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ER stress causes rapid loss of intestinal epithelial stemness through activation of the unfolded protein response

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Abstract

Stem cells generate rapidly dividing transit-amplifying cells that have lost the capacity for self-renewal but cycle for a number of times until they exit the cell cycle and undergo terminal differentiation. We know very little of the type of signals that trigger the earliest steps of stem cell differentiation and mediate a stem cell to transit-amplifying cell transition. We show that in normal intestinal epithelium ER stress and activity of the unfolded protein response (UPR) is induced at the stem cell to transit-amplifying cell transition. Induction of ER stress causes loss of stemness in a Perk-eIF2α-dependent manner. Inhibition of Perk-eIF2α signaling results in stem cell accumulation in organoid culture of primary intestinal epithelium. Our findings show that the UPR plays an important role in the regulation of intestinal epithelial stem cell differentiation.

Introduction

Intestinal stem cells or so-called crypt base columnar (CBC) cells are morphologically recognizable as slender columnar cells that lie interspersed with Paneth cells at the base of the crypt and express stem cell markers such as Lgr5, Olfm4, and Ascl2 (Barker et al., 2007; van der Flier et al., 2009). Stem cells divide and form transit-amplifying cells (TA-cells) which are localized higher up in the crypts just above the level of the uppermost Paneth cell. TA cells cycle a number of times and differentiate into the different epithelial lineages of the small intestine.

During cellular differentiation, a broad range of specialized transmembrane and secreted proteins is produced, that requires processing in the endoplasmic reticulum (ER). The accumulation of nascent proteins in the ER attract chaperones such as Grp78, that are normally bound to the ER-membrane (Ni and Lee, 2007). This shifts Grp78 away from binding to three distinct transmembrane receptors, Ire1α, Atf6, and Perk (Harding et al., 2002) and is one of the mechanisms through which these receptors activate an ER stress response called the unfolded protein response (UPR). Ire1α activates transcription factor Xbp1 and Atf6 is cleaved to generate a transcriptionally active fragment. The resulting transcriptional response increases the capacity of the ER. The PKR-like ER-Kinase (Perk) phosphorylates the translation initiation factor eIF2α and thereby causes temporary translation attenuation. Altogether, UPR signals from the ER are critical to resolve ER stress and restore homeostasis in the ER. Or, if ER stress remains unresolved, apoptosis is induced (Harding et al., 2002).

In the intestine the UPR transcription factor Xbp1 is involved in the maintenance of secretory cell lineages and has been associated with the risk of developing inflammatory bowel disease (Kaser et al., 2008). However, the role of ER stress and UPR signaling in the intestinal epithelium remains incompletely understood. Here we use a combination of genetic and cellular techniques
to characterize their function. Our data reveal a role for ER stress and Perk-eIF2α signaling in mediating differentiation of intestinal epithelial stem cells.

**Results**

**ER-stress is low in intestinal stem cells compared to TA cells**

To localize the occurrence of ER stress and UPR signaling in the normal intestine we examined the expression of components and targets of the UPR. The chaperone Grp78 acts as a repressor of the UPR (Bertolotti et al., 2000), and is one of its major transcriptional targets and therefore widely used as readout for ER stress signaling (Mao et al., 2004). Analysis by immunohistochemistry (IHC) showed that Grp78 expression was low in the stem cells and high in the TA cells higher up in the crypt and in differentiated cells on the villus (Figure 1A and 1B). Expression of components and targets of the three arms of the UPR followed a similar pattern. Levels of both Xbp1 (Figure 1C and 1D) and phospho-eIF2α (Figure 1E and 1F) were very low in crypt base columnar cells compared to TA cells higher up in the crypt. It was previously shown that differentiation of Paneth cells depends on UPR signaling (Kaser et al., 2008). We found that expression of UPR components was heterogeneous in these cells with only a subset of Paneth cells expressing high levels of Grp78, Xbp1 and phospho-eIF2α (Figure S1). This suggests that activation of the UPR may regulate a specific stage of Paneth cell differentiation or activation. To confirm the difference in levels of ER stress and UPR signaling between stem cells and TA cells we analyzed markers of ER stress in gene arrays performed on sorted intestinal epithelial stem cells (Munoz et al., 2012). In this experiment we used the intestinal epithelium of Lgr5-eGFP mice, sorted in 5 different populations of cells based on the intensity of eGFP expression. We marked the highest expressing population as +5 and the lowest eGFP expressors as +1. Differential analysis of these cell populations showed that stem cell markers Lgr5, Ascl2 and Olfm4 were strongly enriched in the eGFP +5 population compared to the +1 population as expected. Markers of ER stress and components of the UPR clearly showed an inverse correlation with markers of stemness (Figure 1G). Taken together these results suggest that stem cells are low in ER stress and that activation of the UPR occurs in TA cells and differentiated cells.
Figure 1. Components of the UPR are expressed from the level of transit amplifying cells upwards. (A-F) immunohistochemistry (A and B) expression of Grp78. Black arrowheads point at crypt base columnar (CBC) cells at the stem cell position, white arrowheads point to TA cells and dashed lines delineate crypts. The asterisk in B marks a single high expressing Paneth cell. (C and D) IHC for Xbp1, activity of Xbp1 can be seen by nuclear localization of the protein which is high in TA cells (white arrowheads) compared to CBC stem cells (black arrowheads). (E and F) Phospho-specific detection of eIF2α shows a similar differential expression between TA cells (white arrowheads) and CBC stem cells (black arrowheads). (G) 2 log fold change values of indicated genes in sorted cell populations normalized to their expression in population +5 (Lgr5hi cells). Expression gradients of markers of stemness and the activity of the UPR are inversely correlated. Each row depicts one probe on the microarray. Original magnifications: 200x in A,C,E; 400x in B,D,F.

Induction of ER stress causes loss of the stem cell signature in vitro
Since we observed a differential activity of the UPR between stem cells and TA cells, we examined the consequence of increased ER stress on intestinal epithelial stemness. For in vitro experiments
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we used the LS174T colon cancer cell line, since its transcriptional profile resembles that of stem cells and TA cells (van de Wetering et al., 2002). These cells have been successfully used in the initial screens that have identified intestinal epithelial stem cell markers such as Lgr5 and Ascl2 (Barker et al., 2007; van der Flier et al., 2007; van der Flier et al., 2009). To induce ER stress in LS174T cells we treated them with thapsigargin. This resulted in upregulation of targets of the UPR such as CHOP and GRP78 as expected (Figure 2A). Treatment with thapsigargin resulted in marked repression of stem cell markers LGR5 and ASCL2 whereas the expression of differentiation markers VILLIN1 and P21 was not affected (Figure 2A). To confirm this pharmacological approach, we used subtilase cytotoxin (SubAB) to deplete cells of GRP78. This bacterial toxin specifically inactivates GRP78 inside the ER by proteolysis (Paton et al., 2006). Apart from being a target of the UPR, GRP78 serves as an important repressor of the UPR and as an important ER localized chaperone (Bertolotti et al., 2000; Pfaffenbach and Lee, 2011). Similar to treatment with thapsigargin, SubAB-mediated GRP78 depletion resulted in cell cycle arrest (not shown) and upregulation of targets of the UPR and reduction of expression of both LGR5 and ASCL2 whereas expression of VILLIN1 and P21 was not affected (Figure 2B). LS174T cells express the intestinal epithelial stemness signature but these cells are colorectal cancer cells. We therefore used cultured organoids of primary intestinal epithelium (Sato et al., 2009) to confirm the effect of activation of the UPR on stem cell markers in untransformed cells. Organoids have crypt and villus domains, contain normal numbers of stem cells per crypt and serve as a useful model for the study of cellular differentiation. We treated organoids with SubAB, which activated the UPR and resulted in rapid and near complete loss of expression of stem cell markers Lgr5 and Olfm4 (Figure 2C). Loss of these stem cell markers was already apparent at 8 hours of treatment. Despite the loss of expression of stem cell markers at 24 hours, organoids still looked morphologically normal (Figure S2A). In the next 24 hours crypts were slowly lost from the organoids, whereas the central villus domain was maintained (Figure S2A). At 48 hours of SubAB treatment we observed upregulation of both Villin1 and p21 expression suggesting increased enterocyte differentiation (Figure 2C).

We performed microarray analysis of LS174T cells after treatment with SubAB or protease-dead SubA_{272B} (Figure 2D) and analyzed differentially expressed genes by Gene Ontology analysis. The most significantly activated pathways were ER overload response, Endoplasmic reticulum and UPR (P = 2.9E10 -20 , P = 2.4E10 -16 , P = 4.4E10 -13 respectively). The pathways DNA replication, DNA strand elongation and cell cycle were among the most significantly downregulated pathways (P = 6.0E10 -12 , P = 3.4E -11 , P = 2.5E10 -11 respectively). Thus, depletion of Grp78 can serve as a bona fide model to study ER stress signaling. We further analyzed a set of intestinal stem cell markers in our dataset. For this we used the published list of genes that are high in stem cells isolated from Lgr5-eGFP mice (van der Flier et al., 2009). Gene set enrichment analysis (GSEA) revealed that induction of ER stress causes profound de-enrichment of these intestinal epithelial stem cell markers (normalized enrichment score -2.013, nominal P value <0.001, FDR q-value <0.001) (Figure 2E). This suggests that ER stress signaling reduces intestinal epithelial stemness.
We next assessed whether cells that experience ER stress still possessed the capacity for self renewal. To this end we generated organoids from mice that homozygously carry the conditional allele for Grp78 (Luo et al., 2006). To enable inducible deletion of Grp78, we transduced these organoids with a retrovirus carrying tamoxifen sensitive Cre recombinase (CreERT2). In these organoids, Grp78 can be deleted by treatment with 4-OH tamoxifen (4OHT). We confirmed successful recombination using this approach in Rosa26-zsGreen reporter organoids (Figure S2B). We seeded CreERT2-Grpfl/fl organoids and treated them with 4OHT or vehicle. Whereas vehicle treated organoids exhibited normal expansion of the number of crypts per organoid, 4OHT treated organoids retained their initial size for a number of days and then regressed with cells dying off. (Figure 2F). When 4OHT treated CreERT2-Grp78fl/fl organoids were reseeded, they failed to establish new organoids (Figure 2G) indicating that the stem cells had lost their capacity for self renewal. We next assessed whether loss of self renewal capacity in CreERT2-Grp78fl/fl organoids is accompanied by loss of stem cells. We therefore treated these organoids with vehicle or 4OHT for 24 hours, and harvested them for RNA expression studies 24 hours later. Genes that are highly expressed in crypt base columnar stem cells, such as Lgr5, Olfm4 and Cd44 were significantly downregulated upon deletion of Grp78. Interestingly, genes that mark alternatively proposed stem cell populations, were either upregulated (Bmi1), unaltered (mTert) or decreased (Hopx). Together, these data suggest that knockout of Grp78 causes loss of self renewal capacity, accompanied by loss crypt base columnar stem cells. Genes that mark alternative stem cell populations are not unequivocally altered.

Figure 2. ER stress reduces expression of markers of intestinal epithelial stemness in vitro. (A and B) Quantitative RT-PCR for UPR markers CHOP and GRP78, stem cell markers LGR5 and ASCL2 and differentiation markers VILLIN and P21 in LS174T cells treated for 24 hours with 200 nM thapsigargin versus vehicle or 100 ng/ml SubAB versus SubA322B. (C) Quantitative RT-PCR for UPR markers Chop and Grp78, stem cell markers Lgr5 and Olfm4 and differentiation markers Villin and p21 in organoids at different time points after treatment with 100 ng/ml SubAB or protease-dead SubA322B. (D) Gene expression analysis of SubAB treated LS174T cells results in loss of several stem cell markers and upregulation of UPR target genes. (E) GSEA of Lgr5hi genes in LS174T cells after treatment with SubAB shows profound loss of the stemness signature. (F) Brightfield images of Grp78fl/fl organoids that were treated with either vehicle or 4OHT show reduced growth in organoids that lack Grp78. (G) Quantification of CreERT2-Grp78fl/fl organoids after treatment with vehicle or 4-OH tamoxifen (4OHT). Seven days after treatment, non-recombined organoids were harvested, reseeded and treated with either vehicle or 4OHT, showing growth inhibition of organoids that lack Grp78. (H) Quantitative RT-PCR for UPR markers and a panel of stem cell markers on CreERT2-Grp78fl/fl organoids shows robust loss markers of crypt base columnar stem cell, but not alternatively proposed stem cell markers Bmi1 and mTert. Tg = thapsigargin. Values in (A-C, G, H) are mean ± s.e.m. ** = P < 0.01, *** = P < 0.001.
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Induction of ER stress results in loss of stem cells in vivo

To examine the effect of ER stress on intestinal epithelium in vivo we generated mice in which we conditionally inactivated Grp78. We crossed mice harboring the Grp78 floxed allele (Luo et al., 2006) to Ah1Cre mice (Ireland et al., 2004). In Ah1Cre mice treatment with β-naphtoflavone induces expression of Cre in the crypts and lower part of the villus of the intestinal epithelium but not in Paneth cells (Ireland et al., 2004) (see also Figure S3A). Recombination in the long-lived stem cells that maintain small intestinal epithelium under homeostatic conditions is virtually 100% (Ireland et al., 2004) (see also Figure S3B). To monitor recombination, we used the Rosa26lacZ reporter allele (Soriano, 1999). Littermate Ah1Cre-Grp78+/+ mice were used as controls. In the epithelium of Ah1Cre-Grp78fl/fl, mRNA of targets of the UPR such as Xbp1(s) and Chop was upregulated at day 2 p.i. (Figure 3A). At day 1 p.i. IHC showed nuclear Xbp-1 and phosphorylated-eIF2α in the CBCs in Grp78 mutant animals (Figure S4). Additionally, the ER was expanded and appeared dilated in absorptive enterocytes of Ah1Cre-Grp78fl/fl mutant mice (not shown). Recombination efficiency was high, with >99% LacZ+ crypts at day 1 post induction (p.i.) in both Ah1Cre-Grp78fl/fl and Ah1Cre-Grp78+/+ animals (Figure 3B). In the first two days after induction, the intestine of Ah1Cre-Grp78fl/fl mice appeared grossly normal, but at day 3 p.i. crypts became hypoplastic with thinning of the epithelial layer. Over the next two days, an increasing amount of hyperplastic crypts evolved until the epithelium had regained an almost normal appearance on day 5 (Figure S5). We found that from day 3 onwards, the epithelium showed increasing presence of LacZ negative, Grp78 proficient (non-recombined, wild type) cells until almost the whole epithelium was repopulated by wild type cells at day 5 p.i. (Figure 3B and 3C). These results show that despite low levels of expression, Grp78 serves a critical role in epithelial stem cells. It has previously been demonstrated that Ah1Cre-mediated deletion of genes that are pivotal for stem cell fate causes repopulation by wild type cells that have escaped Cre mediated recombination. Examples include c-Myc (Muncan et al., 2006) and the stem cell specific transcription factor Ascl2 (van der Flier et al., 2009). To further investigate loss of self-renewal capacity of mutant epithelium, we analyzed the presence of stem cells by mRNA in situ hybridization for stem cell marker Olfm4. In Ah1Cre-Grp78fl/fl mice stem cells are already almost entirely lost at day 1 p.i. Between day 3 and 5 p.i. an increased number of Olfm4+ stem cell containing crypts re-appear (Figure 4A). A similar manner of repopulation was observed when we performed IHC for Grp78 (Figure 4B). Serial sections localize these Olfm4+ cells inside crypts that harbor Grp78 proficient (wild type) cells (Figure 4C). Thus induction of ER stress by deletion of Grp78 in the intestinal epithelium confirmed our in vitro experiments in which we found that ER stress leads to rapid loss of intestinal epithelial stemness.
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**Figure 3.** Rapid repopulation by unrecombined cells upon induction of ER stress by Grp78 deletion in vivo. (A) Quantitative RT-PCR for the floxed exon of Grp78 on epithelial scrapings of AhCre1-Grp78<sup>fl/fl</sup> and AhCre1-Grp78<sup>+/+</sup> control animals at day 2 p.i. (n=3 per group) confirms loss of the targeted allele. UPR targets such as the spliced form of Xbp1 (Xbp1(s)) and Chop are upregulated. (B) LacZ staining on sections on day 1, 3 and 5 p.i. shows gradual repopulation of the epithelium of AhCre1-Grp78<sup>fl/fl</sup> mice by wild type cells. (C) Whole mount LacZ staining shows almost complete repopulation in the mutant mouse at 5 days p.i. Values in (A) are mean ± s.e.m. * = P < 0.05, ** = P < 0.01, *** = P < 0.001. WT = AhCre1-Grp78<sup>+/+</sup>, KO = AhCre1-Grp78<sup>fl/fl</sup>. Original magnifications in B: 125x.
Figure 4. Repopulation of the epithelium by wild type stem cells. (A) ISH for Olfm4 shows complete loss of stem cells on day 1 with foci of new stem cells on day 3 and reconstitution of stem cells in all crypts by day 5 p.i. (B) IHC for Grp78 shows loss of Grp78 on day 1, foci of Grp78 positive cells on day 3 and extensively Grp78 positive epithelium by day 5 p.i. (C) ISH for Olfm4 and IHC for Grp78 on consecutive slides on day 3 p.i. shows that Olfm4+ repopulating stem cells are derived from Grp78 positive wild type cells. Original magnifications: A and B: 125x; C: 200x.
Since unresolved ER stress can result in apoptosis, we examined if apoptosis could explain loss of stem cells. We observed a transient modest increase of active caspase-3 and TUNEL positive cells in the crypts around 3 days p.i., two days after the loss of stem cells (Figure S6). Thus, low levels of apoptosis are induced upon loss of Grp78, possibly through unresolved ER stress. This was an unlikely cause for the rapid loss of expression stem cell markers we observed at day 1 p.i. To further characterize the effect of loss of Grp78 on crypt cells we examined their proliferative potential at different time points after recombination. We found that incorporation of BrdU in Grp78 mutant crypts (as determined using consecutive sections stained for Grp78 and BrdU) remained normal at day 1 p.i. to extinguish only at day 3 p.i. (Figure 5A-5C and Figure S5). This suggests that although stem cells are rapidly lost, proliferation of TA cells is unaffected at first and that stem cells may have adopted a TA cell-like phenotype.

**Stressed stem cells are removed by differentiation**

We were unable to detect apoptosis in CBC cells at day 1 p.i. We therefore examined the Grp78 mutant mice for an alternative cause of the disappearance of Olfm4 expression and loss of self-renewal capacity of the mutant epithelium. We analyzed multiple crypt bases by electron microscopy at day 1 p.i. This revealed that in mutant mice, CBC stem cells had either disappeared leaving adjacent Paneth cells, or alternatively had increased width to height ratio and contained increased amounts of ER and mitochondria (Figure 5D-5G). These changes gave mutant CBC cells a TA cell-like appearance.

To further examine the possibility that Grp78 mutant stem cells are lost by differentiation, we performed lineage tracing of Grp78 mutant intestinal stem cells. To that end, we crossed Lgr5-eGFP-ires-CreERT2 mice (Barker et al., 2007) (in short Lgr5-CreERT2) into mice with the Rosa26<sup>LacZ</sup> reporter allele. In these mice Lgr5 positive stem cells are marked by expression of eGFP. Expression of eGFP is mosaic with approximately half the crypts containing eGFP positive stem cells and other crypts being negative. A single injection of tamoxifen induces recombination in a proportion of eGFP-positive stem cells (Barker et al., 2007). Recombination in these cells marks both stem cells and their descendants by expression of LacZ. Thus, both recombined and non-recombined stem cells could be observed (Figure S3C). By crossing the Grp78<sup>fl/fl</sup> allele into these mice, we could directly monitor the influence of ER stress on the fate of Lgr5-positive stem cells. In control mice (Lgr5-CreERT2-Rosa26<sup>lacZ</sup>-Grp78<sup>+/fl</sup>), a single injection of tamoxifen 48 hours prior to analysis marked LacZ positive Lgr5 progeny in a proportion of crypts, mostly reaching up to cell position +4 from the crypt base. In Lgr5-CreERT2-Rosa26<sup>lacZ</sup>-Grp78<sup>fl/fl</sup> mutant mice LacZ+ cells were positioned higher in the crypt and crypt bases were mostly free of LacZ+ cells (Figure 5H and 5I). A double stain for BrdU and LacZ showed that LacZ positive cells maintained their proliferative capacity (Figure 5J and 5K) suggesting that the Grp78 mutant cells shift up in the crypt and adopt a TA cell fate.
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A) Ah1Cre-Grp78^-^- Ah1Cre-Grp78^-^ B) LacZ/BrdU

Day 1 p.i.

C) 

\[ \text{Graph showing cell count over Time} \]

D) Ah1Cre-Grp78^-^- Ah1Cre-Grp78^-^ F) Ah1Cre-Grp78^-^- TA cells

Day 1 p.i.

G) 

\[ \text{Box plot comparing WT and KO} \]

H) Lgr5Cre-Grp78^-^- Lgr5Cre-Grp78^-^ I) 

\[ \text{Bar graph showing cell number over position} \]

J) Lgr5Cre-Grp78^-^- Lgr5Cre-Grp78^-^ K) 

\[ \text{Bar graph showing %BrdU/LacZ cells} \]

L) Lgr5Cre-Grp78^-^- Lgr5Cre-Grp78^-^ M) 

\[ \text{Bar graph showing GFP+ cells/LacZ crypt} \]

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Figure 5. Mutant stem cells are removed by differentiation. (A) Olfm4 ISH and BrdU IHC double staining on day 1 p.i. (B) Double staining of LacZ and IHC for BrdU shows loss of BrdU positive cells in remaining Grp78 mutant LacZ positive crypts at day 5 p.i. (C) Counting of BrdU positive cells in recombined crypts (n ≥ 3/group, >20 crypts counted/animal). (D) Slender inter-Paneth CBCs on electron micrographs in Grp78 wild type animals. (E) In Grp78 mutant mice CBC cells display an increased width and have adopted the morphological appearance of wild type TA cells (shown in F). White dashed lines demarcate cell borders. (G) Width/height ratio of CBC cells and TA cells in control animals and of CBC cells in Ah1Cre-Grp78fl/fl mice at day 1 p.i. (H) LacZ staining on Lgr5-CreERT2-Rosa26lacZ-Grp78fl/+ control mice two days after a single injection with 4 mg/kg tamoxifen shows stem cell progeny, marked by LacZ filling the crypts from the CBC cell position upwards. In Lgr5-CreERT2-Rosa26lacZ-Grp78fl/fl mutant mice LacZ+ cells at the CBC cell position at the crypt base have been lost and progeny is seen in the upper half of the crypt. (I) Quantification of the distribution of cell positions at day 2 p.i. shows an upwards shift of Grp78 mutant cells, away from the crypt base. (J) Double staining for LacZ and BrdU in wild type shows both double positive CBC cells (black arrow head) and TA cells (white arrowhead). In mutant mice LacZ cells shift up the crypt but remain BrdU positive indicating a conversion to TA cell phenotype. (K) Quantification of the % of BrdU positive LacZ cells at day 2 p.i., n=3 animals per group, >15 crypts counted per genotype. (L) LacZ and Lgr5eGFP IHC double staining on Lgr5-CreERT2-Rosa26lacZ-Grp78fl/+ control mice at day 2 p.i. shows the expected LacZ and Lgr5eGFP double positive CBC cells (black arrowheads) giving rise to LacZ+ progeny. In tamoxifen injected Lgr5-CreERT2-Rosa26lacZ-Grp78fl/fl mice LacZ+ (Grp78 mutant) crypts (arrowhead) have lost Lgr5eGFP expression whereas Lgr5eGFP expression is maintained in a LacZ negative (non recombined, Grp78 wild type) crypt. (M) Quantification of the number of eGFP+ cells/LacZ+ crypt at day 2 p.i., n=3 animals per group, >15 crypts counted per genotype. Values in C, G, K and M are depicted as mean ± s.e.m, ** = P < 0.01, *** = P < 0.001. Original magnifications: A and B 400x, H, J, L 800x.

We used Lgr5 driven eGFP expression as a surrogate for expression of Lgr5. We observed the expected LacZ-eGFP double staining cells in control mice at 48 hours p.i. Examination of LacZ+ cells in crypts of Lgr5-CreERT2-Rosa26lacZ-Grp78fl/+ mutant mice showed that these cells were almost entirely negative for eGFP (Figure 5L and 5M). Thus at 48 hours after injection of tamoxifen, recombined cells have shifted up the crypt, lost Lgr5 driven expression of eGFP but maintain their proliferative capacity as indicated by their capacity to incorporate BrdU. This is consistent with a stem cell to TA cell conversion. We concluded therefore, that induction of ER-stress by means of loss of Grp78 expression causes CBC stem cells to lose self-renewal capacity and to exit the stem cell pool. Most of these cells adopt a TA cell fate, differentiate and migrate up the crypt.

Loss of stemness occurs in a PERK-eIF2α dependent manner.

To analyze whether ER stress causes loss of stemness through UPR signaling, we generated LS174T colon cancer cell lines harboring stable knockdown against UPR components. We treated cells with thapsigargin or SubAB for and analyzed expression of stem cell markers. No rescue was observed in LS147shXBPI or LS174shATF6 cells (Figure S7). Knockdown of PERK partially rescued loss of expression of stem cell markers LGR5 and ASCL2 after induction of ER stress (Figure 6A). While PERK knockdown was efficient (88% ± 11%), stress induced phosphorylation of eIF2α was only partially prevented (45% ± 17% reduction in phosphorylation). To obtain complete dephosphorylation of eIF2α we therefore created a cell line which expressed a constitutively active fragment of GADD34 (GADD34ca), the phosphatase that specifically dephosphorylates
eIF2α (Novoa et al., 2001; Oyadomari et al., 2008). This completely rescued loss of stem cell markers in thapsigargin or SubAB treated LS174T cells (Figure 6A). Interestingly, upon induction of ER stress with SubAB, LS174GADD34<sup>−/−</sup> cells not only rescued stem cell markers, but actually increased expression of these genes. This may reflect a disturbed balance between the UPR components IRE1 and PERK, which are known to have opposing effects on cell viability (Lin et al., 2009). These data show that ER stress induced loss of the stemness signature critically depends on phosphorylation of eIF2α.

![Figure 6.](image)

**Figure 6.** Loss of stem cell markers occurs in an eIF2α dependent fashion.  
(A) Quantitative RT-PCR for stem cell markers LGR5 and ASCL2 at 24 hours after start of treatment. LS174T were infected with lentiviral constructs as indicated and treated with 100 ng/ml protease dead control SubA<sub>272</sub>B versus SubAB or treated with 200 nM thapsigargin versus vehicle. (B) Immunoblots for c-MYC at one hour after the start of treatment. Note that expression of c-MYC protein is inversely correlated with phosphorylation of eIF2α. Graphs show mean ± s.e.m. * = P < 0.05, *** = P < 0.001.

Intestinal stem cell fate depends on at least one protein with a very short half life. c-MYC has a half life of around 30 minutes (Hann and Eisenman, 1984), is critical to maintain expression of a core set of Wnt target genes (Sansom et al., 2007) and known to play a key role in maintenance of intestinal epithelial stem cells (Muncan et al., 2006). To maintain adequate expression levels, proteins such as c-MYC are highly dependent on continuous mRNA translation. Therefore
translation attenuation following eIF2α phosphorylation could affect stem cell fate rapidly and profoundly by blocking translation of these proteins. We performed immunoblots for presence of c-MYC after induction of ER stress and found indeed that within 1 hour protein expression is almost completely lost. In agreement with the rescue of stem cell markers, c-MYC protein translation was rescued partially upon knockdown of PERK. Complete rescue was achieved upon expression of Gadd34ca (Figure 6B). These results suggest that the translation inhibition caused by phosphorylation of eIF2α results in a rapid loss of short lived proteins with an important role in stem cell fate such as c-MYC.

**Perk signaling is required for stem cell differentiation**

We next examined whether Perk signaling is not only sufficient for stem cell differentiation but also required for normal intestinal differentiation. We therefore adapted a recently described method to transduce organoids with murine stem cell virus (Koo et al., 2011) and generated organoids transduced with lentiviral constructs containing shRNA directed against Perk (Figure 7A). To protect cells during infection organoids were cultured on medium containing the Gsk3β inhibitor CHIR-90221 until one week after infection. This hyperactivates Wnt signaling and expands the precursor cell compartment causing a cystic shape of the organoids (Sato et al., 2011). One week after CHIR-90221 withdrawal, we observed that the majority of shControl organoids had reverted to a budding shape, which indicates the normal establishment of a differentiated domain of cells at the core of the organoids surrounded by crypt like structures. In contrast, shPerk organoids remained cystic for a longer period (Figure 7A and 7B), suggesting a less differentiated phenotype of organoids that lack Perk. Three weeks after CHIR-90221 withdrawal, cystic organoids were rare in both groups and shape was stable. Morphologically, shPerk organoids consisted of more and larger crypts, budding was more frequent and the proliferative compartment in crypts was larger (Figure 7C). Transduction with three distinct shRNAs against Perk resulted in downregulation of Perk expression and upregulation of stem cell markers Lgr5 and Olfm4 (Figure 7D). Thus under homeostatic conditions, Perk-eIF2α signaling facilitates stem cell differentiation. We therefore tested whether preventing eIF2α dephosphorylation with the small molecule salubrinal (Boyce et al., 2005) affected the expression of stem cell markers. Treatment with 25 μM salubrinal for 24 hours resulted in increased expression of the Perk-eIF2α target Chop and reduced expression of stem cell markers Lgr5 and Olfm4. We conclude that ER stress is not only sufficient for stem cell differentiation but that physiological ER stress signaling plays a role in differentiation of stem cells under homeostatic conditions.
Figure 7. Perk signaling regulates stem cell differentiation under homeostatic conditions. 
(A) Quantification of the % of budding organoids in at indicated time points (mean of 4 wells per time point). (B) Brightfield images of shControl and shPerk organoids one week after withdrawal of CHIR-99021 shows the cystic shape of shPerk organoids. (C) BrdU incorporation in shControl and shPerk organoids. (D) Quantitative RT-PCR for Perk, Lgr5 and Olfm4 in shPerk transduced organoids. (E) Quantitative RT-PCR for Chop, Lgr5 and Olfm4 in organoids treated with 25 μM salubrinal for 24 hours. Graphs show mean + s.e.m. 
* = P < 0.05, ** = P < 0.01, *** = P < 0.001.

Discussion

Our data reveal that levels of ER stress and activation of the UPR are low in stem cells compared to transit amplifying cells. Activation of Perk-eIF2α signaling is both sufficient and necessary for intestinal epithelial stem cell differentiation.

ER stress and activation of the UPR are associated with differentiation (Iwakoshi et al., 2003; Lee et al., 2005; Wu and Kaufman, 2006) and differentiation of several secretory cell types has been show to rely on an intact UPR (Kaser et al., 2008; Lee et al., 2005). The possibility
that signaling by the UPR may not only be the result of cellular differentiation but itself be a driving force in cell fate decisions has received little attention. The differential expression of markers of ER stress and components of the UPR between stem cells and TA cells in the intestinal epithelium suggest that in the intestinal epithelium ER stress may in fact mediate a very early and critical step in differentiation, i.e. loss of the capacity for self renewal. Multiple independent lines of evidence support our conclusion that ER stress is sufficient for intestinal stem cell differentiation. (1) Induction of ER stress results in loss of the stem cell signature in vitro in colon cancer cells and organoids. (2) Deletion of Grp78 results in loss of self-renewal capacity in vivo as was demonstrated by the rapid repopulation of Grp78 mutant epithelium by wild type cells. (3) Stressed stem cells adopt a TA-cell like phenotype, characterized by increased cell size and organelle content, while having lost expression of stem cell marker Olfm4. (4) Linage tracing demonstrated that Grp78 mutant stem cells lose Lgr5 promoter activity and migrate up the crypt while remaining BrdU positive consistent with a stem cell to TA cell conversion. The accumulation of stem cells in Perk deficient organoids supports the notion that ER stress is not only sufficient but also necessary for normal stem cell differentiation.

Our finding that Lgr5 positive stem cells are exquisitely sensitive to ER stress is reminiscent of the sensitivity of these cells to gamma irradiation (Yan et al., 2012). Potentially, this indicates converging mechanisms by which stem cells respond to environmental stressors.

We observed a remarkable rate of repopulation with wild type cells after recombination of Grp78 with the Ah1Cre. Based on the high efficiency of stem cell recombination in the Ah1Cre that has previously been described, the almost complete loss of stem cells that we observe at 24 hours after recombination and the absence of extensive crypt fissioning during the repopulation process we feel that this may have to be explained by alternative mechanisms than incomplete stem cell recombination. In this light it is interesting to note that it has recently been demonstrated that Dll1 positive partially committed progenitors of the secretory lineage can dedifferentiate and reacquire stem cell characteristics in situations of damage and repair (van Es et al., 2012). Alternatively, it has recently been suggested that a population of cells that is positive for Paneth cell markers may behave as a quiescent stem cell population (Roth et al., 2012). Since the Ah1Cre does not recombine Paneth cells, such cells could be responsible for repopulation in our model.

In conclusion, our data show that there is differential activity of ER stress between stem cells and TA cells in the intestinal epithelium and suggest that the ER may be an important early regulator of intestinal epithelial stem cell differentiation.

Methods

Animal experiments
All animals were housed in the Leiden University Medical Center experimental animal center or in the Academic Medical Center Animal Research Institute and were handled in accordance
with guidelines of the local experimental committee. The Grp78<sup>fl/fl</sup> allele (Luo et al., 2006), the Rosa<sup>lacZ</sup> allele (Soriano, 1999), the Ah1Cre allele (Ireland et al., 2004) and the Lgr5-eGFP-ires-CreERT<sub>2</sub> allele (Barker et al., 2007) have been described previously. In Ah1Cre-mice, Cre was induced by intraperitoneal injections with β-naptoflavone in corn-oil (80 mg/kg) three times in 12 hours. In Lgr5-eGFP-ires-CreERT<sub>2</sub> mice, Cre was induced by a single intraperitoneal injection with tamoxifen (4 mg/mouse).

**Immunohistochemistry, TUNEL staining, in situ hybridization and X-gal staining.**

The small intestine was divided into three equal parts, proximal, middle and distal. The analysis described in this report was performed on the middle intestine. For many of the observations made we sampled both proximal and distal intestine to confirm observations made in the middle intestine and did not find any major differences (data not shown). Tissue was fixed overnight in 10% formalin, embedded in paraffin and sectioned. For immunohistochemistry, sections were deparaffinized using xylene and rehydrated in a series of ethanols. Endogenous peroxidases were blocked using methanol with 0.3% H<sub>2</sub>O<sub>2</sub>. For antigen retrieval, tissue was cooked in 0.01 M Sodium Citrate solution (pH 6.0) for 20 minutes or, alternatively, in 0.1 M Sodium EDTA (pH 9.0) for 20 minutes. For Xbp1 IHC, slides were additionally blocked for 30 minutes in TENG-T (10 mM Tris, 5 mM EDTA, 0.15 mM NaCl, 0.25% gelatin, 0.05% Tween 20, pH 8.0). Subsequently, slides were incubated with a primary antibody in PBS with 1% BSA and 0.1% Triton X-100. Sections were then washed and incubated with a PowerVision secondary antibody (Immunologic) for one hour. Slides were washed in PBS. Chromagen substrate consisted of diaminobenzidine (Sigma), according to the manufacturer’s instructions. For immunohistochemistry, the following antibodies were used: anti-Xbp1 (SC 7160, Santa Cruz), anti-Grp78 (3177, Cell Signaling), anti-phospho-eIF2α (3597, Cell Signaling), anti-BrdU (BMC 9318, Roche), anti-lysozyme (A0099, Dako) and anti-Ascl2 (MAB4418, Millipore).

For TUNEL staining we used the In situ cell death detection kit from Roche (ref number: 11 684 817 910) according to the manufacturers instructions.

For in situ hybridization, DNA templates of in situ probes were made by amplification the mRNA of interest. Amplicons were cloned into T-Easy Vector (Promega), according to instructions. Subsequently, dig labeled probes were made using dig labeled dUTP (Roche) and transcribed with T7 or Sp6 RNA polymerase (Promega) according to manufacturer’s instructions. For hybridization, 4 or 8 µm formalin fixed paraffin embedded sections were used. Sections were deparaffinized and rehydrated in H<sub>2</sub>O, incubated for 10 minutes in 1 M HCl, digested with proteinase K for 20 minutes, refixed in 4% paraformaldehyde for 10 minutes and acetylated with acetic anhydride. Slides were then pre-hybridized for an hour in a mix of 2% Blocking Powder (Roche), 0.05% Chaps, 50% formamide, 5x SSC pH 4.5, 5 mM EDTA, 100 µg/ml heparin (Sigma) and 100 µg/ml yeast RNA (Ambion). Subsequently slides were incubated for 72 hours at 68°C with a dig labeled antisense cRNA probe. After incubation, slides were washed three times for 20 minutes.
at 65°C in a stringency wash buffer containing 50% formamide and 2x SSC pH 4.5. Slides were then rinsed in TBS with 0.1% Tween-20, blocked with 0.5% Blocking Powder (Roche) in TBS-T and incubated overnight with sheep anti-dig alkaline phosphatase conjugated Fab fragments (Roche). Staining was developed with NBT/BCIP substrate (Sigma) over several hours to several nights.

X-Gal staining was performed by fixing freshly isolated tissues for 90 minutes at 4°C in PBS containing 1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP-40. Tissue was washed in ice cold PBS subsequently and stained overnight in the dark using PBS containing 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂, 1 mg/ml X-Gal and 0.02% NP-40.

Isolation of Lgr5-eGFP+ cells by FACS
Preparation of intestines and subsequent isolation of different populations of eGFP positive cells based on the level of eGFP expression by FACS was performed as described previously (van der Flier et al., 2009).

Electron Microscopy and morphometric analysis
1 mm³ sized pieces of intestine were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C temperature for several days to several weeks, postfixed in osmium tetroxide for one hour at 4°C, dehydrated in a graded ethanol series and embedded in an epoxy resin. 110 nm sections were contrasted with uranyl acetate and lead citrate, and viewed and imaged with a FEI Tecnai 12 transmission electron microscope, operated at 120 kV and equipped with an Eagle 4kx4k camera (FEI, Eindhoven, the Netherlands). For measurement of crypt base columnar cells, at least four animals per genotype were analyzed. Per animal, pictures of 2-10 crypts were measured. Crypt base columnar cells were identified by morphological appearance and localization between Paneth cells. For the width measurement, the mid-nuclear length was taken, and divided by the straight distance from the basolateral to the apical side of the cell. Measurements were performed using Image-J version 1.43U (NIH, USA).

Cell culture experiments and lentiviral transductions
Cells were grown in DMEM with 10% FCS and 1% penicillin/streptomycin. Lentiviral shRNA constructs were obtained from the Mission shRNA library (Sigma). The pLV-ca-GADD34 construct was made by subcloning the GADD34 fragment into the pLV vector. Virus was produced according to the manufacturer’s instructions. Cells were plated in 1 cm² wells until 25% confluency (approximately 10⁵ cells per well) and infected with an m.o.i. of 5. Subsequently, cells were cultured with 5 μg/ml puromycin (Invitrogen) for a week and expanded for experiments.
Organoid culture and transduction

We generated organoids as previously described (Sato et al., 2009). Organoids were kept on Egf, Noggin, Rspondin1 (ENR) medium. This medium contains N2, B27 supplements (Invitrogen), n-acetylcysteine, 50 ng/ml Egf (Invitrogen), Noggin-Fc conditioned medium (20%, equivalent to 200 ng/ml) and Rspo1-Fc conditioned medium (the Rspo1-Fc expressing cell line was a kind gift from Dr. Calvin Kuo, Stanford). Noggin-Fc conditioned medium was generated by cloning the murine Noggin cDNA into the pFuse plasmid containing the human IgG1 fragment (InvivoGen) to obtain a Noggin-Fc expression vector. Next, 150cm² flasks containing Hek293T cells were transiently transfected with 45 μg Noggin-Fc plasmid per flask, using polyethyleneimine (PEI, Brunswick scientific) in DMEM medium containing 10% FCS. The next day medium was changed to DMEM advanced medium without FCS and left for 7 days after which supernatant was harvested. This Noggin-Fc conditioned medium contains an equivalent of 1μg/ml of Noggin-Fc.

For lentiviral transductions, we adapted previously described methodology (Koo et al., 2011). Organoids were split to obtain approximately 50 organoids in 20 μl matrigel covered with ENR medium, supplemented with 10 mM nicotinamide (Sigma) and 10 μM CHIR-99021 (Axon Medchem) to generate cystic hyperproliferative organoids. Two days later, hyperproliferative organoids were harvested, disrupted with a Pasteur pipet and spun down to remove supernatant and matrigel fragments. Next, crypts were trypsinized for 3 minutes to generate a single cell suspension to which we added high titer lentivirus in ENR containing nicotinamide, CHIR-90221, 10 μM ROCK inhibitor Y27632 (Sigma) and 8μg/ml polybrene (Sigma). Cells were transduced, using one hour spinoculation at 600 RCF at 32°C. Transduced cells were incubated under normal culturing circumstances to recover for 4 hours, resuspended in matrigel and covered with ENR medium containing nicotinamide, CHIR-90221 and Y-27632. After three days, transduced cells were selected using puromycin (4 μg/ml) in ENR medium containing nicotinamide, CHIR-90221 and Y-27632 for 7 days. Surviving cells were further grown on ENR medium without additives.

Immunoblotting

Cells were lysed in cell lysis buffer (Cell Signaling Technology, Leiden, Netherlands), and boiled in sample buffer containing 0.25 M Tris-HCl pH 6.8, 8% SDS, 30% glycerol, 0.02% bromophenol blue and 1% β-mercaptoethanol. Separation was done on 10% SDS-PAGE, and proteins were transferred to a PVDF membrane. Specific detection was done by incubating the blot overnight in TBS with 0.1% Tween-20 with 1% BSA. Antibody binding was visualized using the Lumi-Light western blotting substrate (Roche). For primary detection same antibodies were used as for immunohistochemistry with addition of the following antibodies: anti-Actin (SC1616R, Santa Cruz), anti-Perk (5683, Cell Signaling), anti-eIF2α (2106, Cell Signaling) and anti Chop (2895, Cell Signaling).
RNA extraction and quantitative RT-PCR
Cells or tissue were lysed in 1 ml trizol. Tissue was homogenized and RNA extraction was performed according to manufacturer’s instructions. For organoid RNA preparations, cells in matrigel were resuspended in 350 µl RLT buffer (RNeasy, Qiagen) and stored for later use, RNA extraction was performed according to manufacturer’s instructions. For cDNA synthesis, 1 µg of RNA was transcribed using Revertaid (Fermentas). Quantitative RT-PCR was performed using SybrGreen (Qiagen) according to manufacturers’ protocol on a BioRad iCycler using specific primers for the mRNA of interest (available upon request).

RNA-microarray experiments
Cells were harvested in Trizol. RNA was extracted according to the manufacturer’s protocol. RNA cleanup was performed using RNeasy kit (Qiagen). For microarray analysis, RNA was labeled using cRNA labeling kit for illumina arrays (Ambion) and hybridized with Illumina HT12 Arrays. Differentially expressed genes were extracted using ANOVA test ($P < 0.05$) and FDR post-analysis correction. GSEA were done using GSEA software (Broad Institute of MIT and Harvard). The gene set used is the full list of genes published as table S1 of the original article describing $Lgr5$-eGFP-ires-CreERT2 sorted cells (van der Flier et al., 2009). Heat maps were generated using TreeView software generated by the Eisen lab (Stanford, California).

Statistics
All data are presented as mean ± standard error of the mean. Cell culture experiments were repeated at least three independent times. Statistical analysis of cell culture experiments was performed by 2-way ANOVA analysis. For animal experiments, student’s t-test, 1-way ANOVA tests or 2-way ANOVA tests were used. All ANOVA tests were followed by Bonferroni’s post hoc test for multiple comparisons.

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Reference List


ER stress causes rapid loss of intestinal epithelial stemness through activation of the unfolded protein response.


**Figure S1.** Heterogeneous expression of markers of ER stress in Paneth cells, related to Figure 1. Immunohistochemistry for Grp78 (A), p-eIF2α (B) and XBP-1 (C). A subset of the Paneth cells shows high expression of these markers of ER stress (positive cells indicated by black arrows). Original magnifications: left panels 400x, right panels 800x.
ER stress causes rapid loss of intestinal epithelial stemness through activation of the unfolded protein response

**Figure S2.** Organoids treated with SubAB and transduction with CreERT2

**(A)** Bright field images of same budding organoids at 24 and 48 hours after treatment with 100 ng/ml SubAB or SubA<sub>A272B</sub>. Organoids treated with SubA<sub>A272B</sub> grow new crypts (black arrowhead). SubAB treated organoids have normal appearing crypts at 24 hours (white arrowhead) when expression of stem cells markers is already completely lost. Crypts start to disappear in the next 24 hours (white arrowhead). 

**(B)** CreERT2 mediated recombination in organoids containing the Rosa26-<i>zsGreen</i> reporter allele. These organoids were transduced with retroviral Cre<sup>ERT2</sup> and treated with either vehicle or 4-OH tamoxifen (4OHT). Above panels depict increase of <i>zsGreen</i> fluorescence upon induction of Cre<sup>ERT2</sup> with 4OHT. Some autofluorescence is observed in untreated organoids. This is seen in all organoids in our hands, whether or not they carry a reporter allele. Below panels show increase in <i>zsGreen</i>-expressing cells by FACS analysis.
Figure 3. Ah1Cre mediated deletion occurs in stem cells and TA cells, not Paneth cells related to Figure 3. (A) Double staining for LacZ and IHC for the Paneth cell marker lysozyme. Ah1Cre-RosaLacZ mice were injected with 3 x 80 mg/kg of β-naphtoflavone in 12 hours and sacrificed 24 hours later. LacZ staining shows blue staining of recombined crypt base columnar cells (white arrowheads). Lysozyme positive Paneth cells (black arrowheads) are LacZ negative. (B) LacZ staining of Ah1Cre-RosaLacZ control animal 30 days after injecting 3 x 80 mg/kg of β-naphtoflavone in 12 hours shows long-term recombination of the epithelium and the appearance of LacZ positive Paneth cells (black arrowheads) derived from mutant precursor cells. Original magnifications: a left panel, b upper panel 125x; a right panel, b lower panel 800x. (C) Cre mediated recombination occurs in a subset of GFP positive Lgr5 cells in Lgr5-eGFP-Ires-CreERT2-Rosa26<sup>lacZ</sup> mice, related to Figure 5. Lgr5-eGFP-Ires-CreERT2-Rosa26<sup>lacZ</sup> mice were injected with a single dose of 4 mg/kg tamoxifen and sacrificed 48 hours later. Black arrowheads show crypts with GFP/LacZ double staining.
indicating Cre mediated recombination in Lgr5+ (GFP+) stem cells. White arrowheads indicate Lgr5+ (GFP+) cells in which recombination has not occurred (LacZ-). Original magnification 200x.

**Figure S4.** Activation of Xbp-1 and phosphorylation of elf2α in Grp78 mutant animals, related to Figure 3. (A) IHC for phosphorylated elf2α shows that at day 1 p.i. the phosphorylated form of elf2α extends towards the crypt base in β-naftoflavone injected Ah1Cre-Grp78fl/fl animals compared to β-naftoflavone injected controls. (B) IHC for Xbp-1 at day 1 p.i. shows increased nuclear accumulation of Xbp-1 in the TA cells in the upper part of the crypt in β-naftoflavone injected Ah1Cre-Grp78fl/fl animals compared to β-naftoflavone injected controls. The nucleus of CBCs of control animals (black arrow heads) is Xbp1 negative. In contrast, several Xbp-1 positive CBCs can be seen in Grp78 mutant animals (white arrowheads). Original magnifications: A, B upper panel 200x; B lower panel 800x.
**Figure S5.** Crypt regeneration occurs in 5 days in Grp78 mutant animals, related to Figure 3. H&E and BrdU staining of mid small intestine of Grp78 mutant and wild type animals. Arrows indicate hypoplastic crypts with thinning epithelium at day three after recombination. Asterisk indicates hyperplastic crypts that are already in the process of repopulation. Original magnification upper rows 200x, lower rows 400x.
Figure S6 Low levels of apoptosis at day 3 p.i, related to Figure 5. Representative examples of IHC for the activated form of caspase-3 (A) and TUNEL staining (B) at different time points after recombination. Quantification of the number of caspase-3 (C) and TUNEL (D) positive cells/crypt at different time points after recombination (n ≥ 3/group, >20 crypts counted per animal). Original magnifications: A upper panel 125x, A lower panel 800x, B upper panel 200x, B lower panel 400x. Values in (C, D) are mean ± s.e.m. * = P < 0.05, *** = P < 0.001.
Figure 7 Knockdown of ATF6 or XBP1 has no effect on markers of stemness, related to Figure 6. Quantitative RT-PCR for stem cell markers LGR5 and ASCL2 upon 24 hour treatment with 200 nM Tg in LS174T cells stably transduced with lentiviral plasmids containing shRNA's directed against XBP1 and ATF6.