Ribosome impairment regulates intestinal stem cell identity via ZAKɑ activation

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The small intestine is a rapidly proliferating organ that is maintained by a small population of Lgr5-expressing intestinal stem cells (ISCs). However, several Lgr5-negative ISC populations have been identified, and this remarkable plasticity allows the intestine to rapidly respond to both the local environment and to damage. However, the mediators of such plasticity are still largely unknown. Using intestinal organoids and mouse models, we show that upon ribosome impairment (driven by Rptor deletion, amino acid starvation, or low dose cyclohexamide treatment) ISCs gain an Lgr5-negative, fetal-like identity. This is accompanied by a rewiring of metabolism. Our findings suggest that the ribosome can act as a sensor of nutrient availability, allowing ISCs to respond to the local nutrient environment. Mechanistically, we show that this phenotype requires the activation of ZAKɑ, which in turn activates YAP, via SRC. Together, our data reveals a central role for ribosome dynamics in intestinal stem cells, and identify the activation of ZAKɑ as a critical mediator of stem cell identity.

The mammalian intestine is an extremely proliferative organ. The entire epithelium turns over in around 3–5 days, and this proliferation is maintained by cells within the intestinal crypt. The crypt is home to a well-defined stem cell niche that supports a population of rapidly cycling ISCs, found at its base1. These stem cells express Lgr52, and are reliant on WNT ligands that are produced by various cells in the intestine, primarily the Paneth cell and various stromal cells3,4. It is known however, that the Lgr5-positive population is dispensable for normal intestinal growth5, and that multiple cell types with stem cell capacity exist in the intestine, including those marked by MEX3A, mTERT, LRG1, HOPX, DCLK1, and DLL16. For example, ALPI, a marker of the enterocyte lineage, labels cells that normally migrate up the intestinal villus and are shed into the lumen within days7. Upon the loss of Lgr5-positive cells however, these cells can undergo dedifferentiation into stem cells, demonstrating the remarkable plasticity of the organ8. More recently, a population of cells that appear to regain markers of the fetal intestine has been identified9, and has been shown to be crucial in a number of contexts, including after helminth infection10, and extracellular matrix remodeling11,12. They have also been shown to be a colitis-associated ISC that contributes to mucosal repair in this condition13,14.

While these various populations have been characterized, there is still a lack of understanding of the specific signaling pathways that determine alternative ISC identity. The canonical Lgr5-positive ISC is well studied, and the role of WNT, EGF, Notch, Bmp, and Hedgehog have been well defined (reviewed in ref. 15). This is not the case for...
other ISC types however, and although some physical determinants of ISC identity have been identified\textsuperscript{,22,26} the signaling cascades that define various phenotypes are largely unknown.

In several organs, mRNA translation has been shown to play a key role in stem cell fate\textsuperscript{27,28}. In hematopoiesis for example, simply increasing or decreasing protein synthesis in stem cells by 30% is sufficient to completely alter cell fate decisions\textsuperscript{29}, the intestinal altered translation is known to be crucial for intestinal tumorigenesis\textsuperscript{30,31}, and while several studies have demonstrated the importance of mTOR signaling in ISCs (particularly under stress conditions)\textsuperscript{32,33}, there are relatively few studies that directly analyze the role of translation in these cells\textsuperscript{34,35}.

In this study, we observe that the ISC is very responsive to the inhibition of RNA translation. Following a decrease in protein synthesis we see a loss of Lgr5-positivity and the induction of a fetal-like phenotype. This is accompanied by a translationally regulated switch in the metabolic identity of the cells, from oxidative phosphorylation to glycolysis. We show that this phenotype switch is driven by ribosome impairment. Although such translational issues have been shown to be a common occurrence\textsuperscript{36-38}, their biological relevance is currently unknown. Here, we provide insights into a biologically relevant change in cell fate caused by such impairment. Furthermore, we show that this phenotype switch occurs in a ZAKa-dependent manner, via activation of SRC and YAP, and that amino acid restriction results in the same ISC-identity change both in vitro and in vivo. We thus show that ribosome dynamics regulate intestinal cell fate, and may act as a sensor of nutrient availability, allowing the intestine to adapt to low amino acid levels by altering ISC identity.

**Results**

**Translation inhibition causes a cell and metabolic identity switch in the ISC**

In order to examine the role of RNA translation in the ISC, we generated organoids from the intestines of VilCre\textsuperscript{ERT2};Rptor\textsuperscript{fl/fl} mice\textsuperscript{30-32} (Supp. Fig. 1a). Raptor is an essential component of mTORC1, and we have previously shown that this complex regulates RNA translation in WNT-high intestinal cells\textsuperscript{33}. As expected, upon tamoxifen induction there was a loss of Raptor and mTORC1 signaling (as measured by S6 phosphorylation), and \textsuperscript{35}S-methionine incorporation showed that these organoids had decreased levels of protein synthesis (Fig. 1a, b). Surprisingly, the organoids took on a strikingly different morphology, losing the expected budding phenotype, and becoming cystic (Fig. 1c), which was reminiscent of organoids that were enriched for stem cells\textsuperscript{34}. However, qPCR analysis revealed that the organoids did not express markers commonly associated with the fetal intestine (Supp. Fig. 1b) in a specifically fetal manner, the induction of a fetal-like stem cell identity, there was an increase in the expression of the RiboTag allele specifically within the Lgr5-positive ISC population (Fig. 2d). Twenty four hours after tamoxifen induction, the RiboTag\textsuperscript{HA/HA} allele was expressed exclusively in the crypt base, and could be used to isolate ribosomes specifically from these cells (Fig. 2e). We could then use this material to carry out an in vivo ribosome profiling experiment, comparing Lgr5Cre\textsuperscript{ERT2};Riptor\textsuperscript{fl/fl};RiboTag\textsuperscript{HA/HA} animals\textsuperscript{35}. This allowed the in vivo deletion of Riptor and the inducible expression of the RiboTag allele specifically within the Lgr5-positive ISC population (Fig. 2d). Twenty four hours after tamoxifen induction, the RiboTag\textsuperscript{HA/HA} allele was expressed exclusively in the crypt base, and could be used to isolate ribosomes specifically from these cells (Fig. 2e). We could then use this material to carry out an in vivo ribosome profiling experiment, comparing Lgr5Cre\textsuperscript{ERT2};Riptor\textsuperscript{fl/fl};RiboTag\textsuperscript{HA/HA} animals, thus measuring the impact of Riptor deletion specifically within the ISC in vivo (Supp. Fig. 5a–e). As we observed in organoids, deletion of Riptor in vivo results in the upregulation of the fetal signature, and an increase in the ribosome association of mRNAs associated with glycolysis (Fig. 2f), showing that the same process occurs in vivo.

**Ribosome impairment causes the adult to fetal-like ISC identity switch**

Upon further analysis of this data, we observed that there was a striking increase in ribosome occupancy specifically on stop codons following Riptor deletion, suggestive of stalling ribosomes on these codons (Fig. 2g & Supp. Fig. 6). Such stalling (and subsequent ribosome collisions) around stop codons has been observed previously\textsuperscript{29}, however the biological relevance of this is unknown. Stalled ribosomes are known to have wide-ranging effects on translation dynamics, via recruitment of the ribosome quality control machinery\textsuperscript{35,36}, activation of GCN2\textsuperscript{37,38}, and an increase in ribosome collisions\textsuperscript{39,40}. As both stalled and collided ribosomes are known to activate cellular signaling, we assessed the ability of cycloheximide to drive the same adult to fetal-like stem cell switch that we observed following Riptor deletion. We used both low (0.005 \(\mu\)g/ml) and high (100 \(\mu\)g/ml) doses of cycloheximide, as low doses inhibit the elongation of some ribosomes, causing ribosome collisions, whereas high doses stall all ribosomes, allowing us to differentiate between the two situations\textsuperscript{38,39}. Importantly, both doses result in similar inhibition of protein synthesis (Supp. Fig. 7a). However, exclusively at low doses, cycloheximide treatment phenocopied Riptor loss, resulting in a loss of Lgr5-positivity and an increase in markers of fetal-like stem cells (Fig. 3a). This suggests that the ribosome impairment caused by Riptor deletion includes an increase in ribosome collisions (particularly around the stop codons), and that this may be causing the observed phenotype.

Ribosome collisions and other impairments of translational elongation are thought to be common events, and are known to have...
different outcomes depending on the driver and circumstances of the problem. For example, translation continues after some collisions, while mRNA and nascent peptide degradation occurs after others. They are also a natural response to many stimuli, including damaged or misfolded mRNA, and amino acid depletion. As the intestine is a major nutrient sensing organ, we asked whether translational impairment driven by amino acid depletion could also drive the same alternation in ISC populations. We therefore grew the organoids in a medium driven by amino acid depletion could also drive the same nutrient sensing orga.

The most well characterized detector of amino acid availability is GCN2, which is known to recognize uncharged tRNAs, and drive the cellular response to amino acid limitation. GCN2 has also previously been linked to the detection of ribosome stalling, which caused an activation of the integrated stress response and eIF2α phosphorylation. However, Rptor deletion, cycloheximide treatment and, most surprisingly, amino acid restriction did not result in an increase in eIF2α phosphorylation. Furthermore, inhibition of GCN2 was not sufficient to block the stem cell identity switch, suggesting that the canonical nutrient sensing pathways are not used to respond to low levels of amino acids in the ISC, nor is this pathway used to detect ribosome impairment.

**Phenotype switch is regulated via a ZAKα-mediated signaling cascade.** Recent studies have pinpointed the long isoform of the MAPKKK family member ZAK (ZAKα) as a key mediator of cellular responses to ribosome stalling and collisions. We therefore assessed ZAKα phosphorylation, following Rptor deletion, cycloheximide treatment, or glutamine/leucine deprivation. This analysis showed that there was an increase in the phosphorylation of ZAKα in all of these conditions. To understand the importance of ZAKα in ISC identity, we depleted it in organoids using shRNA. It is important to note that the shRNA used is specific to the long isoform of ZAK, and the levels of ZAKβ remain unchanged. Strikingly, ZAKα inhibition completely blocked the ability of cycloheximide to activate the fetal signature, and the metabolic switch that accompanies it.

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**Fig. 1 | Inhibition of translation drives stem cell identity changes.** a Western blot analysis confirming reduced Raptor and pS6 (Ser235/236) protein levels in cells from 3 different animals treated with 4-Hydroxymethylxen. β-actin and Ponceau serve as loading control. b Incorporation of 3S-methionine shows decreased protein synthesis in Rptorfl/fl organoids compared to WT. Mean and standard deviation are shown (n = 4 biological replicates each accessed in technical duplicates). p values were determined using a two-tailed t test. c Representative images showing morphological differences in organoids upon Rptor loss, compared to WT. Pictures were acquired using 5x (left panel) and 20x (right panel) magnifications. d RT-qPCR analysis of individual genes related to stem (Lgr5, Muska and Axin2), differentiation (Lyz1, Mc2 and AlpR), and fetal-like state (Tasc1td2, Sca1, Spp1 and Cx43) of Rptorfl/fl organoids compared to WT, using Hprt as a reference. Mean and standard error of the mean are shown (n = 3 biological replicates each accessed in technical triplicates). p values were determined using a two-tailed t test. e Representative 3D-reconstructed confocal images of wild type and Rptorfl/fl organoids show significant reduction of adult stem cell marker OLFM4 (left panel, green) and activation of fetal marker Tacstd2, Sca1, Spp1 and Cx43 (right panel, green). Dapi is used to visualize the nuclei (magenta). Scale bar is 50 μm. f OCR and ECAR analyses reveal decreased respiration and increased glycolysis in Rptorfl/fl organoids compared to WT. Mean and standard deviation are shown (n = 2 biological replicates each accessed in technical quadruplicates). Refer to Supp. Fig. 1 for quantifications. g Gene Set Enrichment Analysis based on RNASeq differential expression data comparing Rptorfl/fl organoids to WT (n = 4 from 2 biological replicates for each). Enrichment is shown for transcriptional signatures related to stemness, glycolysis, mTORC1 signaling and fetal-like state. p values were determined using the clusterProfiler package.
This was also the case for Raptor deletion, and amino acid restriction (Fig. 3f–h). Additionally, Vemurafenib has been shown to be a potent inhibitor of ZAK\(^5\)\(^6\)\(^7\). As with shRNA mediated silencing of ZAK\(\alpha\), treatment with Vemurafenib also blocked ISC identity switch, as well as the accompanying metabolic switch (Supp. Fig. 8a–d).

As we had already shown that this effect is GCN2-eIF2\(\alpha\) independent, we measured the activation of the JNK and p38 signaling pathways, as these are known to mediate the downstream consequences of ZAK\(\alpha\) activation\(^8\). There was no activation of JNK signaling following Raptor deletion (Supp. Fig. 7f), and inhibition of this pathway with JNK\(-\)inhibitor of ZAK\(\alpha\)–FLAG, both wild-type and Raptor\(-\)deficient organoids, followed by mass spectrometry (IP-MS) (Supp. Fig. 9a). While we could only identify a small number of proteins that were precipitated with ZAK\(\alpha\)-FLAG in our fetal-like system. To identify other potential substrates of ZAK\(\alpha\), by defining its specific interactome in our fetal-like system. To do this, we carried out immunoprecipitations of FLAG tagged ZAK\(\alpha\) in both wild-type and Raptor\(-\)deficient organoids, followed by mass spectrometry (IP-MS) (Supp. Fig. 9a).

We therefore set out to identify other potential substrates of ZAK\(\alpha\), by defining its specific interactome in our fetal-like system. To do this, we carried out immunoprecipitations of FLAG tagged ZAK\(\alpha\) in both wild-type and Raptor\(-\)deficient organoids, followed by mass spectrometry (IP-MS) (Supp. Fig. 9a). While we could only identify a small number of proteins that were pulled down with ZAK\(\alpha\) in our control cells (Supp. Fig. 9b), Raptor loss caused a dramatic increase in the number of proteins that co-precipitated with ZAK\(\alpha\), highlighting the importance of this kinase in mediating the cellular response to impaired translation (Fig. 4A). Among these interactors we identified SRC, a well known YAP activator which has been recently implicated in injury-induced regeneration in the intestine\(^11\). In that study, the authors showed that upon tissue damage the intestinal epithelium is converted into a fetal-like state, with decreased levels of differentiation markers, which seems to be mediated by increased FAK/SRC signaling and a resulting YAP/TAZ activation. To validate the interaction of ZAK\(\alpha\) with SRC in our other fetal-like models, we performed a Co-IP of ZAK\(\alpha\)-FLAG

promoter, both in the presence and absence of Raptor. 24 h after tamoxifen induction, the ribosomes are recombined with the HA tag exclusively in the intestinal stem cells, allowing the capture and further study of their translome by RIboSeq. e Immunohistochemistry staining of Lgr5\(\text{Cre}^{\text{ERT2}}\)RPL22.HA intestines show that HA staining is restricted to ISCs. Stainings were done in 3 biological replicates. Scale bar: 20 \(\mu\)m. f Gene Set Enrichment Analysis based on differential translatome of intestinal stem cells from Rptor\(-\)fl/fl and WT mice (n = 3 biological replicates). Enrichment is shown for transcriptional signatures related to oxidative phosphorylation and glycolysis. p values were determined using the clusterProfiler package. g Distribution of RPFs along transcripts show an accumulation of reads in the stop codon in the Lgr5\(\text{Cre}^{\text{ERT2}}\)Rptor\(-\)deficient organoids, compared with the Lgr5\(\text{Cre}^{\text{ERT2}}\)Rptor\(\text{fl/fl}\) intestines (gray). Barplot depicts RPF abundance change (log2FC) between the two conditions and lines show total RPF abundance (%), for which RPFs are grouped based on their A-site position with stop-codon as reference.
To understand the functional consequences of such interaction, we measured the levels of the active form of SRC by looking at the phosphorylation status of its residue Tyr416. To assess whether ribosome-mediated activation of ZAK occurs in infected organoids which were cultured with either low dose Chx or in the absence of glutamine, and saw that in both cases ZAK interacts with SRC (Fig. 4B & Supp. Fig. 9c). This interaction is decreased in the control organoids, highlighting its potential role in sensing ribosome collisions (Fig. 4C).

As there is a significant overlap between genes included in the YAP genset used for this analysis and in the genset used to identify fetal-like cells, we analyzed YAP-target genes that were not part of the fetal signature, Myof and Ankrd1. Both genes were significantly upregulated upon Rptor deletion and this increase is mediated by ZAK, supporting the idea that YAP/TAZ is activated upon ribosome impairment (Supp. Fig. 9d). In order to test whether the ZAK-mediated activation of SRC and YAP caused the observed cell fate switch, we treated Rptor-deficient organoids with either a SRC inhibitor (dasatinib) or a YAP inhibitor (veretopirin). Both treatments could block the adult to fetal-like switch (Fig. 4G). Together, these results indicate that ribosome impairment causes a signaling cascade that results in the YAP-dependent activation of a fetal-like transcriptional program.

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infected organoids which were cultured with either low dose Chx or in the absence of glutamine, and saw that in both cases ZAK interacts with SRC (Fig. 4B & Supp. Fig. 9c). This interaction is decreased in the control organoids, highlighting its potential role in sensing ribosome collisions (Fig. 4C).

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To assess whether ribosome-mediated activation of ZAKa caused the same cell identity switch in human tissue, we used human colonic
organoids, which we grew in either full media or media deprived from leucine. In line with our previous findings, we saw that amino acid restriction led to an activation of the fetal signature which could be rescued when we inhibited Zak (Supp. Fig. 9e).

Amino acid restriction in vivo drives the same phenotype switch

Finally, as the intestine is an extremely complex tissue that cannot be fully recapitulated in organoid culture, we carried out an amino acid depletion study in WT and Zak knock-out mice (Fig. 4H & Supp. Fig. 9f). Zak deletion has no overt phenotype, and food intake is unchanged (Supp. Fig. 9g). We maintained animals on a leucine-deficient diet for 9 days (Fig. 4H) and measured the levels of Lgr5 and markers of the fetal intestine (Tacstd2, Cx31, Cx43, Ctgf) and YAP signature (Ankrd1) in the colons of these animals\(^5\). As observed in organoids, in vivo amino acid depletion resulted in an increase in fetal markers (Fig. 4I). Crucially however, deletion of Zak prevented the appearance of these cells, demonstrating that the change in cell fate caused by leucine starvation is a ribosome impairment-mediated effect (Fig. 4I). It’s worth noting that both in the human (Supp Fig. 8d) and mouse (Fig. 4E) colon samples, leucine depletion does not seem to affect the Lgr5 + population. This may be due to the fact that this cellular population is rarer in the colon compared to the small intestine, and the effect of restricting amino acids may be underestimated as a result.

Discussion

Ribosome stalls and collisions have long been known to cause mRNA and nascent protein degradation\(^6,7\). As they can be caused by defective mRNA, it is clearly advantageous to a cell to remove the problem. The development of disome seq showed that collisions were far more common than expected, and confirmed their importance in the correct folding of peptides, underlining the fact that they play a role in normal biology, alongside their role in sensing faulty mRNA\(^8,9\). Recent work has taken this a step further, and has shown that ribosome collisions may act as key signaling hubs that can determine cellular fate

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**Fig. 4 | ZAKα activates the SRC-YAP axis during ribosome impairment and results in ISCs identity switch in vivo during leucine deprivation.**

A Volcano plot depicting differentially enriched interactors of ZAKα in Rptor\(^\alpha\) organoids, using RIME-MS (n = 2 biological replicates). An empty vector tagged FLAG bait is used as a control. Significance when p-value ≤ 0.05 (t-test, two-tailed). B Immunoprecipitation of ZAKα-FLAG confirms interaction with SRC in organoids treated with cycloheximide (0.015 ug/ml) and deprived from glutamine. EV-FLAG is used as a control. One biological animal was used. C Quantification of the western blot shows enrichment of ZAKα-SRC binding in organoids treated with cycloheximide (0.015 ug/ml) and deprived from glutamine, compared with wild type cells. D Western blot showing activation of SRC in Rptor\(^\alpha\), low dose cycloheximide and glutamine-deprived organoids compared to wild type. This activation is dependent on ZAKα activity, as inhibition with vemurafenib abolishes SRC phosphorylation (T416). β-actin serves as a loading control. Blue panel has a higher exposure. Blots were done on one animal. E Western blot showing activation of YAP in Rptor\(^\alpha\), low dose cycloheximide and glutamine-deprived organoids compared to wild type. This activation is dependent on ZAKα, as inhibition with dasatinib abolishes YAP phosphorylation (Y357). β-actin serves as a loading control. Blue panel has a higher exposure. Blots were done on one animal. F Gene Set Enrichment Analysis based on RNASeq differential expression data comparing Rptor\(^\alpha\) organoids to WT (n = 4 from 2 biological replicates for each). Enrichment is shown for transcriptional signatures related to YAP target genes. p-values were determined using the clusterProfiler package. G RT-qPCR analysis of genes related to stem (Lgr5) and fetal-like state (Tacstd2 and Scal) of WT and Rptor\(^\alpha\) organoids treated with inhibitors for SRC (vemurafenib, 100 nM, 24 h) and YAP (verapamil, 3 μM for 24 h). Hprt is used as a reference. Mean and SEM are shown (n = 1 biological replicates accessed in technical triplicates). p-values were determined using a two-tailed t-test. H Experimental workflow of the dietary interventions performed in WT and ZAK KO mice. I RT-qPCR analysis of genes related to stem (Lgr5) and fetal-like state (Tacstd2 and Scal) of colons of WT and ZAK KO mice. Hprt is used as a reference. Mean and SEM are shown (n = 5 (WT) and 9 (ZAK KO) biological replicates each accessed in technical triplicates). p-values were determined using a two-tailed t-test.

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by recruiting different players which activate specific pathways depending on the “severity” of the damage. Indeed, studies have now started to dissect the downstream effects of ribosome impairment, and it appears that several factors, such as elongation rate, “strength” of stall, and the activity of the ribosome quality control mechanisms can subtly alter the signaling that results. However, the extent of the in vivo relevance of ribosome impairment is currently unknown.

Here we show that ribosome-induced signaling through ZAKα is a crucial determinant of ISCs identity (Supp. Fig. 9h). Unlike canonical ZAKα signaling however, it appears to be independent of both JNK and p38 signaling, and instead it seems to be mediated by a SRC-YAP axis. Indeed, it has been shown that p38 activation is important for maintaining adult ISC identity, so it is not surprising that ZAKα activation of p38 does not mediate a switch to a fetal-like phenotype. Furthermore, contrary to what has previously been reported, we find that GCN2 and the ISR are not activated downstream of ZAKα. However, Vind et al. have also found that GCN2 is not involved in ZAKα-mediated signaling, and a recent study has shown in yeast that GCN2-mediated ribosome quality control (RQC) is more likely to result from high levels of mRNA damage compared to low levels, demonstrating a coordination (and indeed antagonism) between the RQC and ISR. Crucially, activation of the ISR via ER stress is known to cause the differentiation of ISC, and thus would lead to the loss of the stem cell pool if it were activated by ribosome collisions.

Our results suggest an alternative mechanism through which intestinal stem cells can sense perturbations in ribosome dynamics that prompt them to switch to a fetal-like state. We describe a pathway activated by ribosome impairment, whereby ZAKα phosphorylates SRC, triggering a YAP/TAZ transcriptional reprogramming which converts adult ISCs into a fetal-like state. Several studies have implicated both YAP and TAZ in the maintenance of cell identity during different stress conditions. The switch to a fetal-like state has also been observed in several stress conditions. Yui et al. demonstrated that upon tissue damage, cells convert into a fetal-like state as a result of increased FAK/SRC signaling and consequent YAP/TAZ activation. However, it is still not clear which specific damages and signaling cascades lead to this transcriptional reprogramming. In this study, we propose that ribosome collisions may act as sensors for different stress conditions, allowing for the emergence of a fetal-like population of ISCs through the activation of ZAKα.

Further studies are clearly needed to understand the role of ribosomes and ZAKα in the ISC. The ISC is known to be a plastic population, and this plasticity is especially important under various stress conditions, when the cells can take on a new identity in order to overcome this. A number of these stressors are known to result in the inhibition of translation, particularly calorie and nutrient deprivation. Indeed, it has been shown that amino acid levels can have a significant effect on ISCs, although studies have focused on amino acid supplementation rather than depletion, and have been very limited in scope. Here, we show that both deprivation of leucine or glutamine in vitro and leucine in vivo are sufficient to drive a ZAKα-mediated ISC identity switch, suggesting that ribosome dynamics may be used as a nutrient sensor to alter stem cell fate in response to local nutrient availability. As dedicated nutrient sensing machinery exists in the cell, it is surprising that the ribosome would be used in this way in the ISC. However, as mentioned above, activation of the ISR is detrimental to ISCs, and must be avoided if the stem cell population is to be maintained. It is possible therefore that the recognition of amino acid depletion via changes in ribosome dynamics evolved in order to allow the cell to respond to such depletion, without activating the ISR, thus maintaining the vital ISC population. Although it has been shown that Gcn2 deletion enhances intestinal inflammatory phenotypes, the specific role of GCN2 in the ISC was not analyzed in that manuscript, however it is safe to say that the response to amino acid depletion in the intestine is a complex one. Interestingly, it has been shown that calorie restriction (as opposed to specific amino acid depletion) leads to an increase in the number of ISCs, rather than a decrease. This process appears to be regulated in a non-autonomous manner, with Paneth cells working as direct sensors or calorie availability in this context, and activating pathways to augment ISC number. In our model, ribosome impairment via amino acid restriction, cycloheximide treatment, or inactivation of mTORC1 leads to a striking decrease of Paneth cells, perhaps explaining the difference between our findings and those related to calorie restriction.

The ability of ISCs to detect and respond to amino acid depletion is crucial to the well-being of the organ, and the sensor of this would be central to intestinal biology. Indeed, the fetal-like ISC phenotype has been shown to be important following helminth infection, extra-cellular matrix remodeling, and has been suggested to allow Wnt-independent growth in intestinal cancer. We do not know whether ribosome collisions and ZAKα play a role in these models, but due to the diversity of triggers of this phenotype, it is possible that independent mechanisms can result in the same outcome.

In all, we show that ribosome impairment is a major result of translation inhibition in the intestine. This impairment results in a dramatic change in ISC identity both in vitro and in vivo, and we show that activation of a ZAKα-SRC-YAP axis is central to this. Considering the many stimuli that can drive such impairment, we propose that the dynamics of translation elongation represent a major cell fate decision checkpoint in the intestine, and a previously undescribed mechanism through which Lgr5-expressing cells can be targeted by therapy.

Methods
Ethical approval
All experiments were carried out with the approval of the relevant ethical bodies: NKI Animal Welfare Body, University of Copenhagen Institutional Animal Care and Use Committee, and the NKI-AVL Institutional Review Board (IRB).

Mouse colonies
Animals for this study were bred in-house at the Netherlands Cancer Institute and all experimental protocols were approved by the NKI Animal Welfare Body.

C57BL/6 female and male mice between 8 and 12 weeks of age were used for experiments.

Animals were generated as previously described. Briefly, for the recombination of VillinCreERT2/Rptorfl, VillinCreERT2/RPL22.HA and VillinCreERT2/Rptorfl/RPL22.HA animals, two consecutive injections of 80 mg/kg tamoxifen were performed and samples were taken after 120 h. For Lgr5CreERT2/RPL22.HA and Lgr5CreERT2/Rptorfl/RPL22.HA animals, a single intraperitoneal injection of 120 mg/kg tamoxifen and samples were taken after 24 h, in order to account for differences in recombination efficiency and total amount of cells.

For the leucine-deficient diet in vivo, WT and ZAK KO male mice around 10–11 weeks old were fed a full synthetic diet for 3 weeks. After 3 weeks, 5 mice (of each genotype) continued eating a full synthetic diet, and inactivation of mTORC1 leads to a striking decrease of Paneth cells, perhaps explaining the difference between our findings and those related to calorie restriction.

The ability of ISCs to detect and respond to amino acid depletion is crucial to the well-being of the organ, and the sensor of this would be central to intestinal biology. Indeed, the fetal-like ISC phenotype has been shown to be important following helminth infection, extra-cellular matrix remodeling, and has been suggested to allow Wnt-independent growth in intestinal cancer. We do not know whether ribosome collisions and ZAKα play a role in these models, but due to the diversity of triggers of this phenotype, it is possible that independent mechanisms can result in the same outcome.

Cryp

Cryp
Organoids were cultured in complete medium with growth factors and stem-cell inducing factors: 10 μM Rho kinase inhibitor Y-27632 (Cayman), 1 mM VPA (Biovision), 1 μM Jagged-1 (AnaSpec) and 6 μM CHIR99021 (Cayman). After 2 days in culture, organoids were then dissociated into single cells with TrypLE Express enzyme (Thermo Fisher Scientific). For the Raptor knock out organoids StemRad Actinase (Thermo Fisher Scientific) was used instead. Cells were resuspended in complete medium, containing growth factors, stem-cell inducing factors and 8 μg/ml polybrene and laid over wells covered with BME. The virus was added and cells were incubated in normal culture conditions. After 24 h of infection a layer of BME was put on top and the media was refreshed. The next day selection with 2 μg/ml puromycin was added. After a further 10 days selection the organoids were put in BME plugs and kept in normal culture conditions.

Protein synthesis analysis

Organoids were grown in normal ENR media and taken at Day 4 for analysis. Protein synthesis rates were measured, as described previously. Briefly, cells were treated with DMEM methionine-free media (Thermo Fisher Scientific #2103024) for 20 min and incubated with 30 μCi/ml [35S]-methionine label (Hartmann Analytic) for 1 h. After washing the samples with PBS, proteins were extracted with lysis buffer (50 mM TrisHCl pH 7.5, 150 mM NaCl, 1% Tween-20, 0.5% NP-40, 1% protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma Aldrich)) and precipitated onto filter paper (Whatmann) with 25% trichloroacetic acid and washed twice with 70% ethanol and twice with acetone. Scintillation was then read using a liquid scintillation counter (Perkin Elmer) and the activity was normalized by total protein content. All experiments were done in technical triplicates for each biological unit.

RNA isolation and RT-qPCR

RNA was isolated by chloroform extraction followed by centrifugation, isopropanol and glycogen precipitation and 75% ethanol washing. Pellets were resuspended in nuclease-free water and RNA was quantified with Nanodrop. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following manufacturer’s instructions. qPCR was performed with SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) in technical triplicates and relative gene expression was calculated using the comparative CT method by normalization to Hprt. The primer sequences used were: Hprt forward (5′-CTGGTGAAGACCTCTCG-3′), Hprt reverse (5′-TGAAGTACTCTATATGCAAGGGA-3′), Lgr5 forward (5′-ACCAGGGAGGAGGTGCG-3′), Lgr5 reverse (5′-GAGTTTGTGTGCGTCCAG-3′), Axin2 forward (5′-GAGACCAAGGCAACCAG-3′), Axin2 reverse (5′-GGGCGCCAGCTCATCAG-3′), αT3 forward (5′-ACCCACACACACACAA-3′), αT3 reverse (5′-GGCTTGGGGCAAGGAGG-3′), Mexca forward (5′-ACACCGACGACGCGTC-3′), Mexca reverse (5′-ATGGTTTTGGCCTCAGA-3′), E(spl)-Cbx1 forward (5′-GAGAGTCCCGCCGTCCT-3′), E(spl)-Cbx1 reverse (5′-TCGCCAGGTATCATTGGA-3′), Tacstd2 forward (5′-GAAGGT CATGACCTCTCAC-3′), Tacstd2 reverse (5′-CAACCAAGTGGGAGAAGAAC-3′), Cnx3 forward (5′-GGGACCAAGGAGATGAAAAAGGGAGG-3′), Cnx3 reverse (5′-GGGACCAAGGAGATGAAAAAGGGAGG-3′), MyoD forward (5′-GCGCGCGCTACTATCTCTCAC-3′), MyoD reverse (5′-CAGTCATGGAATGCTAGAAAATAG-3′), Cnx2 reverse (5′-TTGGAGAACCTGTT-3′).

Generation of intestinal organoids with ZAKα knock down, EV-FLAG and ZAKα-FLAG

shRNA targeting Zaka was chosen from the Open Biosystems Expression Arrest™ TRC library (target sequence CACGATACTCTGAACCTGT). As negative control an shRNA containing a scramble sequence was used (CACCAAGTAGAAGGACCA). These shRNAs were inserted into a plKO.1 vector (Addgene).

The viral vectors were transfected into HEK293T cells (obtained from the ATCC) with third-generation packaging plasmids (pVS-V, pRSV-REV and pMDL.RRE). The viral supernatant was filtered through a 0.45 μm filter and concentrated using Lentix Concentrator (Takara).

For the affinity purification experiments, lentiviral vectors expressing FLAG, pLV[Exp]Bsd-mPGK > 3xFLAG/Stuffer_300bp (Ev-FLAG; ID = VB210329-1386htq) and pLV[Exp]Bsd-mPGK > mMap3k20/NM_023057.5/3xFLAG (ZAKα-FLAG; ID = VB210329-1381jwy), were constructed and packaged by VectorBuilder. Detailed information can be retrieved on vectorbuilder.com using the identifications.

For generating stem cell enriched (SCe) cultures, organoids were grown in ENR media supplemented with 10 μM of CHIR 99021 (Cayman Chemical) and 1.5 mM of valproic acid.

For the human organoids, a piece of normal colon tissue, of which the muscle layer and fat were removed, was cut in small pieces followed by EDTA treatment to release epithelium from underlying mesenchyme. The isolated epithelium was embedded in BME CultiPrep and grown at 37 °C with ENR media, including Wnt conditioned media, until the organoids were fully formed. All patients gave written informed consent to have organoids generated from their left-over tissue, and agreed to have these included in a biobank for future scientific research. Human material cannot be shared due to ethical and privacy reasons. Protocols were approved by the NKI-AVL Institutional Review Board (IRB).

Cycloheximide (Sigma Aldrich) treatment was done using high (100 μg/ml) and low (0.015 μg/ml) doses for 30 min. GCN2 was inhibited using A-A2 (2 μM for 24 h), p38 was inhibited with SB203580 (10 μM, 24 h) and JNK was inhibited with JNK-IN-8 (1 μM for 24 h). Dasatinib was used to inhibit SRC(100 nM, 24 h) and verteporfin was used to inhibit VAP (3 μM, 24 h). Finally, for inhibition of ZAK, Vemurafenib was added to the media at a final concentration of 1 μM and organoids were treated for 1 h.

Immunofluorescence

Immunofluorescence of intestinal organoids was performed as previously described. DAPI was combined with appropriate Alexa Fluor labeled secondary antibodies: Chicken anti-Rabbit Alexa Fluor 647 (Thermo Fisher Scientific #A21443) 1:500; Donkey Anti-Rat Alexa Fluor 555 (Abcam #ab150154) 1:500; Donkey anti-Goat Alexa Fluor Plus 555 (Thermo Fisher Scientific #A32816) 1:500. Images were collected on an inverted Leica TCS SP8 confocal microscope (Mannheim, Germany) in 12-bit with 25Xwater immersion objective (HC FLUOTAR L N.A. 0.95 W VISIR 0.17 FWD 2.4 mm). Imaris software (version 9.3.1, Oxford Instruments) was used for 3D reconstruction of images. The organoids were incubated with the following primary antibodies overnight: anti-Ly-6A (Sca1) (Biolegend #108101) 1:200, anti-Olfm4 (Cell Signaling #39141) 1:100, anti-Aldolase (Abcam #ab75751) 1:300, anti-Lysozyme (Agilent #A0099) 1:400, and anti-Mouse TROP-2 Antibody (R&D systems #AF1122) 1:50. Alexa Fluor labeled secondary antibodies (ThermoFisher Scientific #A22287) were combined with DAPI.

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AGAGAAG-3'); Zakβ forward (5'-GGCCATCGTCAAGCAAATC-3'), Zakβ reverse (5'-GATAGGGAAAACCGCTTAAA-3').

RNA sequencing
Total RNA was isolated from wild type, Raptorfl/fl and SC-enriched organoids as described above. Quality of the samples was measured with the 2100 Bioanalyzer using a RNA Nanochip (Agilent) and accepted for downstream analysis when showing an RNA integrity number (RIN) above 8. Libraries were generated with the TrueSeq Stranded mRNA kit (Illumina) and sequenced using the HiSeq2500 equipment.

Affinity purification and quantitative mass spectrometry
Rapid Immunoprecipitation mass spectrometry of endogenous proteins (RIME) was used to identify ZAKu interactors as previously described30, with some minor modifications. Briefly, wild type and Raptorfl/fl organoids transfected with Ev-FLAG and ZAKα-FLAG constructs were crosslinked with 1% formaldehyde for 10 min and quenched with glycine to stop the reaction. Cells were lysed with LB3 and left in rotation for 10 min at 4 °C. Lysates were sonicated for 10 cycles of 30 s ON/30 s OFF and cleared by centrifugation at max speed for 12 min, at 4 °C. Samples were incubated with pre-washed FLAG-conjugated beads, overnight at 4 °C. For mass spectrometry, peptide mixtures were prepared and measured as previously described30, with the following preparations. Peptide mixtures (10% of total digest) were loaded directly onto the analytical column and analyzed by nanoLC-MS/MS on an Orbitrap Fusion Tribrid mass spectrometer equipped with a Proxeon nLC1200 system (Thermo Scientific). Solvent A was 0.1% formic acid/water and solvent B was 0.1% formic acid/80% acetonitrile. Peptides were eluted from the analytical column at a constant flow of 250 nL/min in a 104 min gradient, containing a 84-min stepped increase from 2% to 24% solvent B, followed by an 20 min wash at 80% solvent B.

Raw data were analyzed by MaxQuant (version 2.0.3.0)73 using standard settings for label-free quantitation (LFQ). MS/MS data were searched against the Swissprot Mus musculus database (17,073 entries, standard settings for label-free quantitation (LFQ). MS/MS data were searched against the Swissprot Mus musculus database (17,073 entries, standard settings for label-free quantitation (LFQ).

Bioenergetics Analysis
Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using Seahorse Bioscience XFe24 Analyzer (Agilent). Organoids were first seeded in normal plates, in order to diminish growth variations. At Day 3, organoids were resuspended in 40uL of BME, re-plated in XF24 Seahorse Cell Culture microplates (Agilent) and cultured for 16–24 h before the analysis, which was done according to the manufacturer’s instructions in DMEM (Sigma-Aldrich) supplemented with 2 mM L-glutamine for the ECAR experiments and additional 5.5 mM D-glucose for the OCR measurements. For the ECAR analysis, the following reagents were added: glucose (10 mM), oligomycin (1 μM) and 2-deoxy-D-glucose (50 mM). For OCR measurements, the following reagents were added: oligomycin (1 μM), FCCP (0.4 μM), and rotenone (1 μM) and antimycin A (1 μM). When applicable, cells were pre-treated with either vemurafenib (1 μM) or cycloheximide (0.015 μg/μL) for the desired time. All results were normalized to DNA content. Briefly, cells were scraped from the microplates and centrifuged for 3 min at 4,000 rpm at 4 °C. Pellets were then washed with cold PBS and resuspended in lysis buffer (75 mM NaCl, 50 mM EDTA, 0.02% SDS, 0.4 mg/mL Protease K). Samples were incubated at 56 °C for 2 h. Isopropanol (1 volume) was then added and mixed and incubated at 4 °C overnight. Tubes were centrifuged at 8,000 rpm for 30 min at 4 °C and pellets washed with 70% ethanol and air dried. DNA was resuspended in H2O and quantified using Nanodrop.

Ribosome profiling
Sample preparation. Samples were prepared as described previously74. Briefly, for in vitro analyses, organoids were generated from VillinCreERT2RPL22.HA and VillinCreERT2Rptor/fl RPL22.HA animals. Around 120–150 plugs of 30 μL BME were used for each replicate. Cells were treated with 100 μg/ml cycloheximide for 3–5 min at 37 °C and immediately incubated on ice for the remainder of the experiment. After collecting the cells, pellets were washed twice with cold PBS supplemented with 100 μg/ml cycloheximide, resuspended in ice-cold lysis buffer (20 mM Tris HCl pH 7.4, 10 mM MgCl2, 150 mM KCl, 1% NP-40, 100 μg/ml cycloheximide and 1x EDTA-free proteinase inhibitor cocktail (Roche)) and incubated for 20 min on ice. Lysates were then centrifuged at max speed for 20 min at 4 °C and the supernatants were collected.

For in vivo analyses, Lgr5CreERT2RPL22.HA and Lgr5CreERT2Rptor/fl RPL22.HA animals were euthanized by CO2 and small intestines were immediately dissected, flushed with cold PBS supplemented with 100 μg/ml of cycloheximide and snap frozen using liquid nitrogen. Frozen samples were then homogenized by pestle and mortar while submerged in liquid nitrogen and the resulting powder was resuspended in ice-cold lysis buffer and incubated on ice for 30 min. Finally, lysates were centrifuged at max speed for 20 min at 4 °C and the supernatants were collected.

HA pulldown. All lysates were incubated with Pierce™ Control Agarose Matrix (ThermoFisher) for 20 min at 4 °C in order to remove any non-specific binding, following by an incubation with pre-washed AntiHA.II Epitope Tag Affinity Matrix (BioLegend) for 4 hours (in vitro samples) or overnight (in vivo samples) at 4 °C. Tagged ribosomes were then
eluted from the beads by incubating with 200 μg/mL HA peptide (ThermoFisher Scientific) for 15 min at 30 °C with constant agitation. Non-protected RNA was digested with 10 μl of RNase I (ThermoFisher Scientific) for 40 min at 25 °C and the reaction was stopped by adding 13 μl of SUPERASE (ThermoFisher Scientific). Ribosome protected fragments (RPFs) were finally purified using miRNeasy minikit (Qiagen) according to manufacturer’s instructions.

**Library preparation.** The library preparation was performed as previously described with some modifications. To discard undigested RNA fragments, RPFs were run in a 10% TBE-Urea polyacrylamide gel and size selected between 19 nt and 32 nt using marker RNAs. 3′ ends were dephosphorylated using T4 poly-nucleotide kinase (PNK) (NEB) and 1.5xMES buffer (150 mM MES-NaOH, 15 mM MgCl2, 15 mM β-mercaptoethanol and 450 mM NaCl, pH 5.5) and incubated at 37 °C for 4 h. After purifying RNAs with Trizol, 3′ adapters were ligated with T4 RNA ligase I (NEB) and incubated overnight at 24 °C. After size-selecting the ligated products, 5′ ends were phosphorylated with T4 PNK and incubated at 37 °C during 30 min. 5′ adapters were then ligated with T4 RNA ligase I and incubated 37 °C for 2.5 h. The RPFs containing both adapters were then size-selected one more time and rRNA depletion was performed by first denaturing the samples at 100 °C for 1 min followed by incubation with 100x TAE-biotinylated UCA-CAGAUUGACCCACCCUCGGC-3′; 5′-GCGGCUC-3′; 5′-GCCGGACGGGGGGAGAGGGA-3′; 5′-ACGGACGGUCAGACAACACCCGGCCACGC-5′; 5′-AGCAAGUGCGU-3′; 5′-GGCGGACGGGUUAGUAAUGGG-3′; 5′-CGCGAUCAGCAGGCGUGGUGGUUCG-3′; 5′-GGCGGACGGGCGUCGGCUAGAAGCUUAGUGCUACGUACGACGAAACCCCGACC-3′; 5′-GGCGGACGGGGGCCGGUGGUGCGCCCUCGGC-3′; 5′-UCGAAGUGUCGAUGAUC-3′; 5′-GCGGCUC-3′; 5′-GCUGAACUAGCAGGCGUGGUGGUUCG-3′; 5′-ACGGACGGUCAGACAACACCCGGCCACGC-5′; 5′-ACGGACGGUCAGACAACACCCGGCCACGC-5′. The RPFs were purified using magnetic Dynabeads and re-suspended in 8 μL of RNase-free water. cDNA was synthesized with SuperScript III (ThermoFisher Scientific) according to manufacturer’s instructions and using the RTP primer (seq). After purification with G50 columns (Merck), cDNA was amplified using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific) for 15 min at 37 °C. Pre-washed MyOne Streptavidin C1 DynaBeads (ThermoFisher Scientific) were then incubated with samples in wash/bind buffer (2 M NaCl, 1 mM EDTA, 5 mM Tris and 0.2% Triton X-100) for 30 min at 37 °C with agitation. Supernatants were collected and RPFs were purified and re-suspended in 8 μL of RNase-free water. cDNA was synthesized with SuperScript III (ThermoFisher Scientific) for 15 cycles, with primers containing different indexes to allow for sequencing. PCR products were purified using the QiAquick PCR purification kit (Qiagen) followed by a final size selection with a E-Gel SizeSelect II 2%. All samples were accessed with the Agilent 2100 Bioanalyzer to assure high quality and quantify the molarity and the libraries were sequenced on the Illumina HiSeq2500.

**Data analysis.** Initial quality control of all sequencing data was performed using the FastQC tool. Adapter trimming and size selection marker cleaning of the raw Ribo-seq data was performed using the cutadapt tool. After Ribo-seq reads were cleaned from rRNA fragments, QC plots were generated with RiboCode 7 for which Ribo-seq reads were mapped to mm10 genome using the STAR aligner 8. For the quantification of transcript abundances with RNAseq, Salmon 9 was used with protein-coding transcript sequences obtained from gencode vM21 annotation. To measure ribosome occupancy with Ribo-seq, the same tool was used with a different reference sequence set, where UTRs were trimmed from the same mRNA sequences and duplicated sequences were removed prior to the run. Differential expression and ribosomal occupation analyses were performed in R environment, using the DESEQ2 package 10 with Salmon outputs. Differential translation efficiency analysis was performed with RiboDIF 11 for which the input consisted of Ribo-seq and RNAseq transcript quantifications (NumReads column in salmon output) of primary transcripts, excluding the genes with low sequencing depth. Primary transcripts were decided based on Ensembl 96 APPRIS annotation. Gene Set Enrichment Analyses were performed in R using the clusterProfiler package 12 for which the genestes were taken from MSigDB v7.0 with additional genestes from published sources 13. For the differential codon usage analysis performed with Ribo-seq data, we use the assumption that RPFs’ P-site is at the nucleotide position 12. 13 & 14 for all in-frame reads, with a correction of +1 or –1 for out-of-frame reads.

**Reporting summary** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The sequencing datasets generated and analysed during the current study are available in the NCBI Gene Expression Omnibus repository, and can be accessed using the GSE111535 accession id. The proteome datasets are available in the PRIDE repository, and can be accessed using the PXD03122 accession id. Source data are provided with this paper.

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Author contributions

J.S. and W.J.F. conceived the study and wrote the manuscript. F.A. carried out all the bioinformatic analysis. J.S., S.R., G.S., S.P., A.K.G., S.H.-P., R.v.d.K., D.B., L.H., and S.J.E.S. carried out experiments. W.J.F., M.A., W.Z., and S.B.J. supervised experiments.

Competing interests

The authors declare no competing interests.

Additional information

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