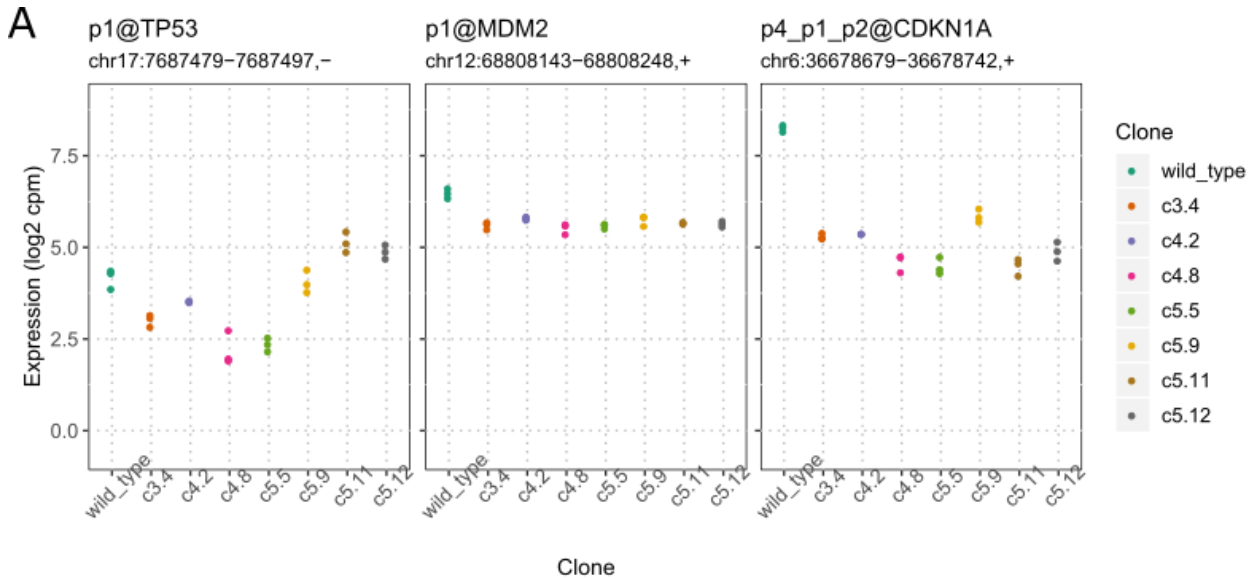
327

328 We prepared the combined expression table for all samples by summing the number of
329 reads per CAGE clusters based on the FANTOM5 promoter set (see Methods). After
330 filtering for lowly expressed promoters and normalizing the expression levels, we arrived at
331 the expression counts table of 19,931 CAGE clusters representing 14,994 genes (12,475
332 known) (Supplementary Table 2). With the normalized expression table, we first visualized
333 the expression changes of *TP53*, *MDM2*, and *CDKN1A*, the core regulatory partners in the
334 p53 pathway (Figure 2a) (Enge et al., 2009). While the expression levels of *TP53* did not
335 show any consistent patterns across different clones, *MDM2* and *CDKN1A* showed
336 consistent down-regulation in all of the mutant clones compared to the wild type. It is
337 important to note that CAGE measures the transcription initiation events at the promoters
338 only, and does not cover the entire transcript body as RNA-seq would. Since the sgRNAs
339 targeted exon 5, the mutations did not result in abolishing of the transcription initiation
340 events; in some clones, we even observed higher levels of expression than the wild type.
341 Instead, the p53 transcripts produced in the mutant clones likely contain nonsense
342 mutations that result in truncated proteins with reduced functionality. Conversely, we could
343 clearly observe the effects of the mutation in the expression patterns of p53's direct
344 interacting partners *MDM2* and *CDKN1A*: *MDM2* expression was reduced approximately
345 by half, and *CDKN1A* showed greater than 3-fold decrease on average compared to the wild
346 type. Other genes known to interact with p53 or *MDM2* show down-regulation in the
347 mutant clones as well (Supplementary Figure 4a) (Basu and Haldar, 1998; Bensaad et al.,
348 2006; Meng et al., 2016; Serrano et al., 2013; Xiong et al., 2001). We also examined the
349 expression levels of *ITGAV* and *ITGB3*, and confirmed that all mutant clones show
350 expression levels similar to or greater than that of the wild type, as observed previously
351 with RT-PCR. Especially, clone 4.8 showed notably elevated expression in *ITGAV* and
352 *ITGB3*, suggesting that this clone may exhibit higher integrin presence on the cell surface
353 than the other ones (Supplementary Figure 4b).

354

355 **Figure 2.** Comparison of the transcriptome profiles of the mutant clones against the wild
356 type can be used to infer the degree of genomic changes caused by the respective mutation
357 in each clone. (a) Normalized expression levels of *TP53*, *MDM2*, and *CDKN1A*. Both

358 *MDM2* and *CDKN1A* exhibit down-regulation in the mutant clones. (b) PCA plot of the
359 sample transcriptomes, with first two dimensions shown. Clones c3.4, c5.5, c5.9 and c5.12
360 cluster closely with the wild type.
361



362

363

364 With the confirmation that the key marker genes show consistent changes across the mutant
365 clones, we proceeded to evaluate the overall transcriptome similarity of each mutant clone
366 to the wild type using principal components analysis (PCA) (Figure 2b). By reducing the
367 dimensionality of the entire transcriptome through orthogonal projection, PCA allowed us
368 to visualize the proximity of different samples and infer their similarity to one another.

369 When visualized using the first two dimensions of the PCA, clones c3.4, c5.5, c5.9, and
370 c5.12 clustered closely with the wild type, whereas the clones c4.2, c4.8, and c5.11 showed
371 higher separation from one another as well as the other clones. As expected, the replicates
372 of the same clone type formed the tightest clusters. Hierarchical clustering of the samples
373 confirmed these results, with clones c4.2, c4.8 and c5.11 forming the outer branches of the
374 clustering dendrogram (Supplementary Figure 5). The results suggest that the mutations in
375 these ‘outer’ clones affected more widespread gene expression changes than the ‘inner’
376 clones, potentially leading to additional phenotype changes from the wild type such as the
377 faster growth rate in clone 4.2 we observed.

378
379 To investigate the differences between clones in more detail, we performed differential
380 expression analysis to identify the promoters in each mutant clone showing significant up
381 or down regulation compared to the wild type expression levels (Supplementary Figure 6,
382 Supplementary Table 3). The number of observed differentially expressed promoters were
383 low in all clones. c5.5 showed the lowest number (37 up, 44 down) of both up- and down-
384 regulated promoters, while c5.9 had the highest number of down-regulated promoters (417
385 down) and c5.12 the highest number of up-regulated promoters (126 up). The other clones
386 showed similar numbers of differentially expressed promoters to one another, and overall
387 there was no obvious relationship between the numbers of differentially expressed
388 promoters and how they clustered together. This suggests that while numerous genes
389 undergo minor but still significant expression changes due to the loss of p53 function, there
390 may be a subset of genes with more drastic changes that contribute to the overall
391 transcriptome differences to a higher degree. To examine whether the differentially
392 expressed genes found were likely to be regulated by p53, we retrieved ChIP-seq binding
393 data from ChIP-Atlas and analyzed whether there was an association between the
394 differentially expressed genes and evidence of TP53 binding in their promoters
395 (Supplementary Table 4). After determining which genes had TP53 binding evidence, we
396 calculated the mean of the log fold change of expression of those genes in the mutant
397 clones vs. the wild type and observed a consistent down-regulation of expression, further
398 supporting the disruption of the p53-regulated genes resulting from its mutation (Figure
399 3a). We also made computational predictions of off-target regions affected by our sgRNAs
400 and compared the results against our list of differentially expressed genes (Supplementary
401 Table 5). Of the 32 candidate genes located within 1k base pairs from the affected regions,
402 none were differentially expressed in the mutant clones vs. the wild type. The only overlap
403 observed was from the list of differentially expressed genes between the fast growth clone
404 c4.2 vs. the other mutant clones, where *TRABD2B* and *PDE7B* were found in our potential
405 off-target gene list.

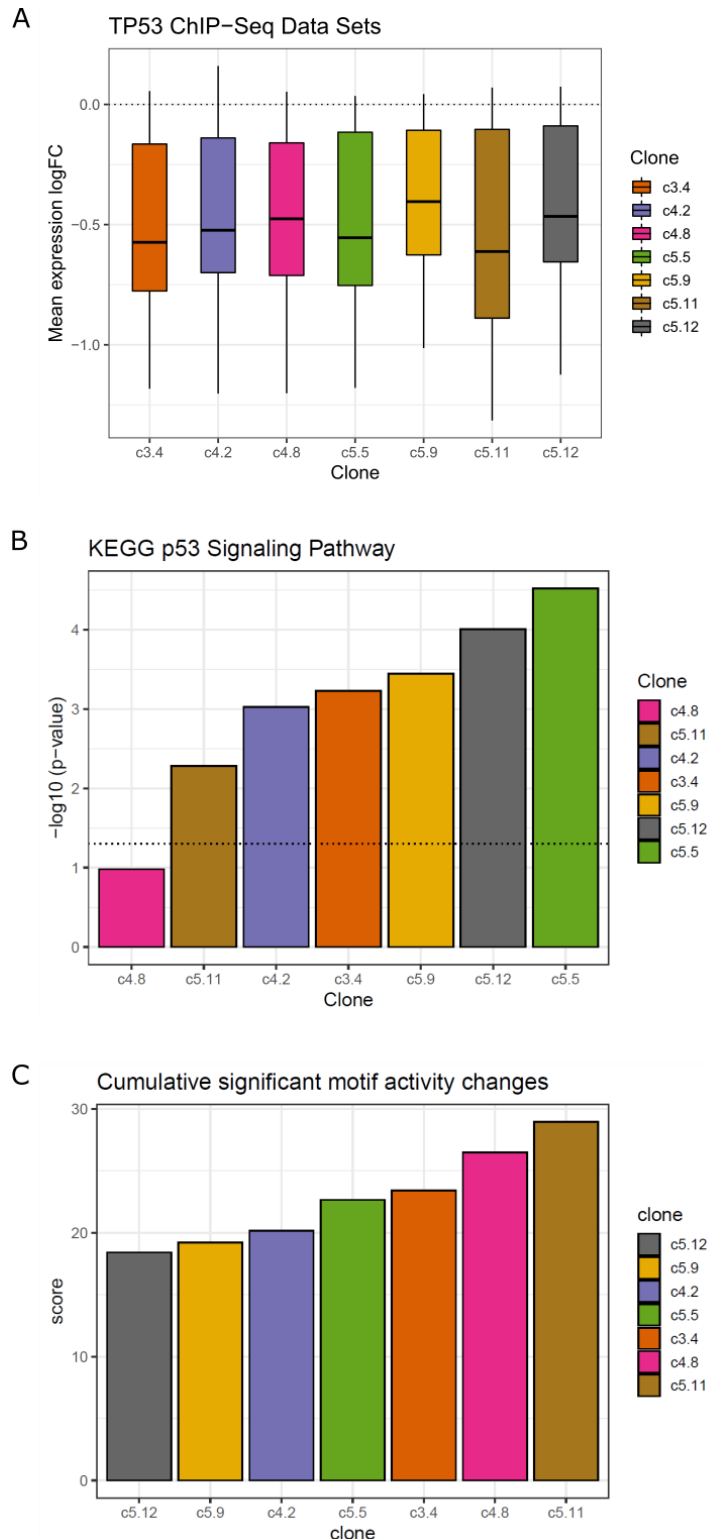
406
407 In order to determine which cellular pathways are disrupted with these differentially
408 expressed genes in each mutant clone, we performed functional analysis by identifying the
409 enriched KEGG pathways in the list of differentially expressed genes, and examined in
410 which group of genes the p53 signalling pathway was enriched in (Supplementary Table 6).

411 As expected, we found that the genes contributing to the p53 signalling pathway were
412 significantly enriched in the list of down-regulated genes among all comparisons ($p < 0.05$),
413 except for the clone 4.8, which was placed away from the wild type samples in the PCA
414 plot (Figure 3b, Supplemental Figure 7). When ranking the clones by the p53 signalling
415 pathway enrichment scores (negative logarithm of enrichment p-values), we observed that
416 the clones c4.8 and c5.11 scored the lowest while the clones c5.5, c5.12 and c5.9 scored the
417 highest, in agreement with our previous observation from the PCA and hierarchical
418 clustering. Further examining the top 10 pathways ordered by their median enrichment
419 scores across the samples, we observed that the ‘inner’ clones show higher enrichment for
420 cytokine-cytokine interaction pathways than the ‘outer’ clones, which is interesting as the
421 link between p53 and inflammation has been previously reported (Figure 3b) (Gudkov et
422 al., 2011). c5.5, which showed the highest enrichment for the p53 signalling pathway,
423 showed elevated scores for other pathways as well, even compared to other ‘inner’ clones.
424

425 Finally, we performed Motif Activity Response Analysis (MARA) to probe the underlying
426 regulatory factors behind the gene expression changes brought on by the mutations
427 (Balwierz et al., 2014). MARA combines the predicted transcription factor (TF) binding
428 sites (represented by TF motifs) found in promoters with the gene expression changes to
429 calculate the motif activities, which can be used to estimate the responsible TFs for their
430 regulation. When we applied the analysis using the SwissRegulon collection of TF motifs
431 on our promoter regions (300 bp downstream and 100 upstream from each CAGE cluster),
432 we detected a drastic decrease of TP53 motif activities in all the mutant clones compared to
433 the wild type (Supplementary Figure 8, Supplementary Table 7). Besides the TP53 motif,
434 those with significant activity variation included the motifs for NFY series of TFs and
435 NFKB1/REL/RELA, key components of inflammation regulation (Ji et al., 2019). Another
436 interesting motif was for PITX1, whose activity was down in the ‘outer’ clones c4.8 and
437 c5.11, and is known to activate p53 by binding to its promoter (Liu and Lobie, 2007).
438 Ideally, the clones selected for the purpose of this study should exhibit minimal regulatory
439 changes from the wild type, except for TP53. To rank the clones based on this criterium, we
440 first calculated the differences in motif activities between each mutant clone and the wild
441 type, then summed these values for all significant motifs in each mutant clone, except for
442 TP53. This creates a scoring system where the mutant clones with less regulatory
443 differences would be assigned smaller values, and vice versa (Figure 3c). Consistent with
444 previous observations, the ‘outer’ clones c4.8 and c5.11 obtained the highest scores, while
445 the ‘inner’ clones c5.12 and c5.9 scored the lowest. One exception was c4.2, which
446 obtained the third lowest score despite being an ‘outer’ clone.
447

448 **Figure 3.** Down-regulated genes in the mutant clones vs. the wild type are enriched in p53
449 regulatory signals. (a) Genes with evidence of TP53 ChIP-seq binding show consistent
450 down-regulation of expression across all samples compared to the wild type. The boxplot
451 represents the mean log fold change of expression of the TP53-bound genes in mutant
452 clones vs. the wild type. (b) Down-regulated genes show enrichment of the KEGG p53

453 signalling pathway. Barplot represents the negative log₁₀ of p-values of pathway
454 enrichment for each mutant clone. The clones are sorted in the order of increasing values,
455 and the dotted line represents the p-value threshold of 0.05. (c) Greater motif activity
456 differences between the ‘outer’ clones and the wild type suggests more extensive regulatory
457 changes compared to the ‘inner’ clones. The differences in the motif activities between
458 each mutant clone and the wild type for the significant set of motifs are summed and sorted
459 to produce the overall ranking of the regulatory changes from the wild type.
460



461

462

463 **3.3 Phenotypic comparison of selected clones to wild type**

464

465 The transcriptome analysis confirmed that the major transcriptional changes in the mutant
466 clones were associated with disrupted p53 function. However, it is clear that clone-specific
467 transcriptomic differences also exist, with a subset of clones showing higher divergence
468 from the wild type. To fully characterize how these differences will be manifested, we set

469 out to perform further phenotype comparison experiments on the mutant clones. The
470 phenotype comparison experiments performed in this study were designed to confirm 3
471 aspects of the mutant clones that would be crucial for the mutants to be useful for ADC
472 development: disruption of TP53-MDM2 interaction, maintenance of cellular uptake and
473 localization through surface integrins, and maintenance of tumorigenicity *in vivo*. Those
474 mutant clones that meet all 3 criteria would qualify as the final candidates for ADC testing
475 platform.

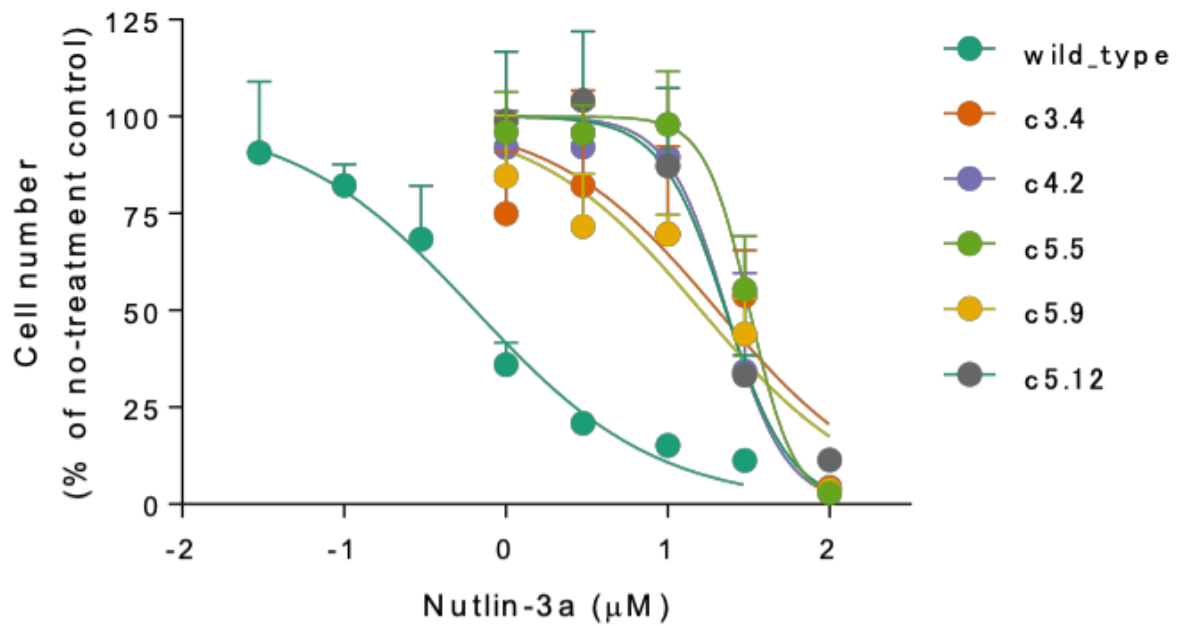
476
477 Before embarking on the experiments, however, we attempted to predict which clones were
478 likely to show smaller or bigger phenotype changes compared to the wild type based on the
479 transcriptome analysis results. We first separated out the clones c4.8 and c5.11 from the
480 rest, as they exhibited consistently divergent behaviours from the wild type or other mutant
481 clones. Clone c4.2 was not proximal to the ‘inner’ group clones in PCA, and obtained lower
482 enrichment score for the p53 signalling pathway. However, its regulatory signal divergence
483 from the wild type was at similar levels to those of the ‘inner’ group. The best performing
484 clones were c 5.5, c5.9, and c5.12, but while c5.5 showed the highest enrichment for the
485 p53 signalling pathway, it also exhibited higher regulatory changes than c5.9 and c5.12.

486
487 Taking these observations together, we decided to subject the following subset of mutant
488 clones to further phenotypic studies and compare their behaviours against the wild type:
489 c3.4, c5.5, c5.9, c5.12, and c4.2. We predicted that the clones c5.9 and c5.12 would prove
490 to be the best candidates with the least phenotypic differences from the wild type. As for
491 c4.2, while it did not appear to be proximal to the wild type, we were nonetheless interested
492 in observing its phenotypic behaviour compared to the other clones, due to its conflicting
493 transcriptomic signals and the previously noted rapid *in vitro* growth rate compared to other
494 samples.

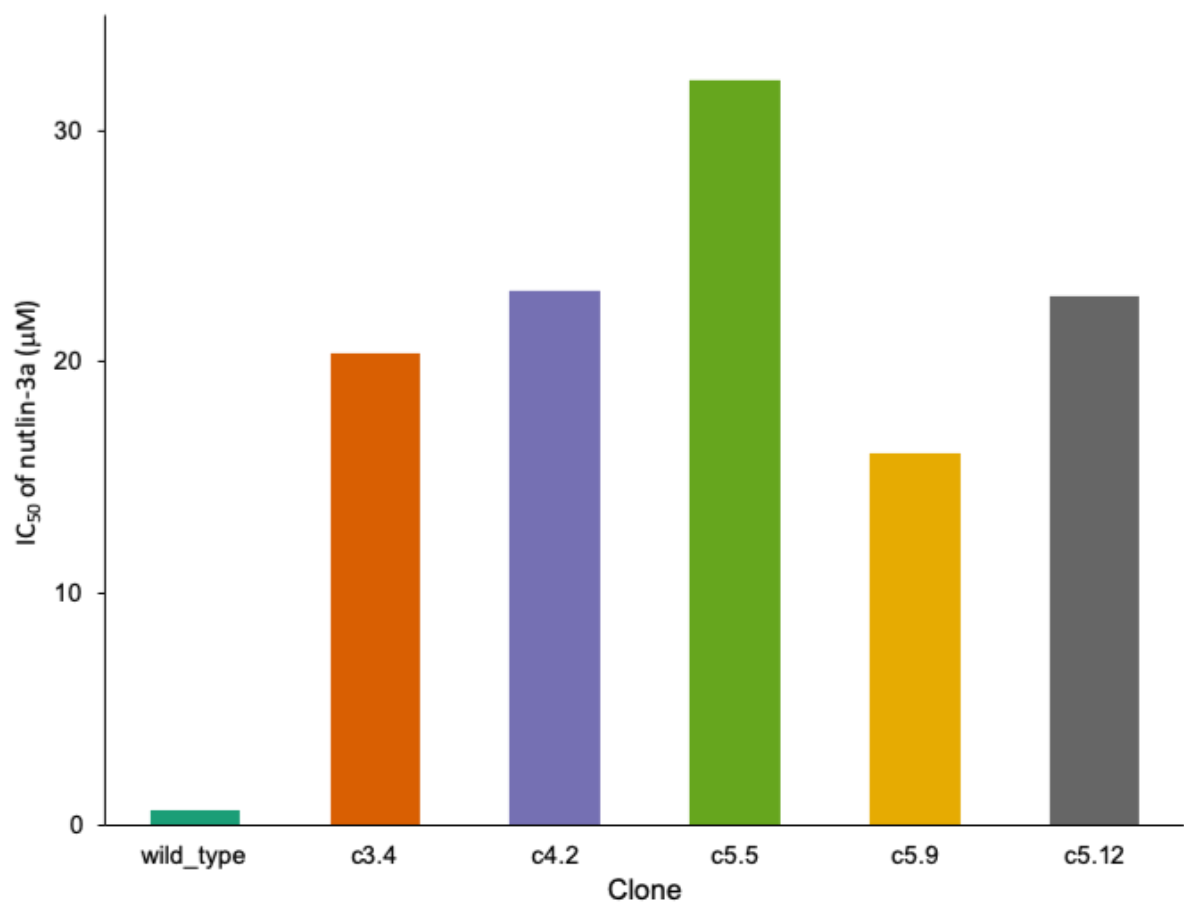
495
496 **Figure 4.** Cell proliferation assay results support successful disruption of p53-MDM2
497 interaction leading to decreased sensitivity to inhibitory drug nutlin-3a in mutant clones
498 compared to the wild type. (a) Cell counts of nutlin-3a-treated groups with increasing drug
499 dosage measured with WST-8 assay. Cells were incubated with nutlin-3a at 0 to 100 μ M
500 for 5 days at 37 °C. The count ratios of nutlin-3a-treated and non-treated cells in 4 replicates
501 were plotted against different nutlin-3a concentrations (log scale). Results are expressed as
502 the mean \pm standard deviation. (b) IC₅₀ values for nutlin-3a. All mutant clones show IC₅₀
503 values > 20-fold over the wild type.

504

A



B



505

506

507 First, we wanted to confirm that the mutation in each clone led to successful disruption of
508 the p53 protein's capacity to interact with MDM2 and regulate cell growth. Nutlin-3a acts

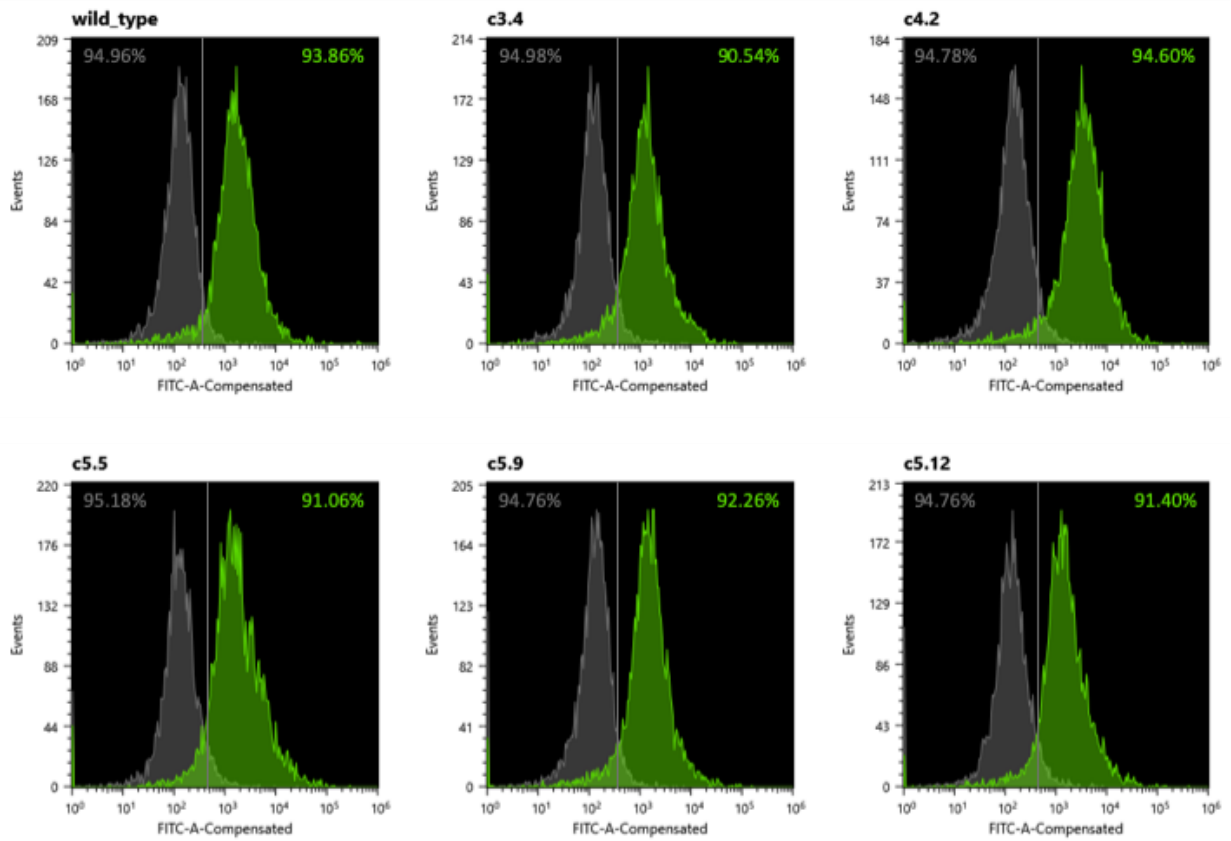
509 as an inhibitor of p53 and MDM2 interaction by blocking the p53-binding pocket of
510 MDM2, and is known to activate the p53 pathway in cancer cells, leading to cell cycle
511 arrest and apoptosis (Villalonga-Planells et al., 2011). Thus, we expected the wild type cells
512 to have higher sensitivity to the inhibitory effects of nutlin-3a than the mutant clones,
513 leading to slower growth rate at lower doses of the drug. After incubating the cells for 5
514 days with increasing dosage of nutlin-3a ranging from 0 to 100 μM , we performed
515 colorimetric assay with WST-8 to measure the resulting relative cell concentration by
516 taking the ratio of the absorbance against that of the no-treatment control group (Figure 4a).
517 As expected, wild type U87MG cells showed high sensitivity to nutlin-3a, with decrease in
518 cell number observed even at low doses of 0.1 μM , whereas the mutant clones required
519 greater than 1 μM before any decrease could be observed. Clones c4.2, c5.5, and c5.12
520 showed the greatest resistance to the drug, requiring greater than 10 μM of dosage before
521 significant decrease in cell counts could be observed. The IC_{50} values for nutlin-3a, which
522 are half maximal inhibitory concentration representing the potency of an inhibitory drug,
523 were more than 20-fold higher in the mutant clones than in the wild type cells (Figure 4b).

524

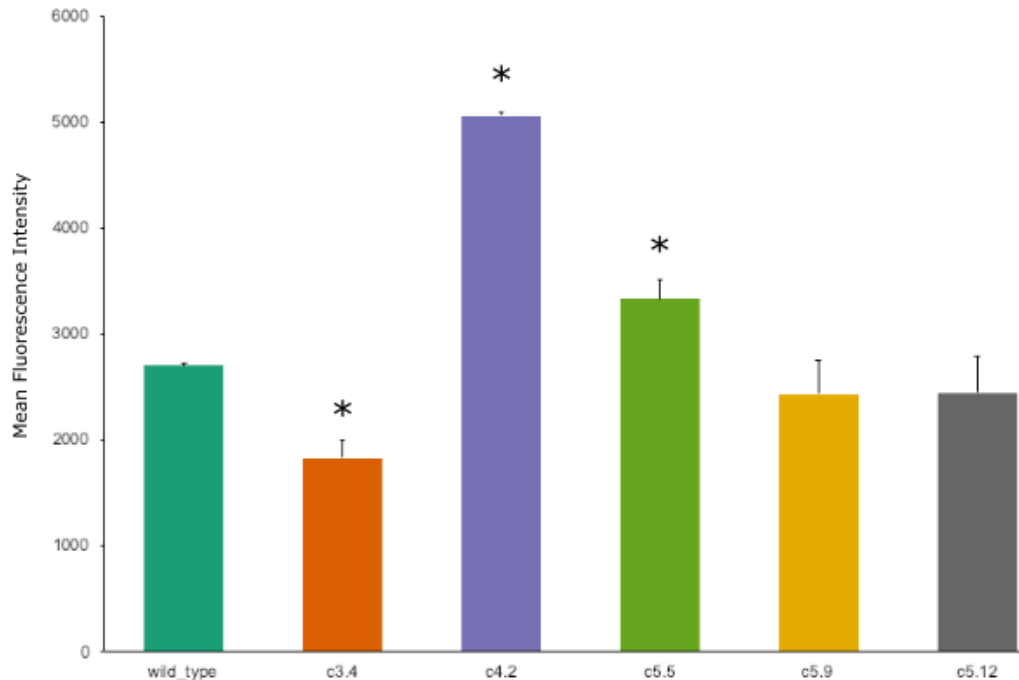
525 **Figure 5.** Flow cytometric analysis after incubation with FITC-RGD peptide shows similar
526 levels of cellular uptake in both the wild type and mutant clones. The cells were incubated
527 with the peptide at a final concentration of 20 μM for 4 h at 37°C. (a) Histograms of non-
528 treated cells (gray) and FITC-RGD-treated cells (green). Vertical line represents the 95%
529 mark of non-treated cells, and the fluorescein-positive ratios of cells can be seen on the
530 right of the line. (b) Mean fluorescence intensity (MFI) of FITC-RGD-treated cells as an
531 index of cellular uptake. The MFI values are calculated by subtracting the fluorescence
532 intensities of the treated samples from those of the non-treated samples, and the results are
533 expressed as the means \pm standard deviations of 3 replicate samples. * $P < 0.05$ compared
534 with the wild type group.

535

A



B



536

537

538 Next, we explored whether the mutant clones retained the same capacity for cellular uptake

539 and intracellular localization of compounds that bind to the surface integrin $\alpha\beta3$. As the

540 goal of creating the mutants was to utilize them as testing platforms for ADC development,

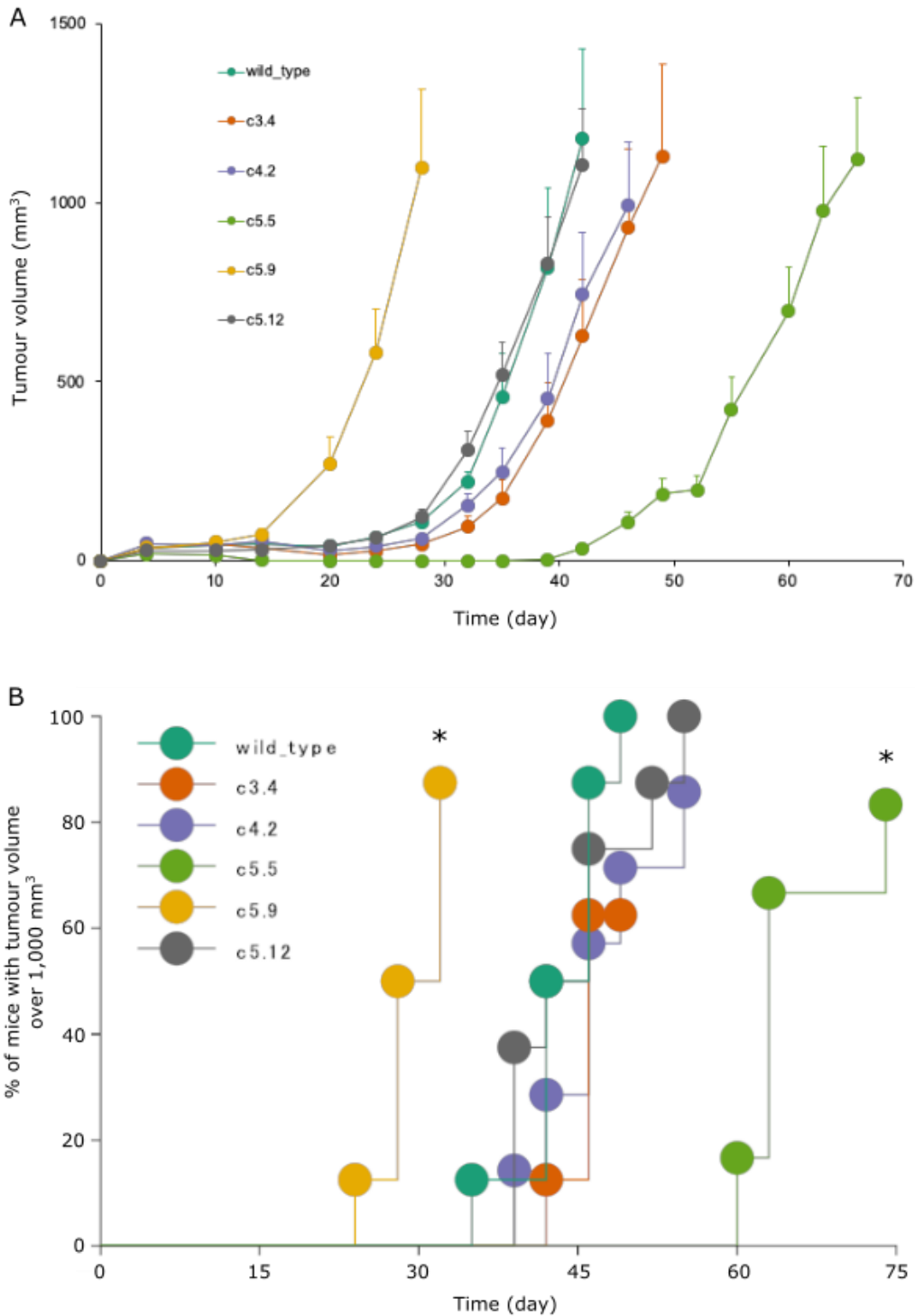
541 it was important to ascertain that not only was the integrin expressed at similar levels, as

542 previously seen through fluorescence imaging (Supplementary Figure S2), but that their
543 binding initiated the same level of endocytosis into the cells. As integrin $\alpha\beta3$ is
544 characterized by the exposed arginine-glycine-aspartic (RGD) tripeptide sequence,
545 fluorescein isothiocyanate (FITC) can be conjugated to RGD peptides and induce its
546 cellular uptake (Zheng et al., 2014). By performing cytometric analysis based on the
547 fluorescence observed after the uptake, we could infer whether the activity of the expressed
548 integrins is maintained across the mutant clones compared to the wild type U87MG. After
549 incubation with FITC-RGD peptide, both the wild type cells and mutant clones showed
550 fluorescence intensity shifts of greater than 90%, indicating they take up FITC-RGD
551 peptides with similar efficiencies (Figure 5a). Clones c5.9 and c5.12 showed similar levels
552 of mean fluorescence intensity (MFI) as the wild type (Figure 5b). Clones c3.4 and c5.5
553 showed small but still significant changes in the MFI levels from the wild type. c4.2, the
554 outlier in our group, showed a significantly higher level compared to the rest of the
555 samples. When we confirmed the localization of the fluorescence signals in the cells, they
556 all showed punctuate patterns that co-localize with lysosome locations, suggesting that the
557 FITC-RGD peptides were transported to lysosomes after their cellular uptake
558 (Supplementary Figure 9).

559

560 **Figure 6.** Tumour growth in BALB/c nu/nu mice after subcutaneous inoculation reveals the
561 differences in the *in vivo* growth rates. (a) Increase in tumour volume over time. Results are
562 expressed as a mean \pm standard error of 8 mice (wild type, c5.9, c5.12 and c3.4), 7 mice
563 (c4.2), or 6 mice (c5.5). Of the mutant clones tested, clones c3.4, c4.2 and c5.12 exhibit
564 similar growth rates as the wild type. (b) Kaplan-Meier plots with the ratio of mice with
565 tumour volume over 1,000 mm³ again show that the clones c5.5 and c5.9 exhibit different
566 tumour growth patterns from the other samples. (* $P < 0.05$ compared with the U87MG
567 tumour bearing mice group)

568



569

570

571 Finally, as we wanted to confirm the usability of the mutants for drug delivery system
572 testing *in vivo*, we injected the cells subcutaneously into 5-week old mice and measured the
573 tumour growth over the course of 70 days and examined whether there were any
574 differences in their behaviour. Of the 5 mutant clones tested, clones c3.4, c4.2 and c5.12
575 achieved similar tumour growth as the wild type U87MG cells, while the clone 5.9
576 exhibited faster growth rate and c5.5 slower growth rate (Figure 6a). Most of the mutant
577 clones and the wild type resulted in tumour growth in all the mice they were inoculated

578 into, with the exception of clone c4.2 (7 out of 8 mice with tumour growth) and c5.5 (6 out
579 of 8 mice with tumour growth) (Supplementary Table 8). One interesting observation was
580 that the clone c4.2 showed similar *in vivo* growth as the wild type, even though *in vitro*,
581 they exhibited higher growth rate. The same pattern of tumour growth rate differences
582 could be observed when we measured the percentage of inoculated mice with tumour
583 volumes greater than 1,000 mm³ over time (Figure 6b). Mice inoculated with clones c3.4,
584 c4.2 and c5.12 exhibited similar rates of tumour growth, with greater than 50% of the mice
585 exceeding 1,000 mm³ between 40 to 50 days after inoculation. Mice injected with the
586 clones c5.9 and c5.5 differed significantly ($P < 0.05$), with those with clone c5.9 exceeding
587 this figure before 30 days, while those with c5.5 needed more than 60 days.

588
589 In summary, the phenotypic comparison experiments strongly support the successful
590 creation of the p53 mutant clone c5.12 to be utilized for p53-specific *in vitro* testing
591 platforms. This clone not only demonstrated disrupted interaction between p53 and MDM2
592 (Figure 4), but also maintained the capacity for cellular uptake through the surface integrin
593 $\alpha\beta3$ (Figure 5) and *in vivo* tumour growth as the wild type (Figure 6). While clones c3.4
594 and c4.2 also exhibited similar *in vivo* growth as the wild type, signs pointing to their
595 significantly different cellular uptake prevented us from including them in the group of top
596 candidate clones (Figure 5).

597

598 **4 Discussion**

599

600 In development of novel targeted therapeutics, there is an acute need for efficient
601 procedures for establishing cell line-based testing platforms where the differences between
602 the test and control samples are limited to the gene being targeted in order to avoid
603 unwanted secondary effects stemming from other genomic differences. In order to improve
604 upon the current situation where many researchers have to rely on two dissimilar cell lines,
605 we employed CRISPR-Cas9 system to create targeted mutation of p53 in the U87MG cell
606 line, while minimizing other genomic changes that may complicate interpretations of
607 experimental results. While such endeavours have been previously made, we here took the
608 extra step of performing detailed transcriptome profiling using CAGE and classified the
609 clones into ‘inner’ and ‘outer’ groups, based on the amount of accompanying genetic
610 disruptions compared to the wild type. Of the ‘inner’ group members, we further predicted
611 that the clones c5.9 and c5.12 would be the best candidates to be used in the testing
612 platform. We followed up with phenotype analysis experiments designed to fully
613 characterize the mutant clones created and evaluate our predictions. The cell proliferation
614 assay with p53-MDM2 interaction inhibitor nutlin-3a confirmed reduced interaction in the
615 mutant clones. The FITC-RGD peptide uptake assay confirmed that the cellular uptake and
616 localization through integrin $\alpha\beta3$ remains intact in the mutant clones. Finally, tumour
617 xenograft experiments in mice showed that 3 of the 5 mutant clones have similar *in vivo*
618 growth rates as the wild type.

619

620 Based on the overall analysis results, we select the clone c5.12 as the best candidates for
621 serving as the testing platform with the parent U87MG cell line as the control. Not only do
622 this clone show relatively low genomic changes stemming from the p53 mutation, it is
623 highly resistant to nutlin-3a, indicating strong inactivation of p53-MDM2 interaction, while
624 still exhibiting similar levels of integrin functionality and *in vivo* growth behaviours. c5.12
625 was one of our two predicted candidates based on transcriptomic analysis. While c5.9, the
626 other top predicted candidate, proved to have more rapid *in vivo* growth rate than the wild
627 type, it performed comparatively similar in most of the other phenotypic measures.

628

629 This does not mean, however, that other clones are not of further research interests, as
630 individual differences manifested through their transcriptome and phenotype changes
631 present intriguing prospects of further investigation into their causes, and their inclusion in
632 the development pipeline provides additional opportunities to control for clone specific
633 effects. The CAGE technology used in this study offers many attractive advantages, as the
634 data can be used to identify differentially expressed enhancers and various noncoding
635 RNAs, both annotated and unannotated (Andersson et al., 2014; Hon et al., 2017). p53 is
636 known to have complex regulatory relationships with many other genes, some of which are
637 of noncoding type (Dangelmaier et al., 2019; Hu et al., 2018; Khan et al., 2019; Li et al.,
638 2017). Some of these noncoding regulatory partners of p53 may be included in the list of
639 differentially expressed promoters and enhancers we have identified in this study, which
640 would serve as interesting research directions to follow upon.

641

642 Moving forward, we believe that we can employ the strategy used in this study for tailor-
643 building of control-mutant cell line pairs targeting many different genes, both for the
644 surface marker and the intracellular target. Being able to build a repertoire of these cell line
645 platforms would greatly facilitate the ongoing development efforts for novel targeted
646 therapeutics and their delivery methods. The newly created mutant clones are available as a
647 resource, and can be provided upon request. The transcriptomic profiles of the clones in the
648 form of CAGE data can be downloaded from GEO series **GSE155461** for those interested
649 in further analysis.

650

651 **5 Conclusion**

652

653 Using the CRISPR-Cas9 system, we have successfully performed targeted mutation of
654 TP53 in U87MG for the purpose of building an *in vitro* drug testing platform, and through
655 transcriptomics and phenotypic studies, demonstrate that only limited biological changes
656 from the wild type are found.

657

658 **Author Contributions**

659

660 Conceptualization, EA, HM, ShT, HS; methodology, AK, KM, SaT, TA, MT, BK;
661 software, AK, BK; investigation, AK, KM, SaT, TA, MT; validation, SaT, TA, KM, MT;
662 formal analysis, AK, KM, BK; data curation, AK; writing—original draft preparation, AK,
663 KM, SaT, TA; writing—review and editing, AK, KM, SaT, TA, BK, HS, ShT, HM, EA;
664 visualization, AK, KM, SaT, TA; supervision, EA, HM; project administration, AK, EA,
665 MF; funding acquisition, EA, ShT, HM, HS, MF.

666

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672

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674

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677

678

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680

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