Grp78 is required for intestinal Kras-dependent glycolysis proliferation and adenomagenesis

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In development of colorectal cancer, mutations in APC are often followed by mutations in oncogene K\textsuperscript{+}. The latter changes cellular metabolism and is associated with the Warburg phenomenon. Glucose-regulated protein 78 (Grp78) is an important regulator of the protein-folding machinery, involved in processing and localization of transmembrane proteins. We hypothesize that targeting Grp78 in Apc and K\textsuperscript{+}-mutant intestines interferes with the metabolic phenotype imposed by K\textsuperscript{+} mutations. In mice with intestinal epithelial mutations in Apc, K\textsuperscript{+} mutations and heterozygosity for Grp78 (AK-Grp78\textsuperscript{HET}) adenoma number and size is decreased compared with AK-Grp78\textsuperscript{WT} mice. Organoids from AK-Grp78\textsuperscript{WT} mice exhibited a glycolysis metabolism which was completely rescued by Grp78 heterozygosity. Expression and correct localization of glucose transporter GLUT1 was diminished in AK-Grp78\textsuperscript{HET} cells. GLUT1 inhibition restrained the increased growth observed in AK-mutant organoids, whereas AK-Grp78\textsuperscript{HET} organoids were unaffected. We identify Grp78 as a critical factor in K\textsuperscript{+}-mutated adenomagenesis. This can be attributed to a critical role for Grp78 in GLUT1 expression and localization, targeting glycolysis and the Warburg effect.

**Introduction**

Colorectal cancer (CRC) is one of the leading causes of cancer-related death in Western countries (Global Burden of Disease Cancer Collaboration, 2017). Development of CRC results from accumulation of a distinct set of mutations causing stepwise development from healthy tissue to adenomas and, subsequently, carcinomas, a sequence known as the adenoma-to-carcinoma sequence (Vogelstein et al, 1988; Fearon & Vogelstein, 1990). Activating mutations in the KRAS gene are among the major oncogenic drivers and occur as a second hit after APC mutations. KRAS is mutated in over 40% of all CRC cases and in CRCs that have no mutations in KRAS, mutations in BRAF may occur (Bos et al, 1987; Forrester et al, 1987; Samowitz et al, 2005; Normanno et al, 2009; Ogino et al, 2009). KRAS mutations succeed loss of tumor suppressor gene APC or alternatively, after activating mutations in \(\beta\)-catenin (Forrester et al, 1987; Rubinfeld et al, 1993; Su et al, 1993). Sole mutations in KRAS, which do not arise in the context of prior \(\beta\)-catenin-activating mutations are exceedingly rare in adenomas, and in a murine model, a single KRAS mutation resulted in senescence as opposed to hyperproliferation seen in the Apc-Kras mutational sequence (Bennecke et al, 2010; Feng et al, 2011; Smit et al, 2020). In colorectal adenomas, occurrence of a KRAS mutation on top of \(\beta\)-catenin signal-activating mutations was associated with the transition of small to large adenomas (Fearon & Vogelstein, 1990; Feng et al, 2011).

In the murine process of adenoma development, Apc and K\textsuperscript{+} synergistically stimulate proliferation and stemness and usher development of small adenomas into larger adenomas (Fearon & Vogelstein, 1990; Janssen et al, 2006; Sakai et al, 2018). To meet the increased demand for cellular growth, K\textsuperscript{+}-mutant cells have a higher protein translation capacity and an altered metabolic profile (Kimmelman, 2015). The molecular CRC subtype CMS3 that relies on activated (and frequently mutated) KRAS is also characterized by high metabolic activity (Guinney et al, 2015). In addition, we and others have shown that K\textsuperscript{+} potentiates global protein translation in organoids with homozygous loss of Apc (Smit et al, 2020; Knight et al, 2021). Furthermore, mutant KRAS promotes the expression of glucose uptake receptor GLUT1 and thereby increases glycolysis (Racker et al, 1985; Yun et al, 2009; Ying et al, 2012). In line with this, restoring KRAS to WT in CRC cells reduces glycolysis and growth. K\textsuperscript{+}-driven tumors are thus dependent on glycolysis and targeting...
the glycolytic pathway decreases tumor proliferation in preclinical studies (Xie et al, 2014; Sheng & Tang, 2016).

In previous work, we have pinpointed a role for the endoplasmic reticulum (ER)-resident chaperone glucose-regulated protein 78 (Grp78) as a potential target to reduce intestinal adenoma formation in mice. Grp78 is a critical mediator of the unfolded protein response (UPR) and activation of the UPR, as seen after deletion of Grp78, leads to temporary inhibition of protein translation and increases the ER size (Ma & Hendershot, 2001; Harding et al, 2002; Hetz, 2012). In addition, UPR activation results in increased transcription and translation of Grp78 in a stimulatory feedback loop. Deletion of a single copy of Grp78 in haploinsufficient mice, in which the threshold for activation of the UPR is lowered (van Lidth de Jeude et al, 2018; Rangel et al, 2021). In Apc-heterozygous mice, that over time develop intestinal adenomas, Grp78 heterozygosity was associated with reduced adenomagenesis, and organoids of these mice exhibited reduced protein translation (van Lidth de Jeude et al, 2018).

Besides its role in the UPR and protein synthesis, GRP78 is known as a glucose-sensing protein (Li et al, 2015). Up-regulation of Grp78 in solid tumors results from poor vascularization and glucose deprivation and Grp78 up-regulation results in increased expression of glucose transporter GLUT1 (Toyoda et al, 2018). GLUT1 and Grp78 are reported to be up-regulated in several cancers and a high GLUT1 expression corresponds with a poor prognosis in CRC (Yamamoto et al, 1990; Haber et al, 1998; Li & Lee, 2006).

We hypothesized that Grp78 heterozygosity would challenge CRC growth, because of altered protein translation and glucose consumption. This would result in inhibition of growth, specifically in the context of Apc and Kras mutations compared with a single Apc mutation. We thus set out to determine the effect of Grp78 haploinsufficiency in Apc-Kras mutant intestinal adenomagenesis.

Results

Grp78 heterozygosity reduces colon adenoma initiation and progression in Apc-Kras<sup>G12D</sup> mice

We modeled human disease by generating mice that harbored an inducible deletion in Apc and mutant Kras<sup>G12D</sup>. Apc and Kras mutations synergize to augment the number and size of intestinal adenomas and adenoma localization is shifted towards the colon (Sakai et al, 2018). All mice were crossed into VillinCre<sup>ERT2</sup> mice and Cre-mediated recombination occurred in the small intestinal and colonic epithelia after tamoxifen injections. VillinCre<sup>ERT2-Apc<sup>fl/fl</sup>-Kras<sup>G12D</sup>/+</sup> mice, were crossed into Grp78 heterozygously floxed mice (further referred to as AK-Grp78<sup>HET</sup>) and Grp78 WT littermates were used as control animals (AK-Grp78<sup>WT</sup>). To assess the contribution of the mutant Kras allele, VillinCre<sup>ERT2-Apc<sup>fl/fl</sup>-Grp78<sup>fl/fl</sup>-/+</sup> mice (further referred to as A-Grp78<sup>WT</sup>) were used as comparison.

To confirm recombination of Grp78 throughout the intestine, we combined Grp78 immunohistochemistry with specific visualization of the novel Grp78<sup>Δ7</sup> by Basescope (Fig 1A). As expected, immunostaining for Grp78 was present in AK-Grp78<sup>HET</sup> mice, resulting from staining of the protein product of the remaining Grp78 allele. In contrast, because of up-regulation of Grp78<sup>Δ5-7</sup> mRNA transcription in AK-Grp78<sup>HET</sup> mice, specific expression of the floxed Grp78<sup>Δ5-7</sup> mRNA was abundant. In addition, we confirmed recombination efficacy of the Kras allele throughout the intestine by immunohistochemistry for the mutant KRAS (Fig 1A). In addition, β-catenin was highly expressed as a consequence of mutated Apc (Fig 1A). Next, we analyzed Grp78 protein expression in epithelial cells from AK-Grp78<sup>WT</sup> and AK-Grp78<sup>HET</sup> mice. To this end, we generated organoids from mice with these genotypes. To investigate the specific role of Grp78 in combination with Kras, we compared these organoid with lines without mutant Kras (A-Grp78<sup>WT</sup> and A-Grp78<sup>HET</sup>) (van Lidth de Jeude et al, 2018). The resulting Grp78<sup>Δ7</sup> gene product has an open reading frame that is out of frame, resulting in no detectable protein being made. Thus, Grp78 protein from Grp78<sup>HET</sup> cells reflects expression from the single WT allele in these cells (Luo et al, 2006). Using immunoblots, we confirmed that Grp78 protein levels were diminished in AK-Grp78<sup>HET</sup> compared with AK-Grp78<sup>WT</sup> organoids (Fig 1B).

In mice of both AK-Grp78<sup>WT</sup> and AK-Grp78<sup>HET</sup> genotypes, we investigated cell fate by analysis of proliferation and apoptosis. Using a 2-h BrdU incorporation pulse, we found that enterocytes of AK-Grp78<sup>HET</sup> animals exhibited modestly reduced proliferation compared with their WT counterparts (8.3 versus 6.5 P < 0.05, Fig 1C and D). Apoptosis, visualized by cleaved caspase immunohistochemistry, was not significantly reduced (13.8 versus 9.2 cells per 100 crypts, P = 0.09) (Fig S1A). From the moment of recombination, both AK-Grp78<sup>WT</sup> and AK-Grp78<sup>HET</sup> animals lost weight compared with A-Grp78<sup>WT</sup> animals, but weight did not differ between AK-Grp78<sup>HET</sup> and AK-Grp78<sup>WT</sup> animals (Fig S1B). Of all AK-Grp78<sup>WT</sup> mice, 80% developed a rectal prolapse, indicating severe rectal adenoma formation, whereas AK-Grp78<sup>HET</sup> mice were markedly protected from development of rectal prolapse (20%, P = 0.054, Fig 1E and F) (Colnot et al, 2004). As expected, colon of A-Grp78<sup>WT</sup> mice contained no adenomas (Fig 1G). In the colon, AK-Grp78<sup>WT</sup> and AK-Grp78<sup>HET</sup> animals harbored multiple adenomas (Fig 1G–I). AK-Grp78<sup>WT</sup> animals exhibited markedly increased adenoma numbers and growth compared with AK-Grp78<sup>HET</sup> animals (total adenomas [of which large] = 61.4 [31.6] versus 40.6 [17], P < 0.01, Fig 1I and J). In the small intestine, adenoma numbers were very low and there was a nonsignificantly reduced adenoma number in AK-Grp78<sup>HET</sup> animals (Fig S1C and D).

The above results display that Grp78 levels are important for initiation and growth of Apc and Kras mutant adenomas, especially in the large intestine.

Grp78 levels are critical for proliferation, protein production, and stemness

To analyze how adenomagenesis was reduced in AK-Grp78<sup>HET</sup> mice, we further analyzed intestinal epithelial organoids of AK-Grp78<sup>WT</sup> and AK-Grp78<sup>HET</sup> genotypes. After recombination, increased growth of AK-Grp78<sup>WT</sup> organoids was observed as expected (Fig 2A). However, AK-Grp78<sup>HET</sup> organoids did not increase in size, suggesting that Grp78 is a rate-limiting factor, responsible for growth upon
accumulating oncogenic mutations in Apc and Kras (Fig 2A and B). We next analyzed proliferation by EdU incorporation and quantified organoid size. Interestingly, the increased growth that was observed in AK-Grp78WT versus A-Grp78WT was almost completely nullified in AK-Grp78HET organoids (Fig 2C–E).

Heterozygosity of master ER chaperone Grp78 decreases the threshold for ER stress, causing up-regulation of ER chaperones Grp78 and Grp94, thereby regulating constant levels of Grp78 mRNA (Bertolotti et al, 2000). Using mRNA primers that did not distinguish between WT and floxed mRNA transcripts, we indeed observed unaltered total Grp78 mRNA expression levels in AK-Grp78HET organoids, whereas Grp94 mRNA was increased (Fig S2A and B). To confirm deletion of a single Grp78 allele, we used a specific primerset to measure expression of the truncated (incapacitated) Grp78 transcript derived from the Grp78Δ5–7-floxed allele (van Lidth de Jeude et al, 2018). The floxed Grp78Δ5–7 was only observed in in the AK-Grp78HET organoids, confirming the loss of a single allele of Grp78 (Fig 2F).

We recently reported a significant rise in global protein translation when Apc-mutant cells acquire a Kras mutation (Smit et al, 2020). We therefore tested whether reduced GRP78 levels in AK-Grp78HET organoids would negatively impact protein translation. Indeed, translation capacity was significantly increased in AK-Grp78WT organoids compared with A-Grp78WT organoids (Fig 2G). Strikingly, the increased protein translation that was observed in AK-Grp78WT crypts was largely (52%) reduced in AK-Grp78HET organoids (P < 0.01).

Thus, AK-Grp78HET organoids exhibited reduced size, proliferation, and protein translation. We next analyzed whether these alterations coincided with reduced stemness. Previously, we...
found reduced mRNA expression of stem cell markers in A-Grp78 WT organoids compared with A-Grp78 HET organoids (van Lidth de Jeude et al., 2018). Moreover, adenoma progression from the Apc mutant to Apc-Kras double-mutant state, is marked by an increase in stemness (Janssen et al., 2006). In AK-Grp78 HET organoids, a significant decrease in transcription of intestinal stem cell markers Lgr5 and Olfm4 was observed compared with AK-Grp78 WT organoids (Fig 2H). Thus, Grp78 remains critical for the increase in stemness that is observed in Apc-mutant neoplastic crypts and Grp78 is a rate-limiting factor during progression from Apc to Apc-Kras double-mutant organoids. To examine stem cell clonogenicity on a functional level, we performed single-cell seeding experiments. Single cells without an oncogenic Kras G12D mutation exhibit limited self-renewal capacity and hardly grew into organoids (Fig 2I and J). In agreement with reduced mRNA expression of stem cell markers, AK-Grp78 HET cells had a significantly reduced capacity for organoid outgrowth (64 versus 100, P < 0.05) and grew into smaller organoids than AK-Grp78 WT controls (Fig 2I).
In the context of an additional KrasG12D mutation, we find that Grp78 is required for growth, stemness, and protein translation. Grp78 heterozygosity almost completely abrogates the effect of a Kras mutation on growth and translation.

**Grp78-dependent glycolysis and proliferation in AK organoids is fueled by Glut1-mediated glucose influx**

Under normal culture conditions, we noticed that growth medium acidified more rapidly in AK-Grp78WT organoids compared with AK-Grp78HET organoids. Acidification may be the result of increased glycolysis and consequent production of lactic acid under aerobic conditions. CRC cell lines with mutant KRAS depend on glycolysis (ECAR), a phenomenon known as the Warburg effect (Warburg, 1956). It has been shown that restoration of WT KRAS reverts the glycolytic Warburg effect towards metabolism in which oxidative phosphorylation (OCR) is dominant (Yun et al, 2009). Thus mutations in Kras that lead to constitutive Kras activation may impose a metabolic switch towards the Warburg effect. We next analyzed the influence of Grp78 levels on Kras-induced Warburg metabolism.

**Figure 3. GLUT1-dependent glycolysis in Apc-Kras mutant organoids is regulated by Grp78.**

(A) Oxygen consumption rate in organoids of indicated genotypes, measured by Agilent Seahorse. (B) Basal respiration, ATP-linked respiration, and maximum respiration in the indicated organoids. (C) Glycolytic function in the organoids, measured by Agilent Seahorse. (D) Glycolysis and glycolytic capacity in the indicated organoids. (E) Grp78 mRNA and Glut1 mRNA expressions derived from microarray analysis of indicated mouse organoids (GSE142509). (F) Immunoblots of Grp78 and GLUT1 from indicated organoids. (G) Flow cytometric immunostaining of membrane-localised GLUT1 protein on live cells, percentage Alexa 647+ve of the parent (single cells, PI negative). (H) Glucose consumption upon treatment with vehicle, 10, and 100 nM of GLUT1-inhibitor BAY-876. (I) Lactate production upon treatment with vehicle, 10, and 100 nM of GLUT1-inhibitor BAY-876. (J) Quantitative RT–PCR analysis for Pgc1a mRNA on organoids of indicated genotypes upon treatment with vehicle, 10, and 100 nM of GLUT1-inhibitor BAY-876.

*P < 0.05, **P < 0.01, ***P < 0.001, ns, nonsignificant.
organoids, we could corroborate previous data that revealed that mutations in Kras introduce the metabolic switch which results in a transition from oxidative phosphorylation to predominant glycolysis (Yun et al., 2009; Ying et al., 2012). Interestingly, AK-Grp78WT organoids had comparable levels of oxidative phosphorylation with AK-Grp78Het organoids, although glycolysis and glycolytic capacity were markedly reduced (Fig 3A–D). Glycolytic function in AK-Grp78Het organoids was comparable with A-Grp78Het organoids, suggesting sufficient Grp78 is critical for Apc-deficient cells to undergo the glycolytic switch towards the Warburg effect after mutation of Kras (Fig 3C and D). We thus pinpoint Grp78 as a critical factor for Kras-induced Warburg metabolism.

To further understand the molecular basis of Grp78-dependent glycolysis in AK-Grp78Het organoids, we analyzed glucose influx. It has been shown that Kras-mutant CRC cells exhibit increased levels of one of the most important glucose transporters GLUT1 and depend on GLUT1 for their proliferative capacity (Yun et al., 2009). As knockdown of Grp78 has been shown to reduce Glut1 mRNA levels, we hypothesized that reduced glycolysis in AK-Grp78Het organoids may result from an inability of these organoids to induce sufficient levels of Glut1 (Li et al., 2015). We analyzed both Grp78 and Glut1 mRNA expression from our previously published mRNA microarray experiments (GSE143509) in Apc-mutant versus Apc-Kras-mutant organoids (Smit et al., 2020). Interestingly, we observed a gradual increase in Glut1 mRNA expression upon acquisition of Apc and KrasG12D mutations, respectively, which correlated with Grp78 expression ($r = 0.92; P < 0.001$) (Fig 3E). This was also observed in an analysis of GRP78 and GLUT1 expression in a micro array of colon normal tissue versus tumor tissue (GSE33114) (de Sousa et al., 2011). On the level of protein expression, A-Grp78Het and A-Grp78HET organoids also exhibited low levels of GLUT1, potentially contributing to their low levels of glycolysis under normal conditions (Fig 3F). In AK-Grp78Het organoids however, we found that both Grp78 protein and Glut1 protein were abundantly expressed (Fig 3F). This supports a potential role for Grp78 as a tumor marker during adenoma progression (Ma et al., 2015; Shen et al., 2019). Heterozygosity for Grp78 resulted in marked decrease of both Grp78 as GLUT1 protein levels, although expression levels of GLUT1 were still higher than seen in A-Grp78Het organoids (Fig 3F). These data suggest some, but not full normalization of GLUT1 expression in AK-Grp78Het organoids compared with A-Grp78Het organoids.

We next analyzed the expression of GLUT1 protein in a set of 10 paired random samples of human colon carcinomas with unknown mutation status, with their adjacent normal colonic mucosa (Fig S3B). In seven out of 10 pairs, we observed strongly increased GLUT1 levels in carcinomas compared with their adjacent normal mucosa. These results suggest that GLUT1-mediated glycolysis may be equally important in both large adenomas and carcinomas.

Because reductions in Grp78 levels may cause nascent proteins to remain inside the endoplasmic reticulum instead of translocating to their functional site, we next assessed whether GLUT1 protein could properly localize towards the plasma membrane in AK-Grp78Het cells (Toyoda et al., 2018). Using flow cytometry on live cells with an antibody directed against GLUT1, we could specifically detect surface GLUT1 (Figs 3G and 3A–C). Consistent with total GLUT1 levels, membrane-localized GLUT1 protein levels were low in Apc-mutant organoids regardless of Grp78 levels, but were increased on the surface of AK-Grp78Het cells. Membrane localization of GLUT1 in AK-Grp78Het organoids was fully restored to a degree similar to organoids that lacked mutant Kras. Thus, although we found total GLUT1 protein to be up-regulated in AK-Grp78Het and AK-Grp78Het organoids compared with A-Grp78 counterparts, Grp78 heterozygosity resulted in strongly reduced membrane translocalization of this protein.

To further investigate the functional role of GLUT1 in cellular proliferation, we treated organoids with GLUT1 inhibitor (BAY-876), thereby disrupting glucose uptake and glycolysis (Siebeneicher et al., 2016). We assessed glucose consumption, pyruvate consumption, and lactate production as readouts of oxidative phosphorylation and glycolysis (Figs 3H and I). In AK-Grp78Het organoids, however, GLUT1 inhibition resulted in a considerable dose-dependent reduction of glucose consumption, lactate production, and proliferation (Fig 3H–I). In AK-Grp78Het organoids, glucose consumption, lactate production, and proliferation were already on a much lower level than AK-Grp78Het, potentially owing to low levels of surface GLUT1, and additional inhibition of GLUT1 could only marginally decrease growth in AK-Grp78Het organoids.

As we observed a strong reduction of glycolysis in AK-Grp78Het organoids upon inhibition of GLUT1, we examined whether GLUT1 inhibition could revert the metabolic switch from oxidative phosphorylation to glycolysis that is observed in AK-Grp78Het organoids. Inhibition of GLUT1 decreased the medium lactate to pyruvate ratio in AK-Grp78Het organoids, suggesting an increase in oxidative phosphorylation when GLUT1 is inhibited (Fig S5B). To understand how GLUT1 inhibition could revert the Warburg effect in Apc-Kras mutant organoids, we analyzed whether increased mitochondrial biosynthesis could be responsible for accommodating increased oxidative phosphorylation. Indeed, we found that the level of Pgc1a, a transcriptional coactivator that induces mitochondrial biogenesis, was increased in AK-Grp78Het organoids upon inhibition of GLUT1, but not in the other organoids (Fig 3K) (Wu et al., 1999).

Altogether, these data indicate that AK-Grp78Het organoids are skewed towards glycolysis as seen in the Warburg effect and this is impaired when faced with reduced capacity to internalize glucose. GLUT1 inhibition results in reduced glycolysis in AK-Grp78Het organoids, but a transcriptional activation towards oxidative phosphorylation is suggested. In contrast, AK-Grp78Het organoids exhibit low levels of glycolysis and blocking glucose uptake via GLUT1 inhibition had little effect on their metabolism. Increased oxidative phosphorylation in these organoids was not observed.

In conclusion, Grp78 heterozygosity reduces protein and surface expressions of GLUT1 in Apc-KrasG12D organoids. Grp78 heterozygosity reduced glycolysis and thus the growth potential of Apc-KrasG12D organoids by normalizing the enhanced GLUT1 expression in KrasG12D mutant cells.

**Discussion**

The most common initiating mutations of the adenoma to carcinoma sequence, APC and KRAS, increase proliferation, protein...
Grp78 is an important regulator of protein-folding capacity and a glucose-sensing protein. Previously, we showed that Grp78 knockout crypts are not viable, resulting in rapid repopulation by WT crypts, which renders Grp78 knockout an unviable model for adenoma studies. However, heterozygous targeting of Grp78 impacts protein translation, stemness, and tumor growth in mice and organisms with an Apc mutation (Heijmans et al., 2013; van Lidth de Jeude et al., 2017; van Lidth de Jeude et al., 2018). Here, we explore Grp78 heterozygosity in mice and organoids with mutations in both Apc and Kras and we specifically focus on the influence of Grp78 levels on the additional phenotypical, molecular, and metabolic changes that are caused by a Kras mutation in the context of the Apc mutant cell state.

We find that adenoma numbers in colons of AK-Grp78HET mice are reduced compared with AK-Grp78WT controls. Although in earlier studies, adenoma reduction was also observed in A-Grp78HET mice compared with A-Grp78WT controls, AK animals exhibited adenoma growth primarily in the large intestine and effects of Grp78 in these animals thus seem specific for animals harboring an additional Kras mutation (van Lidth de Jeude et al., 2018).

At the root of reduced colonic adenomas in AK-Grp78HET animals, we find decreased proliferation. Effects that we observe emanate from allelic heterozygosity, but because GRP78 activity and thereby stronger anti-tumor effects may be feasible without overt toxicities. In preclinical in vitro studies, these compounds have shown reduced proliferation, similar to our findings in Grp78 heterozygous cells and animals (Hensel et al., 2013; Elfky et al., 2020).

Ex vivo, in organoids with mutant Apc and Kras, we find that Grp78 heterozygosity reduces growth, proliferation, and cell renewal capacity to a level that is similar to organoids with WT Kras. We find that in Apc-Kras mutant cells, increased proliferation and growth result from increased glucose uptake, compared with Apc single-mutant cells, and that these cells switch from oxidative phosphorylation to glycolysis for their energy requirement as is seen during the Warburg effect. This metabolic reprogramming, which is critical for Apc-Kras mutant cells is considered a hallmark of cancer and high-Grp78 levels are required for this switch, because Grp78 heterozygosity reverts this phenotype (Hanahan & Weinberg, 2011). Glucose uptake in Apc-Kras mutant cells is largely mediated by up-regulation of glucose transporter GLUT1 and is reported in several studies to be a prognostic biomarker for worse clinical outcome in multiple malignancies (Yamamoto et al., 1990; Haber et al., 1998). We corroborate earlier findings that Grp78 plays a central role in the production and localization of GLUT1 (Li et al., 2015; Toyoda et al., 2018). Moreover, we show that Grp78 is a rate-limiting factor for the Warburg effect as we find that a mild reduction of GRP78 protein level in Grp78 heterozygous cells already suffices in reducing GLUT1 levels and glycolysis, as opposed to strongly reduced levels as are seen upon knockout or RNAi.

Interestingly, mitochondrial respiration was not altered in AK-Grp78WT organoids compared with their WT counterparts which could not be attributed to glucose or pyruvate consumption, suggesting an altered metabolism which would enable these cells to maintain oxidative phosphorylation at a similar level. Recently, glutamine metabolism was found to play an important role in Kras-mutant tumors and perhaps utilization of glutamine could explain the persistence of oxidative phosphorylation in AK-Grp78HET organoids (Wang et al., 2020; Najumudeen et al., 2021). More studies are needed to evaluate the possible synergistic effects of targeting both metabolic pathways as a therapeutic strategy in (colon) cancer.

Taken together, we identify Grp78 as a critical vulnerability in mutational progression of colorectal tumorigenesis. Our data strengthen the potential of Grp78 as an attractive target for therapy in CRC.

Materials and Methods

Animal experiments

All mouse experiments were performed in the Academic Medical Center Animal Research Institute in accordance with local guidelines. VillinCreERT2, Apcfl, Krasfl2, and Grp78fl alleles were all described previously (El Marjou et al., 2004; Jackson et al., 2001; Luo et al., 2006; Madisen et al., 2010; Sansom et al., 2004; Shibata et al., 1997; Soriano, 1999). All mice had a C57BL/6 background.

For CreERT2-mediated recombination, mice were given daily injections with 1 mg of tamoxifen (T5648; 10 mg/ml in corn oil; Sigma-Aldrich), during 5 consecutive days. 2 h before euthanizing, all mice received 100 mg/kg BrdU intraperitoneally (10 mg/ml in NaCl; Sigma-Aldrich). The mice were euthanized 8 wk after CreERT2 recombination or earlier (week 6, week 7) when the endpoints, according to the Netherlands Association for Laboratory Animal Science, were reached. Rectal prolapse was a common symptom and accorded as a humane endpoint.

Tissue preparation and immunohistochemistry

Paraffin embedding and subsequent (immuno)histochemistry was performed as previously described (Heijmans et al., 2011). Primary antibodies that were used are as follows: mutant specific anti-G12D-RAS 1:100 (14429; Cell signaling) (Muthalagu et al., 2020), anti-Grp78 antibody 1:700 (3177S; Cell signaling), anti-BrdU mouse monoclonal 1:500 (BMC9318; Roche), anti-β-catenin 1:2,000 (AB32572; Abcam), and anti-cleaved-caspase-3 1:400 (9661S; Cell Signaling). Antibody binding was visualized with Powervision (Immunologic) and sub-strate development was performed using diaminobenzidine (D5637-10G; Sigma-Aldrich). Hematoxylin was used as counterstain.

In situ hybridization

RNAscope was performed according to the manufacturer’s protocols. These included the “Formalin-Fixed Paraffin-Embedded Sample Preparation and Pre-treatment for RNAscope 2.5 assay.” For in situ assessment of the Grp78RNAI+ mRNA, The BaseScope Reagent Kit (Advanced Cell Diagnostics) was used as previously described (van Lidth de Jeude et al., 2018).
**Organoid culture**

Experiments were performed with recombined organoids of four different genotypes, referred to as A-Grp78WT and A-Grp78HET and AK-Grp78WT or AK-Grp78HET. In organoid experiments, all Apc mutations were homozygous as opposed to heterozygosity for Apc in mouse experiments.

Crypt harvest and expansion of small intestinal organoid culture were performed as described previously (Heijmans et al., 2013). Recombination of organoids was established by adding 4-hydroxy tamoxifen (4-OHT) (H6278-10MG, Sigma-Aldrich) to culture the medium for 24 h directly after passaging (1 μM).

**FACS-based EdU incorporation**

We used the Click-iT EdU Alexa Fluor 647 (C10634; Thermo Fisher Scientific) according to the manufacturer's protocol. 72 h after recombination, EdU was added for 4 h. Results were analyzed with FlowJo V10 software.

**Flow cytometric immunostaining of GLUT1**

72 h after recombination, organoids were harvested. Matrigel was fractionated using a Pasteur pipette and organoids and matrigel were separated by centrifuging. Crypts were detached to single cells by incubating with TrypLE (Gibco) during 10 min at 37°C. Anti-GLUT1 (forward TGTGTCAAAGCATTTCCAGC, reverse CAGCGTCTTATTGCTGGACTGA, reverse TTCAGCTGTCACTCGGAGAA), Grp78 (forward TTGCTTCAATTCCAAGGTAATCA, reverse TTGCTGACCCCAAGAGGAA) were separated by centrifuging. Crypts were detached to single cells and cysteine into newly translated proteins. Newly translated proteins were shown relative to control samples, after normalizations were homozygous as opposed to heterozygosity for Apc in mouse experiments.

**RNA isolation**

For gene-expression experiments in organoids, mRNA isolation was performed 72 h after recombination using the Bioline ISOLATE II RNA Mini kit (BIO-52073; Bioline) according to the manufacturer’s instructions. For RNA extraction from mouse intestine, tissue was homogenized with a Micrcr D-1 homogenizer in 1 ml Tri-reagent (T9424; Sigma-Aldrich) and RNA extraction was performed according to the manufacturer’s protocol.

**cDNA synthesis, and quantitative RT–PCR**

Synthesis of cDNA was performed using 1 μg of purified RNA using Revertaid reverse transcriptase according to the protocol (Fermentas). Quantitative RT–PCR was performed using sensiFAST SYBR No-ROX Kit (Bio–98020; GC–biotech) according to the manufacturer's protocol on a Bio-Rad iCycler. Primer sequences were ordered as found on Primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primersets were tested and chosen as previously described. Relative gene expression was calculated using the LinReg method. Primers: 36B4 (forward CCACGGAGCCACACTGCTG, reverse ACATG-GCCACAGTTGGGAC), β-actin (forward TTCTTTGACGTCTCTGTT, reverse ATGGAGGGGAATACAGCCC), Grp78 (forward ACTTGGGGACACCTATTCCT, reverse ATGGAGGGGAATACAGCCC), AK-Grp78WT (forward T6278-10MG, Sigma-Aldrich) and AK-Grp78HET (forward T6278-10MG, Sigma-Aldrich) were added to the crypts for 2 d. For EdU experiments, the medium was changed and both EdUs as BAY-876 or vehicle were added for 4 h before continuing with the manufacturer's protocol (C10634; Thermo Fisher Scientific).

**Measuring global translation rates**

For measurement of global protein translation rates, we used previously described measurements in the Materials and Methods section (van Lidth de Jeude et al, 2018). In short, global protein synthesis rates were quantified measuring 35S-labeled methionine and cysteine into newly translated proteins. Newly translated proteins were shown relative to control samples, after normalization to total protein, using BCA Protein Assay Kit (Pierce).

**Human tissue samples**

Tissue samples were taken from CRC patients who underwent resection in the Amsterdam UMC, location AMC. The study protocols were approved by the Medical Ethical Committee of the AMC and all patients provided written informed consent. Healthy colonic samples were taken at least 5 cm from the tumor. Samples were homogenized in cell lysis buffer (Cell Signaling Technology) with protease inhibitors (Roche).

**Immunoblot and quantification**

Organoids were recombined and lysed after 72 h in a lysis buffer (Cell Signaling) containing Protease Inhibitor Cocktail (13538100; Roche). Primary antibody detection was performed overnight; antibodies used for detection were anti-Grp78/BiP (31381, 1:1000; Cell Signaling), anti-Glut1 (135309, 1:1000; Abcam), and anti-β-actin (A1978, 1:1000, Sigma-Aldrich). Secondary antibody detection with HRP-labeled polyclonal antibodies was performed (#P0448; Dako, goat anti-rabbit, #P0447; goat anti-mouse, 12,000), and visualization was done using Lumilight Plus (12015196001; Roche). Bands were quantified using ImageJ software (version 1.5 Image; NIH).

**Seahorse**

Seahorse experiments were performed according to the manufacturer's instructions. 4 d after recombination, organoids were disrupted and transferred to XFe24 cell culture microplates (Agilent). After 2 d in the XFe24 cell culture plate, the organoids were
washed twice. The assay medium (Agilent) was added to the wells. For OCR measurements, the compounds oligomycin A (1 µM), FCCP (0.5 µM), rotenone (1 µM) and antimycin A (1 µM) were used. For ECAR, the compounds glucose (10 mM), oligomycin A (1 µM), and 2DG (50 mM) were used. OCR and ECAR were measured in an XFe24 Seahorse machine (Agilent). The experiment was performed and analyzed by the Seahorse software (Wave). Experiments were normalized to total DNA amount.

**Enzymatic assay for glucose, L-lactate, and pyruvate**

Enzymatic assays for glucose, L-lactate, and pyruvate were performed as described, using a CLARIOstar microplate reader (BMG LABTECH) (Chang et al., 2021). 24 h after recombination, the medium was refreshed with vehicle or BAY-876 and incubated for 48 h. Glucose was measured using a colorimetric assay with glucose oxidase. L-Lactate was measured with lactate dehydrogenase (Roche). Pyruvate was measured by a homovanilic acid-based fluorometric assay with pyruvate oxidase (P-4591; Sigma-Aldrich). Results were normalized to cell count.

**Array analysis**

Publicly available databases were analyzed using R2 platform. A complete description of the bioinformatics tool R2 may be found at https://hgserver1.amc.nl/cgi-bin/r2/main.cgi.

**Statistics**

Statistical analysis was performed using GraphPad Prism version 9.1.0 (GraphPad Software, www.graphpad.com). All values are depicted as the mean ± SEM. In experiments comparing two groups, statistical significance was calculated using a t test. For multiple comparisons, one-way or two-way analysis of variance (ANOVA) was used followed by a Bonferroni post-test. All organoid experiments were done in triplicate, with three wells of organoids per condition. Differences were considered statistically significant at P < 0.05.

**Supplementary Information**

Supplementary Information is available at https://doi.org/10.26508/lsa.202301912.

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**Conflict of Interest Statement**

The authors declare that they have no conflict of interest.

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