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ORIGINAL ARTICLE

Induction of endoplasmic reticulum stress by deletion of *Grp78* depletes *Apc* mutant intestinal epithelial stem cells

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Intestinal epithelial stem cells are highly sensitive to differentiation induced by endoplasmic reticulum (ER) stress. Colorectal cancer develops from mutated intestinal epithelial stem cells. The most frequent initiating mutation occurs in *Apc*, which results in hyperactivated Wnt signalling. This causes hyperproliferation and reduced sensitivity to chemotherapy, but whether these mutated stem cells are sensitive to ER stress induced differentiation remains unknown. Here we examined this by generating mice in which both *Apc* and ER stress repressor chaperone *Grp78* can be conditionally deleted from the intestinal epithelium. For molecular studies, we used intestinal organoids derived from these mice. Homozygous loss of *Apc* alone resulted in crypt elongation, activation of the Wnt signature and accumulation of intestinal epithelial stem cells, as expected. This phenotype was however completely rescued on activation of ER stress by additional deletion of *Grp78*. In these *Apc-Grp78* double mutant animals, stem cells were rapidly lost and repopulation occurred by non-mutant cells that had escaped recombination, suggesting that *Apc-Grp78* double mutant stem cells had lost self-renewal capacity. Although in *Apc-Grp78* double mutant mice the Wnt signature was lost, these intestines exhibited ubiquitous epithelial presence of nuclear β-catenin. This suggests that ER stress interferes with Wnt signalling downstream of nuclear β-catenin. In conclusion, our findings indicate that ER stress signalling results in loss of *Apc* mutated intestinal epithelial stem cells by interference with the Wnt signature. In contrast to many known inhibitors of Wnt signalling, ER stress acts downstream of β-catenin. Therefore, ER stress poses a promising target in colorectal cancers, which develop as a result of Wnt activating mutations.

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INTRODUCTION

Colorectal cancer forms from precursor lesions known as adenomas. These lesions develop as the result of oncogenic mutations that occur in intestinal epithelial stem cells.[1–3] Tight control over intestinal stem cell differentiation is thus required to maintain homeostasis and to protect from cancer development.

We have previously shown that stress emanating from the endoplasmic reticulum (ER) and subsequent activation of the unfolded protein response (UPR) results in differentiation of intestinal epithelial stem cells into transit amplifying cells.[4] Under homeostatic conditions, the ER chaperone Grp78 resides at the ER membrane and inhibits activity of three transmembrane proteins: IRE1, ATF6 and PERK. When misfolded proteins accumulate, chaperones dissociate from these transmembrane proteins and UPR signalling is initiated. PERK, phosphorylates eIF2α resulting in a temporary halt of global protein translation. IRE1α and ATF6 signalling result in activation of a transcriptional programme that expands the capacity of the ER to meet increased demands for protein processing.

Besides its regulatory role in ER homeostasis, UPR activity has increasingly been recognised in cell fate decisions.[5,6]

Using mice in which ER stress was induced in the intestinal epithelium specifically by deletion of chaperone *Grp78*, we have previously found that mutant stem cells were lost and replaced by new stem cells derived from non-recombined cells, a process called repopulation. We identified *Perk-eIF2α* signalling as responsible for ER stress induced stem cell loss. In addition to our work in the intestine, an identical role for ER stress signalling was observed in oesophageal epithelium and in hematopoietic stem cells.[6,7]

Intestinal epithelial stem cells thus exhibit marked sensitivity to ER stress, which can trigger their differentiation. It is however unknown whether this sensitivity applies to cells that have already acquired oncogenic mutations that prime them towards malignancy. We hypothesize that intestinal epithelial stem cells that have obtained tumourigenic mutations are still sensitive to ER stress induced differentiation.

We investigate this using *VillinCre^{ERT2}-Apc$^{fl/fl(IEC)}$* animals in which both alleles of the tumour suppressor gene *Apc* can be deleted at the same time from the intestinal epithelium on injection with tamoxifen. *Apc* is a gatekeeper gene that constrains Wnt signalling activity. In humans, homozygous mutations in APC are found in 90% of sporadic colorectal carcinomas and those tumours that do not carry APC mutations often have activating mutations in Wnt signalling transcription factor β-catenin.[8] Heterozygosity of *Apc* both in mice and humans results in development of multiple adenoma's throughout the intestine, a syndrome in humans known as familial adenomatous polyposis. Homozygous deletion

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of *Apc* from the intestinal epithelium, results in prompt progenitor cell expansion in the crypt, eventually causing adenomatous tissue to develop throughout the intestine.[9] Moreover, hyperactivated Wnt signalling protects against chemotherapeutics.[10] We set out to determine the effect of ER stress on intestinal epithelium in which we homozygously delete *Apc*.

RESULTS

ER stress abrogates Wnt signalling *in vitro* and *ex vivo*

The starting point of these studies was the observation that a large number of Wnt signalling target-genes are downregulated upon induction of ER stress by subtilase cytotoxin (SubAB) treatment *in vitro*.[4] We had previously performed messenger RNA (mRNA) micro-array analysis on LS174T colorectal cancer cells that were treated with SubAB to induce ER stress compared with cells that were treated with enzymatically inactive $SubA_{A272}B$ as control. Treatment with SubAB results in specific proteolysis of GRP78 inside the ER, resulting in UPR activation.[11,12] Upon SubAB treatment we observed downregulation of a large number of Wnt target-genes, such as *LGR5*, *AXIN2*, *CD44*, *EPHB2* and *EPHB3* (Figure 1a).

To further analyse Wnt signalling pathway activity, we performed geneset enrichment analysis (GSEA) for the Wnt signature in these cells. We extracted the LS174T specific Wnt signature from mRNA expression arrays performed with cells in which Wnt signalling was blocked by dominant negative expression of effector transcription factor TCF4.[13] We found that shortly after induction of ER stress in LS174T cells using SubAB, the Wnt signalling gene signature was significantly lost (Figure 1b).

We additionally analysed the effect of ER stress on Wnt signalling in organoids. To this end we generated $Apc^{fl/fl}$ organoids in which treatment with tamoxifen metabolite 4OHT resulted in homozygous deletion of *Apc*. Upon recombination, these organoids exhibit highly activated Wnt signalling and become hyperproliferative. We treated non-recombined (wild type) and recombined ($Apc^{-/-}$) organoids with SubAB and tested a panel of Wnt target-genes. Both in wild type and *Apc* mutant organoids, we found that SubAB induced ER stress results in downregulation of Wnt target-genes (Figure 1c).

To assess the extent of UPR activation upon loss of *Grp78*, we performed microarray analysis of $Grp78^{fl/fl}$ organoids in which treatment with 4OHT had resulted in knockout of *Grp78*. By GSEA we found significant induction of the UPR signature (Supplementary Figure. 1). Further GSEA showed profound loss of both the Wnt signalling geneset and the intestinal epithelial stem cell geneset on deletion of *Grp78*. These results show that induction of ER stress, by loss of *Grp78*, compromises the Wnt signalling gene signature *in vitro* and *ex vivo*.

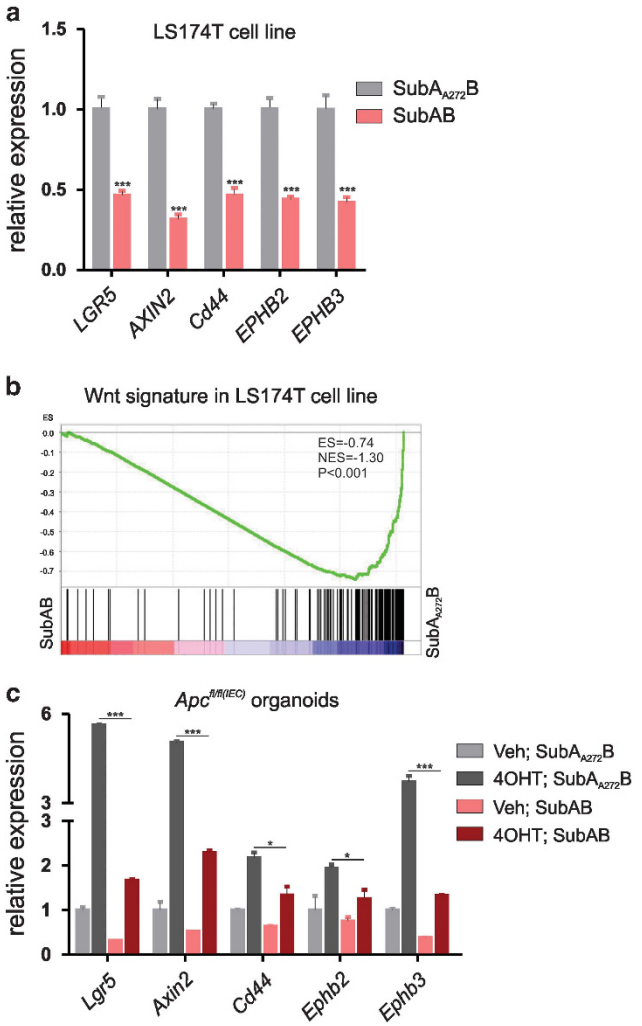

Figure 1. ER stress signalling represses the Wnt signalling signature *in vitro*. (**a**) Relative mRNA levels of a panel of Wnt target-genes from mRNA expression arrays of LS174T cells on 20 h treatment with SubAB (200 ng/ml) or the enzymatically inactive $SubA_{A272}B$ as a control. Values are extracted from mRNA micro-array. (**b**) GSEA of Wnt signalling pathway target-genes on LS174T cells that were treated with either SubAB or $SubA_{A272}B$. (**c**) mRNA expression in $Apc^{fl/fl(IEC)}$ organoids that were either treated with vehicle (veh) or 4OHT for 24 h followed by either 200 ng/ml SubAB or $SubA_{A272}B$ treatment for 20 h. $*P < 0.05$, $***P < 0.001$.

Effects of ER stress on the intestinal epithelium are dominant over the phenotype of hyperactive Wnt signalling

In $Grp78^{fl/fl}$ mice, we previously showed that intestinal epithelial stem cells are lost as soon as 24 h after recombination. In the following days, crypts become hypoplastic and crypt epithelium becomes thin.[4] Conversely, loss of *Apc* leads to increased proliferation and stem cell expansion resulting from hyperactive Wnt signalling. This causes crypt enlargement, which is visible most clearly from day 3 post induction (p.i.).[9,14,15] In a number of days thereafter, the intestine fills with adenomatous tissue, proving that homozygous loss of *Apc* is sufficient for adenomagenesis.[16] To investigate whether the $Apc^{-/-}$ intestinal epithelium is sensitive to ER stress mediated stem cell differentiation, we crossed $Apc^{fl/fl}$ animals to $Grp78^{fl/fl}$ and $Villin^{CreERT2}$ mice.

In these animals, injections with tamoxifen result in recombination restricted to the small intestinal and colonic epithelium.[17] Recombination occurs to a similar extent in stem cells, transit amplifying cells and all differentiated cell types, including Paneth cells that reside at the crypt base and which are reported to play a critical role in maintenance of the stem cell niche.[18] We generated animals in which intestinal epithelial cell (IEC) specific deletion of *Grp78*, *Apc* or both *Grp78* and *Apc* alleles occurred on injections with tamoxifen. In this fashion we could induce IEC specific ER stress ($Grp78^{-/-(IEC)}$), Wnt signalling pathway hyperactivation ($Apc^{-/-(IEC)}$) or both ER stress and Wnt signalling hyperactivation ($Apc^{-/-}Grp78^{-/-(IEC)}$). We compared these animals to littermate controls that either carried the wild type alleles of both *Apc* and *Grp78* or lacked the $Villin^{CreERT2}$ allele. To monitor Cre-mediated recombination on tissue sections we crossed $Rosa26^{LacZ}$ or $Rosa26^{ZsGreen}$ reporter alleles into all animals. We killed animals on day 3 p.i., as effects of both ER stress and Wnt signalling hyperactivation are clearly observed at that time point.

In concordance with previous reports we observed that $Apc^{-/-(IEC)}$ animals displayed crypt elongation and densely

packed crypt cells.[9] Conversely, *Grp78*$^{-/-(IEC)}$ animals displayed epithelial thinning and crypt hypoplasia, similar to our previous findings in *Ah1*^{Cre}-*Grp78*$^{-/-(IEC)}$ animals.[4] Combined knockout in *Apc*$^{-/-}$*Grp78*$^{-/-(IEC)}$ animals resulted in crypts that mostly resembled *Grp78*$^{-/-(IEC)}$ crypts with reduced length and marked thinning of crypt cells (Figures 2a and b).

Analysis of expression of the ZsGreen and LacZ reporter alleles, showed that recombination efficiency was excellent (>95% of all crypts, Supplementary Figure. 2). Also, staining for Grp78 confirmed absence of the protein in *Grp78*$^{-/-(IEC)}$ and *Apc*$^{-/-}$*Grp78*$^{-/-(IEC)}$ mice (Figure 2c). To confirm recombination on the mRNA level we performed quantitative reverse transcription PCR (RT-PCR) analysis of *Grp78* and *Apc* on isolated epithelial fractions. In *Apc*$^{-/-(IEC)}$ and *Apc*$^{-/-}$*Grp78*$^{-/-(IEC)}$ animals, *Apc* mRNA levels were significantly reduced and in *Grp78*$^{-/-(IEC)}$ and *Apc*$^{-/-}$*Grp78*$^{-/-(IEC)}$ animals, *Grp78* mRNA levels were significantly reduced (Figure 2d).

We did not detect morphological differences between *Villin*^{CreERT2}-*Grp78*$^{-/-(IEC)}$ animals and our previously published *Ah1*^{Cre}-*Grp78*$^{-/-(IEC)}$ animals although recombination in *Ah1*^{Cre} excludes Paneth cells. To assess effects of *Grp78* deletion on Paneth cell presence and distribution, we performed staining for lysozyme and found that similar to control animals, Paneth cells were present in all crypts of animals lacking *Grp78* (*Villin*^{CreERT2}-*Grp78*$^{-/-(IEC)}$ and *Villin*^{CreERT2}-*Apc*$^{-/-}$*Grp78*$^{-/-(IEC)}$) with distribution similar to wild type (Supplementary Figure. 3). We therefore concluded that the phenotype obtained upon deletion of *Grp78* is not dependent on its signalling in Paneth cells or on presence of these cells.

Hyperactivation of Wnt signalling results in progenitor cell proliferation and stem cell expansion, whereas deletion of *Grp78* induces stem cell differentiation at 24 h, followed by loss of proliferation at ~72 h.[4,9] We therefore next analysed intestinal proliferation. As expected, *Apc*$^{-/-(IEC)}$ mice had increased amounts of BrdU-positive cells per crypt (18.4 vs 10.3 in controls, $P < 0.001$) whereas the intestines of *Grp78*$^{-/-(IEC)}$ mice displayed reduced proliferation (3.1 vs 10.3 in controls, $P < 0.001$). In *Apc*$^{-/-}$*Grp78*$^{-/-(IEC)}$ mice, BrdU incorporation was reduced to the level of *Grp78*$^{-/-(IEC)}$ mice (2.7 vs 10.3 in controls, $P < 0.001$; Figures 2e and f).

It has previously been described that homozygous deletion of *Apc* results in increased apoptosis of crypt cells.[19] Analysis of cleaved caspase-3 indeed showed increased numbers of apoptotic cells in crypts of *Apc*$^{-/-(IEC)}$ mice (0.878 vs 0.386 in controls, $P < 0.05$), whereas apoptosis was unaltered in *Grp78*$^{-/-(IEC)}$ mice (0.267 vs 0.386 in controls, NS). In *Apc*$^{-/-}$*Grp78*$^{-/-(IEC)}$ animals, numbers of apoptotic cells were low, similar to numbers observed in in *Grp78*$^{-/-(IEC)}$ mice (0.233 vs 0.386 in controls, NS; Figures 2g and h). Taken together, simultaneous deletion of *Grp78* to induce ER stress and *Apc* to induce hyperactive Wnt signalling from the intestinal epithelium results in a phenotype that completely rescues the *Apc*$^{-/-(IEC)}$ phenotype in terms of crypt length, proliferation and apoptosis, and highly resembles the phenotype of *Grp78*$^{-/-}$ epithelium. We

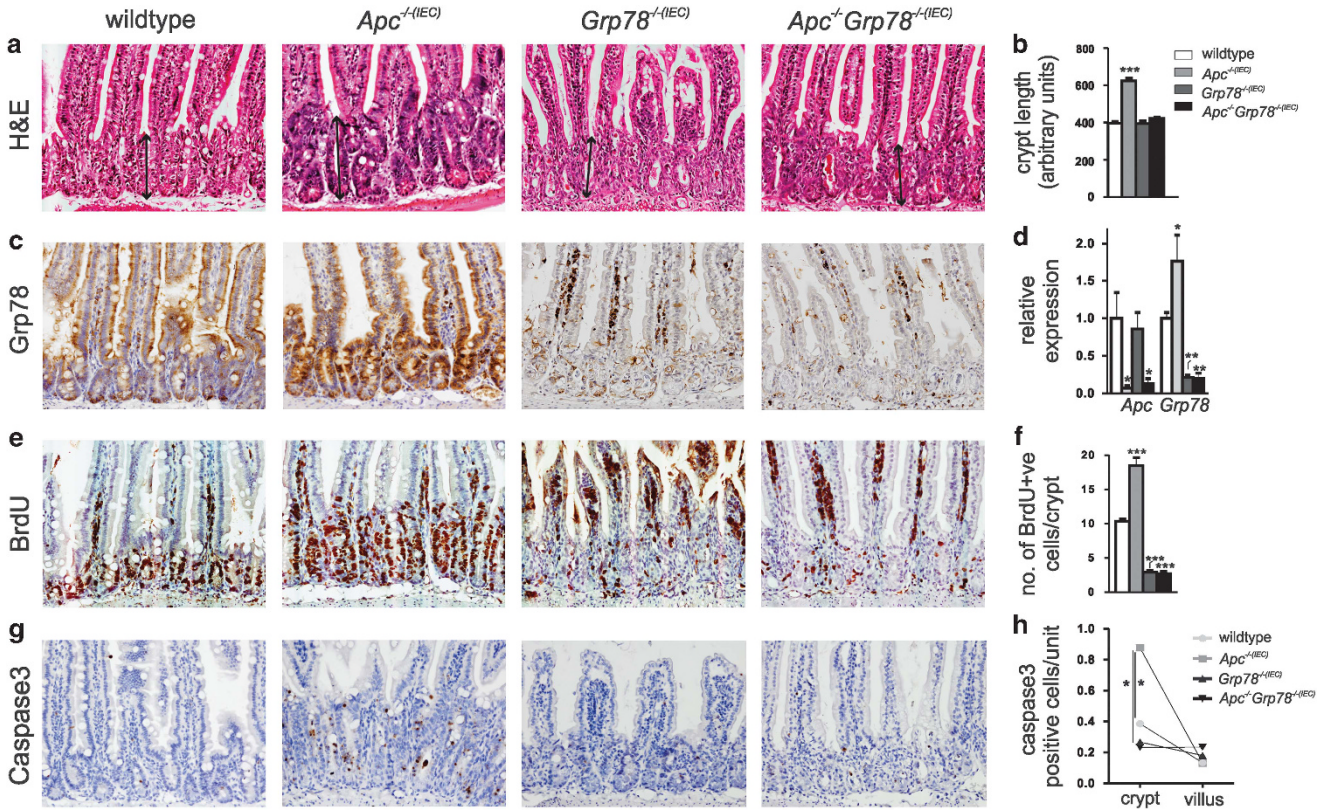

Figure 2. Induction of ER stress by loss of *Grp78* causes *Apc*$^{-/-}$*Grp78*$^{-/-(IEC)}$ crypts to resemble *Grp78*$^{-/-(IEC)}$ crypts. (**a**) Haematoxylin and eosin staining on representative small intestinal sections of animals of indicated genotypes on day 3 after tamoxifen injections. Arrows depict a representative morphometric measurement. (**b**) Relative crypt length of animals of indicated genotype. (**c**) Grp78 staining on small intestinal sections of animals of indicated genotypes. (**d**) Quantitative RT-PCR analysis of *Apc* and *Grp78* from intestinal epithelial fractions. (e) BrdU staining on small intestinal sections of animals of indicated genotypes. (**f**) Quantification of BrdU-positive cells per crypt. (**g**) Cleaved caspase-3 staining on representative small intestinal sections of animals of indicated genotypes. (**h**) Quantification of cleaved caspase-3-positive cells in either crypts or villi. Quantifications were performed in 30 crypts per animal, $N = 5$ animals per genotype. Magnifications ×200. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

thus conclude that activation of the UPR leads to loss of both normal and *Apc* mutant stem cells *in vivo*.

Wnt signalling pathway target-genes are not increased in Apc$^{-/-}$Grp78$^{-/-\,(IEC)}$ epithelium while high expression of ER stress markers is maintained

As intestines of *Apc*$^{-/-}$*Grp78*$^{-/-\,(IEC)}$ mice did not exhibit a histological phenotype of Wnt signalling activation, we next evaluated gene expression levels of Wnt signalling target-genes in epithelium of these mice. To this end we used intestinal organoids of primary epithelium from mice of all three different genotypes and compared all organoids to non-recombined controls. Organoids were recombined by 4OHT treatment for 24 h, and harvested 24 h later. Recombination was excellent, as judged by mRNA levels of *Apc* and *Grp78* after deletion (Figure 3a). All organoids carried the *Rosa26*^{LacZ} reporter allele which enabled verifying recombination efficacy by lacZ staining (Figure 3b). mRNA expression analysis confirmed that all tested Wnt target-genes (*Axin2; CD44; Ephb2; Ephb3*) were upregulated in *Apc*$^{-/-\,(IEC)}$ organoids (Figure 3c). In *Apc*$^{-/-}$*Grp78*$^{-/-\,(IEC)}$ organoids these genes were downregulated. To test whether downregulation of Wnt targets extended from mRNA to the level of protein expression we performed immunohistochemical staining for CD44 and Ephb2 and indeed found upregulation of these proteins in *Apc*$^{-/-\,(IEC)}$ epithelium and downregulation in both *Grp78*$^{-/-\,(IEC)}$ and *Apc*$^{-/-}$*Grp78*$^{-/-\,(IEC)}$ epithelium (Figure 3d and Supplementary Figure. 4). Next, we analysed presence of ER stress and UPR activity in organoids of all genotypes. We found significant upregulation of UPR activation markers *Chop, Xbp1(s), Perk, Atf4 and Erdj4* in *Grp78*$^{-/-\,(IEC)}$ and *Apc*$^{-/-}$*Grp78*$^{-/-\,(IEC)}$ organoids. In *Apc*$^{-/-\,(IEC)}$ organoids, UPR target-genes were not upregulated, in contrast, *Xbp1(s)* was downregulated (Figure 3e). Thus, depletion of *Grp78* from the epithelium results in UPR activation, regardless of *Apc* status. In addition, Wnt signalling is abrogated phenotypically and on the level of target-genes in *Apc*$^{-/-}$*Grp78*$^{-/-\,(IEC)}$ epithelium.

Stem cell marker expression in Apc$^{-/-}$Grp78$^{-/-\,(IEC)}$ epithelium is similar to Grp78$^{-/-\,(IEC)}$ epithelium

Wnt signalling drives stem cell fate and animals that lack epithelial *Grp78* have profoundly reduced expression of Wnt signalling target-genes. To examine presence of stem cells at the crypt base (crypt base columnar cells), we performed *in situ* hybridisation for *Olfm4*.[20] As expected, we observed increased stem cell numbers in *Apc*$^{-/-\,(IEC)}$ animals and stem cells were absent from crypts in *Grp78*$^{-/-\,(IEC)}$ animals. Similarly, in crypts of *Apc*$^{-/-}$*Grp78*$^{-/-\,(IEC)}$

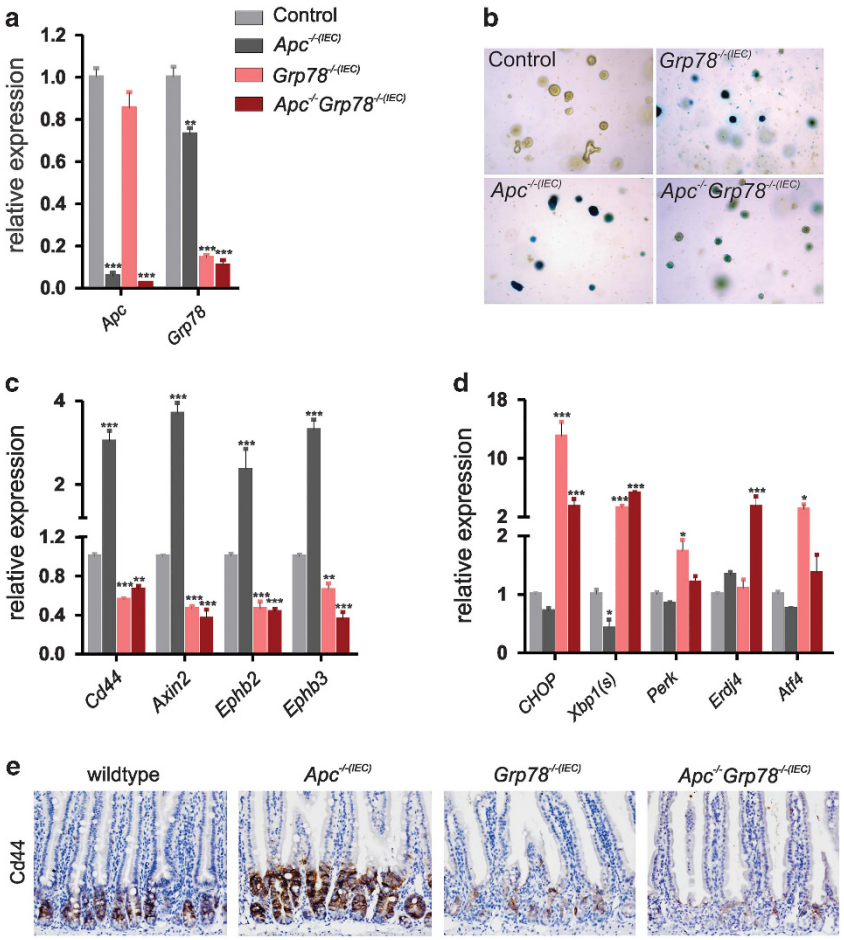

Figure 3. Loss of *Grp78* in *Apc* mutant epithelium dampens the increased expression of Wnt signalling target-genes. (**a**) Quantitative RT-PCR analysis of *Apc* and *Grp78* on either non-recombined organoids (control) or recombined organoids carrying the indicated genotypes. (**b**) Recombination efficiency of LacZ reporter allele assessed in organoids by X-gal staining. (**c**) Quantitative RT-PCR of a panel of markers of ER stress and activation of the UPR. (**d**) Quantitative RT-PCR of a panel of Wnt signalling pathway target-genes. (**e**) CD44 staining on representative small intestinal sections of animals of indicated genotypes. Magnifications × 100 for organoids and × 200 for intestinal sections. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

mice, we observed loss of all *Olfm4*-positive stem cells (Figure 4a). We next performed quantitative RT-PCR on organoids from these animals for stem cell markers *Olfm4* and *Lgr5*. Indeed, mRNA levels of these stem cell markers were high in $Apc^{-/-(IEC)}$ organoids and reduced in both $Grp78^{-/-(IEC)}$ and $Apc^{-/-}Grp78^{-/-(IEC)}$ organoids (Figure 4b). We next analysed alternative stem cell markers *Bmi1, Hopx* and *mTert*.[21–23] We found these transcripts to be upregulated in $Apc^{-/-}$ organoids but expression in $Grp78^{-/-(IEC)}$ and $Apc^{-/-}Grp78^{-/-(IEC)}$ organoids was variable (Figure 4c).

Thus, presence of *Grp78* is required for crypt base columnar stem cell fate. Sensitivity of these cells for ER stress is independent of *Apc* since cells in which *Apc* and *Grp78* are deleted simultaneously are rapidly lost on induction of ER stress.

ER stress induced loss of Wnt signalling occurs downstream of β-catenin

Deletion of both alleles of *Apc* results in accumulation of transcriptionally active, nuclear β-catenin throughout all crypt and villus cells.[9] To delineate the molecular basis of ER stress induced loss of the Wnt signature, we next assessed presence of β-catenin protein in intestines of animals from all four genotypes (Figure 5a). We confirmed *Grp78* deletion by staining for Grp78 on consecutive sections (Figure 5b). Under homeostatic conditions β-catenin is expressed on the membrane of all epithelial cells and nuclear localisation is solely found in cells at the crypt base. As expected, in $Apc^{-/-(IEC)}$ intestine, nuclear localisation of β-catenin extended to all epithelial cells, including those located in the differentiated compartment. Conversely, in crypts of $Grp78^{-/-(IEC)}$ mice we did not observe nuclear β-catenin in transit amplifying cells and villi. Surprisingly, in $Apc^{-/-}Grp78^{-/-(IEC)}$ mice, we found that although the expression of Wnt signalling target-genes was significantly reduced, nuclear β-catenin expression was detectable in all enterocytes. These data suggest that those components required for nuclear translocation of β-catenin upon deletion of *Apc*, are unaltered upon deletion of *Grp78* and that in $Apc^{-/-}Grp^{-/-(IEC)}$ epithelium, the Wnt signature is abrogated downstream of β-catenin.

Expression of c-Myc depends on Grp78 presence

In colorectal cancer cell lines, we previously identified that ER stress induced loss of stemness results from *Perk-eIF2a* signalling.[4] The resulting protein translation attenuation depletes cells of proteins with a short half-life that rely on continuous translation such as the oncogene *c-Myc*.[24] Moreover, *c-Myc* has been shown to be critical in Wnt signalling induced proliferation and for expression of the Wnt signature downstream of β-catenin.[2519] We therefore hypothesized that reduced proliferation and stemness in $Apc^{-/-}Grp78^{-/-(IEC)}$ animals correlated with reduced levels of *c-Myc*. Assessment of *c-Myc* levels on both mRNA and protein levels revealed increased expression in $Apc^{-/-(IEC)}$ mice compared with wild type, and strongly decreased expression in both $Grp78^{-/-(IEC)}$ and $Apc^{-/-}Grp78^{-/-(IEC)}$ mice (Figures 6a and b) despite abundant nuclear β-catenin levels in the double mutant mice.

We thus show that in $Apc^{-/-}Grp78^{-/-(IEC)}$ animals, suppression of Wnt signalling downstream of nuclear β-catenin results in severely compromised expression of *c-Myc*, potentially contributing to loss of stemness.

Loss of self-renewal capacity results in repopulation of $Apc^{-/-}Grp78^{-/-}$ cells by wild-type cells that evaded recombination

In previous experiments using $Ah1^{Cre}$-$Grp78^{-/-(IEC)}$ animals, we observed that *Grp78* mutant cells are replaced by wild-type cells that have evaded recombination, establishing loss of self-renewal capacity in these animals. To investigate self-renewal capacity of $Apc^{-/-}Grp78^{-/-}$ cells, we first analysed longevity of organoids to analyse functional self-renewal capacity. We found that $Apc^{-/-}Grp78^{-/-(IEC)}$ organoids remained small and eventually died within 7 days after recombination, contrasting the unimpaired

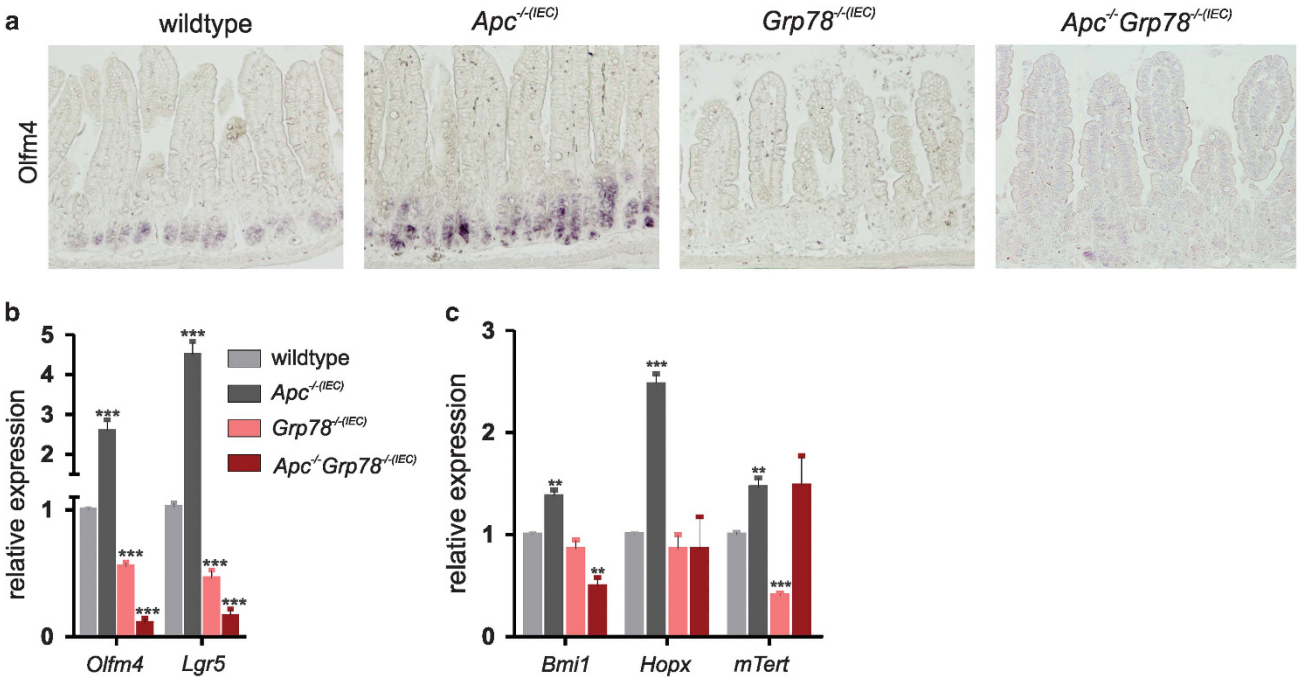

Figure 4. Loss of *Grp78* results in loss of intestinal epithelial stem cells regardless of *Apc* status. (**a**) *In situ* hybridisation for *Olfm4* in mice of indicated genotypes (**b**) Quantitative RT-PCR on recombined organoids with indicated genotypes and non-recombined organoids (control) for stem cell markers *Olfm4* and *Lgr5*. (**c**) Quantitative RT-PCR of a panel of alternative stem cell markers. $^{**}P < 0.01$, $^{***}P < 0.001$. Magnifications × 200.

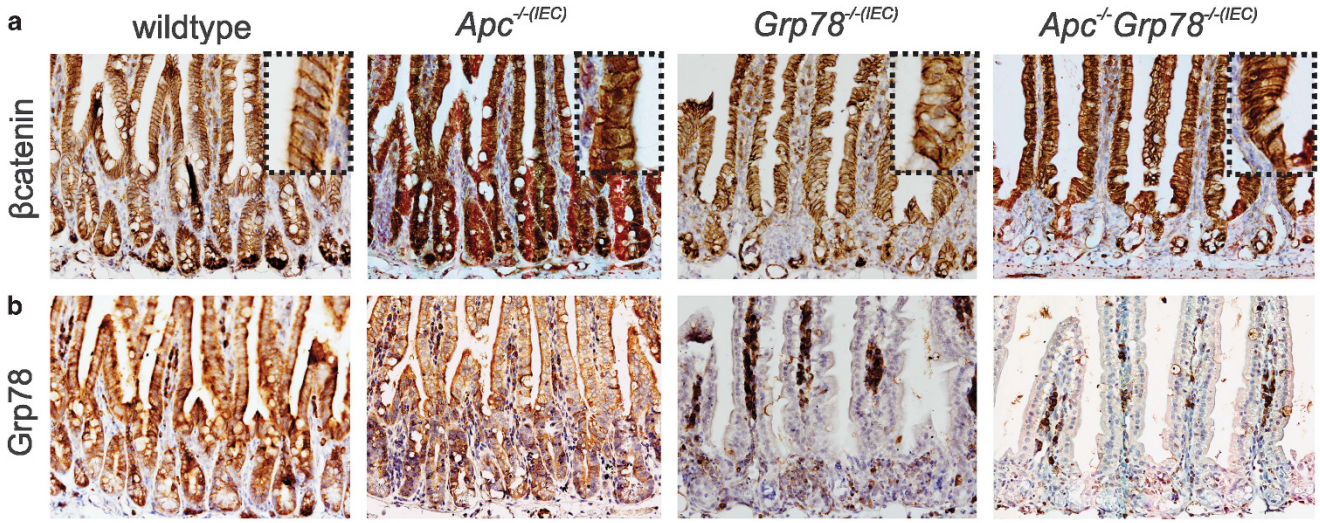

Figure 5. Maintenance of nuclear β-catenin in $Apc^{-/-}Grp78^{-/-(IEC)}$ mice. (**a**) β-catenin staining on small intestinal sections of animals of indicated genotypes. (**b**) Grp78 staining on consecutive slides. Magnifications × 200, zoom × 400.

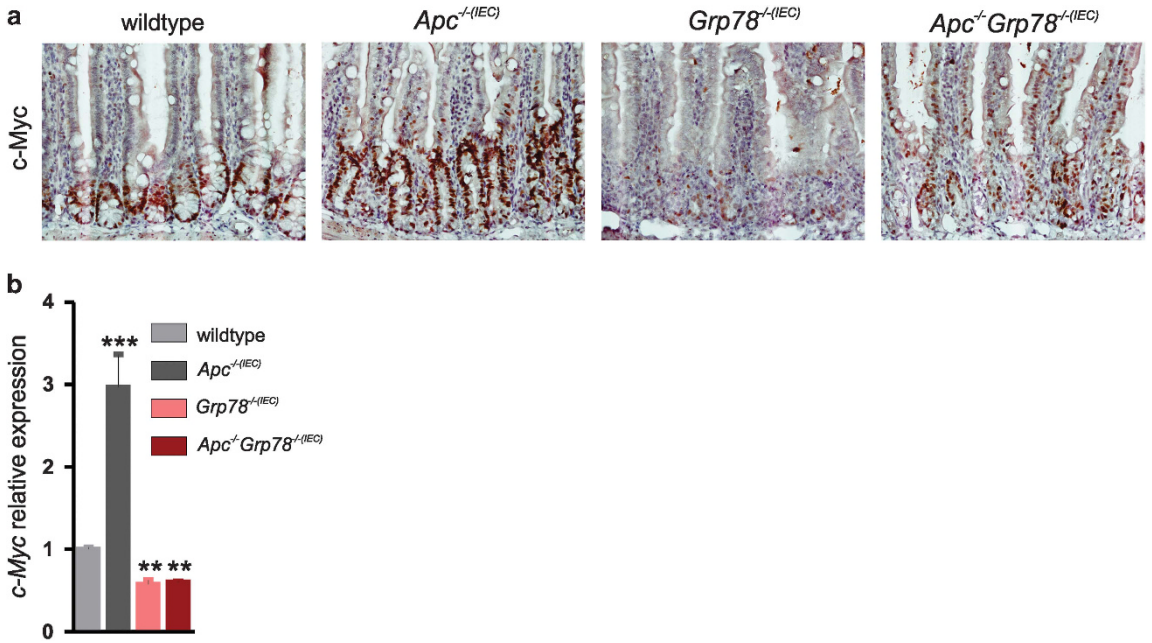

Figure 6. Loss of *Grp78* results in loss of c-Myc regardless of *Apc* status. (**a**) c-Myc staining on representative small intestinal sections of animals of indicated genotypes. Magnifications × 200. (**b**) Quantitative RT-PCR for *c-Myc* on organoids of indicated genotypes. $^{**}P < 0.01$, $^{***}P < 0.001$.

growth in non-recombined organoids (Figure 7a). Reseeding of either $Grp78^{-/-(IEC)}$ or $Apc^{-/-}Grp78^{-/-(IEC)}$ organoids did not result in growth as opposed to non-recombined and $Apc^{-/-}$ organoids, suggesting loss of self-renewal capacity in $Apc^{-/-}Grp78^{-/-(IEC)}$ epithelium *ex vivo*.

We performed an additional mouse experiment in which we gave two tamoxifen injections on consecutive days instead of five injections in 3 days. We killed animals on day 2 to 4 p.i., Similar to $Ah1^{Cre}$-$Grp78^{-/-(IEC)}$ mice, we observed that the epithelium of $Apc^{-/-}Grp78^{-/-(IEC)}$ animals was repopulated by wild type, *Grp78* proficient, cells (Figure 7b). At day 4 p.i., most mutant cells had been replaced by wild-type cells. We confirmed these findings by quantitative RT-PCR for *Grp78* mRNA expression, which increased significantly in the days following recombination (Figure 7c). These results show that self-renewal capacity of double mutant cells is lost and that *Grp78* presence is thus required for self-renewal of cells that have homozygously lost *Apc*.

DISCUSSION

The ER chaperone *Grp78* acts as a suppressor of the UPR. We previously found that activation of the UPR by depletion of *Grp78* results in rapid loss of intestinal epithelial stem cells by forced differentiation to a transit amplifying cell fate *in vivo*. We and others subsequently made similar observations in normal oesophageal and hematopoietic stem cells and in cultures of human colorectal cancer stem cells.[6,7,26] In the present study we used an *in vivo* model of intestinal epithelial adenomatous transformation to examine if activation of the UPR can correct intestinal adenomagenesis. To address this question we generated

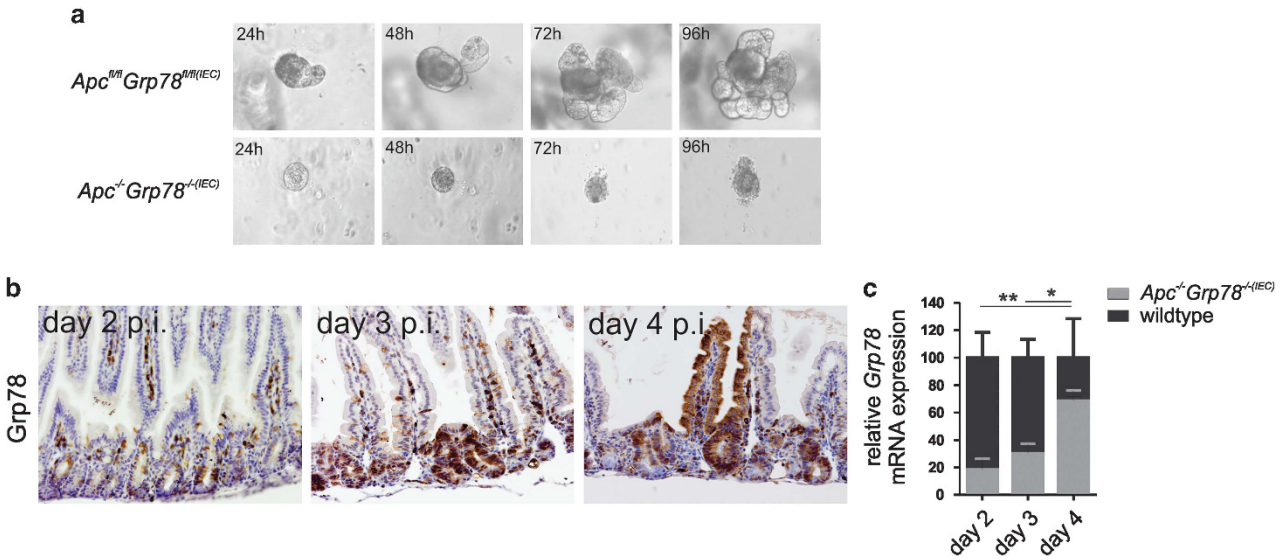

Figure 7. Rapid repopulation of the $Apc^{-/-}Grp78^{-/-(IEC)}$ epithelium by wild-type cells. (**a**) *Ex vivo* culture of organoids from indicated genotypes at indicated time points. (**b**) Immunohistochemical detection of Grp78 on indicated time points in $Apc^{-/-}Grp78^{-/-(IEC)}$ mice. (**c**) Relative expression of *Grp78* mRNA in epithelium derived from indicated mice, detected by quantitative RT-PCR, reflecting presence of wild-type cells. $N = 5$. Magnifications × 200. $^{*}P < 0.05$, $^{**}P < 0.01$.

animals in which homozygous deletion of *Apc* and *Grp78* from the intestinal epithelium occurs on tamoxifen injections. Deletion of *Grp78* completely rescued the *Apc* mutant phenotype, resulting in an intestinal epithelium with shortened crypts, loss of stem cells and loss of proliferation. This shows that activation of the UPR is sufficient to drive loss of *Apc* mutant intestinal epithelial stem cells *in vivo*. Similar to our previous observations in normal intestinal epithelium and oesophageal epithelial cells, we found no evidence that activation of the UPR resulted in epithelial cell loss by apoptosis. Instead, we found that the increased number of apoptotic cells observed in *Apc* mutant crypts was reduced in $Apc^{-/-}Grp78^{-/-(IEC)}$ animals. This suggests that stem cells may be lost by differentiation as was previously observed by lineage tracing in normal intestinal and oesophageal epithelial stem cells.[4,7]

To induce UPR activation we use deletion of *Grp78*. With genetic rescue experiments we have previously shown that stem cell loss on *Grp78* depletion specifically depends on UPR activation. Our current studies add to these observations by showing that in $Grp78^{-/-}$ epithelium, UPR pathway activity is significantly increased.

In contrast to our observations that the UPR rescues the *Apc* mutant phenotype, it was shown that deletion of UPR transcription factor *Xbp1* increases intestinal tumourigenesis.[27] This may be explained by the fact that *Grp78* deletion in our experiments results in activation of all three branches of the UPR instead of isolated perturbation of *Xbp1* signalling.

The definitive proof of loss of self-renewal capacity in $Apc^{-/-}Grp78^{-/-}$ double stem cells was provided by the observation that mutant crypts were repopulated by new stem cells that were negative for the LacZ reporter allele and had thus escaped Cre-mediated recombination. Such repopulation has previously been described for other genes that are critical for stem cell self-renewal such as *c-Myc*,[25] *Brg1*,[28] and the stem-cell-specific transcription factor *Ascl2*.[29] The most rapid repopulation was previously observed in 5 days after deletion of *Grp78* in the intestinal epithelium using the β-naphtoflavone inducible $Ah1^{Cre}$.[4] The time course of repopulation of $Apc^{-}Grp78$ double mutant stem cells is identical to that of *Grp78* single-mutant stem cells, suggesting that loss of stemness is independent of the *Apc* status and may therefore occur at some level below the function of *Apc*. Interestingly, although stem cell repopulation has been shown for multiple genes using $Ah1^{Cre}$ promoter driven recombination in which Paneth cells are not recombined, this phenomenon had not been described using $Villin^{CreERT2}$ mediated recombination, in which efficient Cre-mediated deletion occurs in Paneth cells.[30,31] This suggests that it is unlikely that Paneth cells are the major source of intestinal epithelial repopulation. Instead many different epithelial cell types may have the capability to de-differentiate in the case of massive loss of stem cells.[31] Alternatively repopulating cells are derived from a non-epithelial source such as the stroma or hematopoietic cells.[32]

The pronounced effect of activation of the UPR on intestinal epithelial stemness seems to be related to inhibition of the Wnt signature. It has been described that ER stress can inhibit Wnt signalling by interfering with the glycosylation of Wnt ligands *in vitro*.[33] Interestingly, however, in our experiment Wnt pathway hyperactivation that results from deletion of *Apc* is Wnt ligand independent. Therefore, reduced glycosylation of Wnt ligands cannot explain loss of the Wnt signature. Indeed, we observed nuclear accumulation of β-catenin throughout $Apc^{-/-}Grp78^{-/-(IEC)}$ epithelium despite profound loss of the Wnt signature, intestinal epithelial stem cells and proliferation. This suggests that loss of the Wnt signature occurs at a level downstream of β-catenin. This contrasts most other strategies targeting Wnt signalling upstream of β-catenin and may be an important advantage in treatment strategies directed against colorectal cancer in which β-catenin accumulation in the nucleus is the result of genetic mutations.[34] A similar phenotype to what we have observed in $Apc^{-/-}Grp78^{-/-(IEC)}$ epithelium has previously been described in mice that lacked both *Apc* and *c-Myc*. These animals do not exhibit hyperactive Wnt-signalling phenotypically while maintaining nuclear accumulation of β-catenin throughout the crypt–villus axis.[25] In previous *in vitro* experiments, we found that activation of the UPR results in rapid loss of c-Myc protein expression in a *Perk-eIF2α*-dependent manner. We therefore examined if c-Myc expression was lost in $Apc^{-/-}Grp78^{-/-(IEC)}$ mice and found that despite intact nuclear β-catenin, c-Myc expression was lost from the same cells. Therefore, loss of c-Myc likely contributes to loss of the Wnt signature. The fact that repopulation in $Grp78^{-/-(IEC)}$ and $Apc^{-/-}Grp78^{-/-(IEC)}$ animals occurs much more rapidly than in animals that lack *c-Myc* (5 vs 21 days) suggests loss of additional factors that are pivotal for stem cell fate. Although untransformed stem cells are also affected by *Grp78*

deletion-mediated ER stress, human colon cancer stem cells were more sensitive to chemotherapy *in vitro* and *in vivo* after induction of the UPR while healthy tissue remained unaffected putting forth ER stress signalling as a potential therapeutic target in colorectal cancer.[26] In conclusion, our results show that ER stress signalling suppresses the intestinal epithelial Wnt signature and results in loss of stemness and self-renewal capacity of premalignant $Apc^{-/-}$ stem cells. The UPR mediates this effect below the level of nuclear β-catenin. This is an important observation as to date, limited therapeutic options target Wnt signalling in the presence of *Apc* or *β-catenin* mutations. Our data therefore suggest that modulation of the UPR may be a potent strategy in the prevention or treatment of colorectal cancer.

MATERIALS AND METHODS

Animal experiments

All mouse experiments were performed in the Academic Medical Center Animal Research Institute in accordance with local guidelines. *VillinCre*^{ERT2}, *Rosa26*^{LacZ}, *Rosa26*^{ZsGreen}, *Apc*$^{fl/fl}$ and *Grp78*$^{fl/fl}$ alleles were all described previously.[9,35–39] Group size ($N = 5$ per genotype) was chosen based on previous experiments. Blinding and randomisation was not performed. Investigators were blinded after tissue processing. All mice had a C57BL/6 background, were between 8 and 12 weeks old. Different sexes were equally distributed among all groups.

For *Cre*^{ERT2} mediated recombination, mice were given 5 injections with 50 mg/kg tamoxifen (Sigma-Aldrich, St Louis, MO, USA 10 mg/ml in corn oil), during 3 consecutive days and were killed 24 h thereafter. Two hours before killing all mice received 100 mg/kg BrdU intraperitoneally (Sigma-Aldrich, 10 mg/ml in phosphate-buffered saline (PBS)). For repopulation experiments, mice were given 2 injections with tamoxifen on days 0 and 1, and were killed on day 2 to 4. After killing, intestines were immediately taken out and rinsed in cold PBS.

Immunohistochemistry and tissue preparation

Tissue was fixed in 4% buffered formaldehyde in PBS. The next day, formalin was replaced with 70% ethanol and processed according to standard protocols for paraffin embedding. For immunohistochemistry, 4 μm sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked in 0.3% H_2O_2 in methanol. For antigen retrieval, slides were treated at 96 °C for 10 min in 0.01 M sodium citrate buffer pH 6.0, or for 20 min in 10 mM Tris 1 mM EDTA buffer pH 9.0. Slides were incubated overnight at 4 °C with primary antibody diluted in PBT (PBS, 0.1% Triton X-100, 1% w/v BSA). Primary antibodies: anti-BrdU mouse monoclonal 1:500 (Roche BMC9318), anti-GRP78 rabbit monoclonal 1:200 (Cell Signaling C50B12), anti-β-catenin mouse monoclonal 1:1000 (BD Transduction Laboratories 610154), anti-c-Myc rabbit polyclonal 1:500 (Santa Cruz sc-764), anti-cleaved caspase-3 (Cell Signaling 9661L), anti-lysozyme rabbit polyclonal 1:2000 (Dako A0099), anti-CD44 rat monoclonal 1:500 (AbD Serotec MCA1967) and anti-EphB2 goat polyclonal 1:50 (R&D Systems AF647). Antibody binding was visualised with Powervision (Immunologic) and substrate development was performed using diaminobenzidine (Sigma-Aldrich D5637-10G).

mRNA probe synthesis and *in situ* hybridisation

For probe synthesis, a total of 1 μg of linearized plasmid DNA was transcribed *in vitro* using either SP6 or T7 RNA polymerase (Promega) using RNA DIG labeling mix (Roche). The probe was cleaned subsequently through a column (Qiagen RNEasy 74106) and dissolved in diethylpyrocarbonate (DEPC)-treated water containing 50% v/v formamide.[40]

In situ hybridisation was performed as described previously.[41]

Organoid culture

Intestinal epithelial organoids were obtained from mice with indicated genotypes. Harvest and expansion of intestinal organoid culture was performed as described previously.[4,42,43] Recombination of organoids was established by adding 1 μM 4OHT (Sigma-Aldrich, H6278-10MG) to culture medium for 24 h.

Separation of intestinal epithelial cell fractions

Tissue was harvested in PBS, and 2 cm pieces of whole intestine were used for further processing to obtain pure epithelial fractions separate from mesenchyme.[44] In short, the pieces of intestine are incubated for 7 min in warm EDTA (30 mM) in HBSS, vortexed and centrifuged after which the supernatant contains the epithelial fraction.

RNA isolation

For gene expression experiments in organoids, mRNA isolation was performed 24 h after treatment with 4OHT using the Bioline ISOLATE II RNA Mini kit (BIO-52073, Bioline) according to manufacturers' instructions. Organoids were derived from mice bred in our facility, and were tested mycoplasm free. For RNA extraction from mouse intestine, tissue was homogenised (Miccra D-1 homogenizer) in 1 ml Tri-reagent (T9424, Sigma-Aldrich) and RNA extraction was performed according to manufacturer's protocol.

complementary DNA synthesis and quantative RT-PCR

complementary DNA synthesis was performed using 1 μg of purified RNA using Revertaid reverse transcriptase according to protocol (Fermentas, Vilnius, Lithuania). Quantitative RT-PCR was performed using sensifast SYBR No-ROX Kit (GC-biotech Bio-98020) according to manufacturer's protocol on a BioRad iCycler. Primers sequences were ordered as found on qPrimerdepot (mouseprimerdepot.nci.nih.gov/). All primersets were intron spanning, primer specificity was tested using melting curve analyses. β-actin was used as reference gene. Relative gene expression was calculated using the $2^{-\text{delta Ct}}$ method.

RNA microarray experiments and GSEA

RNA cleaning was performed using RNeasy columns (Qiagen 74106) according to manufacturer's protocol and adequate RNA integrity was confirmed on an Agilent 2100 bioanalyzer. All samples had a RNA integrity number of 7, 5 or higher. For microarray experiments, 500 ng RNA was amplified and labelled using Illumina Totalprep RNA amplification kit (Invitrogen AMIL1791) according to manufacturer's protocol. Of the amplified and biotinylated cRNA 750 ng was hybridised to an Illumina human ref 12BeadChip. The BeadChips were scanned on a BeadArray Reader (Illumina) and processed using Genome Studio software (Illumina). Micro-array results can be found under GEO accession numbers: GSE28467, GSE28466 and GSE83333.

GSEA was performed using software from the Broad Institute. The UPR signature geneset was created by extracting the top 50 upregulated genes (one-way analysis of variance (ANOVA) test, $P < 0.05$) from SubAB treated LS174 cells. The intestinal stem cell signature was previously published.[22] The Wnt signature geneset was created by extracting the top 100 upregulated genes (ANOVA test, $P < 0.05$) from small intestinal epithelium of *Apc* knockout mice.

Statistics

Statistical analysis was performed with GraphPad Prism 5.0 (La Jolla, CA, USA). All values are depicted as the mean ± s.e.m. Statistical significance was analysed using Student's *t*-test. For multiple comparisons, one-way ANOVA was used followed by a Bonferroni post-test. Organoids experiments are shown as triplicate experiments of recombined organoids compared with non-recombined organoids. For microarray analyses, differentially expressed genes were extracted using ANOVA test and false discovery rate post analysis correction. Differences were considered statistically significant at $P < 0.05$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

JFvLdJ, JH, VM, designed experiments, analysed and discussed data. JFvLdJ, BJM, MCBW, BB, SLR, YHS and SM performed the experiments. ASL provided *Grp78*$^{fl/fl}$ mice, JCP and AWP provided SubAB and SubA$_{A272}$BJH, VM and GRvdB supervised the study. JFvLdJ and JH wrote the manuscript with input of all authors.

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