

1 **Serum-dependent and independent regulation of PARP2**

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15 **Abstract:** PARP2 belongs to a family of proteins involved in cell differentiation, DNA
16 damage repair, cellular energy expenditure, chromatin modeling and cell differentiation.
17 In addition to these overlapping functions with PARP1, PARP2 participates in
18 spermatogenesis, T-cell maturation, extraembryonic endoderm formation and
19 adipogenesis. The function(s) of PARP2 is far from complete, and the mechanism(s) by
20 which the gene and protein are regulated are unknown. In this study, we found that two
21 different mechanisms are used *in vitro* to regulate PARP2 levels. In the presence of serum,
22 PARP2 is degraded through the ubiquitin-proteasome pathway, however, when serum is
23 removed, PARP2 is rapidly sequestered into an SDS- and urea-insoluble fraction. This
24 sequestration is relieved by serum in a dose-dependent manner, and again PARP2 is
25 detected by immunoblotting. Furthermore, and despite the presence of a putative serum
26 response element in the *PARP2* gene, transcription is not affected by serum deprivation.
27 These observations that PARP2 is tightly regulated by distinct pathways highlights the
28 critical roles PARP2 plays under different physiological conditions.

29

30 *Key Words:* PARP2, ubiquitin, proteasome, proteolysis.

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33 **Introduction**

34 The poly-ADP-ribose polymerase (PARP) enzymes belong to a large family of
35 proteins, including 17 in humans (Leung 2014), that have integral roles in DNA repair, and
36 thus are studied extensively as targets to inhibit a variety of cancers (Ali et al. 2016).
37 PARPs are also involved in the inflammatory response, transcription, mitochondrial
38 function, oxidative metabolism and heterochromatin function (Ali et al. 2016; Bai and
39 Canto 2012; Chen et al. 2018; Dantzer and Santoro 2013; Gupte et al. 2017; Hottiger 2015;
40 Jeggo 1998; Krishnakumar and Kraus 2010). PARPs catalyze the formation of free poly-
41 ADP-ribose polymers as well as poly-ADP-ribosylated (pARYlated) proteins using NAD⁺
42 as a substrate (Kraus 2015; Nicolas et al. 2010). PARP1 and PARP2 share similar crystal
43 structure and function (Hanzlikova et al. 2017; Oliver et al. 2004), and both proteins homo-
44 and heterodimerize and poly ADP-ribosylate each other (Schreiber et al. 2002). Despite
45 these structural and functional similarities, PARP2 can function in a manner distinct from
46 PARP1 (Bai et al. 2011; Celik-Ozenci and Tasatargil 2013; Dantzer et al. 2006b; Farres et
47 al. 2015; Jha et al. 2009; Kamboj et al. 2013; Nicolas et al. 2010; Tramontano et al. 2007;
48 Yelamos et al. 2006), but both proteins are required for mouse early development
49 (Menissier de Murcia et al. 2003; Nicolas et al. 2010). This early requirement is evident
50 from studies with mouse F9 teratocarcinoma cells, where the loss of PARP1 or PARP2
51 blocks the expression of markers (Quenet et al. 2008) required in the Wnt- and Hedgehog-
52 dependent differentiation of extraembryonic endoderm (Deol et al. 2017; Golenia et al.
53 2017; Hwang and Kelly 2012). Another important role assigned to PARP proteins is their
54 maintenance of telomere integrity (Dantzer et al. 2004), and genome stability through
55 recruiting DNA repair factors to DNA-strand breaks and base-excision lesions resulting

56 from DNA damage (Ame et al. 1999; Riccio et al. 2016; Schreiber et al. 2002). These
57 activities are suspended during apoptosis by caspase-8, which serves to inactivate PARP2
58 (Benchoua et al. 2002). In addition, PARP2 contributes to the translocation of apoptosis-
59 inducing factor (AIF) from the mitochondria to the nucleus during oxidative damage to
60 DNA (Li et al. 2010), and it can control cell death and autophagy linked to oxidative stress
61 (Wyrsh et al. 2012). PARP2, together with the PPAR γ /RXR transcription machinery, is
62 also important in adipocyte differentiation (Bai et al. 2007), in the regulation of surfactant
63 protein B expression in pulmonary cells (Maeda et al. 2006), and for hematopoietic stem
64 cell survival under steady-state conditions and in response to radiation stress (Farres et al.
65 2013). However, the loss of PARP2 can increase apoptosis, contradicting a focal cerebral
66 ischemia report that showed a suppression of AIF in PARP2^{-/-} (Li et al. 2010). Despite
67 these discrepancies, owing to cell specificity and exemplified by the PARP2 depletion
68 results in several cell lines (Boudra et al. 2015), these studies confirm that PARP2 is not
69 entirely functionally redundant with PARP1. Furthermore, they underscore the importance
70 of PARP2 in maintaining a number of key cellular physiological processes. For these
71 reasons, we sought to investigate the mechanism by which PARP2 is regulated under
72 normal and stress-induced conditions.

73 To investigate how PARP2 is regulated, a survey of several cell lines showed robust
74 PARP2 levels that were stable over several hours of cycloheximide treatment. Interestingly,
75 following serum removal, PARP2 signals were absent, but when the cells were returned to
76 complete medium, the protein was detected on immunoblots. Since *PARP2* gene
77 expression was not affected by serum removal, we postulated the loss of PARP2 was the
78 result of post-translational modifications. Analysis using proteolytic inhibitors failed to

79 identify the protease(s) responsible for the loss of PARP2 under serum-free conditions.
80 Finally, our focus turned to the proteasome and the post-translational modification of
81 PARP2 by ubiquitination. *In vitro* assays showed that PARP2 was ubiquitinated and when
82 cells were cultured in complete medium, ubiquitination led to PARP2 degradation in the
83 proteasome. Unexpectedly, inhibiting proteasome activity under serum-free conditions did
84 not prevent PARP2 signals from disappearing, which together with the protease inhibition
85 experiments, suggested the protein was being sequestered to a denaturing-insoluble
86 compartment rather than being degraded by a protease or the proteasome.

87 Together, our findings strongly support the notion that when serum is present, and
88 cells are stimulated to grow, PARP2 is detected under standard SDS-denaturing conditions
89 and thus is available fulfill its roles in maintaining normal cellular physiology. However,
90 in the absence of serum, PARP2 is sequestered to an SDS- and urea-resistant compartment.
91 Regardless of the mechanism, this outcome would serve to reduce global ADP-ribosylation
92 enzyme activity to thereby minimize energy expenditure under adverse conditions.

93

94 **Materials and methods**

95

96 **Antibodies, plasmids and reagents**

97 PARP1/2 (H250), β -actin and ERK antibodies were purchased from Santa Cruz
98 Biotechnology, rabbit anti-mouse PARP2 (Yucatan) from Enzo Life Sciences, Inc., rabbit
99 anti-human PARP2 antibody from Axxora, and GST and HA antibodies from GenScript.
100 HRP-conjugated secondary antibodies were purchased from Pierce. *pBC-GST-PARP2*,
101 *pBC-GST-NLS* and *pEGFP-PARP2* plasmids were gifts of Dr. V. Schreiber (École
102 Supérieure de Biotechnologie Strasbourg, France). The *mRFP-ub* plasmid was a gift of Dr.
103 N. Dantuma (Karolinska Institutet, Sweden; Addgene #11935), and the *pMT123-HA-*
104 *ubiquitin* plasmid was kindly provided by Dr. D. Bohmann (University of Rochester, USA).
105 MG-132 and cycloheximide were from Sigma, and the caspase inhibitor III (BD-FMK),
106 calpeptin and pepstatin A methyl ester (PME) were from Calbiochem (EMD Millipore).
107 Caspase-8 inhibitor (Z-IETD-FMK) was from BD-Biosciences. Alpha-2-macroglobulin
108 was purchased from Enzo Life Sciences and leupeptin from Bio Basic Inc. The HALT
109 protease inhibitor cocktail was from Pierce and GST-PARP2 human recombinant protein
110 from BPS Bioscience. Protein fraction II, HA-ubiquitin, ubiquitin aldehyde and ubiquitin
111 conjugation reaction buffer kits were purchased from Boston Biochem, Inc. The
112 transfection reagent XtremeGene 9 was from Roche Applied Sciences, and Glutathione
113 Sepharose 4B beads were purchased from GE Healthcare Life Sciences. Power SYBR
114 Green PCR master mix was purchased from Invitrogen Thermo Fisher Scientific.

115

116 **Cell culture, treatment and transfection**

117 COS-7, MCF-7, HeLa, NIH3T3, MEF F20 and IMCD cells were maintained in
118 Dulbecco's modified Eagle's medium (DMEM)/F-12 or DMEM supplemented with 10%
119 FBS, 100 units/ml penicillin and 100 mg/ml streptomycin in 5% CO₂ at 37°C. Cells were
120 treated with different protease inhibitors at the concentration and duration as indicated in
121 the figures. Cells were subject to serum starvation or maintained in medium containing
122 different concentrations of sera where stated. Transfections were carried out using X-
123 tremeGENE 9 transfection reagent as per manufacturer's recommendation. To test if the
124 loss of the PARP2 signal on blots under serum starvation conditions was due to
125 sequestration rather than degradation, MCF-7 cells were cultured to approximately 90%
126 confluence in complete medium (CM) and then this was removed and replaced with serum-
127 free (SF) medium containing 50µgml⁻¹ cycloheximide (CHX) to inhibit protein synthesis.
128 The cells were incubated for 7hr, and then the medium replaced with CM containing
129 50µgml⁻¹ CHX. The cells were cultured for 1hr and then lysed in Laemmli sample buffer.
130 Cells serum-starved for 7hr with or without CHX treatment or remaining in CM with or
131 without CHX.

132

133 **End point and quantitative reverse transcription-PCR**

134 MCF-7 cells were cultured in CM until 90% confluence. At this point the medium
135 was removed for one plate and replaced with SF medium. Cells on a second plate were
136 maintained in CM. Following 30 minutes, cells on both plates were lysed in TRIzol
137 (Invitrogen Thermo Fisher Scientific) and total RNA extracted following the
138 manufacturer's instructions. Following preparation of first strand cDNA by reverse
139 transcription with (+) or without reverse transcriptase (-) (control), PCR was performed

140 using primers specific to human *PARP2* (forward 5'- GAATCTGTGAAGGCCTTGCTG-
141 3' and reverse 5'-TTCCCACCCAGTTACTCATCC-3'). PCR products were resolved on
142 1% agarose gels and images captured with a FluorChem IS-8900 Imager (Alpha Innotech
143 Corp.). For q-RT-PCR, MCF-7 cells were cultured in CM, or serum-starved for 15, 30 and
144 60 minutes. F9 cells were also serum-starved for 60 minutes and then treated for additional
145 12 hours with DMSO, or with retinoic acid (RA) 10^{-7} M. For controls, cells were treated
146 for 12 hours in CM containing DMSO or RA 10^{-7} M. In all experiments, total RNA was
147 extracted with the RNeasy mini kit (QIAGEN), and first strand cDNA prepared using
148 qScript cDNA SuperMix (Quanta Bioscience) following the manufacturer's instructions.
149 For MCF-7 cells q-RT-PCR was performed using the abovementioned *PARP2* primers,
150 while mouse *Gata6* primers (forward 5'-ATGGCG TAGAAATGCTGAGG-3' and reverse
151 5'-TGAGGTGGTCGCTTGTGTAG-3') and *Hoxb1* primers (forward 5'-
152 GGGGTCGGAATCTAGTCTCCC-3' and reverse 5'-
153 CCTCCAAAGTAGCCATAAGGCA) were used with the F9 cells. *GAPDH* primers
154 (forward 5'-GTGTTCCCTACCCCCAATGTGT-3' and reverse 5'-
155 ATTGTCATACCAGGAAATGAGCTT-3') were used as the reference primers for MCF-
156 7 cells and mouse *L14* (forward 5'-GGGTGGCCTACATTCCTTCG-3' and reverse 5'-
157 GAGTACAGGGTCCATCCACTAAA-3') for F9 cells. Reactions were performed using
158 a CFX Connect Real-Time PCR Detection System (Bio-Rad), and results were analyzed
159 using the comparative cycle threshold ($2^{-\Delta\Delta C_t}$) method with the internal controls.

160 **Cell free ubiquitination and GST pull-down assays**

161 COS-7 cells were transfected with *pBC-GST-PARP2* or *pBC-GST-NLS* (control)
162 alone or with *pMT123-HA-ubiquitin* plasmids using the X-tremeGENE 9 transfection

163 reagent (Sigma-Aldrich). Twenty-four hours post-transfection, one plate of COS-7 cells
164 transfected with both *pBC-GST-PARP2* and *pMT123-HA-ubiquitin* was treated with 40 μ M
165 MG-132, and the second with DMSO as a vehicle control. After 20hr, cells were washed
166 in PBS (phosphate buffered saline) and proteins harvested by lysing in 1x RIPA buffer
167 (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP-40, 0.5% Na-deoxycholate and 0.1% SDS)
168 supplemented with protease inhibitor cocktail on ice. Cell lysates were stored at -80°C for
169 GST-pull down assays. Protein concentrations were determined using a Bradford assay and
170 equal amounts of total protein from each sample were incubated with Glutathione
171 Sepharose 4B beads overnight at 4°C. Beads were washed 4 x 5 minutes with RIPA buffer
172 and then resuspended in 2x Laemmli sample buffer. Proteins pull-downed in these assays
173 were resolved by 8% SDS-PAGE, and then subjected to immunoblot analysis.

174

175 ***In vitro* ubiquitination assay**

176 GST-PARP2 human recombinant protein (0.4 μ g) was added to a 20 μ l final volume
177 reaction mix containing 1x ubiquitin conjugation reaction buffer, 0.5mM MG-132, 1x
178 ubiquitin aldehyde, 2mM HA-ubiquitin, 1x Mg-ATP and 1 μ l HeLa protein fraction II. For
179 controls, 1 μ l of water was substituted for 1 μ l HeLa protein fraction II. Reactions were
180 carried out at 37°C for 2hr and then inhibited by adding 2 μ l 10x E1 stopping buffer, 4 μ l 5x
181 Laemmli sample buffer and 1.5 μ l of β -mercaptoethanol. Samples were boiled for 5 minutes,
182 and the proteins resolved by 8% SDS-PAGE before immunoblot analysis.

183

184 **Immunoblotting**

185 After a PBS wash, cells were either lysed on ice in 1x Laemmli sample buffer
186 supplemented, or urea lysis buffer (8M Urea, 2M Thiourea, 4% w/v CHAPS). All buffers
187 were supplemented with protease inhibitor cocktail, but without bromophenol blue.
188 Lysates were sonicated for 10sec and boiled for 5 minutes, then centrifuged for 10 minutes
189 at 13,200 rpm at 4°C. To determine protein concentrations using a Bradford assay, aliquots
190 were diluted 200 fold to minimize SDS interference. Equal amount of proteins was
191 resolved by 8% or 10% SDS-PAGE and then transferred to nitrocellulose membranes,
192 which were then blocked in 5% skim milk/Tris-buffered saline/Tween 20 (TBS/T) buffer
193 for 2hr at room temperature. Following an overnight incubation in primary antibody
194 (1:2500 diluted in blocking buffer) at 4°C, membranes were washed in TBS/T buffer and
195 then incubated 1hr with a HRP-conjugated secondary antibody (1:4000 diluted in blocking
196 buffer) at room temperature. Membranes were washed extensively with TBS/T buffer and
197 signals were detected by enhanced chemiluminescence (Pierce Thermo Fisher Scientific).
198 Densitometric analyses were performed using ImageJ software (NIH).

199

200 **Confocal microscopy**

201 HeLa cells cultured on glass cover slips were transfected with *pEGFP-PARP2* and
202 *mRFP-ub* plasmids. At 24hr post-transfection, cells were fixed with 4% paraformaldehyde
203 in PBS for 30 minutes at room temperature. After 3 x 10 minute washes with PBS, cells
204 were mounted in ProLong Gold anti-fade mounting medium (Invitrogen Thermo Fisher
205 Scientific) and viewed with a Zeiss LSM 510 Duo Vario confocal microscope.

206

207 **Statistical analysis**

208 Statistical significance was determined using Student's t-test ($p < 0.05$). Error bars
209 represent standard deviation. Minimum of 3 independent biological replicates were
210 conducted for each experiment. Data was analyzed using SPSS Version 21.0 (IBM Corp.).
211

212 **Results**

213

214 **PARP2 expression is serum responsive but is not regulated by its putative SRE**

215 PARP2 is ubiquitously expressed in mammalian cells and a putative serum
216 response element (SRE) within the promoter of the *PARP2* gene suggested its expression
217 is subject to serum stimulation (Ame et al. 2001; Ame et al. 1999). To test whether this
218 SRE was functional, we first assayed PARP protein levels in HeLa, COS-7, MCF-7,
219 NIH3T3 and inner medullary collecting duct (IMCD) cells cultured in complete medium
220 (CM) or serum-free (SF) medium. Immunoblot analysis with the PARP H-250 antibody
221 revealed a prominent 116kDa band corresponding to PARP1 in all cell lines with the
222 exception of NIH3T3 cells (Fig. 1A). A band at 89kDa, corresponding to the C-terminal
223 fragment of PARP1 (Chaitanya et al. 2010) was detected in COS-7 cells and weakly in
224 MCF-7 cells. The appearance of these bands did not change significantly when cells were
225 cultured in CM or SF medium. The H-250 antibody also recognized a 62kDa band,
226 corresponding to PARP2, in all cells cultured in CM (Fig. 1A). This band, however, was
227 absent when COS-7 or MCF-7 cells were cultured in SF medium (Fig. 1A). ERK staining,
228 used as a loading control in this and other studies (Fernandez-Garcia et al. 2007; Rygiel et
229 al. 2008; Xu et al. 2012) showed that protein was present in all samples, with approximately
230 equal amounts assayed in the COS-7 and MCF-7 lanes under the different culturing
231 conditions (Fig. 1).

232 The loss of the PARP2 signal in cells cultured in SF medium was not due to cell
233 death as within one hour, when cultured in CM, these previously serum-starved COS-7
234 cells had reacquired a PARP2 signal (Fig. 1B). Furthermore, signals were comparable to
235 those in cells that had been continually growing in CM. These results showing that PARP2

236 levels were influenced by the presence or absence of serum implied that regulation was
237 either at the level of the gene, the protein or both. To address whether or not serum had an
238 effect on altering the activity of the *PARP2* gene, and specifically the SRE in its promoter,
239 MCF-7 cells were cultured in CM or SF medium for 30 minutes and then total RNA was
240 extracted and used for cDNA synthesis. Endpoint and quantitative RT-PCR using *PARP2*
241 primers showed that *PARP2* mRNA was available regardless of the treatment (Fig. 1C, D).
242 However, immunoblot analysis showed PARP2 signals in cells cultured in CM, but not in
243 those that were cultured in SF medium (Fig. 1E).

244 To address whether the loss of PARP2 signal under SF conditions had physiological
245 effects on cells, we used F9 cells to test whether the loss of PARP2 affected their
246 differentiation potential. F9 cells cultured in CM and treated with RA upregulated the
247 expression of differentiation makers *Gata6* and *Hoxb1* (Fig. S1). Interestingly, F9 cells
248 cultured under SF conditions and treated with RA showed significantly lower expression
249 of *Gata6* and *Hoxb1* when compared to controls (CM + RA), suggesting that loss of PARP2
250 signal through serum starvation attenuates RA-induced differentiation of F9 cells (Fig. S1).

251 Together these observations indicating that *PARP2* expression was not affected by
252 serum deprivation brought into question the significance of the putative SRE. More
253 importantly, they strongly suggested that the loss of PARP2 signals could be the
254 consequence of the accelerated degradation of the protein itself or its sequestration to a
255 compartment resistant to Laemmli extraction buffer following serum starvation. To test the
256 latter, 2% SDS and 8M urea lysis buffers were used to lyse NIH3T3 cells cultured under
257 CM or SF conditions. Results show PARP2 signals were absent in both SF cells lysed in

258 2% SDS and urea lysis buffer (lanes 2 and 3, respectively, Fig. 1F), and indicate that
259 PARP2 is either sequestered in a detergent-insoluble fraction or proteolytically degraded.

260

261 **PARP2 is long-lived in cells cultured in complete medium**

262 Since results indicated that PARP2 might be a short-lived protein when cells were
263 cultured in SF medium, an *in silico* analysis was done to identify PEST sequences
264 (mobyle.pasteur.fr), which are responsible for the rapid turnover of many short-lived
265 proteins (Belizario et al. 2008). Although no putative sites were identified in PARP2, COS-
266 7 cells were cultured in CM and protein turnover examined when translation was blocked
267 using cycloheximide (CHX). Cells were cultured in the presence of CHX ($50\mu\text{gml}^{-1}$) for 1,
268 3, 5 and 7hr, and then processed for immunoblot analysis to detect PARP2 (Fig. 2A).
269 Contrary to the rapid disappearance seen in SF culture, PARP2 appeared stable over the
270 7hr period. Similar results were seen for PARP1 and ERK, which together would indicate
271 that the loss of the PARP2 signals was serum-dependent.

272 The loss of the 62kDa PARP2 signal in cells cultured in SF medium prompted us
273 to undertake a more detailed investigation on the relationship between serum treatment and
274 the levels of PARP2 (Fig. 2B). COS-7 cells were cultured in SF medium or in medium
275 containing increasing amounts of serum. Immunoblot analysis showed a similar staining
276 pattern for full-length PARP1 and its C-terminal fragment, regardless of whether serum
277 was present or not (Fig. 2B). In contrast, increasing the serum concentration from 0 to 10%
278 resulted in the significant increase in the appearance of PARP2 ($P<0.05$; Fig. 2B). Together
279 these results showed that PARP2, but not PARP1, changes in cells in response to serum.

280 Furthermore, the evidence with that seen in figures 1C and 1D, would suggest that this
281 increase is at the protein level rather than due to increased gene activity.

282 Having determined that PARP2 was available as an SDS soluble protein and this
283 was dependent on the presence of serum, the next question was to address how fast the
284 PARP2 signal would decline when cells were deprived of serum. To determine this, COS-
285 7 cells were cultured in CM for 24hr until 90% confluence, and then the medium was
286 removed and replaced with SF medium. Cell lysates were collected at 15, 30, 45 and 60
287 minutes and then processed for immunoblot analysis with the H-250 PARP antibody.
288 Results showed PARP1 levels were unaffected by the serum conditions, and comparable
289 signals were seen in all lanes (Fig. 2C). In contrast, PARP2 signals were absent within 15
290 minutes after serum deprivation (Fig. 2C). This finding suggested serum starvation
291 activated an efficient mechanism to reduce the PARP2 signal, which could be either
292 proteolytic degradation or sequestration to an insoluble fraction.

293

294 **PARP2 reduction following serum deprivation is not mediated by a known protease**

295 Since caspase-8 is known to cleave PARP2 in apoptotic murine neurons (Benchoua
296 et al. 2002), and caspase activation is seen in osteoblastic cells following serum deprivation
297 (Mogi et al. 2004), this group of proteases was the first to be investigated. COS-7 cells
298 were treated with 40 μ M of either the caspase-8-specific inhibitor, Z-Ile-Glu(OMe)-Thr-
299 Asp(OMe)-FMK (IETD) or the broad-spectrum caspase inhibitor Boc-Asp(OMe)-FMK
300 (CI III), and whole cell lysates were collected for immunoblot analysis to detect PARP2
301 (Fig. 3A). PARP1 levels in cells cultured in SF medium and treated with either of the two
302 inhibitors were comparable to those in cells cultured in CM. The PARP2 signal, however,

303 declined despite the presence of the caspase inhibitors (Fig. 3A). These results, together
304 with those seen in figure 1A, led us to dismiss the notion that the decline in PARP2 levels
305 in cells growing in SF medium was a caspase-dependent, apoptotic-related event.

306 Since the caspase inhibitors had no effect on preventing the disappearance of the
307 PARP2 signal when cells were deprived of serum, the next step was to broaden the search
308 for other proteases that might be involved in the process. MEF F20 (PARP1^{+/+}) cells were
309 selected for these studies since the Yucatan PARP2 antibody provided little to no consistent
310 signal with human PARP2 but is robust in detecting mouse PARP2 (data not shown).
311 Furthermore, comparable levels of PARP2 were observed between F20, COS-7 and MCF-
312 7 cells (data not shown). F20 cells were cultured in CM until 90% confluence and then
313 pretreated with 250 μ M leupeptin (LEU), 30 μ M pepstatin A methyl ester (PME), 30 μ M
314 calpeptin (CAL), or 50 μ gml⁻¹ α -2-macroglobulin (α 2-M). After 5hr, the medium was
315 replaced with SF medium containing the corresponding protease inhibitor. To inactivate a
316 broad spectrum of endo- and exopeptidases, cells were treated with the 1x HALT Protease
317 Inhibitor Cocktail, which contains AEBSF-HCl, aprotinin, bestatin, E-64, leupeptin,
318 pepstatin A and EDTA. After a 15-minute incubation, cell lysates were collected and
319 processed for immunoblot analysis with the Yucatan PARP2 antibody. Results showed that
320 the inhibitors, either alone or in a cocktail (HALT), were not effective in preventing the
321 disappearance of the PARP2 signal (Fig. 3B). This led us to conclude that under serum
322 deprivation, PARP2 levels were not affected by an amino, serine, cysteine, metallo- and
323 aspartic acid protease. After exhausting a broad spectrum of candidate proteases thought
324 to be responsible for degrading PARP2, our attention turned to the ubiquitin-proteasome
325 system (UPS).

326

327 **PARP2 is ubiquitinated**

328 Without finding an inhibitor that would ensure the PARP2 signal was present under
329 SF conditions, and since the UPS is involved in degrading PARP1 (Masdehors et al. 2000;
330 Wang et al. 2008), we considered the same might be true for PARP2. To address this,
331 confocal microscopy was used to examine if EGFP-PARP2 and mRFP-Ubiquitin encoded
332 by vectors transfected into HeLa cells would co-localize. Results showed that PARP2 was
333 present in the nucleus, while ubiquitin was present in the nucleus and cytoplasm (Fig. 4A).
334 Although the co-localization of these proteins in the nucleus was only suggestive that
335 PARP2 was ubiquitinated, further analysis was necessary to provide conclusive evidence.
336 An *in vitro* assay using human recombinant GST-PARP2 and an immunoblot analysis with
337 a GST antibody showed a smear of higher molecular weight PARP2 when Protein Fraction
338 II containing ubiquitination enzymes was present (lane 2, Fig. 4B). Omitting the
339 ubiquitination enzymes served as a negative control (lane 1, Fig. 4B), indicating that
340 PARP2 could be ubiquitinated *in vitro*. To confirm the ubiquitination of PARP2, COS-7
341 cells were co-transfected with *pBCGST-PARP2* and *pMT123HA-ubiquitin* plasmids, and
342 then cultured in CM. Cells transfected without *pMT123HA-ubiquitin* served as a negative
343 control. Cell lysates were collected, and GST pull down assays were performed prior to
344 immunoblot analysis with an anti-HA antibody. Results showed that ectopically expressed
345 mouse PARP2 was ubiquitinated (lane 1, Fig. 4C), and the amount of ubiquitin (smear)
346 accumulated following MG-132 treatment (lane 2, Fig. 4C). As expected, no HA-ubiquitin
347 signal was detected in cells transfected with *pBCGST-PARP2* alone (lane 3, Fig. 4C). A
348 Simian virus 40 nuclear localization signal epitope tagged to GST served as a positive

349 control and was also ubiquitinated (lane 4, Fig. 4C). Thus, when serum was present,
350 exogenously expressed PARP2 was ubiquitinated and despite being a long-lived protein
351 under these conditions (Fig. 2A), this post-translational modification targets PARP2 to the
352 proteasome.

353

354 **PARP2 is degraded by the proteasome in the presence of serum**

355 The ubiquitination results, together with those showing that the proteasome
356 inhibitor MG-132 prevented ectopically expressed PARP2 from degrading, strongly
357 suggested that the UPS was the mechanism used to regulate endogenous PARP2 levels.
358 Unexpectedly, however, results showed that MG-132 had no effect on preventing
359 endogenous PARP2 from being detected under SDS-denaturing conditions in cells cultured
360 under SF conditions (Fig. 5A). As a comparison, immunoblot analysis showed COS-7 cells
361 cultured in CM and treated with MG-132 had consistent PARP2 signals (Fig. 5B). In fact,
362 the signals increased with increasing concentrations of MG-132, which was confirmed by
363 densitometric analysis showing the significant increase over levels in cells cultured in CM
364 alone. Although PARP1 was reported to be ubiquitinated and degraded in the proteasome
365 (Ame et al. 2009), we did not see any appreciable change after MG-132 treatment, which
366 mimicked that seen under SF conditions. Together this data would indicate that when cells
367 were cultured in CM, endogenous PARP2 is ubiquitinated and degraded in the proteasome.
368 The data also points to the fact that different mechanisms of PARP2 regulation exist and
369 that these processes are activated in a manner that depends on the conditions under which
370 the cells are cultured.

371

372 **PARP2 is sequestered to an insoluble fraction after serum deprivation**

373 Having shown PARP2 is degraded through the UPS in cells cultured in CM and
374 having excluded the involvement of many known protease types responsible for its absence
375 after serum withdrawal, we set out to examine if PARP2 was sequestered to an insoluble
376 compartment following serum deprivation. If PARP2 was degraded rather than being
377 sequestered after serum withdrawal, then it was expected that the signals would not
378 reappear, as indicated in figure 1B, after serum was added and protein synthesis inhibited
379 with CHX.

380 Immunoblot analysis with a PARP2 antibody (Axxora) showed, as expected, the
381 62kDA PARP2 signal in cells cultured in CM (Fig. 6A, lane 1). The PARP2 signal was
382 lost when serum was removed and remained so in cells cultured in SF medium and treated
383 with CHX (Fig. 6A, lanes 2 and 3, respectively). Similarly, CHX had no apparent effect on
384 PARP2 when cells were cultured in CM (Fig. 6A, lane 4). However, when cells were
385 treated with CHX, and cultured under serum-free conditions, were transferred to CM
386 containing CHX, the PARP2 signal appeared (Fig. 6A, lane 5). In fact, the signal strength
387 was almost identical to cells cultured without CHX (Fig. 6A, lane 6). These results would
388 suggest serum starvation led to the sequestration of PARP2 to an SDS- (and urea) insoluble
389 compartment, which could recycle back to a soluble form when serum was present. To
390 further understand the capacity of this sequestration, we overexpressed GST-PARP2 in
391 MCF-7 cells cultured in CM or SF medium for 15, 30 and 60 minutes. Immunoblot analysis
392 of these lysates, using a GST antibody, showed that the GST-PARP2 signal was
393 comparable between the cells cultured in CM and SF medium (arrow, Fig. 6B). Lower
394 molecular weight signals were also seen on blots (arrowheads, Fig. 6B), and although not

395 characterized, they may be PARP2 fragments that appeared due to ubiquitin-mediated
396 proteasomal degradation in CM. Thus, the evidence would suggest that either the
397 sequestration capacity was limiting, or the GST tag conferred solubility to PARP2, either
398 way with most of the ectopically expressed PARP2 free in the SDS-soluble fraction.
399 Together, the results indicate that serum withdrawal does not lead to PARP2 degradation.

400

401 **Discussion**

402 PARP proteins were first identified as being players in DNA repair (Gupte et al.
403 2017; Wei and Yu 2016), and this involves initiating their poly ADP-ribosylation
404 polymerase activity (Barzilai and Yamamoto 2004; Dantzer et al. 2006a; Huber et al. 2004).
405 In addition to this crucial role, PARP proteins serve other functions involved in, but not
406 limited to the regulation of gene activity, cell death, the immune system, cellular
407 metabolism and differentiation (Bai 2015; Vida et al. 2017). PARP2 is required for
408 initiating the differentiation of F9 cells into primitive extraembryonic endoderm (Quenet
409 et al. 2008), and our results showing *Gata6* and *Hoxb1* expression under serum-free
410 conditions (Fig. S1) would indicate that PARP1 was unable to act in a functionally
411 redundant manner (Quenet et al. 2008) and implicating PARP2 in differentiation.

412 Whatever the case, and given this diversity, the mechanism(s) to control the level
413 and activity of these enzymes must be tightly regulated. In the case of PARP1 and PARP2,
414 basal activities are low, while in response to DNA damage this changes rapidly (Bai and
415 Canto 2012; Krishnakumar and Kraus 2010; Langelier et al. 2014). At the level of the gene,
416 the presence of a putative serum response element (SRE) in the *PARP2* promoter region
417 (Ame et al. 2001) suggested that transcriptional activity might be influenced by growth

418 factor and/or mitogen stimulation. To investigate this, we cultured different cell lines in
419 the presence or absence of serum and then assayed PARP2 levels. The loss of the PARP2
420 signal in cells cultured in SF medium (Fig. 1A), and its return when serum was replaced
421 (Fig. 1B) supported the notion that the *PARP2* gene was serum responsive. Given this on-
422 off appearance at the protein level, and the presence of the putative SRE, we had expected
423 to see changes in *PARP2* mRNA expression under the different culturing conditions. This,
424 however, was not the case and the presence of a *PARP2* amplicon in cells cultured in the
425 SF medium indicated that the message was available (Fig. 1C). Furthermore, the fact that
426 the quantitative-RT-PCR results showed no significant differences in expression in cells
427 cultured under the different conditions (Fig. 1D) indicated that the regulation of PARP2 in
428 cells deprived of serum was not due to the direct regulation of the gene, at least within the
429 time frame of our investigation. Thus, the presence of *PARP2* mRNA under the SF
430 conditions did not seem to contribute to the fast return (within one hour) of the PARP2
431 signal after serum was added to cells incubated in CHX to block new protein synthesis (Fig.
432 6A). Also, the reappearance of the PARP2 signal when serum was added was dose-
433 dependent (Fig. 2B) and not reliant on increased transcription that was dependent on the
434 putative SRE in the *PARP2* promoter (Fig. 1D). These results implied that PARP2 under
435 serum-free conditions was either being rapidly degraded (Fig. 2C) or sequestered in an
436 insoluble compartment.

437 Serum withdrawal in different cultured cells activates many proteolytic, and
438 proteolytic-related proteins including caspases, calpains, autophagy-related proteins,
439 ubiquitin, and proteasome subunits (Fuertes et al. 2003; Kilic et al. 2002; Mogi et al. 2004;
440 Nakashima et al. 2005; Schamberger et al. 2005). The fact that caspases can cleave PARP2

441 (Benchoua et al. 2002) placed this family of cysteine-dependent aspartate-directed
442 proteases at the forefront of candidates responsible for the serum-dependent changes seen
443 with the different cell types (Fig. 1). Unfortunately, our analysis using a caspase-specific
444 and a broad-spectrum caspase inhibitor (Fig. 3A) as well as a proteolytic inhibitor cocktail
445 (Fig. 3B) ruled out the possibility that caspases were responsible for the proteolysis.
446 Subsequent experiments were designed to explore the ability of serine, cysteine, metallo-
447 and aspartic proteases to alter PARP2 levels (Fig. 3B). As with the caspase inhibitors, those
448 routinely employed to prevent proteolysis did not have an effect on the PARP2 signals
449 when cells were cultured in serum-free conditions (Fig. 3B). Furthermore, the reports that
450 the cysteine protease cathepsin L is present in the nucleus, like PARP2, and is involved in
451 cell-cycle progression (Puchi et al. 2010) prompted us to block its activity to see what effect
452 it would have on endogenous PARP2 levels in serum-deprived cells. Leupeptin, an
453 inhibitor of endosomal trypsin-like serine and cysteine proteases (Simmons et al. 2005)
454 had no effect, which ruled out the involvement of cathepsin L. Details of the inhibitor
455 studies suggested that the disappearance of the PARP2 signal may not be the result of
456 proteolytic degradation, but instead and as noted above, due to the sequestration of the
457 protein to an insoluble form under serum-free conditions (Fig. 2B).

458 The detergent solubility of constituents within a cell is determined by the chemical
459 properties of the substrates as well as the detergents. Some cellular entities are naturally
460 resistant to extraction by detergents (Horigome et al. 2008; Takata et al. 2009) while other
461 proteins may be converted to detergent-soluble or insoluble forms following various
462 stimulations (Peters et al. 2012; Reis-Rodrigues et al. 2012). For instance, serum starvation
463 causes the translocation of dynein to a more detergent-soluble compartment in NRK cells,

464 and this change is reversed when serum is added (Lin et al. 1994). Serum withdrawal also
465 leads to sequestration of caspase-9 into detergent-insoluble cytoskeletal structures in rat
466 423-cells (Schamberger et al. 2005). The same is true for IM-9 cells, where detergent
467 insolubility of growth hormone receptors occurs due to ligand-induced formation of cross-
468 linked disulfide bonds (Goldsmith et al. 1997). These reports and our results led us to
469 investigate if serum deprivation caused an SDS solubility change in PARP2. Furthermore,
470 they raised the question of what in serum ameliorates the SDS solubility of PARP2?
471 Conversely, why does PARP2 become SDS insoluble when cells are serum-deprived and
472 what process initiates this sequestration or biochemical change? More importantly, what
473 physiological function does this regulation serve in cells, or does it even have biological
474 significance? Despite the similarities between PARP1 and PARP2, that the solubility in
475 SDS of the former did not change following serum deprivation would indicate that serum
476 withdrawal activated a PARP2-specific mechanism.

477 PARP1 regulation has been studied extensively and the protein can be found in
478 lamin-enriched or DNA-bound detergent-resistant fractions (Frouin et al. 2003; Vidakovic
479 et al. 2004). Furthermore, PARP1 changes its solubility in NP-40 detergent when modified
480 by sumoylation following heat shock (Martin et al. 2009). Several other proteins involved
481 in regulating DNA replication and repair, e.g. PCNA, P21, OGG1, XRCC1 and CAF-1
482 P150 are also found in DNA-bound detergent-resistant fractions (Amouroux et al. 2010;
483 Campalans et al. 2013; Frouin et al. 2003; Okano et al. 2003). Although PARP2 functions
484 in DNA repair, this association with DNA would only enhance its resistance to some
485 nonionic detergents including Triton and NP-40, but not to anionic detergents like SDS.
486 Thus, despite the link between serum starvation to activate the DNA damage response

487 pathway in some cancer cells and to induce DNA fragmentation in normal cells (Lu et al.
488 2008; Shi et al. 2012), and based on the chemistry noted above, it is questionable that
489 PARP2 would be insoluble to SDS (and urea) if serum starvation had caused it to bind to
490 DNA lesions.

491 PARP2 can also localize to the cytosol in gonocytes, spermatogonia and spermatids
492 in mice (Gungor-Ordueri et al. 2014), and proteins are known to change their detergent
493 solubility when associated with either glycosylphosphatidyl inositol enriched
494 microdomains, the cytoskeleton or when posttranslationally modified (Brown and Rose
495 1992; Fujita et al. 2011; Ledesma et al. 1994; Paladino et al. 2002; Refolo et al. 1991;
496 Waelter et al. 2001). Moreover, under pathological conditions proteins can become SDS-
497 insoluble, as in the mouse model of Alzheimer's disease where amyloid β protein ($A\beta$)
498 changes to SDS-insoluble forms of $A\beta_{42}$ and $A\beta_{40}$ (Kawarabayashi et al. 2001) or in
499 Huntington's disease where the mutated Huntingtin protein with its polyglutamine repeat
500 expansion is resistant to SDS extraction (Heiser et al. 2000; Scherzinger et al. 1999). These
501 conversions to SDS-insoluble forms are disease-dependent and the combinatory effects of
502 conformational changes and oligomerization and fibril formation that appear irreversible
503 in patients with specific neurodegenerative diseases (Cruz et al. 1997; Diaz-Hernandez et
504 al. 2005; Dolev and Michaelson 2004; Wong et al. 2008). The conversion of PARP2
505 between soluble and insoluble forms under different serum conditions that we observed
506 suggests the protein adopts a physiological conformation in either condition, linked to a
507 stress response. This is evident in nematodes and yeast where the accumulation of SDS-
508 insoluble proteins in cells is indicative of aging (Peters et al. 2012; Reis-Rodrigues et al.
509 2012). The accumulation of SDS-insoluble proteins is accelerated by nitrogen starvation

510 even in young yeast cells, where Tor1 kinase plays a regulatory role in this accumulation
511 of a novel autophagic cargo preparation process (Peters et al. 2012). It is unlikely, however,
512 that the loss or sequestration of PARP2 is a mechanism to prepare it for autophagic
513 degradation since inhibiting lysosomal enzymes with leupeptin had no apparent effect (Fig.
514 3B). Serum deprivation and the way cells cope, however, is indicative of a stress response,
515 and although our results suggest that PARP2 participates in these events, it does not explain
516 why the protein cannot be detected following urea denaturation (Fig. 1F). Urea is often
517 used to recover proteins from inclusion bodies (Burgess 2009), but there is no single
518 method of solubilization for every protein (Singh et al. 2015).

519 Given the shortcomings on being unable to find a sequestration mechanism noted
520 above, our data does indicate that PARP2 is degraded through the UPS in cells when serum
521 is present (Fig. 5B). PARP1, the structural and functional relative of PARP2 is
522 ubiquitinated and degraded through the 26S proteasome, and the ubiquitination site is
523 mapped to its N-terminal DNA-binding domain (Wang et al. 2008). Two E3 ubiquitin
524 ligases, Iduna and CHFR, ubiquitinate and target PARP1 for proteasomal degradation in a
525 poly-ADP-ribose (PAR)-dependent manner (Kang et al. 2011; Kashima et al. 2012; Liu et
526 al. 2013). Interestingly, Iduna binds and ubiquitinates a panel of DNA damage repair
527 proteins including PARP2 (Kang et al. 2011). It is not known, however, if CHFR
528 ubiquitinates PARP2, but it is recruited to DNA lesions through binding to the PAR moiety
529 of pARylated PARP1, and is the first E3 ligase engaged in protein ubiquitination at DNA
530 damage sites (Liu et al. 2013). When this occurs PARP1 dissociates from DNA lesions and
531 is subsequently degraded in the proteasome. This degradation prevents cells from ATP
532 deprivation due to persistent poly-ADP-ribosylation (pARylation) (Liu et al. 2013) that

533 occurs even by ubiquitinated PARP1 (Wang et al. 2008). Although PARP2 accounts for
534 only 10-15% of the total PARP activity in cells (Bai and Canto 2012), the remaining
535 pARYlation and recruitment of CHFR to DNA lesions seen in PARP1 knockdown cells
536 (Liu et al. 2013) is likely the result of other DNA-dependent PARPs, possibly PARP2. If
537 so and in response to DNA damage, the presence of autoPARYlated PARP2 may be the
538 result of it binding through the PBZ domain in CHFR. As attractive as this sounds, as serum
539 removal would have initiated mechanisms for cell cycle arrest (Bertoli et al. 2013), it is
540 unlikely with our cell lines that PARP2 ubiquitination and degradation contributes to cell
541 cycle arrest, as noted for CHFR-mediated PARP1 (Kashima et al. 2012). Our rationale for
542 disputing the degradation of PARP2 under these conditions comes from the MG-132
543 studies (Fig. 5A), where cells should have retained some of the PARP2 protein when they
544 were transferred to serum-free medium.

545 In summary, PARP2 was shown to be a long-lived protein that is continually
546 degraded by the ubiquitin proteasome system in cells cultured in medium containing serum.
547 However, in the case of a cell stress response when serum is removed, PARP2, through an
548 unknown mechanism, is no longer in a SDS or urea soluble form, and it is not known if
549 this is a prelude to apoptosis or to some other physiological requirement. Nevertheless,
550 alleviating this cellular stress by the addition of serum recycles PARP2 to a form, thereby
551 allowing it to resume its enzymatic role involved in the pARYlation of target substrates.

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972 **Figure legends**

973

974 **Fig. 1.** PARP2 is not detected in serum-deprived cells. (A) Immunoblots of protein lysates
975 from HeLa, COS-7, MCF-7, NIH3T3, and IMCD cells cultured in CM and probed with the
976 H-250 PARP1/2 antibody to detect PARP1 (top panel) and PARP2 (middle panel), and
977 with an ERK antibody for a loading control. For the COS-7 and MCF-7 lines, cells were
978 cultured in CM until 90% confluent, and then the medium was replaced with SF medium
979 for 1 hour. (B) The loss of the PARP2 signal in COS-7 cells cultured in SF medium was
980 not due to cell death since adding medium containing serum (CM) (lane 2), allowed cells
981 to reacquire the PARP2 signal. MCF-7 cells were cultured in CM until 90% confluent and
982 then cultured for 30 minutes in either SF medium or maintained in CM. RNA was extracted
983 from each sample and (C) endpoint and (D) quantitative RT-PCR was done with human
984 *PARP2* specific primers. The + lane had reverse transcriptase added to the first strand
985 reaction, and the - lane was the control for genomic contamination. Analysis from qRT-
986 PCR data did not reveal any significant differences between cells cultured in CM compared
987 with those cultured for various times in the absence of serum. (E) Proteins collected from
988 MCF-7 cells cultured as in (C), were used for immunoblotting analysis with antibodies
989 against PARP1/2 and ERK. The reappearance of the PARP2 signal when serum was
990 replaced was indicative that the cells had not undergone apoptosis. (F) Proteins collected
991 from NIH3T3 cells cultured as in (C), were used for immunoblotting analysis with
992 antibodies against PARP1/2 and β -ACTIN. PARP2 signal was absent in SF cells when
993 SDS or urea extractions were used. Unless stated otherwise, results are representative of
994 three independent experiments.

995 **Fig. 2.** PARP2 is a long-lived serum-dependent protein. (A) COS-7 cells were cultured in
996 CM until 90% confluent, and then cycloheximide (CHX) was added. Cell lysates were
997 collected after 1, 3, 5 or 7hr (lanes 2, 3, 4 and 5, respectively) and probed with antibodies
998 against PARP1/2 and ERK. Results show that there were no significant differences in the
999 PARP1 or PARP2 levels following CHX treatment. (B) COS-7 cells were cultured in SF
1000 medium or medium containing increasing amounts of serum as indicated. Immunoblot
1001 analysis of lysates probed with antibodies against PARP1/2 and ERK revealed significant
1002 increase in levels of PARP2 corresponding to increased serum levels in the medium. (C)
1003 COS-7 cells were cultured in CM until 90% confluent, and then the medium was replaced
1004 with SF medium. Immunoblot analysis of lysates from cells harvested at 15-minute
1005 intervals for 60 minutes and probed with antibodies against PARP1/2 and ERK, show the
1006 rapid decline in PARP2 levels following starvation. * $P < 0.05$.

1007

1008 **Fig. 3.** Proteolytic inhibitors do not affect the loss of PARP2 protein. A) COS-7 cells were
1009 cultured in CM until 90% confluent and then treated for 5hr with: DMSO vehicle control
1010 (lanes 1 and 2), the caspase-8 inhibitor, IETD (lane 3), or the broad-spectrum caspase
1011 inhibitor, CI III (lane 4). Following treatment, the medium was replaced with DMSO in
1012 CM (lane 1) or SF medium (lane 2), or SF medium containing IETD (lane 3) or CI III (lane
1013 4). Cells were cultured for 15 minutes and then lysates collected for immunoblot analysis
1014 with antibodies against PARP1/2 and ERK. Results show that the PARP2 signal disappears
1015 when caspase enzymes-inhibited cells were cultured in SF medium. (B) Mouse embryonic
1016 fibroblast F20 cells were cultured in CM until 90% confluent, and then for 5hr in CM
1017 containing leupeptin (LEU, 250 μ m), Pepstatin A methyl ester (PME, 30 μ m), calpeptin

1018 (CAL, 30 μ m), α 2-macroglobulin (α 2-M, 50 μ gml⁻¹) or 1X HALT protease inhibitor
1019 cocktail containing 1.25 μ M EDTA. Following treatment, the medium was replaced with
1020 SF medium containing the same protease inhibitors and the cells cultured for an additional
1021 15 minutes. Cells continually cultured in CM served as controls. Cell lysates from all
1022 treatments were collected for immunoblot analysis with an antibody specific to PARP2
1023 (Yucatan) or β -ACTIN. Results show that inhibition of a broad spectrum of proteases was
1024 not able to preserve the PARP2 signal in cells cultured in SF medium. The asterisk denotes
1025 non-specific staining.

1026

1027 **Fig. 4.** PARP2 protein is ubiquitinated. (A) HeLa cells were transfected with *pEGFP-*
1028 *PARP2* and *RFP-ubiquitin* and then cultured in CM for 24hr. To visualize the fluorescence,
1029 cells were fixed in 4% paraformaldehyde in PBS, mounted in ProLong Gold antifade
1030 medium, and viewed on a Zeiss LSM 510 confocal microscope. Results show PARP2
1031 staining in the nucleus (panel B). Ectopically expressed ubiquitin was seen in the cytoplasm
1032 and nucleus (panel A), and the overlap in PARP2 and ubiquitin staining is seen in yellow
1033 when the images were merged (panel C). (B) Human GST-PARP2 recombinant
1034 protein/Mg-ATP +/- Ubiquitin was incubated without Fraction II (lane 1) or with Fraction
1035 II (lane 2) and resolved by SDS-PAGE. Proteins were transferred to blots and probed with
1036 a GST-specific antibody. The smear of higher molecular weight PARP2 seen in lane 2 was
1037 indicative that the protein had been ubiquitinated by enzymes in Fraction II. The band
1038 around 150kD in lane 1 was GST-PARP2 dimers present in commercial recombinant GST-
1039 PARP2 protein. The intense signal seen in the input lane was GST-PARP2 smear from
1040 COS-7 cells overexpressing *pBC-GST-PARP2*. (C) COS-7 cells were transfected with

1041 *pBCGST-PARP2* or *pBC-GST-NLS*, with or without *pMT123HA-ubiquitin* and then treated
1042 with MG-132 or left untreated as a control. Cells were lysed in 1x RIPA buffer and the
1043 lysates used in GST-pull down assays. Results show that PARP2 was ubiquitinated (lane
1044 1) and the levels accumulated when proteasome activity was inhibited with MG-132 (lane
1045 2). The input lane was protein from COS-7 cells that were overexpressing GST-PARP2
1046 and HA-ubiquitin. Cells transfected with *pBC-GST-PARP2* alone did not show an HA
1047 signal (lane 3), while the Simian virus 40 nuclear localization signal and positive control
1048 was ubiquitinated (lane 4). The GST immunoblot (right panel) confirmed the presence of
1049 the GST-epitope tagged proteins in the samples probed with the HA antibody (left panel).
1050 The asterisk represents GST fragments from the recombinant proteins.

1051

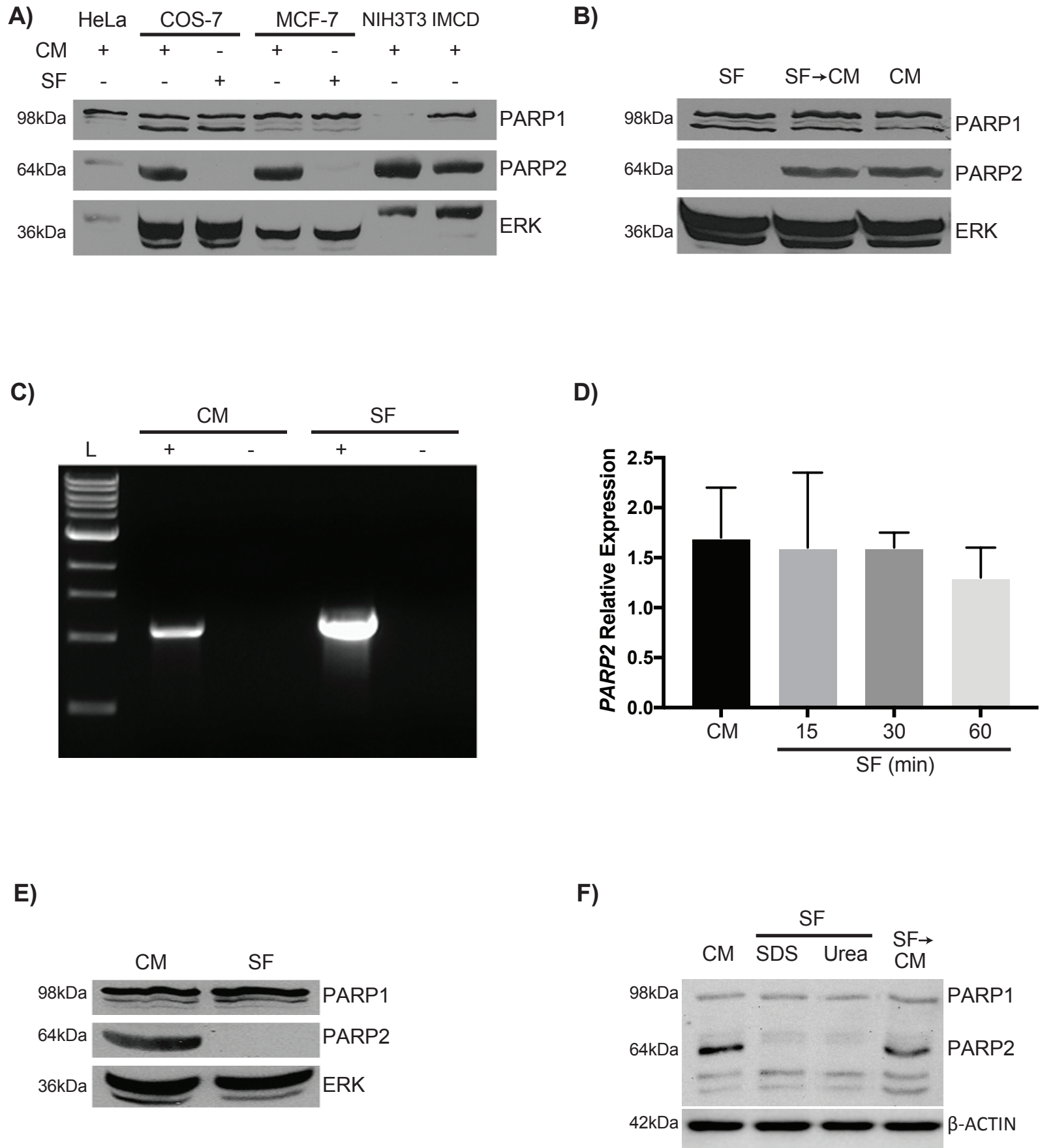
1052 **Fig. 5.** The ubiquitin-proteasome system (UPS) is involved in PARP2 degradation under
1053 serum conditions. (A) COS-7 cells were cultured in CM until 90% confluent and then
1054 transferred and cultured for 5hr in CM containing MG-132. Cells were cultured for 15
1055 minutes in SF medium containing MG-132, and lysates collected for immunoblot analysis
1056 with antibodies against PARP1/2 and ERK. Inactivating proteasome activity had no effect
1057 on preserving the PARP2 signal. (B) COS-7 cells were cultured in CM for 24hr and then
1058 in SF (lane 1), CM (lane 2), or CM containing different concentrations of MG-132 (lanes
1059 3-5). Immunoblot analysis with antibodies against PARP1/2 and ERK of lysates from cells
1060 maintained for 24hr under these conditions shows significant increase in levels of PARP2,
1061 but only in cells cultured in CM with MG132. * $P < 0.05$.

1062

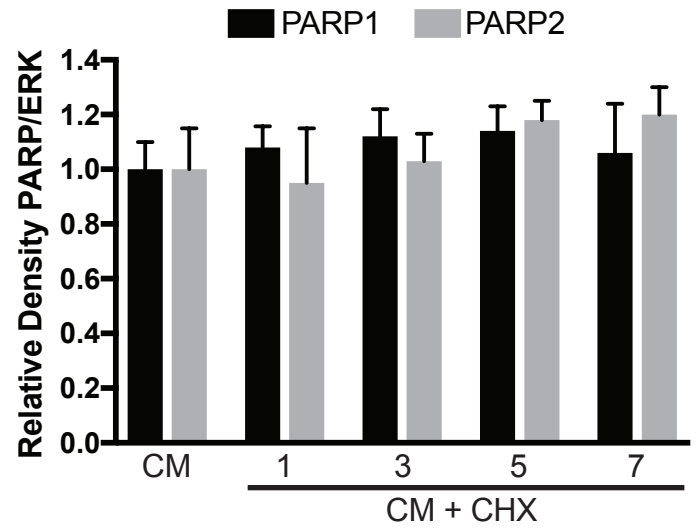
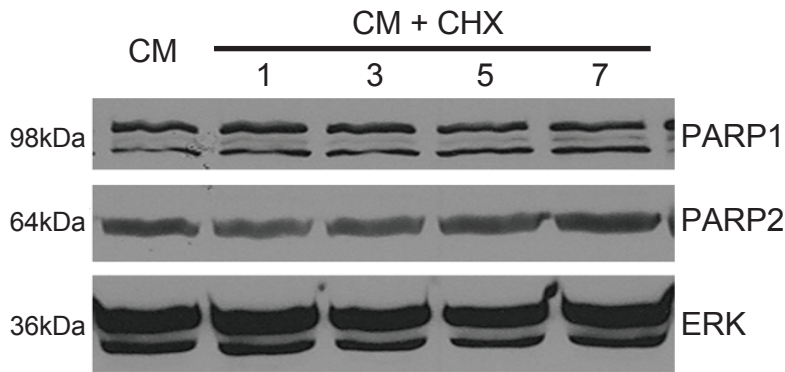
1063 **Fig. 6.** PARP2 is sequestered to a SDS-insoluble fraction following serum starvation. (A)
1064 MCF-7 cells were cultured in CM until 90% confluent and then medium was replaced with
1065 SF medium containing CHX or in CM with CHX. After 7hr, cells in lanes 1-4 were lysed
1066 in 1x Laemmli extraction buffer. Cells in lanes 5 and 6 were cultured in CM or CM and
1067 CHX, respectively, for an additional hour and then lysed in Laemmli buffer. All samples
1068 were probed with antibodies against PARP2 (Axxora) and ERK. Though no apparent
1069 differences were seen in the PARP2 signals between samples in lanes 4-6, the presence of
1070 a band in lane 5, under conditions where protein synthesis was inhibited, would indicate
1071 that PARP2 had been sequestered into an SDS-insoluble fraction resulting from serum
1072 deprivation. (B) MCF-7 cells were transfected with *pBC-GST-PARP2* and after 24hr, CM
1073 was replaced with SF medium. Cells were serum starved for 15, 30 or 60 minutes, and
1074 analyzed with antibodies against GST and ERK, show that the GST-PARP2 signal was
1075 comparable between the cells cultured in CM and SF medium (arrow). The asterisk in panel
1076 A denotes non-specific staining, and minor bands seen on blots (arrowheads, panel B) may
1077 be PARP2 fragments from ubiquitin-mediated proteasomal degradation in CM.
1078

1079 **Supplementary Figure legend**
1080

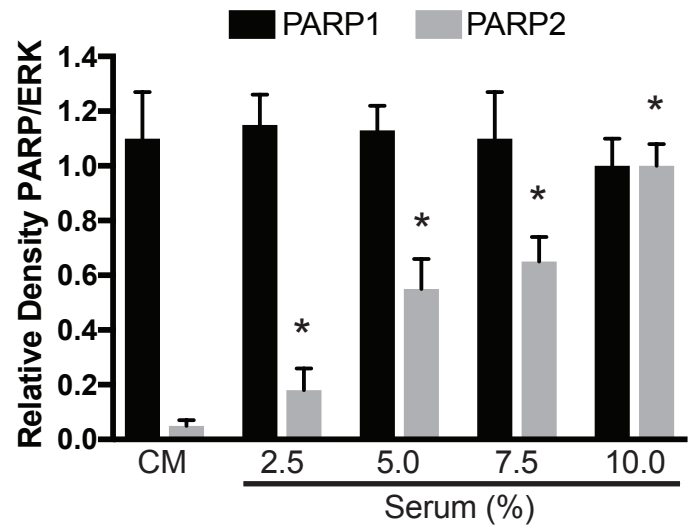
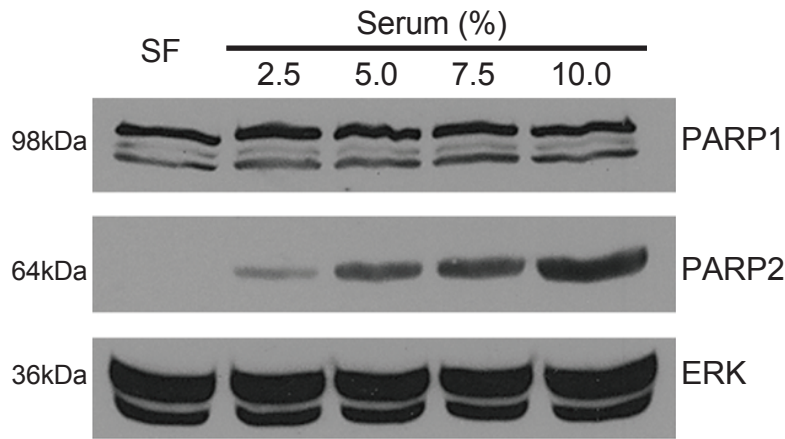
1081 **S1.** Loss of PARP2 under SF conditions attenuates RA-induced differentiation of F9 cells.
1082 F9 cells were cultured in CM for 24 hours and then cultured for 60 minutes in either SF
1083 medium or maintained in CM. Media was then changed to either contain DMSO or RA,
1084 and either in CM or SF conditions for an additional 12 hours. RNA was extracted from
1085 each sample and quantitative RT-PCR was done with mouse *Gata6* and *Hoxb1*-specific
1086 primers. Analysis of quantitative RT-PCR showed that F9 cells treated with RA under SF
1087 conditions had significantly lower *Gata6* and *Hoxb1* expression.



A)



B)



C)

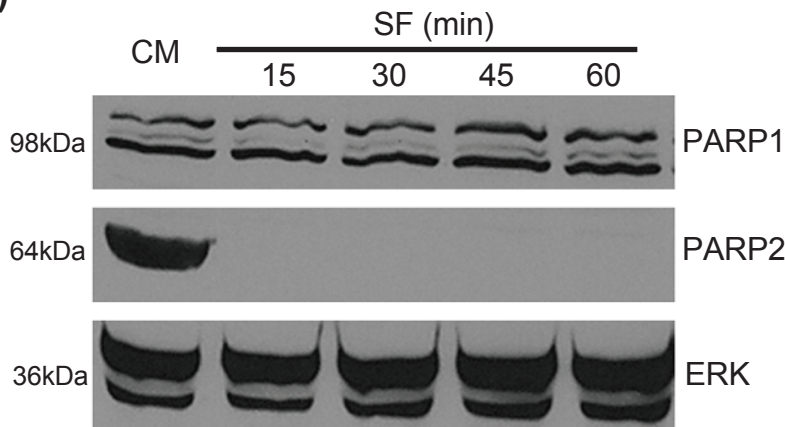
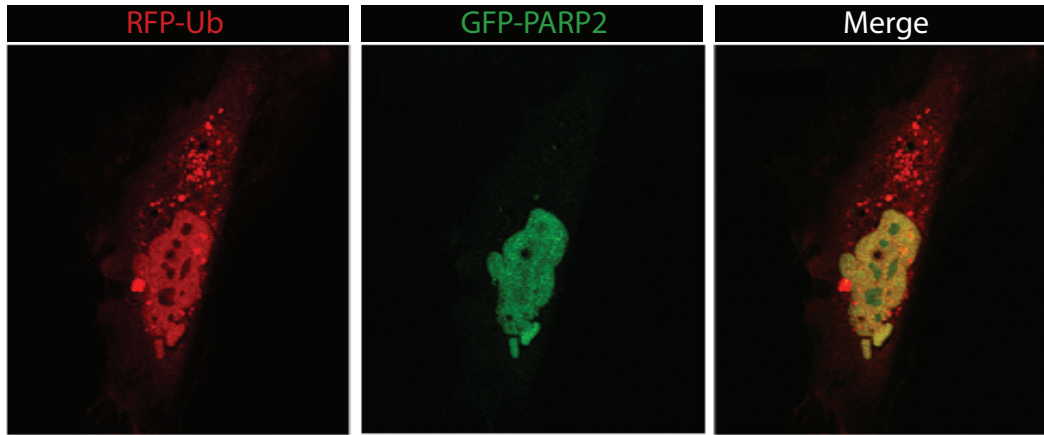
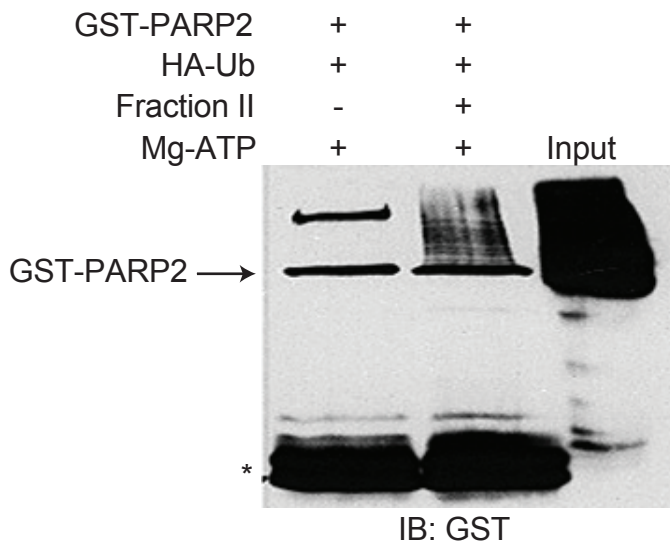


Figure 4

A)



B)



C)

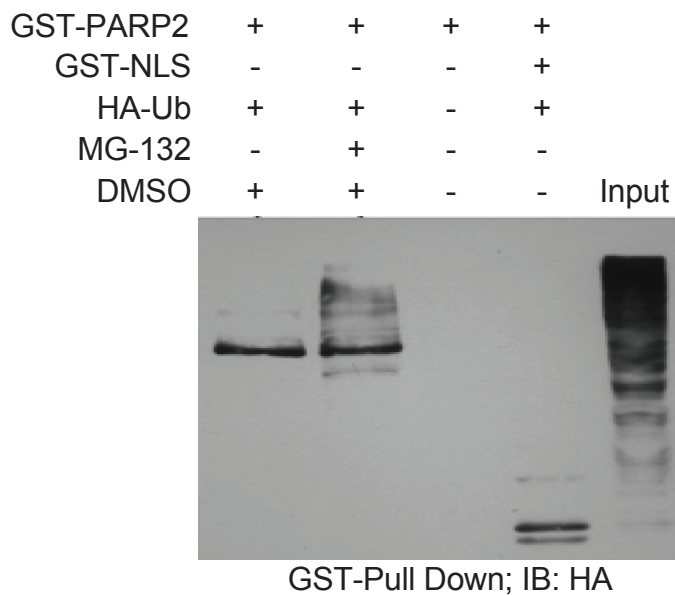
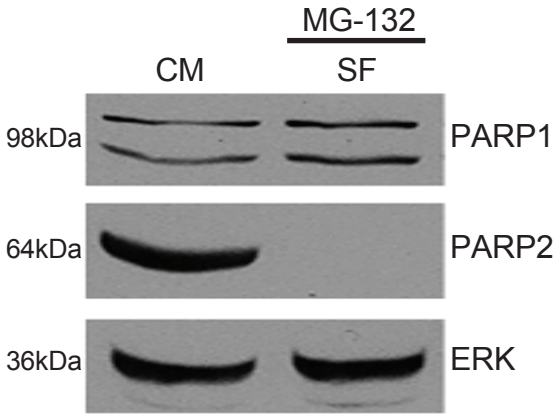


Figure 5

A)



B)

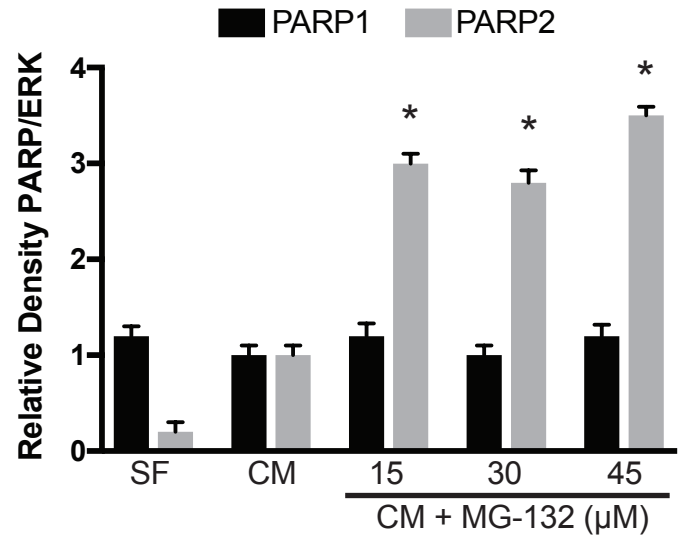
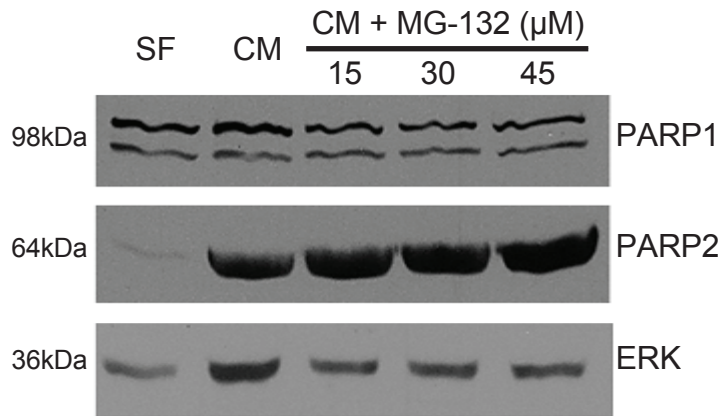
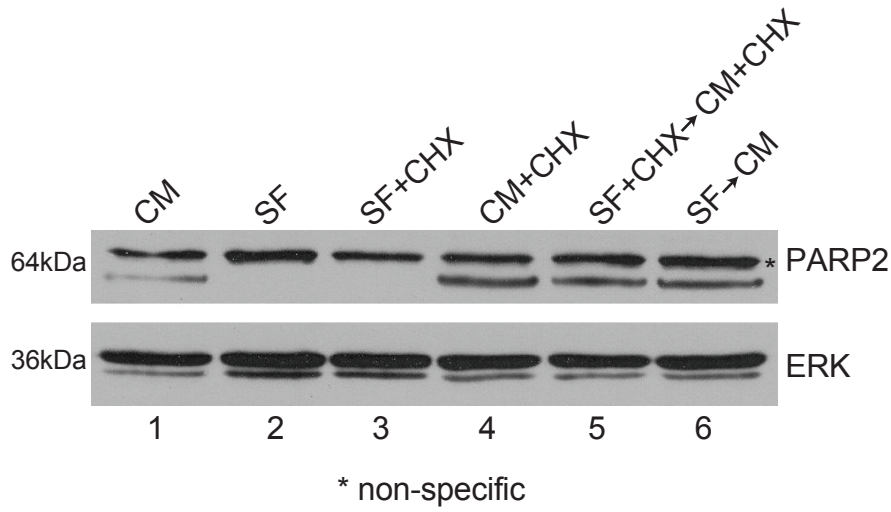
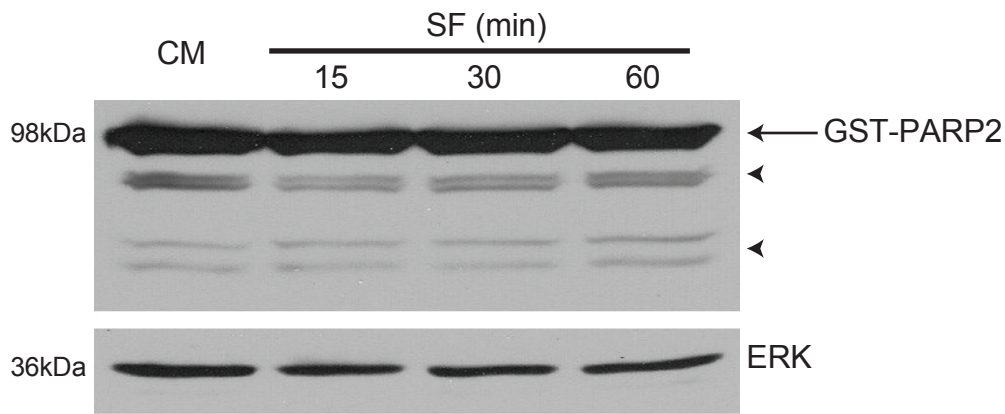


Figure 6

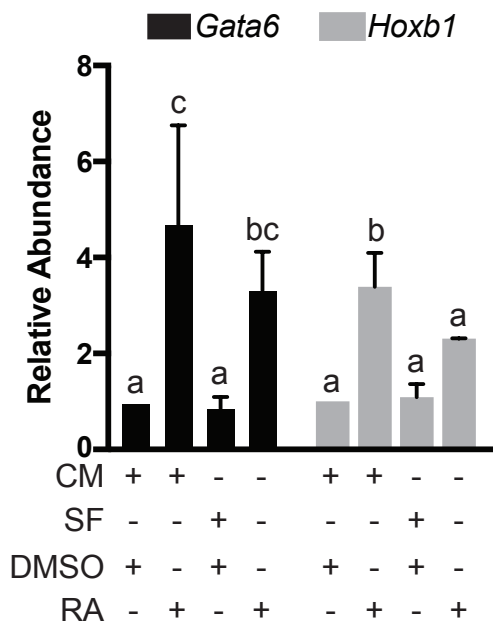
A)



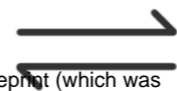
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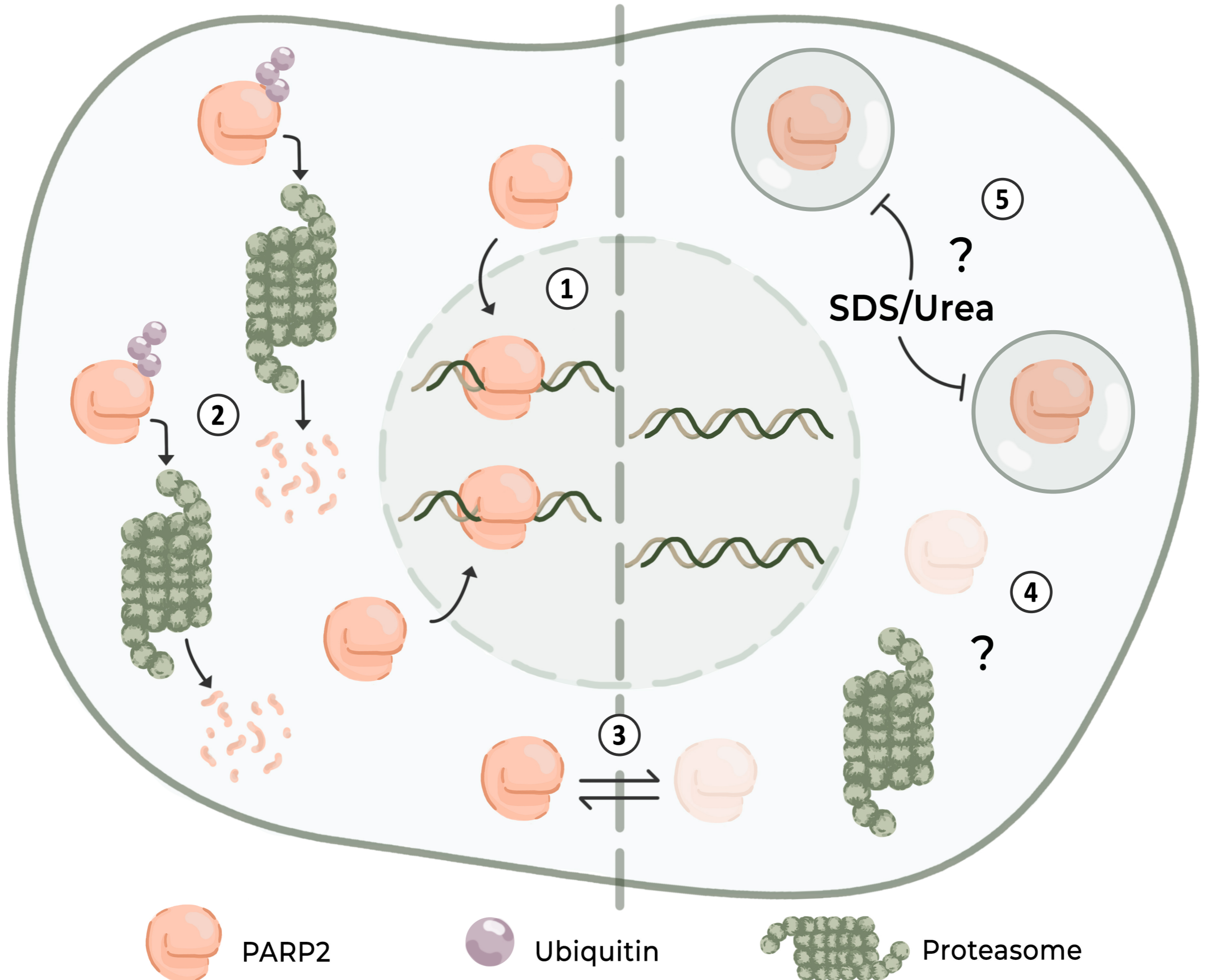
Supplementary Figure 1



Serum



Serum-Free



Graphical Abstract Legend

Fig. 1. Schematic representation of PARP2 regulation under different growing conditions. PARP2 maintains genomic stability by recruiting DNA repair machinery to sites of DNA damage (1), and when not required is ubiquitinated and degraded by the proteasome (2). Under stress condition (3), such as serum-starvation, PARP2 is either degraded in a proteasome-independent manner (4) or packaged into a SDS/urea-insoluble fraction (5).