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

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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 cells[1], 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[2-

50   4]. In absence of functional p53 pathway, ribosome biogenesis dysfunction also triggers

51   p53-independent mechanisms[5]. 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 pathway[12],

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 regulator[13],

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   eukaryotes[14-16] and involved in the late steps of maturation and subsequent export of the

76   60S particle[17-20]. In previous studies, we showed that the role of *Nle* in the maturation of

77   the large ribosomal subunit is conserved in the mouse[21], 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   differentiation[22]. 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-CreERT2 [23]. 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   biomedicals) 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'- agacttggggtctggactca -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/µL 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   Puromycilated 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 1µM, 5µM and 10µM 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^cKO. 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 macroapotome Zeiss Axiozoomer and analyzed

196 using the Zen software.

197

198 **Statistical analysis.** All experiments were performed with a sample size n≥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 ± 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 0.43 [26] with default options. Rounded estimated counts were used as input to DESeq2 [27].

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 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 ~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 0.6 [28]) 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$^{flox/+}$*; *Nle$^{flox/+}$*), Apc^{cKO} (Villin-CreERT2$^{tg/0}$;

253   *Apc$^{flox/flox}$*; *Nle$^{flox/+}$*) and Apc^{cKO}; Nle^{cKO} (Villin-CreERT2$^{tg/0}$; *Apc$^{flox/flox}$*; *Nle$^{flox/null}$*) 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^{flox}* and *Nle^{flox}*

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^{flox}* and *Nle^{flox}* alleles was efficient in the

258   intestinal epithelium from Control, Apc^{cKO} and Apc^{cKO}; Nle^{cKO} mice (Figure 1B). Apc^{cKO}

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^{cKO} (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 cells[10] and enterocyte hypertrophia were similarly

269   observed in Apc^{cKO}; Nle^{cKO} (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^{cKO}; Nle^{cKO} intestinal epithelia,

276   we monitored nuclear β-catenin on small intestine sections. Accumulation of nuclear β-

277   catenin was similarly observed in Apc^{cKO} and Apc^{cKO}; Nle^{cKO} intestines (Figure 2A).

278   Moreover, upregulation of the Wnt targets *c-Myc* and *Axin2* was found in both Apc^{cKO} and

279   Apc^{cKO}; Nle^{cKO} 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   progenitors[22]. 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 villi[22]. 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 cells [22] 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 epithelium[22]. 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 reports[29]. 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   cells[22]. 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 growth [46], 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 cancer[32] and increased translational elongation is key for

472 intestinal tumorigenesis[33]. 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 rates[34]. 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 initiation[33]. 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 cells[22]. 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 progenitors[40]. On a different note, it has
519 been shown that the Wnt pathway modulates protein stoichiometry of polysomal
520 ribosomes during mouse neocortex development[41], which, in turns could potentially
521 result in significant modulation of the proteome[42]. 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 expression[43], 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 pathway[44].

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 cells[6-8]. 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

560

561   **Competing interests :** The authors declare no competing interest.

562

563

REFERENCES

1   Ruggero D, Pandolfi PP. Does the ribosome translate cancer? *Nat Rev Cancer* 2003; **3**: 179–192.

2   Fumagalli S, Ivanenkov VV, Teng T, Thomas G. Suprainduction of p53 by disruption of 40S and 60S ribosome biogenesis leads to the activation of a novel G2/M checkpoint. *Genes & Development* 2012; **26**: 1028–1040.

3   Bursać S, Brdovčak MC, Pfannkuchen M, Orsolić I, Golomb L, Zhu Y *et al.* Mutual protection of ribosomal proteins L5 and L11 from degradation is essential for p53 activation upon ribosomal biogenesis stress. *Proceedings of the National Academy of Sciences* 2012; **109**: 20467–20472.

4   Donati G, Peddigari S, Mercer CA, Thomas G. 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint. *Cell Rep* 2013; **4**: 87–98.

5   Challagundla KB, Sun X-X, Zhang X, DeVine T, Zhang Q, Sears RC *et al.* Ribosomal protein L11 recruits miR-24/miRISC to repress c-Myc expression in response to ribosomal stress. *Mol Cell Biol* 2011; **31**: 4007–4021.

6   Bywater MJ, Poortinga G, Sanij E, Hein N, Peck A, Cullinane C *et al.* Inhibition of RNA Polymerase I as a Therapeutic Strategy to Promote Cancer-Specific Activation of p53. *Cancer Cell* 2012; **22**: 51–65.

7   Peltonen K, Colis L, Liu H, Trivedi R, Moubarek MS, Moore HM *et al.* A targeting modality for destruction of RNA polymerase I that possesses anticancer activity. *Cancer Cell* 2014; **25**: 77–90.

8   Kim DW, Wu N, Kim Y-C, Cheng P-F, Basom R, Kim D *et al.* Genetic requirement for Mycl and efficacy of RNA Pol I inhibition in mouse models of small cell lung cancer. *Genes & Development* 2016; **30**: 1289–1299.

9   Brannon AR, Vakiani E, Sylvester BE, Scott SN, McDermott G, Shah RH *et al.* Comparative sequencing analysis reveals high genomic concordance between matched primary and metastatic colorectal cancer lesions. *Genome Biol* 2014; **15**: 454.

10   Andreu P, Colnot S, Godard C, Gad S, Chafey P, Niwa-Kawakita M *et al.* Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. *Development* 2005; **132**: 1443–1451.

11   Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP *et al.* Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes & Development* 2004; **18**: 1385–1390.

12   Sansom OJ, Meniel VS, Muncan V, Phesse TJ, Wilkins JA, Reed KR *et al.* Myc deletion rescues Apc deficiency in the small intestine. *Nature* 2007; **446**: 676–679.

602   13   van Riggelen J, Yetil A, Felsher DW. MYC as a regulator of ribosome biogenesis and
603   protein synthesis. *Nat Rev Cancer* 2010; **10**: 301–309.

604   14   Royet J, Bouwmeester T, Cohen SM. Notchless encodes a novel WD40-repeat-
605   containing protein that modulates Notch signaling activity. *Embo J* 1998; **17**: 7351–
606   7360.

607   15   Cormier S, Le Bras S, Souilhol C, Vandormael-Pournin S, Durand B, Babinet C *et al.*
608   The Murine Ortholog of Notchless, a Direct Regulator of the Notch Pathway in
609   Drosophila melanogaster, Is Essential for Survival of Inner Cell Mass Cells. *Mol Cell*
610   *Biol* 2006; **26**: 3541–3549.

611   16   Gazave E, Lapébie P, Richards GS, Brunet F, Ereskovsky AV, Degnan BM *et al.* Origin
612   and evolution of the Notch signalling pathway: an overview from eukaryotic
613   genomes. *BMC Evol Biol* 2009; **9**: 249.

614   17   la Cruz de J, Sanz-Martínez E, Remacha M. The essential WD-repeat protein Rsa4p is
615   required for rRNA processing and intra-nuclear transport of 60S ribosomal subunits.
616   *Nucleic Acids Research* 2005; **33**: 5728–5739.

617   18   Ulbrich C, Diepholz M, Baßler J, Kressler D, Pertschy B, Galani K *et al.*
618   Mechanochemical Removal of Ribosome Biogenesis Factors from Nascent 60S
619   Ribosomal Subunits. *Cell* 2009; **138**: 911–922.

620   19   Matsuo Y, Granneman S, Thoms M, Manikas R-G, Tollervey D, Hurt E. Coupled GTPase
621   and remodelling ATPase activities form a checkpoint for ribosome export. *Nature*
622   2014; **505**: 112–116.

623   20   Barrio-Garcia C, Thoms M, Flemming D, Kater L, Berninghausen O, Baßler J *et al.*
624   Architecture of the Rix1-Rea1 checkpoint machinery during pre-60S-ribosome
625   remodeling. *Nat Struct Mol Biol* 2015; **23**: 37–44.

626   21   Le Bouteiller M, Souilhol C, Cormier S, Stedman A, Burlen-Defranoux O, Vandormael-
627   Pournin S *et al.* Notchless-dependent ribosome synthesis is required for the
628   maintenance of adult hematopoietic stem cells. *Journal of Experimental Medicine*
629   2013; **210**: 2351–2369.

630   22   Stedman A, Beck-Cormier S, Le Bouteiller M, Raveux A, Vandormael-Pournin S,
631   Coqueran S *et al.* Ribosome biogenesis dysfunction leads to p53-mediated apoptosis
632   and goblet cell differentiation of mouse intestinal stem/progenitor cells. *Cell Death*
633   *Differ* 2015; **22**: 1865–1876.

634   23   Marjou El F, Janssen K-P, Chang BH-J, Li M, Hindie V, Chan L *et al.* Tissue-specific and
635   inducible Cre-mediated recombination in the gut epithelium. *Genesis* 2004; **39**: 186–
636   193.

637   24   Guo J, Longshore S, Nair R, Warner BW. Retinoblastoma protein (pRb), but not p107
638   or p130, is required for maintenance of enterocyte quiescence and differentiation in
639   small intestine. *J Biol Chem* 2009; **284**: 134–140.

640 25 Léguillier T, Vandormael-Pournin S, Artus J, Houlard M, Picard C, Bernex F *et al.*
641   Omcg1 is critically required for mitosis in rapidly dividing mouse intestinal
642   progenitors and embryonic stem cells. *Biol Open* 2012; **1**: 648–657.

643 26 Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq
644   quantification. *Nat Biotechnol* 2016; **34**: 525–527.

645 27 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for
646   RNA-seq data with DESeq2. *Genome Biol* 2014; **15**: 550.

647 28 Bezanson J, Edelman A, Karpinski S, Shah VB. Julia: A Fresh Approach to Numerical
648   Computing. *SIAM Review* 2017; **59**: 65–98.

649 29 Reed KR, Meniel VS, Marsh V, Cole A, Sansom OJ, Clarke AR. A limited role for p53 in
650   modulating the immediate phenotype of Apc loss in the intestine. *BMC Cancer* 2008;
651   **8**: 162.

652 30 Pelletier J, Thomas G, Volarevic S. Ribosome biogenesis in cancer: new players and
653   therapeutic avenues. *Nat Rev Cancer* 2017; **4**: a003681.

654 31 Bastide A, David A. The ribosome, (slow) beating heart of cancer (stem) cell.
655   *Oncogenesis* 2018; **7**: 34.

656 32 Tsoi H, Lam KC, Dong Y, Zhang X, Lee CK, Zhang J *et al.* Pre-45s rRNA promotes colon
657   cancer and is associated with poor survival of CRC patients. *Oncogene* 2017.
658   doi:10.1038/onc.2017.86.

659 33 Faller WJ, Jackson TJ, Knight JRP, Ridgway RA, Jamieson T, Karim SA *et al.* mTORC1-
660   mediated translational elongation limits intestinal tumour initiation and growth.
661   *Nature* 2015; **517**: 497–500.

662 34 Bruno PM, Liu Y, Park GY, Murai J, Koch CE, Eisen TJ *et al.* A subset of platinum-
663   containing chemotherapeutic agents kills cells by inducing ribosome biogenesis
664   stress. *Nature Medicine* 2017; **23**: 461–471.

665 35 Cole AM, Myant KB, Reed KR, Ridgway RA, Athineos D, van den Brink GR *et al.* Cyclin
666   D2–Cyclin-Dependent Kinase 4/6 Is Required for Efficient Proliferation and
667   Tumorigenesis following Apc Loss. *Cancer Research* 2010; **70**: 8149–8158.

668 36 Ashton GH, Morton JP, Myant KB, Phesse TJ, Ridgway RA, Marsh V *et al.* Focal
669   adhesion kinase is required for intestinal regeneration and tumorigenesis
670   downstream of Wnt/c-Myc signaling. *Dev Cell* 2010; **19**: 259–269.

671 37 Holik AZ, Young M, Krzystyniak J, Williams GT, Metzger D, Shorning BY *et al.* Brg1
672   loss attenuates aberrant wnt-signalling and prevents wnt-dependent
673   tumourigenesis in the murine small intestine. *PLoS Genet* 2014; **10**: e1004453.

674 38 Eom T-Y, Jope RS. GSK3 beta N-terminus binding to p53 promotes its acetylation. *Mol
675   Cancer* 2009; **8**: 14.

676   39   Kulikov R, Boehme KA, Blattner C. Glycogen synthase kinase 3-dependent
677   phosphorylation of Mdm2 regulates p53 abundance. *Mol Cell Biol* 2005; **25**: 7170–
678   7180.

679   40   Pfister AS, Keil M, Kühl M. The Wnt Target Protein Peter Pan Defines a Novel p53-
680   independent Nucleolar Stress-Response Pathway. *Journal of Biological Chemistry*
681   2015; **290**: 10905–10918.

682   41   Kraushar ML, Viljetic B, Wijeratne HRS, Thompson K, Jiao X, Pike JW *et al.* Thalamic
683   WNT3 Secretion Spatiotemporally Regulates the Neocortical Ribosome Signature
684   and mRNA Translation to Specify Neocortical Cell Subtypes. *Journal of Neuroscience*
685   2015; **35**: 10911–10926.

686   42   Genuth NR, Barna M. Heterogeneity and specialized functions of translation
687   machinery: from genes to organisms. *Nat Rev Genet* 2018; **13**: 1.

688   43   Cole AM, Ridgway RA, Derkits SE, Parry L, Barker N, Clevers H *et al.* p21 loss blocks
689   senescence following Apc loss and provokes tumourigenesis in the renal but not the
690   intestinal epithelium. *EMBO Mol Med* 2010; **2**: 472–486.

691   44   Teng T, Mercer CA, Hexley P, Thomas G, Fumagalli S. Loss of tumor suppressor
692   RPL5/RPL11 does not induce cell cycle arrest but impedes proliferation due to
693   reduced ribosome content and translation capacity. *Mol Cell Biol* 2013; **33**: 4660–
694   4671.

695   45   Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE *et al.* Single
696   Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche.
697   *Nature* 2009; **459**: 262–265.

698   46   Bohin N, Carlson EA, Samuelson LC. Genome Toxicity and Impaired Stem Cell
699   Function after Conditional Activation of CreERT2 in the Intestine. *Stem Cell Reports*
700   2018; **11**: 1337–1346.

701

FIGURE LEGENDS

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

Figure 2 *Nle* loss-of-function limits the expansion of the proliferative compartment expansion despite Wnt pathway hyperactivation. (A) β-catenin immunostaining (brown) counterstained with hematoxylin (blue) on intestinal epithelium sections from Control, Apc^{cKO} and Apc^{cKO}; Nle^{cKO} intestines at day 2 pi. The second lane shows magnified views of framed regions. Scale bars, 50 μm. (B) RT-qPCR performed on total RNA from Control, Nle^{cKO}, Apc^{cKO} and Apc^{cKO}; Nle^{cKO} intestinal epithelium extracts at day 2 pi. Graphs represent the mean fold changes ± S.E.M. for Wnt transcriptional target genes *c-Myc* and *Axin2*. n ≥ 3 for each genotype. *, p<0.05 **, p<0.01 ***, p<001 according to Student's t-test. (C) BrdU immunostaining (brown) counterstained with hematoxylin (blue) on intestinal epithelium sections from Control, Apc^{cKO} and Apc^{cKO}; Nle^{cKO} intestines at day 2 pi harvested 2 hours after BrdU injection. Black bars indicate the extension of the proliferative compartment. Scale bars, 50 μm. (D) Histogram showing the mean proportion (± S.E.M.) of BrdU-positive cells in the proliferative compartment in Control, Apc^{cKO} and Apc^{cKO}; Nle^{cKO} small intestines. Twenty-five transverse crypt sections were scored per mouse, n = 4 for each genotype. The means were not significantly different among genotypes according to Mann-Whitney Wilcoxon test.

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 μm. (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 μm. (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 μm. (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 μm. (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≥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 ± S.E.M. for differentiation markers *Mucin 2* (*Muc2*) and
754 *Chromogranin A* (*ChromoA*). n ≥ 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 ± 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 μm. (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
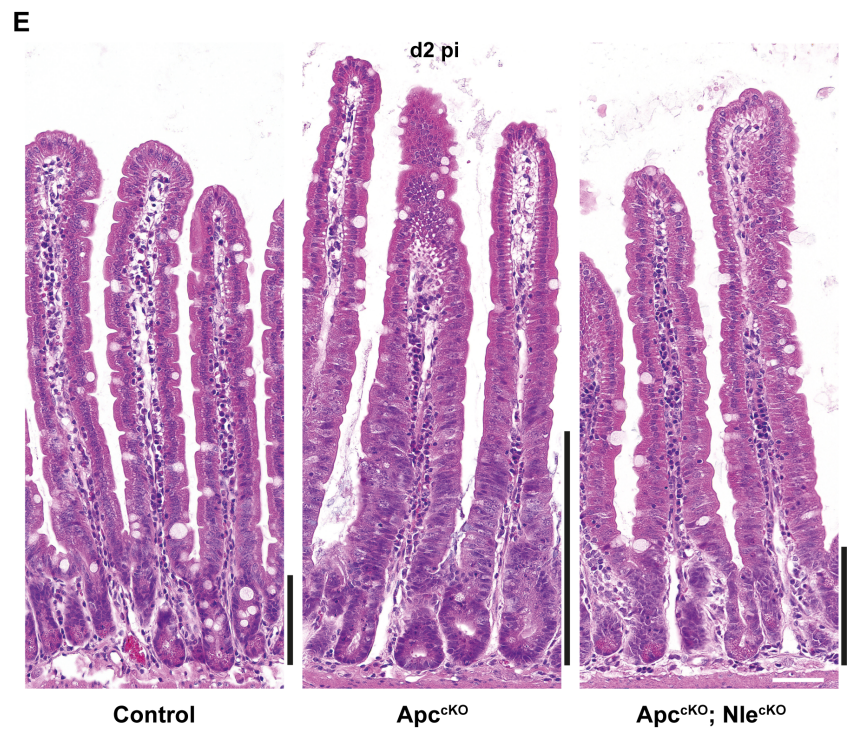
771   for Apc[cKO]. Scale bars, 50 μm.
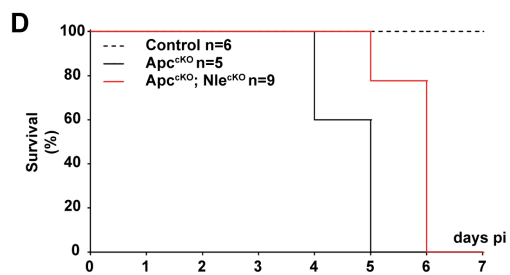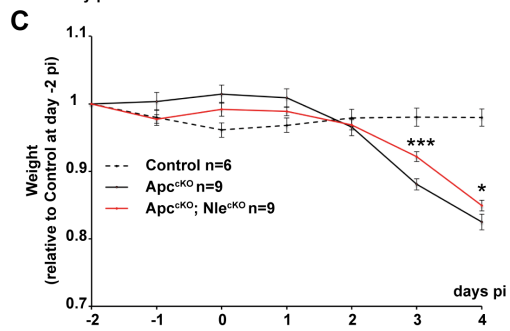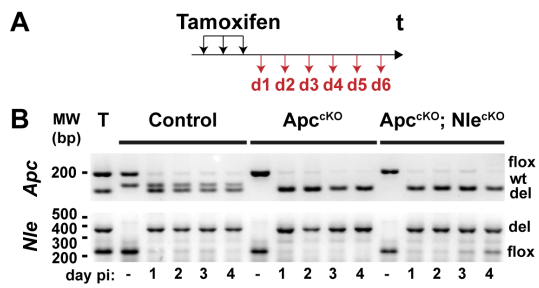
772

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 ± 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 ± S.E.M from the immunoblot shown in (B). n = 4 for each

802 genotype. *, p<0.05 according to Mann–Whitney Wilcoxon test.

803

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 ± S.E.M. for ISC markers

807 Lgr5 and Olfm4. n = 4 for each genotype. *, p<0.05 **, p<0.01 ***, p<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 μm. (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 μm. (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 μm.

817

818 **Figure 8 Apc[cKO] intestinal organoids show increased tolerance to *Nle*-deficiency and**

819 **p53 stabilization.** (A) Histogram showing the mean percentage (± 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 ≥ 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 (± 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 10μM

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 μm.

A

Tamoxifen   t

d1 d2 d3 d4 d5 d6

B

MW (bp)	T	Control	Apc^cKO	Apc^cKO; Nle^cKO	

Apc   200 —   flox
                                                                 wt
                                                                 del

Nle   500 —   del
         400 —
         300 —   flox
         200 —

day pi: – 1 2 3 4   – 1 2 3 4   – 1 2 3 4

C

Weight (relative to Control at day -2 pi)

- - - Control n=6
—— Apc^cKO n=9
—— Apc^cKO; Nle^cKO n=9

***

*

days pi

D

Survival (%)

- - - Control n=6
—— Apc^cKO n=5
—— Apc^cKO; Nle^cKO n=9

days pi

E

d2 pi

Control   Apc^cKO   Apc^cKO; Nle^cKO

A β-catenin | d2 pi

Control | Apc^cKO | Apc^cKO; Nle^cKO

B

c-Myc | Axin2

Control
Apc^cKO
Apc^cKO; Nle^cKO

Relative expression

C BrdU | d2 pi

Control | Apc^cKO | Apc^cKO; Nle^cKO

D

Control
Apc^cKO
Apc^cKO; Nle^cKO

BrdU+/total

A **BrdU** d3 pi

2h chase | 48h chase | 2h chase | 48h chase | 2h chase | 48h chase

Control | Apc^cKO | Apc^cKO; Nle^cKO

B **Caspase3** d2 pi

Control | Apc^cKO | Apc^cKO; Nle^cKO

C **Muc2** d2 pi

Control | Apc^cKO | Apc^cKO; Nle^cKO

D **ChromoA** d2 pi

Control | Apc^cKO | Apc^cKO; Nle^cKO

E

F

Control
Apc^cKO
Apc^cKO; Nle^cKO

Figure 3

A

47S

its1 its2

21S 32S

nucleolus 12S
cytoplasm

18S 5.8S 28S

B

Relative expression

Control
Apc^cKO
Apc^cKO; Nle^cKO

*
*
**
***

its1 its2 its2/its1

C

its1 d2 pi

its2

Control Apc^cKO Apc^cKO; Nle^cKO

D

p53 d2 pi

Control Apc^cKO Apc^cKO; Nle^cKO

A

PC2 : 12.77% explained

PC1 : 58.79% explained

B

Cluster: 10 — 529 genes
Cluster: 9 — 602 genes
Cluster: 8 — 1129 genes
Cluster: 7 — 678 genes
Cluster: 6 — 1450 genes
Cluster: 5 — 1413 genes
Cluster: 4 — 2594 genes
Cluster: 3 — 1574 genes
Cluster: 2 — 2211 genes
Cluster: 1 — 2935 genes

C24 C48 N24 N48 A24 A48 AN24 AN48

C

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
[-|N|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 expression variation due to both Nle loss-of-function and Apc loss-of-function but gene expression in the Apc^{cKO};Nle^{cKO} is not the sum of Apc^{cKO} and Nle^{cKO}

D

d1 pi
d2 pi

[-\|-\|-] 6557 4462
[A\|-\|-] 7291 6346
[-\|N\|-] 198 873
[A\|N\|-] 378 1577
[-\|-\|D] 12 119
[A\|-\|D] 23 886
[-\|N\|D] 5 51
[A\|N\|D] 5 344

E

[A\|N\|-]
Max normalised expression

C48 N48 A48 AN48

Genes	GO terms / Associated phenotypes
Bmi1, RNApolI, Brix1, Dkc1, Bax, Puma	Ribosome biogenesis, p53 signaling pathway, Abnormal survival
Fos	Cellular homeostasis
CyclinE1, Brca1	Cell cycle replication, Abnormal cell physiology
	Small molecules metabolic process, Abnormal cell differentiation

[A\|N\|D]
Max normalised expression

C48 N48 A48 AN48

Genes	GO terms / Associated phenotypes
CyclinD1	-
Hopx	Regulation of cell proliferation, Embryo phenotype
Perp, Fas	Cell death
Olfm4, Mcm6, Mcm10	DNA replication, Abnormal survival
	Anatomical structure development
	-

F

d2 pi

Apc^{cKO}; Nle^{cKO}
Apc^{cKO}
Nle^{cKO}

Stem Cell markers:
Pw1, Lrig1, Krt15, Mex3a, Sox9, Hopx, Tert, Bmi1, Olfm4

Wnt targets:
Lgr5, Ascl2, Musashi, Cd44, CyclinD2, CyclinD1, Fz6, Axin2, cMyc

p53 targets:
Perp, Fas, Puma, NoxA, Bax, Mdm2

Cell cycle inhibitors:
p21, p57, p18, p29, p27

Fold change (0.1, 1, 10, 100)

A

Relative expression

* (18S)
* (28S)
** (28S/18S)

Control
Apc^{cKO}
Apc^{cKO}; Nle^{cKO}

B

Size (kDa)

Control   Apc^{cKO}   Apc^{cKO}; Nle^{cKO}

130 –
100 –
70 –
55 –
35 –

IB: puromycin   d2 pi

C

Relative intensity

Control
Apc^{cKO}
Apc^{cKO}; Nle^{cKO}

*** *

puromycin

A

B *Olfm4* ISH   d2 pi

Control   Nle^cKO   Apc^cKO   Apc^cKO; Nle^cKO

C p53
d2 pi   d3 pi   d4 pi

Apc^cKO; Nle^cKO

D BrdU 2h chase   d2 pi
Control   Nle^cKO

Apc^cKO   Apc^cKO; Nle^cKO

A

% growing organoids at day 4 pi

Control
Nle^cKO
Apc^cKO
Apc^cKO; Nle^cKO

B

day4

day4

day6

Control   Nle^cKO   Apc^cKO   Apc^cKO; Nle^cKO

C

% healthy organoids after 48h

Control
Apc^cKO

Mock   1 µM   5 µM   10 µM

D

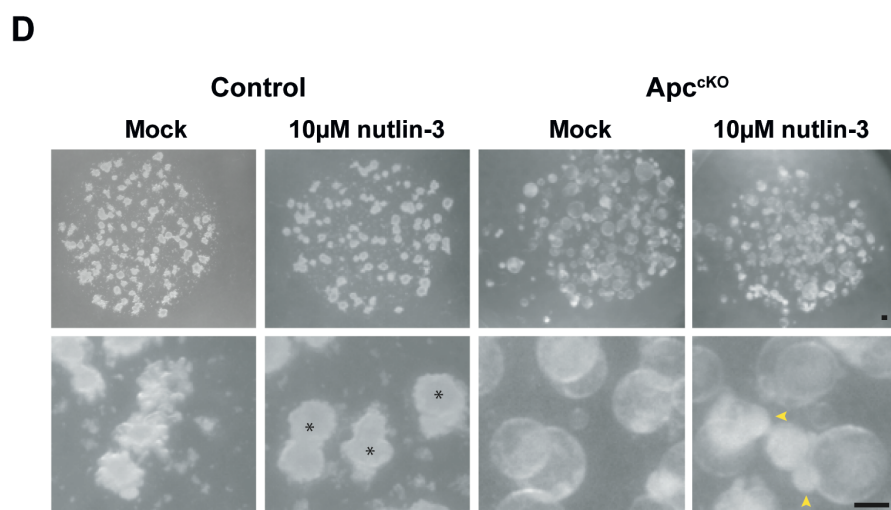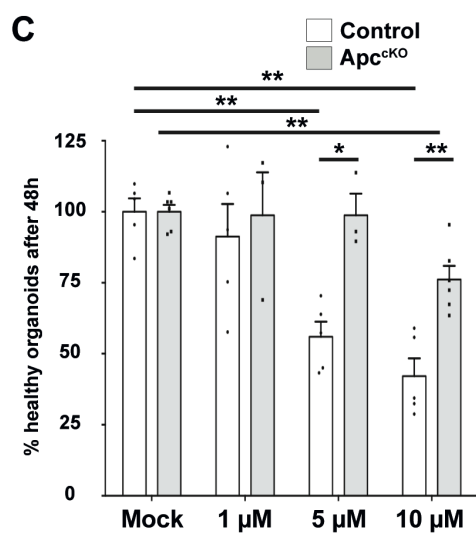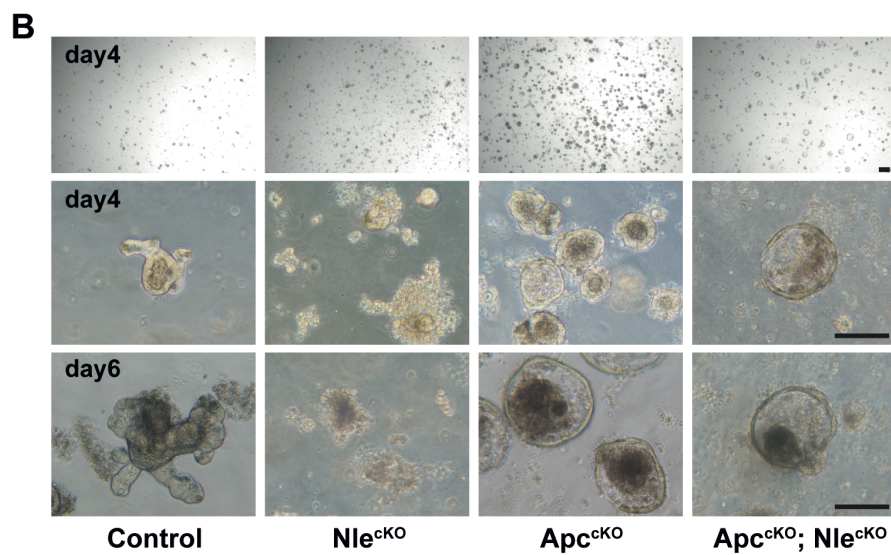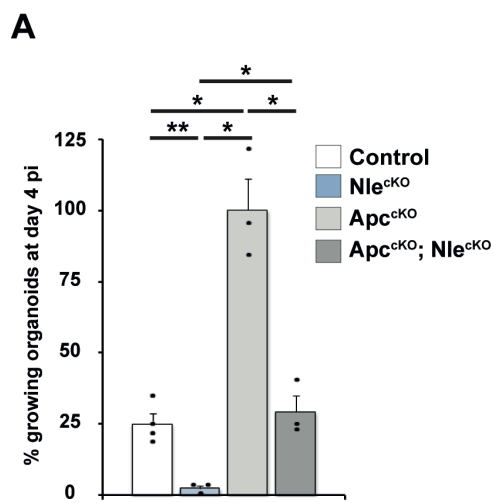Control   Apc^cKO

Mock   10µM nutlin-3   Mock   10µM nutlin-3

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

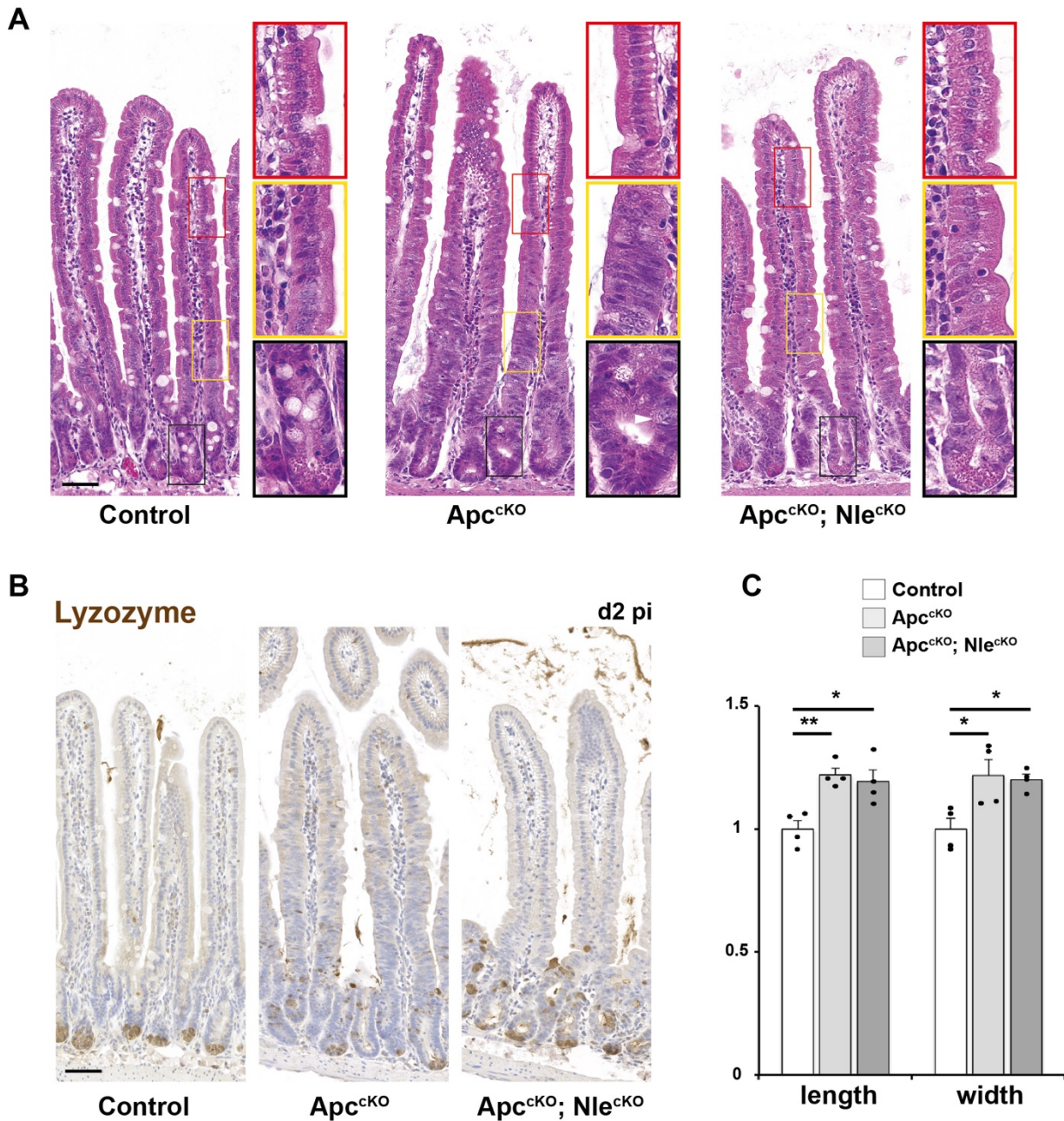**Supplementary Table 2: Sequences of RT-qPCR primers**

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 µm. For each genotype, a portion of the villus is magnified (top) to highlight enterocyte hypertrophia, 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 µm. (C) Histogram showing the mean (± 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.
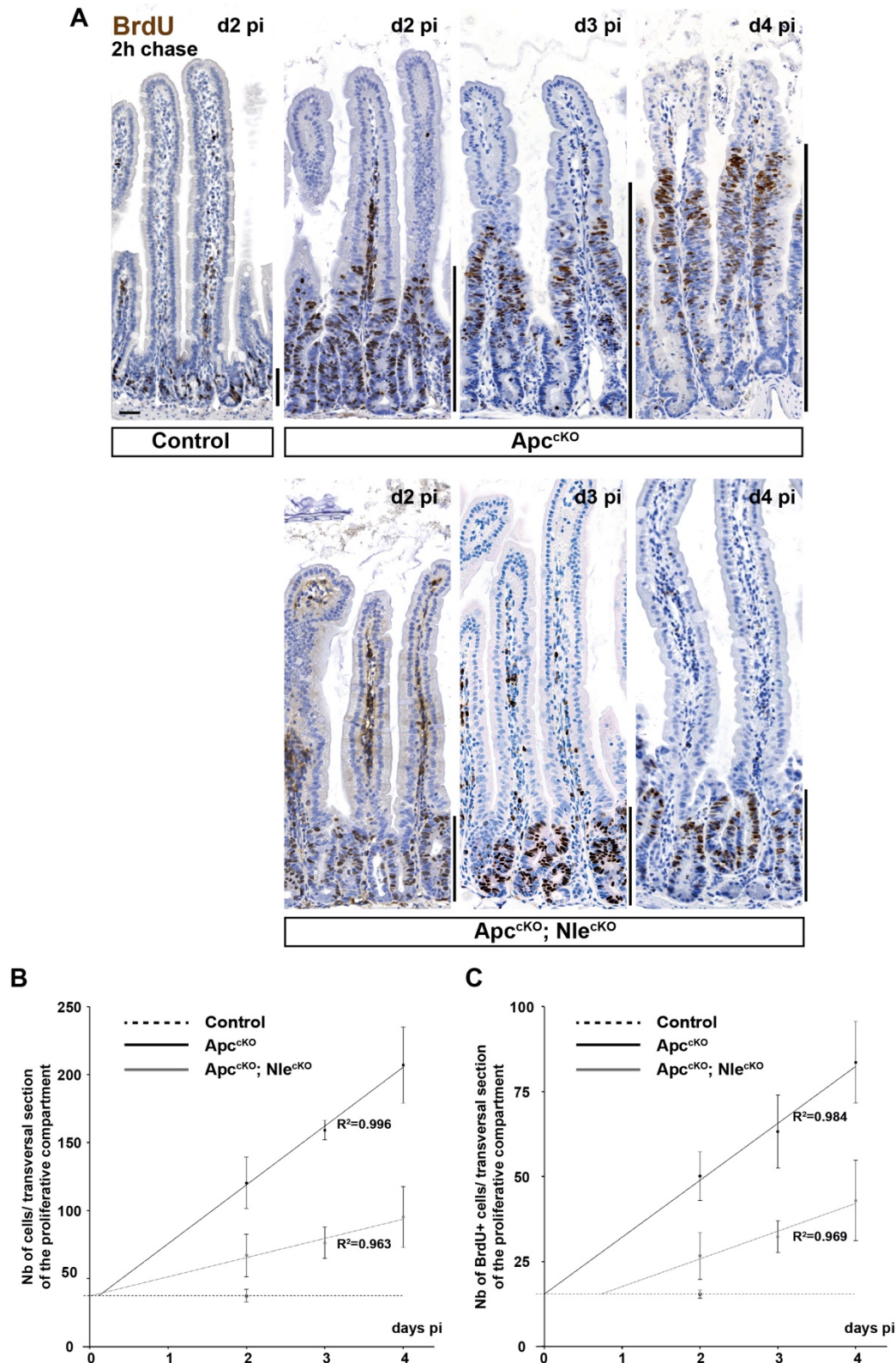
Figure S2 *Nle* **loss-of-function reduces proliferative compartment expansion rate in the** *Apc*-**deficient epithelium**. (A) BrdU immunostaining (brown) counterstained with hematoxylin (blue) on intestinal epithelium sections from Control (day 2 pi), Apc^{cKO} and Apc^{cKO}; Nle^{cKO} (days 2, 3 and 4 pi) intestines harvested 2 hours after BrdU injection. Scale bars, 50 μm. (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}.
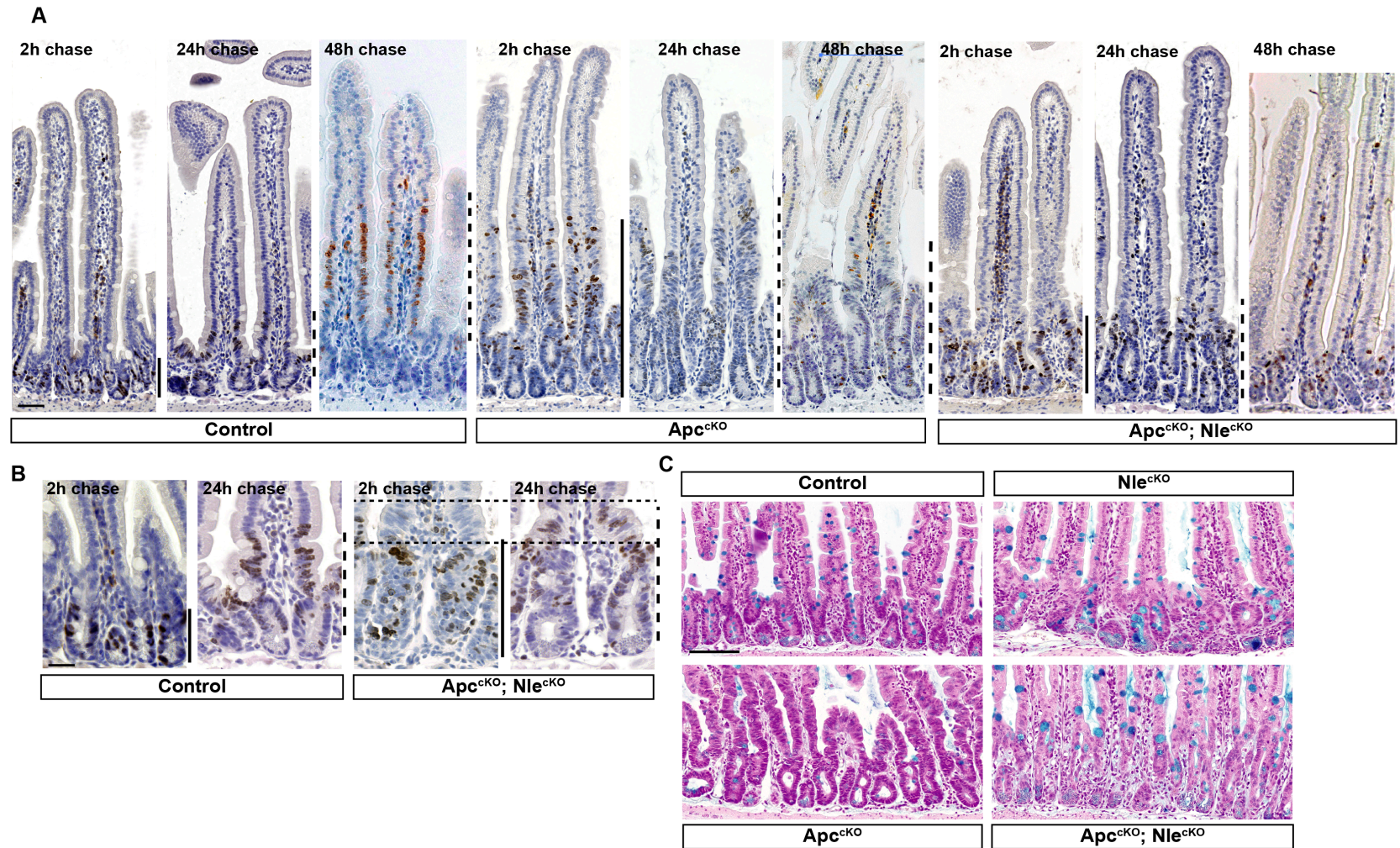
Figure S3 Cell cycle exit is delayed in the double mutant compared to the control. (A) BrdU immunostaining (brown) counterstained with Hematoxylin (blue) on intestinal epithelium sections from Control and Apc^{cKO}; Nle^{cKO} intestines at day 2 pi harvested 2, 24 or 48 hours after BrdU injection. Plain black bars indicate the proliferative compartment. Dotted black bars indicate the range of proliferative cell migration within 24 or 48 hours. Scale bars, 50 µm. (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 µm. (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 µm.
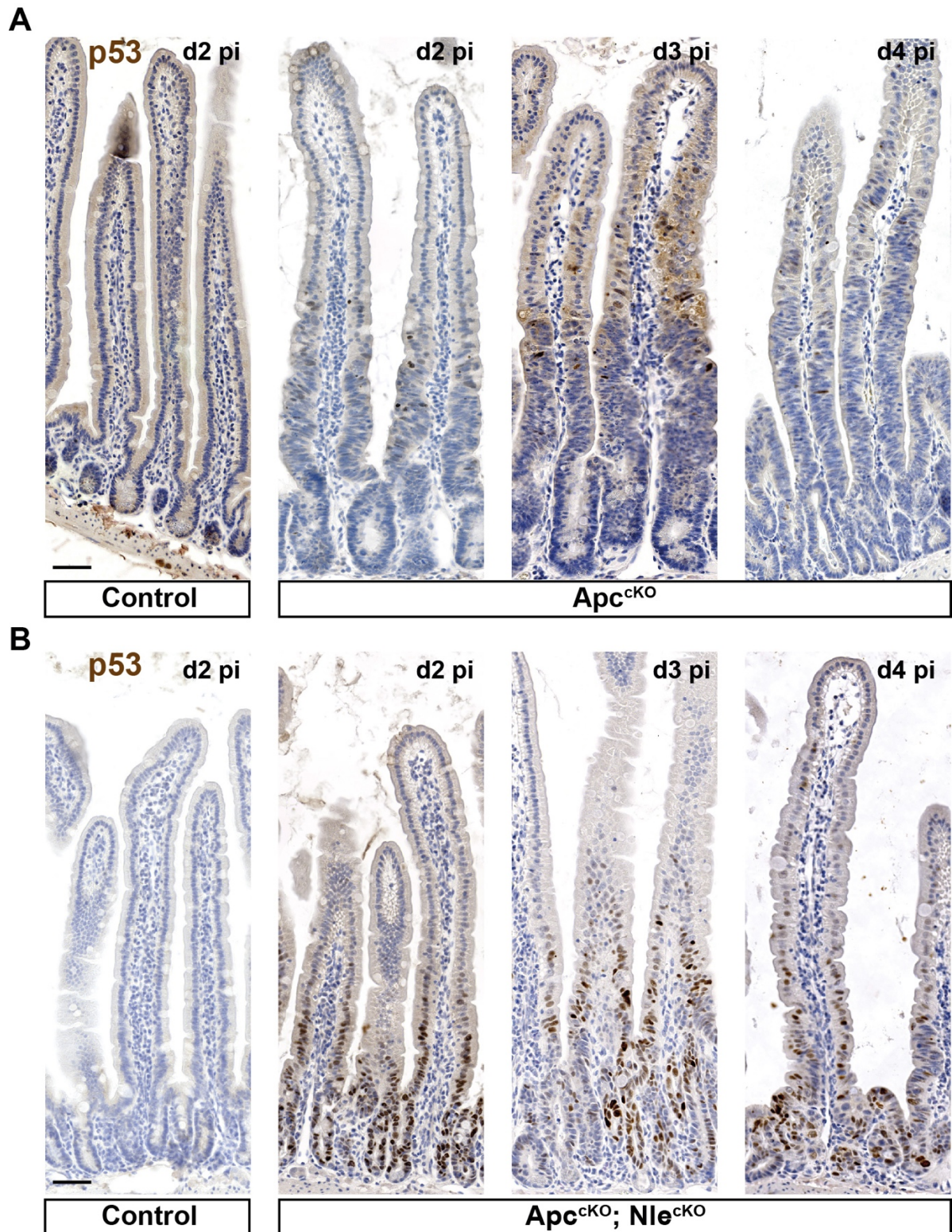
Figure S4 *Nle* loss-of-function induces persistent p53 stabilization in the *Apc*-deficient epithelium. (A) p53 immunostaining (brown) counterstained with Hematoxylin (blue) on intestinal epithelium sections from Control (day 2 pi) and Apc^{cKO} (days 2, 3 and 4 pi) intestines. Scale bars, 50 μm. (B) 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.

genes

GO terms
Associated phenotypes (Mousemine)

[AI-I-]
Max normalised expression

c-Myc *Ascl2*	Nucleic acid metabolism
Axin2 *Sox9*	Abnormal survival
Fz6	
Ppan	

- - - - - - - - - - - - - -   - - - - - - - - - - - - - -

p29	Nucleic acid metabolism
Pw1	Homeostasis phenotype
Tert	

[-INI-]
Max normalised expression

p21	G1/S transition
Mdm2	Tissue development
Caspase14	Abnormal survival

- - - - - - - - - - - - - -   - - - - - - - - - - - - - -

p27	Cell Cycle
p18	Abnormal survival
CyclinA2	
CyclinB2	

[-I-ID]
Max normalised expression

Alcam	Cell adhesion
Cercam	

- - - - - - - - - - - - - -   - - - - - - - - - - - - - -

Adrbk2	Regulation of
F2r	metabolism

[AI-ID]
Max normalised expression

- - - - - - - - - - - - - -   - - - - - - - - - - - - - -

Mcm2, Mcm3, Mcm4	DNA replication
Mcm5, Mcm8, Lgr5,	Abnormal survival
Mex3a, Lrig1, Musashi	

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Gata4	Nucleic acid metabolism
	Immune response
	Abnormal homeostasis

[-INID]
Max normalised expression

Krt15	Cellular response
CyclinD2	to DNA damage

- - - - - - - - - - - - - -   - - - - - - - - - - - - - -

Cd44	-

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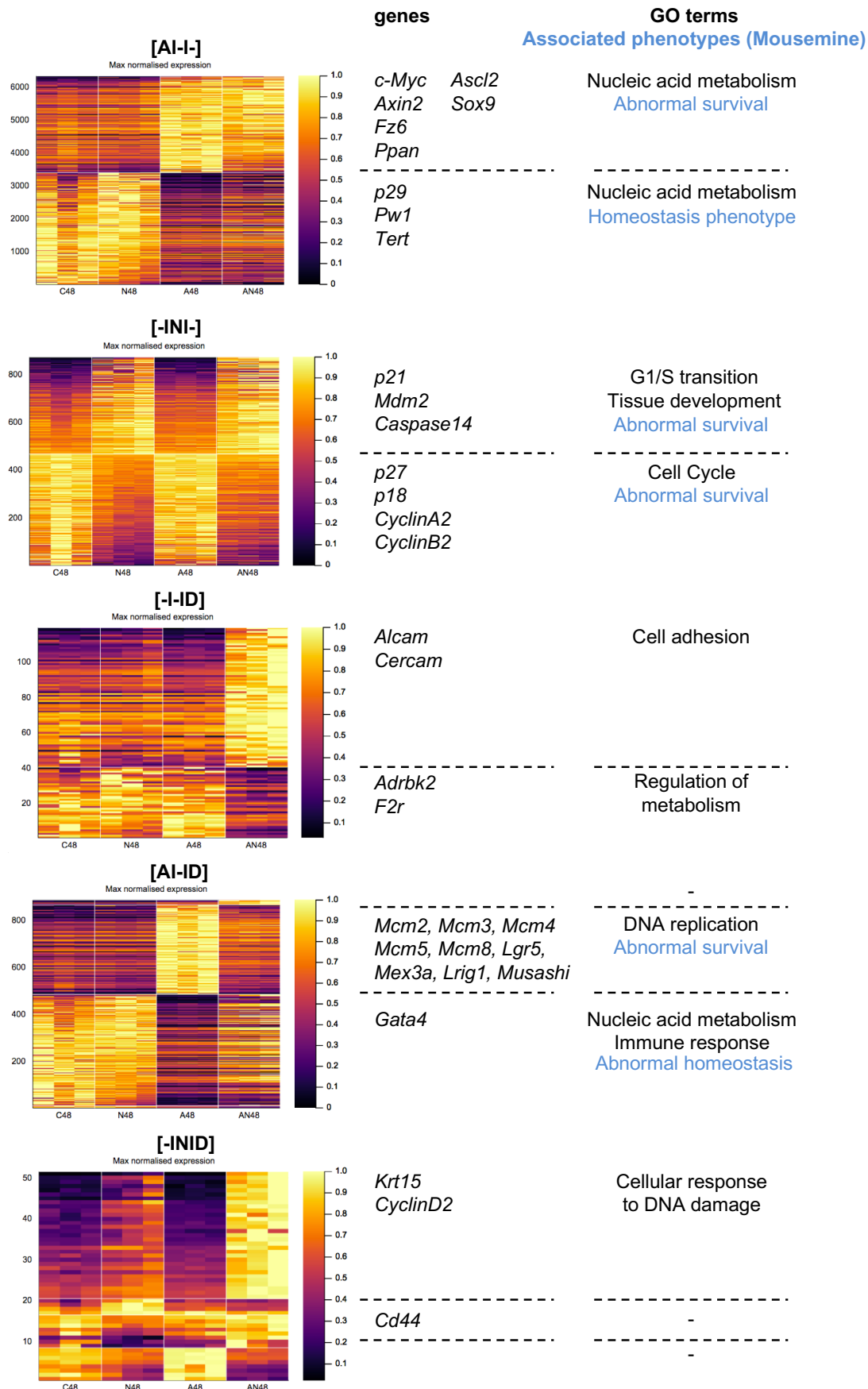
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Figure S5 Functional analysis of gene clusters does not indicate signaling pathway interference between *Apc* loss-of-function and *Nle* loss-of-function. Genes in the [A|N|-] and [A|N|D] categories were separated into clusters by the direction of 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.
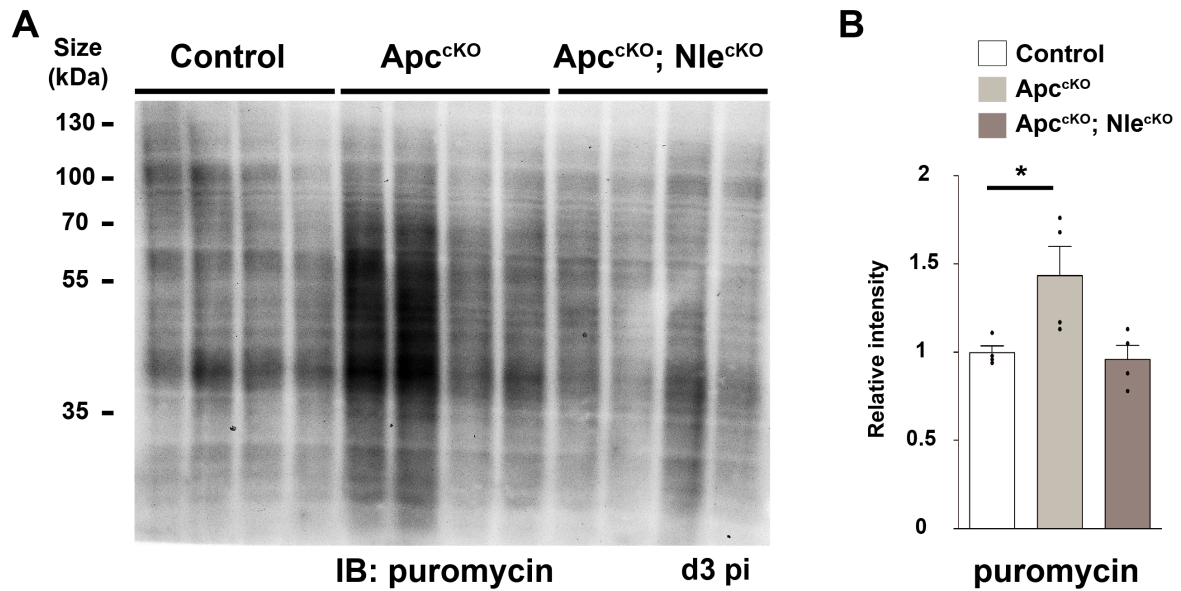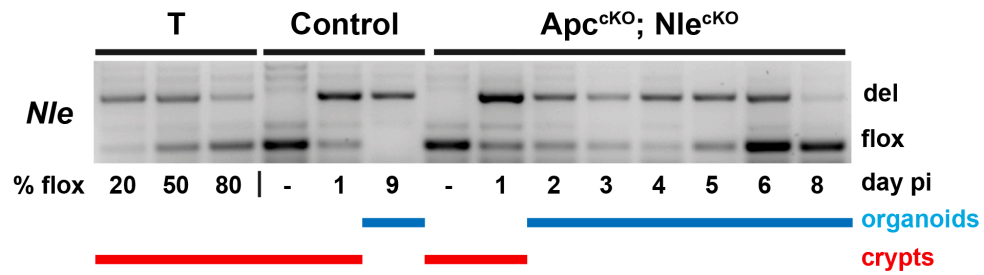
Figure S6 Global protein synthesis at day 3 pi. (A) Anti-puromycin immunoblotting of protein extracts for identical number of crypts cells from Control, Apc^{cKO} and Apc^{cKO}; Nle^{cKO} intestines at day 3 pi. (B) Histogram showing the mean normalized signal intensity ± S.E.M from the immunoblot shown in (A). n = 4 for each genotype. *, p<0.05 according to Mann–Whitney Wilcoxon test.

A

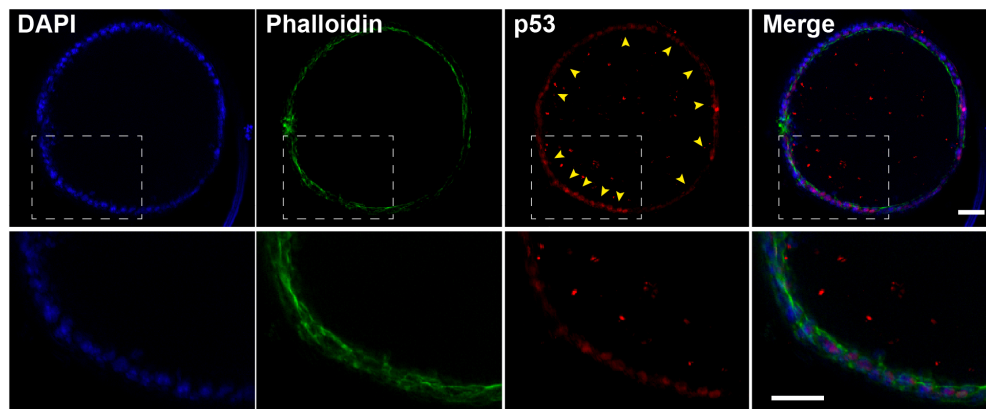**B**

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 *et al.*, 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
Olfm4	ATCAGCGCTCCTTCTGTGAT	AGGGTTCTCTCTGGATGCTG
Lgr5	ACATTCCCAAGGGAGCGTTC	ATGTGGTTGGCATCTAGGCG
Muc2	CAAGGGCTCGGAACTCCAG	CCAGGGAATCGGTAGACATCG
ChromoA	AGTCATCTCCGACTCGCTGT	GGTGTCGCAGGATAGAGAGG
c-Myc	AAGGCCCCCAAGGTAGTG	TGCTCGTCTGCTTGAATGGA
Axin 2	GATTCCCCTTTGACCAGGTGG	CCATTACAAGCAAACCAGAAGT
Its1	TCTGACCTCGCCACCCTA	CCTCGTAGACACGGAAGAGC
Its2	TGTGTGTGTTTGGGTCTTGC	GGATACCACCTCTCTCCGTTC
28S	TCATCAGACCCCAGAAAAGG	GATTCGGCAGGTGAGTTGTT
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT