Small Molecule Activators of the Mitochondrial Protease ClpP Induce Senescence in Triple-Negative Breast Cancer Cells and Sensitize Cells to the Bcl-2 Inhibitor Venetoclax

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Running title: ClpP activators induce senescence and synergize with venetoclax

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Abstract:

ONC201 is a first-in-class, FDA approved small molecule activator of the mitochondrial ATP-dependent caseinolytic peptidase P (ClpP). This and other related small molecules referred to as ClpP agonists, exert antiproliferative effects in several cancer cell types. We report that ONC201 and highly potent second generation ClpP agonists (TR-57, TR-107), promote induction of senescence in triple-negative breast cancer (TNBC) cell lines. Senescence was determined by increased β-galactosidase activity, downregulation of phosphorylated Rb, c-Myc (Myc), and lamin B1, upregulation of senescent-associated secretory phenotype (SASP), and extended cell proliferation assays. These responses were not observed in ClpP knockout cell lines, demonstrating ClpP-dependence. Proteomics analyses identified multiple events related to the development of senescence including cell cycle arrest and mitochondrial dysfunction. Flow cytometry confirmed an S-phase arrest; DNA damage was detected by Comet assay, 53BP1, phospho-S*Q, and γH2A.X immunostaining. In parallel with this, activation of the ATM pathway and phosphorylation of Chk2 was observed. We determined that ClpP agonist-induced senescence was irreversible in both *in vitro* and *in vivo* studies. Following TR-57 treatment and drug washout, cells remained growth arrested which coincided with the loss of Myc protein. By contrast, cells treated with the cell cycle inhibitor and senescence inducer, abemaciclib rapidly regained p-Rb and Myc expression and cell proliferation following washout. This response was reproduced *in vivo* wherein senescent 4T1-Luc cells did not develop tumors following injection into mice. Finally, the combination of a ClpP agonist with a known senolytic (venetoclax), synergistically increased the amount of cell death observed. Combining a ClpP agonist with a PARP inhibitor (olaparib) produced an additive effect. In summary, we show that ClpP activators stably induce an irreversible senescence in a ClpP-dependent manner that synergizes with venetoclax in TNBC cells.

Introduction:

Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer with a significantly lower survival rate than other subtypes (1–3). Since TNBC cells lack estrogen and progesterone hormone receptors or enrichment of the receptor tyrosine kinase HER2, targeted therapeutics like tamoxifen and trastuzumab are largely ineffective (2,4). Current treatments for TNBC patients are limited to surgical intervention and traditional chemotherapies, and third-line therapies include immunotherapy, PARP inhibitors, and antibody drug conjugates (5–7). While there are potential targeted therapies in clinical trials for TNBC, none of them have reached FDA approval, therefore there is an unmet need for improved therapies for TNBC patients (7,8)

ONC201 was the first imipridone molecule to show strong anti-cancer properties. ONC201(Dordaviprone) is in multiple clinical trials for a variety of aggressive cancers including blood, breast, and endometrial cancers among others and recently received FDA approval for the treatment of diffuse midline glioma (9–14). Although initially believed to be a TRAIL inducer and/or dopamine receptor D2 antagonist, Greer, et al. (2018), determined that ONC201 induces mitochondrial dysfunction (15). Further studies identified ONC201 and related molecules as highly selective activators of the mitochondrial protease ClpP (referred to as ClpP agonists hereafter). This includes ONC206, ONC212, the highly potent TR compounds, and related small molecules (15–21). Of these, the TR compounds exemplified by TR-57, TR-107 and others (19,22–25) (Madera Therapeutics), are the most potent and selective ClpP agonists known (19,22–25). TR-57 and TR-107 were primarily evaluated in this study because they have been well characterized as selective ClpP activators with anti-cancer properties in TNBC (19,23,24,26).

ONC201 and the TR compounds directly bind and activate ClpP, a mitochondrial matrix protease (19,21), part of the multimeric ClpXP protein complex. ClpXP includes the ATP-dependent

84 unfoldase ClpX and the serine protease subunit ClpP. ClpP regulates mitochondrial protein

85 homeostasis (27) and has been implicated in degrading proteins as part of the mitochondrial

86 unfolded protein response (28–30). Small molecule ClpP agonists bind to the hydrophobic H site

87 cleft of the tetradecameric form of ClpP, preventing ClpX binding and inducing conformational

88 change and activation of ClpP (23,27). ClpP activation induces degradation of multiple

89 mitochondrial proteins leading to increased mitochondrial stress responses (19), inhibition of

90 mitochondrial transcription (25), disruption of TCA cycle metabolism, OXPHOS, (22,24,31), and loss

91 of mitochondrial DNA (15). These mitochondrial-related events induce the integrated stress

92 response, inhibit protein synthesis, and arrest cancer cell proliferation (19,22,32). While many of the

93 direct results of ClpP activation have been identified, the mechanisms that inhibit cancer cell

94 proliferation are only partially understood. Moreover, various cancer types respond differently to

95 ClpP agonists (19,33–36), illustrating the need to better understand the specific effects of these anti-

96 cancer compounds.

97 Cellular senescence is characterized by permanent cell cycle arrest, DNA damage,

98 decreased expression of pro-apoptotic proteins, resistance to apoptosis, and increased secretion of

99 cytokines and proteins known as the senescence-associated secretory phenotype (SASP) (37–40).

100 DNA damage, expression of anti- or pro-apoptotic proteins, and the SASP components vary based

101 on the senescence inducer and tissue type (39,41). For example, cancer drugs such as abemaciclib

102 and palbociclib induce senescence through inhibition of the cell cycle without inducing DNA damage

103 (42–45). Increased SASP is a common feature of senescent cells, but the phenotype varies in

104 replicative, DNA damage-induced, oncogene-induced, and mitochondrial dysfunction-induced

105 senescence (39,43,46–48).

106 In this study, we examined the effects of highly selective ClpP agonists on growth inhibition

107 and senescence of TNBC cell models. We observed that ClpP activation induces DNA damage, cell

108 cycle arrest, SASP, and senescence in response to ClpP agonist treatment. Compared to other

109 senescence inducers, ClpP agonist-induced senescence was not reversible. Consistent with

110 increased senescence, the combination of ClpP agonists with an established senolytic (venetoclax)

111 resulted in a synergistic increase in cell death. Thus, these studies could have important implications

112 for the use of ClpP agonists in the treatment of TNBC.

113

114 **Methods:**

115 Chemicals

116 The TR-57 and TR-107 compounds were supplied by Madera Therapeutics, LLC. Other compounds

117 were obtained as follows: ONC201 (SelleckChem, S5716), Abemaciclib (SelleckChem, S5716),

118 Irinotecan (MedChemExpress, HY-16562), Venetoclax (MedChemExpress, HY-15531), Olaparib

119 (SelleckChem, S1060), and KU-60019 (MedChemExpress, HY12061).

120 Cell Culture

121 The human TNBC cell line, SUM159, was obtained from Dr. Gary Johnson at UNC-CH. MDA-MB-231,

122 including ClpP knockout (KO) cells, were generous gifts from Yoshimi Greer and Stan Lipkowitz at the

123 NCI. SUM159 cells were cultured in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12

124 supplemented with 5% fetal bovine serum, 5µg/mL insulin, 1µg/mL hydrocortisone, and 1%

125 antibiotic−antimycotic (Gibco, 15240062). MDA-MB-231 cells were cultured in RPMI 1640 media

126 supplemented with 10% FBS and 1% antibiotic−antimycotic. 4T1-Luciferase cells were cultured in

127 RPMI 1640 supplemented with 10% FBS, 1% antibiotic-antimycotic, and 4µg/mL Blasticidin

128 (InvivoGen, BLL-44-07). Cell lines were incubated at 37°C and 5% CO_2 and periodically tested for

129 mycoplasma.

130 Generation of ClpP null cells using CRISPRi

131   To generate sgRNA for CRISPRi, primer pairs for each individual sgRNA were first annealed and later

132   ligated to a digested VDB783 vector (50ng/µL). Primer sequences are listed in Table 1. Each ligation

133   product was then transformed into DH5α by mixing 3µL of DNA into 25µL of competent cells.

134   Cells/DNA mixture was incubated on ice for 30 min and heat shocked at 42°C for 45 seconds.

135   Bacteria were spun at 10 000xg for 1 min at room temperature on a tabletop centrifuge. The resulting

136   bacterial pellet was resuspended in 30µL of LB, spread on an LB-Amp plate and incubated at 37°C

137   overnight. Colony PCR was performed to check for positive clones of each sgRNA. Single positive

138   clones were grown in 5mL of LB supplemented with Ampicillin at 37°C overnight. Cultures were

139   miniprepped the next day using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's

140   protocol. Lentiviruses were produced in HEK293T cells. Transfection and clonal isolation of the

141   CRISPR null mammalian cells was done as previously described in SUM159 cells (22).

142   <u>Generation of 4T1-luciferase (4T1-Luc) cells</u>

143   4T1 cells were purchased from the Tissue Culture Facility at UNC-Chapel Hill and maintained in

144   RPMI-1640 containing 1% penicillin-streptomycin, 10% heat-inactivated FBS, HEPES (pH 7.0), 1mM

145   sodium pyruvate, and 0.075% sodium bicarbonate. Cells were transduced with pTK1261 (LV-CMV-

146   FLuc-IRES-GFP/BSD) wherein the firefly luciferase and the fusion GFP/blasticidin marker gene were

147   expressed under the control of a CMV promoter; cells were selected with blasticidin (4µg/ml) and

148   were clonally selected for high luciferase expression (BMG Clariostar Plate reader, Cary, NC, USA).

149   Identity of the parental and luciferase-expressing clones were verified by Short Tandem Repeat

150   Analysis (Powerplex 16HS, Promega), and cells were routinely monitored every two weeks for

151   mycoplasma Mycostrip detection kit (Invivogen). Prior to *in vivo* experiments, growth rates of parental

152   and luciferase-expressing clones were confirmed to be similar (CellTiter Aqueous One MTS assay,

153   Promega).

154   <u>Senescence-associated β-galactosidase (β-gal) staining</u>

155   SUM159 and MDA-MB-231 cells were stained for β-gal activity using an adjusted protocol from Dimri

156   et al., 1995 (49). After treatment with senescence inducing compounds, the cells were washed twice

157   with Dulbecco's phosphate buffered saline (DPBS, Gibco, 14190-144) and fixed with fixing solution

158   (2% formaldehyde, 0.2% glutaraldehyde in DPBS) for 3-5 min. After fixation, cells were washed gently

159   3 times with DPBS. Next, the cells were stained with staining solution (1mg/mL X-gal (Roche, XGAL-

160   RO), 40mM citric acid/sodium phosphate buffer (pH 6.0), 5mM potassium ferrocyanide, 5mM

161   potassium ferricyanide, 150mM sodium chloride, and 2mM magnesium chloride). Plates were

162   covered with parafilm and incubated for 13 and 16 h for SUM159 and MDA-MB-231 respectively at

163   37°C without CO_2. After incubation, the cells were washed with DPBS and imaged in DPBS on the

164   Olympus IX70 inverted microscope (Tokyo, Japan) at 10x magnification. β-gal positive cells were

165   counted on ImageJ with >100 cells/condition.

166   <u>Dose-Response and Drug Washout Proliferation Assays</u>

167   *Dose-Response*: Proliferation assay was performed as described previously in Daglish et al. (2023)

168   with modifications based on treatment duration (25). Briefly, SUM159 (200 cells/well) or MDA-MB-

169   231 (500 cells/well) were plated in a black with clear bottom 96-well plate and allowed to adhere

170   overnight. Cells were treated with varying concentrations of TR-57 and incubated at 37°C for 3 days

171   or 30 days. Staining, imaging, and quantification steps are described below.

172   *Drug Washout:* SUM159 (1 000 cells/well), MDA-MB-231 (2 000 cells/well), or 4T1-Luc cells (500

173   cells/well) were seeded and allowed to adhere overnight. Cells were treated with TR-57 or controls

174   for 24 or 48h and then drugged media was removed and replaced with regular media which remained

175   for the duration of the experiment. To determine cell count, Hoechst 33342 (Thermo Fisher Scientific,

176   H3570) was diluted in DPBS to 2.5µg/mL and 100µl of the stain was added to each well and plate was

177   incubated for 20 mins at 37°C. Total cell number was determined by imaging and quantified using the

178   Celigo Imaging Cytometer (Nexcelom, Lawrence, MA, USA).

179   <u>Immunoblotting</u>

180   Cells were plated in a 6-well plate or 10 cm dish and treated with compounds as described above.

181   Following treatment, cells were lysed with RIPA buffer, pH 7.4 [no SDS, 2mM Na(VO3)4, 10mM NaF,

182   0.0125μM calyculin A, and complete protease inhibitor cocktail (Roche, 11873580001)] and lysates

183   immunoblotted as described previously (19). For secreted protein immunoblots, cell culture media

184   was collected and 4X Laemmeli buffer was added before immunoblotting as described

185   previously(19). Nitrocellulose membranes were first incubated in 1% fish gelatin (Sigma-Aldrich,

186   G7041) for 1 h and then incubated with the indicated primary antibody (Table 2) in 1% fish gelatin

187   overnight at 4°C. After incubation, the membranes were washed 3 times for 5 min with Tris-buffered

188   saline (supplemented with 0.1% Tween-20 (TBST). Membranes were then incubated with the

189   respective secondary antibody for 1 h at room temperature. After incubation, the membranes were

190   washed 3 times for 5 min with TBST prior to incubation in enhanced chemiluminescence reagent

191   (BioRad, 1705061) for 1 min and imaged using a Chemidoc MP (BioRad, Hercules, CA, USA). Images

192   acquired were analyzed using Image Lab software (BioRad).

193   <u>RNA extraction and cDNA synthesis</u>

194   Total RNA was extracted and purified using RNeasy Plus Mini Kit (Qiagen, 74136) according to the

195   manufacturer's protocol. RNA concentration was determined using the NanoDrop One

196   spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized from reverse

197   transcription on 2.0μg total RNA in a 20μL reaction using High-Capacity cDNA Reverse Transcription

198   Kit (Applied Biosystems, 4368814) and T100 thermal cycler (BioRad), according to the

199   manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR)

201 The cDNA was analyzed by qRT-PCR using iTaq Universal SYBR Green Supermix (BioRad, 1725121)

202 on an Applied Biosystems 7500 Fast Real-Time PCR System. For each reaction, 1 µL of cDNA was

203 mixed with 12.5 µl of 2 x SYBR mix, 8 µL of nuclease-free water, 1.75 µl of Forward primer and 1.75 µl

204 of Reverse primer. Expression of 18S was used to normalize expression of genes of interest. Every

205 biological replicate was analyzed in technical duplicate. Primer targets and sequences are listed in

206 Table 3.

207 Proteomics

208 *Sample Preparation for Proteomics:* MDA-MB-231 cells were plated in 10cm plates at a density of 2

209 000 000 cells per plate and allowed to adhere overnight. Plates were then treated with either 0.1%

210 DMSO or 150nM TR57 for 48 h. Cells were washed thrice with 5mL of ice-cold DPBS. Harvesting was

211 completed by mechanical scraping (Corning, 3008) on ice in DPBS, centrifugation at 3000 rpm for 3

212 min, and resuspension of the pellet in 100µl 8M urea lysis buffer (8M urea, 50 mM Tris (pH 7.4),

213 2.5 mM Na3VO4, 1 mM NaF, 1X protease inhibitor cocktail (Sigma, 11836170001), 12.5nM Calyculin

214 A). Samples were incubated on ice for 20 min and then clarified by centrifugation at 15000 rpm for 10

215 min. Protein concentration was quantified by Bradford assay (BioRad, 5000006) and normalized to

216 1µg/µl using molecular biology grade water. Samples were then flash frozen in liquid nitrogen and

217 submitted on dry ice to the UNC Metabolomics and Proteomics Core. Cell lysates (n=12 per group)

218 were subjected to S-trap digestion, as previously described (50). Approximately 35 µg of sample were

219 reduced with 20 mM dithiothreitol (Pierce) for 10 min at 56 °C, and alkylated with 40 mM

220 iodoacetamide (Pierce) for 30 min at room temperature. Samples were then loaded onto S-trap Micro

221 columns (Protifi) according to manufacturer's recommended protocol. Samples were subjected to

222 on-column digestion using trypsin (Promega) for 1 h at 47 °C at a 1:10 enzyme:protein ratio. Eluates

223   were dried via vacuum centrifugation (Labconco, Kansas City, MO, USA) and peptide concentration

224   was quantified using BCA Fluorometric Peptide Assay (Pierce). Peptide clean-up was performed on

225   Evotips as recommended by the manufacturer.

226   *LC/MS/MS Analysis:* Each sample was analyzed by LC-MS/MS using an Evosep One coupled to a

227   Fusion Lumos mass spectrometer (Thermo Scientific). Peptides were separated with the 15 SPD

228   Extended Method, a standardized 88-min method using a ReproSil-Pur C18 column (15 cm × 150

229   μm, 1.9 μm beads, Evosep). The mobile phases for separation were 0.1% formic acid in water for

230   buffer A and 0.1% FA in acetonitrile for buffer B. The Lumos was operated in Data-Independent

231   Acquisition (DIA) mode. A full MS scan (m/z 35–1200 m/z) was collected; resolution set to 120 000

232   with a default charge state of 2, max injection time of 45 ms and automatic gain control (AGC)

233   target of 250%. Following full MS scan, product ion scan was collected at a resolution of 30 000,

234   with higher collision dissociation (HCD) set to 30, AGC target set to 2000%, maximum injection

235   time to set to 54 ms, 31 m/z precursor isolation windows.

236   *Data Analysis:* Raw data were analyzed using directDIA within Spectronaut (v18, Biognosys). Data

237   were searched against the reviewed human proteome database from Uniprot (downloaded January

238   2024; 20,441 protein sequences); appended with a common contaminants database (MaxQuant;

239   245 protein sequences). The following settings were used: enzyme specificity set to trypsin, up to

240   two missed cleavages allowed, cysteine carbamidomethylation set as a fixed modification,

241   methionine oxidation and N-terminal acetylation set as variable modifications. A false discovery

242   rate (FDR) of 1% was used to filter all data; and proteins identified by only one peptide were

243   excluded from the results. Within Spectronaut, log2 fold change ratios of each pairwise comparison

244   was calculated, along with a q-value (FDR-corrected p-value from Student's t-test). R (version

245   4.4.1) was used for further analysis and figure generation (PCA plot). VolcanoseR was utilized for

246   generation of volcano plots (51). GO pathway enrichment analysis was performed using the

247   clusterProfiler package (52). The proteomics datasets generated and analyzed in this study are

248   available in the Proteomics Identification Database (PRIDE) repository under project identifier

249   PXD067842.

250   <u>Apoptotic Antibody Array</u>

251   The Proteome Profiler Apoptosis Array kits were purchased from R&D Biosystems (Cat#ARY009).

252   Cells were plated in 10 cm dishes (1 000 000 cells/plate) and treated with compounds as described

253   above. Cells were manually scraped and pelleted, then lysed with provided lysis buffer as detailed in

254   kit. After lysis and clarification by centrifugation (10 mins at 15 000 rpm), protein concentration was

255   determined by Bradford and 400 ug was added to array membrane. After cell lysis and protein

256   concentration determination, the kit instructions were followed for blotting and imaging. Array

257   membranes were imaged using a Chemidoc MP (BioRad) and images were quantified using Fiji

258   software (ImageJ).

259   <u>Immunofluorescence</u>

260   SUM159 cells were cultured at 5×10^4 cells per well in an 8 well chambered slide (ibidi, 80806). After

261   cells were treated for indicated durations, the cells were fixed with 4% formaldehyde in DPBS for 10

262   min at 37°C. Then the slide was washed thrice for 5 min with DPBS on the orbital shaker. The cells

263   were permeabilized with permeabilization solution (5% bovine serum albumin (BSA), 100mM glycine,

264   5% donkey serum (Sigma, D9663), 2% Triton X-100) for 15 min at room temperature on the shaker.

265   Next the cells were incubated in blocking solution (5% BSA, 100 mM glycine, 5% donkey serum) for

266   30 min at room temperature on the shaker. Primary antibodies were added at indicated dilutions (see

267   Table 2) in 5% BSA and 100mM glycine solution and cells were incubated and shaken in this solution

268   overnight at 4°C covered from light. The next day, cells were washed thrice for 5 min with DPBS. Then

269   the secondary antibody solution (5% BSA, 100mM glycine, indicated dilutions of secondary antibody)

270   was added to the cells and shaken for 1 h covered from light at room temperature. Next, 2nM DAPI in

271   DPBS solution was incubated on the cells for 10 min. The cells were washed twice for 5 min before

272   being stored at 4 °C until ready for imaging. The slides were imaged on a Zeiss LSM700 confocal

273   microscope using an EC Plan-Neofluar 40x/1.30 Oil DIC M27 oil objective with a 38μm optical section

274   (Oberkochen, Germany). Images were collected at 1024 x 1024 pixels with 8 bits per pixel, using 0.2%

275   laser power of a 5mW 405 nm laser, 10mW 488 nm laser, and 10mW 561 nm laser. Images were

276   analyzed using custom Python scripts with at least 50 cells per condition counted. Images were

277   imported and processed utilizing scikit-image. Nuclear segmentation was employed using skimage

278   on DAPI channel, and foci were counted after manual thresholding in red and green channels within

279   the nucleus areas (53). Data was exported, then graphed and statistically analyzed in Prism.

280   <u>Flow Cytometry</u>

281   *Cell Cycle:* The flow cytometry protocol was modified from Mouery, et al. (2024) (54). SUM159 and

282   MDA-MB-231 cells were supplemented with 10 μM EdU for 30 min before being trypsinized and

283   pelleted at 1000×g for 5 min. After a DPBS wash, the cells were fixed for 15 min at room temperature

284   with 4% formaldehyde. Next, the cells were washed with 1% BSA in DPBS and stored in 4°C until

285   staining. Cells were permeabilized with 1% BSA and 0.5% Triton X-100 for 15 min before EdU staining

286   was performed. The staining solution (1mM copper (II) sulfate, 100 mM ascorbic acid, and 1 mM

287   Alexa Fluor 647 Azide (Invitrogen, A10277) in DPBS) was added to permeabilized cells, protected

288   from light, and incubated at room temperature for 30 min. After washing the cell pellet with 1% BSA

289   and 0.5% Triton X-100 in DPBS, the cells were incubated overnight at 4°C in DAPI stain (1μg/mL DAPI,

290   100μg/mL RNAse, 1% BSA, 0.5% Triton X-100 in DPBS). Cells were filtered in 35μm filtered test tubes

291   (Falcon, 352235) before being imaged on the Attune NXT cytometer (New York City, NY, USA). Cell

292   cycle data were analyzed using FCS Express.

293 *Annexin V/Propidium Iodide Experiment:* Protocol was adapted from the Der Lab (UNC (55). TNBC

294 cell lines were treated for 72h with drug combinations, then media was collected, and remaining

295 cells were washed with DPBS then detached with trypsin/EDTA (Gibco, 15400054). Floating cells

296 from media and trypsinized cells were combined and collected by centrifugation. After a DPBS wash

297 and repelleting, the cells were resuspended in 100µl staining solution (5% Annexin V-AF488

298 (Invitrogen, A13201), 1µg/mL propidium iodide (Invitrogen, P1304MP) in Annexin V Binding Buffer

299 (10mM HEPES (pH 7.4), 140mM NaCl, 2.5mM CaCl2)) and left to incubate in the dark at room

300 temperature for 15 min. Next, cells were further diluted into 400µl Annexin V Binding Buffer and

301 filtered into flow test tubes (Falcon, 352235) before being read on the Attune Nxt Cytometer. Data

302 were analyzed utilizing Floreada.io (https://floreada.io, last accessed June 21, 2025)

303 <u>Comet Assay</u>

304 A modified comet assay protocol was adapted from Fagan-Solis, et al. (2020) utilizing the

305 CometAssay Single Cell Gel Electrophoresis Assay kit (R&D Systems, 4250-050-K) (56). Briefly, 5,000

306 cells were mixed with 0.5 mL of LMAgarose and plated on slides. The slides were submerged in

307 CometAssay Lysis solution overnight at 4°C. After 1 h incubation in Alkaline Unwinding Solution

308 (200mM NaOH, 1mM EDTA pH >13), gel electrophoresis was run on the slides with Alkaline

309 Electrophoresis Solution (200mM NaOH, 1mM EDTA, pH >13) at 21V for 1 h at 4 $^{\circ}$C. Slides were

310 suspended in deionized H_2O (diH_2O) twice for 5 min and then with 70% ethanol for another 5 min.

311 Samples were dried for 15 min at 37°C and then incubated with 1X SYBR Gold (Invitrogen, S11494)

312 solution (dissolved in TE buffer (10mM Tris (pH 7.5), 1mM EDTA) for 30 min at room temperature in

313 the dark. Slides were washed with diH_2O before being imaged on a ZEISS Axio Vert.A1 inverted

314 microscope at 10x magnification.

315 <u>Cell Line-Derived Xenograft Mouse Experiment:</u>

316   All animal studies were carried out following approval from the UNC Institutional Animal Care and

317   Use Committee (Protocol# 22.215). Arrive2.0 guidelines were observed, including randomization of

318   mice to study groups and blinding investigators involved in the study. 4T1-Luc cells were treated with

319   150nM TR-57 for 24 or 48h, or 0.01% DMSO for 48h for controls. After treatment, cells

320   were detached with Cell Stripper (Corning, 25-056-CI) before being counted and pelleted. Cells were

321   resuspended at 5x10^6 cells/mL in sterile 1X DPBS and placed on ice until injection. Female BALB/c

322   mice were injected on the left mammary fat pad with 100µl of 4T1-Luc cell suspension. A total of 8

323   mice per condition were tested. Body condition, weight, and tumor formation were

324   monitored 2x/week; tumor volumes were assessed by intravital imaging (IVIS Optical Imager,

325   Waltham, MA, USA) performed on anesthetized mice that were intraperitoneally injected with d-

326   luciferin (75mg/kg). Study endpoints were maximum tumor volume (2000 mm^3) or declining body

327   condition (e.g. weight loss of 20%). Maximum tumor burden was not exceeded in this study. TR-57-

328   pretreated mice did not reach either endpoint and accordingly, the study was terminated after 55

329   days.

330   <u>Synergy Dose-Response and Analysis</u>

331   SUM159 and MDA-MB-231 cells were plated at 1 000 and 2 000 cells/well respectively and plated in

332   black with clear bottom 96 well plates. The Tecan D300e liquid handler (Männedorf, Switzerland) was

333   used to treat the cells with TR-57 and venetoclax or olaparib, then cells were incubated for 72h. 100µl

334   of 5µg/mL propidium iodide and 2.5 µg/mL of Hoechst 33342 stain in DPBS was added to each well.

335   Cells were incubated for 20 min at 37°C. Then cells were imaged on the Celigo Imaging Cytometer

336   and dead and total cell count was determined with the Celigo software. Percent viability was

337   calculated and input into SynergyFinder+ (57). HSA synergy scores were exported and graphed in

338   heatmaps using GraphPad Prism.

Statistical Analysis

340 Statistical calculations for all the data, excluding proteomics, were performed using GraphPad

341 Prism. Data are reported as the mean ± standard deviation which was performed on all datasets to

342 determine positive and negative errors. Unpaired two-tailed student t-test, one-way, or two-way

343 ANOVAs were used to make comparisons between groups, and p values below 0.05 at the 95%

344 confidence level were considered to be statistically significant. The number of significance stars are

345 based on GraphPad reporting style. When brackets are not used to compare experimental groups,

346 the statistical star is for the indicated group compared to the DMSO control at that timepoint.

347

348 **Results:**

349 **ClpP activation induces senescence and increases SASP**

350 We previously observed that exposure of TNBC cell lines to ClpP agonists induced cytostasis but not

351 cell death (19,22). To further investigate this, we examined multiple events associated with cell

352 senescence including β-galactosidase (β-gal) activity, lamin B1 expression, cell proliferation, and

353 SASP markers as shown (Fig. 1A). Incubating MDA-MB-231 cells with three chemically different ClpP

354 agonists (TR-57, TR-107, ONC201) resulted in significant increases in β-gal activity (Fig. 1B, and Supp.

355 Fig. 1A). For simplicity we focused on the potent and selective ClpP activator TR-57 for many of the

356 studies described hereafter. Abemaciclib was used as a positive control for therapy-induced

357 senescence in most cases (44). TR-57 induced significant increases in β-gal staining after 96h

358 treatment in both TNBC cell lines. However, TR-57 did not increase β-gal activity in the ClpP knockout

359 (KO) cell lines (SUM159 KO or MDA-MB-231 KO), confirming ClpP dependence (Fig. 1C-D, Supp. Fig.

360 1B-C). Loss of lamin B1, an established marker of senescence (58), was observed after abemaciclib

361 and TR-57 treatment of SUM159 and MDA-MB-231 cells (Fig. 1E, Supp. Fig. 1D). Furthermore, TR-57-

362 dependent growth inhibition was confirmed by decreased retinoblastoma protein (Rb)

363   phosphorylation and a reduction in c-Myc (Myc) protein after 48 h, whereas lamin B1, Myc, or Rb

364   phosphorylation was unaffected in the ClpP KO cells following TR-57 treatment (Fig. 1E, Supp. Fig.

365   1D). Other ClpP agonists also decreased lamin B1, p-Rb, and Myc protein expression, demonstrating

366   that these senescence markers are consistently affected by chemically different ClpP agonists

367   (Supp. Fig 1G).

368   The secretion of cytokines, chemokines, MMP's, and other proteins (GDF15) is a hallmark of

369   senescence collectively known as the SASP (46,59,60). As determined by qRT-PCR, TR-57 incubation

370   strongly increased IL-6, IL-8, and IL-12 mRNA in MDA-MB-231 cells whereas no significant

371   differences were observed in the MDA-MB-231 ClpP KO cells (Fig. 1F). Western blotting for growth

372   differentiation factor 15 (GDF15), demonstrated increased GDF15 protein levels after TR-57

373   treatment of SUM159 cells (Supp. Fig. 1E). Lastly, we performed long-term incubation assays to

374   determine if ClpP agonists were stably inducing senescence. TNBC cells were cultured for 30 days

375   with TR-57 to determine if growth arrest was permanent under these conditions. After 30 days, no

376   increase or decrease in total cell count was observed at the higher concentrations of TR-57,

377   suggesting that the TNBC cells were neither dying nor proliferating (Fig. 1G). Comparison of the IC_{50}

378   values from the 30-day incubation showed that they were not significantly different from that

379   obtained after 3-day TR-57 incubation (Supp. Fig. 1F). Taken together, these data demonstrate that

380   the ClpP-dependent inhibition of cellular proliferation in TNBC cell models is accompanied by

381   increased markers and evidence of stable cell senescence.

382   **Proteomics data identifies protein changes related to cell cycle arrest and senescence**

383   Because our data suggested that exposure of MDA-MB-231 cells to TR-57 for 48h was sufficient to

384   induce senescence, we performed proteomics analysis on these cells (31). Principle component

385   analysis (PCA) demonstrated excellent clustering of the DMSO and TR-57-treated samples (Fig. 2A).

16

386   Analysis of this data confirmed that TR-57 caused down-regulation of multiple mitochondrial matrix

387   proteins (mitochondrial elongation factor Tu (TUFM), aconitate hydratase (ACO2), Pyrroline-5-

388   carboxylate reductase 1 (PYCR1), small ribosomal subunit protein bS16m (MRPS16), others) as

389   observed earlier (24h TR-57), albeit to a greater extent (31). Volcano plot analyses identified

390   additional upregulated proteins indicating activation of cyclic-AMP dependent transcription factor

391   (ATF4) and the integrated stress response (SLC7A11, asparagine synthetase (ASNS), cystathionine

392   gamma-lyase (CTH), NIBAN1), cell cycle arrest (Sororin (CDCA5), G2/mitotic specific cyclin B1

393   (CCNB1)), and cell senescence (GDF15) (Fig. 2B). We next applied GO pathway enrichment analysis

394   to evaluate the effects of TR-57 on cellular signaling processes. As expected, multiple mitochondrial

395   functions (Tricarboxylic acid (TCA) cycle, oxidative phosphorylation) and mitochondrial-associated

396   pathways (fatty acid oxidation, mitochondrial transcription and translation) were disrupted (Fig. 2C).

397   Additional events related to cell cycle regulation (mitotic nuclear division, chromosome segregation)

398   and regulation of cell death (negative regulation of cell death) were also observed (Fig. 2D).

399   The expression of pro- and anti-apoptotic proteins were examined using commercial

400   antibody arrays. These results demonstrated that the pro-apoptotic proteins Bad, SMAC/Diablo, and

401   others were downregulated in SUM159 and MDA-MB-231 cells after TR-57 treatment (Supp. Fig. 2 A-

402   B). TR-57 also resulted in the downregulation of specific anti-apoptotic proteins, cellular inhibitor of

403   apoptosis protein 1 (cIAP1), Survivin, and others (Supp. Fig. 2A-B). The TR-57-dependent decrease in

404   some of these proteins was confirmed to occur in wildtype (WT) but not ClpP KO TNBC cells by

405   immunoblotting (Supp. Fig. 2C-D). Thus, these results suggested that ClpP agonists affected both

406   pro- and anti-apoptotic protein expression in the TNBC cell lines tested.

407   **TR-57 Induces Cell Cycle Arrest in Breast Cancer Models**

408   To investigate the effects of ClpP agonists on cell cycle progression, flow cytometry was performed

409   on TNBC cells exposed to TR-57. MDA-MB-231 and SUM159 cells were incubated with TR-57 for 24,

410   48, or 72h and analyzed as described in Methods. As shown in Fig. 3A, we observed a profound

411   inhibition of cell cycle progression in the WT, but not the ClpP KO cells. Analysis of the flow data

412   demonstrated a strong decrease in EdU staining in S-phase from both MDA-MB-231 and SUM159 cell

413   lines at 72h, indicating that TR-57 was inducing an S-phase arrest (Fig. 3B, C and Supp. Fig. 3A, B).

414   The absence of EdU staining suggested that ClpP activation was inducing replication stress, wherein

415   DNA replication is stalled and EdU cannot be incorporated (61,62). To further characterize the effects

416   of ClpP agonists on cell cycle arrest, we measured the expression of proteins that regulate cell cycle

417   progression. A decrease in cyclins A2, D3, E1, and S-phase kinase associated protein 2 (Skp2) were

418   observed after TR-57 treatment (Fig. 3D). By contrast, p21 expression markedly increased after 72h.

419   Decreased expression of p21 is associated with S-phase arrest, but p21 expression is known to

420   increase during senescence (63). Similar decreases in cyclin A2, D3, E1, and Skp2 protein expression

421   were observed with TR-107 and ONC201 treatment at 72h, indicating this S-phase arrest is

422   consistent across ClpP agonists (Supp Fig. 3C). Taken together this data provides strong evidence

423   supporting an S-phase arrest.

424   **ClpP agonist treatment induces DNA damage and a DNA damage response**

425   Our previous phosphoproteomics data indicated that TR-57 induced a DNA damage response based

426   on increased phosphorylation of multiple substrates on phospho-S*Q (p-S*Q) sites which are

427   commonly phosphorylated by activated Ataxia-Telangiectasia Mutated (ATM) and Ataxia-

428   Telangiectasia and Rad3-related (ATR) proteins (31). Since DNA damage often precedes the

429   development of senescence and S-phase arrest, we examined the effects of TR-57 on well-

430   established markers of the DNA damage response (DDR). This included 53BP1, γH2A.X, ATM/ATR,

431   and Chk1/2. Western blotting confirmed an increase in Chk2 and H2A.X phosphorylation after TR-57

432 treatment of SUM159 cells. These results were observed in WT but not ClpP KO cells, whereas they

433 were increased in both cell models with the positive control irinotecan (Fig. 4A). Similar results were

434 observed with MDA-MB-231 cells (Supp. Fig. 4A) and with ONC201 and TR-107 (Supp. Fig. 4B).

435 Moreover, TR-57 and ONC201 increased the phosphorylation of ATM in parallel with increased

436 phosphorylation of Chk2, the effects of which were blocked with the ATM inhibitor KU-60019.

437 However, neither TR-57 nor ONC201 induced Chk1 phosphorylation (Supp. Fig. 4C-D).

438 Next the influence of TR-57 on the DDR was further investigated by immunofluorescence

439 microscopy. We observed colocalization of 53BP1 with γH2A.X or the p-S*Q binding motif for ATM

440 and ATR substrates after incubation of SUM159 cells with TR-57 or irinotecan (Fig. 4B-C).

441 Quantification of the images confirmed a significant increase in p-S*Q, 53BP1, and γH2A.X foci after

442 TR-57 treatment compared to DMSO (Fig. 4D). The Mander's coefficient of colocalization for 53BP1

443 signal overlapping with p-S*Q or γH2A.X were both significantly increased (Fig. 4E-F). Colocalization

444 of 53BP1 and γH2A.X demonstrated a strong correlation (higher M2 value) between 53BP1 location

445 and γH2A.X location, suggesting DNA damage was occurring at these foci (Fig. 4F).

446 To validate that ClpP activation induces DNA damage, we performed an alkaline comet assay.

447 This assay identifies double- and single-strand breaks in nuclear DNA (Fig. 4G). As shown in Fig. 4H,

448 the alkaline comet assay showed increased signs of DNA damage after TR-57 incubation with

449 SUM159 cells, confirming that TR-57 induces DNA damage in TNBC cells.

450 **ClpP agonist induced senescence is not reversible**

451 To determine if ClpP-agonist treated TNBC cells were truly senescent, we performed TR-57

452 treatments followed by drug washout and monitored cell proliferation. SUM159 or MDA-MB-231 cells

453 were incubated with DMSO or TR-57 for 24 or 48h at which time the media was replaced with drug-

454 free media (washout) and cells allowed to proliferate. While DMSO-treated cells grew rapidly as

455   expected, cells treated with TR-57 for 24h regained slow growth after washout. By comparison, after

456   48h TR-57 incubation and washout, there was no increase in growth even enough the cells remained

457   viable 6 days after washout (Fig. 5A-B). By contrast, in washout experiments following treatment with

458   the CDK4/6 inhibitor abemaciclib (48h), we observed rapid recovery of cell proliferation almost

459   immediately after drug washout, especially in the SUM159 cells (Fig. 5A-B). Both SUM159 and MDA-

460   MB-231 ClpP KO cells showed no significant changes between DMSO and TR-57-treated growth after

461   drug washout, but abemaciclib growth curves were consistent with the WT abemaciclib treatments

462   as expected (Supp Fig. 5A-B). Finally, the mouse TNBC cell line, 4T1-Luc, was tested and similar

463   results obtained as in the human TNBC cells. Both TR-57 (24h and 48h) treatments were significantly

464   different from DMSO growth following 4 days of washout, but the TR-57 (48h) treated cells never grew

465   past the initial seeding density (Fig. 5C).

466   To investigate the underlying mechanism for the sustained senescence induced by TR-57,

467   we blotted for the cell cycle regulators Rb and Myc. Both TR-57 and abemaciclib strongly

468   downregulated Rb phosphorylation after 72h treatments. Following abemaciclib washout (2, 4, 6

469   days), p-Rb and Myc expression rapidly returned to the pretreatment levels whereas neither Rb, p-Rb

470   or Myc protein levels recovered following TR-57 washout. Instead, these proteins all remained

471   significantly decreased, suggesting a more permanent effect (Supp Fig. 5C). Notably, the absence of

472   TUFM protein indicated mitochondrial function remained impaired even after 6 days of drug washout

473   (Supp. Fig. 5C). Together, this data suggests that TR-57-induced a sustained proliferation arrest after

474   the drug was removed compared to abemaciclib where after drug washout, proliferation returned.

475   **TR-57 induces stable cell senescence in a cell-line derived xenograft model**

476   To further determine if the TR-57-induced senescence was truly irreversible, we treated 4T1-Luc cells

477   with TR-57 for 24 or 48h and performed drug washout as described in Methods. Viable cells from

478 each treatment condition were injected into the mammary fat pads of BALB/c mice and tumor

479 formation monitored by luciferase activity (Fig. 5D). As expected, the control (DMSO) treated cells

480 rapidly grew tumors. TR-57 (24h) treated cells lagged initially but also showed an increase in tumor

481 growth over time. By contrast, 4T1-Luc cells exposed to TR-57 (48h) before washout, did not show

482 tumor growth at any time during this experiment, even though cells were detectable as demonstrated

483 by inset (Fig. 5E). No significant changes in the mouse weight were observed for the duration of the

484 experiment between groups (Supp. Fig. 5D). 4T1-Luc response to ClpP activation was further

485 demonstrated by Western blots that confirmed reduction of Myc and p-Rb expression, similar to

486 changes observed in the human TNBC cell lines (Supp. Fig 5E). This data suggested that once

487 senescence was established (48h, TR-57 treatment), the senescent phenotype was not reversible *in*

488 *vivo*.

489 **Venetoclax synergizes with TR-57 to induce senolysis**

490 Because our data showed that TR-57-treated TNBC cells underwent senescence and not apoptosis

491 (Fig. 1), we investigated whether addition of a senolytic would increase cell death. Venetoclax is a

492 Bcl-2 family inhibitor and established senolytic (64–67). The combination of TR-57 and venetoclax

493 resulted in significantly enhanced inhibition of SUM159 and MDA-MB-231 cell growth after 72h

494 treatments (Fig. 6A, F). Synergy between TR-57 and venetoclax was further confirmed by viability

495 analysis based on the highest single agent (HSA) model (Fig. 6B, G). HSA scores greater than 10

496 indicate a strong chance of synergy. With the largest scores of 32 and 56 for SUM159 and MDA-MB-

497 231 cells respectively, TR-57 and venetoclax demonstrated a synergistic effect on TNBC cell viability.

498 Furthermore, the area under the curve (AUC) indicates a strong combination effect with high doses

499 of venetoclax with TR-57 (Fig. 6C, H). Importantly, this combination resulted in a significant increase

500 in cell death compared to TR-57 or venetoclax alone as measured by annexin V positive staining

501 (Apoptotic cells) (Fig. 6D, I) or propidium iodide (PI) staining (Dead cells) (Fig. 6E, J) in both TNBC cell

502 lines (Supp. Fig. 6A-B). To demonstrate that this synergistic effect was not solely dependent on TR-

503 57, we tested TR-107 and venetoclax and found this combination also significantly increased the

504 percent of apoptotic and dead cells compared to either drug alone (Supp. Fig. 6C).

505 Given the observed increase in the DNA damage response after TR-57 treatment, we

506 examined if co-targeting DNA repair would induce cell death. Specifically, we tested the PARP

507 inhibitor, olaparib in combination with TR-57. Adding increasing concentrations of olaparib and TR-

508 57 demonstrated dose-dependent inhibition of growth of SUM159 and MDA-MB-231 cells after 72h

509 (Fig. 7A, F). Applying synergy calculations (HSA) determined that the highest synergy score was 4 and

510 3 in the SUM159 and MDA-MB-231 cells respectively. HSA synergy scores between 0 and 10 generally

511 indicate an additive effect, suggesting that TR-57 and olaparib are not synergistic, but do have

512 potential beneficial effects as co-treatments (Fig. 7B, G). The significant decrease in AUC with

513 increasing olaparib concentrations supports evidence of an additive effect (Fig. 7C, H). Measuring

514 cell death by flow cytometry demonstrated an increase in cell death in response to this combination

515 that further suggested additive effects in both TNBC cell lines (Fig. 7D-E, 7I-J, Supp Fig. 6B).

516 **Discussion:**

517 Represented by ONC201 and the TR compounds, small molecule ClpP activators (ClpP agonists)

518 have generated considerable interest as anti-cancer agents with a novel mechanism of action. The

519 fact that ONC201 (Dordaviprone) is now an FDA-approved monotherapy, suggests enormous

520 promise for this novel modality of cancer treatment. These compounds show broad activity against

521 multiple cancer types with increased cell death (apoptosis) most commonly observed (33,34,68–70).

522 In this study we demonstrate that senescence is the dominant outcome in TNBC models. We

523 confirmed this by examining a multitude of well-established senescence markers and demonstrating

524 the permanence of this response, both in cell cultures and an animal model. Before TR-57 induces

525   senescence in TNBC cell lines, DNA damage and S-phase arrest were observed, but we were unable

526   to demonstrate that DNA damage or S-phase arrest was the cause of senescence in these cells.

527   However, taking advantage of the senescent response, we show that ClpP agonists can be combined

528   with the known senolytic venetoclax to increase cell death.

529   TR-57 was shown to induce senescence by significantly increasing β-gal activity and SASP

530   cytokines and decreasing Lamin B1 expression and proliferation markers (p-Rb and Myc). A 30-day

531   TR-57 treatment showed no increase in, or loss of cell count, further demonstrating the senescent

532   phenotype. Analysis of our proteomics data characterized the properties of ClpP agonist-induced

533   senescence. This identified well-established markers of senescence (i.e. GDF15) and events known

534   to precede senescence such as increased DNA damage, cell cycle arrest, and cytokine production.

535   Interestingly we also observed protein markers that may be unique to the action of ClpP agonists

536   including upregulation of multiple amino acid transporters and metabolic enzymes. Whether or not

537   these proteins contribute to the increased secretion or altered metabolism of senescent cells

538   remains to be established.

539   An increase in DNA damage following ClpP activation is well supported by our evidence

540   (γH2A.X and 53BP1 foci, comet formation, ATM/CHK2 activation), and the timing of DNA damage

541   correlates with the induction of senescence (Fig 4A, Supp. Fig 4E.) However, the origin of the DNA

542   damage response remains unclear. Unexpectedly, ATR and Chk1 phosphorylation were not observed

543   after ClpP activation, unlike that seen with ATM and Chk2 (Supp. Fig. 4C). A possible explanation is

544   our finding that TR-57 induces S-phase arrest. ATR activity is essential for protecting replication forks

545   during S-phase, and blocking ATR leads to replication stress (62). The lack of ATR/Chk1 activation

546   combined with an S-phase arrest suggests ClpP activation is inducing replication stress. The cause

547   of DNA damage and S-phase arrest was not determined. However, one possibility is that increased

548 ROS is responsible, although this was not directly measured in this study. Moreover, the antioxidant,

549 N-acetylcysteine failed to block γH2A.X phosphorylation, a strong indicator of DNA damage (Supp.

550 Fig. 7A). An alternative hypothesis is that the DNA damage results from nucleotide depletion.

551 Because ClpP activation impairs pyrimidine biosynthesis and limits the availability of nitrogenous

552 bases (31), we tested whether the observed DNA damage was caused by nucleotide depletion.

553 However, supplementation of MDA-MB-231 cells with pyrimidines and/or purines during TR-57

554 treatment did not prevent the DNA damage response, suggesting nucleotide depletion was not the

555 cause of TR-57-induced DNA damage (Supp. Fig. 7B). Studies, including our own, have correlated

556 mitochondrial dysfunction and nuclear DNA damage (71–73), but how mitochondrial dysfunction

557 leads to increased nuclear DNA damage is still unknown.

558 We observed evidence for S-phase arrest in response to ClpP agonists contrary to previous

559 reports identifying a G1 arrest (20,74). Those studies, conducted in lung and colorectal cancers,

560 relied only on DAPI or PI staining, which can make it difficult to distinguish S-phase arrest without the

561 added resolution of EdU incorporation. Thus, it is possible that the S-phase arrest went undetected

562 in those analyses. Alternatively, these differences may reflect cancer type–specific effects of ClpP

563 activation. The observed S-phase arrest was characterized by an inhibition of EdU incorporation,

564 consistent with increased DNA replication stress and arrest. Decreases in cyclin A2, cyclin D3, and

565 Skp2 further indicate a deregulated S-phase. These responses were ClpP-dependent and differed

566 from that observed with abemaciclib (G1 arrest). Thus, the induction of cell cycle arrest by ClpP

567 agonists appears to be unique and may explain the irreversibility observed with these agents.

568 Abemaciclib-induced growth inhibition was rapidly reversed following drug washout, but growth

569 inhibition by ClpP activation was not. This coincided with a drastic decline in Myc protein that was

570 not observed following abemaciclib treatment. Importantly, the loss of Myc was not readily reversed

571 upon ClpP agonist washout, suggesting that Myc downregulation may play an important role in the

24

572   sustained inhibition of cell proliferation and development of senescence. Our *in vivo* model further

573   demonstrates the irreversibility of TR-57-induced senescence, though further studies are needed to

574   determine how ClpP activation causes a permanent proliferation arrest.

575   Therapy-induced senescence is relevant in the context of cancer (42) and many

576   chemotherapeutics induce cancer cell senescence including cisplatin, irinotecan, bleomycin, and

577   doxorubicin (42,75–78). Ionizing radiation increases DNA damage and senescence and is one of the

578   most commonly used methods of inducing senescence in models of aging (79,80). ClpP agonists

579   induce senescence in TNBC cells, whereas apoptosis is more common in other cancers, such as

580   H3K27 mutant gliomas targeted by ONC201. TR-57 did not significantly increase apoptosis in TNBC

581   (Fig. 6D, I), suggesting these cells are uniquely resistant to ClpP-induced apoptosis. The apoptotic

582   arrays (Supp. Fig 2) did not reveal any specific vulnerabilities of TR-57-induced senescent TNBC cells.

583   Instead, we sought to induce cell death in TNBC with co-treatment of ClpP agonists and a well-

584   established senolytic (venetoclax). As shown here and earlier, venetoclax significantly increased cell

585   death in TNBC and leukemia cells (67). Furthermore, synergy was observed between TR-57 and

586   venetoclax as observed by HSA synergy scores. These studies suggest that successful application of

587   ClpP agonists may depend on the pro- and anti-apoptotic properties of the cancer cell types in

588   question.

589   Combination experiments with TR-57 and olaparib had additive effects, suggesting that

590   increasing DNA damage is beneficial to the mechanism of action of ClpP agonists. However, the

591   relationship is not synergistic, and this observation was confirmed by testing other DNA damaging

592   agents (doxorubicin, 5-FU, irinotecan). There was a distinct additive effect to treating TR-57 with DNA

593   damaging agents, but the effects are not synergistic (Supp. Fig. 7D). Ultimately, there may be more

594   therapeutic rationale for combination treatments targeting anti-apoptotic proteins than targeting

595   DNA and DNA repair with ClpP agonists in TNBC.

596   In summary, we describe the effects of novel small molecule activators of ClpP on TNBC cells

597   and the events preceding and/or contributing to the development of senescence. Compared to other

598   cancer models where ClpP agonists induce apoptosis or other forms of cell death (i.e., ferroptosis),

599   senescence predominates in the TNBC models tested here. As such, these studies may have

600   important implications for potential treatments of this specific type of cancer with ClpP agonists.

601   ClpP agonists represent a novel class of anti-cancer agents with promising results in many

602   cancers, exemplified by the FDA-approved ONC201. In TNBC models, ClpP agonists predominantly

603   induce senescence. TR-57-induced senescence is preceded by DNA damage and S-phase arrest,

604   and the associated downregulation of Myc may contribute to the irreversibility of this response.

605   Importantly, TNBC cells are resistant to apoptosis, but combination with the senolytic venetoclax

606   produces significant synergistic cell death, highlighting a promising therapeutic strategy. These

607   findings provide mechanistic insight into ClpP agonist action in TNBC cells and identify a

608   combinatorial approach that may offer new therapeutic options for breast cancer patients with few

609   targeted treatments available.

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622 phase arrest phenotype.

623 **Conflict of Interest:**

624 EJI is currently employed by Madera Therapeutics, LLC. The remaining authors declare no competing

625 financial interests.

626 **Data availability:**

627 The proteomics dataset generated and analyzed during the current study are available in the

628 Proteomics Identification Database (PRIDE) repository under project identifier PXD067842. The

629 remaining datasets generated and/or analyzed during the current study are available from the

630 corresponding author on reasonable request.

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852

853 **Figure Legends:**

854 **Figure 1: ClpP activation induces senescence and the SASP in TNBC cell lines**

855 A) Model depicting initiators of senescence and the consequences on cell markers. B) MDA-MB-

856 231 cells were treated with 0.1% DMSO, 150nM TR-57, 100nM TR-107, 10μM ONC201, or 500nM

857 abemaciclib for 96h. β-galactosidase (β-gal) activity was measured by colorimetric assay and

858   quantified, N=3. C-D) MDA-MB-231 or SUM159 and respective ClpP KO cell lines were treated with

859   0.1% DMSO (96h), 500nM abemaciclib (48h) or 150nM TR-57 (48 or 96h). β-gal activity was

860   measured and quantified, N=3 E) Immunoblots of MDA-MB-231 and ClpP KO cells treated with

861   DMSO (D) for 72h, abemaciclib (A) for 48h, or TR-57 for indicated timepoints. Representative

862   images of N=3. F) qRT-PCR determined relative expression levels of IL-6, IL-8, and IL-12 after

863   treatment in MDA-MB-231 and ClpP KO cells, N=3. G) TNBC cell lines were treated with varying

864   concentrations of TR-57 for 30 days. Representative of N=3. All error bars are representative of the

865   standard deviation (SD).

866

867   **Figure 2: Proteomics analysis indicates TR-57 induces mitochondrial dysfunction, cell cycle**

868   **arrest, and regulation of cell death**

869   MDA-MB-231 cells were treated with 0.1% DMSO or 150nM TR-57 for 48h and whole cell

870   proteomics was performed. A) Principal component analysis (PCA) plot demonstrates distinct

871   clustering of the DMSO and TR-57 samples. B) Volcano plot analyses show significant increases

872   (right) and decreases (left) in protein expression after TR-57 treatment. GO pathway analysis of

873   significantly decreased proteins (C) and increased proteins (D) after TR-57 treatment (48h).

874

875   **Figure 3: ClpP activation induces an S-phase arrest**

876   A) MDA-MB-231 and ClpP KO cells were treated with 0.1% DMSO or 150nM TR-57 for 72h. After a 30

877   min EdU pulse, cells were harvested and analyzed via flow cytometry. Representative images

878   indicate populations of cells in G1, S with EdU incorporation (EdU+), S without EdU incorporation

879   (EdU-) or G2/M. B-C) Graphed percentages of MDA-MB-231 and SUM159 cells in G1, S EdU+, S

880   EdU-, or G2/M, N=2. D) Representative immunoblots of MDA-MB-231 cells treated with DMSO or

881   TR-57 (24-72h) N=3. All error bars are representative of the standard deviation (SD).

882

883   **Figure 4: ClpP activation induces DNA damage and the DNA damage response**

884   A) Representative immunoblotting images of DNA damage markers from SUM159 and ClpP KO cells

885   treated with 0.1% DMSO for 72h (D), 2μM irinotecan for 48hr (I), or 150nM TR-57 for indicated times,

886   N=3. B) Representative immunofluorescence images of SUM159 cells treated with DMSO,

887   irinotecan (Irino), or TR-57 for 72 h. DNA damage signaling was detected with 53BP1 and p-S*Q foci

888   C) Representative images of γH2A.X and 53BP1 foci after 72h treatment with DMSO, Irino, or TR-57

889   in SUM159 cells. D) Quantification of p-S*Q substrate, 53BP1, or γH2A.X foci per cell in each

890   treatment condition described in B and C. E-F) Mander's Coefficient of probability that 53BP1 signal

891   overlaps with p-S*Q or γH2A.X respectively per cell. G) Representative images of DNA from comet

892   assay. H) Quantification of comet assay results. %DNA in Tail and Tail moment were quantified

893   using OpenComet, N=3. All error bars are representative of the standard deviation (SD).

894

895   **Figure 5: TR-57-induced senescence is irreversible *in vitro* and *in vivo***

896   A-B) SUM159 and MDA-MB-231 cells were treated with 0.1% DMSO (48h), 150nM TR-57 (24-48h) or

897   500nM abemaciclib (48h), then drugs were washed out and cell count was monitored for 6 days

898   after drug washout, N=3. C) 4T1-Luc cells were treated with TR-57 for 24 or 48h before drug

899   washout. Cell count was measured for 6 days after drug washout, N=3. D.) Scheme for

900   experimental set up in mouse study. 4T1-Luc cells were treated with 0.1% DMSO (48h) or 150nM

901   TR-57 (24 or 48h). The cells were collected and 500 000 cells injected into each mouse. E.) Tumor

902   formation was monitored by relative luminescence units (RLU) twice per week after injection of 4T1-

903   Luc cells, N=8 mice per condition. All error bars are representative of the standard deviation (SD).

904

905   **Figure 6: Venetoclax synergizes with TR-57 to induce cell death in TNBC cells**

906   A) Dose-response curve of SUM159 cells treated with increasing concentrations of TR-57 titrated

907   with venetoclax, N=3. B) Heat map of HSA synergy scores for graphed combinations of TR-57 and

908   venetoclax in A. C) Area under the curve (AUC) of A. D-E) Flow cytometry was performed on

909   SUM159 cells treated with 0.1% DMSO, 10µM venetoclax (Veneto), 25nM TR-57 or both for 72h.

910   Percent apoptotic and dead cells are graphed respectively, N=3. F) Dose-response curve of MDA-

911   MB-231 cells treated with increasing concentrations of TR-57 titrated with venetoclax, N=3. G) Heat

912   map of HSA synergy scores for graphed combinations of TR-57 and venetoclax in F. H) Area under

913   the curve (AUC) of F. I-J) Flow cytometry was performed on MDA-MB-231 cells treated with DMSO,

914   20µM venetoclax (Veneto), 25nM TR-57 or both for 72h. Percent apoptotic and dead cells are

915   graphed respectively, N=3. All error bars are representative of the standard deviation (SD).

916

917   **Figure 7: Olaparib and TR-57 co-treatment produces additive cell death effects in TNBC cells**

918   A) Dose-response curve of SUM159 cells treated with increasing concentrations of TR-57 titrated

919   with olaparib, N=3. B) Heat map of HSA synergy scores for graphed combinations of TR-57 and

920   olaparib in A. C) Area under the curve (AUC) of A. D-E) Flow cytometry was performed on SUM159

921   cells treated with 0.1% DMSO, 20µM olaparib, 25nM TR-57 or both for 72h. Percent apoptotic and

922   dead cells are graphed respectively, N=3. F) Dose-response curve of MDA-MB-231 cells treated

923   with increasing concentrations of TR-57 titrated with olaparib, N=3. G) Heat map of HSA synergy

924   scores for graphed combinations of TR-57 and olaparib in F. H) Area under the curve (AUC) of F. I-J)

925   Flow cytometry was performed on MDA-MB-231 cells treated with DMSO, 20μM olaparib, 25nM TR-

926   57 or both for 72h. Percent apoptotic and dead cells are graphed respectively, N=3. All error bars

927   are representative of the standard deviation (SD).

928

929   **Supplementary Figure Legends:**

930   **Supplementary Figure 1: ClpP Agonists Induce Senescence in TNBC cells**

931   A) Representative images of quantified β-gal staining data from Figure 1B. B) Representative images

932   of MDA-MB-231 cells from Fig 1C. C) Representative images of SUM-159 cells from Fig 1D. D)

933   SUM159 cells were treated with abemaciclib (48h) or TR-57 for 24-72h and immunoblotting was

934   performed to measure protein expression of senescence markers. Representative images of N=2 E)

935   Immunoblot of increasing concentrations of TR-57 and GDF15 expression in SUM159 cells. N=2 F)

936   Comparison of dose-response curves from 3 or 30 days of TR-57 treatment. IC$_{50}$ values were

937   plotted for both cell lines in graph as shown on the right. Representative curves from N=3 G)

938   Immunoblot of ONC201, TR-57, TR-107, abemaciclib, and irinotecan (2μM) demonstrating changes

939   in protein expression of senescence markers. Representative images of N=2.

940

941   **Supplementary Figure 2: ClpP Agonists Deplete Pro- and Anti-Apoptotic Proteins**

942   A) SUM159 and MDA-MB-231 cells were treated with 0.1% DMSO or 150nM TR-57 for 48h and an

943   immunoblotting array was utilized on cell lysates to visualize proteins known to regulate apoptosis.

944   B) Quantification of SUM159 array. C-D) SUM159 cells and MDA-MB-231 cells respectively were

945   treated with 0.1% DMSO, 100nM staurosporine (S) for 24h, 500nM abemaciclib (A) for 48h, or

946   150nM TR-57 for 48h. Validation of changes in protein expression from array was performed by

947   immunoblotting. Representative images of N=2-3.

948

949   **Supplementary Figure 3: ClpP Agonists Induce S-phase Arrest**

950   A) Gating scheme for cell cycle analysis in Figure 3. B) Representative plots of SUM159, MDA-MB-

951   231 and respective ClpP KO cells treated with 0.1% DMSO or 150nM TR-57 for 24-72h from Figure

952   3B-C. C) MDA-MB-231 cells were treated with 150nM TR-57, 10μM ONC201, or 100nM TR-107 for

953   72h. Immunoblotting was performed on cell-cycle proteins. Representative images of N=2

954

955   **Supplementary Figure 4: ClpP Agonists Induce DNA Damage in TNBC cells**

956   A) MDA-MB-231 and ClpP KO cells were treated with 0.1% DMSO (D) for 48h, 2μM irinotecan (I) for

957   48h, and 150nM TR-57 for 24-72h. Immunoblotting was performed to detect DNA damage markers.

958   Representative images of N=2. B). MDA-MB-231 cells were treated with 150nM TR-57, 10μM

959   ONC201, 100nM TR-107, 500nM abemaciclib, or 2μM irinotecan for 72h. Immunoblotting was

960   performed on DNA damage markers. Representative images of N=2. C-D) SUM159 cells were

961   treated with the indicated drugs for 48h and immunoblotted as shown. A positive control, indicated

962   by (+ Ctrl), consisted of 250μM H_2O_2 for 30 min for all samples except p-Chk1 and Chk1 which was

963   50μM etoposide for 2h. N=2. E) SUM159 cells were untreated (time 0) or treated with 50nM TR-57

964   for the indicated times and immunoblotted as shown. A positive control, indicated by (+), consisted

965   of 100μM H_2O_2 for 30 min for all samples except p-ATR, ATR, and p-Chk1 which was 50μM etoposide

966   for 2 h.

967

968 **Supplementary Figure 5: TR-57-induced senescence is not reversible in *in vitro* and *in vivo***

969 **TNBC models**

970 A-B) SUM159 or MDA-MB-231 ClpP KO cells respectively were treated with 0.1% DMSO, 500nM

971 abemaciclib, or 150nM TR-57 for 48h before drug washout. Cell count was monitored for 6 days

972 after drug washout. N=3. C) MDA-MB-231 cells were treated with above drugs for 72h before drug

973 washout. Cells were collected every 2 days after washout for up to 6 days. Immunoblotting was

974 performed on proliferation markers and TUFM. Representative images of N=2. D) Weights of mice

975 from experiment described in Figure 5D-E. E) 4T1-Luc cells were treated with 0.1% DMSO or 150nM

976 TR-57 for 24-72h and immunoblotting was performed on proliferation markers and TUFM to

977 demonstrate ClpP activation. Representative images of N=2.

978 **Supplementary Figure 6: Venetoclax or Olaparib Co-Treatment with ClpP Agonists Increase**

979 **Cell Death in TNBC Cells**

980 A-B) Example gating scheme and representative plots for SUM159 and MDA-MB-231 cells

981 respectively. C) SUM159 cells were treated with 0.1% DMSO, 10μM venetoclax (Veneto), or 25nM

982 TR-107 for 72h. Annexin V/PI staining was quantified to determine %Apoptotic (Annexin V+) or

983 %Dead (PI+). N=3.

984

985 **Supplementary Figure 7: Effects of TR-57 are not reversed by NAC or nucleotide**

986 **supplementation while doxorubicin co-treatment induces additive effects**

987 A) MDA-MB-231 cells were treated with 0.1% DMSO (D), 1μM Rotenone (R), 150nM TR-57 (TR), and/or

988 5mM N-acetyl cysteine (NAC) for 48h. Immunoblots of γH2A.X were performed to assess changes in

989 DNA damage. Representative images of N=2. B) MDA-MB-231 cells were treated with 0.1% DMSO,

990   50μM Teriflunomide (TF), 150nM TR-57 (TR), and/or 30μM Uridine, 100μM Cytidine, and 100μM

991   Thymidine (U+C+T), or 100μM Adenosine and 100μM Guanosine (A+G) for 72h. Representative

992   images of N=2. C) SUM159 cells were treated with varying concentrations of TR-57 with doxorubicin

993   titrated in. Cell count and viability were measured after 72h of co-treatment. HSA plot indicates

994   additive effects with co-treatment. Representative of N=2 experiments.

A
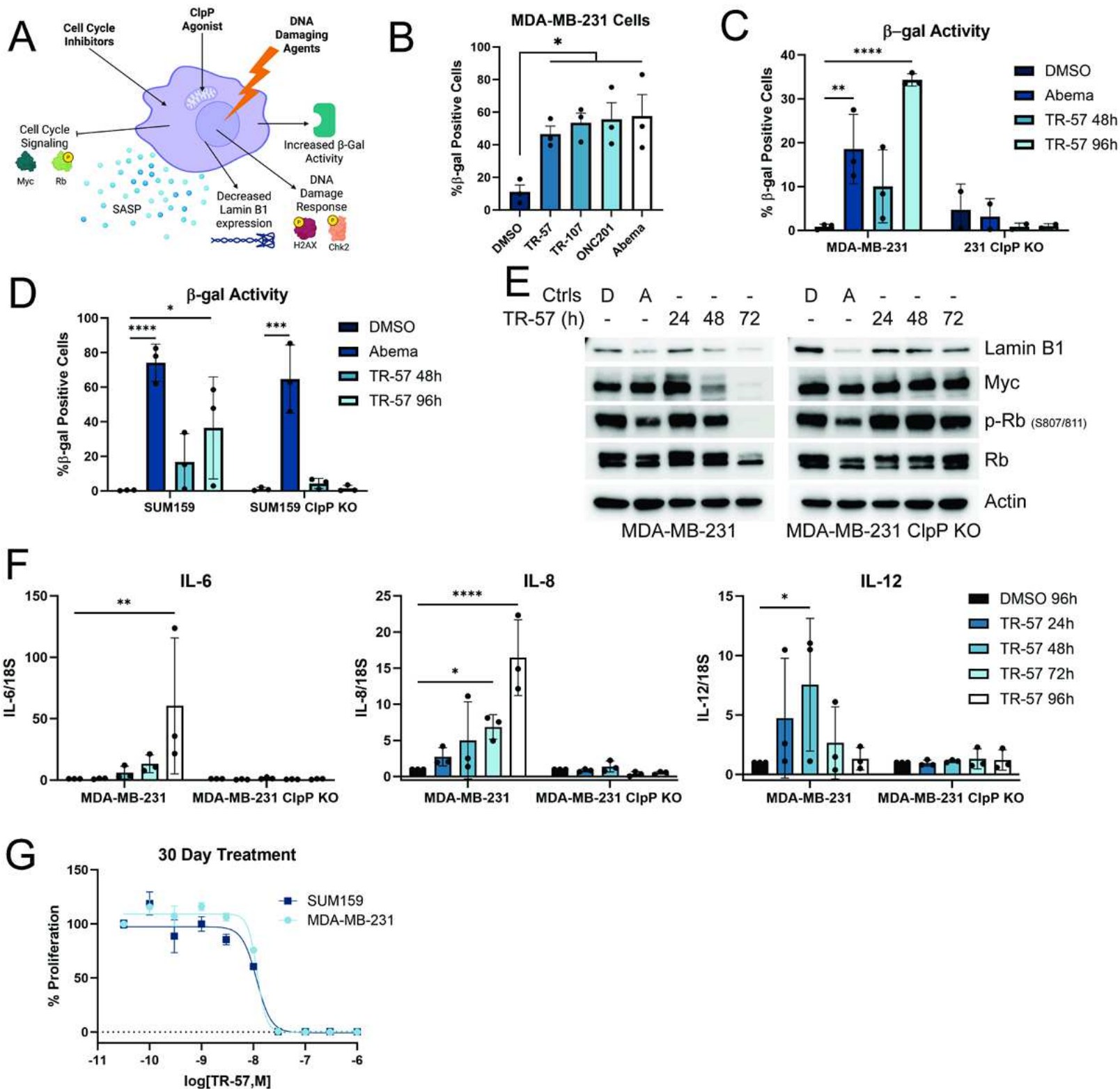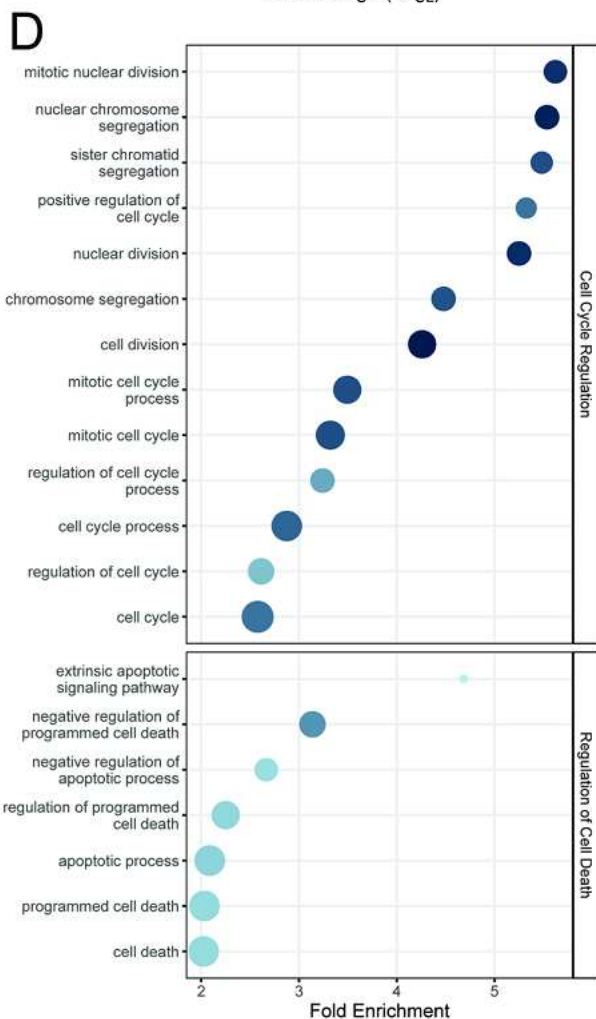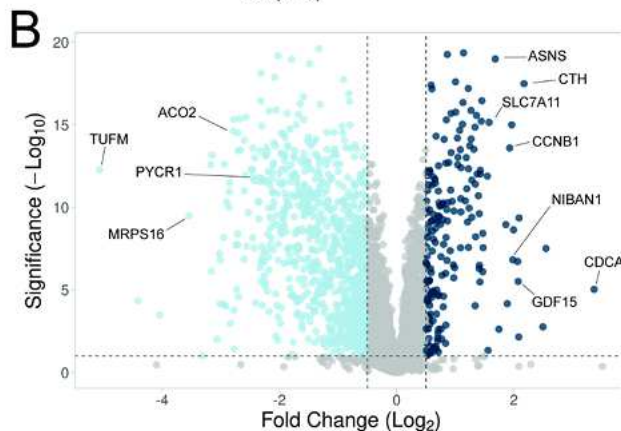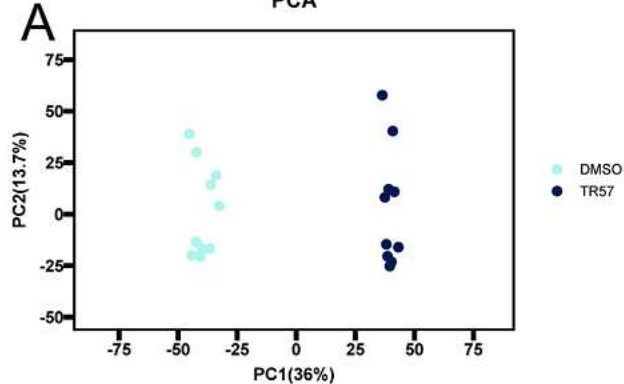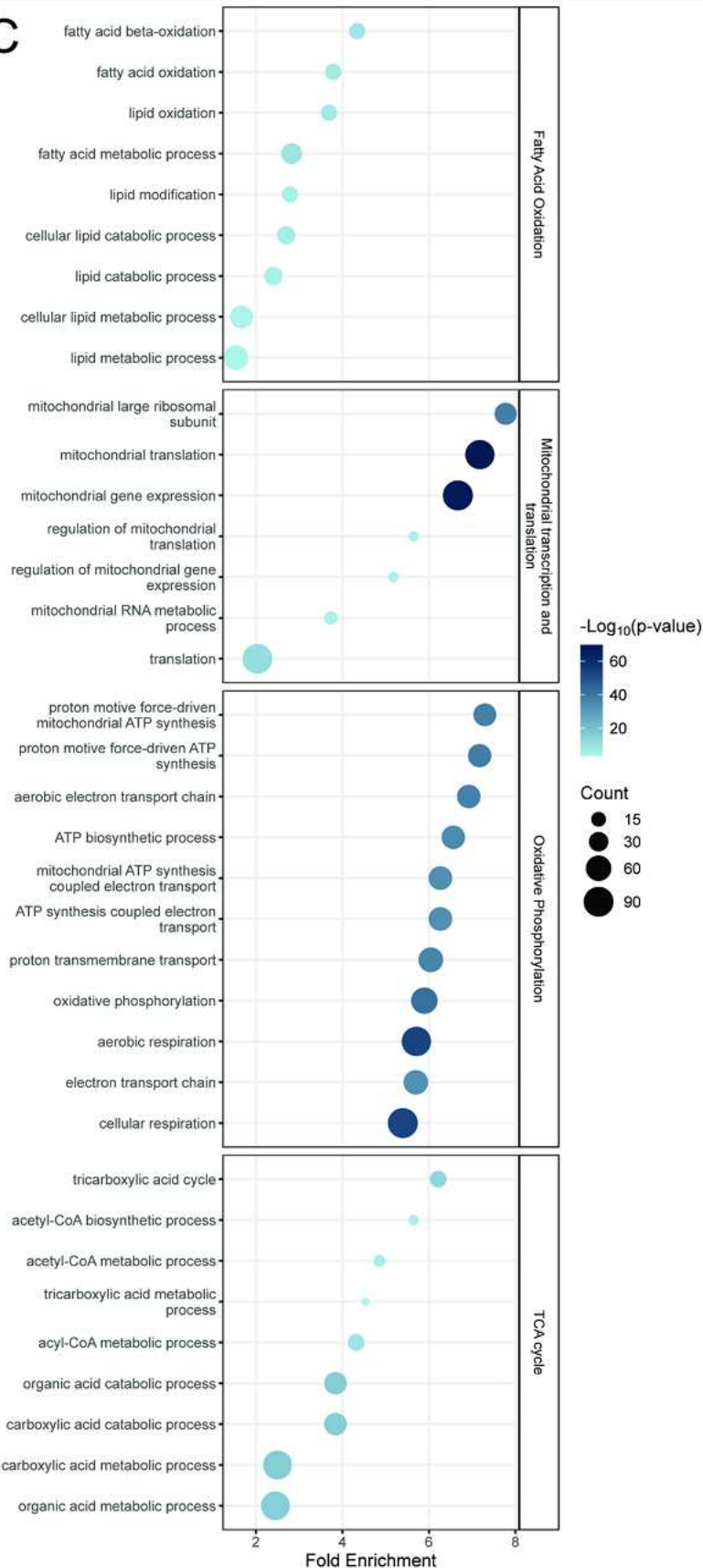
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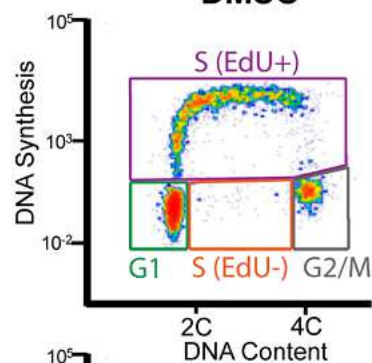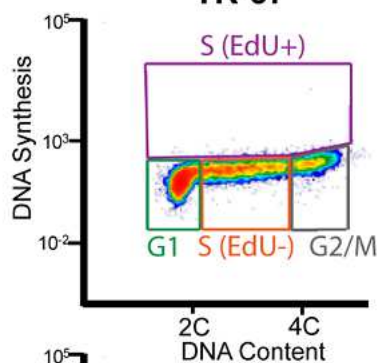
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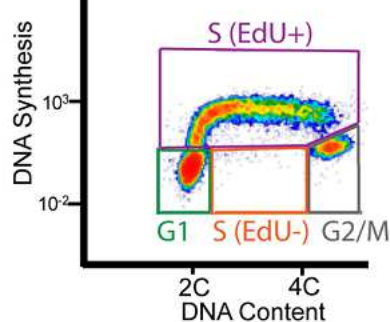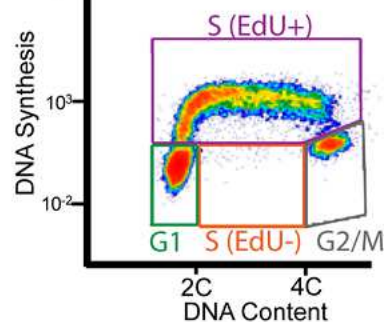
MDA-MB-231 ClpP KO

B

MDA-MB-231   MDA-MB-231 ClpP KO
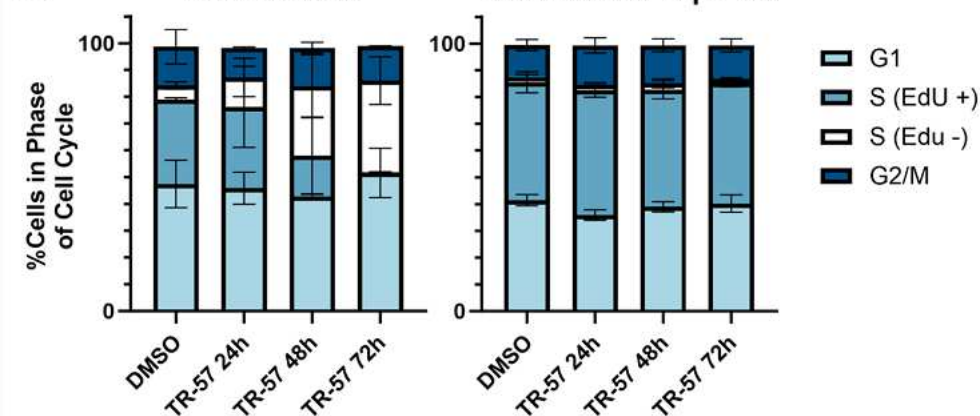
C

SUM159   SUM159 ClpP KO

D

TR-57 (h)   D 24 48 72

Cyclin A2
Cyclin D3
Cyclin E1
Skp2
p21
Vinculin

A Ctrls: D I - - -
TR-57 (h): - - 24 48 72 | D I - - - / - - 24 48 72
p-Chk2 (T68), Chk2, γH2A.X, Vinculin
SUM159 | SUM159 ClpP KO

B p-S*Q | 53BP1 | Merged+Hoechst
DMSO / Irinotecan / TR-57

C γH2A.X | 53BP1 | Merged+Hoechst
DMSO / Irinotecan / TR-57

D DNA damage foci
Foci/Cell
p-S*Q: DMSO, Irino ****, TR-57 ****
53BP1: DMSO, Irino ****, TR-57 ****
γH2A.X: DMSO, Irino ****, TR-57 ****

E M2 (53BP1/p-S*Q)
DMSO, Irino ****, TR-57 ****

F M2 (53BP1/γH2A.X)
DMSO, Irino ****, TR-57 ****

G DMSO | Irinotecan | TR-57

H %DNA in Tail
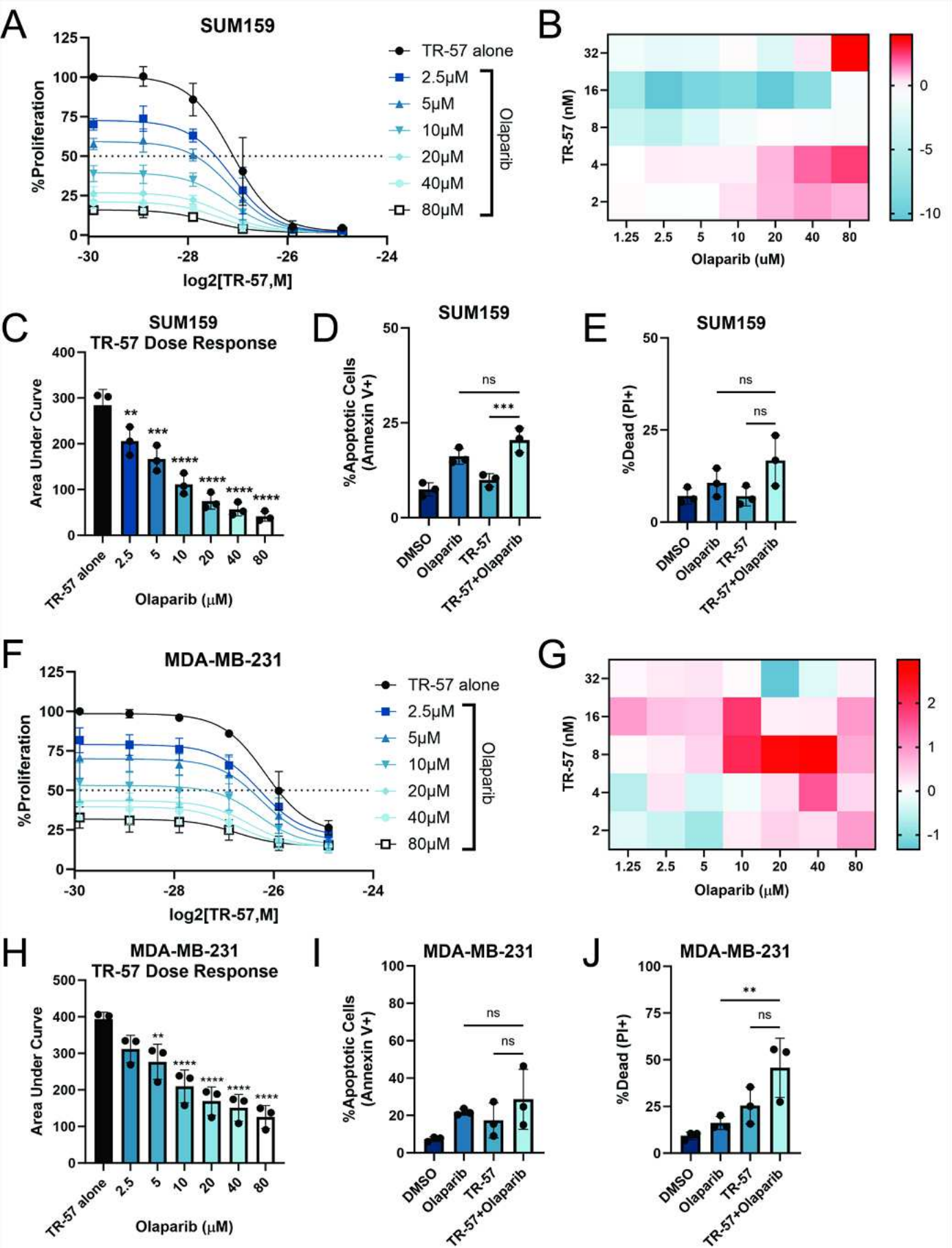DMSO, Irino ****, TR-57 ****

Tail Moment
DMSO, Irino ****, TR-57 ****

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMethodTables.docx
- UncroppedWesternblots.pdf
- SuppFigure7PotentialcausesofDNAdamage.tif
- SuppFigure5WOmousedata.tif
- SuppFigure6TR57VenSynergy.tif
- SuppFigure3SphasearrestFlow.tif
- SuppFigure2ProandAntiApoptoticProteins.tif
- SuppFigure4DNAdamage.tif
- SuppFigure1Senescence.tif