

1 **COLD ATMOSPHERIC PLASMA DIFFERENTIALLY AFFECTS CELL RENEWAL AND**
2 **DIFFERENTIATION OF STEM CELLS AND APC-DEFICIENT-DERIVED TUMOR CELLS**
3 **IN INTESTINAL ORGANOIDS**

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25 **ABSTRACT**

26 Cold atmospheric plasma (CAP) treatment has been proposed as a potentially innovative therapeutic
27 tool in the biomedical field, notably for cancer due to its proposed toxic selectivity on cancer cells versus
28 healthy cells. In the present study, we addressed the relevance of three-dimensional organoid technology
29 to investigate the biological effects of CAP on normal epithelial stem cells and tumor cells isolated from
30 mouse small intestine. CAP treatment exerted dose-dependent cytotoxicity on normal organoids and
31 induced major transcriptomic changes associated with global response to oxidative stress, fetal-like
32 regeneration reprogramming and apoptosis-mediated cell death. Moreover, we explored the potential
33 selectivity of CAP on tumor-like *Apc*-deficient versus normal organoids in the same genetic
34 background. Unexpectedly, tumor organoids exhibited higher resistance to CAP treatment, correlating
35 with higher antioxidant activity at baseline as compared to normal organoids. This pilot study suggests
36 that the *ex vivo* culture system could be a relevant alternative model to further investigate translational
37 medical applications of CAP technology.

38

39 **INTRODUCTION**

40 Cold atmospheric plasma (CAP) is a partially or totally ionized gas including photons, electromagnetic
41 fields, electrons, ions and neutral radicals such as reactive oxygen and nitrogen species (RONS)(1). In
42 the past decade, this innovative technology has generated growing interest for various applications in
43 the biomedical field such as blood coagulation, sterilization, wound healing and anti-cancer therapy(2).
44 Number of studies have reported that CAP exerts cytotoxic effects when applied directly to cultured
45 cells or indirectly through a plasma activated medium (PAM), as well as anti-tumoral activity(3)(4). The
46 cytotoxic effects are mainly attributed to the production of short and long-lived RONS that generate a
47 redox imbalance, leading to increased intracellular oxidative stress along with damage of cellular
48 components, such as proteins, lipids and DNA(5)(6). Metabolically active cancer cells are reported to
49 exhibit higher basal level of oxidative stress as compared to healthy cells, which could explain the
50 reported selectivity of the anti-tumoral effects of CAP (7)(8). However, the underlying molecular

51 mechanisms of CAP tumor selectivity remain to be clarified. Additionally, most *in vivo* studies have
52 focused on immortalized cells, which do not recapitulate the physiological complexity of epithelia,
53 constituted of various cell types devoted to specific functions *in vivo*(7). Lastly, the impact of CAP
54 treatment on healthy tissues that naturally have a high renewal rate has not been fully investigated.

55 The adult intestinal epithelium is one of the most rapidly self-renewing tissues in adult mammals,
56 supported by a pool of Lgr5 intestinal stem cells (ISCs), also called crypt base columnar cells, that
57 reconstitute the whole epithelium in less than 5 days(9). ISCs have the capacity to both self-renew and
58 give rise to transit-amplifying cells which differentiate along the villus architecture into all the cell
59 lineages of the epithelium, (i.e., absorptive enterocytes, mucus-producing goblet cells, hormone-
60 secreting enteroendocrine cells, Paneth cells generating antimicrobial products, and chemosensory type
61 2 immune response-induced tuft cells)(10). The *ex vivo* culture technology has recently been developed
62 to indefinitely grow ISCs in a Petri dish. Upon seeding into a 3D matrix, ISCs self-renew, proliferate
63 and differentiate into the various epithelial lineages present in the normal epithelium; *ex vivo* grown
64 organoids maintain the *in vivo* relative proportion of each cell subtype and its temporal differentiation
65 (11). Recently, this versatile technology has been used in the context of human colorectal(12)(13) and
66 rectal cancer (14) allowing for the accurate prediction of drug responses(15)(16) in a personalized
67 treatment setting.

68 In the present study, using the *ex vivo* culture system, we investigated the impact of an endoscopic
69 helium plasma jet application on mouse ISCs at the morphological, cellular and transcriptomic levels.
70 Moreover, we explored the potential selectivity of CAP application on tumor versus normal organoids
71 originating from the same genetic background. Our data suggest that the *ex vivo* culture system could
72 be a relevant alternative model to further investigate translational medical applications of CAP.

73 **RESULTS**

74 *CAP treatment affects normal growth of intestinal stem cell-derived organoid cultures*

75 To assess the potential benefits and adverse consequences of CAP treatment on normal surrounding
76 tissues in the digestive epithelium, we set out to investigate the biological effects of plasma on mouse
77 ISCs using the plasma jet device as described in Fig. 1a. For this purpose, we first generated mouse
78 intestinal organoid lines from individual mice (Fig. 1b). For the experiments, fully grown organoids
79 were mechanically dissociated and replated in a tridimensional matrix (Matrigel) supplemented with
80 complete culture medium (Fig. 1b). Twenty-four hours after organoid replating (at day 1), CAP was
81 directly applied to the culture plate wells. Different settings were applied, with varying durations (30 s
82 or 60 s) and powers (30 W, 60 W or 80 W). Fresh culture medium was added 24 hours after CAP
83 treatment (at day 2) and at day 4. At the endpoint (day 5), organoid survival and morphology of the
84 grown elements were compared (Fig. 1c, d). Control cultures, and those treated with either helium gas
85 alone (designated “60 s/0 W”) or mild CAP dose (60 s/30 W) followed similar organoid morphological
86 evolution and survival rates. The spheroid-like structures (primarily constituted of proliferating ISCs
87 during the first days of culture), started protruding as crypt-like domains and then differentiated into the
88 various epithelial lineages present in the normal epithelium by day 5. Then, differentiated cells
89 accumulated in the lumen of fully grown organoids (Fig. 1c, d). Conversely, exposure to moderate and
90 higher doses of CAP (60 and 80 W, respectively) significantly reduced organoid budding capacity.
91 Meanwhile, spheroid-like elements were overrepresented as compared to controls at day 5 (Fig. 1c, d).
92 Moreover, under the 80 W-dose condition, dark declining structures were observed, accompanied by
93 overall reduced survival rate tendency as compared to controls [$34.2 \pm 1.6\%$ in CAP 60 s/80 W versus
94 (vs) $44.5 \pm 4.3\%$ in untreated samples, respectively, unpaired t-test $p=0.0698$]. Together, these
95 experiments indicate that direct CAP treatment alters the growth capacity of healthy ISCs in a dose-
96 dependent manner.

97 Next, we investigated the influence of the culture medium on ISC growth at a fixed moderate dose (60
98 s/ 50 W) (see experimental design in Fig. 2a). Direct CAP treatment was applied at day 1 and the treated
99 culture medium was replaced with fresh medium at 30 min, 3 h or 1 day (1 d) later (Fig. 2a). The

100 morphology of grown elements was studied at day 3, an endpoint at which 61.8% of grown elements in
101 untreated cultures showed at least one crypt-like domain (Fig. 2b, c). Removal of CAP-treated medium
102 early after the procedure (30 min and 3 h) did not significantly alter protrusion formation. In contrast,
103 prolonged incubation with CAP-treated culture medium (24 hours = 1 d) was associated with higher
104 proportion of elements grown as spheroids (67%), only 9% of total elements were protruded organoids
105 (Fig. 2b, c). Together, these data indicate that the time of exposure to reactive species generated by CAP
106 treatment affects the capacity of ISCs to normally grow and differentiate. Interestingly, a similar
107 negative impact on organoid budding was observed when “naïve” organoids were indirectly submitted
108 to reactive species present in conditioned media generated either by CAP treatment applied onto another
109 organoid culture (designated as “indirect CAP”) or the Plasma-Activated Medium alone (designated as
110 “PAM”) (Fig. 2a-c). Exposure to Indirect CAP- or PAM-conditioned media 24 hours after the treatment
111 (from day 2 to day 3) also altered organoid protrusion, albeit to a milder degree, thereby indicating that
112 toxic long-lived reactive species were still present in culture supernatants after 24 hours (Supplementary
113 Fig. 1S a-c). Taken together, these data demonstrated that normal ISCs are sensitive to direct as well as
114 indirect CAP/PAM treatment *ex vivo*.

115 *CAP treatment decreases the intestinal stem cell pool and is associated with Apoptosis*

116 To further investigate the molecular and cellular mechanisms of CAP-induced morphological effect on
117 intestinal organoid growth, we analyzed at day 3 the whole transcriptome of organoids treated directly
118 or indirectly with CAP at a moderate dose (50W/60 s) during a period of 24 hours (Fig. 2a). We
119 identified 2462 differentially expressed genes in CAP-treated vs untreated cultures (False Discovery
120 rate 0.01 and Log2-fold change of 1 or above) (Fig 2d). Of these, CAP-treatment induced
121 downregulation of 1195 genes involved in the following biological processes: cell cycle progression,
122 cell division and DNA replication (Fig. 2e). ISC markers like *Lgr5*, *Olfm4*, *Smoc2* and *Axin2* genes were
123 amongst the most downregulated genes (Fig. 2d). In particular, 35% of the genes identifying the
124 intestinal crypt base columnar stem cell signature (i. e. 135 out of 379 genes) reported by Munoz et al,
125 (17) were downregulated in CAP-organoids (Fig. 3a, b). Loss of ISCs in CAP-treated cultures was
126 confirmed by *in situ* hybridization experiments and immunofluorescence staining for *Olfm4*-expressing

127 cells; this correlated with significant drop in canonical Wnt signaling activity (*Axin2* used as reporter
128 gene) (Fig. 3b-d). Consistent with suggested upregulation of the apoptotic process (p value e-49, Fig.
129 2e), the *Pycard* gene, involved in regulation of apoptosis and adaptation to the inflammatory response,
130 and genes associated with DNA repair (*Pclaf*, *Usp49*, *Hmga2*) were found to be downregulated
131 meanwhile apoptosis-mediator/effector genes (Calpains *Capn2/5/12/13*, *Trp53inp2*, *Ctsl*, *Unc5b*, *Cidec*,
132 *Atf3*, *Ddit3*, *Cfap157*) were upregulated in CAP-treated organoids (Fig. 3c, 4a). TUNEL assay
133 performed on organoid sections confirmed the presence of DNA double strand breaks in CAP-treated
134 cultures (Fig. 4b).

135 *CAP treatment induces a global response to reactive species in intestinal stem cell-derived organoids*

136 In addition, CAP induced upregulation of genes involved in modulation of intracellular signal
137 transduction, negative regulation of response to stimulus, and response to oxygen containing compounds
138 as well as in positive regulation of developmental processes (Fig. 2e). In line with response to CAP
139 mediated-injury, pro-survival (*Dsg3*, *Phlda3*) and regeneration-associated genes (*Trop2/Tacstd2*, *Ptgs2*,
140 *Hbegf*, *Ly6a/Sca1*, *Clu*, *Areg*, *Epn3*) were induced as early as 30 min post-direct CAP treatment (Fig.
141 5a). Indeed, 34% of the genes identifying the fetal-like Trop2 regeneration signature (50 out of the 148
142 gene list, (18)) were upregulated in CAP-treated organoids (Fig. 5b). Accordingly, both the number of
143 Trop2-expressing cells and Areg expression were significantly increased in CAP-treated organoids (Fig.
144 5c, d). Furthermore, a global stress response gene expression pattern was detected with early
145 upregulation of oxidative stress-associated transcription factors like *Fos*, *Fosb*, *Egr1*, *Nfe2l1/Nrf1*,
146 *Nupr1*, *Nr4a1* and *Jdp2* (Fig. 5a, Supplementary Fig. 2Sa). In line with reported regulation of oxidative
147 stress by long noncoding RNAs, expression of *Fer1l4*, *Gm20417*, *Neat1*, *Malat1*, *Gm37376* or *Kcnq1ot1*
148 was induced 30 min after CAP-treatment (Supplementary Fig. 2Sa) (19). Effectors of global response
149 to CAP-mediated oxidative stress in organoids were found upregulated. Among these, genes encoding
150 solute/metabolite transporters such as *Slc7a11*, a cysteine/glutamate antiporter xCT, and detoxifying
151 enzymes (*Hmox1*, *Ethe1*, *Gch1*, *Gstk1*) were identified (Fig. 5a,d). Components of the
152 cysteamine/cystamine metabolism (*Gpr5a*, *Vnn1*, *Chac1*, *Ggh*), involved in glutathione redox status,
153 were also induced by the treatment (Supplementary Fig. 2Sb). Cell signaling molecules such as the Polo

154 like kinases (*Plk2*, *Plk3*) reported to exert antioxidant functions, as well as *Mapk11*, *Lif* and *Pmepa1*
155 were also upregulated by CAP application (Fig. 2Sb). Of note, the major cellular enzymatic ROS
156 scavengers (superoxide dismutases, glutathione peroxidases, peroxiredoxins and catalase) were detected
157 at high levels in control organoids but their expression did not substantially differ upon CAP-treatment
158 (Table 1). *Pparg*, a master regulator of lipid metabolism and immune response (20), was also found
159 upregulated in CAP-treated organoid cells (Fig. 5a, d). Inflammatory response genes were significantly
160 modulated in CAP-treated organoids: interferon induced *Ifitm2/Ifitm3* and chemokine *Ccl9* were
161 downregulated whereas expression of *Ifnlr1*, *Ilr1*, *Ddx60* and *Pdlim7* were upregulated (Fig. 2Sb).
162 Moreover, consistent with extensive reshaping of epithelial cells, CAP application was associated with
163 upregulation of cytoskeleton organization, cell motility and biological adhesion processes (Fig. 2e, Fig.
164 2Sb).

165 *Apc deficient-derived organoids exhibit increased resistance to CAP treatment as compared to ISCs-
166 derived organoids*

167 Since ISCs were sensitive to moderate and high doses of CAP, we sought to compare resistance of
168 normal or tumor organoids to this treatment. For this purpose, adult VilCreERT2-Apc^{fl/fl} or
169 VilCreERT2-Apc wild-type (wt) mice were injected with tamoxifen to induce specific deletion of Apc
170 exon 15 in VilCreERT2-Apc^{fl/fl} mice, leading to loss-of-function of this tumor suppressor (designated
171 as Apc Δ). Apc wt and Apc Δ intestinal crypts were isolated and cultured to generate normal and tumor
172 organoid lines, respectively (Fig. 6 a). Efficient recombination in Apc Δ tumor-derived organoids was
173 controlled at initial seeding (Supplementary Fig. 3Sa). Then, upon organoid replating, CAP was directly
174 applied at various doses for 60 s on both kinds of organoids (Fig. 6b). As expected, at day 5, Apc wt
175 organoid survival was substantially reduced at a dose of 50 W and 80 W as compared to untreated
176 organoids (Fig. 6 b,c). Conversely, Apc Δ organoids demonstrated higher survival rates, regardless of
177 the CAP dose (Fig. 6c). Dosage of reactive species in culture supernatants did not demonstrate
178 substantial differences between organoid types at a given CAP-dose, which might have explained the
179 observed higher resistance of Apc Δ vs Apc wt organoids (Supplementary Fig. 3Sb). To further explore
180 the underlying molecular mechanisms, qPCR experiments were performed on samples collected at day

181 5. With the exception of *Olfm4*, expression of markers for active (Lgr5) and quiescent (Hopx) stem cell
182 populations was maintained in tumor organoids, even at the highest CAP dose (80 W) (Fig. 6d).
183 Regarding cell differentiation, mild CAP dose (30 W) in Apc wt organoids had a tendency to decrease
184 the Paneth lineage vs the other cell types whereas Apc Δ-organoids exhibited limited cell differentiation
185 in any tested condition (Fig. 6d). Interestingly, expression of regeneration marker genes was upregulated
186 in Apc wt organoids upon CAP treatment (30 W), and was detected at much higher levels in tumor
187 organoids even under normal conditions (Fig. 7b). Moreover, tumor organoids maintained proliferation
188 capacity and low cell death behavior following CAP treatment (Fig. 7b). Furthermore, Apc Δ organoids
189 demonstrated increased levels of genes (e. g. *Atf3*, *Nuprl1*) involved in early response to reactive species
190 as compared to Apc wt organoids (Fig. 7b). In addition, cellular transporters (*Slc7a11*) and detoxifying
191 enzymes (*Hmox1*), induced in Apc wt organoids by mild CAP treatment, were expressed at significantly
192 higher levels in basal conditions in tumor organoids (Fig. 7b). Differential expression of the membrane-
193 associated aquaporins has also been proposed to contribute to CAP selectivity by facilitating influx of
194 water and hydrogen peroxide into cancer cells(21). In ISCs and healthy intestinal organoids, RNAseq
195 data showed that *Aqp1*, *Aqp4* and *Aqp11* were the most significantly expressed (Supplementary Fig
196 S3c). CAP treatment particularly reduced *Aqp1* and *Aqp4* levels in Apc wt organoids (Supplementary
197 Fig S3c). Interestingly, irrespective of CAP treatment, tumor organoids were expressing 2-fold less
198 *Aqp1*, *Aqp4* and 10-fold less *Aqp3* levels than normal organoids, whereas *Aqp5* expression was
199 increased (Supplementary Fig S3d). Taken together, these experiments indicate that tumor-like
200 organoids deficient for the tumor suppressor Apc demonstrate a higher potential to resist CAP-induced
201 injury as compared to ISC-derived organoids. Such behavior could be, in part, explained by the
202 expression of an oxidative stress resistance program, already active under basal conditions, and reduced
203 cell surface expression of aquaporins.

204 **DISCUSSION**

205 The present study demonstrated the relevance of a 3D organoid culture to investigate the biological
206 effects of CAP on normal epithelial stem cells and tumor cells isolated from the mouse small intestine,
207 and unexpectedly showed higher resistance of tumor organoids to CAP treatment.

208 First, we studied the parameters that determine CAP effects on cells and found that helium, the carrier
209 gas, did not by itself alter organoid growth. We observed toxic effect of direct CAP application to ISC
210 cultures, showing dose-dependency as reported on various cancer lines (21). To determine whether CAP
211 treatment can induce a “bystander effect” as described in radiobiology, in which irradiated cells
212 communicate stress via extracellular signaling to neighboring cells, we also analyzed indirect
213 application of CAP on organoids (Indirect CAP and PAM). In our experimental setting, organoid growth
214 was similarly affected by the three conditions, indicating that the main effect of CAP was mediated by
215 reactive species delivered in the culture medium, with minor contribution of cellular components
216 potentially released from the CAP-treated organoids. Our data also suggest that CAP-mediated
217 cytotoxicity depends in part on long-lived reactive species (typically nitrites, hydrogen peroxide, ozone)
218 since increased levels of nitrites were still detected 24 hours after CAP treatment as compared to controls
219 (Supplementary Fig S3b). On the other hand, it is known that the nature of CAP-generated RONS is
220 conditioned by the composition of the cell culture medium(22). Organoid growth requires a complex
221 medium, including several antioxidant supplements such as N-acetyl cysteine, glutathione, sodium
222 pyruvate and ascorbic acid (23) (24)(25) (26). We hypothesize that the presence of such antioxidants in
223 the medium might have increased the threshold level needed to detect variations in the long-lived ROS
224 species with the DCFH-DA probe (Supplementary Fig S3b).

225 Moderate (50/60 W) to high (80 W) doses of CAP severely impacted ISC self-renewal and
226 differentiation. Although ISCs express important levels of ROS scavenger enzymes (Supplementary
227 Fig. S3e), previous studies have also reported that, under steady-state conditions, ISCs exhibit two-fold
228 increased levels of ROS as compared to the rest of the epithelium(27). Together, these data are in line
229 with the hypothesis that the application of moderate-to-high CAP doses to ISCs reaches a redox status
230 threshold above which toxic accumulation of RONS occurs, similar to what has been previously

231 described for cancer cell lines(7)(8); this ultimately induces DNA damage and cell death by apoptosis.

232 Interestingly, moderate CAP dose also elicited an epithelial response with hallmarks of tissue

233 regeneration, characterized by re-expression of a fetal signature, reported to be induced by a variety of

234 insults in the gastrointestinal tract(18)(28)(29)(30). As part of this fetal reprogramming, upregulation of

235 genes coding for factors mainly released by the surrounding stromal compartment (Areg, Ptgs2, etc..),

236 was observed in epithelial cells *ex vivo*. This suggests that, upon injury, the epithelium has the intrinsic

237 potential to contribute to the repair process. In the same line, CAP treatment on murine cementoblasts

238 has been reported to induce a regeneration process similar to that elicited by enamel matrix derivatives

239 *in vivo*(31). Moreover, CAP treatment induced a global response to oxidative stress in organoids that

240 involved upregulation of RONS-associated transcription factors and effectors known to regulate

241 intracellular ROS balance and reported to be activated upon CAP treatment(32)(33)(34)(35).

242 A major observation of our study relates to CAP application effects on tumoral APC-deficient organoids.

243 The potential of CAP treatment in oncology relies on a proposed selective targeting of cancer cells over

244 healthy cells due to higher intracellular levels of ROS in malignant cells (36). However, compared

245 sensitivity of CAP was never performed on the same cell type and genetic background, with cells

246 growing under the same culture conditions. This was addressed in the present study by comparing the

247 impact of CAP application on normal “healthy” organoids and tumoral Apc-deficient organoids, the

248 latter being used as a colorectal cancer (CRC) model. Contrary to our initial expectations, tumor

249 organoids exhibited higher resistance to CAP treatment than healthy organoids. Such results could be

250 explained, at least in part, by the fact that Apc-deficient organoids exhibited a much higher antioxidant

251 response at baseline than ISC-derived organoids. The potential contribution of differential expression

252 of aquaporins to CAP selectivity could be interesting to address in future *ex vivo* studies.

253 In summary, the present study reveals the potential of organoid technology to further investigate the

254 biological effects of CAP on normal and tumoral tissues. Indeed, organoid cultures faithfully reflect cell

255 heterogeneity in epithelial tissues, they are as amenable to “Omics” studies as the cancer cell lines and,

256 from a translational point of view, they can be used to compare various CAP application settings. Our

257 study highlights the importance of considering CAP toxicity on metabolically active resident stem cells

258 in tissues, like the intestine, undergoing permanent self-renewal, in order to adjust the dose of treatment.

259 Furthermore, CRC development is known to be a multistep process. Following the initial hit mutation

260 in the Apc gene that leads to Wnt signaling overactivation, mutations deregulating other pathways (such

261 as KRAS/TGFb and p53) sequentially accumulate in cancer cells, and correlate with cancer

262 progression(37)(38). Future studies will be needed to investigate the sensitivity of CAP treatment on

263 tumor organoids bearing the aforementioned additional mutations in order to definitely elucidate the

264 potential of CAP for personalized anti-cancer therapy.

265 **MATERIALS AND METHODS**

266 *Experimental animals*

267 Adult mice were from the outbred CD1 strain (Charles River Laboratories). To obtain tumor-derived
268 organoids, we crossed Tg(Vil1-cre/ERT2)^{23Syr/J} (39) and Apc^{tm1Tyj/J} designated Apc^{flox} (40). Adult Vil-
269 cre/ERT2/Apc^{wt/wt} and Vil-cre/ERT2/Apc^{flox/flox} were injected intraperitoneally for 3 consecutive days
270 with tamoxifen (2 mg per 30 g of body weight) to induce recombination in the Apc locus and the small
271 intestine was harvested 2-3 days after the last injection. Tamoxifen was dissolved in a sunflower
272 oil/ethanol mixture (9:1) at 10 mg/ml (both products from Sigma-Aldrich).

273 *Ex vivo culture*

274 To generate intestinal organoids, mouse adult small intestine was dissociated with 5 mM EDTA-in
275 DPBS (Gibco) according to the protocol reported in(41). Briefly, the culture medium consisted of
276 Advanced-DMEM/F12 medium supplemented with 2 mM L-glutamine, N2 and B27 w/o vit.A,
277 gentamycin, penicillin-streptomycin cocktail, 10 mM HEPES (all from Invitrogen), 1 mM N acetyl
278 cysteine (Sigma-Aldrich), 50 ng/ml EGF and 100 ng/ml Noggin (both from Peprotech), and 100 ng/ml
279 CHO-derived mouse R-spondin 1 (R&D System). Culture medium was changed every other day and
280 after 5-6 days in culture, organoids were harvested, mechanically dissociated and replated in fresh
281 Matrigel matrix (catalog 356235 from BD Biosciences). Culture media were supplemented with 10 µM
282 Y-27632 (Sigma Aldrich) in all initial seeding and replating experiments for the first two days. Pictures
283 were acquired with a Moticam Pro camera connected to Motic AE31 microscope.

284 *Cold atmospheric plasma treatment*

285 The cold plasma source was an endoscopic plasma jet allowing for the generation and transport of CAP
286 over long distances as described in our previous work(42). It consists of a tubular DBD chamber made
287 of quartz supplied with helium gas and surrounded by a high-voltage electrode. The power-control
288 source was an AFS (G10S-V) generator, delivering an 18 kHz sinusoidal signal. The discharge chamber
289 was plugged into a polytetrafluoroethylene (PTFE) tube (outer diameter 3 mm, wall thickness 0.75 mm)
290 transporting the plasma post-discharge over > 2 meters. An electrically floating copper wire (diameter
291 0.2 mm) was inserted partially into the dielectric chamber and extended almost until the end of the PTFE

292 tube (5 mm before its end) to allow the maintenance of active plasma for several meters and to sustain
293 a plasma plume at the outlet for potential endoscopic treatment. Treatment was performed under a
294 laminar flow hood using a 1.6 lpm helium flow; thus RONS were created by the mixing of CAP with
295 ambient air (42). The power values (0, 30, 50, 60 or 80 W) were set on the AFS-generator. The catheter
296 was placed vertically in a designated test bench. The tip of the catheter was placed at 3 cm from the top
297 of the 12-well plate containing the media to be treated, i.e. the length of the plasma plume. This allowed
298 plasma-generated RONS to reach the medium without the plasma plume touching it, avoiding any
299 electrical connection that would have increased current and RONS creation in a less controlled manner.
300 Fully-grown organoid cultures were dissociated by mechanical pipetting and replated on Matrigel in 12-
301 well plates (VWR, Belgium). Twenty-four hours later, CAP was applied vertically on organoid cultures
302 at room temperature. Each well was placed successively under the plasma plume for 30 s or 60 s. During
303 treatment, the other wells were protected by a designated cover plate to avoid unwanted RONS diffusion
304 to neighboring wells. Following exposure to CAP, treated samples were placed back at 37 °C in a 5%
305 CO₂ incubator (Binder C150) and the medium was changed as indicated in the results section and
306 different endpoints were applied.

307 *Tissue processing and immunohistochemical analysis*

308 Organoid culture samples were fixed with 10% formalin solution, neutral buffered (Sigma-Aldrich) for
309 20 minutes at room temperature then sedimented through 30% sucrose solution before OCT embedding.
310 Histological protocols as well as immuno-fluorescence/histochemistry experiments on 6 µm sections
311 were carried out as previously described (43). Table 2 lists primary antibodies, TUNEL assay kit and
312 DAPI used. Stained samples were visualized with a DMI600B epifluorescence microscope equipped
313 with a DFC365FX camera (Leica). The number of independent organoid cultures obtained from
314 individual adult mice used for each experiment is reported in Figures and in Figure legends.

315 *Gene expression analysis*

316 qRT-PCR was performed on total RNA extracted from organoid cultures as reported(44). Expression
317 levels of target genes were normalized to that of reference genes (Rpl13, Ywhaz). Table 2 lists the
318 primers used for qPCR studies. *In situ* hybridization experiments were performed according to

319 manufacturer instructions with the RNAscope kit (ACD-Biotechne) (probes listed in Table 2). Stained
320 samples were visualized with a Nanozoomer digital scanner (Hamamatsu).

321 *RNA seq and Gene Set Enrichment Analysis (GSEA)*

322 RNA quality was checked using a Bioanalyzer 2100 (Agilent technologies). Indexed cDNA libraries
323 were obtained using the Ovation Solo (NuGen) or the NEBNext RNA-Seq Systems following
324 manufacturer recommendations. The multiplexed libraries were loaded onto a NovaSeq 6000 (Illumina)
325 using an S2 flow cell and sequences were produced using a 200 Cycle Kit. Paired-end reads were
326 mapped against the mouse reference genome GRCm38 using STAR software to generate read
327 alignments for each sample. Annotations Mus_musculus.GRCm38.90.gtf were obtained from
328 ftp.ensembl.org. After transcripts assembling, gene level counts were obtained using HTSeq.
329 Differentially expressed genes were identified with EdgeR method and further analyzed using GSEA
330 MolSig (Broad Institute)(45). Heatmaps were generated using Heatmapper(46) and common signature
331 genes were identified using Venny 2.0 (47).

332 *Detection of reactive species*

333 Detection of reactive species was performed in organoid culture supernatants 24 hours after CAP
334 treatment, as described in (22). Nitrite concentration was measured using a colorimetric assay with the
335 Griess reagent. Absorbance was determined at 570 nm using the iMark Microplate reader (BioRad).
336 Global ROS were detected using the reporter 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA,
337 D6883 Sigma-Aldrich). The emitted fluorescence of oxidized DFC was detected at 528 nm using the
338 Microwin software on Mithras LB940 reader (Berthold technologies).

339 *Statistical analysis*

340 Statistical analyses were performed with Graph Pad Prism 5. All experimental data are expressed as
341 mean \pm s.e.m unless indicated in Figure legends. The significance of differences between groups was
342 determined by appropriate parametric or non-parametric tests as described in the text or Figure legends.

343 *Data availability statement*

344 The datasets generated and analyzed during the current study are available in the GEODATASET
345 repository [GEO Accession GSE 178148]. Some datasets analyzed during this study were included in a
346 published article(48) and are available in the GEODATASET repository [GSE 135362].

347

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491 **CONFLICT OF INTEREST STATEMENT**

492 The authors have nothing to disclose.

493 **AUTHOR CONTRIBUTION STATEMENT**

494 AH, ML, OB, MT: study concept and design, acquisition of data, analysis and interpretation of data,
495 statistical analysis, drafting of the ms
496 JD, AN, AD, FR: study concept and design, study supervision, critical revision of the ms, obtained
497 funding
498 MIG: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the
499 ms, study supervision, obtained funding.

500 **ETHIC STATEMENTS**

501 Animal procedures complied with the guidelines of the European Union and were approved by the local
502 ethics committee CEBEA from Erasme-Faculty of Medicine under the accepted protocol 713N.

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507 **FIGURE LEGENDS**

508 **Figure 1. CAP treatment affects normal growth of intestinal stem cell-derived organoid cultures.**

509 **a.** The CAP system allows for generation of a helium CAP that can be transported over long distances
510 for applications in endoscopy. **b.** Direct CAP treatment on organoid cultures generated from small
511 intestine isolated crypts. **c.** Representative pictures of a given field showing growth of CAP-treated or
512 untreated organoids at day 1 (before CAP application) and day 5 (endpoint). Survival rate (%) at day 5
513 vs day 1, indicated below the images, is expressed as the mean \pm SEM (n=4 organoid lines generated
514 from different mice). Curved and straight arrows show fully protruded organoid and spheroid,
515 respectively. Arrowheads indicate declining organoids. Scale bars: 500 μ m. **d.** Quantification of
516 organoid complexity at day 5. Representative element categories (Spheroids and Organoids) are defined
517 morphologically on the right. An average number of 195 elements was analyzed per condition per
518 organoid line (n= 3 organoid lines). Data are represented as means \pm sem. Two-way ANOVA:
519 interaction **** P< 0.0001 followed by Tukey's multiple comparisons test: a: compared to Untreated
520 conditions, b: compared to 60 s/ 0 W conditions **P< 0.01; ***P< 0.001; ****P< 0.0001.

521 **Figure 2. Impact of the CAP application method on organoid morphology and global gene**

522 **expression. a.** CAP treatment (50 W/60 s) was applied directly to organoid cultures at day 1 (post-
523 replating) for 30 minutes, 3 hours or 24 hours. Then fresh medium was provided until day 3 (endpoint).
524 Alternatively, naïve organoids were cultured at day 1 for 24 hours with freshly-generated CAP-
525 conditioned media (Indirect CAP) or plasma-activated media (PAM). At day 2 (post-replating), fresh
526 culture medium was provided for a further 24 hours until day 3 (endpoint). **b.** Representative pictures
527 of a given field showing organoid growth at day 1 (before CAP application) and day 3. Arrowheads and
528 triangles show individual elements evolving as protruded organoids and spheroids, respectively.
529 Asterisks indicate dying elements. Scale bars: 500 μ m. **c.** Quantification of organoid complexity at day
530 3. An average number of 100 elements was analyzed per condition per organoid line (n= 4 organoid
531 lines). Data are represented as means \pm sem. Two-way ANOVA: interaction **** P< 0.0001 followed
532 by Tukey's multiple comparisons test: a, d: ****P< 0.0001, b: ***P< 0.001, c: *P< 0.05. (all compared
533 to untreated). Time of exposure to CAP treatment is indicated (30', 3 h, 1 day) ID: Indirect CAP. **d.**

534 Heatmap of differentially regulated genes in CAP-treated vs Untreated (Controls) organoids. Samples
535 were treated with CAP directly (Direct), for 30 min (30') or 24 hours between day 1 and day 2 (1 d);
536 indirectly (Indirect) or with PAM for 24 hours between day 1 and day 2 (1 d) or between day 2 and day
537 3 (2 d). Some of the most-modulated genes are indicated on the right side. **e.** GSEA-Biological processes
538 for upregulated and downregulated gene lists in CAP-treated vs untreated Controls.

539 **Figure 3. CAP treatment decreases the intestinal stem cell pool.** **a.** Venn diagram showing that part
540 of the downregulated gene list in CAP-treated samples vs controls (135 out of 1195 genes) corresponds
541 to the ISC (Intestinal Stem Cell)-associated signature. **b.** Expression levels of some genes of the ISC-
542 associated signature commonly downregulated in CAP-treated organoids vs Untreated controls.
543 CP20M: counts per kilobase of transcript per 20 million mapped reads. Data are represented as means
544 \pm sd. n = 4 and 3 samples in Controls and CAP-treated conditions, respectively. **c.** Expression of *Lgr5*,
545 *Axin2*, and *Pycard* genes detected in organoids by RNAscope at day 3. **d.** Immunofluorescence showing
546 *Olfm4*-expressing cells in organoids at day 3 (red arrows). Cell membranes shown with β -catenin and
547 nuclei counterstained with DAPI. Right panel: Expression levels of *Olfm4* in the various conditions
548 reported in CP20M. Scale bars: 50 μ m (panels c and d).

549 **Figure 4. CAP treatment of organoids is associated with Apoptosis.** **a.** Expression levels of DNA
550 repair and apoptosis-related genes in the various conditions reported in CP20M. Data are represented as
551 means \pm sd. n = 4 and 3 samples in Controls and CAP-treated conditions, respectively. **b.** Organoid
552 sections were stained with TUNEL for apoptotic cells (visualized by pink asterisks). Cell membranes
553 are shown with β -catenin and nuclei were counterstained with DAPI. Scale bars: 50 μ m.

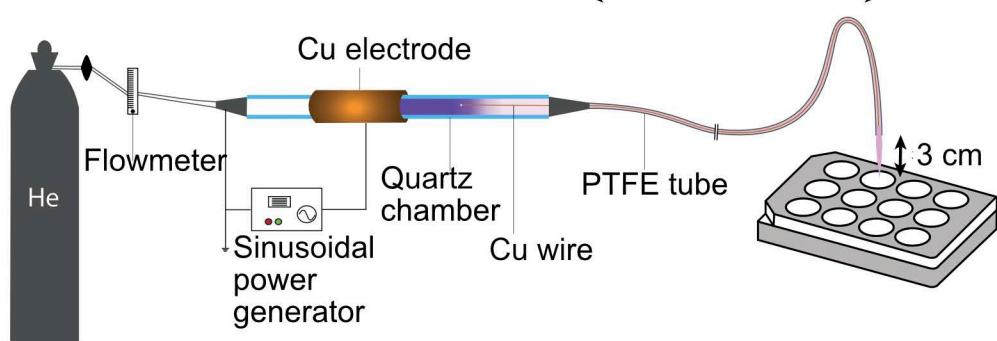
554 **Figure 5. CAP treatment induces a global response to reactive species in intestinal stem cell-
555 derived organoids.** **a.** Expression levels of Pro-survival, regeneration and oxidative stress-associated
556 genes in the various conditions reported in CP20M. Data are represented as means \pm sd. n = 4 and 3
557 samples in Controls and CAP-treated conditions, respectively. **b.** Venn diagram showing that part of the
558 upregulated gene list in CAP-treated vs control organoids (50 out of 1267 genes) corresponds to the
559 Trop-2-associated regeneration signature. **c.** Immunofluorescence showing Trop2-expressing cells
560 (visualized by green asterisks) in organoids at day 3. Cell membranes shown with β -catenin and nuclei

561 counterstained with DAPI. **d.** Expression of *Areg*, *Pparg*, and *Hmox1* genes detected in organoids by
562 RNAscope at day 3. Scale bars: 50 μ m (panels c and d).

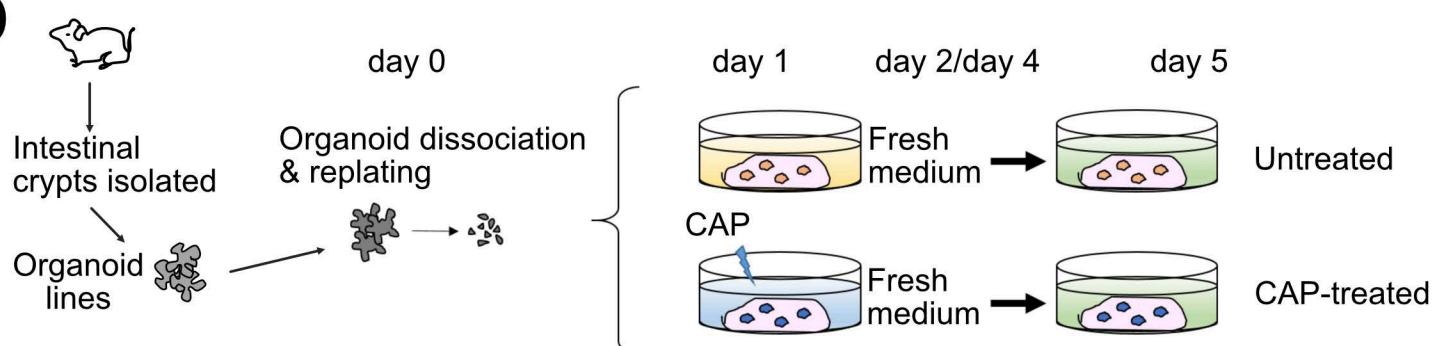
563 **Figure 6. Apc deficient-derived organoids exhibit increased resistance to CAP treatment as**
564 **compared to normal ISC-derived organoids. a.** CAP treatment was applied directly to Apc wt or Apc
565 Δ (Apc-deficient) organoid cultures at day 1 post-replating for 24 hours. Fresh medium was added at
566 days 2 and 4. **b.** Representative pictures of a given organoid type (Apc wt or Apc Δ) at day 5 following
567 CAP treatment at the indicated doses. Scale bars: 500 μ m. **c.** Left panel: organoid survival rate (in %).
568 An average number of 120 elements was studied over time per condition per organoid line (n= 6 Apc
569 wt and 5 Apc Δ organoid lines, respectively). Data are represented as means \pm sem. Two-way ANOVA:
570 CAP dose ***/Genotype **** P< 0.0001 followed by Sidak's multiple comparisons test: a, ***P<
571 0.001; b, ***P< 0.01; c, *P< 0.05; ns: not significant (all compared to Apc wt-0 W), Right panel:
572 quantification of total RNA extracted/sample at day 5. Each symbol corresponds to a given organoid
573 line. Two-way ANOVA: CAP dose * P< 0.05/Genotype **** P< 0.0001 followed by Sidak's multiple
574 comparisons test: a, ****P< 0.0001; b, *P< 0.05; c, **P< 0.01. (all compared to Apc wt-0 W). **d.** Gene
575 expression analysis by qRT-PCR of the indicated stem cell and differentiation markers. Each symbol
576 corresponds to a given organoid line. Values are normalized to Untreated Apc wt levels. Data are
577 represented as means \pm sem. One-way Anova test. **** P< 0.0001; **** P< 0.001; **P< 0.01; *P<
578 0.05; ns: not significant.

579 **Figure 7. Apc deficient-derived organoids exhibit increased resistance to CAP treatment as**
580 **compared to normal intestinal stem cell-derived organoids. a.** Design of the experiment. See Figure
581 Legend Fig. 6a. **b.** Gene expression analysis by qRT-PCR of the indicated markers involved in
582 regeneration, cell growth/apoptosis and response to stress. Each symbol corresponds to a given organoid
583 line. Values are normalized to Untreated Apc wt levels. Data are represented as means \pm sem. One-way
584 Anova test. **** P< 0.0001; **** P< 0.001; **P< 0.01; P< 0.05; ns: not significant.

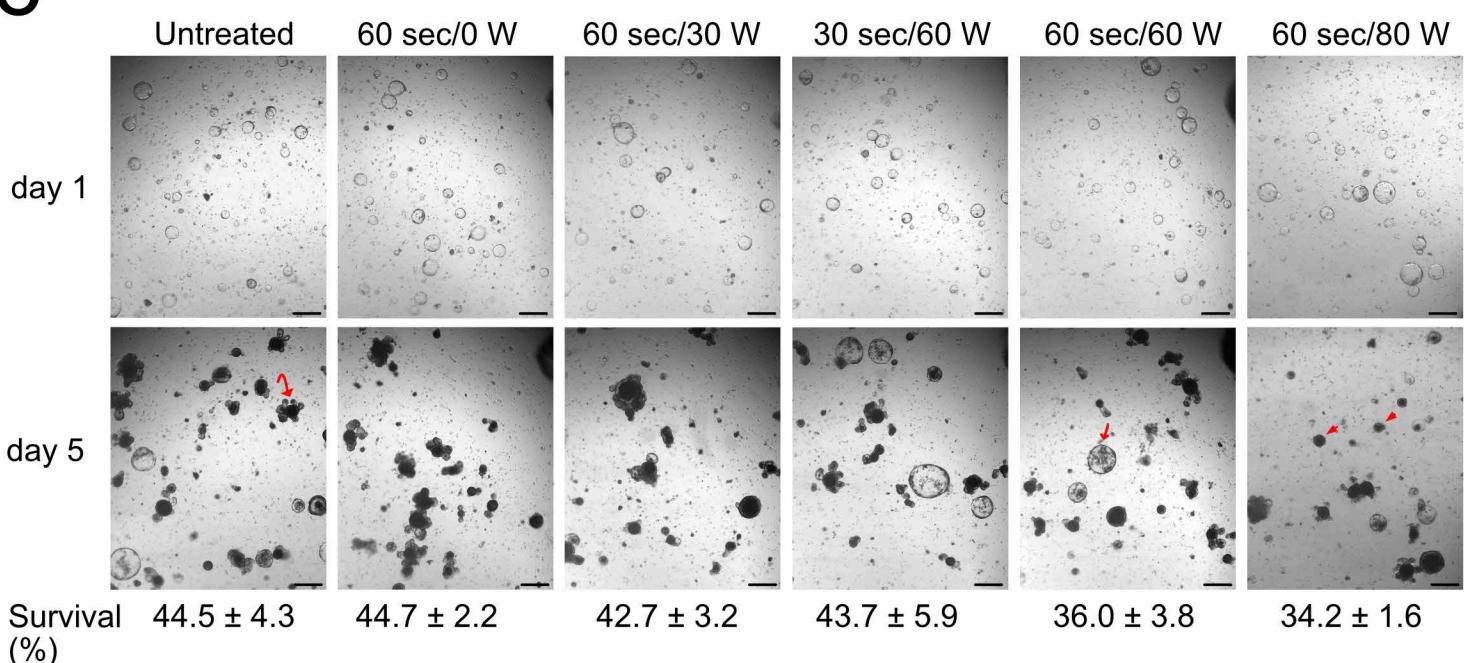
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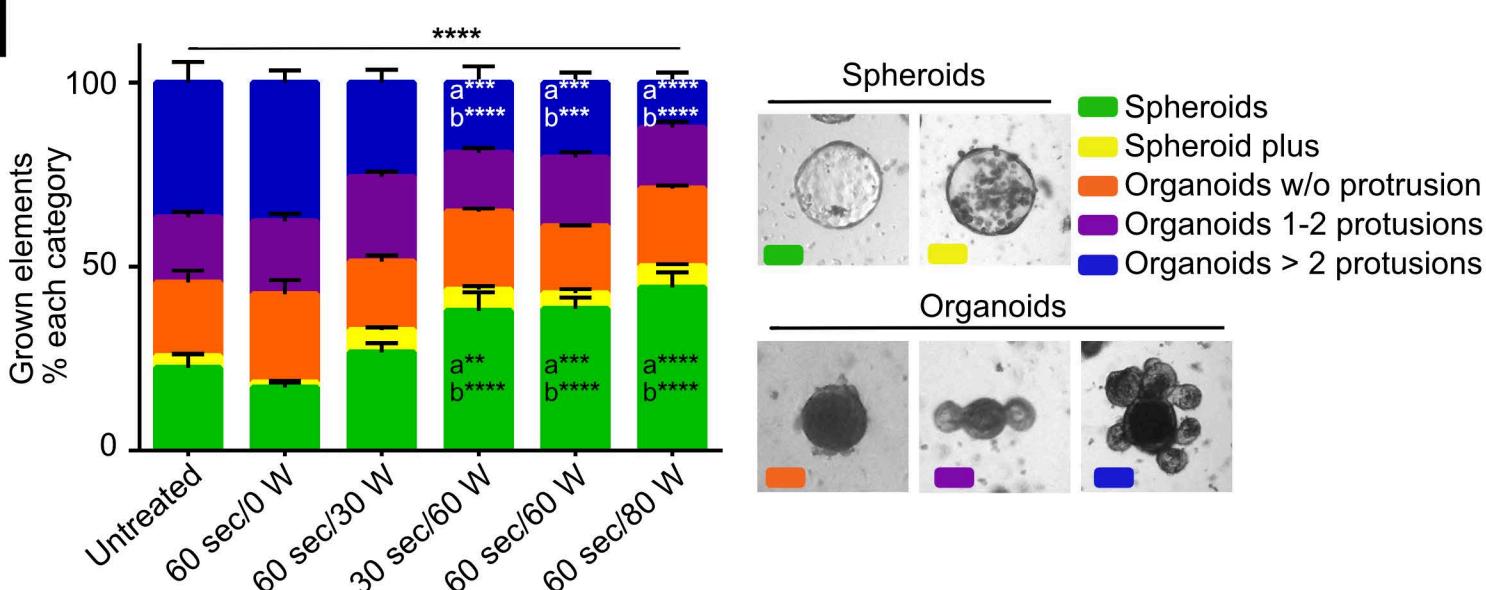
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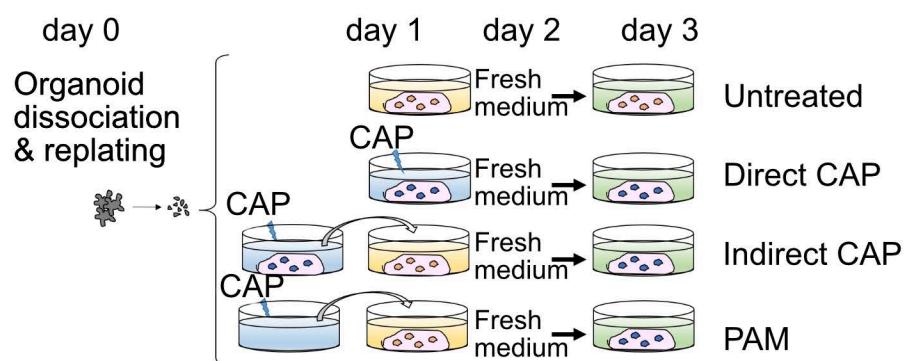
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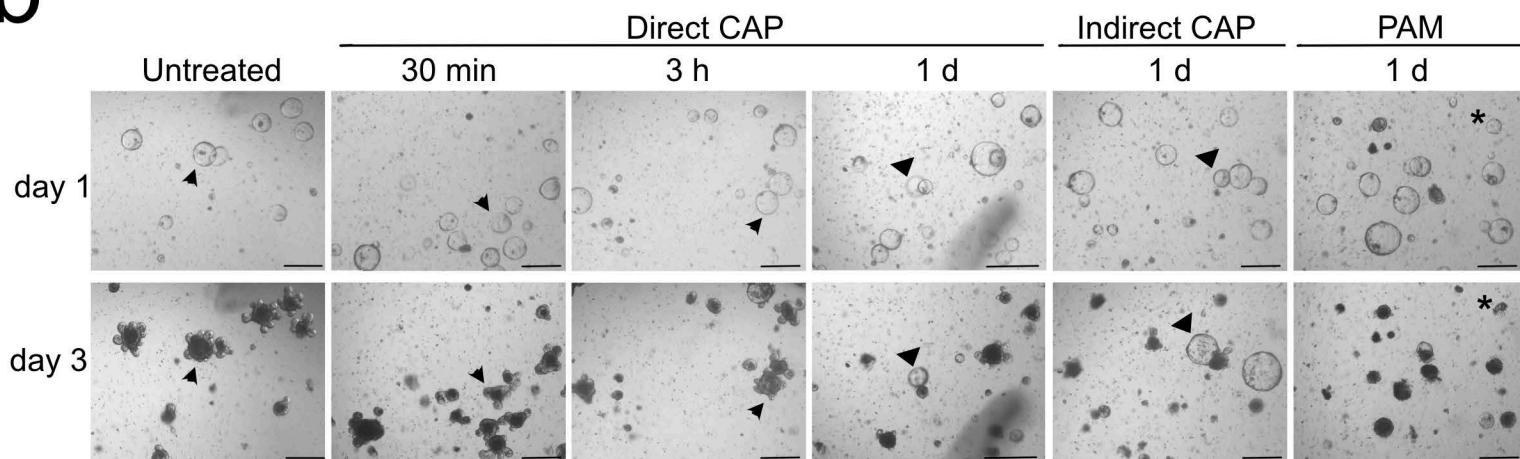
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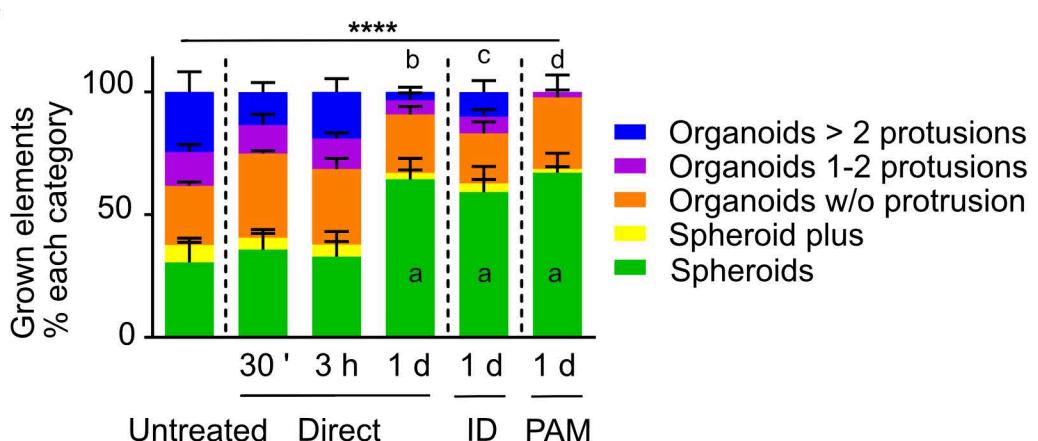
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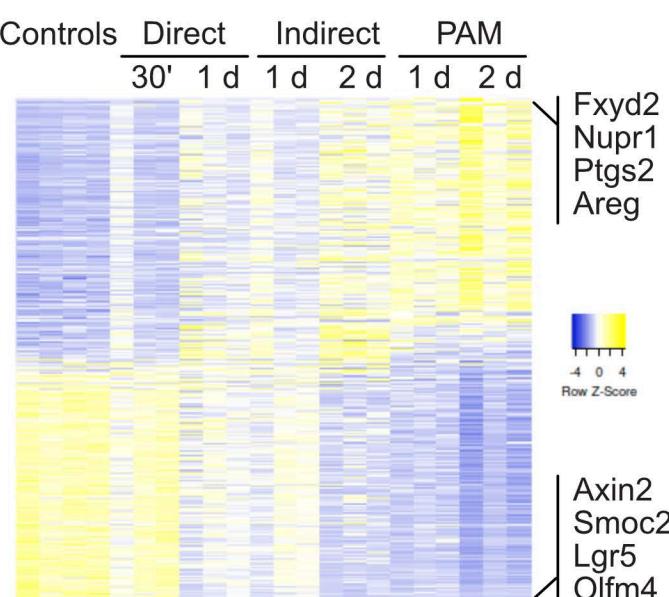
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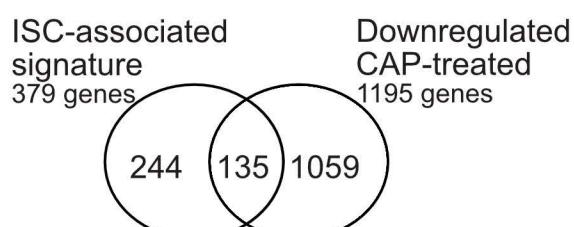
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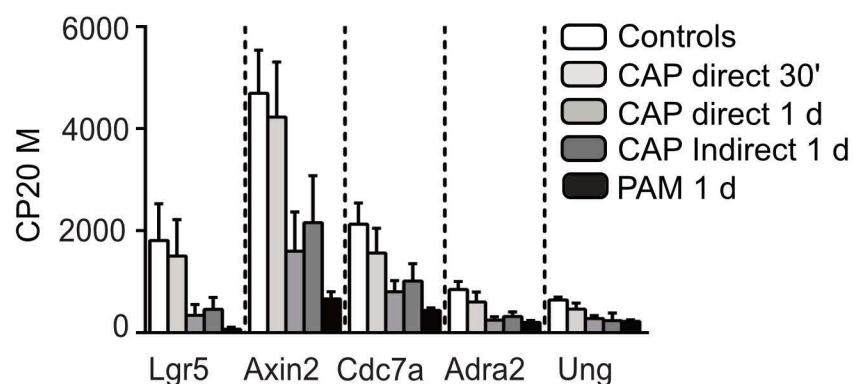
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Biological process	
Upregulated in CAP-treated vs Controls	P-value
Regulation of intracellular signal transduction	e-52
Cytoskeleton organization	e-51
Apoptotic process	e-49
Cell motility	e-47
Negative regulation of response to stimulus	e-47
Response to oxygen containing compounds	e-42
Secretion	e-42
Biological adhesion	e-38
Positive regulation of developmental process	e-38
Regulation of cell differentiation	e-37
regulation of phosphorus metabolic process	e-36
Downregulated in CAP-treated vs Controls	P-value
Cell cycle	e-139
Cell cycle progress	e-122
Chromosome organization	e-101
Cell division	e-83
DNA replication	e-78
Cellular response to DNA damage stimulus	e-69
DNA repair	e-56
Chromosome segregation	e-55
Organelle fission	e-54

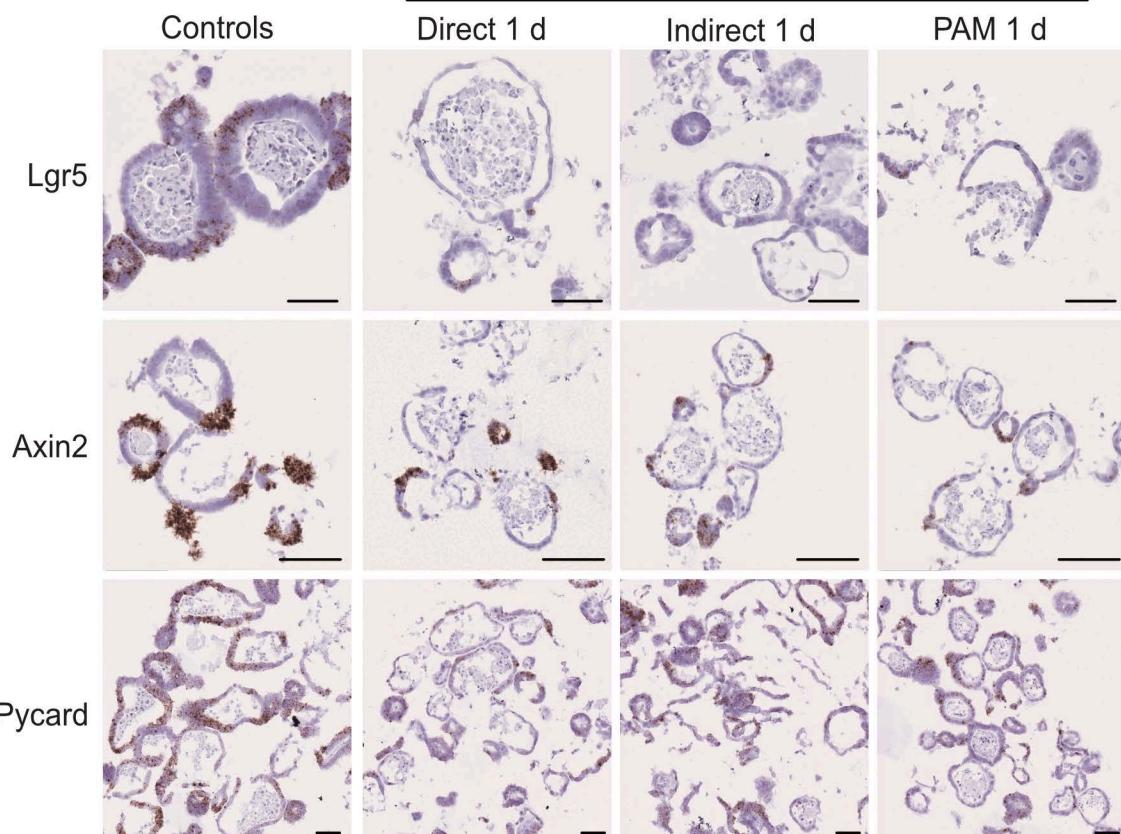
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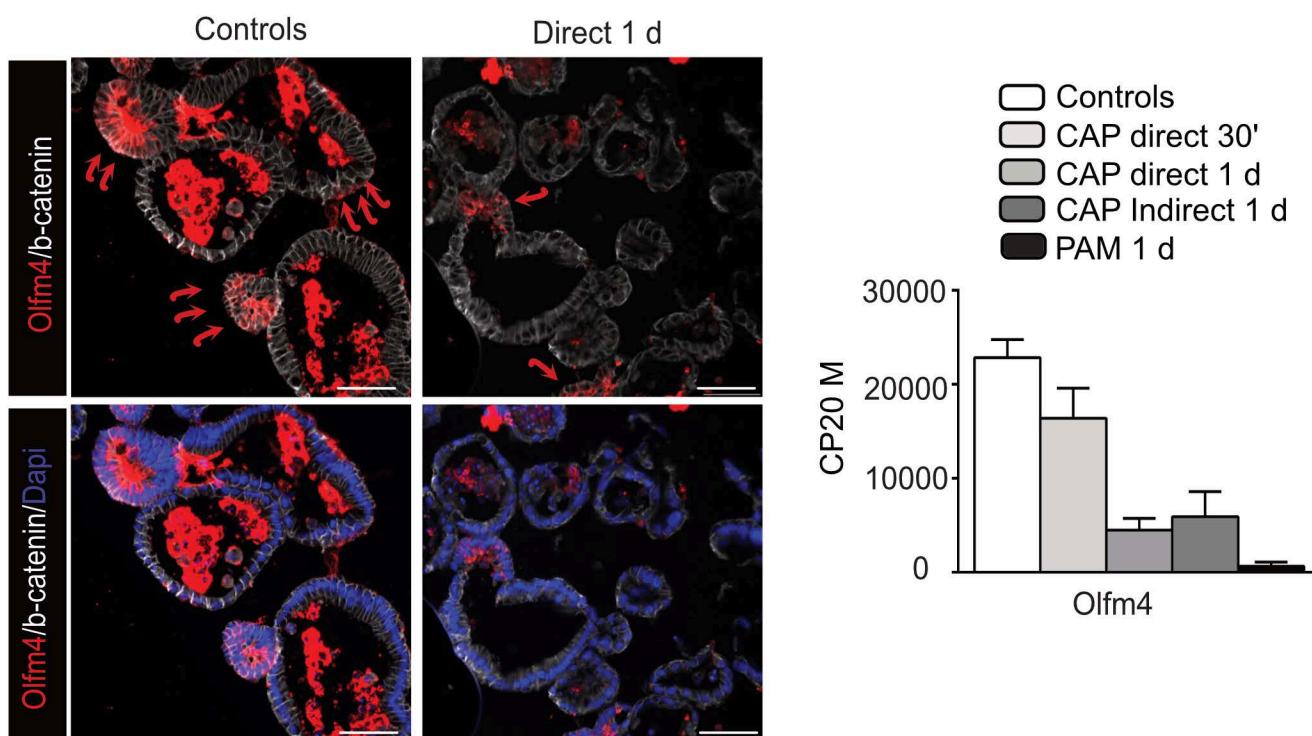
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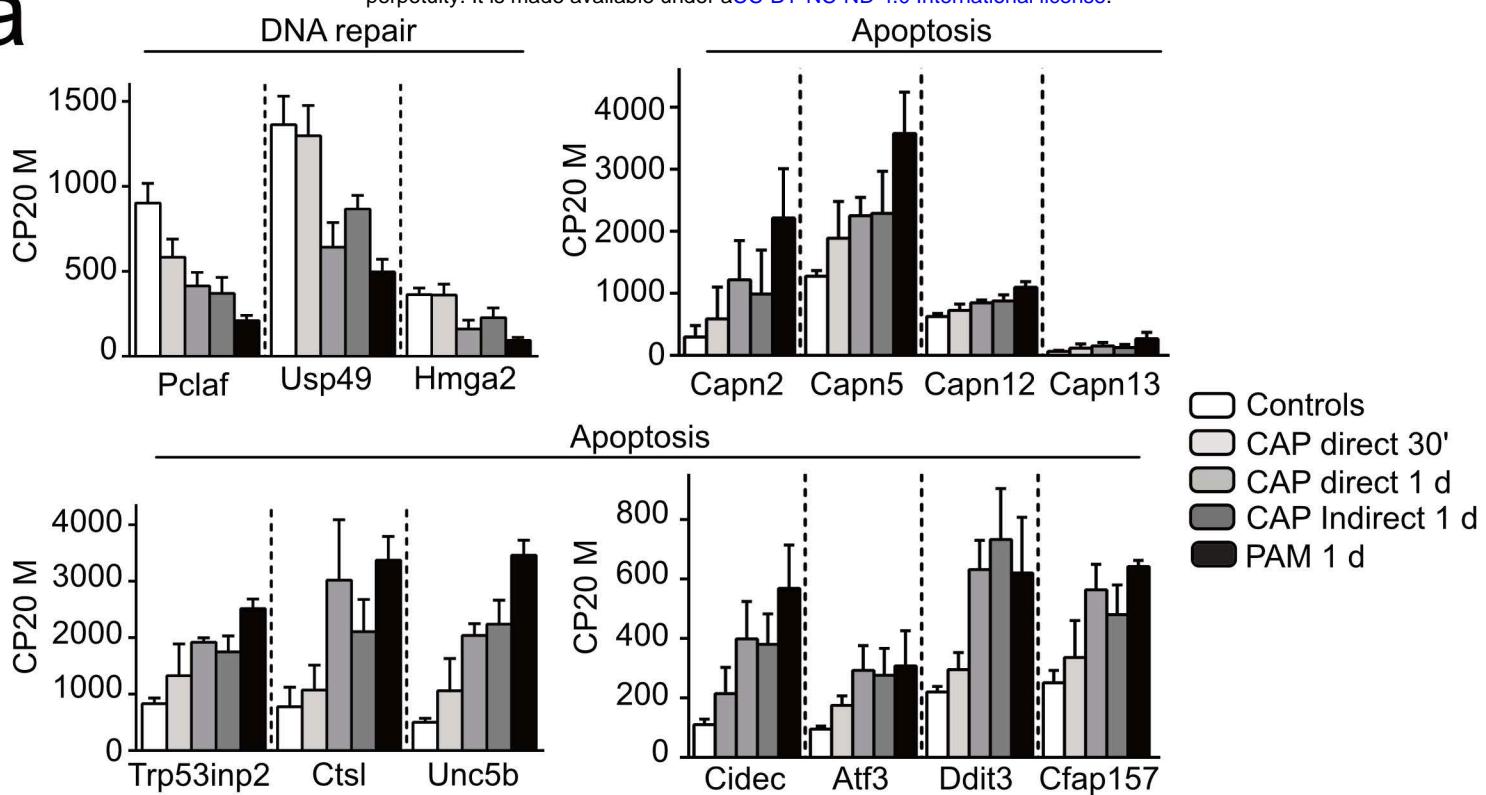
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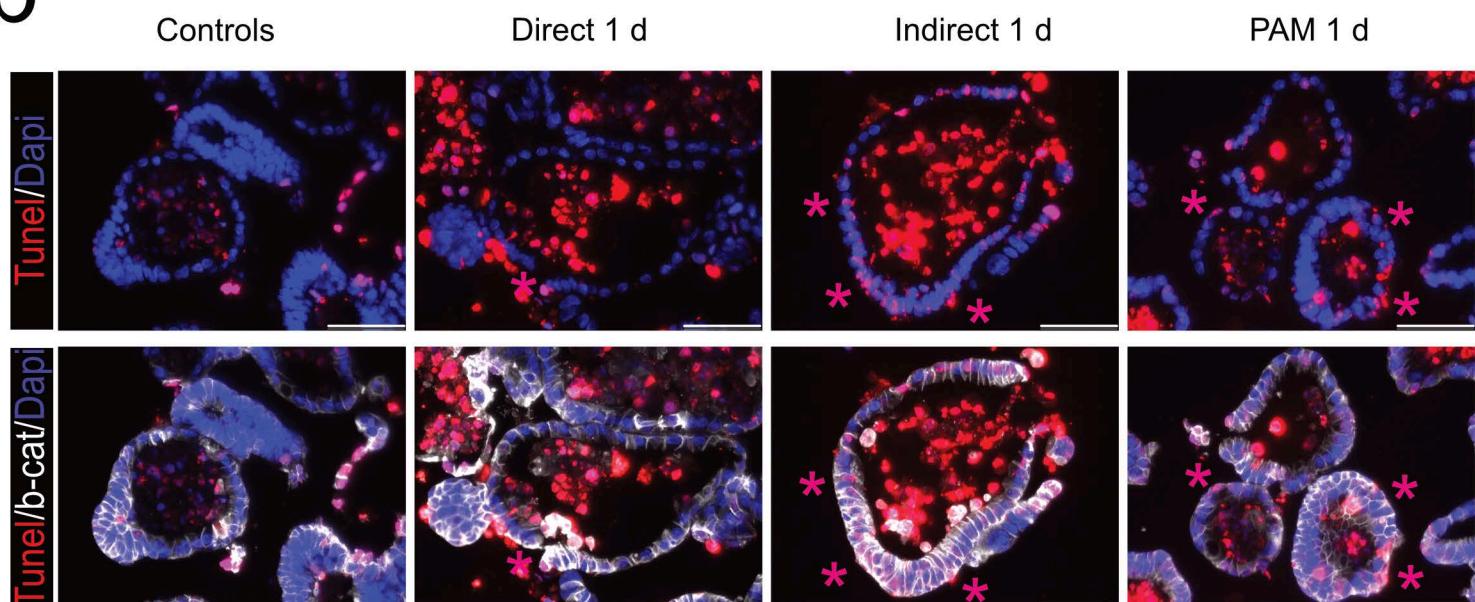
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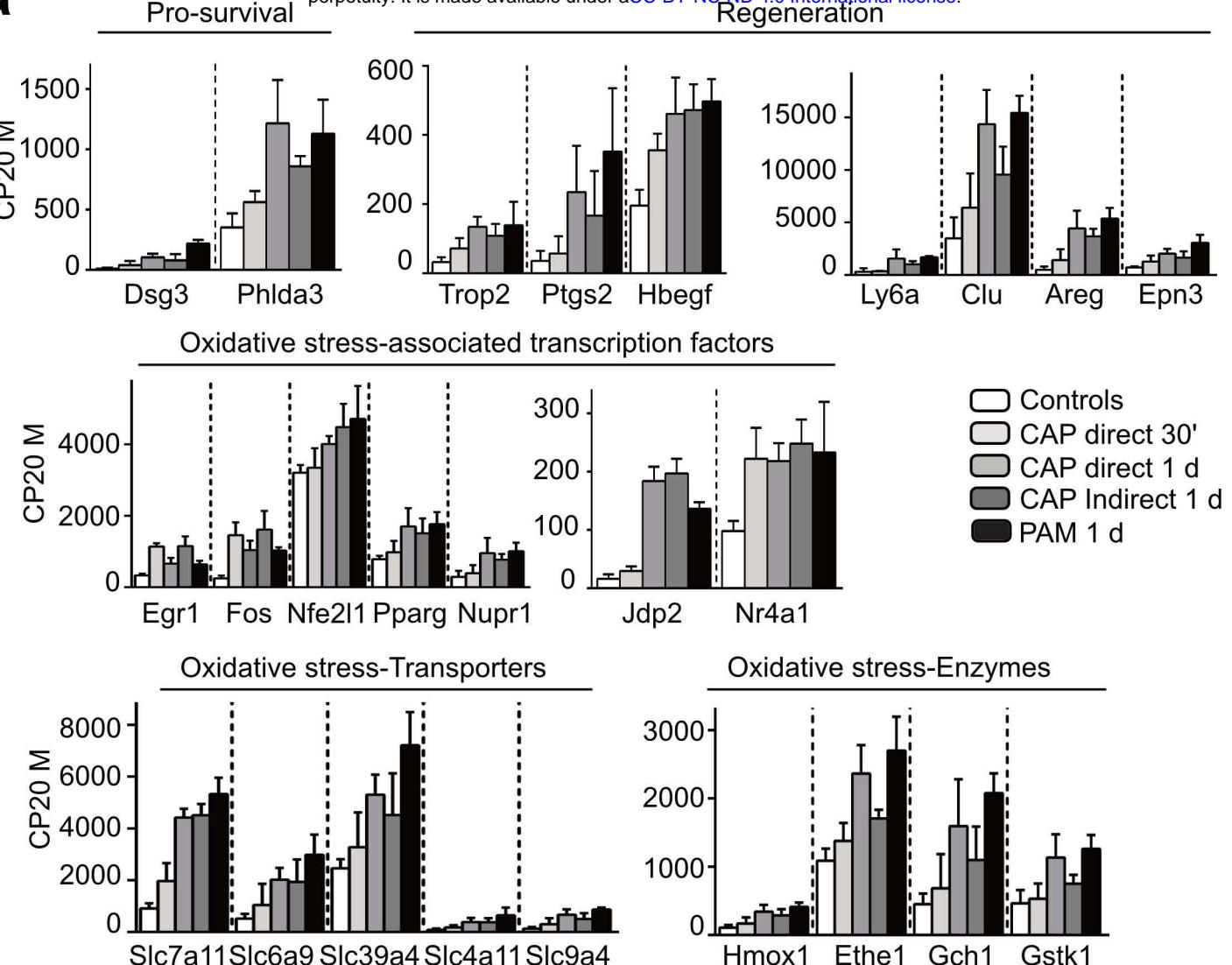
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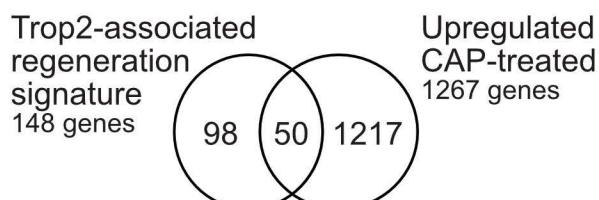
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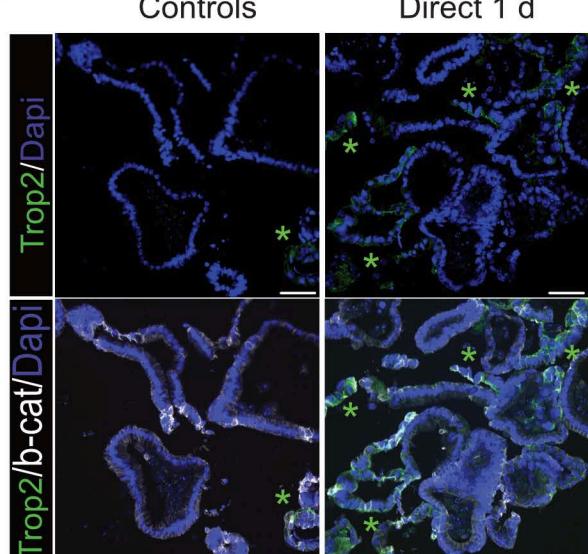
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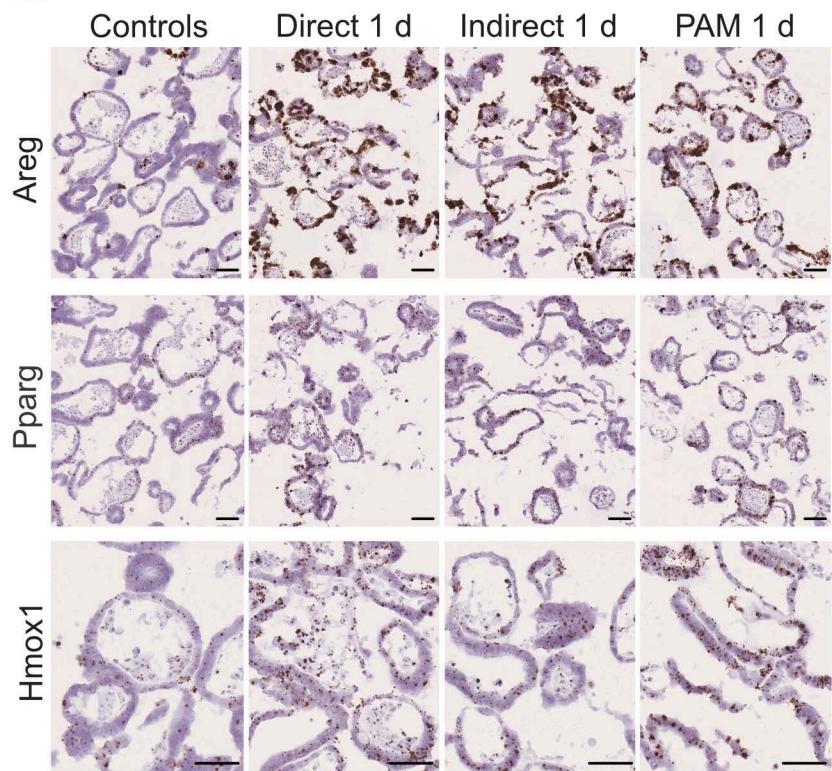
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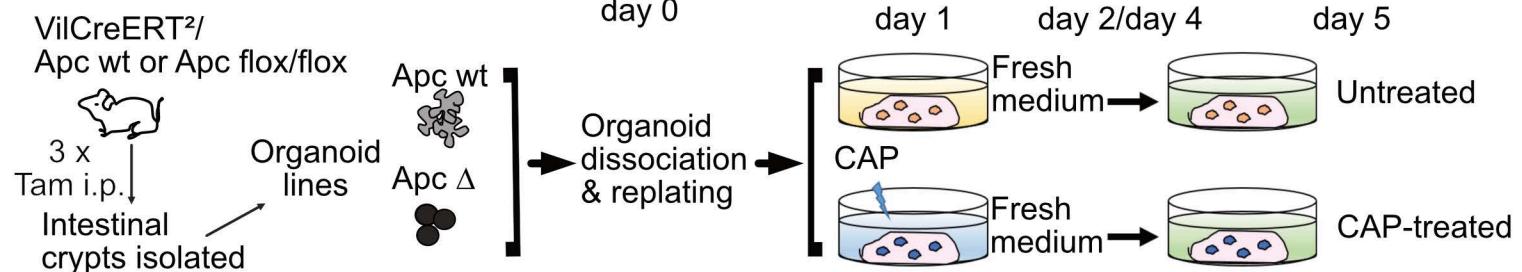
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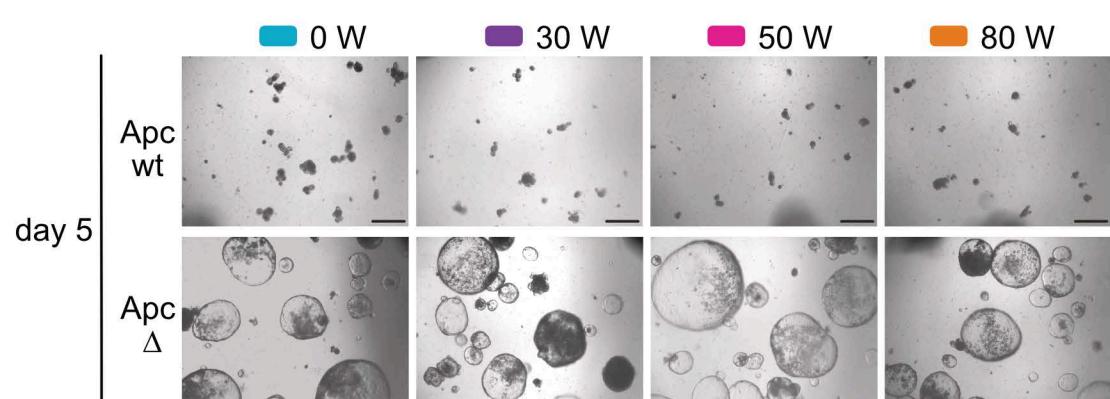
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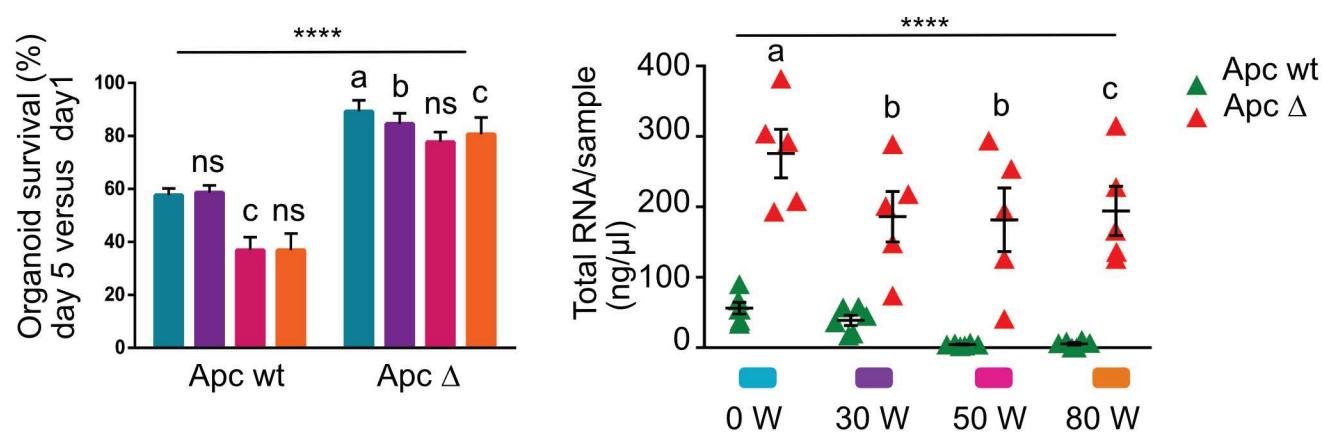
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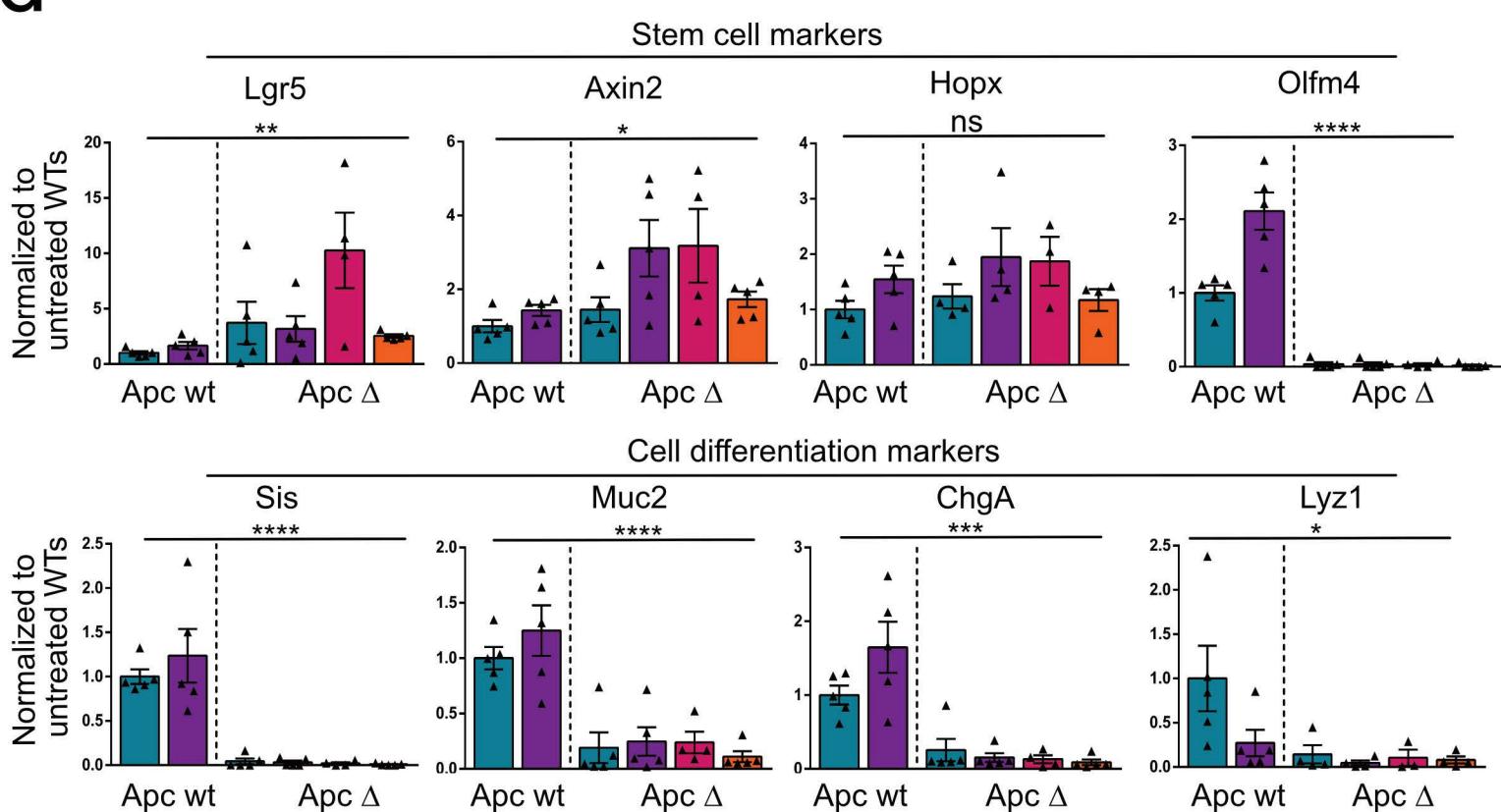
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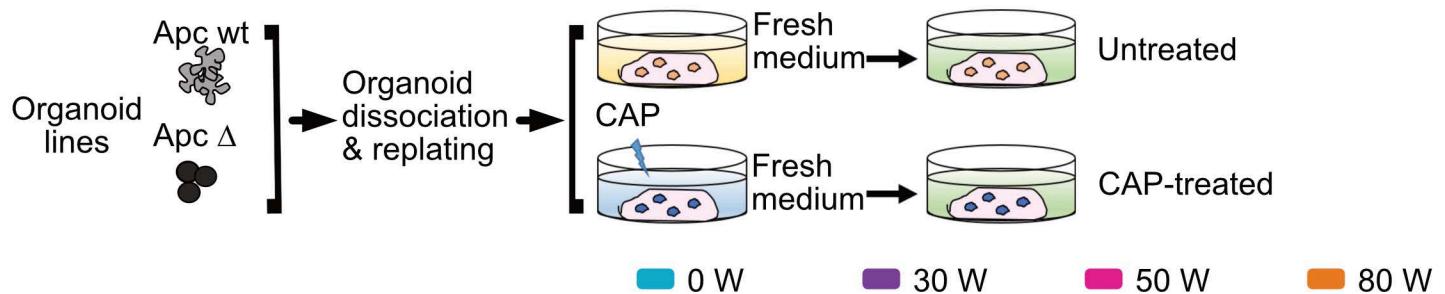
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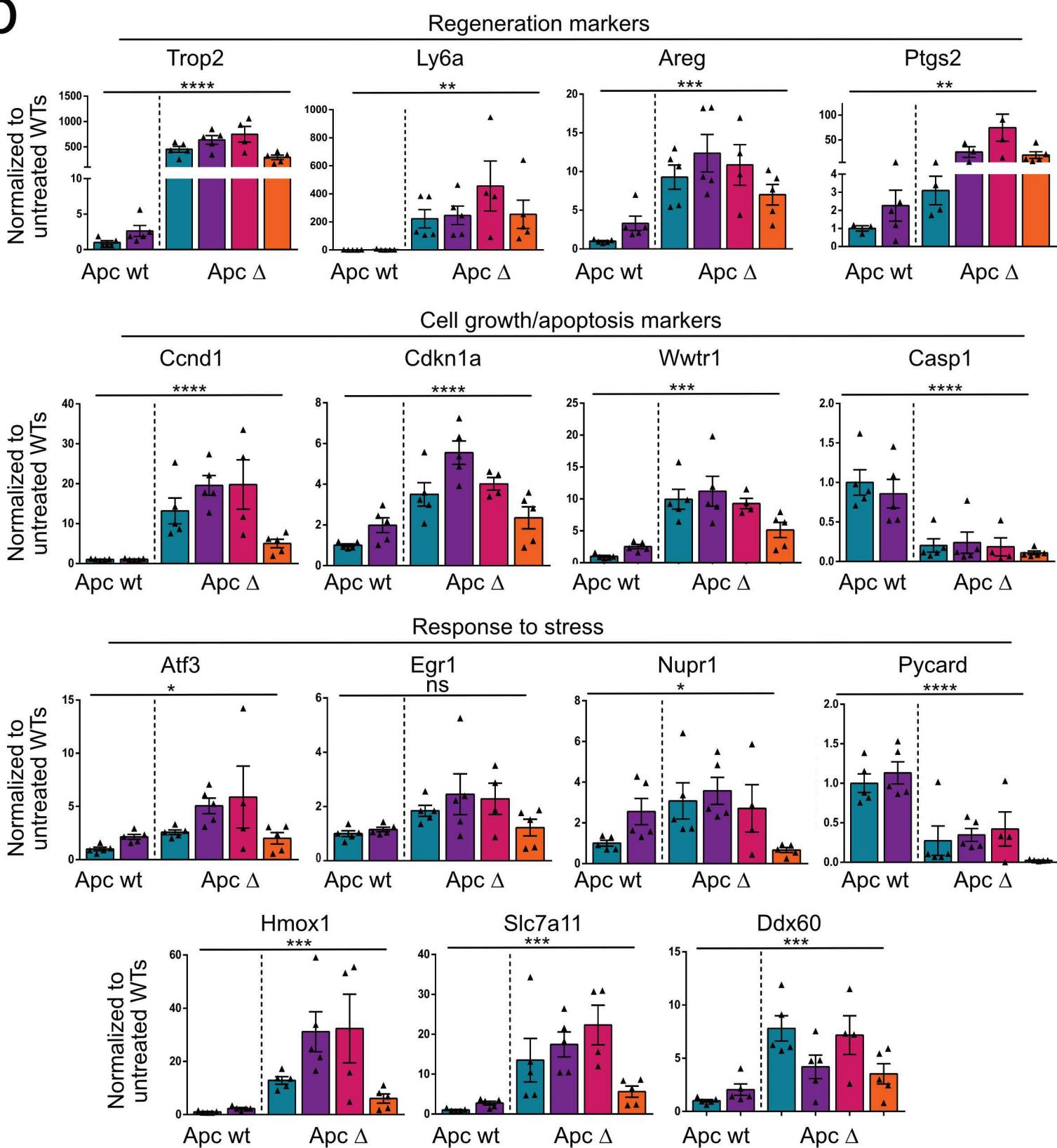


Table 1: List of genes involved in endox control

WPF: **Element** **Container**

ENSMUSG0000000000061704	Cyp2b29	hypothetical
ENSMUSG000000000006190	Cyp2b29	hypothetical
ENSMUSG00000000000618344	Cyp2b26-9s	hypothetical
ENSMUSG00000000000688086	Cyp2b27-9s	hypothetical
ENSMUSG00000000000604605	Cyp2b28	hypothetical

EN1841/10/2000/0003/1803	None3	None
EN1841/10/2000/0003/08062	None4	None
EN1841/10/2000/0003/084605	None5	None
EN1841/10/2000/0003/19130	None6	None

EN16111/0000000064398	Lev	...
EN16111/0000000064393	wt-CuO	...
EN16111/0000000064394	wt-CuO	...
EN16111/0000000064399	wt-CuO	...

TABLE 2. List of material used for immunostainings, *in situ* hybridization and qRT-PCR experiments

Immunostainings	Antibodies	Catalogue number	Manufacturer
	Goat anti-Trop2	AF650	R&D systems
	Mouse anti-Beta catenin	610154	BD transduction laboratories
	Rabbit anti-Olfactomedin 4	#14369	cell signaling tech
	TUNEL assay	12156792910	Roche
	DAPI	D9542	Sigma-Aldrich
RNAscope probes	Gene	Catalogue number	Manufacturer
	m Areg	430501	Advanced cell diagnostics
	m Axin 2	400331	Advanced cell diagnostics
	m Hmox1	498811	Advanced cell diagnostics
	m Lgr5	312171	Advanced cell diagnostics
	m Pparg	513371	Advanced cell diagnostics
	m Pycard	439581	Advanced cell diagnostics
qPCR primers	Gene	Forward primer	Reverse primer
	Aqp1	AGCTGGTACTGTGCGTTCTG	GTAGTCAATGCCAGCAGGT
	Aqp3	TGCCCTGCGCTAGCTACTTT	GAAGCATCTCCCACAACGA
	Aqp4	TGCCCGTAATCTGACTCCCA	AATGTCCACACTTACCCACC
	Aqp5	ATGGGTTGTCGTCGGTCACACT	CCAGAAGACCCAGTGAGAGG
	Areg	GCGAATGCAGATACTCGAGAA	CGCTGTGAGTCTTCATGGATTT
	Atf3	GTCACCAAGTCTGAGGCGG	GTTTCGACACTGGCAGCAG
	Axin2	TGACTCTCCTCCAGATCCCA	TGCCACACTAGGCTGACCA
	Casp1	GAAGGGACCCCTAAGTTTGC	CACCACCTTCAGGATGGCCT
	Ccnd1	CCTGCTACCGCACAACGCAC	GCCTGGCGCAGGCTTGACTC
	Cdkn1a	ATCCTGCAAGAGGCCCTGGAGAGG	CACGAGGTGGACGCCTATGG
	Chga	TCCCCACTGCAGCATCCAGTTC	CCTTCAGACGGCAGAGCTTCGG
	Ddx60	AGTGATGAGCCTTGTGAGGA	TCGTTCATGATGGCATCTCCC
	Egr1	GAGCACCTGACCACAGAGTC	GCGGCCAGTATAAGGTGATGG
	Hmox1	CATAGCCCGAGGCCCTGAATC	CAAATCCTGGGGCATGCTGT
	Hopx	GTGCCTGCGATCTTGGTGGCT	GCCTGACCTTACGTCTGCCCC
	Lgr5	CCTACTCGAAGACTTACCCAGT	GCATTGGGTGAATGATAGCA
	Ly6A	GAAAGAGCTAGGGACTGGAGT	TTAGGAGGGCAGATGGGTAAGCAA
	Lyz1	GAGACCGAAGCACCGACTATG	CGGTTTGACATTGTTGTCG
	Muc2	ATGCCAACCTCTCAAAGAC	GTAGTTCCGTTGGAACAGTGAA
	Nupr1	AATACCAACGCCCTAGCC	TGTGGCTGGCCTTATCTCC
	Olfm4	CAGCTGCCTGGTTGCCTCCG	GGCAGGTCCCAGGCTGTCC
	Ptgs2	CTGACCCCAAGGCTCAAAT	TTAAGTCCACTCCATGGCCC
	Pycard	GAGCAGCTGCAAACGACTAA	CTGGTCCACAAAGTGTCTGT
	Rpl13	CCCCTGGCGATTGTGAA	TCATTGTCCTCTGTGAGGTT
	Sis	TTCAAGAAATCAAACATTCAATTACTAG	CTAAAACTTCTTGACATTGAGCAA
	Slc7a11	AGCTAACTGACTGCCCTGG	ACTCAGAGGTGTGTTCAAGCC
	Trop2	AGAACCGCGTCGAGAAGGGC	CGGGCGGCCATGAACAGTGA
	Wwtr1	CGGTTCCGGGGATGTAAGAG	GAACTGACGAGCTGGAACCC
	Ywhaz	TGCAACGATCTACTGTCTTTG	CGGTAGTAGTCACCCATTTC

COLD ATMOSPHERIC PLASMA DIFFERENTIALLY AFFECTS CELL RENEWAL AND DIFFERENTIATION OF STEM CELLS AND APC-DEFICIENT-DERIVED TUMOR CELLS IN INTESTINAL ORGANOIDS

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SUPPLEMENTARY FIGURES Content

Figure S1. Impact of the CAP application method on organoid morphology.

Figure S2. Impact of the CAP application method on global gene expression of intestinal organoids.

Figure S3. Apc deficient-derived organoids exhibit increased resistance to CAP treatment as compared to normal intestinal stem cell-derived organoids.

Figure S1. Impact of the CAP application method on organoid morphology. **a.** CAP-conditioned media and PAM generated by treatment with 50 W for 60 s at day 1 were applied directly to organoid cultures at day 2 post-replating for 24 hours (until day 3). **b.** Representative pictures of a given field showing growth of organoids at day 2 (before CAP application) and day 3 (endpoint of the experiment). Triangles show individual elements evolving as protruded organoids and spheroids in untreated and CAP-treated cultures, respectively. Right panels: insets of the pictures at day 3. Scale bars: 500 μ m. **c.** Quantification of organoid complexity at day 3. An average number of 100 elements was analyzed over time per condition per organoid line (n= 4 organoid lines). Data are represented as means \pm sem. Two-way ANOVA: interaction ** P< 0.01 followed by Dunnett's multiple comparisons test: *** P< 0.001, ** P< 0.01, * P< 0.05, ns not significant (all compared to untreated).

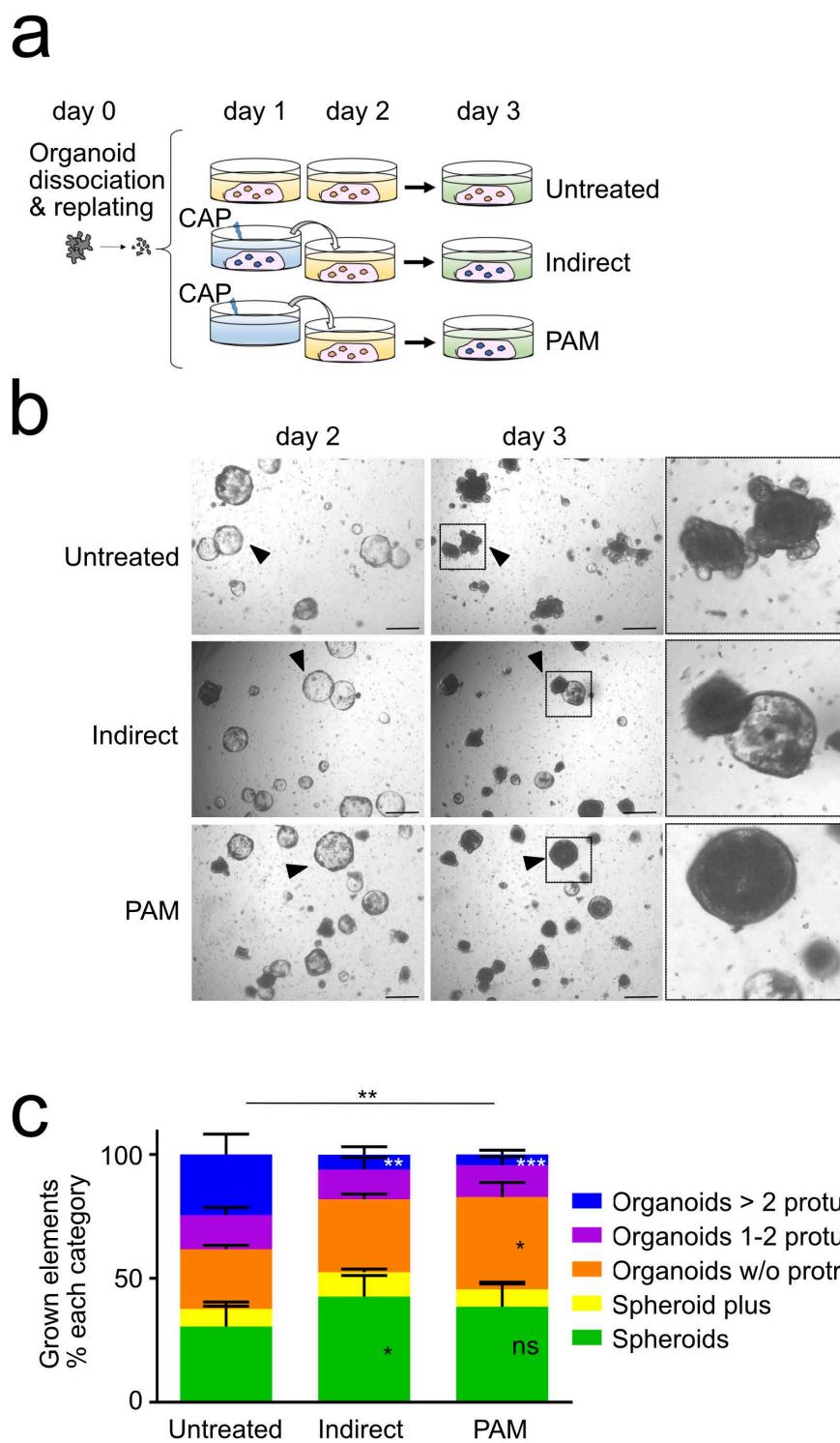


Figure S2. Impact of the CAP application method on global gene expression of intestinal organoids. **a.** Heatmap of the most differentially regulated genes in CAP-treated versus Untreated (Controls) organoids at the early time post-treatment (30 min). **b.** Expression levels of Cys metabolism-, cell signaling-, inflammation- and cytoskeleton organization-associated genes in the various conditions. Data are represented as means \pm sd. n = 4 and 3 samples in Controls and CAP-treated conditions, respectively. CP20M: counts per kilobase of transcript per 20 million mapped reads.

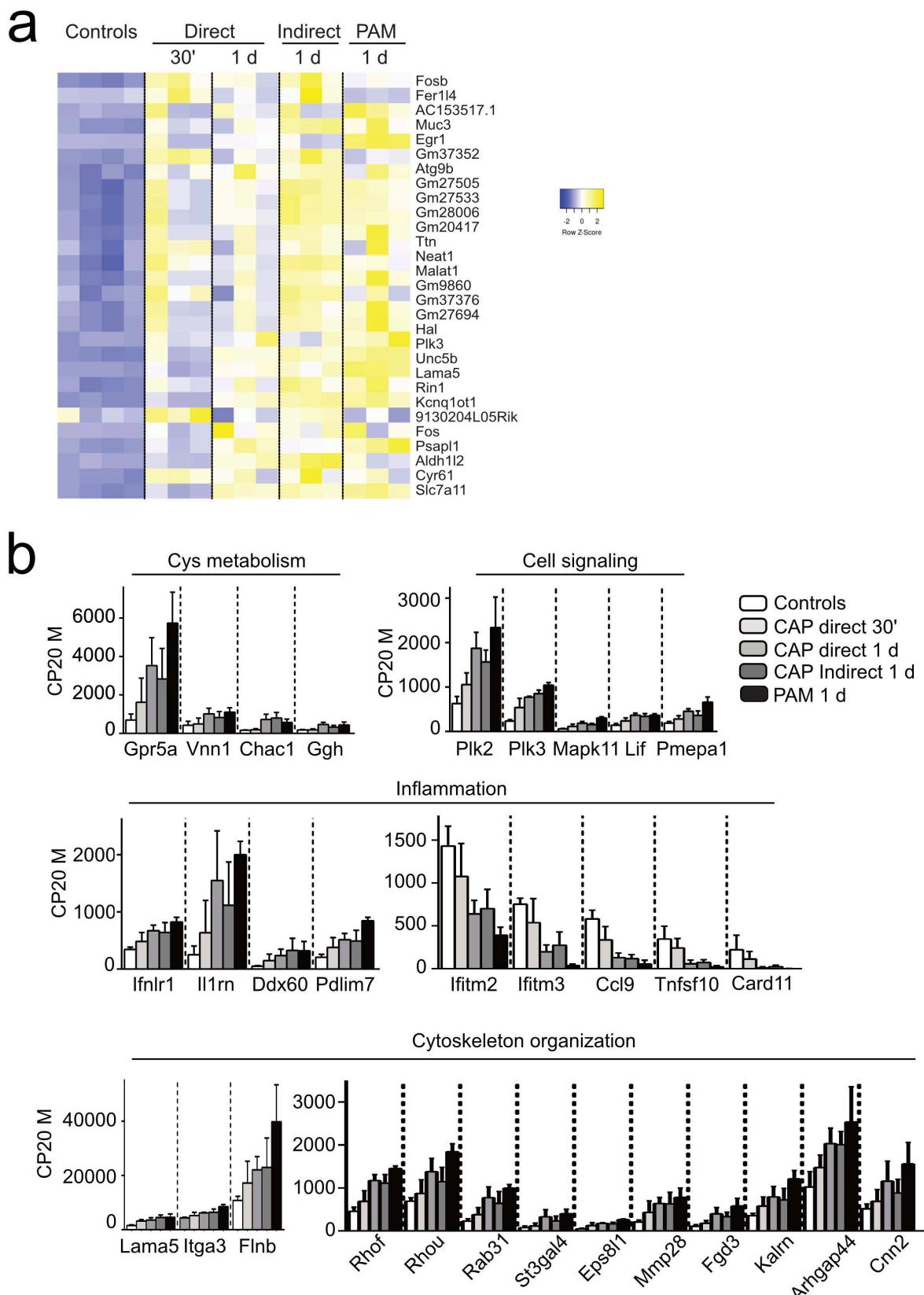


Figure S3. Apc deficient-derived organoids exhibit increased resistance to CAP treatment as compared to normal intestinal stem cell-derived organoids.

a. Organoid growth of Apc wt and Apc Δ crypts upon initial seeding in culture media containing ENR (EGF, Noggin, Rspsondin1) or EN (EGF, Noggin). Representative pictures of a given field showing organoid morphology at day 1 and day 4. Apc Δ organoid efficiently grow in EN conditions as compared to Apc wt organoids. Scale bars: 500 μ m. **b.** Measurement of reactive species in culture supernatants of Apc wt and Apc Δ organoids 24 hours after direct CAP treatment at the indicated doses. DCFDA dye was used to measure ROS levels. A.U. Arbitrary Units. Each symbol corresponds to a given organoid line. Data are represented as means \pm sem. One-way Anova test. *** P < 0.0001. **c.** Expression levels of aquaporin-encoding genes. Left panel: Aqp expression in Lgr5+ve ISC. Data were analyzed from the Gene Expression Omnibus GSE135362 dataset; Right panel: Aqp expression in control and CAP-treated organoids (this work). CP20M: counts per kilobase of transcript per 20 million mapped reads (n = 2 samples). **d.** Gene expression analysis by qRT-PCR of Aquaporins in Apc wt and Apc Δ organoids. Each symbol corresponds to a given organoid line. Values are normalized to Untreated Apc wt levels. Data are represented as means \pm sem. One-way Anova test. *** P < 0.0001; ** P < 0.001. **e.** Expression levels of ROS scavenger enzymes in Lgr5+ve ISC. Data were analyzed from the Gene Expression Omnibus GSE135362 dataset. CP20M: counts per kilobase of transcript per 20 million mapped reads (n = 2 samples).

