

1 **Title:** Intestinal Bacteria are Necessary for Doxorubicin-induced Intestinal Damage but not  
2 for Doxorubicin-induced Apoptosis.

3

4 **Running Title:** Doxorubicin-induced damage dependent on intestinal bacteria

5

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21

22 **Abbreviations:** Doxorubicin (DOXO), intestinal epithelial cell (IEC), conventionalized  
23 (CONV), germ free (GF), intraperitoneal (IP), hematoxylin and eosin (H&E)

24

25 **Abstract**

26 Doxorubicin (DOXO) induces significant, but transient, increases in apoptosis in the stem cell  
27 zone of the jejunum, followed by mucosal damage involving a decrease in crypt proliferation,  
28 crypt number, and villus height. The gastrointestinal tract is home to a vast population of  
29 commensal bacteria and numerous studies have demonstrated a symbiotic relationship  
30 between intestinal bacteria and intestinal epithelial cells (IEC) in maintaining homeostatic  
31 functions of the intestine. However, whether enteric bacteria play a role in DOXO-induced  
32 damage is not well understood. We hypothesized that enteric bacteria are necessary for  
33 induction of apoptosis and damage associated with DOXO treatment. Conventionally raised  
34 (CONV) and germ free (GF) mice were given a single injection of DOXO, and intestinal tissue  
35 was collected at 6, 72, and 120 h after treatment and from no treatment (0 h) controls.  
36 Histology and morphometric analyses quantified apoptosis, mitosis, crypt depth, villus height,  
37 and crypt density. Immunostaining for muc2 and lysozyme evaluated Paneth cells, goblet  
38 cells or dual stained intermediate cells. DOXO administration induced significant increases  
39 in apoptosis in jejunal epithelium regardless of the presence of enteric bacteria; however, the  
40 resulting injury, as demonstrated by statistically significant changes in crypt depth, crypt  
41 number, and proliferative cell number, was dependent upon the presence of enteric bacteria.  
42 Furthermore, we observed expansion of Paneth and goblet cells and presence of  
43 intermediate cells only in CONV and not GF mice. These findings provide evidence that  
44 manipulation and/or depletion of the enteric microbiota may have clinical significance in  
45 limiting chemotherapy-induced mucositis.

46

47 **Introduction**

48 The small intestinal epithelium is one of the most rapidly proliferating tissues in the  
49 body. This property renders intestinal epithelial cells (IEC) particularly susceptible to  
50 chemotherapy-induced damage which is reported in up to 40% of patients who receive  
51 chemotherapy. <sup>1</sup> Chemotherapy-induced cytotoxicity within the gastrointestinal tract  
52 manifests as mucositis, characterized by gross ulcerations of the intestinal mucosa. The  
53 development of mucositis is a limiting factor in administration of chemotherapeutic agents,  
54 and therefore strategies to reduce this side-effect are urgently sought.

55 Doxorubicin (DOXO) is a common chemotherapeutic utilized for sarcomas, select  
56 breast cancers, and several metastatic cancers. We previously reported that a single injection  
57 of DOXO given to mice induced significant, but transient, increases in apoptosis in the stem  
58 cell zone of the jejunum, followed by mucosal damage involving a decrease in crypt  
59 proliferation, crypt number, and villus height. <sup>2</sup> Subsequently, repair occurred, characterized  
60 by crypt hypertrophy, Paneth cell hyperplasia and, ultimately, return of the intestinal mucosa  
61 to normal morphology. <sup>3</sup>

62 The gastrointestinal tract is home to a vast population of commensal bacteria and  
63 numerous studies have demonstrated the importance of the symbiotic relationship between  
64 intestinal bacteria and IECs in maintaining homeostatic functions of the intestine, including  
65 nutrient generation and metabolism and proper development of the innate immune system.  
66 <sup>4,5,6,7</sup> Evidence in models of intestinal damage, indicate distinct effects of bacteria in different  
67 regions of the gastrointestinal tract. For example, in colon of mice that lack TLR signaling  
68 (MyD88<sup>-/-</sup>), restitution and repair following epithelial injury is impaired. <sup>8</sup> Similarly, germ free  
69 (GF) mice have shorter colonic crypts <sup>9</sup> and are more susceptible to chemical-induced injury,  
70 <sup>10</sup> providing additional support for a role of commensal microbiota in sustaining epithelial  
71 integrity or promoting mucosal repair in the colon. In contrast, in the small intestine, the

72 presence of enteric bacteria and/or bacterial products appears to exacerbate damage. For  
73 example, in both TLR4<sup>-/-</sup> and MyD88<sup>-/-</sup> mice, intestinal damage after exposure to  
74 indomethacin was abrogated.<sup>11</sup> Similarly, deficiency of TLR2 or TLR9 expression or inhibition  
75 of TLR9 activity were associated with significantly decreased DOXO-induced damage to the  
76 small intestine.<sup>12</sup> Additionally, the microbiota promotes inflammation and fibrosis following  
77 small bowel resection,<sup>13</sup> reflective of the 'double edged-sword' role of intestinal microbiota  
78 associated-signaling in mucosal repair. These data led us to question whether enteric  
79 bacteria play a role in mucosal damage associated with chemotherapeutic agents like DOXO.

80 In this report, we tested the hypothesis that enteric bacteria were necessary for  
81 induction of apoptosis and mucosal damage associated with DOXO treatment. Our data show  
82 that DOXO administration induces apoptosis regardless of the presence of enteric bacteria,  
83 but that the resulting damage, as demonstrated by changes in crypt depth, crypt number, and  
84 proliferative index, is enteric bacteria-dependent. These findings provide evidence that  
85 manipulation and/or depletion of the enteric microbiota may have clinical significance in  
86 limiting chemotherapy-induced mucositis.

87

88 **Results**

89 **DOXO treatment rapidly induced apoptosis in both GF and CONV small intestine.**

90 We previously reported that DOXO exposure rapidly induces apoptosis in crypt epithelium of  
91 CONV mice and that apoptosis peaked at 6h and remained elevated relative to control mice  
92 out to 120h after DOXO treatment.<sup>2</sup> In the current study, similar levels of apoptosis were  
93 observed in crypt epithelium of both GF and CONV mice, as assessed by both H&E staining  
94 and cleaved caspase 3 (Fig. 1A), at 6h following DOXO treatment. However, by 72h post  
95 DOXO treatment apoptosis had returned to baseline levels (Fig. 1B). Consistent with prior  
96 findings<sup>2</sup> cell positional analysis revealed that DOXO-induced apoptosis occurred primarily  
97 in cell positions 3-6, indicative of involvement of the intestinal stem cell zone (Fig. 1C).  
98 Although not significantly different from CONV, there may be an indication that apoptosis is  
99 occurring in cell positions higher up the crypt in GF, compared with CONV mice. This may  
100 suggest that cells of different lineages and/or differentiation status are more susceptible to  
101 DOXO-induced apoptosis in GF vs. CONV mice, or that DOXO-induced apoptosis induces a  
102 higher turnover in GF mice.

103 **Alterations in villus-crypt morphometry in CONV, but not GF mice, following DOXO.**

104 We previously demonstrated that an increase in crypt depth 120h following DOXO  
105 treatment was a hallmark of the repair phase and that villus blunting was evident at that time  
106 as well.<sup>2</sup> Though GF crypts were significantly shorter than CONV crypts at baseline, GF mice  
107 showed no significant change in crypt depth throughout the entire time course after DOXO  
108 treatment (Fig. 2A and B). In contrast, by 120h after DOXO, crypts were significantly deeper  
109 in CONV mice compared to both control CONV mice and to GF mice at 120h after DOXO.  
110 GF mice showed no significant change in villus height throughout the entire time course after  
111 DOXO treatment (Fig. 2C). CONV mice, while not statistically different, demonstrated a trend  
112 toward shorter villi after DOXO treatment similar to our previous report.<sup>2</sup>

113 Our previous studies demonstrated a significant increase in proliferative index during  
114 repair following DOXO-induced damage which marked the movement of the intestinal  
115 mucosa in the repair phase.<sup>2</sup> Immunohistochemistry revealed a significant increase in the  
116 number pHH3<sup>+</sup> cells in crypt epithelium of CONV mice at 120h following DOXO treatment,  
117 indicating an increase in crypt cell proliferation and this increase was not observed in GF  
118 mice (Fig. 3A and B). Similarly, we previously observed increases in the number of mitotic  
119 figures in crypt epithelium during repair.<sup>2</sup> Evaluation of the number of mitotic figures per crypt  
120 in both GF and CONV mice following DOXO revealed significantly decreased numbers in  
121 both GF and CONV crypts at 6h following DOXO, consistent with induction of DNA damage  
122 and cell cycle arrest (Fig. 3C). The number of mitotic figures per crypt remained significantly  
123 decreased in CONV mice but returned to control levels in GF mice at 72h following DOXO.  
124 A return of mitotic figure number to control levels in CONV mice was observed by 120h  
125 following DOXO treatment. Of note, at baseline the number of mitotic figures per crypt was  
126 significantly greater in CONV compared to GF mice.

127 We previously demonstrated that DOXO-treatment of CONV mice resulted in significant  
128 crypt loss by 72h and this loss of crypts can be used as an indicator of loss of intestinal stem  
129 cells.<sup>2</sup> Although apoptosis and cell cycle arrest were observed in both GF and CONV crypt  
130 epithelium following DOXO treatment, only in CONV mice was this followed by significant  
131 loss of crypt number assessed by reduced crypt density. By 72h following DOXO treatment,  
132 crypt density was significantly decreased in CONV mice compared to control CONV mice,  
133 and compared to GF mice at 72h after treatment with DOXO (Fig. 4). This decrease in crypt  
134 density was still observed at 120 h after DOXO in CONV mice, but trended toward restitution  
135 of crypt number similar to our previous findings.<sup>2</sup>

136

137 **Expansion of the Paneth cell compartment is absent in GF mice following DOXO**  
138 **treatment.**

139 We previously reported an expansion of the Paneth cell compartment in jejunal crypts  
140 following DOXO-induced damage in CONV mice which in turn increases the intestinal stem  
141 cell niche. <sup>2</sup> This expansion included an increase in total lysozyme-positive cell and  
142 intermediate cell (muc2 and lysozyme expressing cell) numbers suggesting alteration in  
143 secretory cell lineage allocation or maturation. <sup>3</sup> As Paneth cells play a critical role in sensing  
144 and responding to enteric bacteria <sup>14</sup> we wished to evaluate whether the absence of enteric  
145 bacteria in GF mice would alter the Paneth cell compartment. Following DOXO treatment,  
146 staining sections for lysozyme and muc2 revealed an expansion of lysozyme-positive cells at  
147 the crypt base 120 h after DOXO in CONV mice. In addition, staining revealed the presence  
148 of muc2- and lysozyme-expressing intermediate cells in CONV mice following DOXO  
149 treatment (Fig. 5). This is in contrast to the lack of expansion of lysozyme-positive cell  
150 numbers and presence of intermediate cells is observed in GF crypts following DOXO  
151 treatment (Fig. 5).

152 **Discussion**

153 This study demonstrates that enteric bacteria are necessary for the initiation and  
154 maintenance of mucosal damage and repair observed in the jejunum of CONV mice following  
155 DOXO treatment, which includes crypt loss and villus blunting, followed by an increase in  
156 proliferating cells, and crypt hyperplasia. In contrast, enteric bacteria do not appear to be  
157 necessary for the rapid DOXO-mediated induction of apoptosis which was induced to similar  
158 levels in both GF and CONV raised mice, and in a similar cell position distribution. Likewise,  
159 DOXO treatment results in a decreased number of mitotic figures in the crypt epithelium of  
160 both GF and CONV mice. Together, these data suggest that the damage-associated crypt  
161 loss and subsequent crypt regeneration documented in CONV mice is *not* secondary to the  
162 rapid induction of apoptosis observed in crypt epithelial cells following DOXO treatment; but,  
163 rather, is coupled to the presence of enteric bacteria. While DOXO is a widely used  
164 anticancer drug, its mechanism of action is not completely understood. Classically, DOXO is  
165 described as a topoisomerase II inhibitor and, as such, inhibits the re-ligation of cleaved DNA  
166 strands which have been unwound for transcription and replication. This inhibition results in  
167 DNA double strand breaks, and ultimately, apoptosis of the cell.<sup>15</sup> Other mechanisms of  
168 induction of apoptosis by DOXO have been suggested, as well, including inhibition of DNA  
169 and RNA synthesis and formation of free radicals or formaldehyde-mediated DOXO-DNA  
170 adducts.<sup>16</sup> Nonetheless, regardless of the mechanism of action of DOXO within the small  
171 intestinal epithelium, our data demonstrate that DOXO induces apoptosis *independent of* the  
172 presence of enteric bacteria. What is unclear are the events that occur after the initial  
173 induction of apoptosis, which culminate in crypt loss, villus shrinking, crypt hyperplasia, and  
174 subsequent restitution of normal small intestinal mucosa, and moreover, what specific roles  
175 enteric bacteria play in this damage and repair process.



176 One possibility is that DOXO treatment elicits a direct effect upon intestinal bacteria,  
177 causing a rapid dysbiosis which, in turn, causes direct damage to the intestinal epithelium,  
178 as has been shown for methotrexate.<sup>17</sup> A similar theory has been put forth for the role of  
179 dysbiosis in susceptibility for inflammatory bowel disease.<sup>18,19</sup> Though it is used in human  
180 medicine primarily for its antineoplastic properties, DOXO is a natural anthracycline antibiotic  
181 product of *Streptomyces peucetius* var. *casieus*.<sup>20</sup> Since DOXO targets rapidly dividing cells,  
182 and bacteria tend to replicate frequently, bacteria may, indeed, be potential primary targets.  
183 Interestingly, studies suggest that unconjugated DOXO may be available to small intestinal  
184 enteric bacteria within a few hours after dosing.<sup>21</sup> These studies were performed on isolated  
185 perfused rat liver, and showed that approximately 30% of a dose of DOXO (equivalent to a  
186 20 mg/kg intravenous dose) was excreted from isolated liver as unconjugated DOXO into bile  
187 within 3 h of dosing.<sup>21</sup> However, *in vitro* studies indicate that DOXO has little direct impact  
188 on bacterial growth. These prior findings provide no evidence that DOXO induces a dysbiosis  
189 of the enteric bacterial census, however they do not inform about potential effects on specific  
190 bacteria. Also, our findings do not rule out the possibility that dysbiosis occurs following  
191 DOXO-induced damage by an indirect, but DOXO-dependent, mechanism. Other  
192 chemotherapeutic drugs have been shown to be reactivated by microbial  $\beta$ -glucuronidases,  
193 leading to direct toxicity to mucosal cells.<sup>22,23,24</sup> Therefore, further studies to evaluate  
194 microbial-dependent mucositis following DOXO treatment are underway.

195 Another potential mechanism of enteric bacteria-mediated intestinal damage following  
196 DOXO treatment is disruption of the physical barrier that separates the intestinal epithelium  
197 from luminal bacteria. In healthy intestine under homeostatic conditions, a barrier of mucin  
198 serves to minimize the direct contact of luminal bacteria with the mucosa.<sup>25</sup> In addition,  
199 Paneth cells secrete a cadre of antimicrobial factors including:  $\alpha$ -defensins,  $\alpha$ PLA2, and  
200 lysozyme.<sup>26</sup> However, DOXO may alter barrier function, permitting an increase in the direct

201 association of bacteria with the epithelium, followed by initiation of a bacteria-dependent  
202 signaling cascade via TLR or NOD receptors. An absence of bacteria, therefore, would fail to  
203 trigger this cascade. Of note, Nigro et al. demonstrated an increase in DOXO-induced  
204 apoptosis and dampened repair in Nod2 knock out mice suggesting that the presence of  
205 bacterial products such as muramyl-dipeptide (MDP) might be protective during damage.<sup>27</sup>  
206 However, because Nod2 was knocked out in the entire mouse it is not clear whether the  
207 protective effect came from Nod2 signaling within intestinal epithelium or lamina propria-  
208 derived cells. Other pro-mucositis chemotherapeutic agents, such as irinotecan, have been  
209 shown to impact mucin secretion,<sup>28</sup> and closer association of microbes with mucosa offers  
210 increased opportunities for activation of TLR and/or NODs on or within epithelial cells and  
211 immune cells intimately associated with the epithelial barrier.<sup>29,30</sup> Likewise, our previous  
212 studies demonstrate alteration in secretory cell allocation within the intestinal crypt, resulting  
213 in increased intermediate cell (both muc2 and lysozyme positive) number which may alter the  
214 mucin barrier.<sup>2,3</sup> This finding is echoed by the increase in muc2<sup>+</sup>/lysozyme<sup>+</sup> cells observed  
215 in crypts of CONV mice at five days after DOXO in the current study. The fact that increases  
216 in muc2<sup>+</sup>/lysozyme<sup>+</sup> cells were not observed in GF mice after DOXO treatment may reflect  
217 an absence of damage and regenerative response or indicate that the alterations in lineage  
218 allocation that follow DOXO result from an increased interaction between enteric bacteria and  
219 epithelium.

220 Alternatively, DOXO-driven disruption of the mucosal barrier may not increase the  
221 association between bacteria and epithelia, but instead allow direct penetration of bacteria or  
222 bacterial products through a more permeable epithelium and into the lamina propria,  
223 facilitating direct interaction with resident leukocytes. Sun et al. demonstrated an increase in  
224 epithelial permeability of rat small intestinal epithelium following treatment with DOXO.<sup>31</sup> This  
225 increase allowed particles as large as albumin to move from the intestinal lumen to the lamina

226 propria, suggesting that an increase in permeability following DOXO may allow bacteria  
227 and/or bacterial products to penetrate the IEC barrier. DOXO treatment may also result in an  
228 inflammatory response following penetration of the epithelial barrier by bacterial products, or  
229 via another distinct pathway, such as the AKT-dependent inflammation that leads to  
230 cardiomyopathy following DOXO, or the CCL2-dependent inflammation that leads to renal  
231 fibrosis following DOXO.<sup>32,33</sup> To elucidate these mechanisms, future investigations will  
232 evaluate epithelial permeability, as well as the role of the inflammatory response, following  
233 DOXO. We hypothesize that treatment with DOXO causes an increase in the permeability of  
234 the epithelial barrier (due to a transient induction of increased apoptosis), concomitant with  
235 increased association of enteric bacteria with the mucosa, allowing bacterial products to  
236 penetrate and induce an inflammatory response, resulting in the significant mucosal damage  
237 (and repair) observed in our studies.

238 The findings of the current study demonstrate that DOXO-induced apoptosis in small  
239 intestinal crypts occurs independent of the presence of bacteria while mucosal damage after  
240 DOXO is dependent upon the presence of bacteria. These findings have translational  
241 implications supporting that manipulation of the intestinal microbiota during DOXO-based  
242 chemotherapy may reduce damage to the intestinal mucosa. This could allow more effective  
243 anticancer therapies with fewer adverse effects

## 244 **Materials and Methods**

245 *Animals.* Conventionally raised (CONV) adult female C57BL/6 mice were purchased from  
246 Jackson Laboratories and used between 8-10 weeks of age. Adult female C57BL/6 mice  
247 were raised under germ free (GF) conditions in the National Gnotobiotic Research Center at  
248 the University of North Carolina at Chapel Hill. Experimental procedures were approved by  
249 the Institutional Animal Care and Use Committee of The University of North Carolina at  
250 Chapel Hill. Mice were given a single intraperitoneal (IP) injection of DOXO (Pharmacia &  
251 Upjohn Co.) at a dose of 20 mg/kg body weight. We have previously reported that this dose  
252 induces a reproducible sequela of intestinal damage in mice.<sup>2</sup> Animals were killed at 6 (n=3  
253 for CONV, n=3 for GF), 72 (n=3, n=3), or 120 h (n=3, n=2) after DOXO treatment and  
254 compared with no treatment controls (n=3, n=6). Small intestine was flushed with ice-cold  
255 phosphate buffered saline (pH 7.4) and a piece of middle jejunum was fixed in 10% buffered  
256 formalin and embedded in paraffin.

257 *Histology.* Formalin-fixed paraffin embedded specimens were oriented to provide sections  
258 perpendicular to the long axis of the bowel, and 5 $\mu$ m sections were used for evaluating  
259 general morphology. Longitudinal sections of crypts or villi were selected for scoring on the  
260 basis that a single, continuous layer of epithelium followed from crypt base to villus base and  
261 from the crypt-villus junction to the villus tip, respectively. For scoring cell position, each crypt  
262 was divided in half and cells were numbered sequentially from crypt base to crypt-villus  
263 junction, with cell position "one" being occupied by the first cell at the base of each half crypt,  
264 as previously described.<sup>2</sup> Apoptosis was scored by H&E staining based on the presence of  
265 one or more pyknotic bodies at a given cell position and confirmed by immunofluorescence for  
266 cleaved caspase 3 (CC3).<sup>34</sup> Number of cells in G2-M phase per crypt was assessed by  
267 immunohistochemistry for phosphohistone H3 (pHH3) and counting the number of pHH3  
268 positive cells per crypt. To directly quantify mitosis, the number of mitotic figures per crypt

269 was counted. Crypt density for each animal was calculated by averaging the number of crypts  
270 contained within five-500  $\mu\text{m}$  lengths of mucosa. Villus height and crypt depth were measured  
271 using Axio Imager software on images captured using an Axio Imager A1 microscope and an  
272 AxioCam MRC 5 high-resolution camera (Carl Zeiss Microimaging, Inc.).

273 *Immunostaining.* For immunohistochemistry, slides were deparaffinized, rehydrated, and  
274 incubated in 3% hydrogen peroxide for 15 min at room temperature (RT) to quench  
275 endogenous peroxidase activity. Sections were treated to heat-induce epitope retrieval  
276 (Antigen Unmasking Solution cat. # H-3300, Vector Laboratories) and allowed to cool to RT.  
277 Primary antibody (rabbit anti-phospho histone H3 cat. 9701S Cell Signaling Technology) was  
278 applied to each section at 1:300 dilution and incubated for 1h at RT. Sections were then  
279 washed and incubated with biotinylated goat anti-rabbit secondary antibody for 30-60 min at  
280 RT. After washing, slides were incubated in Vectastain ABC reagent (PK-4000, Vector  
281 Laboratories) for 30 min and then developed in a DAB substrate solution. Quantification of  
282 pHH3<sup>+</sup> cells was performed on 25-30 crypts. Data are expressed as number of positive cells  
283 per crypt. For immunofluorescence, slides were deparaffinized, rehydrated, treated to antigen  
284 retrieval in 10mM sodium citrate (pH 6.0) with 0.05% Tween 20 for 30 min, and allowed to  
285 come to RT. Sections were washed and incubated with primary antibodies as previously  
286 reported.<sup>3</sup> Primary antibodies used were as follows: anti-lysozyme (sc-27958, Santa Cruz  
287 Biotechnology, 1:100 dilution), anti-mucin2 (Muc2; sc-15334, Santa Cruz Biotechnology,  
288 1:200 dilution), and anti-active caspase 3 (cat. no. 9661, Cell Signaling Technology, 1:200  
289 dilution). Sections were washed and incubated with corresponding fluorescently conjugated  
290 secondary antibodies. Finally, sections were mounted using Vectashield Mounting Medium  
291 with DAPI (H-1200 Vector Laboratories) and evaluated using an Axio Imager A1 microscope  
292 and an AxioCam MRC 5 high resolution camera.

293 *Statistics.* All quantitative results are presented as mean  $\pm$  standard error (SE). All data were  
294 subjected to one-way ANOVA with correction for multiple comparisons using the Fisher's  
295 procedure. For all comparisons, a *P* value of  $\leq 0.05$  was considered significant.

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303 Figure Legends

304

305 **Figure 1.** DOXO induces apoptosis in intestinal epithelium irrespective of the presence of  
306 enteric bacteria. **A.** H&E images demonstrating mitotic bodies and immunofluorescence  
307 staining indicating the presence of active caspase 3-positive cells (green) 6h following DOXO  
308 treatment in both CONV and GF mice. Arrows indicated apoptotic cells. **B.** Quantitation of  
309 the number of apoptotic cells per crypt, for a total of 20 crypts per animal, in CONV and GF  
310 jejunal tissue from control mice and 6, 72, and 120h after DOXO treatment. \* indicates values  
311 significantly different from their respective 0h controls  $p \leq 0.05$ . **C.** Positional analysis of  
312 apoptotic bodies in jejunal epithelium from CONV and GF mice 6h following DOXO treatment.  
313 Scale bar: 30  $\mu\text{m}$ .

314

315 **Figure 2.** DOXO treatment does not alter crypt depth or villus height in GF mice. **A.**  
316 Micrographs of representative H&E stained sections from GF and CONV mice of control  
317 tissue and 6, 72 and 120h following DOXO treatment. **B.** Quantitation of crypt depth on 10-  
318 15 crypts/villi in CONV and GF jejunal tissue from control mice and 6, 72, and 120h after  
319 DOXO treatment. \* indicates values significantly different from their respective controls  
320  $p \leq 0.05$ . # indicates values significantly different within a particular time point  $p \leq 0.05$ . **C.**  
321 Quantitation of villus height in CONV and GF jejunal tissue from control mice and 6, 72, and  
322 120h after DOXO treatment. Scale bar: 50  $\mu\text{m}$ .

323

324 **Figure 3.** DOXO treatment does not impact proliferation or mitotic index in jejunal epithelium  
325 of GF mice. **A.** Micrograph showing representative staining for pHH3 on jejunal sections from  
326 GF and CONV mice. **B.** Quantitation of pHH3+ cells in CONV and GF jejunal tissue from  
327 control mice and 6, 72, and 120h after DOXO treatment. \* indicates values significantly  
328 different from their respective controls  $p \leq 0.05$ . # indicates values significantly different within  
329 a particular time point  $p \leq 0.05$ . **C.** Quantitation of mitotic index in CONV and GF jejunal tissue  
330 from control mice and 6, 72, and 120h after DOXO treatment. # indicates values significantly  
331 different within a particular time point  $p \leq 0.05$ . ND means “not detected”. Scale bar: 50  $\mu\text{m}$ .

332



333 **Figure 4.** DOXO treatment does not alter crypt density in jejunal mucosa of GF mice. \*  
334 indicates values significantly different from their respective controls  $p \leq 0.05$ . # indicates  
335 values significantly different within a particular time point  $p \leq 0.05$ .

336

337 **Figure 5.** Expansion of the Paneth cell compartment and allocation of intermediate cells are  
338 not observed in GF mice following DOXO. Immunofluorescent detection of lysozyme (red),  
339 muc2 (green), and nuclei (blue) in jejunal sections from GF and CONV mice. Arrows indicate  
340 'intermediate cells', characterized by their co-expression of lysozyme (red) and muc2 (green).

341 Scale bar: 50  $\mu\text{m}$ .

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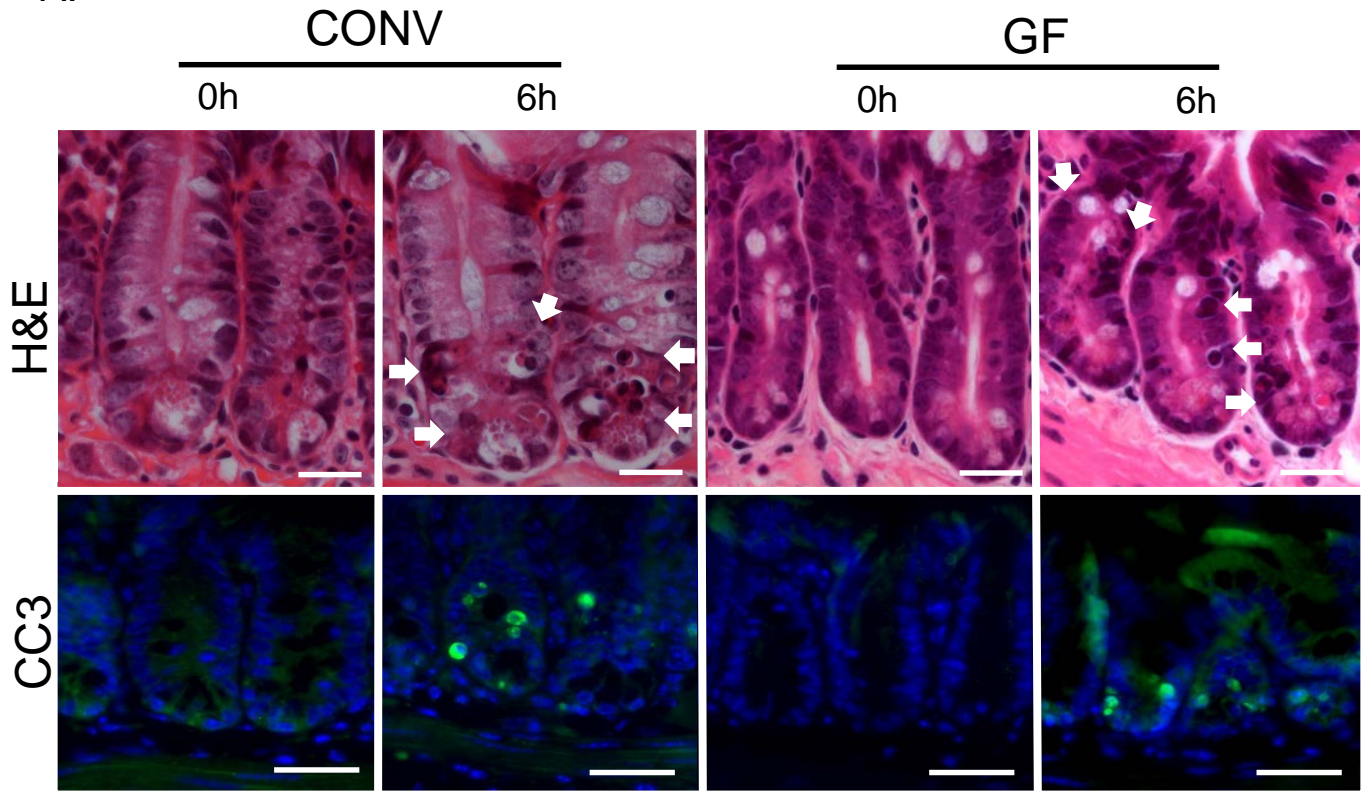
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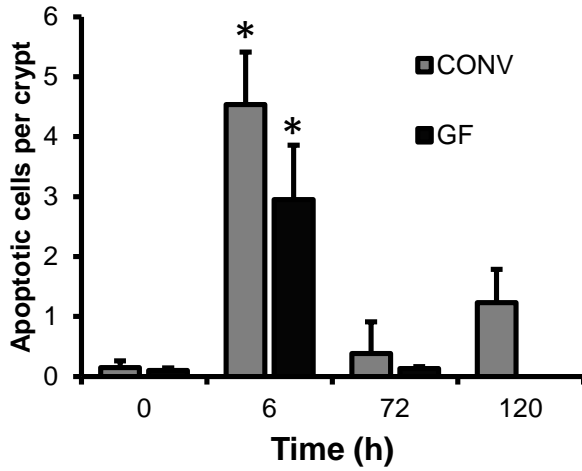
440

Figure 1

A.



B.



C.

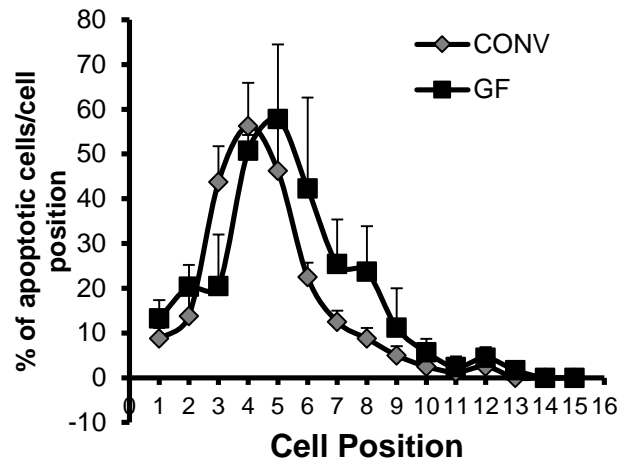
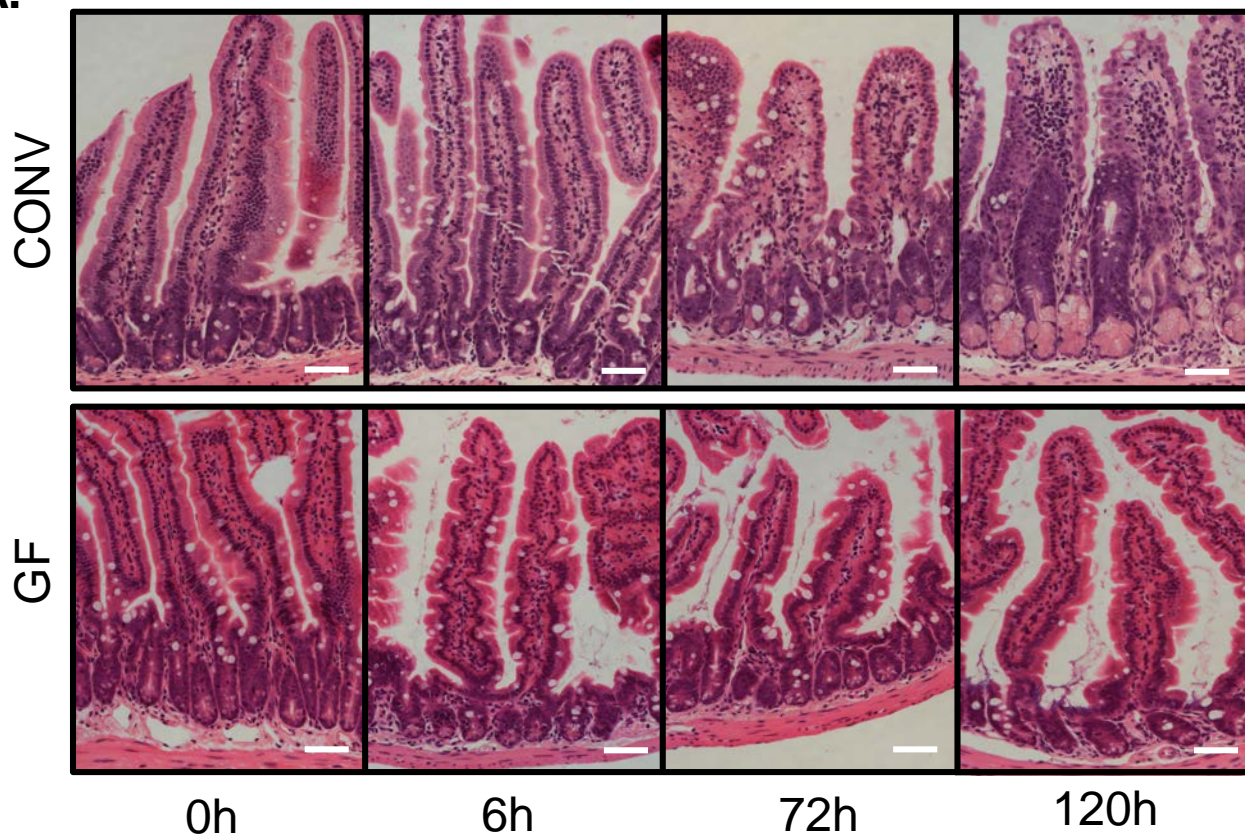
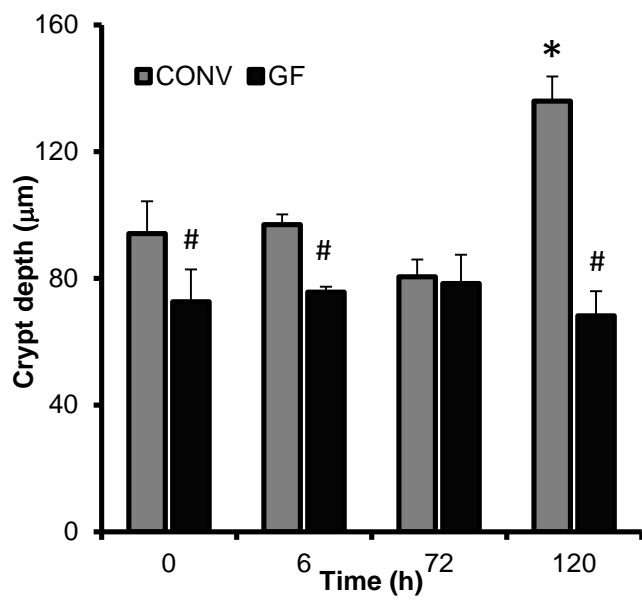


Figure 2

A.



B.



C.

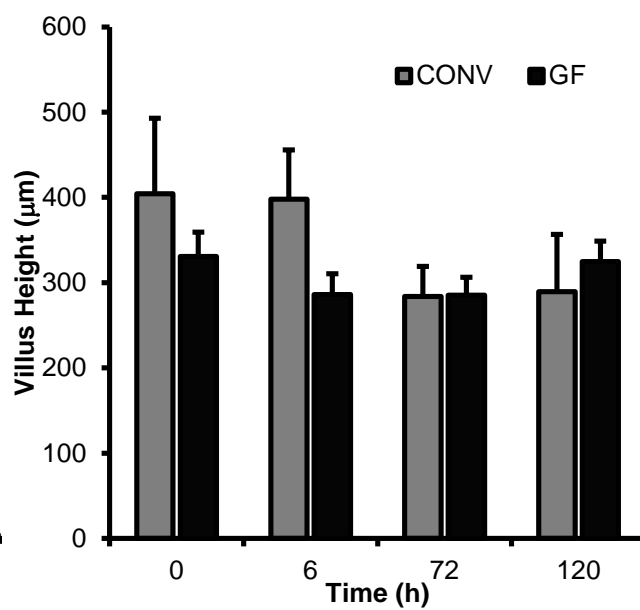
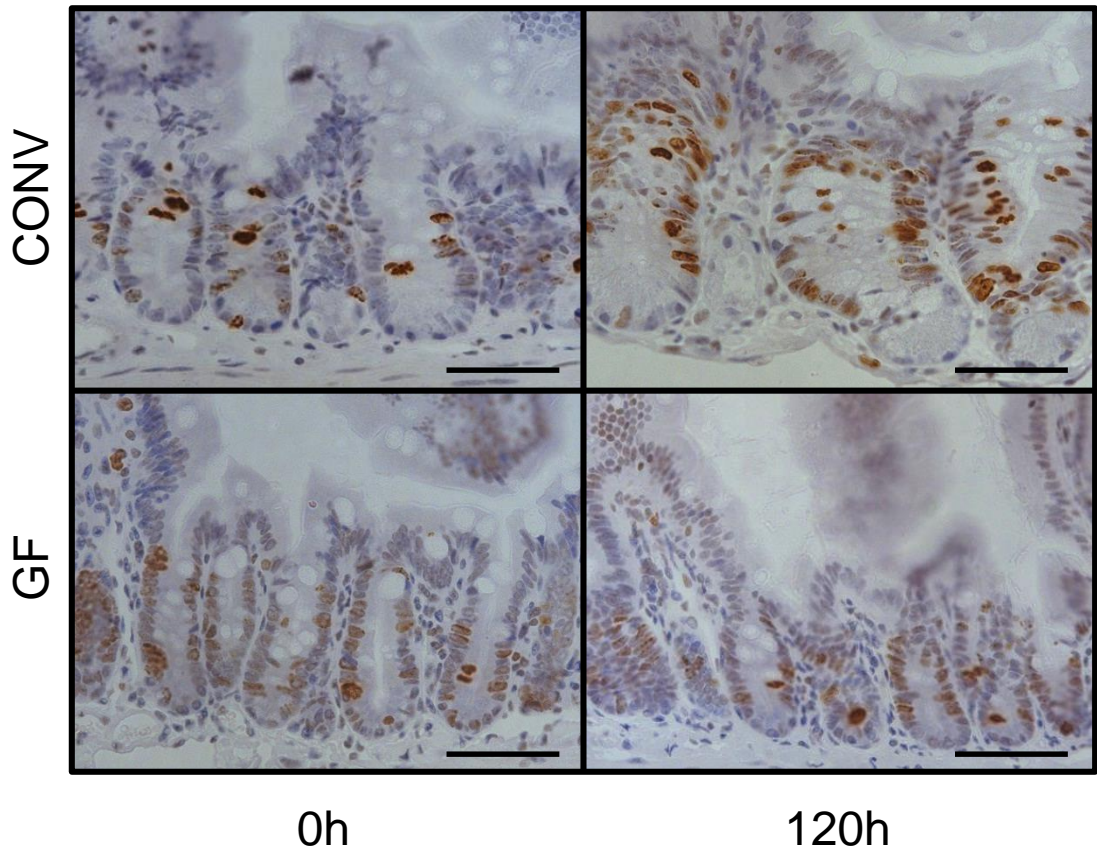


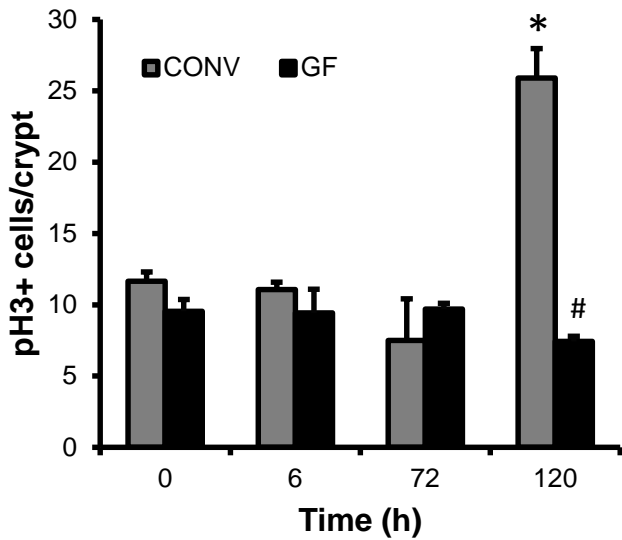
Figure 3

A.



B.

Proliferative index



C.

Mitoses

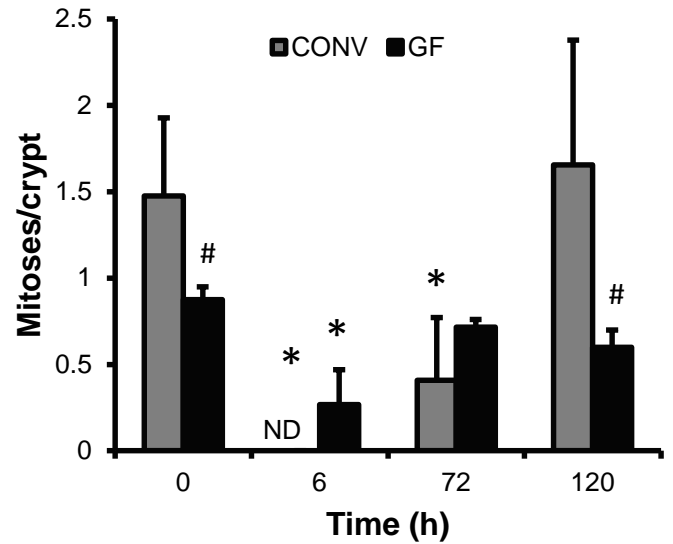




Figure 4

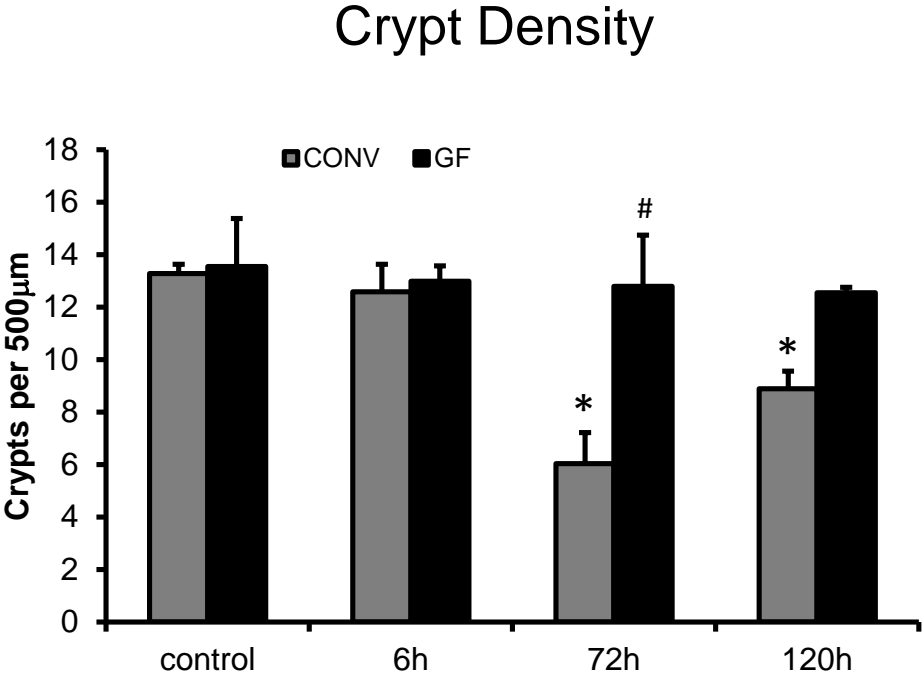


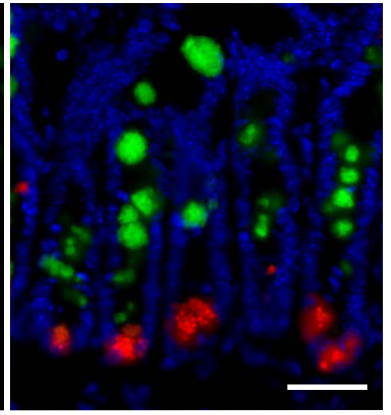
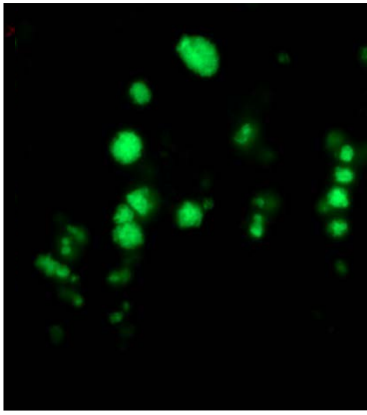
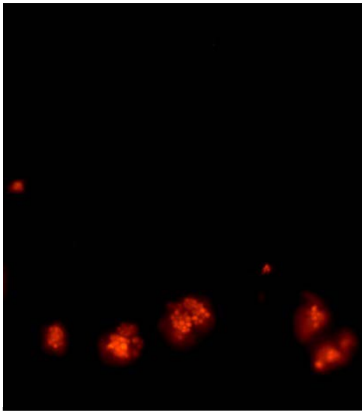
Figure 5

Lysozyme

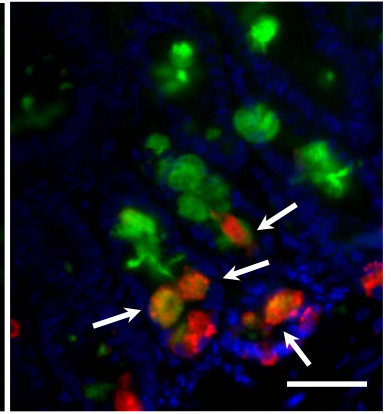
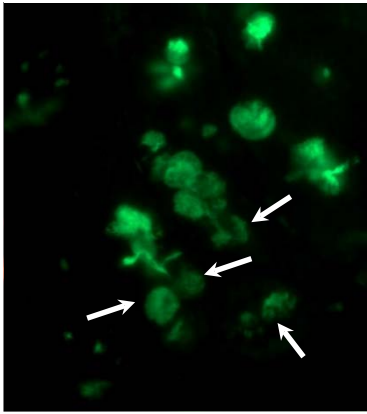
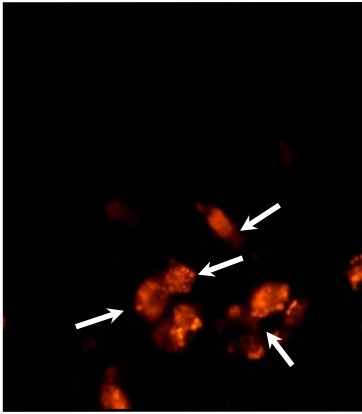
Muc2

Combination

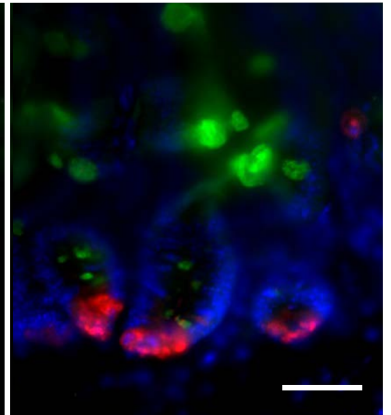
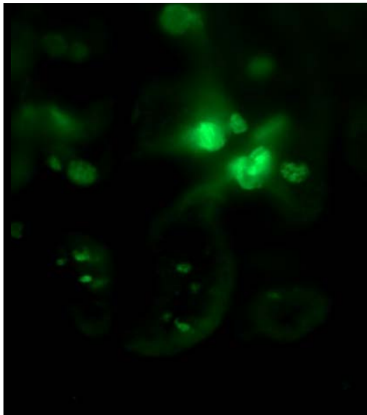
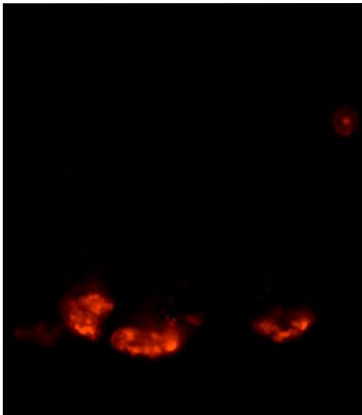
Conv 0h



Conv 120h



GF 0h



GF 120h

