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1 **Compensation between Wnt-driven tumorigenesis and cellular responses to**
2 **ribosome biogenesis inhibition in the murine intestinal epithelium**

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22

23 **Running title:** Ribosome biogenesis defects and tumor initiation

24 **Keywords :** Ribosome biogenesis; Wnt signaling; Colorectal cancer; p53.

25

26 **Abstract**

27 Ribosome biogenesis inhibition causes cell cycle arrest and apoptosis through the
28 activation of tumor suppressor-dependent surveillance pathways. These responses are
29 exacerbated in cancer cells, suggesting that targeting ribosome synthesis may be beneficial to
30 patients. Here, we characterize the effect of the loss-of-function of *Notchless (Nle)*, an essential
31 actor of ribosome biogenesis, on the intestinal epithelium undergoing tumor initiation due to
32 acute *Apc* loss-of-function. We show that ribosome biogenesis dysfunction strongly alleviates
33 Wnt-driven tumor initiation by restoring cell cycle exit and differentiation in *Apc*-deficient
34 progenitors. Conversely Wnt hyperactivation attenuates the cellular responses to surveillance
35 pathways activation induced by ribosome biogenesis dysfunction, as proliferation was
36 maintained at control-like levels in the stem cells and progenitors of double mutants. Thus, our
37 data indicate that, while ribosome biogenesis inhibition efficiently reduces cancer cell
38 proliferation in the intestinal epithelium, enhanced resistance of *Apc*-deficient stem and
39 progenitor cells to ribosome biogenesis defects may be an important concern when using a
40 therapeutic strategy targeting ribosome production for the treatment of Wnt-dependent
41 tumorigenesis.

42

43 **INTRODUCTION**

44

45 Ribosome biogenesis inhibition has emerged as a promising therapeutic strategy
46 against cancer. Indeed, in addition to targeting the production of the ribosomes required
47 for the increased protein synthesis demand of cancer cells¹, RNA Pol I inhibition or
48 deficiencies in ribosome biogenesis factors were shown to trigger the binding of a 5S
49 rRNA/RPL11/RPL5 inhibitory complex to MDM2, thereby preventing p53 degradation<sup>2-
50 4</sup>. In absence of functional p53 pathway, ribosome biogenesis dysfunction also triggers
51 p53-independent mechanisms⁵. Taking advantage of specific RNA Pol I inhibitors, several
52 *in vitro* and xenograft studies have shown that lymphoma ⁶, melanoma, osteosarcoma,
53 breast, colon ⁷ and lung ⁸ cancer cells display a strong and specific sensitivity to ribosome
54 biogenesis inhibition, that induces potent p53-dependent or independent stress
55 responses, which are not observed in normal cells^{6,7}. As a therapeutic strategy, targeting
56 the translational apparatus of cancer cells has two major advantages. Firstly, it is non-
57 genotoxic and therefore minimizes the risk of inducing novel mutations in the
58 surrounding cells. Secondly, this approach displayed so far a clear differential effect
59 between normal cells and the tested cancer cell lines, making it theoretically possible to
60 establish a dosage that reduces deleterious side effects while still being efficient. However,
61 the impact of ribosome biogenesis alterations on tissues undergoing tumorigenesis *in vivo*
62 has insufficiently been assessed until now.

63 In humans, APC is mutated in the vast majority of colorectal cancers ⁹. In the mouse,
64 the acute inactivation of *Apc* in the epithelium of the small intestine results in constitutive
65 activation of the Wnt/ β -catenin pathway, leading to an abnormal expansion of the
66 proliferative compartment at the expense of differentiated cells, reminiscent of early
67 events of adenoma formation^{10,11}. It has been shown that *Myc* deletion largely rescues the
68 *Apc* loss-of-function phenotype through downstream shutdown of the Wnt pathway¹²,
69 suggesting that Wnt-driven tumor initiation is also *Myc*-driven. Interestingly, the
70 aforementioned Pol I inhibitors were demonstrated to be particularly efficient on *Myc*-
71 driven cancer cells^{6,8}. Since c-MYC is a major ribosome biogenesis positive regulator¹³,
72 this raised the possibility that ribosome biogenesis inhibition could also attenuate the
73 phenotype caused by *Apc* deficiency.

74 *Notchless (Nle)* encodes a WD40 repeats-containing protein highly conserved in
75 eukaryotes¹⁴⁻¹⁶ and involved in the late steps of maturation and subsequent export of the

76 60S particle¹⁷⁻²⁰. In previous studies, we showed that the role of *Nle* in the maturation of
77 the large ribosomal subunit is conserved in the mouse²¹, and that *Nle* loss-of-function
78 leads to p53 activation in the intestinal epithelium, resulting in rapid stem cell and
79 progenitor exhaustion through cell cycle arrest, apoptosis and premature goblet cell
80 differentiation²². This genetic model offers a unique opportunity to study the impact of
81 ribosome biogenesis perturbations specifically in the intestinal epithelium under
82 pathological conditions. Here, by combining *Nle* and *Apc* conditional loss-of-function, we
83 show that defective ribosome biogenesis strongly attenuates Wnt hyperactivation-driven
84 proliferative compartment expansion in the intestinal epithelium through restoration of
85 cell cycle exit and differentiation. Conversely, we show that Wnt hyperactivation
86 alleviates the *Nle* loss-of-function phenotype, as proliferation is maintained in double
87 mutant crypts and in the stem cell compartment despite broad and persistent p53
88 stabilization.
89

90 **MATERIAL AND METHODS**

91

92 **Mice.**

93 All experiments were conducted at the Institut Pasteur according to the French and
94 European regulations on care and protection of laboratory animals (EC Directive 86/609,
95 French Law 2001-486 issued on June 6, 2001) and were approved by the Institut Pasteur
96 ethics committee (n° 2016-0106 and 2017-0044).

97 The alleles used were as follows: *Nle^{flox}*²¹, *Nle^{null}*¹⁵, *Apc^{flox}*¹⁰, Villin-CreERT2²³. All
98 experimental animals were generated in a mixed C57BL/6Nx129/Sv genetic background.
99 Mice at 5 to 10 weeks of age were injected intraperitoneally with 56mg/kg tamoxifen for
100 three consecutive days. For proliferation assays, mice were injected with BrdU (100
101 mg/kg) 2h, 24h or 48h before sacrifice. Injection order was randomized between animals
102 and genotype was only assessed after the experiments.

103 Genotyping was performed by PCR after tissue lysis in 50 mM Tris, pH 8.5, 100 mM NaCl,
104 0.5% Tween 20, and 100 mg/ml proteinase K at 56°C overnight, followed by a 10-min
105 incubation at 96°C. PCR was performed using 1.0 unit of Taq DNA polymerase (MP
106 biomedical) under the following conditions: 95 °C for 30 s; 32 cycles at 95 °C for 30 s,
107 60 °C for 30 s, 72 °C for 60 s; 72 °C for 10 min. For *Nle*, PCR amplification with primers
108 mNleQF3 (5'- ctgtactctttctcatccgaccac -3'), CondF (5'- agacttggggctctggactca -3'), and
109 CondR2 (5'- ccctaactaagacaaccaaga -3') allowed us to discriminate *Nle^{wt}* (100 bp), *Nle^{Flox}*
110 (200 bp), and *Nle^{del}* (500 bp) alleles. For *Apc*, PCR amplification with primers APCint13F
111 (5'- ctgttctgcagtatgttatca -3'), APCext14R (5'- ctatgagtcaacacaggatta -3'), and APCext15R
112 (5'- caatataatgagctctgggcc -3') allowed us to discriminate *APC^{wt}* (180 bp), *Apc^{Flox}* (230 bp),
113 and *Apc^{del}* (160 bp) alleles. PCR products were separated by electrophoresis on 2% high-
114 resolution (NuSieve 3:1 agarose) agarose gels.

115

116 **Tissue extracts.**

117 For paraffin sections, the intestinal tract was dissected, then flushed with ice-cold PBS to
118 remove feces and perfused with ice-cold 4% paraformaldehyde (PFA). The small intestine
119 was rolled up from the proximal to the distal end in concentric circles, fixed in 4% PFA at
120 4°C overnight and embedded in paraffin wax. For RT-qPCR on total epithelium, 5 cm of
121 duodenum was harvested in 10mL EDTA (10mM in PBS, pH=8.0). After 30 min incubation
122 on ice and 2x5 min vortex at maximum speed at 4°C, the muscle layer of the intestine was

123 manually removed and the epithelium was pelleted at 300 rcf, 4°C for 5 min, then
124 resuspended in 1 mL Trizol (Invitrogen, Carlsbad, CA, USA). For crypts and villi isolation,
125 5–10 cm of jejunum were collected, opened longitudinally and processed as previously
126 described ²⁴.

127

128 **Histology and immunostaining.**

129 Histology and immunostaining were performed as described previously ²⁵. Specific
130 antibody binding was detected using biotinylated secondary antibodies and
131 Streptavidin/HRP complexes (Dako, Glostrup, Denmark). For β -catenin immunostaining,
132 M.O.M. and ABC kits (Vector) were used. Bright field microscopy was performed using a
133 Axio Scan.Z1 equipped with a 20x objective lens. The system was set to run in automated
134 batch mode with automated focus and tissue finding. Primary and secondary antibodies
135 used in this study are listed in Supplementary material Supplementary Table S1.

136

137 **In situ hybridization.**

138 For Fluorescent In Situ Hybridization (FISH), the hybridization step was performed as
139 previously described. Conjugated FISH probes were purchased from Eurogentec: its1-
140 Cy5: tagacacggaagagccggacgggaaaga; its2-Cy3: gcgattgatcgtcaaccgacgctc and validated in
141 a previous study ²¹. ISH for Olfm4 was carried out with the RNAscope 2.5 BROWN kit
142 (Advanced Cell Diagnostics) according to the manufacturer's instructions (Olfm4 probe
143 reference: 311831).

144

145 **RT-qPCR.**

146 RNA extraction was performed according to the Trizol-chloroform extraction protocol
147 provided by Invitrogen. For epithelium, crypts and villi, the chloroform and ethanol steps
148 were repeated twice. Reverse-transcription was performed using the Superscript II kit
149 (Invitrogen) according to the manufacturers' instructions. Real-time PCR was carried on
150 using custom-designed primers (Supplementary Table S2) and LightCycler 480 SYBR
151 Green I Master mix (Roche Life Sciences) on a LightCycler 480 (Roche Life Sciences).
152 Expression levels were normalized using TBP and Aldolase as reference genes.

153

154 **Western blot.**

155 Proteins were extracted in a buffer containing 10mM Tris-HCl pH 7.5, 5mM EDTA, 150mM
156 NaCl, 30mM Sodium Pyrophosphate, 50mM Sodium Fluoride, 10% Glycerol, 1% NP40,
157 supplemented with antiproteases (Roche Life Sciences) and 2.5U/ μ L Benzonase (Sigma-
158 Aldrich). The extracts were incubated on ice for 30 min, then centrifuged 10 min at 10000
159 rcf, 4°C. The supernatant was recovered and its protein content was determined using a
160 Bradford assay. Proteins were denatured in Laemmli buffer at 95 °C for 5 min before being
161 loaded on a 12% polyacrylamide gel. After migration, proteins were transferred onto a
162 nitrocellulose membrane (Biorad, Hercules, CA, USA) and incubated overnight at 4°C with
163 the primary antibodies. Membranes were incubated with peroxidase-labeled secondary
164 antibodies at RT for 80 min and rinsed in TBS-0.1% Tween. Signals were visualized using
165 ECl 2 (Pierce Biotechnology, Rockford, IL, USA) and quantified on a Typhoon Instrument.
166 Primary and secondary antibodies used in these experiments are listed in Supplementary
167 Table S1.

168

169 **Quantification of protein synthesis.**

170 Mice were injected intraperitoneally with 2mg of puromycin in PBS, 10min before
171 sacrifice. Intestines were rapidly harvested in ice-cold PBS containing emetine to block
172 further puromycin incorporation during the procedure. Crypts were isolated as
173 previously described ²⁴ and counted before being lysed in protein extraction buffer.
174 Puromycolated peptide chains were quantified on a western blot against puromycin by
175 measuring the pixel intensity through the whole length of each lane using a Typhoon
176 instrument.

177

178 **Crypts culture.**

179 Isolated crypts were cultured as previously described ⁴⁵. In brief, crypts were embedded
180 in growth factor reduced matrigel (Corning LifeSciences, Tewksbury, MA, USA) with
181 culture medium (Advanced DMEM/F12; Invitrogen) containing EGF (Peprotech, Rocky
182 Hill, NJ, USA), R-spondin 1 (R&D Systems, Minneapolis, MN, USA), Noggin (Peprotech) and
183 B27 (Invitrogen). For nutlin-3 treatment, 4- or 8-days old organoids derived from Control
184 or APC^{CKO} crypts were incubated with culture medium containing DMSO (mock condition)
185 or nutlin-3a (Sigma) at 1 μ M, 5 μ M and 10 μ M concentrations. After 2 days of treatment, the
186 number of healthy organoids was scored manually. Images of representative organoids
187 were acquired with a bright-field Leica MZ16 binocular (Leica, M165FC). Healthy

188 organoids were defined as budding structures with at least 3 buds and visible lumen for
189 Control, and smooth spheres with visible lumen for APC^{ck0}. 2 mice per genotype and 2 to
190 3 technical replicates per condition were assessed. For immunofluorescence, organoids
191 were fixed in 4% formaldehyde for 2 hours at RT, permeabilized with Blocking Buffer (5%
192 fetal calf serum, 0.5% Triton X-100 in 1X PBS) and incubated with appropriate primary
193 and secondary antibodies. Phalloidin (1/50; Thermofisher) and DAPI (1/1000; Sigma)
194 were used to detect cell membranes and nuclei, respectively. Samples were washed 3
195 times in PBS before being imaged on the macroapoptome Zeiss Axiozoomer and analyzed
196 using the Zen software.

197

198 **Statistical analysis.** All experiments were performed with a sample size $n \geq 4$ (each
199 sample is an individual mouse) so as to dampen the influence of outliers on means. No
200 animal was excluded from the analysis. For mean comparisons, all bar graphs with pooled
201 data show means \pm S.E.M. Statistical analyses were performed using the parametric
202 Student's t-test and the non-parametric Mann-Whitney Wilcoxon test to account for the
203 possibility of non-normal distributions. $p < 0.05$ was considered significant. Tests were
204 always two-sided.

205

206 **RNA sequencing.** Crypts were harvested at day 1 and day 2 pi from 3 mice of each
207 genotypes and total RNA was prepared using Trizol-chloroform extraction. PolyA-
208 enriched library preparation and PE150 sequencing were performed by Novogene Co. Ltd.
209 Approximately 20 million sequences were generated per sample and used for the analysis.
210 The sequence data have been deposited in NCBI's Gene Expression Omnibus under
211 accession number GSE144233.

212

213 **Computational Methods**

214 *RNA-seq Data Processing and Differential Expression*

215 Paired end RNA-seq reads were quantified against the mm9 transcriptome using Kallisto
216 0.43²⁶ with default options. Rounded estimated counts were used as input to DESeq2²⁷.
217 Genes with at least 10 normalized counts in all replicates of at least one condition were
218 considered for differential expression analysis. For all differential expression tests
219 DESeq2 was run without independent filtering and without any fold change shrinkage,
220 genes with $\text{padj} < 0.05$ are considered differentially expressed. Samples at 24h and 48h

221 were processed separately and subjected to the same analysis. We performed Wald tests
222 under the model $\sim Nle + Apc + Nle:Apc$, where Nle is a factor indicating Nle^{cKO} and Apc is
223 a factor indicating Apc^{cKO} . Roughly, for each gene, the fold changes in Nle^{cKO} samples (N)
224 and in Apc^{cKO} samples (A) were calculated. The interaction term (D) indicates that, for a
225 given gene, the fold change in $Apc^{cKO};Nle^{cKO}$ samples differs from $N*A$. We tested the
226 significance of the Nle , Apc and the interaction term.

227 *K-means clustering of RNA-seq Data*

228 Response patterns of genes were identified using k-means clustering (using the
229 Clustering package in Julia 0.6²⁸) on normalised read counts. We clustered the mean
230 expression per timepoint and condition, normalised to the maximum expression of each
231 gene. We found $k = 10$ provided a balance, both generalising broad behaviours and
232 identify specific double cKO results.

233 *Gene/phenotype ontology enrichment analysis*

234 Genes were annotated with gene ontology (GO) terms (transitively closed to include
235 parent terms) from the MGI GO annotation (date : 26/01/2017). Enrichment tests were
236 applied to gene lists resulting from differential expression analysis. In all cases,
237 enrichment tests were performed using χ^2 tests for all terms with at least 5 genes in the
238 gene list of interest against a background of annotations of all genes tested for differential
239 expression. χ^2 test p-values were adjusted by the Benjamini-Hochberg method, those with
240 $padj < 0.05$ where reported.

241 The different gene clusters from the model-based analysis were also analyzed using the
242 MouseMine database. For each gene list, Mammalian Phenotype Ontology Enrichment
243 terms were searched with Max p-value = 0.05 and Holm-Bonferroni correction. When
244 significantly enriched terms were found, those relevant to our mutant phenotypes were
245 selected, and the term with the lowest p-value for each category was indicated on figures
246 5E and S5. For every analysis, $p < 0.05$ is considered significant.

247

248 **RESULTS**

249

250 ***Nle* loss-of-function improves survival of *Apc*-deficient mice and limits expansion of**
251 **the crypt compartment.**

252 Control (Villin-CreERT2^{tg/0}; *Apc*^{fl^{ox}/+}; *Nle*^{fl^{ox}/+}), *Apc*^{ck⁰} (Villin-CreERT2^{tg/0};
253 *Apc*^{fl^{ox}/fl^{ox}}; *Nle*^{fl^{ox}/+}) and *Apc*^{ck⁰}; *Nle*^{ck⁰} (Villin-CreERT2^{tg/0}; *Apc*^{fl^{ox}/fl^{ox}}; *Nle*^{fl^{ox}/null}) mice were
254 subjected to three daily intraperitoneal tamoxifen injections and analyzed at various time
255 points post last tamoxifen injection (pi) (Figure 1A). Conversion of the *Apc*^{fl^{ox}} and *Nle*^{fl^{ox}}
256 alleles into *Apc*^{del} and *Nle*^{del} alleles respectively, was monitored by genomic PCR on both
257 loci. We found that recombination of the *Apc*^{fl^{ox}} and *Nle*^{fl^{ox}} alleles was efficient in the
258 intestinal epithelium from Control, *Apc*^{ck⁰} and *Apc*^{ck⁰}; *Nle*^{ck⁰} mice (Figure 1B). *Apc*^{ck⁰}
259 mice lost weight and died rapidly following tamoxifen injection. Weight loss was
260 significantly reduced in double mutant mice (Figure 1C), and although persisting, their
261 death was significantly delayed compared to the *Apc*^{ck⁰} (Figure 1D).

262 Consistent with previous reports^{10,11}, hematoxylin-eosin staining showed that *Apc*
263 loss-of-function resulted in rapid expansion of the crypt compartment at the expense of
264 the villus (Figure 1E), loss of typical enterocyte polarity and abnormal nuclear
265 morphology (Figure S1A). *Nle* loss-of-function strongly dampened these histological
266 alterations (Figure 1E), as the enlarged crypt compartment was not observed, and cell
267 polarity was restored in the villus (Figure S1A). Other aspects of the *Apc* loss-of-function
268 phenotype such as the ectopic paneth cells¹⁰ and enterocyte hypertrophy were similarly
269 observed in *Apc*^{ck⁰}; *Nle*^{ck⁰} (Figure S1A-C). Collectively, these data indicate that *Nle* loss-
270 of-function restores crypt and villus architecture in the *Apc*-deficient epithelium but does
271 not fully rescue the *Apc* loss-of-function clinical and histological phenotype.

272

273 ***Nle* loss-of-function attenuates proliferative compartment expansion by restoring**
274 **cell cycle exit and differentiation despite Wnt pathway hyperactivation.**

275 To verify that Wnt hyperactivation was effective in *Apc*^{ck⁰}; *Nle*^{ck⁰} intestinal epithelia,
276 we monitored nuclear β -catenin on small intestine sections. Accumulation of nuclear β -
277 catenin was similarly observed in *Apc*^{ck⁰} and *Apc*^{ck⁰}; *Nle*^{ck⁰} intestines (Figure 2A).
278 Moreover, upregulation of the Wnt targets *c-Myc* and *Axin2* was found in both *Apc*^{ck⁰} and
279 *Apc*^{ck⁰}; *Nle*^{ck⁰} epithelia (Figure 2B), confirming that hyperactivation of the Wnt/ β -catenin
280 pathway consecutive to *Apc*-deficiency was not modified by *Nle* loss-of-function.

281 We then performed a 2hrs BrdU chase to visualize proliferation in the intestinal
282 epithelium at day 2, 3 and 4 pi (Figure 2C and S2A). As previously reported^{10,11},
283 proliferation in *Apc*^{ckO} intestines abnormally extended beyond the histological crypt
284 compartment. Strikingly, the proliferation pattern was restored in *Apc*^{ckO}; *Nle*^{ckO}, as BrdU
285 positive cells restricted to the histological crypt like in Control intestines (Figure 2C). The
286 proportion of BrdU-positive cells within the proliferative compartment was similar for all
287 genotypes (Figure 2D), suggesting that the structure of the cell cycle is not dramatically
288 perturbed in *Apc*^{ckO} and *Apc*^{ckO}; *Nle*^{ckO} compared to Control. We observed a linear growth
289 of their proliferative compartment overtime (Figure S2B-C), primarily due to enlarged
290 crypt growth in *Apc*^{ckO}, whereas in *Apc*^{ckO}; *Nle*^{ckO}, it was caused by histological crypt
291 hyperplasia (Figure S2A). Taken together, these data suggest that *Nle* loss-of-function
292 reduces the expansion of the proliferative compartment consecutive to *Apc* loss-of-
293 function without affecting the proliferation rate of *Apc*-deficient intestinal progenitors.

294 We next tested whether such attenuation could be due to a restoration of cell cycle
295 exit by comparing the position of BrdU-positive cells after short and long periods of chase
296 at day 2 and 3 pi. After 48h of chase, while BrdU-positive cells had exited cell cycle and
297 were found in the villus in Control, all *Apc*^{ckO} BrdU-positive cells remained in the
298 proliferative compartment (Figure 3A and S3A), thereby contributing to its expansion. Of
299 note, BrdU staining was fainter in *Apc*^{ckO} than in Control, suggesting that *Apc*^{ckO}
300 progenitors underwent more cell divisions than Control cells. In *Apc*^{ckO}; *Nle*^{ckO} intestine,
301 BrdU-positive cells reached the villus after 48h chase at day 3pi. However, at day 2pi,
302 BrdU-positive cells were found in the crypt and at the boundary between the crypt and
303 the villus (Figure S3A-B) suggesting that though restored, cell-cycle exit is delayed in
304 *Apc*^{ckO}; *Nle*^{ckO}. *Nle*-deficiency induced a potent apoptotic response in normal intestinal
305 progenitors²². Caspase 3 staining on intestinal sections showed a similar proportion of
306 Caspase-3-positive cells in both *Apc*^{ckO} and *Apc*^{ckO}; *Nle*^{ckO} proliferative compartments
307 (Figure 3B, E), indicating that apoptosis is unlikely to be an important driver for the
308 reduction of the proliferative compartment in the *Apc*^{ckO}; *Nle*^{ckO}. Finally, to evaluate the
309 differentiation status of *Apc*^{ckO} and *Apc*^{ckO}; *Nle*^{ckO} epithelia, we performed Mucin-2 and
310 Chromogranin-A immunohistochemistry (Figure 3C-D) and Alcian Blue coloration
311 (Figure S3C). Enteroendocrine and goblet cells were absent from the expanded
312 proliferative compartment of the *Apc*^{ckO} as previously reported^{10,11}. In contrast, both cell
313 types were found in *Apc*^{ckO}; *Nle*^{ckO} villi and crypts. Consistently, *Muc2* and *ChromoA* mRNA

314 levels in $Apc^{cKO}; Nle^{cKO}$ crypts were intermediate compared to Apc^{cKO} and Controls (Figure
315 3F). Collectively, our results indicate that *Nle* loss-of-function attenuates Wnt
316 hyperactivation-driven expansion of the proliferation compartment by partially restoring
317 the capacity of *Apc*-deficient progenitors to exit cell cycle and differentiate.

318

319 ***Nle* loss-of-function leads to ribosome biogenesis defects and p53 stabilization in**
320 **the *Apc*-deficient epithelium.**

321 The immediate consequences of *Apc* loss-of-function on ribosome biogenesis and
322 protein synthesis in intestinal epithelium have not been described so far. No difference in
323 the levels of nucleolar rRNA intermediates of the small (*its1*) and the large (*its2*)
324 ribosomal subunits was observed in Apc^{cKO} compared to Control crypts at day 2 pi (Figure
325 4A-B) indicating that rRNA synthesis was not affected in *Apc*-deficient progenitors.
326 Performing FISH with *its1* and *its2* probes, we previously showed increased ribosome
327 biogenesis in crypts compared to villi²². Consistent with the invasion of proliferative cells
328 into the villus region, FISH staining of Apc^{cKO} showed high pre-rRNAs levels in a region
329 corresponding to the expanded proliferative compartment (Figure 4C). These data
330 indicate that ribosome production in *Apc*-deficient proliferative cells is similar to that of
331 *Apc*-proficient intestinal progenitors.

332 *Nle* loss-of-function was shown to affect large ribosomal subunit biogenesis in
333 crypt cells²² leading to the specific increase in *its2*, but not *its1*, levels. A similar
334 phenotype was detected in $Apc^{cKO}; Nle^{cKO}$ crypts at day 2 pi (Figure 4B-C), however *its2*
335 accumulation was no longer restricted to crypt cells but largely extended to villi (Figure
336 4C). A possible explanation for this observation would be that $Apc^{cKO}; Nle^{cKO}$ progenitors,
337 unlike Nle^{cKO} (*Villin-CreERT2^{tg/0}; Nle^{flox/null}*) progenitors, survived and continued to
338 proliferate despite dysfunctional ribosome biogenesis, thereby giving rise to
339 differentiated cells with increased nucleolar ribosomal pre-particles.

340 We next asked whether these defects activated a p53 stress response, as in a
341 normal epithelium²². In Apc^{cKO} , p53 was transiently stabilized at day 2 pi in a few cells
342 located at the distalmost part of the expanded proliferative compartment (Figure 4D and
343 S4A) consistent with previous reports²⁹. In $Apc^{cKO}; Nle^{cKO}$, strong and persistent p53
344 nuclear staining was observed in most cells of the crypt and of the lower part of the villus
345 (Figure 4D and S4B), like *its2* accumulation (Figure 4C). Importantly, robust p53
346 stabilization was observed in $Apc^{cKO}; Nle^{cKO}$ progenitors in absence of cell cycle arrest

347 (Figures 2A and S2) or massive apoptotic response (Figure 3B) contrary to Nle^{cKO} crypt
348 cells²². Collectively, our data show that *Nle* loss-of-function leads to defective ribosome
349 biogenesis and p53 stabilization in *Apc*-deficient progenitor cells without triggering the
350 cell cycle arrest and apoptotic responses normally observed in *Apc*-proficient cells.

351

352 **The double mutant transcriptome is essentially the sum of single mutant**
353 **transcriptomes.**

354 We performed RNAseq on Control, Nle^{cKO} , Apc^{cKO} and $Apc^{cKO}; Nle^{cKO}$ crypts at day
355 1 and 2 pi (Figure 5; Supplementary Table S3). Principal component analysis clearly
356 distinguished samples according to their genotype (Figure 5A), with PC1 (58.79% of the
357 variance) and PC2 (12.77% of the variance) segregating the samples according to the *Apc*
358 or *Nle* genotype respectively. $Apc^{cKO}; Nle^{cKO}$ samples were undistinguishable from Apc^{cKO}
359 according to PC1 and from Nle^{cKO} according to PC2, indicating that $Apc^{cKO}; Nle^{cKO}$ is
360 essentially the linear combination of Nle^{cKO} and Apc^{cKO} . Using K-means clustering, we
361 found that the 15115 genes expressed in our dataset could be spread into 10 clusters with
362 distinct gene expression variation patterns (Figure 5B; Supplementary Table S4).
363 Strikingly, the vast majority of the genes had the same expression pattern in $Apc^{cKO}; Nle^{cKO}$
364 as in Apc^{cKO} , with only four clusters (6, 7, 9 and 10) comprising 3259 (21%) genes that
365 were differentially regulated in $Apc^{cKO}; Nle^{cKO}$ compared to Apc^{cKO} samples. Gene
366 expression pattern varied little according to the timing.

367 To distinguish additive from non-additive interactions between the two mutations,
368 we performed a model-based analysis where timings were considered separately (Figure
369 5C-D). Considering all samples at a given time point, a “*Nle*-related”, an “*Apc*-related” fold-
370 change and an interaction term describing the deviation of the double mutant from a
371 linear combination of the two mutants were calculated for each gene. Examining cases
372 where the effects of the double mutant were either an independent combination of both,
373 or exhibited a dependence, we distinguished 8 gene categories (Figure 5C; Supplementary
374 Table S5), four of which encompassed genes that were subject to non-additive
375 interactions (*ie* have a “D” term). Consistent with the principal component analysis, *Apc*
376 loss-of-function was largely dominant compared to *Nle* loss-of-function (91% vs 38% of
377 misregulated genes at day 2 pi) (Figure 5D). Besides genes that were either unaffected in
378 all genotypes ([-|-|-]) and those affected only by *Apc* or *Nle* loss of function ([A|-|-] and [-
379 |N|-]), a minority of genes was subject to either additive ([A|N|-], 15% of misregulated

380 genes at day 2 pi) or non-additive interactions ([-|-|D], [-|N|D], [A|-|D], and [A|N|D], 14%
381 of misregulated genes at day 2 pi) (Figure 5D). Gene and phenotype ontology enrichment
382 analysis (Figures 5E and S5) showed that *Apc* or *Nle* loss of function upregulated genes
383 involved in ribosome biogenesis in an additive manner (Figure 5E, [A|N|-]). Interestingly,
384 the sub-cluster of genes that are expressed at intermediate levels in *Apc*^{ckO}; *Nle*^{ckO} due to
385 the additive effect of *Apc*-related upregulation and *Nle*-related downregulation is
386 significantly enriched in genes involved in cell cycling and proliferation (Figure 5E,
387 [A|N|-]). Overall, hypothesizing that the observed phenotypic differences arises from
388 differential gene regulation at the transcriptional level, these results suggest that the
389 phenotype of the *Apc*^{ckO}; *Nle*^{ckO} essentially results from additive gene-by-gene effects of
390 *Apc* and *Nle* loss-of-function rather than signaling pathway transcriptional activation or
391 shutdown.

392 We next examined a few sets of genes whose differential expression might be
393 relevant to the *Apc*^{ckO}; *Nle*^{ckO} phenotype (Figure 5F). Upregulation of cell cycle inhibitor
394 genes in *Apc*^{ckO}; *Nle*^{ckO} progenitors compared to *Apc*^{ckO} might explain their enhanced
395 capacity to stop proliferating. However, *p21* was the only cell cycle inhibitor upregulated
396 in *Apc*^{ckO}; *Nle*^{ckO} to levels similar to *Nle*^{ckO}. This was likely due to p53 activation since *p21*
397 is a well-known p53 target. Other p53 targets were equally upregulated in *Apc*^{ckO}; *Nle*^{ckO}
398 and *Nle*^{ckO}, including those that were downregulated in the *Apc*^{ckO} (*Fas*, *Perp*). Thus, Wnt
399 hyperactivation does not dramatically impact *p53* transcriptional response. Conversely,
400 most Wnt targets were similarly upregulated in *Apc*^{ckO}; *Nle*^{ckO} and *Apc*^{ckO}, suggesting that
401 *Nle* loss-of-function only marginally modulates the Wnt hyperactivation transcriptional
402 response. Finally, intestinal stem cell marker expression in *Apc*^{ckO}; *Nle*^{ckO} resulted from
403 either additive (*Ascl2*, *Sox9*, *Tert*, *Pw1*, *Bmi1*) or non-additive (*Olfm4*, *Lgr5*, *Mex3a*, *Lrig1*,
404 *Krt15*, *Hopx*) effects. Strikingly, *Nle* loss-of-function attenuated Wnt hyperactivation-
405 driven *Lgr5* upregulation but also participated to the synergic upregulation of the reserve
406 stem cell markers *Krt15* and, to a lesser extend *Hopx* and *Bmi1*. This suggests that the
407 reserve intestinal stem cell population might be particularly stimulated in the
408 *Apc*^{ckO}; *Nle*^{ckO} intestine.

409

410 ***Nle* loss-of-function leads to a reduction of mature rRNAs and protein synthesis**
411 **activity in *Apc*-deficient crypts.**

412 We next asked whether alterations in ribosome content and protein synthesis
413 activity could contribute to the phenotype. We first quantified the levels of mature rRNAs
414 (18S and 28S) by RT-qPCR (Figure 4A). No difference in rRNA levels was observed in
415 Apc^{cKO} crypts compared to Control at day 2 pi (Figure 6A) indicating that, like pre-rRNA,
416 ribosome content was not affected in *Apc*-deficient progenitors. We also monitored
417 protein synthesis by puromycin incorporation *in vivo*²². Surprisingly, quantification of
418 puromycylated nascent chains after a short *in vivo* pulse of puromycin revealed an
419 increased number of active ribosomes in *Apc*-deficient progenitors compared to the
420 control at day 2 and day 3 pi (Figure 6B-C, S6). Taken together with the aforementioned
421 analyses of pre-rRNA levels, these data indicate that upregulation of protein synthesis,
422 but not ribosome biogenesis, is an early feature of *Apc*-deficient progenitors.

423 Interestingly, contrary to the Nle^{cKO} , mature rRNA levels were significantly
424 decreased in $Apc^{cKO}; Nle^{cKO}$ crypts (Figure 6A). It can be hypothesized that progenitor
425 survival and proliferation despite ribosome biogenesis defects in the $Apc^{cKO}; Nle^{cKO}$ gives
426 rise to cells with decreased ribosome content. Strikingly, the number of active ribosomes
427 in $Apc^{cKO}; Nle^{cKO}$ crypt cells reduced to normal level compared to Apc^{cKO} (Figure 6B-C, S6).
428 Altogether, these data suggest that, in the context of *Apc*-deficient intestinal epithelium,
429 *Nle* loss-of-function leads to a reduction of ribosome content consecutive to ribosome
430 biogenesis defects, which, in turn, decreases translational activity of *Apc*-deficient
431 progenitors to control-like levels. Such impairment of translation stimulation might
432 contribute to induce cell cycle exit in $Apc^{cKO}; Nle^{cKO}$ progenitors.

433

434 **Wnt hyperactivation prevents p53-induced rapid depletion of intestinal stem and** 435 **progenitor cells in *Nle*-deficient crypts.**

436 We then examined the fate of intestinal stem cells in $Apc^{cKO}; Nle^{cKO}$ mice. RT-qPCR
437 confirmed the transcriptomic data as *Lgr5* was upregulated in the Apc^{cKO} and restored to
438 control levels in the $Apc^{cKO}; Nle^{cKO}$ at day 2 pi, whereas *Olfm4* was downregulated in both
439 Nle^{cKO} and $Apc^{cKO}; Nle^{cKO}$ compared to Control (Figure 7A). *In situ* hybridization indicated
440 that, as expected, *Olfm4* expression confined to cells at the crypt bottom in Control,
441 extended upward in Apc^{cKO} and was markedly decreased in Nle^{cKO} (Figure 7B). In
442 $Apc^{cKO}; Nle^{cKO}$, *Olfm4* staining was similar to Apc^{cKO} but varied from crypt to crypt and was
443 generally fainter than both Control and Apc^{cKO} . Interestingly, nuclear p53 protein was
444 detected in crypt base columnar (CBC) cells, recognizable by their shape and location at

445 the crypt base, at every tested time point (Figure 7C) suggesting that, like progenitors,
446 *Apc*-deficient stem cells were less sensitive to ribosome biogenesis defects and p53
447 activation than *Apc*-proficient stem cells. Indeed, contrary to *Nle*^{ckO}, proliferating CBCs
448 were present at the bottom of *Apc*^{ckO}; *Nle*^{ckO} crypts (Figure 7D). Collectively, these data
449 show that intestinal stem and progenitor cells are maintained in *Apc*^{ckO}; *Nle*^{ckO} mice
450 despite defective ribosome biogenesis and robust p53 activation.

451 To confirm this point, we derived organoids from control, *Nle*^{ckO}, *Apc*^{ckO} and
452 *Apc*^{ckO}; *Nle*^{ckO} crypts harvested at day 1 pi (Figure 8A-B). Contrary to *Nle*^{ckO} crypts,
453 *Apc*^{ckO}; *Nle*^{ckO} crypts grew into organoids at the same rate as Control (Figure 8A). Of note,
454 *Apc*^{ckO}; *Nle*^{ckO} organoids grew as spheroids like *Apc*^{ckO} (Figure 8B). Over time,
455 *Apc*^{ckO}; *Nle*^{ckO} organoids were progressively populated by *Nle*-proficient cells that had
456 escaped recombination at the *Nle* locus. This, together with the fact that continuous Cre
457 induction is detrimental to organoid growth⁴⁶, prevented us to assess the behavior of
458 *Apc*^{ckO}; *Nle*^{ckO} organoids at later time points. In order to highlight possible increased
459 resistance to p53 activation of *Apc*^{ckO} stem and progenitor cells compared to Control, we
460 cultured organoids with increasing doses of nutlin-3, a small compound that activates p53
461 through inhibition of Mdm2. *Apc*^{ckO} organoids were more resistant to nutlin-3 treatment
462 than Control, as viability of Control was strongly reduced at 5μM nutlin-3 while that of
463 *APC*^{ckO} organoids was almost unaffected (Figure 8D) despite robust p53 stabilization
464 (Figure S7B). Collectively, these results show that *Apc*^{ckO}; *Nle*^{ckO} intestinal stem and
465 progenitor cells display enhanced survival to p53 activation.

466

467 **DISCUSSION**

468

469 Colorectal cancers, like many cancers, are characterized by increased ribosome
470 biogenesis and protein translation^{30,31}. High expression of pre-rRNA is associated with
471 poor prognosis in colorectal cancer³² and increased translational elongation is key for
472 intestinal tumorigenesis³³. Interestingly, oxaliplatin, a drug commonly used to treat
473 colorectal cancers, was shown to affect ribosome biogenesis and trigger p53 induction
474 and, consistent with this, cancer cells sensitive to oxaliplatin display higher ribosome
475 biogenesis and mRNA translation rates³⁴. Here, we showed that *in vivo* gut epithelium-
476 specific inhibition of ribosome biogenesis strongly attenuates the expansion of the
477 proliferative compartment during tumor initiation.

478 Inactivation of some key target genes of the Wnt/ β -catenin pathway such as *c-*
479 *Myc*¹², *cyclinD2*³⁵ and *Fak1*³⁶ or of the chromatin remodeling factor *Brg1*³⁷ were also
480 shown to dampen the expansion of the proliferative compartment in *Apc*^{CKO} mice. In all
481 these situations, dampening was accompanied by a marked alteration of the Wnt
482 hyperactivation transcriptional signature. In contrast, *Nle* loss-of-function only
483 marginally modifies the transcriptomic response to *Apc* loss-of-function. The partial
484 rescue of *Apc*^{CKO}; *Nle*^{CKO} mice phenotype is thus rather due to the compensation between
485 the antagonistic effects of Wnt-driven tumorigenesis and ribosome biogenesis inhibition.

486 In *Apc*^{CKO}; *Nle*^{CKO} mice, attenuation likely results from the activation of the
487 RP/MDM2/p53 ribosome biogenesis surveillance pathway. Upon *Apc* inactivation, p53
488 stabilization is observed only locally and transiently in a small population of cells and the
489 intestinal phenotype of *Apc* loss-of-function is not modified in absence of p53 (this study
490 and ²⁹). In contrast, high levels of p53 are detected in *Apc*^{CKO}; *Nle*^{CKO} crypt and villus cells
491 showing dysfunctional ribosome biogenesis, recognizable by their nucleolar
492 accumulation of precursor rRNAs of the large ribosomal subunit. Contrary to *Apc*-
493 deficient progenitors, *Apc*^{CKO}; *Nle*^{CKO} progenitors are able to exit cell cycle and
494 differentiate. Upregulation by p53 of *p21* could be contributing to the restoration of cell
495 cycle arrest and attenuation of the expansion of the proliferative compartment. Another
496 explanation for this attenuation is that *Apc*^{CKO}; *Nle*^{CKO} progenitors are unable to upregulate
497 protein synthesis at a level sufficient for tumorigenesis. We show here that increased
498 protein translation is an immediate hallmark of *Apc* loss-of-function that is no longer
499 observed in *Apc*^{CKO}; *Nle*^{CKO} progenitors. Enhanced translation likely increases the

500 production of important rate-limiting cell cycle regulators and could influence the
501 capacity of cells to divide or not. Similar to *Nle* loss-of-function, genetic or pharmacologic
502 inhibition of mTORC1-dependent translational stimulation limits the expansion of the
503 proliferative compartment during tumor initiation³³. The reduction of the translational
504 potential of *Apc*^{cKO}; *Nle*^{cKO} progenitors may therefore contribute to their eventual cell
505 cycle exit and differentiation.

506 *Apc*-deficiency dramatically modifies the cellular responses of intestinal cells to
507 defective ribosome biogenesis. Indeed, while *Apc*^{cKO}; *Nle*^{cKO} stem cells and progenitors
508 express high levels of nuclear p53 and display a clear p53 transcriptional signature, they
509 do not elicit the strong proliferation arrest and apoptotic responses normally observed in
510 crypt cells²². Crosstalk between the Wnt/ β -catenin pathway and p53 have been
511 previously reported and involve the direct or indirect modulation of p53 stability by
512 GSK3^{38,39}. However, the regulation of p53 activity by GSK3 leads to p53 degradation,
513 suggesting that this is not the mechanism through which *Apc* loss-of-function attenuates
514 p53 activation in *Apc*^{cKO}; *Nle*^{cKO} crypt cells. Rather, the fate of stem and progenitor cells
515 with an activated RP/MDM2/p53 ribosome biogenesis surveillance pathway may be
516 modified by the transcriptional response to Wnt/ β -catenin hyperactivation, such as for
517 example increased expression of *Ppan*, which has been previously shown to elicit anti-
518 apoptotic activities in intestinal stem cells and progenitors⁴⁰. On a different note, it has
519 been shown that the Wnt pathway modulates protein stoichiometry of polysomal
520 ribosomes during mouse neocortex development⁴¹, which, in turns could potentially
521 result in significant modulation of the proteome⁴². If it holds true in the intestine, such
522 qualitative changes in translation may also contribute to the different behaviors of
523 intestinal cells to p53 activation.

524 It is unclear why intestinal progenitors eventually exit cell cycle in *Apc*^{cKO}; *Nle*^{cKO}
525 mice. Disequilibrium between the antagonistic actions of Wnt hyperactivation and
526 defective ribosome biogenesis may trigger proliferation arrest. The extended
527 proliferative compartment of the *Apc* mutant is in many respects heterogeneous, in
528 particular regarding p53 stabilization (this study and ²⁹) and p21 expression⁴³, which
529 concern a small population of cells located at the leading edge of the proliferative
530 compartment. It is therefore possible that while leaving the crypt domain, *Apc*^{cKO}; *Nle*^{cKO}
531 progenitors experience a suprainduction of p53 and p21, which, together with increased
532 concentration of pro-differentiation cues such as BMPs, trigger cell cycle exit. At the same

533 time, *Apc*^{CKO}; *Nle*^{CKO} progenitors located in the crypt domain are able to proliferate despite
534 defective ribosome biogenesis, giving rise to cells with reduced numbers of ribosomes.
535 Such decrease may also be interpreted as a signal for proliferation arrest, as suggested by
536 the impaired proliferation of primary human cell lines with a defective RP/MDM2/p53
537 ribosome biogenesis surveillance pathway⁴⁴.

538 Our work also unravels major modifications of the *Nle* loss-of-function phenotype
539 by *Apc*-deficiency. Importantly, our demonstration that *Apc*-deficient stem cells and
540 progenitors are resistant to defective ribosome biogenesis challenges the notion that
541 cancer cells, and in particular c-MYC-driven cancer cells, are more sensitive to ribosome
542 biogenesis inhibition than normal cells⁶⁻⁸. Moreover, enhanced survival of stem cells to
543 ribosome biogenesis stress under Wnt hyperactivation conditions may be a major
544 limitation for therapeutic strategies targeting ribosome production, not only for
545 colorectal cancers, but also for other cancers associated with deregulation of the Wnt/ β -
546 catenin pathway such as cutaneous melanoma, breast carcinoma, hepatocarcinoma or
547 pancreatic ductal adenocarcinoma.

548

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560

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562

563

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- 701

702 **FIGURE LEGENDS**

703

704 **Figure 1 *Nle* loss-of-function improves survival of *Apc*-deficient mice and limits**
705 **crypt hyperplasia.** (A) Scheme of tamoxifen injection and analysis. (B) Detection of the
706 nonrecombined (flox) and the recombined (del) *Apc* (top) and *Nle* (bottom) alleles by PCR
707 performed on DNA extracts from Control, *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} small intestinal
708 epithelium. Two bands of similar intensity are amplified from *Apc*^{flox/del} (top) and *Nle*^{flox/del}
709 (bottom) DNA (T). The wt allele appears on the gel for *Apc* but not for *Nle*. (C) Weight
710 curve of Control, *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} mice following tamoxifen injection. *, p<0.05
711 ***, p<0.001 according to Student's t-test. (D) Kaplan-Meier survival curve of Control,
712 *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} mice following tamoxifen injection. The difference between
713 *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} is significant with p<0.05 according to Mann-Whitney Wilcoxon
714 test. (E) Hematoxylin–eosin staining of intestinal epithelium sections from Control, *Apc*^{CKO}
715 and *Apc*^{CKO}; *Nle*^{CKO} small intestines at day 2 pi. Black bars indicate the crypt compartment
716 (enlarged in the *Apc*^{CKO}). Scale bars, 50 μm.

717

718 **Figure 2 *Nle* loss-of-function limits the expansion of the proliferative compartment**
719 **expansion despite Wnt pathway hyperactivation.** (A) β-catenin immunostaining
720 (brown) counterstained with hematoxylin (blue) on intestinal epithelium sections from
721 Control, *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} intestines at day 2 pi. The second lane shows magnified
722 views of framed regions. Scale bars, 50 μm. (B) RT-qPCR performed on total RNA from
723 Control, *Nle*^{CKO}, *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} intestinal epithelium extracts at day 2 pi. Graphs
724 represent the mean fold changes ± S.E.M. for Wnt transcriptional target genes *c-Myc* and
725 *Axin2*. n ≥ 3 for each genotype. *, p<0.05 ***, p<0.01 ***, p<0.001 according to Student's t-
726 test. (C) BrdU immunostaining (brown) counterstained with hematoxylin (blue) on
727 intestinal epithelium sections from Control, *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} intestines at day 2
728 pi harvested 2 hours after BrdU injection. Black bars indicate the extension of the
729 proliferative compartment. Scale bars, 50 μm. (D) Histogram showing the mean
730 proportion (± S.E.M.) of BrdU-positive cells in the proliferative compartment in Control,
731 *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} small intestines. Twenty-five transverse crypt sections were
732 scored per mouse, n = 4 for each genotype. The means were not significantly different
733 among genotypes according to Mann-Whitney Wilcoxon test.

734

735 **Figure 3 *Nle* loss-of-function restores cell cycle exit and differentiation in the *Apc*-**
736 **deficient epithelium.** (A) BrdU immunostaining (brown) counterstained with
737 Hematoxylin (blue) on intestinal epithelium sections from Control, *Apc*^{ckO} and
738 *Apc*^{ckO}; *Nle*^{ckO} intestines at day 3 pi harvested 2 hours or 48 hours after BrdU injection.
739 Plain black bars indicate the proliferative compartment. Dotted black bars indicate the
740 range of proliferative cell migration within 48 hours. Scale bars, 50 μ m. (B) Cleaved-
741 Caspase 3 immunostaining (brown) counterstained with Hematoxylin (blue) on intestinal
742 epithelium sections from Control, *Apc*^{ckO} and *Apc*^{ckO}; *Nle*^{ckO} intestines at day 2 pi.
743 Arrowheads indicate Caspase 3-positive cells. Scale bars, 50 μ m. (C) MUC2
744 immunostaining (brown) counterstained with Hematoxylin (blue) on intestinal
745 epithelium sections from Control, *Apc*^{ckO} and *Apc*^{ckO}; *Nle*^{ckO} intestines at day 2 pi. Scale
746 bars, 50 μ m. (D) Chromogranin A immunostaining (brown) counterstained with
747 Hematoxylin (blue) on intestinal epithelium sections from Control, *Apc*^{ckO} and
748 *Apc*^{ckO}; *Nle*^{ckO} intestines at day 2 pi. Scale bars, 50 μ m. (E) Histogram showing the mean
749 number (+/-SEM) of Caspase 3 positive cells per crypt in Control, *Apc*^{ckO} and
750 *Apc*^{ckO}; *Nle*^{ckO} intestines at day 2 pi. 30 transverse crypts were scored per mouse, $n \geq 3$ for
751 each genotype. *, $P < 0.05$ Mann-Whitney Wilcoxon test. (F) RT-qPCR performed on total
752 RNA from Control, *Apc*^{ckO} and *Apc*^{ckO}; *Nle*^{ckO} intestinal crypt extracts at day 2 pi. Graphs
753 represent the mean fold changes \pm S.E.M. for differentiation markers *Mucin 2* (*Muc2*) and
754 *Chromogranin A* (*ChromoA*). $n \geq 3$ for each genotype. *, $p < 0.05$ **, $p < 0.01$ according to
755 Mann-Whitney Wilcoxon test.

756

757 **Figure 4 *Nle* loss-of-function leads to ribosome biogenesis defects and p53**
758 **stabilization in the *Apc*-deficient epithelium.** (A) Simplified diagram illustrating the
759 main steps of ribosome biogenesis in eukaryotic cells. Blue arrows represent the primers
760 used to measure the levels of ribosomal RNAs by RT-qPCR. FISH probes used to detect
761 *its1* (red) and *its2* (green) sequences from precursors of the small and large ribosomal
762 subunits, respectively, are indicated. (B) RT-qPCR performed on total RNA from Control,
763 *Apc*^{ckO} and *Apc*^{ckO}; *Nle*^{ckO} intestinal crypt extracts at day 2 pi. Graphs represent the mean
764 fold changes \pm S.E.M. for the different amplicons. $n = 4$ for each genotype. *, $p < 0.05$ **,
765 $p < 0.01$ ***, $p < 0.001$ according to Student's t-test. (C) FISH for *its1* (red) or *its2* (yellow)
766 counterstained with Hoechst (blue) on intestinal epithelium sections from Control, *Apc*^{ckO}
767 and *Apc*^{ckO}; *Nle*^{ckO} intestines. Scale bars, 50 μ m. (D) p53 immunostaining (brown)

768 counterstained with Hematoxylin (blue) on intestinal epithelium sections from Control,
769 Apc^{cKO} and $Apc^{cKO}; Nle^{cKO}$ intestines at day 2 pi. Lateral bars indicate the position of the
770 crypt domain for Control and $Apc^{cKO}; Nle^{cKO}$ and of the enlarged crypt-like compartment
771 for Apc^{cKO} . Scale bars, 50 μ m.

772

773 **Figure 5 The double mutant transcriptome is the sum of simple mutant**
774 **transcriptomes.** (A) Principal Component Analysis (PCA) of the samples. PC2 is plotted
775 against PC1. Apc^{cKO} (A) and $Apc^{cKO}; Nle^{cKO}$ (AN) are distinguishable from Control (C) and
776 Nle^{cKO} (N) according to PC1, whereas N and AN are distinguishable from C and A according
777 to PC1, meaning that PC1 corresponds to *Apc* loss-of-function and PC2 corresponds to *Nle*
778 loss-of-function. (B) K-means clustering on all genes normalized by their maximum
779 expression among the samples. (C) List and description of the 8 theoretical categories of
780 genes according to the model-based analysis. -: no change; N: change in all Nle^{cKO} samples
781 including double mutant ones; A: change in all Apc^{cKO} samples including double mutant
782 ones; and D: change in double mutant $Apc^{cKO}; Nle^{cKO}$ different from that predicted by the
783 additive combination of N and A. (D) Histogram showing the distribution of genes among
784 the 8 categories at day 1 pi and day 2 pi. The number of genes assigned to each category
785 is indicated at both timings. (E) Genes in the [A|N|-] and [A|N|D] categories were
786 separated into clusters by the direction of the A, N and D fold changes at day 2 pi. Gene
787 number is plotted on the left of each heatmap. For each cluster, genes relevant to the
788 analyzed phenotypes are indicated, as well as the most significantly enriched gene (black)
789 and phenotype (blue) ontology terms from MouseMine. (F) Histograms showing the
790 expression variation at day 2 pi of some cell cycle inhibitor-coding mRNAs (blue), p53
791 transcriptional targets (red), Wnt transcriptional targets (purple) and transcripts
792 enriched in ISCs (red). ns, fold change not significant.

793

794 **Figure 6 *Nle* loss-of-function causes a diminution of mature rRNA levels and global**
795 **protein synthesis in the *Apc*-deficient epithelium.** (A) RT-qPCR for 18S and 28S rRNA
796 performed on total RNA from Control, Apc^{cKO} and $Apc^{cKO}; Nle^{cKO}$ intestinal crypt extracts
797 at day 2 pi. Graphs represent the mean fold changes \pm S.E.M. for the different amplicons.
798 $n = 4$ for each genotype. *, $p < 0.05$ **, $p < 0.01$ according to Student's t-test. (B) Anti-
799 puromycin immunoblotting of protein extracts for identical number of crypts cells from
800 Control, Apc^{cKO} and $Apc^{cKO}; Nle^{cKO}$ intestines at day 2 pi. (C) Histogram showing the mean

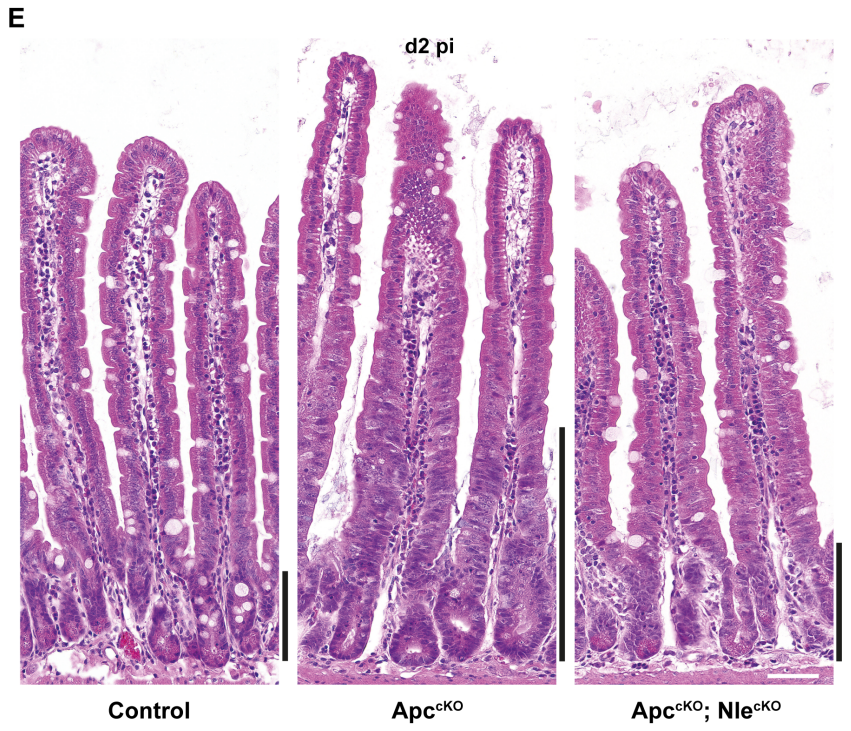
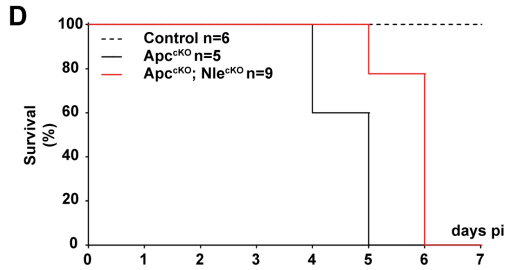
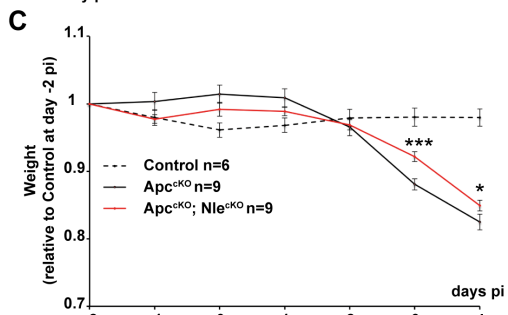
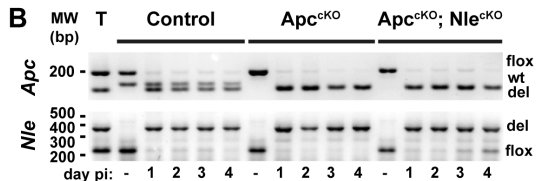
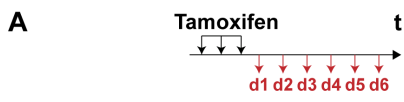
801 normalized signal intensity \pm S.E.M from the immunoblot shown in (B). n = 4 for each
802 genotype. *, p<0.05 according to Mann-Whitney Wilcoxon test.

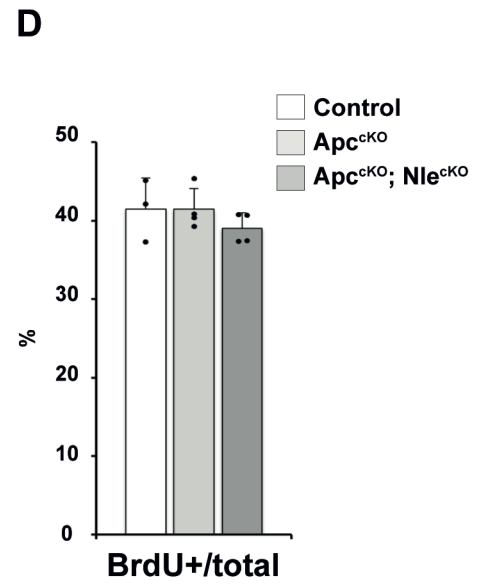
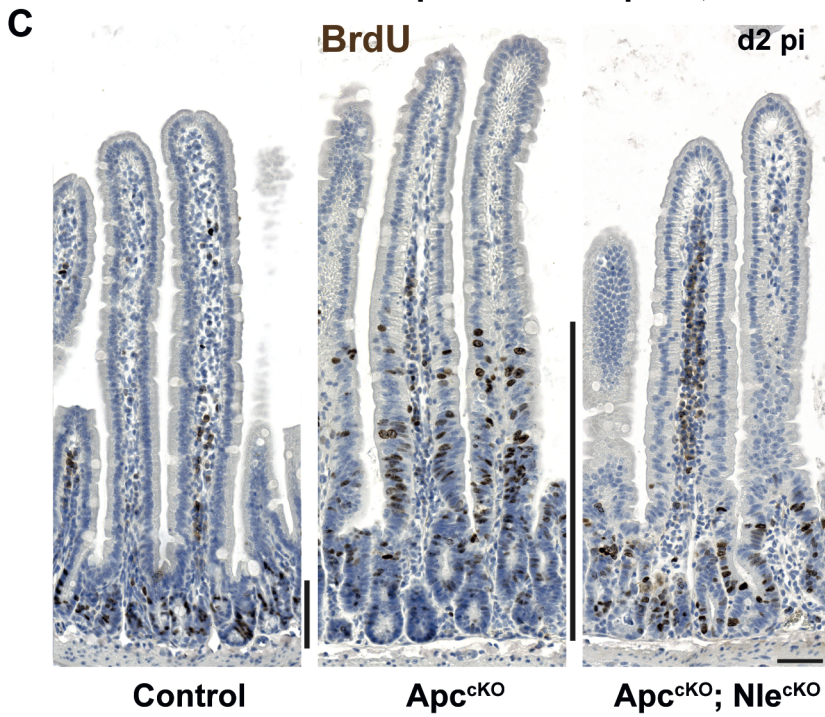
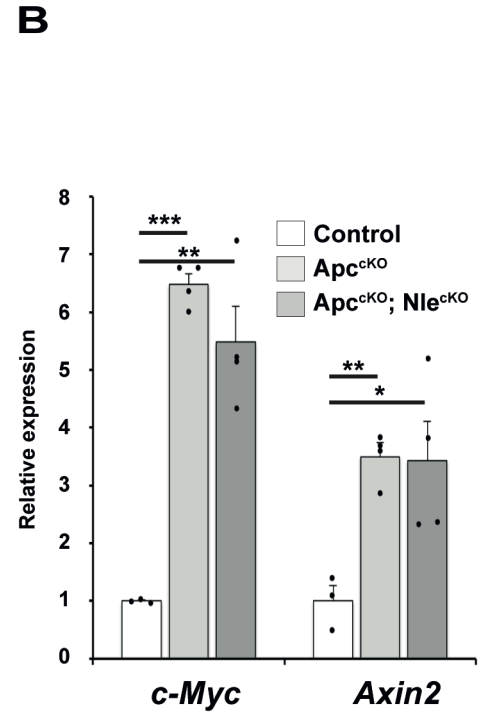
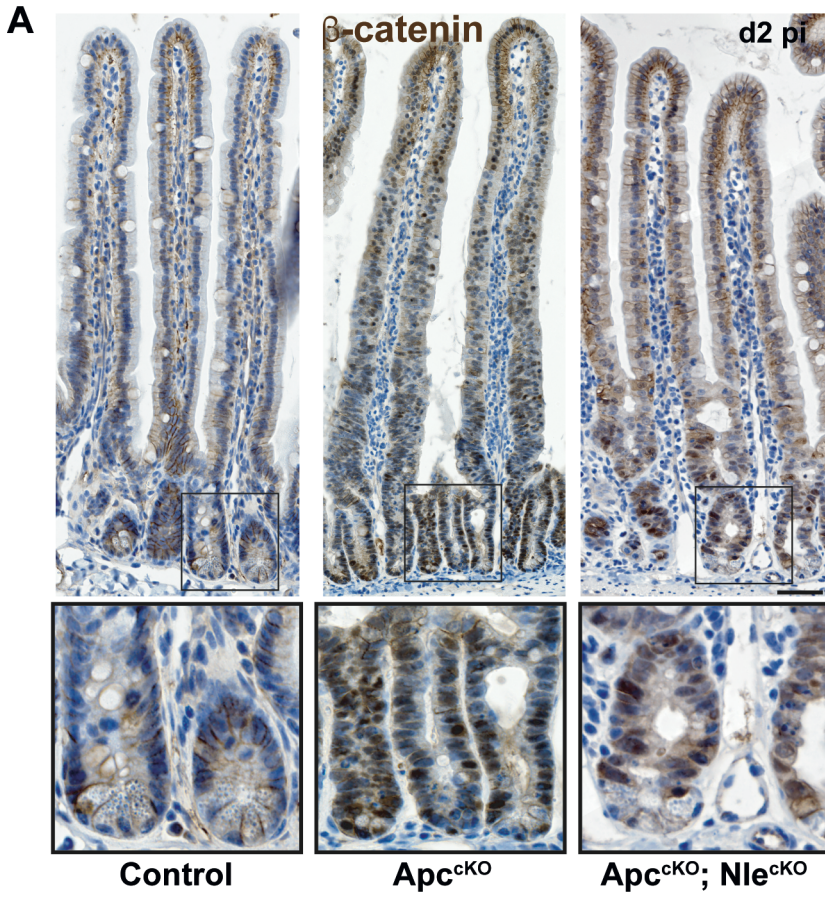
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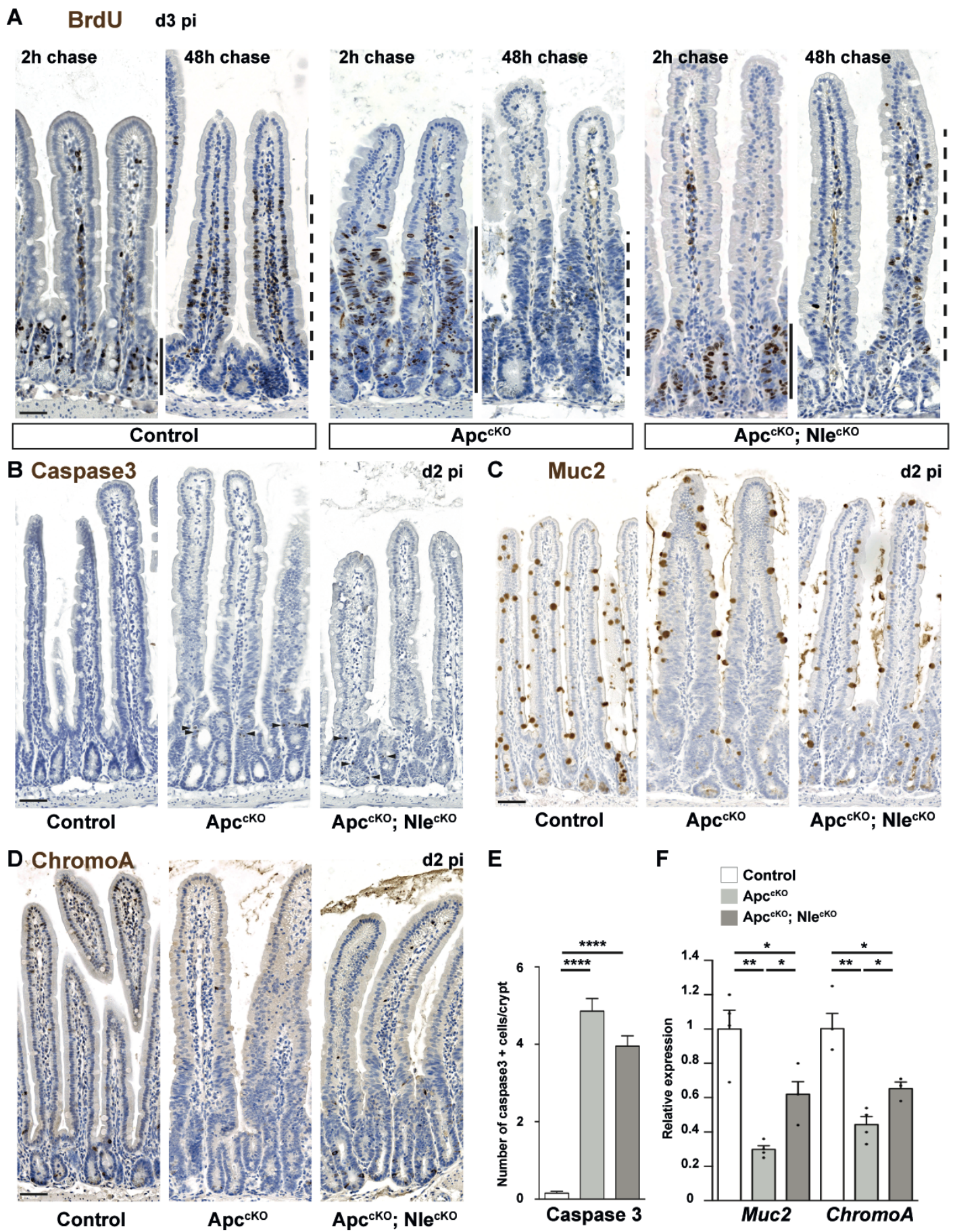
804 **Figure 7 *Apc* loss-of-function attenuates stem cell loss following *Nle* deletion.** (A) RT-
805 qPCR performed on total RNA from Control, *Nle*^{CKO}, *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} intestinal
806 crypt extracts at day 2 pi. Graphs represent the mean fold changes \pm S.E.M. for ISC markers
807 *Lgr5* and *Olfm4*. n = 4 for each genotype. *, p<0.05 **, p<0.01 ***, p<0.001 according to
808 Student's t-test. (B) In situ hybridization for *Olfm4* mRNA (brown) counterstained with
809 Hematoxylin (blue) on intestinal epithelium sections from Control, *Nle*^{CKO}, *Apc*^{CKO} and
810 *Apc*^{CKO}; *Nle*^{CKO} intestines. The second lane shows magnified views of framed regions. Scale
811 bars, 50 μ m. (C) p53 immunostaining (brown) counterstained with Hematoxylin (blue)
812 on crypt sections from *Apc*^{CKO}; *Nle*^{CKO} intestines at days 2, 3 and 4 pi. Asterisks indicate
813 p53-positive CBCs. Scale bars, 25 μ m. (D) BrdU immunostaining (brown) counterstained
814 with Hematoxylin (blue) on crypt sections from Control, *Nle*^{CKO}, *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO}
815 intestines at day 2 pi harvested 2 hours after BrdU injection. Asterisks indicate BrdU-
816 positive CBCs. Scale bars, 25 μ m.

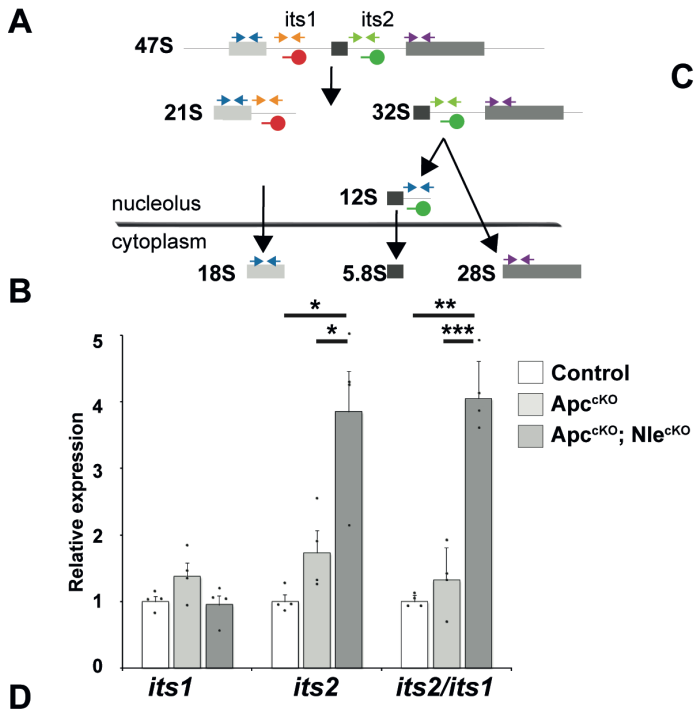
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818 **Figure 8 *Apc*^{CKO} intestinal organoids show increased tolerance to *Nle*-deficiency and**
819 **p53 stabilization.** (A) Histogram showing the mean percentage (\pm S.E.M.) of organoid
820 formation and survival at day 4 pi from an initial culture of 400 crypts per well from
821 Control, *Nle*^{CKO}, *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} intestines harvested at day 1 pi. n \geq 3
822 experiments for each genotype. (B) Pictures of organoids obtained from Control, *Nle*^{CKO},
823 *Apc*^{CKO} and *Apc*^{CKO}; *Nle*^{CKO} crypts. The top lane shows a global view of a well for each
824 genotype at day 4 pi. The middle lane (resp. bottom lane) shows the morphology of
825 organoids of the different genotypes at day 4 (resp. 6) pi. (C) Histogram showing the mean
826 percentage (\pm S.E.M.) of healthy organoids after 48h culture with various concentration
827 of nutlin-3. (D) Pictures of Control and *Apc*^{CKO} organoids after 48h in mock and 10 μ M
828 nutlin-3 culture conditions. Under nutlin-3 treatment, many Control organoids displayed
829 abnormal morphology (star) with absence of budding, swelling and increased opacity
830 likely resulting from increased accumulation of dead cells in the lumen while some *Apc*^{CKO}
831 spheroids were of irregular shape, opaque and collapsed (arrowhead). Such unhealthy
832 structures were usually degenerating in the following days of culture. *, p<0.05 **, p<0.01
833 according to Mann-Whitney Wilcoxon test. Scale bars, 150 μ m.

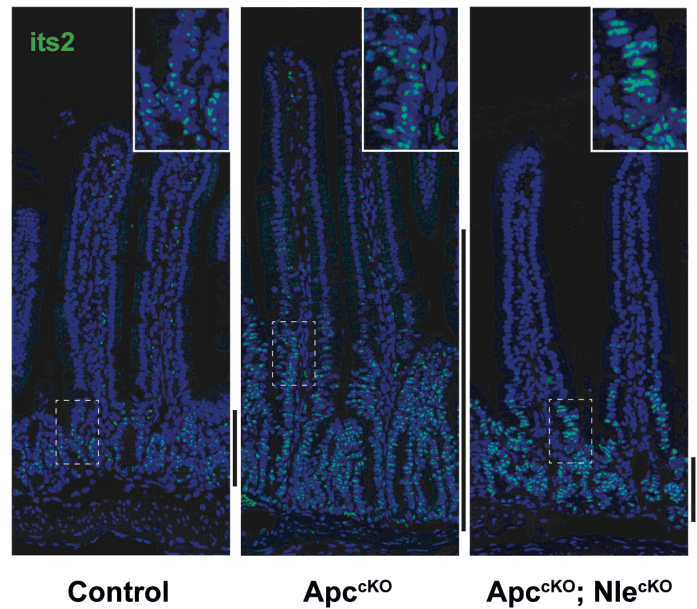
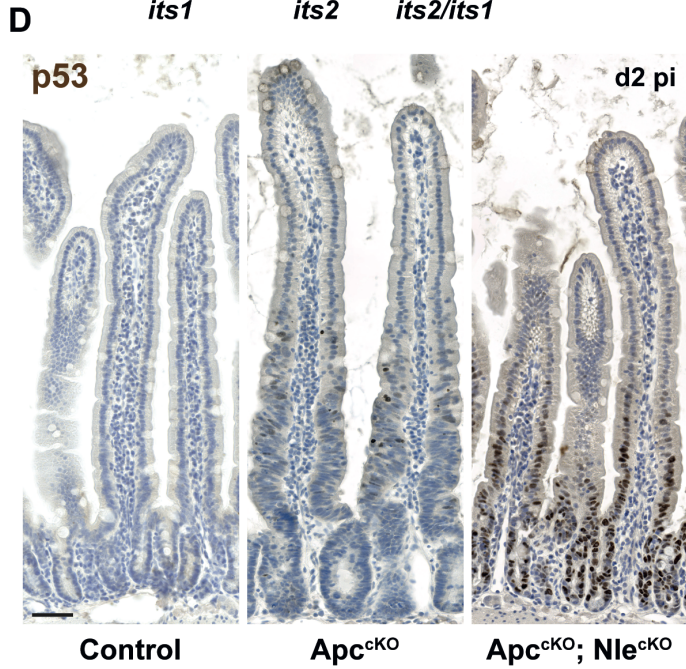
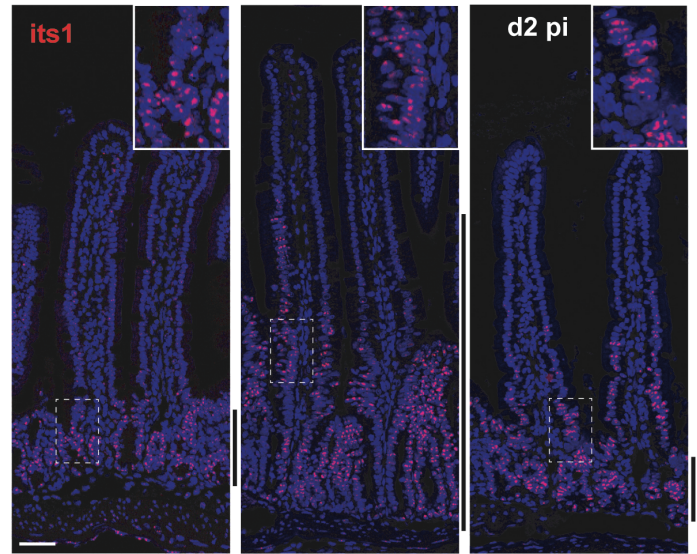


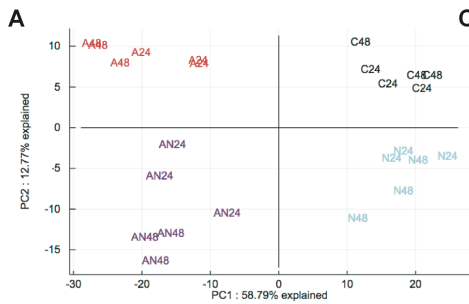






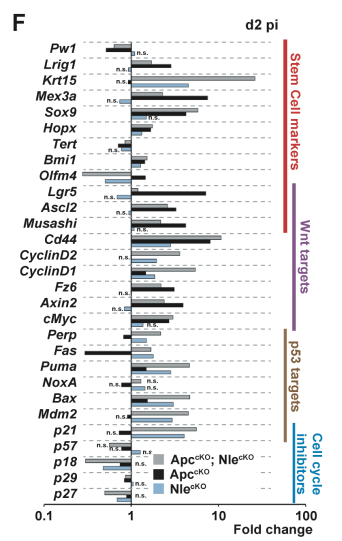
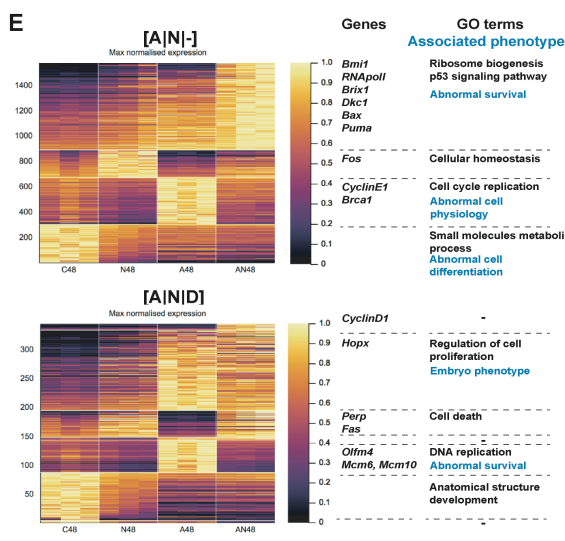
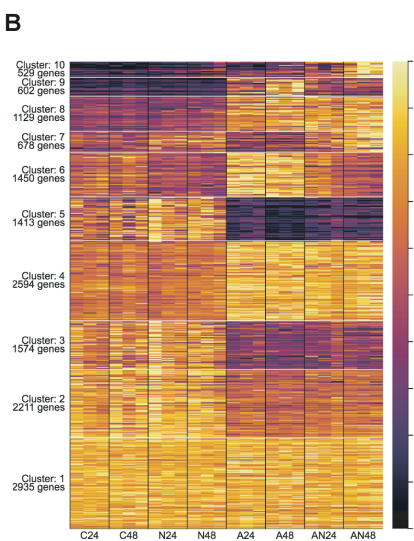
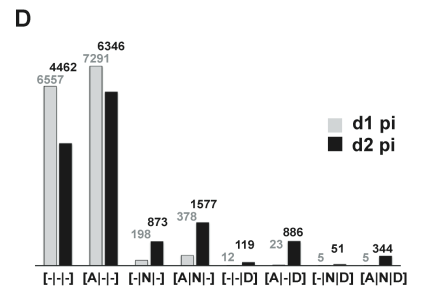
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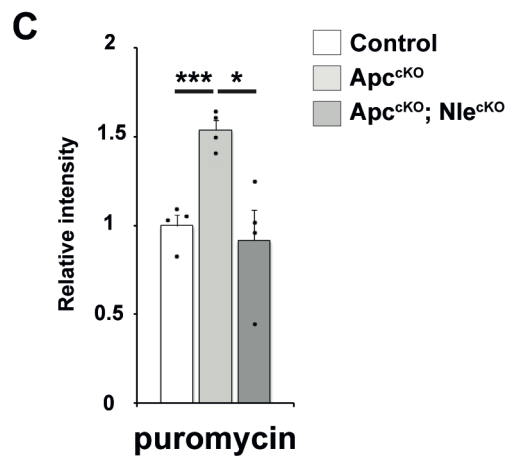
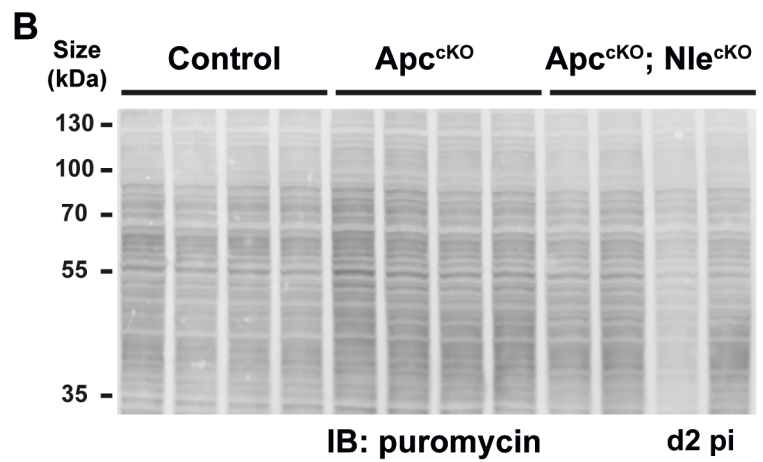
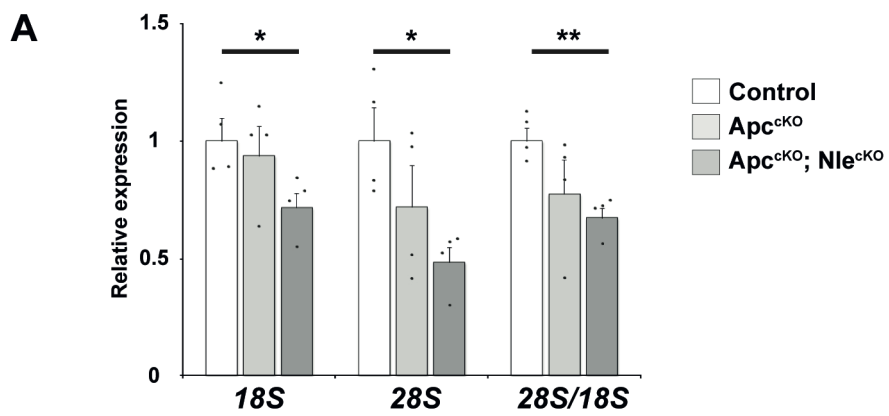


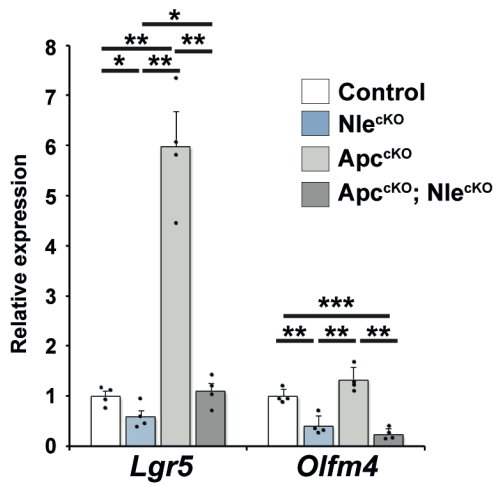
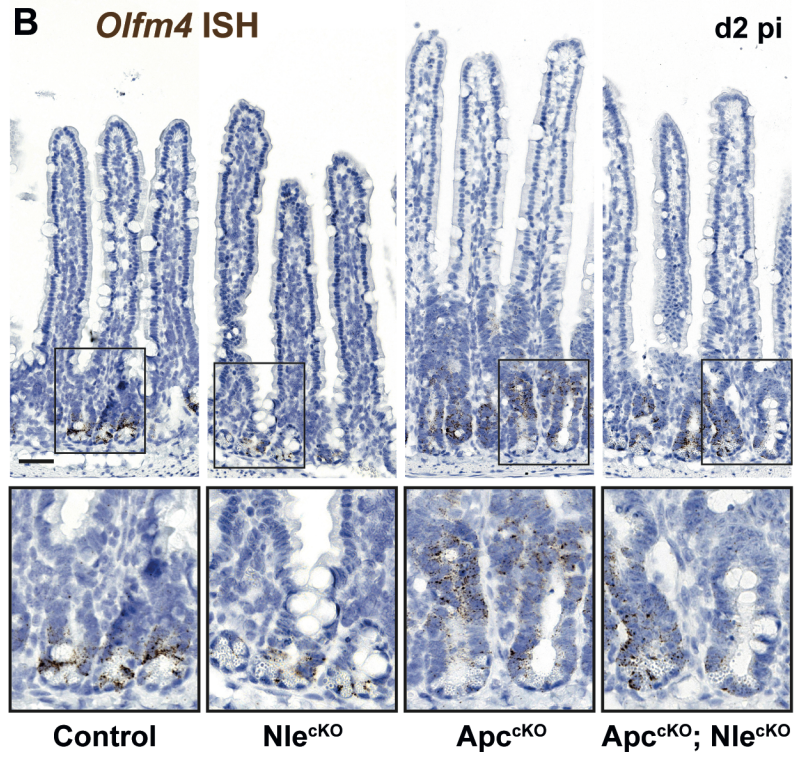
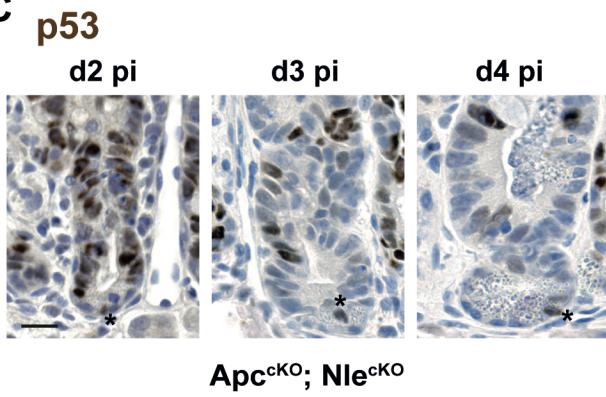
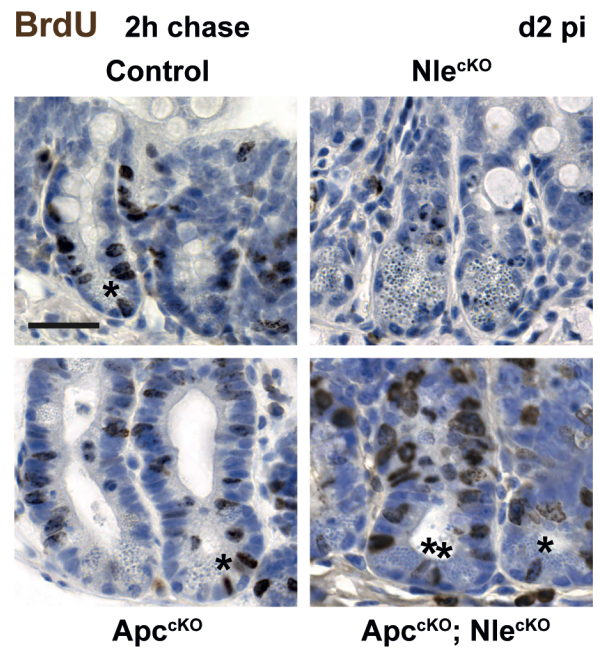


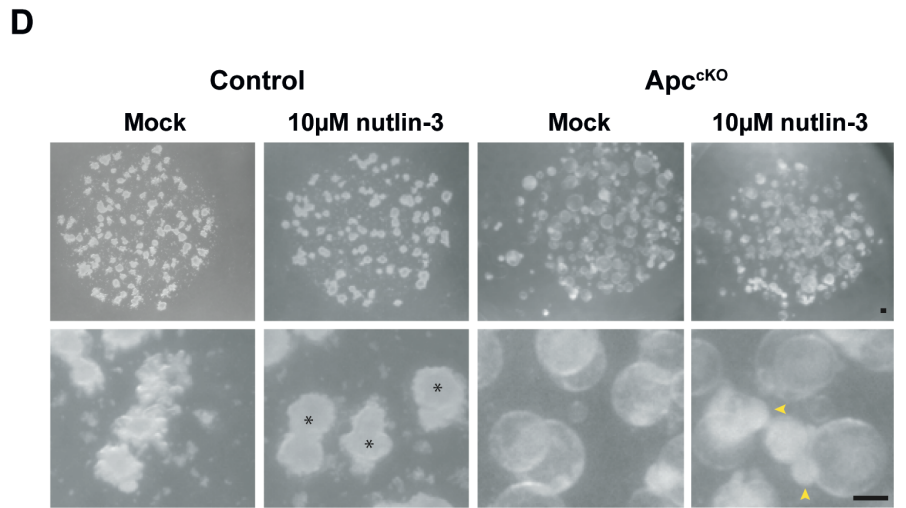
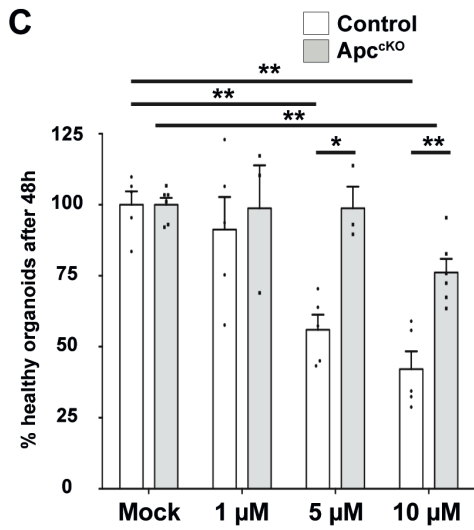
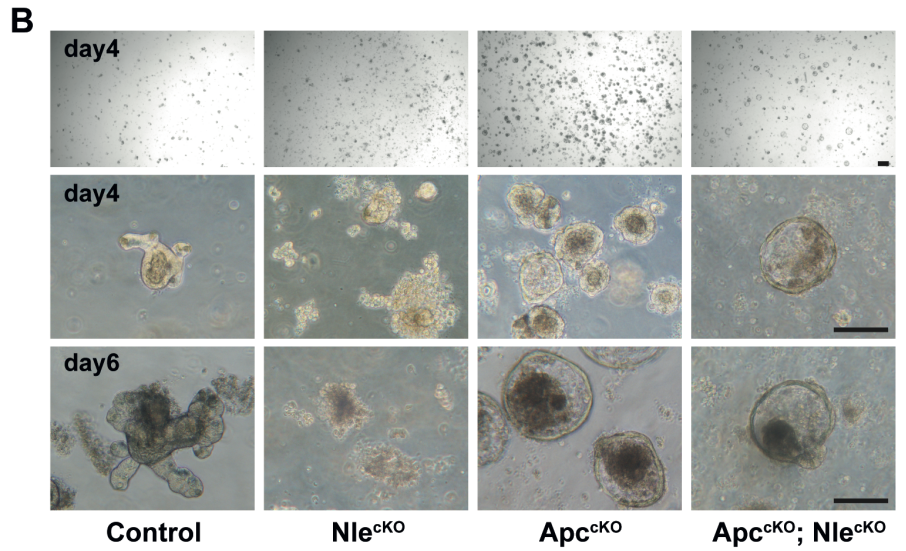
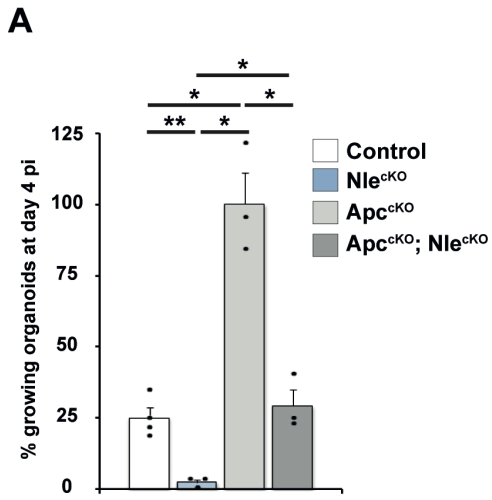
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Label	Description
[- -]	No variation according to the genotype
[A -]	Gene expression variation due only to Apc loss-of-function in Apc ^{CKO} and in Apc ^{CKO} ;Nle ^{CKO}
[- N]	Gene expression variation due only to Nle loss-of-function in Nle ^{CKO} and in Apc ^{CKO} ;Nle ^{CKO}
[A N]	Gene expression variation due to both Nle loss-of-function and Apc loss-of-function. Gene expression in the Apc ^{CKO} ;Nle ^{CKO} is the sum of Apc ^{CKO} and Nle ^{CKO}
[- -D]	Gene misregulated only in the Apc ^{CKO} ;Nle ^{CKO}
[A -D]	Gene misregulated in the Apc ^{CKO} ; Nle loss-of-function modulates gene expression in the Apc-deficient epithelium but not in the Apc-proficient epithelium
[- -D]	Gene misregulated in the Nle ^{CKO} ; Apc loss-of-function modulates gene expression in the Nle-deficient epithelium but not in the Nle-proficient epithelium
[A N-D]	Gene misregulated in the Nle ^{CKO} ; Apc loss-of-function but gene expression in the Apc ^{CKO} ;Nle ^{CKO} is not the sum of Apc ^{CKO} and Nle ^{CKO}





A**B****C****D**



Supplementary Figures

Compensation between Wnt-driven tumorigenesis and cellular responses to ribosome biogenesis inhibition in the murine intestinal epithelium

Aurélien Raveux, Aline Stedman, Sabrina Coqueran, Sandrine Vandormael-Pournin, Nick Owens, Béatrice Romagnolo and Michel Cohen-Tannoudji

This file contains 7 supplementary figures and 2 supplementary tables :

Figure S1 : *Nle* loss-of-function only partially restores epithelial histology in the *Apc*-deficient intestine.

Figure S2: *Nle* loss-of-function reduces proliferative compartment expansion rate in the *Apc*-deficient epithelium.

Figure S3: Cell cycle exit is delayed in the double mutant compared to the control.

Figure S4: *Nle* loss-of-function induces persistent p53 stabilization in the *Apc*-deficient epithelium.

Figure S5: Functional analysis of gene clusters does not indicate signaling pathway interference between *Apc* loss-of-function and *Nle* loss-of-function.

Figure S6: Global protein synthesis at day 3 pi

Figure S7: Derivation and nutlin-3 treatment of intestinal organoids

Supplementary Table 1: list of primary and secondary antibodies

Supplementary Table 2: Sequences of RT-qPCR primers

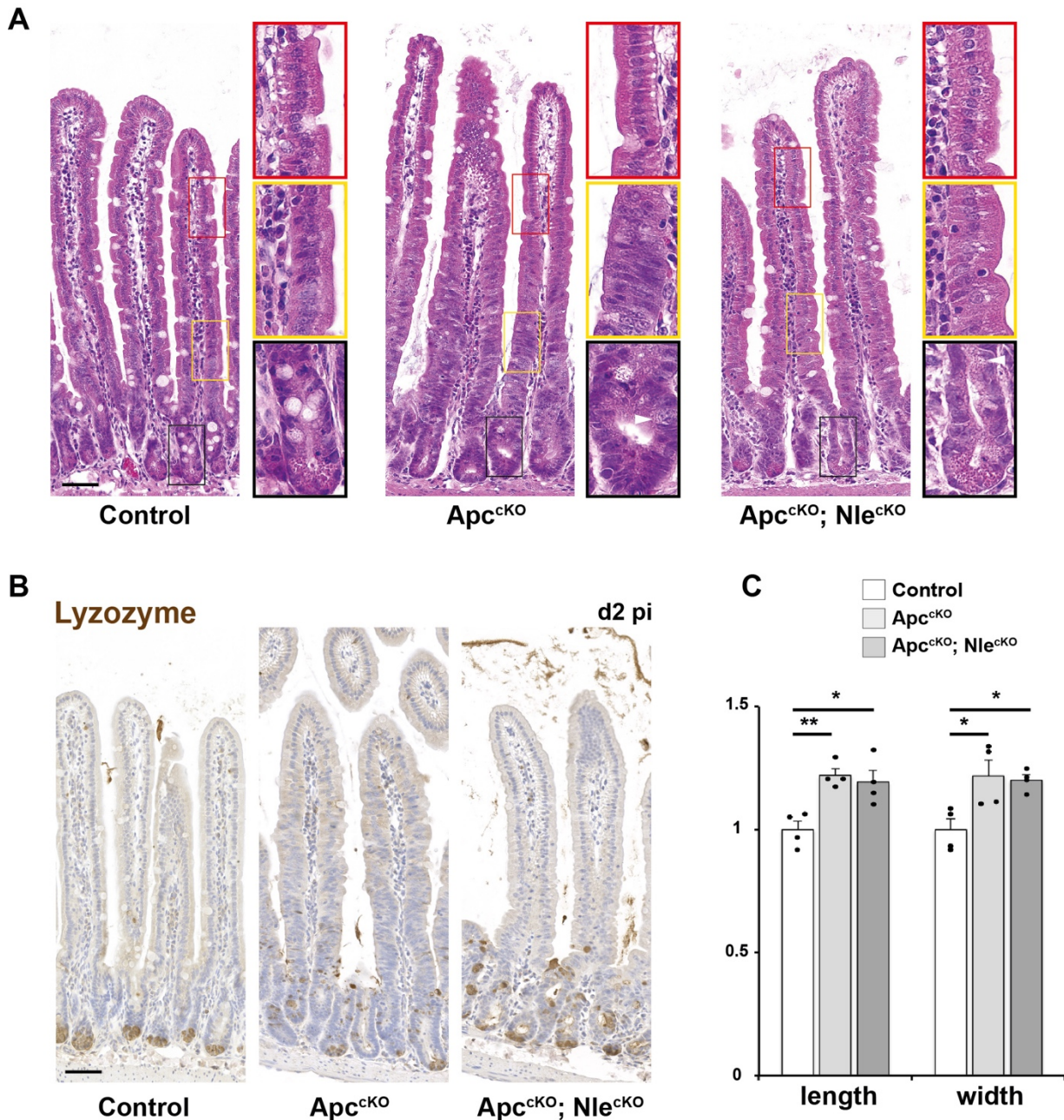


Figure S1 *Nle* loss-of-function only partially restores epithelial histology in the *Apc*-deficient intestine. (A) Hematoxylin–eosin staining of intestinal epithelium sections from Control, *Apc^{cKO}* and *Apc^{cKO}; Nle^{cKO}* small intestines at day 2 pi. Scale bars, 50 μ m. For each genotype, a portion of the villus is magnified (top) to highlight enterocyte hypertrophy, as well as a portion of the enlarged crypt compartment in the *Apc^{cKO}* and of the corresponding villus section in the other genotypes (middle) to highlight epithelial architecture restoration in the *Apc^{cKO}; Nle^{cKO}*, and a magnification of the histological crypt (bottom) is shown to highlight the presence of cells with granules (arrowheads) in the *Apc^{cKO}* and *Apc^{cKO}; Nle^{cKO}*. (B) Lysozyme immunostaining (brown) counterstained with Hematoxylin (blue) on intestinal epithelium sections from Control, *Apc^{cKO}* and *Apc^{cKO}; Nle^{cKO}* intestines at day 2 pi. Scale bars, 50 μ m. (C) Histogram showing the mean (\pm S.E.M.) length (defined as the lateral membrane section length) and width (defined as the apical or basal membrane section length) of enterocytes in Control, *Apc^{cKO}* and *Apc^{cKO}; Nle^{cKO}* villi. Twenty-five transverse sections of enterocytes homogeneously distributed along the jejunum were scored per mouse, $n = 4$ mice for each genotype. Means were normalized according to mean control measurements. *, $p < 0.05$ **, $p < 0.01$ according to Student's t-test. Differences that were significant according to Student's t-test were also significant according to Mann-Whitney Wilcoxon test with $p < 0.05$.

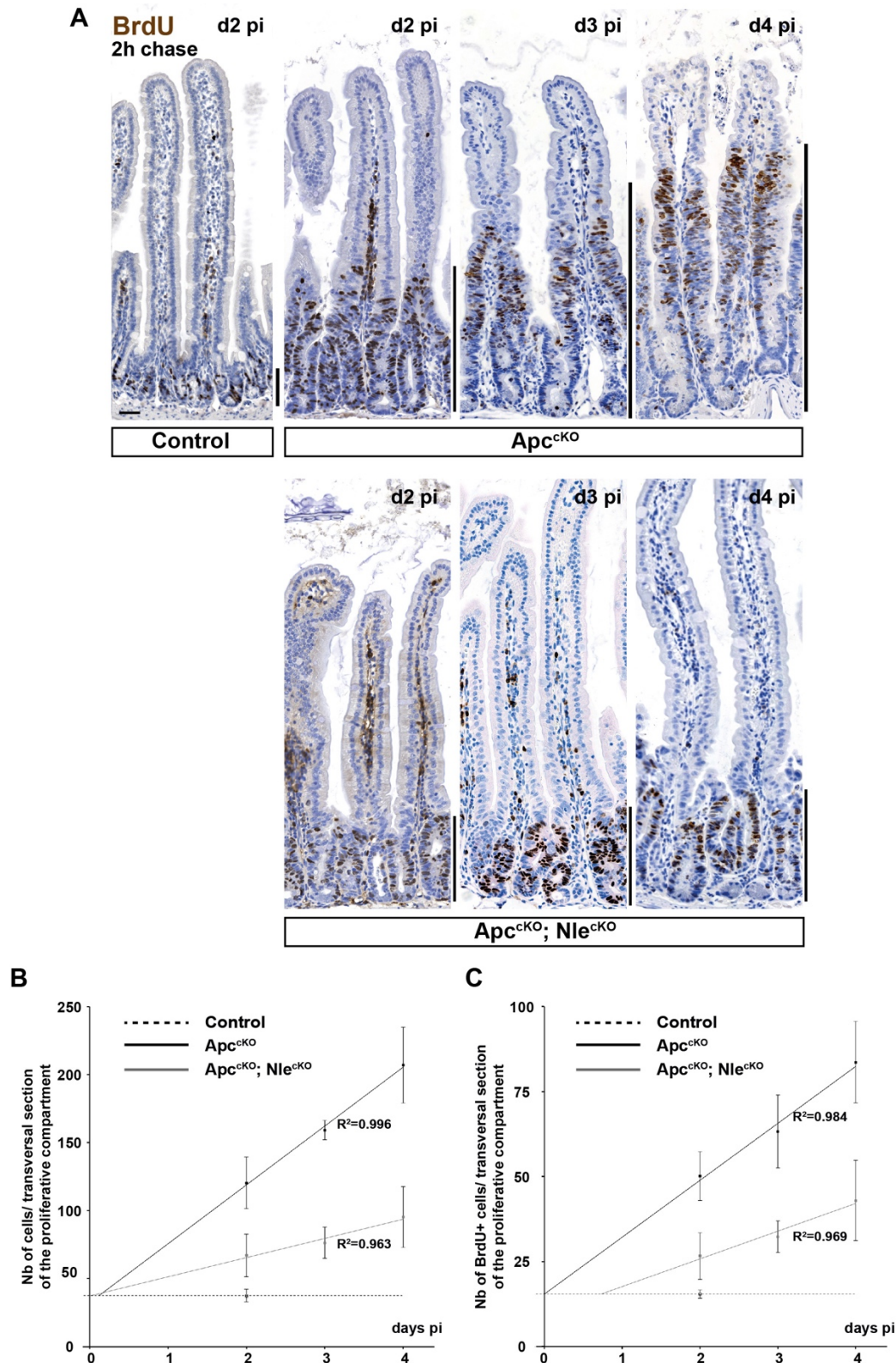


Figure S2 *Nle* loss-of-function reduces proliferative compartment expansion rate in the *Apc*-deficient epithelium. (A) BrdU immunostaining (brown) counterstained with hematoxylin (blue) on intestinal epithelium sections from Control (day 2 pi), *Apc^{cKO}* and *Apc^{cKO}; Nle^{cKO}* (days 2, 3 and 4 pi) intestines harvested 2 hours after BrdU injection. Scale bars, 50 μ m. (B-C) Graphs showing the mean total cell number (B) and the mean number of BrdU-positive-cells (C) in a transverse section of the proliferative compartment of Control, *Apc^{cKO}* and *Apc^{cKO}; Nle^{cKO}* intestines at several timing post tamoxifen injection. Twenty-five transverse crypt sections were scored per mouse, $n = 4$ for each genotype and each time point. R^2 , correlation coefficient with a linear model (plain regression lines). The dotted horizontal line shows baseline Control levels according to day 2 pi measurements. Hypothesizing linear growth, proliferative compartment expansion begins at 0 day pi in the *Apc^{cKO}* as previously reported [10] and in the *Apc^{cKO}; Nle^{cKO}*.

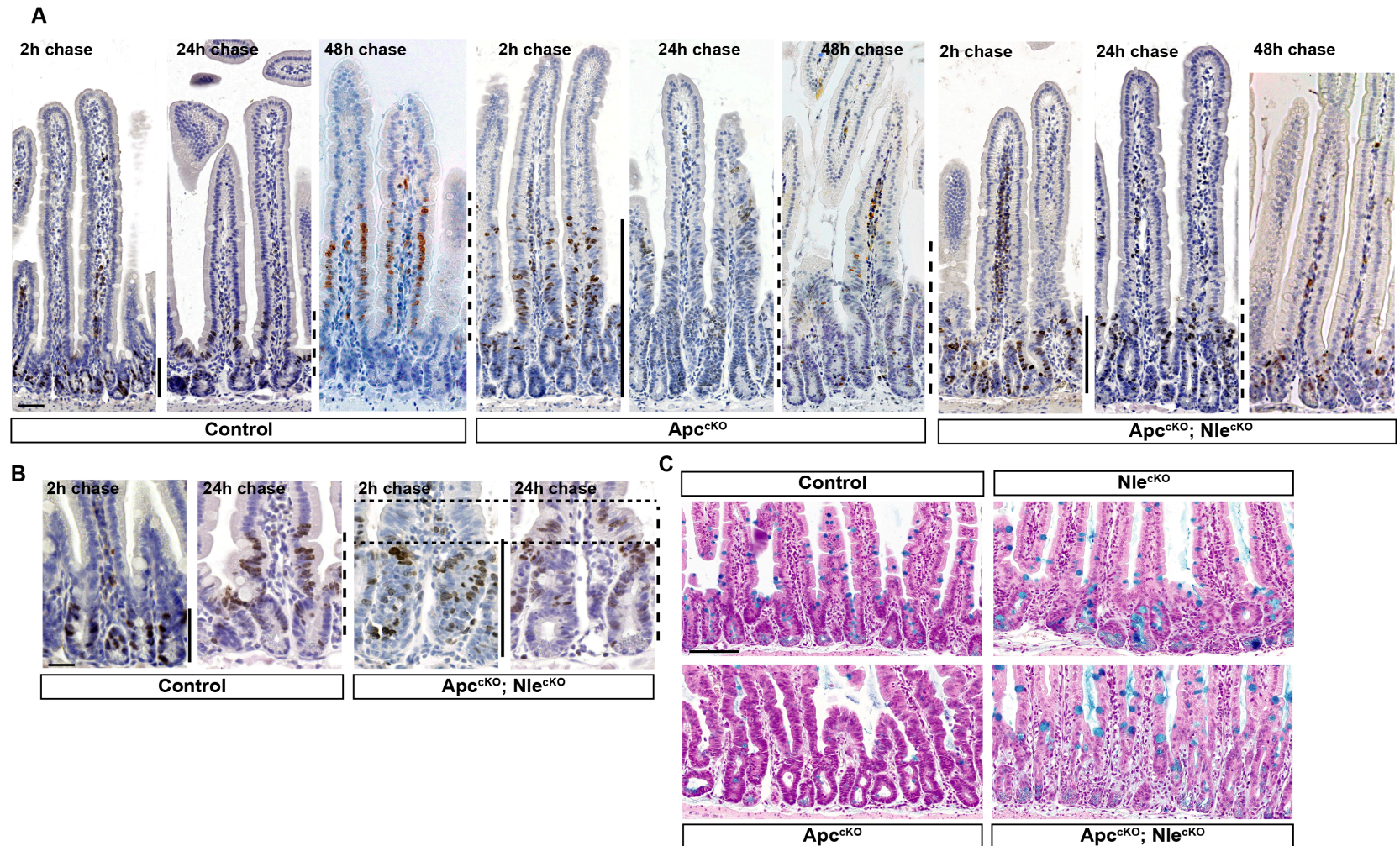


Figure S3 Cell cycle exit is delayed in the double mutant compared to the control. (A) BrdU immunostaining (brown) counterstained with Hematoxylin (blue) on intestinal epithelium sections from Control and Apc^{cKO} ; Nle^{cKO} intestines at day 2 pi harvested 2, 24 or 48 hours after BrdU injection. Plain black bars indicate the proliferative compartment. Dotted black bars indicate the range of proliferative cell migration within 24 or 48 hours. Scale bars, 50 μ m. (B) BrdU immunostaining (brown) counterstained with Hematoxylin (blue) on crypt sections from Control and Apc^{cKO} ; Nle^{cKO} intestines at day 2 pi harvested 2 hours or 24 hours after BrdU injection. Plain black bars indicate the proliferative compartment. The dotted horizontal lines indicate the limits of the double-mutant specific compartment (see text). Scale bars, 25 μ m. (C) Alcian blue coloration counterstained with Nuclear Fast Red on intestinal epithelium sections from Control, Nle^{cKO} , Apc^{cKO} and Apc^{cKO} ; Nle^{cKO} intestines at day 3 pi. Scale bars, 100 μ m.

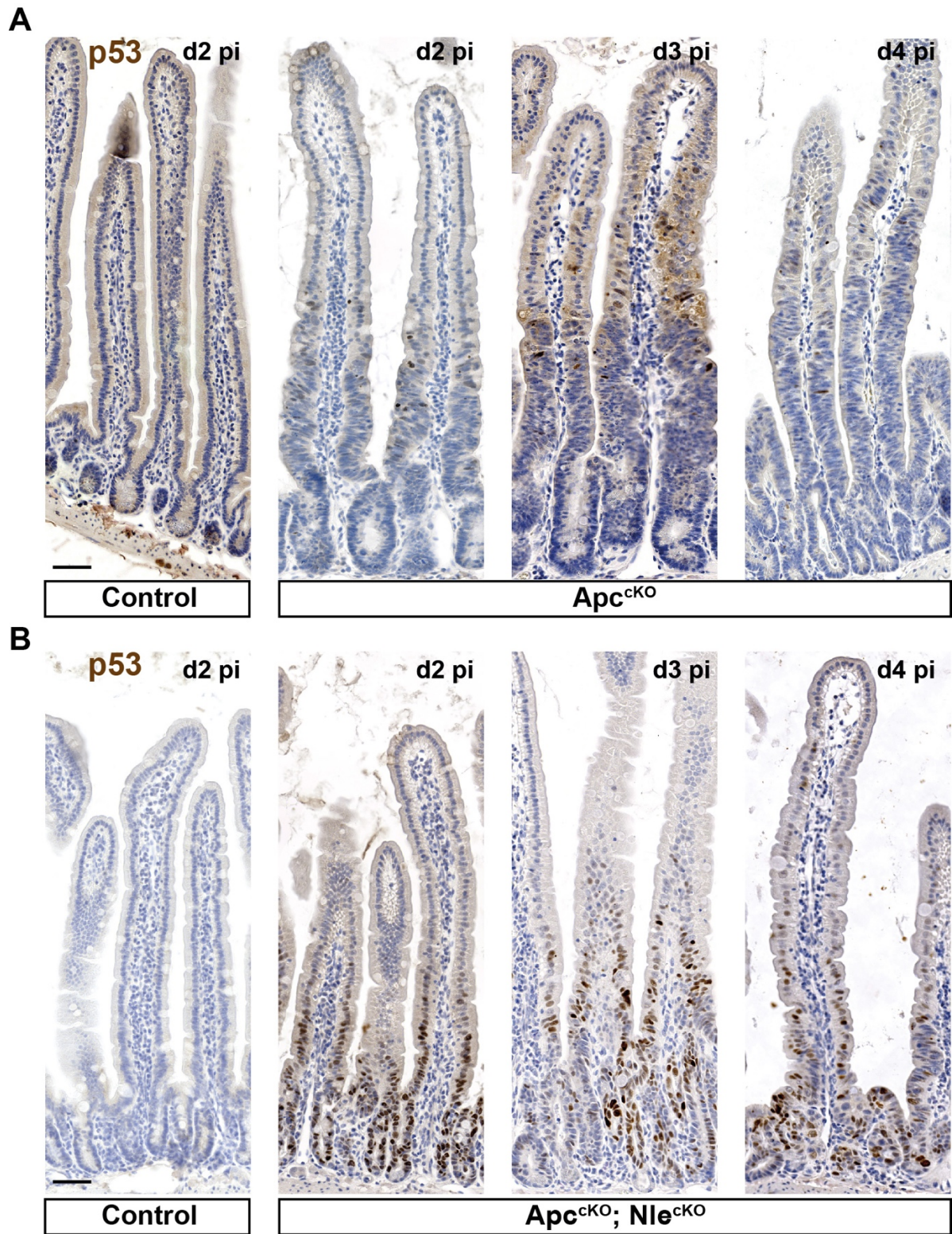


Figure S4 *Nle* loss-of-function induces persistent p53 stabilization in the *Apc*-deficient epithelium. (A) p53 immunostaining (brown) counterstained with Hematoxylin (blue) on intestinal epithelium sections from Control (day 2 pi) and *Apc*^{ckO} (days 2, 3 and 4 pi) intestines. Scale bars, 50 μm. (B) p53 immunostaining (brown) counterstained with Hematoxylin (blue) on intestinal epithelium sections from Control (day 2 pi) and *Apc*^{ckO}; *Nle*^{ckO} (days 2, 3 and 4 pi) intestines. Scale bars, 50 μm.

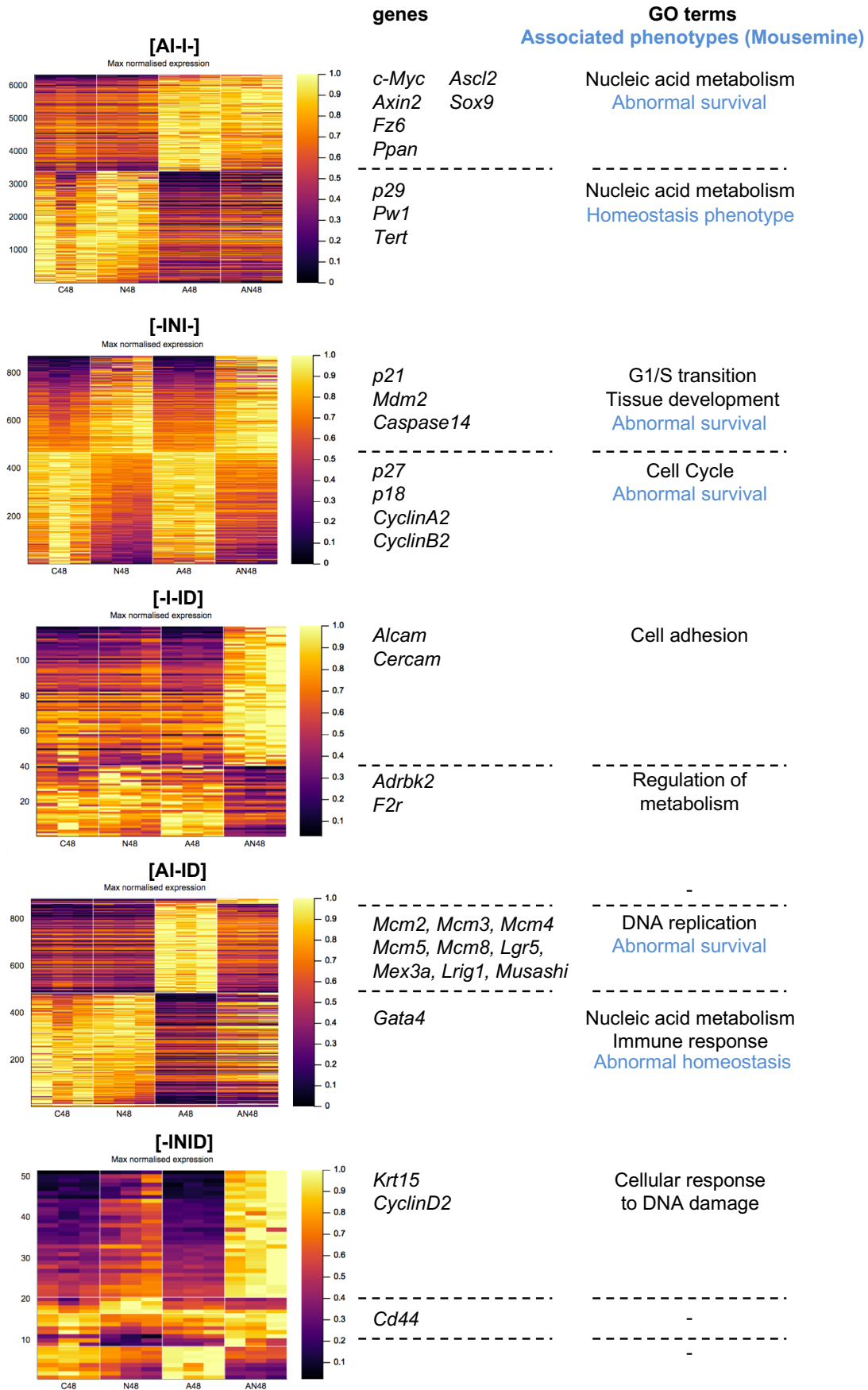


Figure S5 Functional analysis of gene clusters does not indicate signaling pathway interference between *Apc* loss-of-function and *Ne* loss-of-function. Genes in the [A|N|-] and [A|N|D] categories were separated into clusters by the direction of the A, N and D fold changes at day 2 pi. Gene number is plotted on the left of each heatmap. For each cluster, genes relevant to the analyzed phenotypes are indicated, as well as the most significantly enriched gene (black) and phenotype (blue) ontology terms from MouseMine.

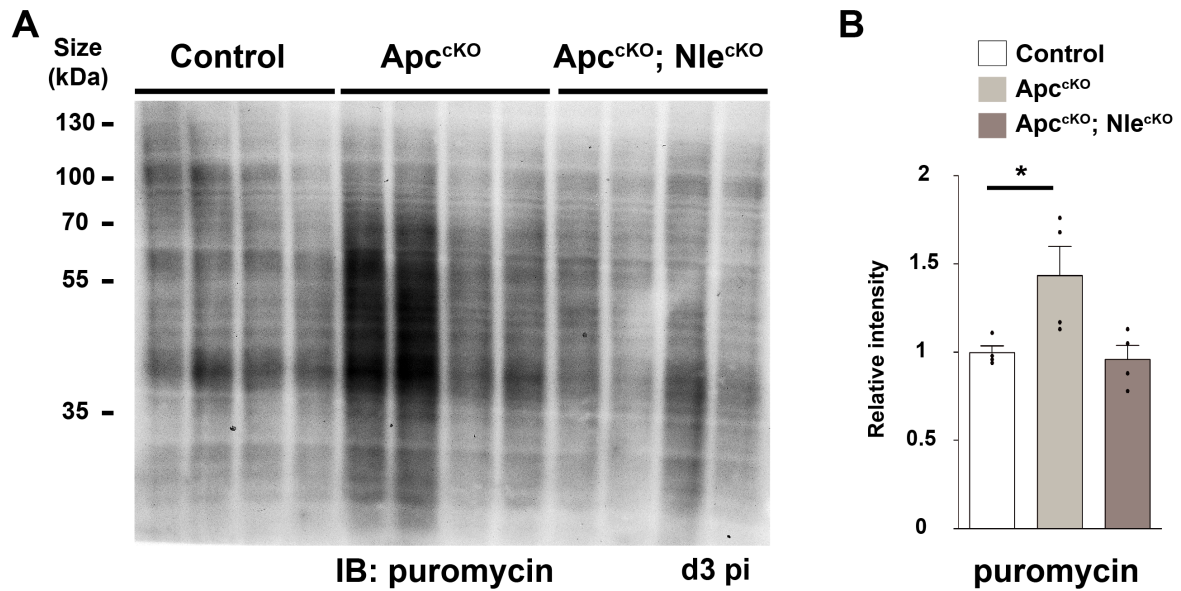


Figure S6 Global protein synthesis at day 3 pi. (A) Anti-puromycin immunoblotting of protein extracts for identical number of crypts cells from Control, *Apc^{ckO}* and *Apc^{ckO}; Nle^{ckO}* intestines at day 3 pi. (B) Histogram showing the mean normalized signal intensity \pm S.E.M from the immunoblot shown in (A). $n = 4$ for each genotype. *, $p < 0.05$ according to Mann-Whitney Wilcoxon test.

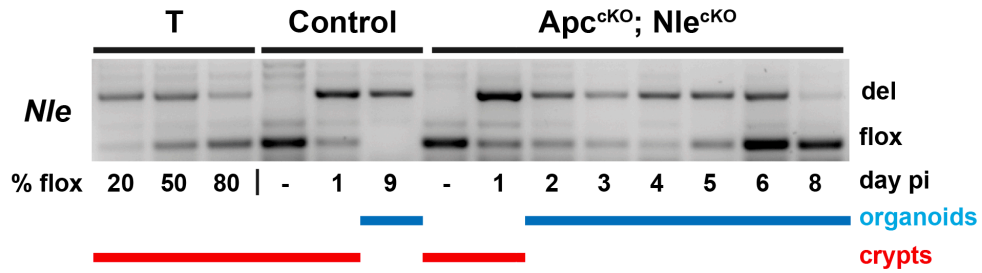
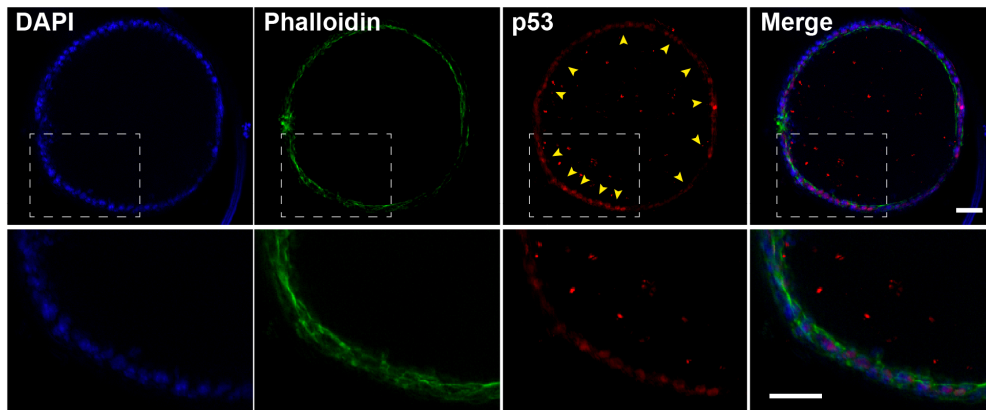
A**B*****Apc^{cKO}* organoid 5μM nutlin-3 for 48h**

Figure S7 Derivation and nutlin-3 treatment of intestinal organoids (A) Detection of the nonrecombined (flox) and the recombined (del) *Nle* alleles by PCR performed on DNA extracts from Control and *Apc;NleVilcKO* wells at different time points. Control DNAs with 20%, 50% and 80% flox alleles obtained by mixing *Nle^{flox/+}* and *Nle^{flox/del}* DNAs are presented. The *Nle⁺* and *Nle^{null}* alleles are not shown on the gel. (A) p53 immunostaining of an *APC^{cKO}* organoid cultured for 48h with 5μM nutlin-3. A single optical section is shown. Note the presence of numerous p53 positive nuclei on the whole circumference of the spheroid. Bright dots correspond to non specific staining of debris present in the lumen of the organoid. Bar: 50μM.

Supplementary Table 1: list of primary and secondary antibodies

Antibodies	Reference	Dilution used
anti-cleaved Caspase 3	9661L Ozyme	1/200
anti-BrdU	347580 BD	1/150
anti- β -catenin	C19220 BD	1/50
anti-p53	CM5-p Leica	1/500
anti-lysozyme	A0099 Dako	1/1000
anti-Muc2	sc-15334 Santa-Cruz	1/200
anti-ChromoA	sc-1488 Santa-Cruz	1/300
anti-puromycin	5B12, David <i>et al.</i> , JCB 2012	1/5000
Biotinylated goat anti-rabbit IgG	E0432 Dako	1/400
Biotinylated goat anti-mouse IgG	E0433 Dako	1/400

Supplementary Table 2: Sequences of RT-qPCR primers

Target gene	Forward primer	Reverse primer
<i>Olfm4</i>	ATCAGCGCTCCTTCTGTGAT	AGGGTTCTCTCTGGATGCTG
<i>Lgr5</i>	ACATTCCCAAGGGAGCGTTC	ATGTGGTTGGCATCTAGGCG
<i>Muc2</i>	CAAGGGCTCGGA ACTCCAG	CCAGGGAATCGGTAGACATCG
<i>ChromoA</i>	AGTCATCTCCGACTCGCTGT	GGTGTCGCAGGATAGAGAGG
<i>c-Myc</i>	AAGGCCCCCAAGGTAGTG	TGCTCGTCTGCTTGAATGGA
<i>Axin 2</i>	GATTCCCCTTTGACCAGGTGG	CCATTACAAGCAAACCAGAAGT
<i>Its1</i>	TCTGACCTCGCCACCCTA	CCTCGTAGACACGGAAGAGC
<i>Its2</i>	TGTGTGTGTTTGGGTCTTGC	GGATACCACCTCTCTCCGTTC
<i>28S</i>	TCATCAGACCCCAAGAAAGG	GATTCCGGCAGGTGAGTTGTT
<i>18S</i>	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT