Mechanical forces inducing oxaliplatin resistance in pancreatic cancer can be targeted by autophagy inhibition

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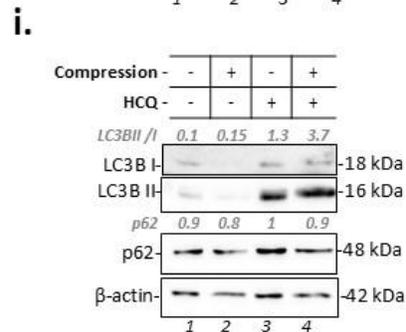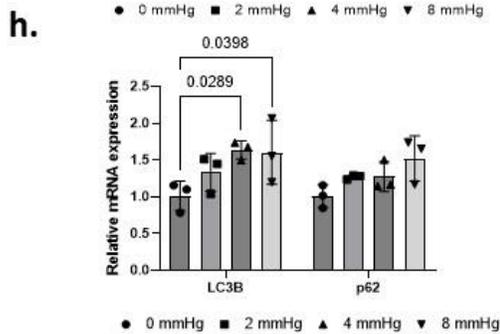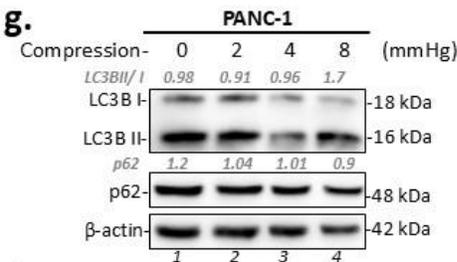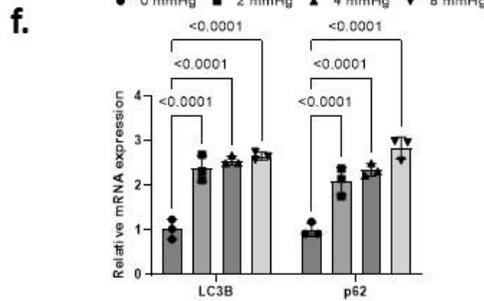
[2] Materia Therapeutics, Las Vegas, NV, United States

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14 **Supplementary Figures**

a.

b.

c. KPC

Compression- 0 2 4 8 (mmHg)

LC3BII/I 0.49 2.73 1.3 1.52

LC3B I- —18 kDa
LC3B II- —16 kDa

p62 0.85 1.21 0.82 0.62

p62- —48 kDa

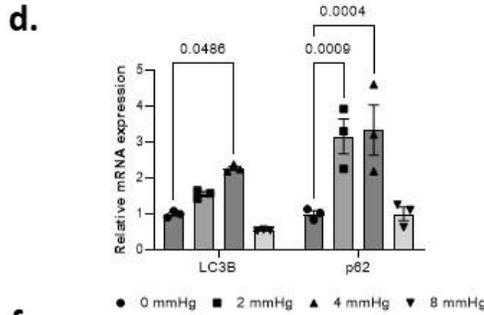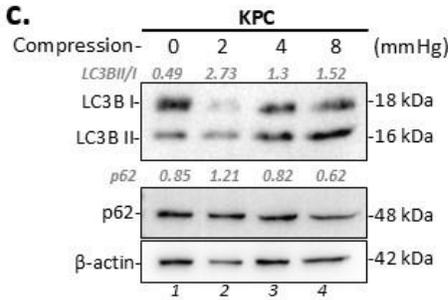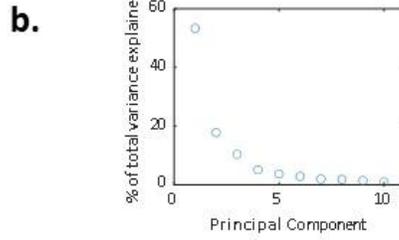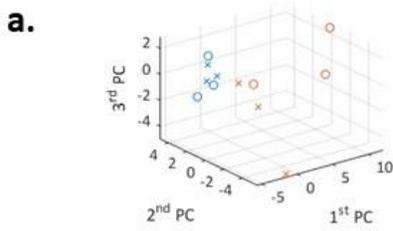β-actin- —42 kDa

1 2 3 4

d.

0.0486

0.0004

0.0009

Relative mRNA expression

LC3B   p62

• 0 mmHg ■ 2 mmHg ▲ 4 mmHg ▼ 8 mmHg

e. Pan02

Compression- 0 2 4 8 (mmHg)

LC3BII/I 0.46 0.9 0.8 0.9

LC3B I- —18 kDa
LC3B II- —16 kDa

p62 1.06 1.08 0.98 0.8

p62- —48 kDa

β-actin- —42 kDa

1 2 3 4

f.

<0.0001
<0.0001   <0.0001
<0.0001   <0.0001
<0.0001

Relative mRNA expression

LC3B   p62

• 0 mmHg ■ 2 mmHg ▲ 4 mmHg ▼ 8 mmHg

g. PANC-1

Compression- 0 2 4 8 (mmHg)

LC3BII/ I 0.98 0.91 0.96 1.7

LC3B I- —18 kDa
LC3B II- —16 kDa

p62 1.2 1.04 1.01 0.9

p62- —48 kDa

β-actin- —42 kDa

1 2 3 4

h.

0.0398
0.0289

Relative mRNA expression

LC3B   p62

• 0 mmHg ■ 2 mmHg ▲ 4 mmHg ▼ 8 mmHg

i.

Compression - - + - +
HCQ - - - + +

LC3BII /I 0.1 0.15 1.3 3.7

LC3B I- —18 kDa
LC3B II- —16 kDa

p62 0.9 0.8 1 0.9

p62- —48 kDa

β-actin- —42 kDa

1 2 3 4

15

16

17 **Suppl. Fig. 1.** Principal component analysis of RPPA dataset from MIA PaCa-2 cells which subjected to either
18 zero or four mmHg pressure for 16 hours or 48 hours. **a,** Representation of the RPPA dataset into a 3D space

19   defined by the first three principal components. **b,** Percent of total variance explained by each principal
20   component. **c, e, g,** Representative immunoblotting showing the levels of LC3BI, LC3BII and p62 in KPC (c),
21   Pan02 (e) and PANC-1 (g) cells exposed to different levels of compression for 24 hours. Antibody against β-
22   actin was used as a loading control. Quantification of each antibody compared with loading control was
23   quantified by ImageJ. Numbers in gray font represent the ratio of LC3BII/I (indicator of autophagic vacuoles)
24   and p62 (indicator of autophagic degradation) expression normalized to β-actin. **d, f, h** *LC3B* and *p62* mRNA
25   expression was quantified by qPCR in control and compressed KPC (d), Pan02 (f) and PANC-1 (h) cells. Each
26   bar indicates the mean fold change ±SE (n=3 independent samples). Differences were statistically significant
27   when P < 0.05 in two-way ANOVA analysis (Tukey's multiple comparisons test). **i,** Representative
28   immunoblotting showing the levels of LC3BI, LC3BII and p62 in MIA PaCa-2 cells exposed to 4 mmHg of
29   compression for 24 hours in the presence or not of 10 μM HCQ. Antibody against β-actin was used as a
30   loading control. Quantification of each antibody compared with loading control was quantified by ImageJ.
31   Numbers in gray font represent the ratio of LC3BII/I and p62 expression normalized to β-actin.
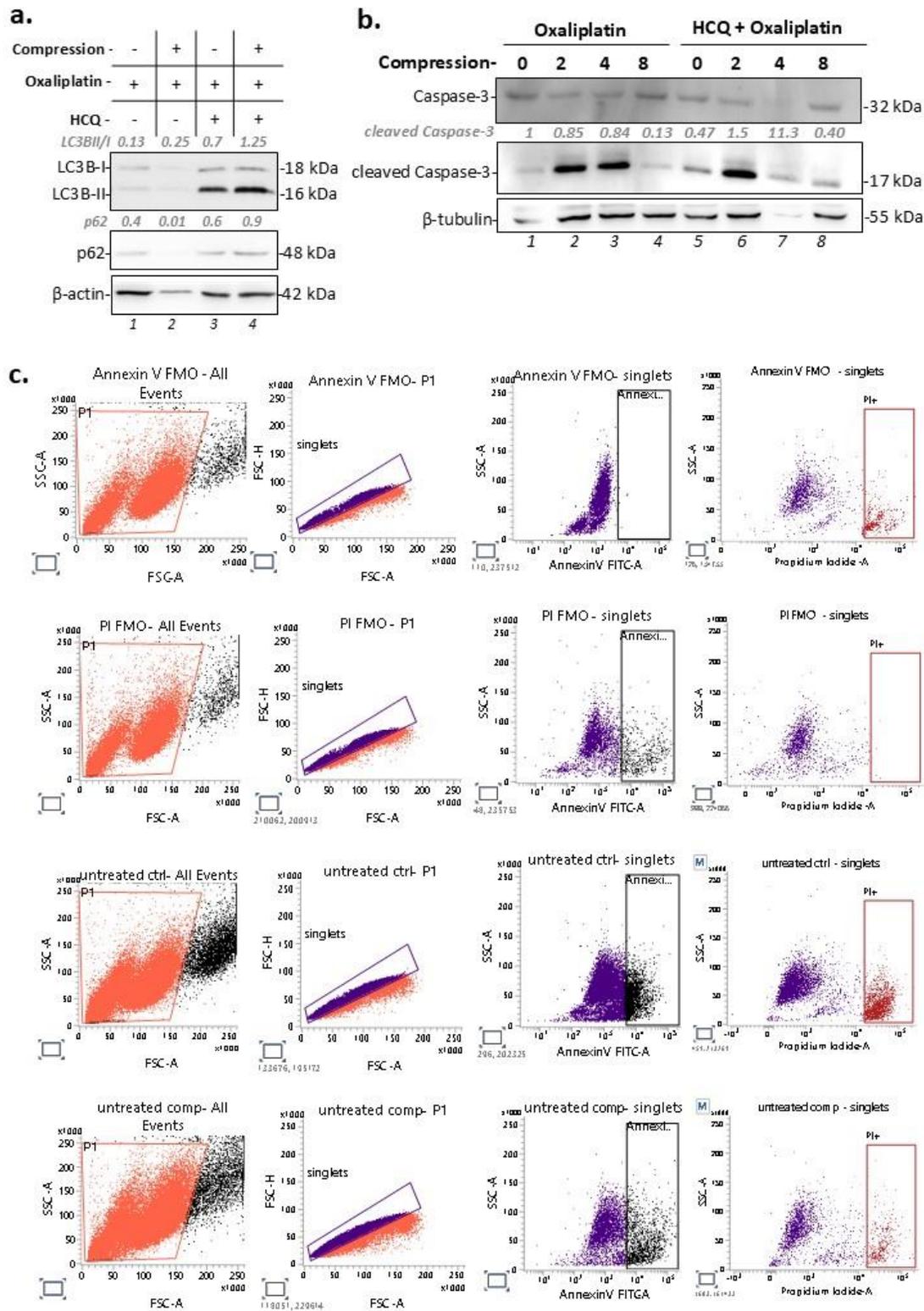
32
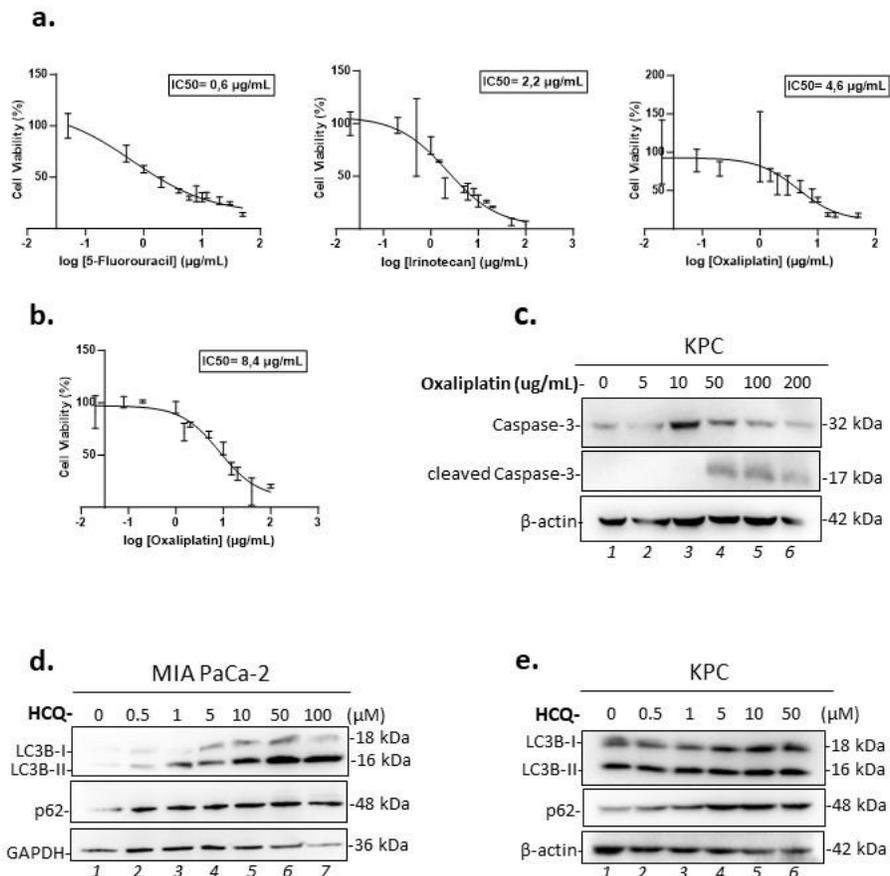
33

34   **Suppl. Fig. 2.** Identification of working concentrations of Oxaliplatin and HCQ in MIA PaCa-2 and KPC cells
35   through IC50 curves and western blotting. **a-b**, MIA PaCa-2 (a) or KPC (b) cells were treated with indicated
36   concentrations of each drug for 24 hours. Resazurin assay was then performed, and graphs represent the

37 average fold change of % difference ±SE in Resazurin reduction using as a reference the untreated cells (n=3
38 independent samples). **c**, Representative immunoblotting showing the levels of caspase 3 in KPC cells
39 treated with the indicated concentrations of oxaliplatin for 24 hours. Antibody against β-actin was used as
40 a loading control. **d-e**, Representative immunoblotting showing the levels of LC3BI, LC3BII and p62 in MIA
41 PaCa-2 (d) and KPC (e) cells treated with the indicated concentrations of HCQ for 24 hours. Antibody against
42 GAPDH (d) and β-actin (e) was used as a loading control.

43

44

a.

Compression -	-	+	-	+
Oxaliplatin -	+	+	+	+
HCQ -	-	-	+	+

LC3BII/I 0.13 0.25 0.7 1.25

LC3B-I- ―18 kDa
LC3B-II- ―16 kDa

p62 0.4 0.01 0.6 0.9

p62- ―48 kDa

β-actin- ―42 kDa

1   2   3   4

b.

Oxaliplatin   HCQ + Oxaliplatin

Compression- 0 2 4 8   0 2 4 8

Caspase-3- ―32 kDa

cleaved Caspase-3 1 0.85 0.84 0.13 0.47 1.5 11.3 0.40

cleaved Caspase-3- ―17 kDa

β-tubulin- ―55 kDa

1 2 3 4 5 6 7 8

c.

45

Suppl. Fig. 3. a, Representative immunoblotting showing LC3BI, LC3BII and p62 protein levels in control and compressed MIA PaCa-2 cells treated with oxaliplatin and/ or HCQ for 24 hours. Antibody against β-actin was used as a loading control. Quantification of each antibody compared with loading control was quantified

49 by ImageJ. Numbers in gray font represent the ratio of LC3BII/I and p62 expression in each condition
50 normalized to β-actin. **b,** Representative immunoblotting showing Caspase 3 and cleaved Caspase 3 protein
51 levels in control and compressed KPC cells treated with HCQ (10 µM) combined or not with Oxaliplatin (100
52 µg/mL) for 24 hours. Antibody against β-tubulin was used as a loading control. Quantification of each
53 antibody compared to loading control was quantified by ImageJ. Numbers in gray font represent the average
54 ratio of cleaved Caspase 3 / β-actin protein expression in each condition (n=2 replicates from 2 independent
55 experiments). **c**, Gating strategy for Annexin V+ and PI+ cells. The first two rows represent the Fluorescent
56 Minus One (FMOs) control samples, for the selection of Annexin V+ and PI+ cells. The other two rows
57 represent the selection strategy of the Annexin V+/ PI+ untreated control and compressed cells, respectively.

58
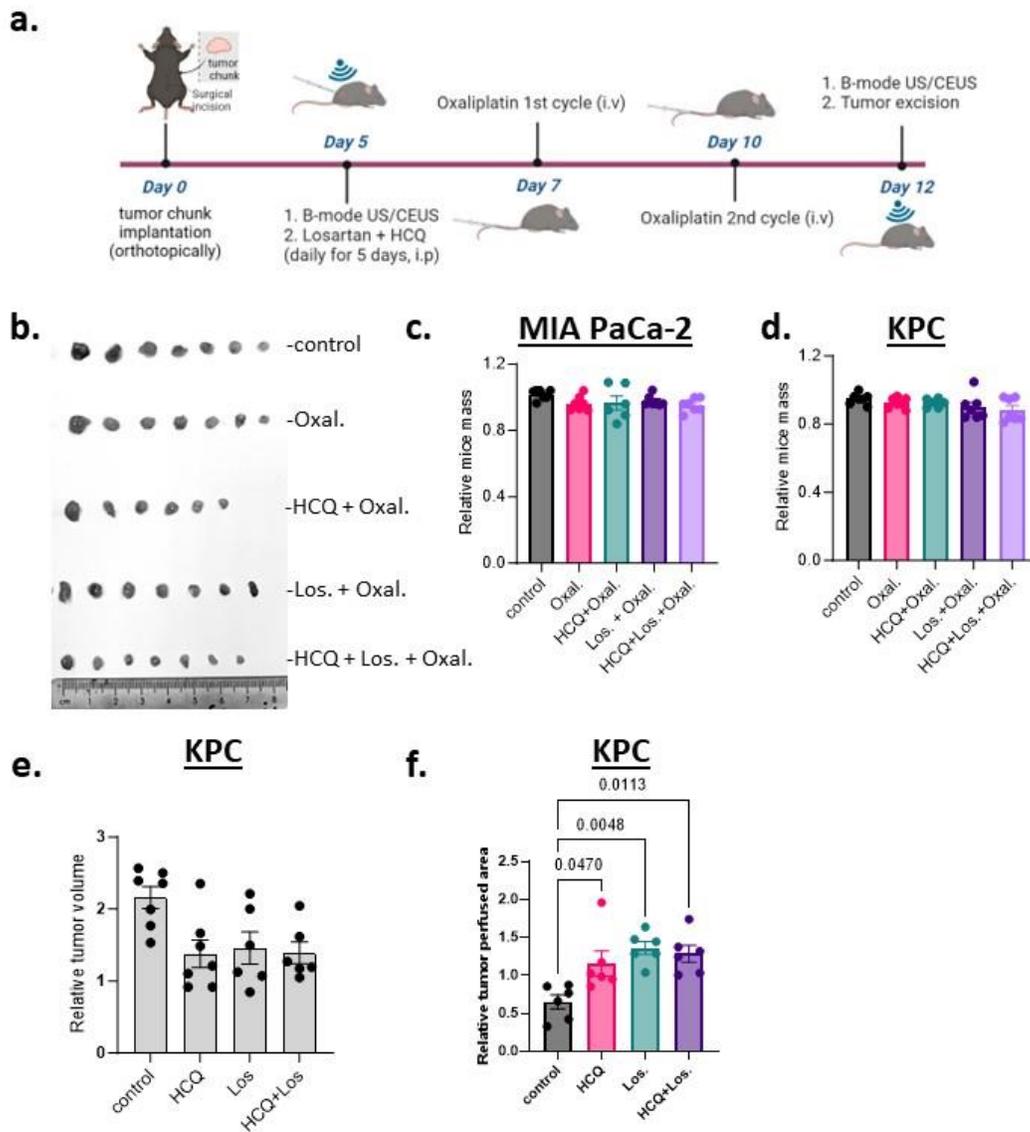
59

60 **Suppl. Fig. 4. a,** Study treatment protocol for the KPC tumor model. Losartan and HCQ were administered
61 at 40- and 50 mg/kg daily, respectively, via intraperitoneal injection (i.p.) once tumor size reached an

62 average volume of 50 mm^3. Two cycles of Oxaliplatin treatment (5 mg/kg) were administered via intravenous
63 injection (i.v.) starting on day 7, allowing Losartan to prime the TME. **b,** Images of MIA PaCa-2 tumors derived
64 from mice of indicated treatments that were excised at day 22. **c-d,** Graphs represent relative mice mass
65 changes in MIA PaCa-2 and KPC bearing mice treated as indicated (n=7 mice per group; n=6 mice in the
66 HCQ+Oxal.-treated group). Data are presented as mean ± SE. Statistical analyses were performed by
67 comparing means between two independent groups using the two-way ANOVA test with Tukey's multiple
68 comparison test. P-values less than 0.05 are denoted on the bar graph. **e,** The effect of HCQ, Losartan or
69 their combination on the relative tumor growth of KPC models (n=6 or 7 mice per group). Data are presented
70 as mean ± SE. Statistical analyses were performed by comparing means between two independent groups
71 using the two-way ANOVA test (Tukey's multiple comparisons test). No statistically significant differences
72 were observed. **f,** The effect of HCQ, Losartan or their combination on the relative tumor perfusion as
73 measured by CEUS (between Day 5 and Day 12) of KPC model (n=6 mice per treatment group). Data are
74 presented as mean ± SE. Statistical analyses were performed by comparing means between two
75 independent groups using the two-way ANOVA test (Tukey's multiple comparisons test). P-values less than
76 0.05 are denoted on the bar graph. Panel (**a**) was created with BioRender.com.

77
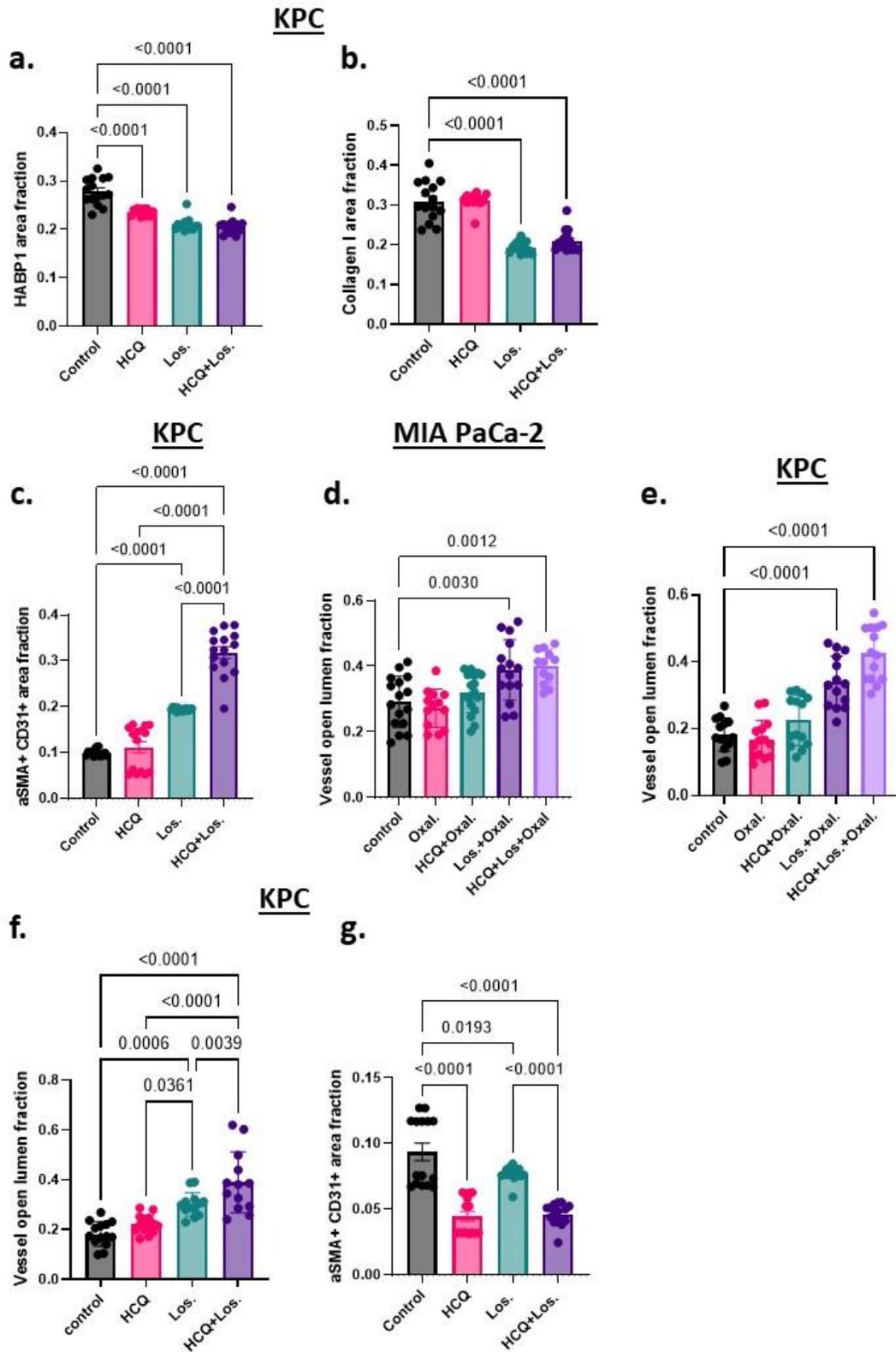
Suppl. Fig. 5. a-b, Quantification of the fraction of area positive for HABP1 (a) and Collagen I (b) stain

normalized to DAPI stain in KPC tumor tissue sections. **c,** Quantification of vessel pericyte coverage as

80 indicated by CD31 and a-SMA overlapping staining normalized to DAPI stain in KPC tumors. **d-f,**
81 Quantification of vessel open lumen fraction as indicated by CD31 staining in MIA PaCa-2 (d) and KPC (e,f)
82 tumors of the indicated treatments. **g,** Quantification of a-SMA stain normalized to DAPI stain in KPC tumors.
83 Data are presented as mean ± SE (n=2-3 tumors per condition; at least 5 image fields per condition).
84 Statistical analyses were performed by comparing means between two independent groups using the two-
85 way ANOVA multiple comparison test (Tukey's correction). P-values less than 0.05 are denoted on the bar
86 graphs.

87

88

89

Supplementary Table 1. Primer sequences for qPCR				
Target gene	Human		Murine	
	Forward	Reverse	Forward	Reverse
LC3B	GAGAAGCAGCTTCCTGTTCTGG	GTGTCCGTTCACCAACAGGAAG	CCACGTGAACATGAGCGAG	AACCATGTACAGGAAGCCGT
p62	CCAGAGAGTTCCAGCACA	CCGACTCCATCTGTTCCT	GGACAGCCAGAGGAACAGAT	GGGAGATGTGGGTATAGGGC
actin	CGAGCACAGAGCCTCGCCTTTGCC	TGTCGACGACGAGCGCGGCGA	GACGGCCAGGTCATCACTAT	AAGGAAGGCTGGAAAAGAGC

90

Supplementary Table 2. p-value with multiple way ANOVA		
	Different time points	Different pressures
1st principal component	0.14	0.096
2nd principal component	0.39	0.0081*
3rd principal component	0.48	0.77

91

92

93

94   **Western Blotting Original Images**

95   <u>**Figure 1g.**</u>

96   **Blotting with a-LC3B only**

97

-35 kDa
-25 kDa
-18 kDa
-16 kDa
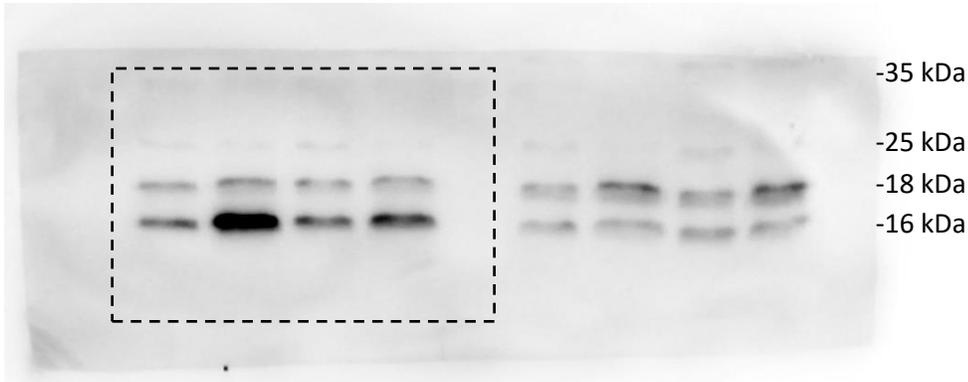
98   **Blotting with anti-p62 only**

99

-48 kDa
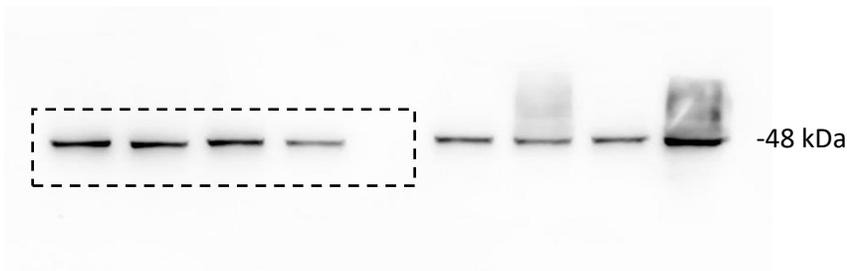
100   **Blotting with anti-b-actin on p62**

-48 kDa
-42 kDa
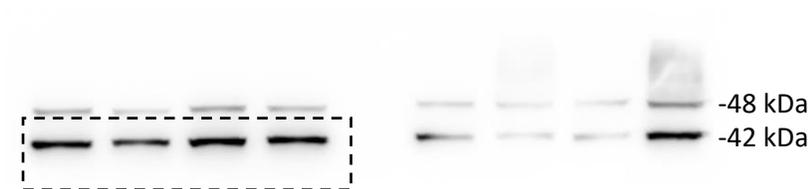
101

102   <u>**Figure 1h**</u>

103   **Blotting with anti-Bcl-2**

-26 kDa
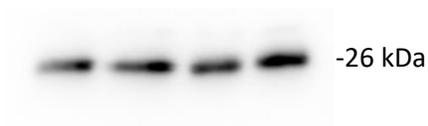
104

105   **Blotting with anti-b-actin on Bcl-2**

-42 kDa

106

107 **Blotting with Bcl-xL**

-26 kDa

108

109 **Blotting with anti-b-actin on Bcl-xL**

-42 kDa

110

111

112 <u>**Suppl. Fig. 1c**</u>

113 **Blotting with anti-LC3B only**   **Blotting with anti-p62 only**

-35 kDa

-25 kDa
-18 kDa
-16 kDa

-55 kDa

-48 kDa

114
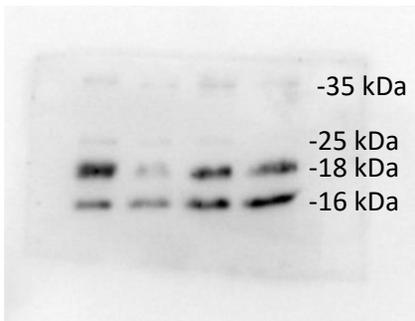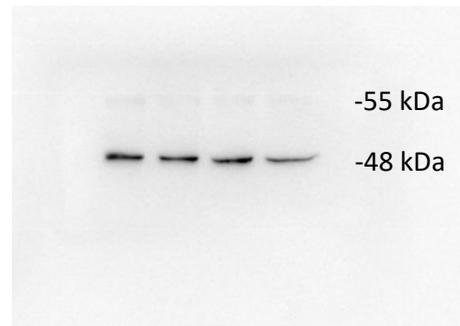
115 **Blotting with anti-beta-actin on p62**

-42 kDa
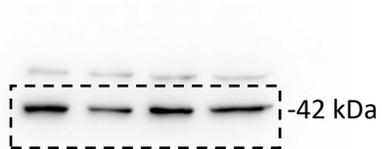
116

117 <u>**Suppl. Fig. 1e**</u>

118 **Blotting with anti-LC3B only**
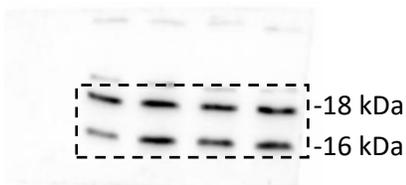
-18 kDa
-16 kDa

119

120

121

122

Blotting with anti-p62

-48 kDa
-42 kDa

124

Blotting with anti-b-actin

-48 kDa
-42 kDa

126

Supplementary Fig. 1g

Blotting with anti-LC3B only

-35 kDa

-18 kDa
-16 kDa

129

Blotting with anti-p62 only

-48 kDa

131

Blotting with anti-b-actin on p62

-48 kDa

-42 kDa

133

134

135
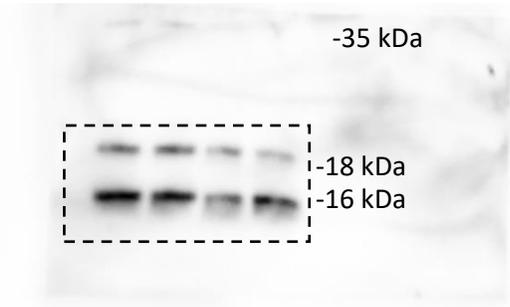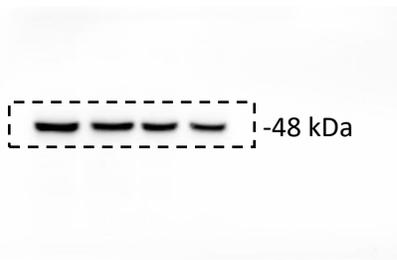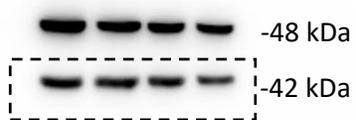
136 **Blotting with anti-LC3B** *(enhanced contrast/brightness for LC3BI)*

-18 kDa
-16 kDa
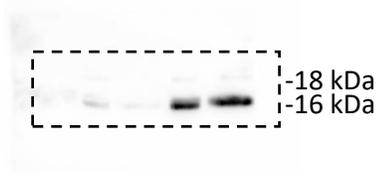
137

138 **Blotting with anti-p62**

-48 kDa
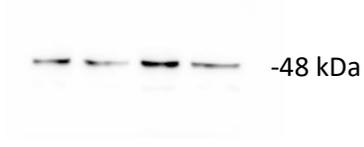
139

140 **Blotting with anti-b-actin on p62**

-48 kDa
-42 kDa
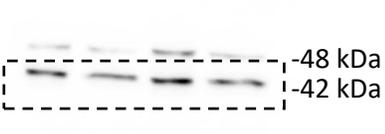
141

142 **Figure 2c**

143 **Blotting with anti-LC3B only**

-35 kDa

-18 kDa
-16 kDa
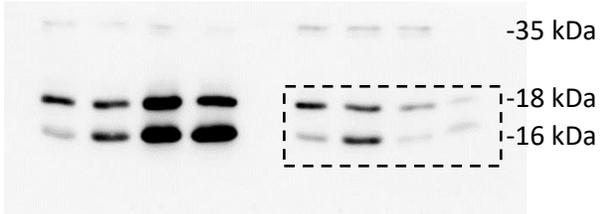
144

145

146 **Blotting with anti-p62 only**

147

-48 kDa
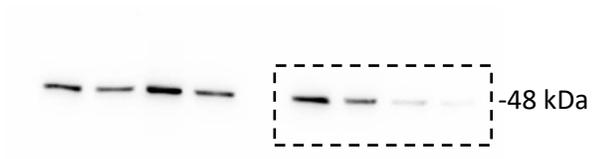
148

149 **Blotting with anti-b-actin on p62**

150

-42 kDa

151

152
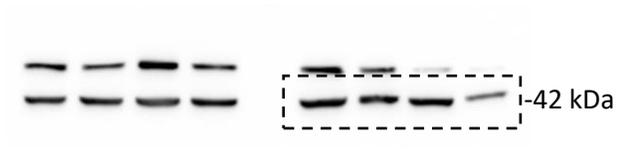
Supplementary Fig. 2d

Blotting with anti-LC3B

-18 kDa

-16 kDa

155

Blotting with anti-p62

-48 kDa

-35 kDa

157
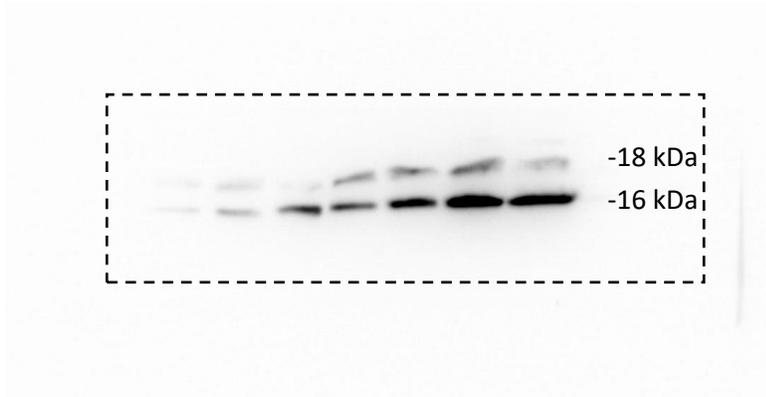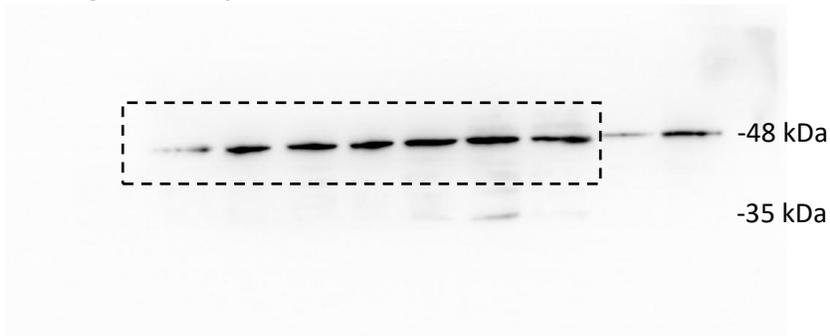
Blotting with anti-GAPDH on anti-p62

-55 kDa

-48 kDa

159

160
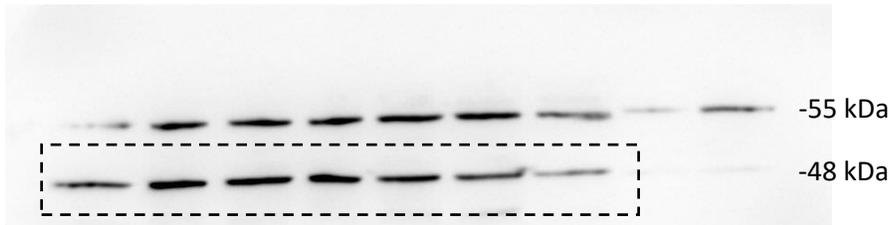
Supplementary Fig.2e

Blotting with anti-LC3B only

-35 kDa

-25 kDa

-18 kDa

-16 kDa
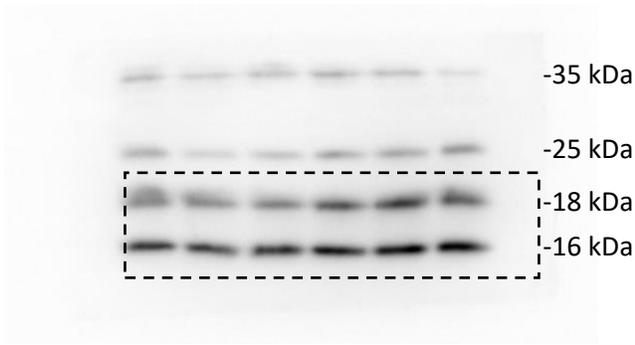
163

164   **Blotting with anti-p62**

-55 kDa

-48 kDa
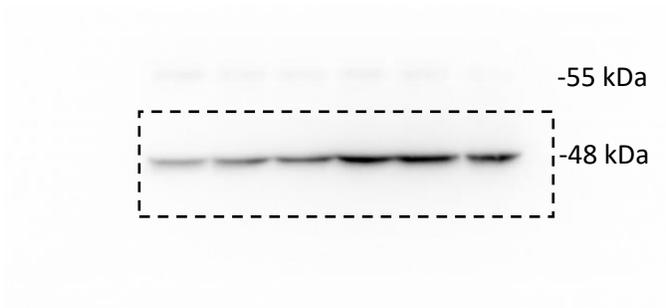
165

166   **Blotting with anti-beta-actin on anti-p62**

-55 kDa

-48 kDa

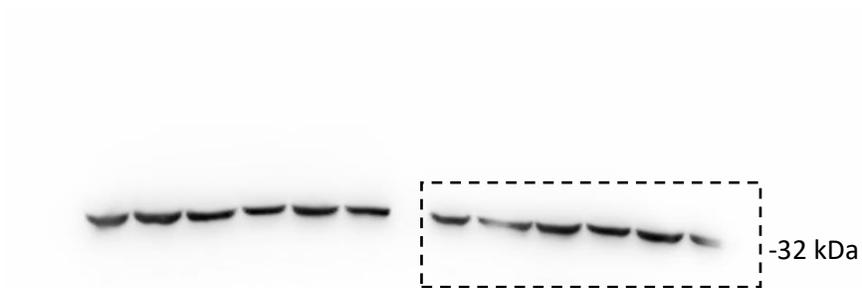-42 kDa
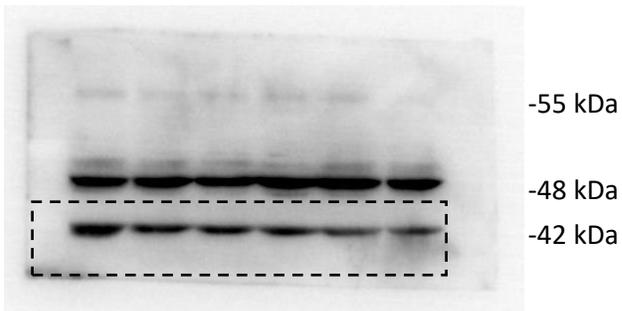
167

168

169   **Supplementary Fig. 2c**

170   **Blotting with anti-Caspase 3**
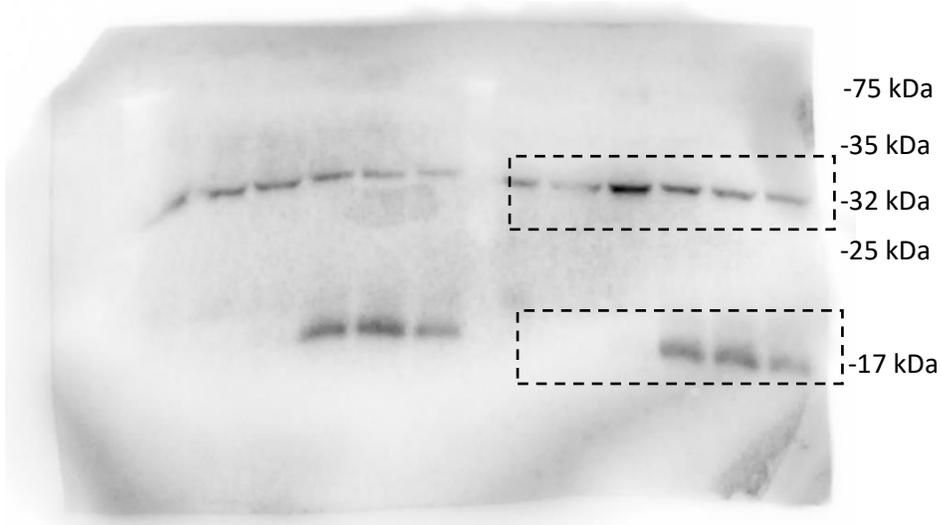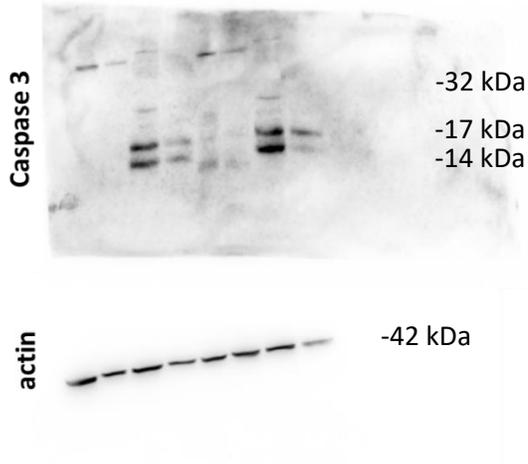
171

172 **Blotting with actin on anti-Caspase 3**

173

174

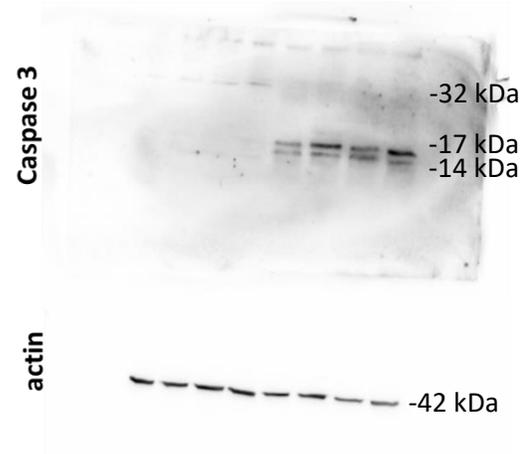175 <u>**Figure 3b (2 blots from 2 biological replicates)**</u>

176 **#1**   **#2**

Compression	-	-	+	-	+	-	+	-	+
Oxaliplatin	-	-	-	+	+	-	-	+	+
HCQ	-	-	-	-	-	+	+	+	+

Caspase 3

-32 kDa
-17 kDa
-14 kDa

actin

-42 kDa

Compression	-	-	+	-	+	-	+	-	+
HCQ	-	-	-	+	+	-	-	+	+
Oxaliplatin	-	-	-	-	-	+	+	+	+

Caspase 3

-32 kDa
-17 kDa
-14 kDa

actin

-42 kDa

177

178

179

180

181

182

183

184

185

186

187 **Suppl. Fig. 3a**

188 **Blotting with anti-LC3B only**

-18 kDa
-16 kDa
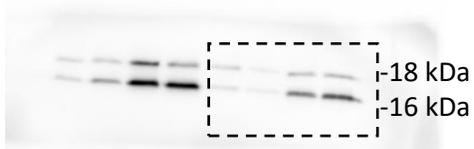
189

190 **Blotting with anti-p62 only**

-48 kDa

191

192 **Blotting with anti-b-actin on p62**

-55 kDa

-42 kDa

193

194

195 <u>**Suppl. Fig. 3b**</u>

196 **Blotting with caspase 3**

197

-55 kDa

-32 kDa

-17 kDa
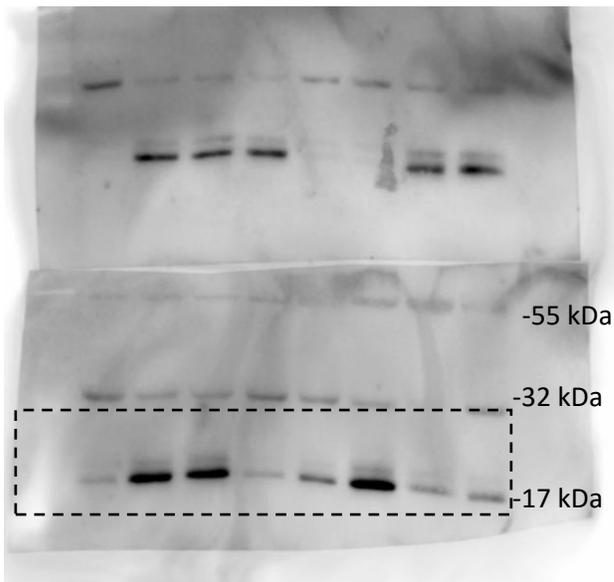
198

199 **Blotting with b-tubulin**

-60 kDa
-55 kDa
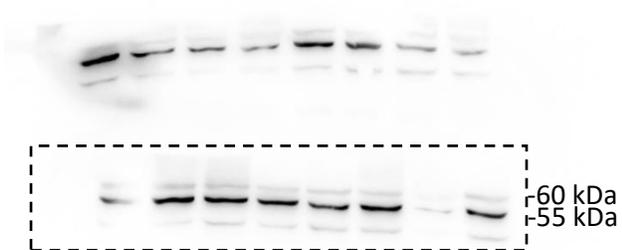
200

201

202

203 <u>**Figure 7c. (MIA PaCa-2)**</u>

204   **Blotting with anti-caspase 3**

-25 kDa

-17 kDa
-14 kDa
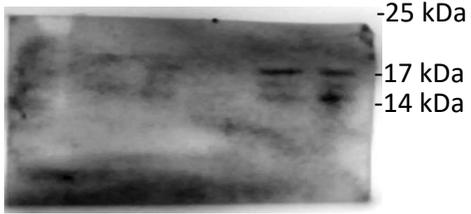
205

206

207   **Blotting with anti-PARP1**

-89 kDa
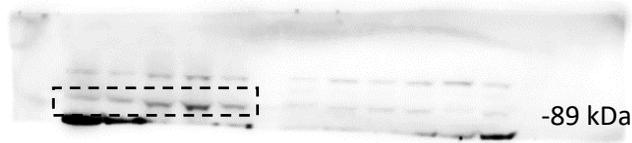
208

209   **Blotting with anti-b-actin on Caspase 3**
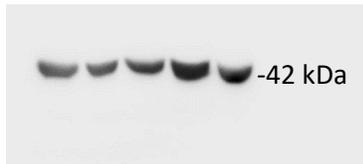
-42 kDa

210

211

212   **Blotting with anti-Bcl-2**

-26 kDa
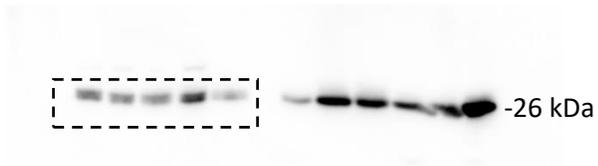
213

214   **Blotting with anti-b-tubulin on Bcl-2**

-75 kDa
-55 kDa

215

216

217   **Blotting with anti-Bcl-xL**

-75 kDa
-55 kDa

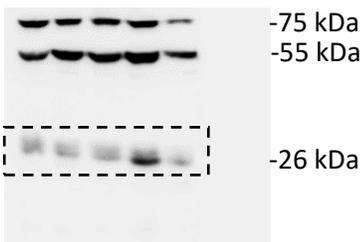-26 kDa

218

219   **Blotting with anti-b-actin on Bcl-xL**

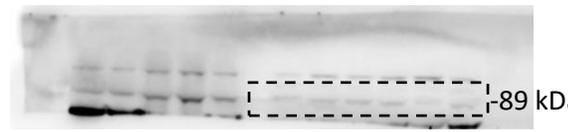-55 kDa

-42 kDa

220

221

222   **Figure 7c (KPC)**

223   **Blotting with anti-caspase 3**

-48 kDa

-17 kDa
-14 kDa

224

225   **Blotting with anti- PARP1 (signal was then enhanced using ImageJ for all samples)**

-89 kDa

226

227   **Blotting with anti-b-tubulin on PARP1**

-55 kDa

228

229

230

231   **Blotting with Bcl-2**

-26 kDa

232

233   **Blotting with anti-b-actin on Bcl-2**

-42 kDa

234

235

236   **Blotting with anti Bcl-xL**

-26 kDa

237

238 **Blotting with b-actin on Bcl-xL**

-42 kDa

-26 kDa

239
240
241