Hyperaminoacidemia induces pancreatic α cell proliferation via synergism between mTORC1 and CaSR-Gq signaling pathways

Yulong Gong

Institute of Hydrobiology

Ding-Dong Zhang

Nanjing Agricultural University

Zihan Tang

Vanderbilt University

Katie Coate

Vanderbilt University Medical Center

Walter Siv

Vanderbilt University Medical Center

Linlin Yin

Vanderbilt University

Brittney Covington

Vanderbilt University

Ravi Patel

Vanderbilt University

Bingyuan Yang

Vanderbilt University

Liu Yang

Vanderbilt University

Danielle Dean

Vanderbilt University Medical Center

Alvin Powers

Vanderbilt University Medical Center

Wenbiao Chen (✉ wenbiao.chen@vanderbilt.edu)

Vanderbilt University <https://orcid.org/0000-0002-0750-8275>

Article

Keywords: hyperaminoacidemia, glucagon, mTORC1, CaSR

Posted Date: August 20th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-798563/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Nature Communications on January 16th, 2023. See the published version at <https://doi.org/10.1038/s41467-022-35705-4>.

Abstract

Glucagon has emerged as the main regulator of extracellular amino acid homeostasis. Insufficient glucagon signaling results in hyperaminoacidemia, which drives adaptive proliferation of glucagon-producing α cells. Aside from mammalian target of rapamycin complex 1 (mTORC1), the role of other amino acid sensors in the α cell proliferation has not been described. Here, using *gcgr*-deficient zebrafish and cultured mouse islets, we show that α cell proliferation requires the calcium sensing receptor (CaSR) and downstream extracellular signal-regulated protein kinase (ERK1/2). Inactivation of *casr* dampened α cell proliferation, which can be rescued by re-expression of CaSR or activation of the downstream Gq, but not Gi, signaling in α cells. CaSR was also unexpectedly necessary for mTORC1 activation in α cells. Furthermore, co-activation of Gq and mTORC1 induced α cell proliferation independent of hyperaminoacidemia. These results reveal another amino acid sensitive mediator, and identify major pathways necessary and sufficient for hyperaminoacidemia-induced α cell proliferation.

Introduction

Amino acids are one of the main building blocks and fuel sources of life. As such, plasma amino acid levels are tightly regulated¹ and remain constant during protein restriction and short-term fasting². Glucagon has emerged as a key hormone regulating plasma amino acid homeostasis during the past a few years^{3,4}. Glucagon stimulates amino acid catabolism by activating ureagenesis and gluconeogenesis in the liver⁵. Impaired glucagon signaling leads to increased plasma amino acid levels (hyperaminoacidemia), which in turn leads to compensatory proliferation of the glucagon-producing α cells and hyperglucagonemia^{6–8}. Liver specific inactivation of *Gcgr*⁹ or its downstream *Gnas1*¹⁰ also leads to α cell hyperplasia in mice. Persistent loss of glucagon signaling eventually results in glucagonoma, tumors of α cells^{11,12}. These recent studies thus reveal an α cell-liver axis in plasma amino acid homeostasis. Glucagon receptor antagonism (GRA) has been shown to be very effective in lowering blood glucose in preclinical diabetes models. This axis is a roadblock for clinical use of GRA therapy for diabetes¹³. However, how hyperaminoacidemia induces α cell proliferation is not fully understood.

A few mediators have been implicated in hyperaminoacidemia-induced α cell proliferation. Unsurprisingly, the amino acid sensor mTORC1 is necessary for the hyperaminoacidemia-induced α cell proliferation^{14–16}. The preferential response of α cells to hyperaminoacidemia is due in part to an mTORC1-dependent induction of a glutamine transporter *Slc38a5* as its ablation dampens the proliferation^{14,15}. In the *Slc38a5*^{-/-} mice and in humans where *SLC38A5* expression is absent in α cells, however, hyperaminoacidemia still causes α cell proliferation^{14,15}. Furthermore, although genetic activation of mTORC1 alone in mouse α cells induces α cell proliferation in adult mice, no change is detected in newborn¹⁷. As activated mTORC1 enhances α cell secretion^{7,18}, leading to hyperglucagonemia and downregulation of hepatic GCGR signaling, the increased α cell proliferation may be secondary to hepatic glucagon resistance. Therefore, other cell intrinsic signaling pathways may also be necessary for the compensatory α cell proliferation.

CaSR is a class C G-protein-coupled receptor (GPCR) that senses L-amino acids and Ca^{2+} , among other endogenous ligands^{19,20}. It was initially identified because its loss of function and gain of function mutations caused familial hypocalciuric, hypercalcaemia and autosomal dominant hypocalcaemia, respectively²¹. In addition to calcitropic tissues (for example, parathyroid glands, kidneys and breast), CaSR is also expressed in noncalcitropic tissues including enteroendocrine and pancreatic endocrine cells¹⁹. In the pancreatic islet, CaSR is highly expressed in the α and β cells^{22,23} and regulates pancreatic endocrine cell secretion^{23,24}. CaSR couples to multiple heterotrimeric G-protein subtypes (Gq/11, Gi/o, and G12/13) to activate intracellular signal transduction cascades^{25,26}. These cascades lead to increased cytosolic calcium concentrations and activation of the mitogen-activated protein kinase (MAPK) pathway, which ultimately results in phosphorylation and activation of ERK1/2^{25,27}. Of note, CaSR has also been shown to regulate cell proliferation^{28,29}. Its mutation or aberrant activation is associated with various types of cancer and altered pancreatic islet mass^{24,30,31}. However, whether CaSR contributes to the hyperaminoacidemia-induced pancreatic α cell proliferation remains unclear.

In the present study, using zebrafish and cultured mouse islets as models, we set out to determine the molecular mechanism of hyperaminoacidemia-induced pancreatic α cell proliferation (Fig. 1a). We deciphered that the CaSR-Gq-ERK1/2 pathway is another mediator of hyperaminoacidemia-induced pancreatic α cell proliferation. We show that CaSR activated MEK1/2 also contributes to mTORC1 activation. Importantly, co-activation of CaSR-Gq and mTORC1 in α cells causes proliferation in the *gcgr*-intact background. These results identify the major pathways that are necessary and sufficient for hyperaminoacidemia-induced α cell proliferation, and illustrate a previously unidentified physiological role of CaSR.

Results

Activation of mTORC1 is insufficient for α cell proliferation

We previously demonstrated that the α cell-liver axis is conserved in zebrafish. In zebrafish lacking both *gcgra* and *gcgrb* (*gcgr*^{DKO}), there are more α cells due to increased proliferation³². In addition, the hyperplasia is rapamycin-sensitive and depends on *slc38a5b*¹⁴. To fully characterize the phenotypic conservation, we determined the free amino acid levels in whole larvae and in adult serum. Compared to the control groups, the free amino acid level was markedly elevated in larvae and in adult serum of *gcgr*^{DKO} zebrafish (Supplementary Fig 1a, b). We also assessed mTORC1 activity in α cells using serine 240/244 phosphorylation of S6 ribosomal protein (pS6) as a readout. The number of pS6 positive α cells and pS6 signal intensity were both increased in the α cells of *gcgr*^{DKO} zebrafish (Supplementary Fig 1c, d and e). These data confirm that loss of glucagon signaling causes hyperaminoacidemia and activates mTORC1 in α cells^{14-16,33}.

Whether mTORC1 activation is sufficient for α cell proliferation is unknown. To answer this question, we established two transgenic lines, *Tg(gcga:MTOR^{L1460P}, cryaa:tagRFP)* and *Tg(gcga:Rheb^{S16H}, cryaa:YFP)* (*Tga^{CA-MTOR}* and *Tga^{CA-Rheb}* hence forward), that express a constitutively active mTOR^{L1460P} (CA-MTOR) or Rheb^{S16H} (CA-Rheb) only in α cells. Lens fluorescent markers were used to facilitate genotyping. As expected, the pS6 signal was overtly increased in both transgenic lines (Supplementary Fig 1f), indicating functional transgene expression. We used EdU incorporation to label the dividing and newly divided α cells³². However, there was no significant increase in either total α cell number or EdU-positive α cells in either of the transgenic lines (Fig 1b, c, e, and f). Nonetheless, α cells in both transgenic lines exhibited hypertrophy compared to the control (Fig 1d). These results indicate that mTORC1 activation leads to α cell hypertrophy but not hyperplasia. However, adult *Tga^{CA-MTOR}* fish did have significantly increased α cell area compared to WT control (supplementary Fig 1g, h), likely a secondary effect of hyperglucagonemia and subsequent glucagon resistance. Since high concentrations of amino acid are sufficient to induce α cell proliferation in primary islets¹⁴⁻¹⁶, we hypothesize that other amino acid-sensitive pathway(s) may also be required for hyperaminoacidemia-induced α cell proliferation.

Hyperaminoacidemia-induced α cell proliferation requires CaSR cell-autonomously

Amino acids are capable of stimulating certain GPCRs which subsequently, can induce cell proliferation. We assessed 3 amino acid-sensitive GPCRs, *gprc6a*, *tas1r3* and *casr*, all of which have previously been implicated in the regulation of endocrine cell function, to determine if these GPCRs impact α cell proliferation³⁴⁻³⁷. Using efficient CRISPR mutagenesis³⁸, we knocked down *gprc6a*, *tas1r3* and *casr* in *gcgr^{DKO}* zebrafish individually. The results revealed only the *casr* knockdown significantly reduced α cell number down to that of the control group (Fig 2a), suggesting that CaSR is involved in α cell proliferation. Hence, we established 2 independent *casr* mutant lines for cross validation (Fig 2b). Loss of *casr* function did not affect gross appearance or body length at 5 dpf (Supplementary Fig 2a, b), the stage most of the analyses were performed at in this study. As expected, the mutants have hypercalcemia at 7 dpf (Supplementary Fig 2c) and impaired calcium deposition in the notochord at 10 dpf (Supplementary Fig 2d, e). In 2-month-old adult zebrafish, *casr* mutants displayed skeletal dysplasia (Supplementary Fig 2f, g) and marked growth retardation (Supplementary Fig 2h, i). The mutant lines did not survive past 3 months of age. These results are reminiscent of *casr*-deficient mice^{39,40}, supporting that the mutations are loss of function.

To delineate the role of CaSR in α cell proliferation, we first evaluated α cell proliferation levels in *casr* mutants. Compared to wild-type and heterozygotes (*casr*^{+/−}), there were significant decreases in the total α cell number and EdU-labelled α cell number in *casr*^{−/−} zebrafish (Supplementary Fig 3a, b, c and d). These results indicate that CaSR plays a role in α cell proliferation in normal aminoacidemia. To test whether CaSR is required for hyperaminoacidemia-induced α cell proliferation, we crossed the *casr*

mutant into *gcgr*^{DKO} background to generate the triple mutants (*casr*^{-/-};*gcgr*^{DKO}). The α cell number in 5 dpf *casr*^{-/-};*gcgr*^{DKO} fish was significantly reduced compared to that of *gcgr*^{DKO}, and was similar to that of wild-type controls (Supplementary Fig 3e, f). These results indicate that CaSR is essential for hyperaminoacidemia-induced α cell proliferation.

It is unknown whether the α cell function of CaSR is cell autonomous. Therefore, we re-expressed the human CASR gene (*CASR*)⁴¹ specifically into α cells using *Tg(gcga:CASR,cryaa:tagRFP)* (*Tga*^{CaSR}) in *casr*^{-/-};*gcgr*^{DKO} zebrafish. We observed that both total α cell number and the number of EdU-labelled α cells in *Tga*^{CaSR};*casr*^{-/-};*gcgr*^{DKO} zebrafish were restored to the levels of the *gcgr*^{DKO} zebrafish (Fig 2c-f). To determine whether CaSR's role was conserved in mammals, we used Calhex 231⁴² to inhibit CaSR in primary mouse islets cultured in high amino acid medium (HAA)¹⁴. We observed that Calhex 231 significantly suppressed HAA-induced α cell proliferation (Fig 2g). These results suggest that CaSR functions cell-autonomously in hyperaminoacidemia-induced α cell proliferation and this function is conserved in mammals.

Gq mediates hyperaminoacidemia-induced α cell proliferation

We used designer receptors exclusively activated by designer drugs (DREADDs) to determine the CaSR downstream signaling pathways involved in hyperaminoacidemia-induced α cell proliferation⁴³. CaSR can couple to Gq and Gi to activate intracellular signals^{25,26}. To determine whether Gq or Gi mediates α cell proliferation, we established 2 transgenic lines, *Tg(gcga:hM3Dq, cryaa:tagRFP)* and *Tg(gcga:hM4Di, cryaa:tagRFP)* (*Tga*^{Gq} and *Tga*^{Gi}). These engineered receptors can be activated by CNO (Clozapine N-oxide) to initiate Gq and Gi signaling, respectively. We bred these transgenes into the *casr*^{-/-};*gcgr*^{DKO} and *gcgr*^{DKO} background and treated the progenies with 20 μ M CNO for 48 h from 3 to 5 dpf (Fig 3a). CNO treatment restored α cell number and proliferation in the *Tga*^{Gq};*casr*^{-/-};*gcgr*^{DKO} group to that of the *gcgr*^{DKO} group, but did not increase α cell number and proliferation further in the *Tga*^{Gq};*gcgr*^{DKO} group (Fig 3b, c, d and e). However, CNO did not increase either α cell number or α cell proliferation in the *Tga*^{Gi};*casr*^{-/-};*gcgr*^{DKO} group (Supplementary Figure 4a, b, c, and d). Overall, these results suggest that activation of Gq, not Gi, signaling leads to α cell proliferation and CaSR may signal through Gq to induce α cell proliferation.

Synergism of CaSR and mTORC1 pathways is sufficient to induce α cell proliferation

To determine whether activation of mTORC1 and Gq pathways are sufficient to cause α cell proliferation independent of hyperaminoacidemia, we crossed *Tga*^{Gq} to *Tga*^{CA-Rheb}. We treated the progeny with 1 μ M

or 20 μ M CNO for 48 hours from 3 to 5 dpf to activate the Gq pathway in α cells. Activation of Gq pathway with 1 μ M CNO was insufficient to cause α cell proliferation in *Tga^{Gq}* fish, but significantly increased α cell number in *Tga^{Gq};Tga^{CA-Rheb}* (Fig 4a). These results suggest that Gq and mTORC1 pathways act synergistically to induce α cell proliferation. Interestingly, 20 μ M CNO was sufficient to increase α cell number and proliferation in *gcgr*-intact fish to the same level of the *gcgr^{DKO}* group (Fig 4b, c, d and e). This degree of activation is likely supraphysiological as the ligand concentration is over 1000-fold of its EC50. This indicates that Gq hyperactivation is sufficient for α cell proliferation.

CaSR-Gq induces α cell proliferation via the ERK1/2 Pathway

GPCR coupling of Gq activates multiple intracellular transduction cascades²⁵. Pertinent to cell proliferation is the MAPK pathway that leads to phosphorylation and consequent nuclear translocation of ERK1/2⁴⁴. Hence, we next explored whether ERK1/2 is activated in α cells by hyperaminoacidemia. We found that the pERK1/2 positive α cells and nuclear pERK1/2 signal intensity were markedly increased in the *gcgr^{DKO}* group compared to that of the control group, but not in the *casr^{-/-};gcgr^{DKO}* group (Fig 5a, b and c). These results indicate that hyperaminoacidemia-induced activation of CaSR activates ERK1/2. To determine whether CaSR activates ERK1/2 through Gq, we assayed the effect of Gq activation on pERK1/2 levels in α cells by using DREADD.

We found that CNO treatment significantly increased the pERK1/2 positive α cell percentage and nuclear pERK1/2 signal intensity in *Tga^{Gq};casr^{-/-};gcgr^{DKO}* fish to the same levels of the *gcgr^{DKO}* fish (Fig 5d, e and f). Interestingly, CNO even increased the pERK1/2 positive α cell percentage further in the *Tga^{Gq};gcgr^{DKO}* group (Fig 5d, e). These results indicate that Gq activation leads to ERK1/2 phosphorylation in α cells. Therefore, hyperaminoacidemia activates the CaSR-Gq-ERK1/2 pathway in α cells.

It is unknown whether ERK1/2 activation is essential in hyperaminoacidemia-induced α cell proliferation. To answer this question, we utilized the MEK1/2 inhibitors U0126 and PD0325901^{45,46} to suppress ERK1/2 phosphorylation in control, *gcgr^{DKO}* and *casr^{-/-};gcgr^{DKO}* zebrafish for 48h (Fig 6a). ERK1/2 is the only known physiological substrate of MEK1/2⁴⁷. As expected, both of U0126 and PD0325901 decreased the nuclear pERK1/2 levels in α cells of *gcgr^{DKO}* islets (Supplementary Fig 5a). Both inhibitors also reduced the α cell number and proliferation in *gcgr^{DKO}* fish to that of control and *casr^{-/-};gcgr^{DKO}* groups (Fig 6b, c, d and e and Supplementary Fig 5b, c, d, e). Notably, U0126 even reduced α cell number and proliferation in the control fish (Fig 6b, c, d and e).

It is unknown whether MEK1/2 is also necessary for amino acids induced a cell in mammalian α cells. We therefore examined the effect of U0126 on HAA-stimulated α cell proliferation in cultured mouse islets¹⁴. We found that U0126 significantly decreased the percentage of Ki67 positive α cells in the islets cultured in HAA medium (Fig 6f, g). These results indicate ERK1/2 activation is essential for hyperaminoacidemia-induced α cell proliferation. Taken together, these findings suggest that CaSR-Gq mediates hyperaminoacidemia-induced α cell proliferation via MEK1/2-ERK1/2.

Hyperaminoacidemia-induced mTORC1 activation in α cells requires CaSR and MEK1/2

Previous studies demonstrated an essential role of mTORC1 in hyperaminoacidemia- induced α cell proliferation^{14-16,33}. Since α cell proliferation also depended on CaSR, we tested whether mTORC1 activation is intact in *casr*^{-/-};*gcgr*^{DKO} fish. Unexpectedly, pS6 levels decreased significantly in *casr*^{-/-};*gcgr*^{DKO} fish when compared to *gcgr*^{DKO} fish (Fig 7a, b, c), suggesting that CaSR is required for mTORC1 activation. To establish if mTORC1 activation requires the downstream MEK1/2, we treated *gcgr*^{DKO} fish with U0126. U0126 decreased the percentage of pS6 positive α cells and the intensity of α cell pS6 signal in *gcgr*^{DKO} fish to the same levels of wild-type fish (Fig 7d, e and f). These results suggest hyperaminoacidemia-induced mTORC1 activation in α cells requires CaSR and MEK1/2.

Gq hyperactivation-induced α cell proliferation requires mTORC1 activity

As Gq hyperactivation caused α cell hyperplasia in *gcgr*-intact animals, we examined whether it also required mTORC1. Indeed, rapamycin abolished α cell hyperplasia induced by Gq hyperactivation (Fig 8a, b). Consequently, Gq hyperactivation also increased pS6 levels in *Tga*^{Gq};*casr*^{-/-};*gcgr*^{DKO} fish (Fig 8c, d and e), and further amplified the percentage of pS6 positive α cells in *Tga*^{Gq};*gcgr*^{DKO} fish compared to untreated controls (Fig 8c and d). Furthermore, the Gq hyperactivation even increased pS6 levels in *gcgr*-intact zebrafish, although the pS6 positive α cell number was still less than *gcgr*^{DKO} group (Fig 8f, g and h). Therefore, Gq hyperactivation simultaneously activates ERK1/2 and mTORC1, and both are necessary for α cell proliferation.

Together, these data revealed activation of ERK1/2 and mTORC1 is necessary and sufficient for inducing α cell proliferation. The study also uncovered a previously unidentified role of the CaSR.

Discussion

Glucagon is a key hormone which regulates plasma amino acid homeostasis by stimulating amino acid catabolism in the liver³⁻⁵. Impaired glucagon signaling leads hyperglucagonemia and

hyperaminoacidemia, leading to compensatory α cell proliferation^{7-9,18,32}. Here, by combining gain-of-function and loss-of-function interventions, both in zebrafish and cultured mouse islets, we found that the CaSR-Gq-ERK1/2 pathway is also essential for mediating hyperaminoacidemia-induced α cell proliferation. We showed that synergy between CaSR/Gq and mTORC1 pathways is sufficient to cause α cell proliferation independent of hyperaminoacidemia. We also demonstrated that mTORC1 activation is CaSR-Gq-ERK/2 dependent during α cell proliferation. Therefore, we deciphered the major pathways that are necessary and sufficient for α cell proliferation and uncovered a previously unidentified physiological role for the CaSR (Fig 9).

We have illustrated activation of mTORC1 is insufficient to induce α cell proliferation in WT larvae. We observed that mTORC1 activated α cells are not hyperplastic but hypertrophic, similar to a recent study in which mTORC1 is activated by knocking out *Tsc2* in mouse α cells¹⁷. In the mouse study, no α cell hyperplasia is observed in neonatal islets¹⁷, consistent with our results. However, adult *Tga^{CA-MTOR}* zebrafish had significantly increased α cell area, similar to adult mice with α cell specific mTORC1 activation¹⁷. In mice, this is due to the secondary effect of liver glucagon resistance resulting from increased glucagon secretion and hyperglucagonemia and is thus not an α cell autonomous effect. The increased α cell area in adult *Tga^{CA-MTOR}* fish is likely due to the same secondary effect (Fig S1g). We speculate that glucagon resistance has not been established in the liver at 5 dpf in zebrafish.

We discovered CaSR is cell autonomously required for hyperaminoacidemia-induced α cell proliferation. Since mTORC1 activation is insufficient to cause α cell proliferation cell autonomously, we speculated a role of other amino acid sensitive pathways and identified CaSR. Through loss of function and rescue studies, we demonstrated that CaSR is required cell-autonomously for hyperaminoacidemia-induced α cell proliferation (Fig 2c, d, e and f). We further confirmed a necessary role of CaSR in high amino acid induced α cell proliferation in mouse primary islets. Although it has been shown that CaSR is highly expressed in pancreatic endocrine cells^{19,22,23}, we established a role for CaSR in hyperaminoacidemia-induced α cell proliferation for the first time. Previous studies also revealed a role for CaSR in other proliferating cell types *in vitro*^{30,48}, while our study showed a necessary role for CaSR in α cell proliferation *in vivo*.

Although CaSR is known to be sensitive to amino acids based on *in vitro* pharmacology^{49,50}, to our knowledge, the present study is the first to genetically demonstrate an amino acid sensitive function of CaSR *in vivo*. L-Phe and L-Trp are slightly more potent in activating CaSR in the presence of physiological Ca^{2+} ⁵¹, however, their concentrations in blood are lower than 100 μM , even under conditions of hyperaminoacidemia, much lower than the EC50 of 2.5 mM⁵¹. Therefore, activation of CaSR by

hyperaminoacidemia *in vivo* results from the action of all amino acids, particularly Gln and Ala, the two major amino acids preferentially increased under conditions of glucagon signaling interruption^{14,15}. In normal conditions, blood amino acid levels are tightly maintained within a small range, and thus unlikely to play a role in regulating CaSR in most tissues. However, the amino acid concentration increases substantially in the GI tract after feeding. This surge likely activates CaSR in the enteroendocrine cells¹⁹. Indeed, several *in vitro* studies have demonstrated a role of CaSR in the secretion of gut hormones^{50,52,53}. Confirmation of these studies *in vivo* by genetic means will establish additional physiological roles of amino acid sensing by CaSR.

Our study suggests that CaSR acts through Gq signaling to induce α cell proliferation. CaSR regulates cell proliferation in many tissues²⁸⁻³⁰. However, few reports have determined which G protein mediates CaSR action in cell proliferation. Using DREADDs, we tested the 2 major CaSR activated pathways and found that activation of Gq, but not Gi, induced α cell proliferation (Fig 3b, c, d and e; Supplementary Fig 4a, b, c and d). These results suggest that the Gq pathway may be responsible for CaSR mediated proliferation. However, we cannot rule out a role for the Gs pathway, which can be activated by CaSR in some cell types^{54,55}. We recognize that the CaSR-Gq connection is based on correlative evidence. Interestingly, Gq and Gs activation increases β cell mass and proliferation^{56,57}, while Gi activation inhibits β cell proliferation⁵⁸. The positive and negative effects on β cell proliferation correlate with the stimulation and inhibition of insulin secretion, respectively. This correlation does not hold true in α cells since several Gi-coupled GPCRs stimulate glucagon secretion⁵⁹. The reason why Gi activation fails to promote α cell proliferation is unknown and may be a subject for future investigation.

Downstream of Gq, CaSR is necessary for hyperaminoacidemia-mediated MEK1/2-ERK1/2 activation in α cell proliferation. MEK1/2 phosphorylation of ERK1/2 and subsequent nuclear localization is essential for cell proliferation and is implicated in many cancer types^{60,61}. As expected, we found that increased nuclear pErk1/2 correlates with α cell hyperplasia (Fig 5) and inhibition of MEK1/2 significantly reduced α cell proliferation both in *gcgr*-deficient zebrafish and in mouse islets cultured in high amino acid medium (Fig 6; Supplementary Fig 5). Although it is unsurprising that MEK1/2 activity is required for hyperaminoacidemia-induced α cell proliferation, it is interesting that CaSR is necessary for their activation. We propose that CaSR is a component of the amino acid sensor in α cells that maintains amino acid homeostasis.

A surprising finding is that CaSR is necessary for hyperaminoacidemia-mediated mTORC1 activation. mTORC1 activation is known to be required for hyperaminoacidemia-induced α cell proliferation, and it is thought that increased intracellular amino acids caused by amino acid transporters are the primary driver

of mTORC1 activation by hyperaminoacidemia^{14-16,33}. However, we found that CaSR, presumably activated by increased extracellular amino acids and acting through Gq-MEK1/2, is also required for mTORC1 activation. A previous study showed that the α 1A adrenergic receptor promotes mTORC1 signaling via the Gq pathway⁶². This may result from ERK1/2, PI3K and TSC2 activation⁶³⁻⁶⁵. The exact mechanism of CaSR mediated mTORC1 activation in α cells requires further studies.

We demonstrated that Gq and mTORC1 pathways act synergistically to induce α cell proliferation (Fig 4 a). Activation individually failed to cause α cell proliferation in *gcgr*-intact fish. However, a high concentration of CNO alone was also sufficient for α cell proliferation in *gcgr*-intact *Tga^{Gq}* fish (Fig4 b, c, d and e). We do not think such degree of activation is achievable in physiological conditions. In the transgenic fish, the Gq-DREADD is under the strongest promoter in α cells and is likely expressed at much higher levels than endogenous GPCRs. The high agonist concentration would likely achieve supraphysiological levels of Gq activation that is sufficient to turn on both MEK1/2-ERK1/2 and mTORC1 signaling.

In summary, we revealed that activation of mTORC1 and CaSR-Gq-ERK1/2 is necessary and sufficient for α cell proliferation and identified a previously unknown physiological role of CaSR (Fig 9). Using zebrafish and cultured mouse islets, the present study demonstrated that the CaSR-Gq-ERK1/2 pathway is another mediator of hyperaminoacidemia-induced α cell proliferation and CaSR-Gq-MEK1/2 also contributes to mTOR activation. Importantly, co-activation of CaSR-Gq and mTORC1 in α cells causes proliferation independent of hyperaminoacidemia. Future studies should decipher the mechanism of the interplay between Gq-ERK1/2 and mTORC1 during hyperaminoacidemia-induced α cell proliferation. Understanding how hyperaminoacidemia induces α cell proliferation may provide new insights for glucagonoma treatment and for mitigating the undesired effects of the proposed GRA therapy for diabetes.

Methods

Zebrafish maintenance.

Zebrafish (*Danio rerio*) were raised at 28°C in an Aquatic-Habitats system and embryos were raised at 28.5°C in an incubator on a 14/10-hour light/dark cycle. All animals were staged by days postfertilization (dpf). In this study, *Tg(gcga:EGFP)*⁶⁶ was used to mark α cells for imaging and/or counting α cells. Transgenic and mutant strains used for this study are listed in Supplementary Table 1. The *gcgra*^{-/-};*gcgrb*^{-/-} fish were described previously³². Mutations in *casr* were generated using CRISPR-Cas9 as described previously⁶⁷, by using recombinant Cas9 (PNA Bio). Two mutant lines (8 bp deletion and 11 bp insertion) in *casr* were characterized for an initial cross-validation before selecting the 8 bp deletion

line for all the experiments. Transgenic lines expressing constitutively active mTOR^{L1460P} or Rheb^{S16H} and CaSR, hM3Dq or hM4Di in α cells were generated using the Tol2 System⁶⁸.

Free amino acids assay.

Free amino acids level was determined using the published method⁶⁹. Briefly, a pool of forty larvae or 10 μ L serum of adult fish was homogenized in perchloric acid (Weight ratio: 1:50). The homogenate centrifuged at 15,000 g for 10 min. The supernatant was neutralized with 0.8 M KOH (using a volume of KOH 1/19th of the supernatant). After the addition of KOH, the samples were cooled on ice water for about 10 min and centrifuged at 15,000 g for 10 min. 10 μ L of supernatant were mixed with 3 mL of OPA-MET reagent. After 2–10 min the fluorescence above the OPA-MET reagent was read in cuvettes on a spectrofluorometer using an excitation wavelength of 340 nm and an emission wavelength of 440 nm. In order to calculate the concentration of the amino acids in the sample, 10 μ L of the standard mixture of amino acids (prepared as described above and diluted 1 : 10, 1 : 100 and 1 : 1,000) were mixed with 3 mL of OPA-MET reagent and the fluorescence was read as for the sample. Six 40-fish pools and 6 adult fish sera of each genotype were measured respectively.

Proliferation analysis in zebrafish.

Proliferation was analyzed using the Click-iT EdU Alexa Fluor 594 Imaging Kit (C10339; Invitrogen). To identify proliferating α cells, embryos were pericardially injected with 1-2 nl of 0.1 mM 5-ethynyl-2-deoxyuridine (EdU) at 4 dpf. After 24 h, they were euthanized and fixed. EdU was detected according to modified manufacturer's instructions. All images were collected using Zeiss LSM780 (Carl Zeiss, Jena, Germany) and analyzed by Imaris (Oxford Instruments).

Isolation and culture of mouse pancreatic islets.

All mice used in these studies were 10-18-week-old C57BL/6J males. Mouse pancreata were digested with collagenase P (Roche, Basel, Switzerland), and islets were isolated via density gradient centrifugation as previously described¹⁴. The islets were then handpicked to > 99% purity and cultured in media with high or normal amino acids, and with an inhibitor or vehicle for 3 days. The islet culture was based on a RPMI 1640 media formula except that each amino acid concentration was individually formulated to mimic the concentrations found in the serum of either wildtype (Low AA) or *Gcgr*^{-/-} (High AA) mice¹⁴ with 10% Fetal Bovine Serum (Atlanta Biologicals), 1% Penicillin/Streptomycin (GIBCO), and 5.6mM D-glucose. To inhibit CaSR, 10 μ M Calhex 231 (Cayman) was used. To inhibit MEK1/2, 10 μ M U0126 was used.

After culture, islets were washed in 2mM EDTA and dispersed in 0.025% Trypsin-2 mM EDTA (GIBCO Life Technologies or HyClone GE Healthcare) for 5-10 min by gentle pipetting to obtain single or very small cell clusters. Dispersed islet cells were recovered by centrifugation in RPMI media containing 5.6 mM glucose, 10% FBS, and 1% Penicillin/Streptomycin. The resulting cell pellet was resuspended in 100 µl of media and centrifuged onto a glass slide using a cytospin (Thermo Scientific, Waltham, MA) centrifuge. Air-dried slides were stored at -80°C until use, thawed, and immediately fixed in 4% paraformaldehyde before immunofluorescence. Slides were mounted with Aqua-Poly/Mount (Warrington, PA).

Immunofluorescence and imaging.

Immunofluorescence on larval zebrafish islet-containing sections was performed as below: zebrafish larvae were fixed in 4% paraformaldehyde (PFA) at 5 dpf, equilibrated in 20% sucrose and embedded into O.C.T. for making cryosections. Islet-containing cryosections (12 µm) were selected for immunofluorescence. Immunofluorescence of cultured mouse islets was performed as described¹⁴.

Primary antibodies used were Anti-GFP (1:300, Thermo fisher scientific, A-11120, Mouse), Anti-pS6 (Ser240/244) (1:300, Cell signaling technology, 5364, Rabbit), Anti-pERK1/2 (Thr202/Tyr204) (1:150, Cell signaling technology, 4370, Rabbit), Anti-glucagon (1:200, Cell signaling technology, 2760, Rabbit) and Anti-Ki67 (1:150, Abcam ab15580, Rabbit). Followed by Alexa Fluor 488 or Alexa Fluor 568 conjugated secondary antibody for visualization (1:1,000, Thermo Fisher Scientific). Nuclei were stained with Hoechst 33342 (0.5 µg/ml, Thermo Fisher Scientific). The coverslips were mounted on slides with ProLong Diamond mounting medium (Thermo Fisher Scientific). Images were collected using a Zeiss LSM880 (Carl Zeiss, Jena, Germany) and analyzed by Imaris (Oxford Instruments).

Ca²⁺ concentration assay, alizarin red S (ARS) staining and Mirco-CT.

Ca²⁺ concentration was measured by ICP-MS. Briefly, 1.5 mL polypropylene microcentrifuge tubes (Fisher Scientific) were immersed and washed three times in 10% nitric acid (67–69%, Optima™; Fisher Chemical) overnight. After acid treatment, tubes were rinsed thoroughly with ultrapure water (Processed by Milli-Q™). Afterward, embryos (7 dpf) were washed 3 times with cold ultrapure water, and four 20-fish pools were placed in tubes for each group. For metal measurement, 200 µL nitric acid was added to digest samples at 85°C until dry. Samples were then suspended in nitric acid and dried again. 200 µL hydrogen peroxide (30%, Optima™; Fisher Chemical) was added and evaporated at 85°C twice. Eventually, samples were diluted in 1.5 mL 2% nitric acid. Empty tubes, processed identically as tubes containing embryos, work as mock treatments. Ca²⁺ level was normalized to sulfur, and the standard curves were generated to measure the content of Ca²⁺ by Agilent 7700 series ICP-MS (Agilent Technologies).

ARS staining was used for larval zebrafish skeletal mineralization. Briefly, zebrafish were fixed in 4 % PFA at 10 dpf, then stained for 15 min with 0.01 % ARS (3,4-Dihydroxy-9,10-dioxo-2-anthracenesulfonic acid sodium salt, from Sigma-Aldrich, St. Louis, MO) dissolved in 70 % ethanol. After rinsing 3 times/5 min in PBS, the samples were imaged immediately under 510 nm excitation fluorescent with Zeiss LSM780 (Carl Zeiss, Jena, Germany) and analyzed by Imaris (Oxford Instruments).

3 zebrafish (60 dpf) were sacrificed for each group and kept in 75% alcohol at -20 °C until use. The sample was then placed in a sample holder in which a sponge was used to fix sample. Micro-CT scanning was run via μ CT 50 (Scanco Medical, Switzerland). High-resolution scans (5 μ m voxel resolution) were obtained using the following parameters: X-ray energy 45 kVp, current 88 μ A, sampling rate 2048, 1000 proj/180°, integration time 300 ms, which were modified according to the previously published methods⁷⁰. Image processing was performed using custom software and the bone development of zebrafish remodeled by micro-CT-scan was described as total volume (TV), bone volume (BV), bone volume fraction (BV/TV) and tissue mineral density (TMD).

Chemical treatment in zebrafish experiment.

All drugs were made in 1,000X stock solution and stored in light-protected Eppendorf tubes at -20°C. CNO (20 mmol/L; Tocris), PD0325901 (10 mmol/L; Sigma-Aldrich), U0126 (10 mmol/L; LC laboratories) and Rapamycin (2.5 mmol/L; Cayman) were dissolved in DMSO at the indicated concentrations. Zebrafish were exposed to 20 μ M or 1 μ M CNO, 10 μ M PD0325901 or U0126 and 2.5 μ M Rapamycin for 2 days, respectively, starting at 3 dpf and injected with EdU at 4 dpf. Then larvae were fixed and stained at 5 dpf for a cell number and proliferation assay.

Statistics.

For group comparisons, either one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) post hoc test or two-tailed unpaired Student's t test was used. A *P* value less than 0.05 was considered statistically significant. Values represent means $\pm$ SEM. Analyses were done using GraphPad Prism 8.

Declarations

Study approval.

The animals were handled in compliance with guidelines approved by the Vanderbilt University Institutional Animal Care and Use Committee protocols (protocol no. M1700143-01).

Acknowledgements.

We thank members of the Chen, Powers and Dean groups for helpful discussions. The CASR-Tango plasmid with codon optimized human CaSR coding sequence was provided by Brian Roth via Addgene (plasmid # #66235). The coding sequence for constitutively active rat RHEB was provided by Mustafa Sahin via Addgene (plasmid # 32520). The coding sequence for constitutively active human MTOR was provided by David Sabatini via Addgene (plasmid # 69006). Human islets were from IIDP. Confocal imaging and image analysis were performed in part through the use of the Vanderbilt Cell Imaging Shared Resource. IP-MS was done at the Vanderbilt Mass Spectrometry Research Center (MSRC). Micro-CT was performed at the VUMC Institute of Imaging Science (VUIIS) Center for Small Animal Imaging (CSAI). This study is supported by DK117147 (WC and ACP) and DK117969 (EDD). YG is supported by China Scholarship Council (#201904910575). BAC is supported by the METP training grant (T32 DK 7563-32). All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Author Contributions.

Conceptualization: WC. Funding acquisition: WC, ACP, EDD; Investigation: YG, DZ, ZT, KCC, WAS, KS, BAC. Methodology: BY, LYin, LYang, RSP. Supervision: WC, ACP, EDD. Visualization: YG, DZ, KCC, WAS. Writing – original draft: YG, WC; Writing – review & editing: KCC, EDD, BAC, ACP.

References

1. Cynober, L. A. Plasma amino acid levels with a note on membrane transport: Characteristics, Regulation, and Metabolic Significance. *Nutrition* **18**, 761–766 (2002).
2. Kalhan, S. C. *et al.* Metabolic and genomic response to dietary isocaloric protein restriction in the rat. *The Journal of biological chemistry* **286**, 5266–5277, doi:10.1074/jbc.M110.185991 (2011).
3. Michael R. Charlton, D. B. A., K. Sreekumaran Nair. Evidence for a catabolic role of glucagon during an amino acid load. *Journal of Clinical Investigation* **98**, 90–99 (1996).
4. Dean, E. D. A Primary Role for α -Cells as Amino Acid Sensors. *Diabetes* **69**, 542–549, doi:10.2337/dbi19-0021 (2020).
5. Holst JJ, W. A. N., Pedersen J, Knop FK.. Glucagon and amino acids are linked in a mutual feedback cycle: The liver- α -cell axis. *Diabetes* **66**, 235–240, doi:10.2337/db16-0994 (2017).

6. Zhou, C., Dhall, D., Nissen, N. N., Chen, C. R. & Yu, R. Homozygous P86S mutation of the human glucagon receptor is associated with hyperglucagonemia, alpha cell hyperplasia, and islet cell tumor. *Pancreas* **38**, 941–946, doi:10.1097/MPA.0b013e3181b2bb03 (2009).
7. Gelling, R. W. *et al.* Lower blood glucose, hyperglucagonemia, and pancreatic alpha cell hyperplasia in glucagon receptor knockout mice. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1438–1443, doi:10.1073/pnas.0237106100 (2003).
8. Tellez, K. & Hang, Y. In vivo studies of glucagon secretion by human islets transplanted in mice. **2**, 547–557, doi:10.1038/s42255-020-0213-x (2020).
9. Longuet, C. *et al.* Liver-specific disruption of the murine glucagon receptor produces α -cell hyperplasia: evidence for a circulating α -cell growth factor. *Diabetes* **62**, 1196–1205, doi:10.2337/db11-1605 (2013).
10. Chen, M. *et al.* Increased glucose tolerance and reduced adiposity in the absence of fasting hypoglycemia in mice with liver-specific Gs alpha deficiency. *The Journal of clinical investigation* **115**, 3217–3227, doi:10.1172/jci24196 (2005).
11. Sandru, F. *et al.* Glucagonoma: From skin lesions to the neuroendocrine component (Review). *Experimental and therapeutic medicine* **20**, 3389–3393, doi:10.3892/etm.2020.8966 (2020).
12. John, A. M. & Schwartz, R. A. Glucagonoma syndrome: a review and update on treatment. *Journal of the European Academy of Dermatology and Venereology: JEADV* **30**, 2016–2022, doi:10.1111/jdv.13752 (2016).
13. Davidson, J. A. *et al.* Glucagon therapeutics: Dawn of a new era for diabetes care. *Diabetes/metabolism research and reviews* **32**, 660–665, doi:10.1002/dmrr.2773 (2016).
14. Dean, E. D. *et al.* Interrupted Glucagon Signaling Reveals Hepatic alpha Cell Axis and Role for L-Glutamine in alpha Cell Proliferation. *Cell metabolism* **25**, 1362–1373 e1365, doi:10.1016/j.cmet.2017.05.011 (2017).
15. Kim, J. *et al.* Amino Acid Transporter Slc38a5 Controls Glucagon Receptor Inhibition-Induced Pancreatic α Cell Hyperplasia in Mice. *Cell metabolism* **25**, 1348–1361.e1348, doi:10.1016/j.cmet.2017.05.006 (2017).
16. Solloway, M. J. *et al.* Glucagon Couples Hepatic Amino Acid Catabolism to mTOR-Dependent Regulation of α -Cell Mass. *Cell reports* **12**, 495–510, doi:10.1016/j.celrep.2015.06.034 (2015).
17. Bozadjieva Kramer, N. *et al.* Glucagon Resistance and Decreased Susceptibility to Diabetes in a Model of Chronic Hyperglucagonemia. **70**, 477–491, doi:10.2337/db20-0440 (2021).
18. Hayashi, Y. *et al.* Mice deficient for glucagon gene-derived peptides display normoglycemia and hyperplasia of islet {alpha}-cells but not of intestinal L-cells. *Molecular endocrinology* **23**, 1990–1999, doi:10.1210/me.2009-0296 (2009).
19. Hannan, F. M., Kallay, E., Chang, W., Brandi, M. L. & Thakker, R. V. The calcium-sensing receptor in physiology and in calcitropic and noncalcitropic diseases. *Nature reviews. Endocrinology* **15**, 33–51, doi:10.1038/s41574-018-0115-0 (2018).

20. Stephanie M. Busque, J. E. K., John P. Geibel and Karl Insogna. L-Type amino acids stimulate gastric acid secretion by activation of the calcium-sensing receptor in parietal cells. *Am J Physiol Gastrointest Liver Physiol* **289**, G664–G669, doi:10.1152/ajpgi.00096.2005.-Parietal (2005).
21. Hannan, F. M. & Thakker, R. V. Calcium-sensing receptor (CaSR) mutations and disorders of calcium, electrolyte and water metabolism. *Best practice & research. Clinical endocrinology & metabolism* **27**, 359–371, doi:10.1016/j.beem.2013.04.007 (2013).
22. Regard JB, S. I., Coughlin SR. Anatomical profiling of G protein-coupled receptor expression. *Cell* **135**, 561–571 (2008).
23. Squires PE, H. T., Persaud SJ, Curtis SB, Buchan AM, Jones PM. The extracellular calcium-sensing receptor on human b-cells negatively modulates insulin secretion. *Diabetes* **49**, 409–417 (2000).
24. Babinsky, V. N. *et al.* Mutant Mice With Calcium-Sensing Receptor Activation Have Hyperglycemia That Is Rectified by Calcilytic Therapy. *Endocrinology* **158**, 2486–2502, doi:10.1210/en.2017-00111 (2017).
25. Conigrave, A. D. & Ward, D. T. Calcium-sensing receptor (CaSR): pharmacological properties and signaling pathways. *Best practice & research. Clinical endocrinology & metabolism* **27**, 315–331, doi:10.1016/j.beem.2013.05.010 (2013).
26. Alex Rojas Bie, T., Sanela, S. & Hans, B.-O. Novel Strategies in Drug Discovery of the Calcium-Sensing Receptor Based on Biased Signaling. *Current Drug Targets* **13**, 1324–1335, doi:http://dx.doi.org/10.2174/138945012802429642 (2012).
27. Hofer, A. M. & Brown, E. M. Extracellular calcium sensing and signalling. *Nature Reviews Molecular Cell Biology* **4**, 530–538, doi:10.1038/nrm1154 (2003).
28. Jin-Oh Kwak, J. K., Hyun-Woo Kim, Kwang-Jin Oh, Yun Tai Kim, Sun-Mi Jung and Seok Ho Cha. The extracellular calcium sensing receptor is expressed in mouse mesangial cells and modulates cell proliferation. *Experimental and Molecular Medicine* **37**, 457–465 (2005).
29. Oda, Y. *et al.* Combined Deletion of the Vitamin D Receptor and Calcium-Sensing Receptor Delays Wound Re-epithelialization. *Endocrinology* **158**, 1929–1938, doi:10.1210/en.2017-00061 (2017).
30. Fang, Y. *et al.* Calcium-sensing receptor promotes tumor proliferation and migration in human intrahepatic cholangiocarcinoma by targeting ERK signaling pathway. *European journal of pharmacology* **872**, 172915, doi:10.1016/j.ejphar.2020.172915 (2020).
31. Kim, W. & Wysolmerski, J. J. Calcium-Sensing Receptor in Breast Physiology and Cancer. *Frontiers in physiology* **7**, 440, doi:10.3389/fphys.2016.00440 (2016).
32. Li, M. *et al.* Glucagon receptor inactivation leads to α -cell hyperplasia in zebrafish. *The Journal of endocrinology* **227**, 93–103, doi:10.1530/joe-15-0284 (2015).
33. Galsgaard, K. D. *et al.* Disruption of glucagon receptor signaling causes hyperaminoacidemia exposing a possible liver- α -cell axis. *American journal of physiology. Endocrinology and metabolism* **314**, E93–e103, doi:10.1152/ajpendo.00198.2017 (2018).
34. Wei, J., Hanna, T., Suda, N., Karsenty, G. & Ducy, P. Osteocalcin promotes β -cell proliferation during development and adulthood through Gprc6a. *Diabetes* **63**, 1021–1031, doi:10.2337/db13-0887

(2014).

35. Kojima, I. *et al.* Return of the glucoreceptor: Glucose activates the glucose-sensing receptor T1R3 and facilitates metabolism in pancreatic β -cells. *Journal of diabetes investigation* **6**, 256–263, doi:10.1111/jdi.12304 (2015).
36. Squires, P. E., Jones, P. M., Younis, M. Y. & Hills, C. E. The calcium-sensing receptor and β -cell function. *Vitamins and hormones* **95**, 249–267, doi:10.1016/b978-0-12-800174-5.00010-7 (2014).
37. Rasschaert, J. & Malaisse, W. J. Expression of the calcium-sensing receptor in pancreatic islet B-cells. *Biochemical and biophysical research communications* **264**, 615–618, doi:10.1006/bbrc.1999.1577 (1999).
38. Jao, L. E., Wente, S. R. & Chen, W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 13904–13909, doi:10.1073/pnas.1308335110 (2013).
39. Tu, Q. *et al.* Rescue of the skeletal phenotype in CasR-deficient mice by transfer onto the Gcm2 null background. *The Journal of clinical investigation* **111**, 1029–1037, doi:10.1172/jci17054 (2003).
40. Chang, W., Tu, C., Chen, T. H., Bikle, D. & Shoback, D. The extracellular calcium-sensing receptor (CaSR) is a critical modulator of skeletal development. *Science signaling* **1**, ra1, doi:10.1126/scisignal.1159945 (2008).
41. Kroeze, W. K. *et al.* PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. *Nature structural & molecular biology* **22**, 362–369, doi:10.1038/nsmb.3014 (2015).
42. Petrel, C. *et al.* Modeling and mutagenesis of the binding site of Calhex 231, a novel negative allosteric modulator of the extracellular Ca(2+)-sensing receptor. *The Journal of biological chemistry* **278**, 49487–49494, doi:10.1074/jbc.M308010200 (2003).
43. Zhu, H. & Roth, B. L. DREADD: a chemogenetic GPCR signaling platform. *The international journal of neuropsychopharmacology* **18**, doi:10.1093/ijnp/pyu007 (2014).
44. Lavoie, H., Gagnon, J. & Therrien, M. ERK signalling: a master regulator of cell behaviour, life and fate. *Nature reviews. Molecular cell biology* **21**, 607–632, doi:10.1038/s41580-020-0255-7 (2020).
45. Droebner, K., Pleschka, S., Ludwig, S. & Planz, O. Antiviral activity of the MEK-inhibitor U0126 against pandemic H1N1v and highly pathogenic avian influenza virus in vitro and in vivo. *Antiviral research* **92**, 195–203, doi:10.1016/j.antiviral.2011.08.002 (2011).
46. Shannon, S. *et al.* Inhibition of glioblastoma dispersal by the MEK inhibitor PD0325901. *BMC cancer* **17**, 121, doi:10.1186/s12885-017-3107-x (2017).
47. Roskoski, R., Jr. ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacological research* **66**, 105–143, doi:10.1016/j.phrs.2012.04.005 (2012).
48. Mao, F. X., Luo, C. H., Chen, H. J., Zhang, Y. X. & Zhang, Q. CaSR is required for ischemia-induced proliferation and differentiation of white matter progenitor cells from neonatal rats. *Brain research bulletin* **154**, 116–126, doi:10.1016/j.brainresbull.2019.11.004 (2020).

49. Mun, H. C., Leach, K. M. & Conigrave, A. D. L-Amino Acids Promote Calcitonin Release via a Calcium-Sensing Receptor: Gq/11-Mediated Pathway in Human C-Cells. *Endocrinology* **160**, 1590–1599, doi:10.1210/en.2018-00860 (2019).
50. Wang, H., Murthy, K. S. & Grider, J. R. Expression patterns of L-amino acid receptors in the murine STC-1 enteroendocrine cell line. **378**, 471–483, doi:10.1007/s00441-019-03074-y (2019).
51. Conigrave, A. D., Quinn, S. J. & Brown, E. M. L-amino acid sensing by the extracellular Ca²⁺-sensing receptor. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 4814–4819, doi:10.1073/pnas.97.9.4814 (2000).
52. Wang, Y. *et al.* Amino acids stimulate cholecystokinin release through the Ca²⁺-sensing receptor. *Am J Physiol Gastrointest Liver Physiol* **300**, G528-537, doi:10.1152/ajpgi.00387.2010 (2011).
53. Acar, I. & Cetinkaya, A. The role of calcium sensing receptors in GLP-1 and PYY secretion after acute intraduodenal administration of L-Tryptophan in rats. **23**, 481–489, doi:10.1080/1028415x.2018.1521906 (2020).
54. Mamillapalli, R., VanHouten, J., Zawalich, W. & Wysolmerski, J. Switching of G-protein usage by the calcium-sensing receptor reverses its effect on parathyroid hormone-related protein secretion in normal versus malignant breast cells. *The Journal of biological chemistry* **283**, 24435–24447, doi:10.1074/jbc.M801738200 (2008).
55. Mamillapalli, R. & Wysolmerski, J. The calcium-sensing receptor couples to Galpha(s) and regulates PTHrP and ACTH secretion in pituitary cells. *The Journal of endocrinology* **204**, 287–297, doi:10.1677/joe-09-0183 (2010).
56. Kim, H. *et al.* Serotonin regulates pancreatic beta cell mass during pregnancy. *Nature medicine* **16**, 804–808, doi:10.1038/nm.2173 (2010).
57. Guettier, J. M. *et al.* A chemical-genetic approach to study G protein regulation of beta cell function in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 19197–19202, doi:10.1073/pnas.0906593106 (2009).
58. Berger, M. *et al.* Gai/o-coupled receptor signaling restricts pancreatic β -cell expansion. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 2888–2893, doi:10.1073/pnas.1319378112 (2015).
59. Winzell, M. S. & Ahrén, B. G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes. *Pharmacology & therapeutics* **116**, 437–448, doi:10.1016/j.pharmthera.2007.08.002 (2007).
60. Maik-Rachline, G., Hacoheh-Lev-Ran, A. & Seger, R. Nuclear ERK: Mechanism of Translocation, Substrates, and Role in Cancer. *International journal of molecular sciences* **20**, doi:10.3390/ijms20051194 (2019).
61. Scholl, F. A. *et al.* Mek1/2 MAPK kinases are essential for Mammalian development, homeostasis, and Raf-induced hyperplasia. *Developmental cell* **12**, 615–629, doi:10.1016/j.devcel.2007.03.009 (2007).

62. Ballou, L. M., Jiang, Y. P., Du, G., Frohman, M. A. & Lin, R. Z. Ca(2+)- and phospholipase D-dependent and -independent pathways activate mTOR signaling. *FEBS letters* **550**, 51–56, doi:10.1016/s0014-5793(03)00816-0 (2003).
63. Suire, S., Hawkins, P. & Stephens, L. Activation of phosphoinositide 3-kinase gamma by Ras. *Current biology: CB* **12**, 1068–1075, doi:10.1016/s0960-9822(02)00933-8 (2002).
64. Carriere, A. *et al.* ERK1/2 phosphorylate Raptor to promote Ras-dependent activation of mTOR complex 1 (mTORC1). *The Journal of biological chemistry* **286**, 567–577, doi:10.1074/jbc.M110.159046 (2011).
65. Pearce, L. R., Komander, D. & Alessi, D. R. The nuts and bolts of AGC protein kinases. *Nature reviews. Molecular cell biology* **11**, 9–22, doi:10.1038/nrm2822 (2010).
66. Zecchin, E. *et al.* Distinct delta and jagged genes control sequential segregation of pancreatic cell types from precursor pools in zebrafish. *Developmental biology* **301**, 192–204, doi:10.1016/j.ydbio.2006.09.041 (2007).
67. Yin, L., Maddison, L. A. & Li, M. Multiplex Conditional Mutagenesis Using Transgenic Expression of Cas9 and sgRNAs. **200**, 431–441, doi:10.1534/genetics.115.176917 (2015).
68. Kawakami, K. *et al.* A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. *Developmental cell* **7**, 133–144, doi:10.1016/j.devcel.2004.06.005 (2004).
69. Fisher, G. H. *et al.* A fast and sensitive method for measuring picomole levels of total free amino acids in very small amounts of biological tissues. *Amino acids* **20**, 163–173, doi:10.1007/s007260170057 (2001).
70. Hur, M. *et al.* MicroCT-based phenomics in the zebrafish skeleton reveals virtues of deep phenotyping in a distributed organ system. *Elife* **6**, doi:ARTN e26014
71. 7554/eLife.26014 (2017).

Figures

Figure 1

Activation of mTORC1 is insufficient for α cell proliferation. a. Representative images of α cells in control, TgaCA-MTOR (Tg(gcga:MTORL1460P, cryaa:tagRFP)), TgaCA-Rheb (Tg(gcga:RhebS16H, cryaa:YFP)), and gcgrDKO (gcgr^{-/-};gcgr^{-/-}) larvae at 5 dpf (scale bar, 10 μ m). b. Quantification of α cell number in control, TgaCA-MTOR, TgaCA-Rheb and gcgrDKO larvae at 5 dpf (Data represent means $\pm$ SEM, n = 15 for each group). c. Quantification of α cell size in control, TgaCA-MTOR, TgaCA-Rheb and gcgrDKO larvae at

5 dpf (Data represent means $\pm$ SEM, n = 16 for each group). d. Representative images of EdU staining in control, TgaCA-MTOR, TgaCA-Rheb and gcgrDKO larvae at 5 dpf. EdU (Red) positive α cells are indicated by arrows (scale bar, 10 μ m). e. Quantification of EdU positive α cell number in control, TgaCA-MTOR and TgaCA-Rheb larvae at 5 dpf (Data represent means $\pm$ SEM, n = 9 for each group). ***P < 0.001, ****P < 0.0001, NS means no significant difference (One-way ANOVA, Tukey's multiple comparisons test).

Figure 2

Hyperaminoacidemia-induced α cell proliferation requires CaSR cell-autonomously. a. Quantification of α cell number in 5 dpf larvae of control, gcgrDKO and gcgrDKO with knockdown of amino acid sensing GPCRs (gprc6a, tas1r3, casr) (Data represent means $\pm$ SEM, n = 12, 12 5 8,16 for each group). b. Diagram of casr CRISPR mutagenesis including target location, target sequence (underlined), PAM (bold) and characterized mutations. Lower case letters indicate insertion. hyphens represent deletion. c. Representative images of α cells in control, gcgrDKO, casr-/-; gcgrDKO and TgαCASR(Tg(gcga:CaSR));casr-/-;gcgrDKO larvae at 5 dpf (scale bar, 10 μ m). d. Quantification of α cell number in control, gcgrDKO, casr-/-;gcgrDKO and TgαCASR; casr-/-;gcgrDKO larvae at 5 dpf (Data represent means $\pm$ SEM, n = 12 for each group). e. Representative images of EdU staining in control, gcgrDKO, casr-/-;gcgrDKO and TgαCASR;casr-/-;gcgrDKO larvae at 5 dpf. EdU (Red) positive α cells are indicated by arrows (scale bar, 10 μ m). f. Quantification of EdU positive α cell number in control, gcgrDKO, casr-/-;gcgrDKO and TgαCASR;casr-/-;gcgrDKO larvae at 5 dpf (Data represent means $\pm$ SEM, n = 9 for each group). g. Quantification of the percentage of Ki67 positive α cells in mouse islets cultured with LAA, HAA, HAA + Calhex 231 mediums. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS means no significant difference (One-way ANOVA, Tukey's multiple comparisons test).

Figure 3

Activation of a Gq-DREADD in α cells restores proliferation in *casr*^{-/-}; *gcgr*^{DKO} zebrafish. a. Diagram of CNO treatment, 20 μ M for 48h. b. Representative images of α cells in control, *Tga*^{Gq}(*Tg*(*gcga*:hM3Dq)); *casr*^{-/-}; *gcgr*^{DKO} and *Tga*^{Gq}; *gcgr*^{DKO} larvae with 20 μ M CNO presence or absence at 5 dpf (scale bar, 10 μ m). c. Quantification of α cell number at 5 dpf in control, *Tga*^{Gq}; *casr*^{-/-}; *gcgr*^{DKO} and *Tga*^{Gq}; *gcgr*^{DKO} larvae treated with 20 μ M CNO or vehicle for 48 hours (Data represent means $\pm$ SEM, n = 12 for each group). d. Representative images of EdU staining at 5 dpf in

control, *TgaGq;casr^{-/-};gcgrDKO* and *TgaGq; gcgrDKO* larvae treated with 20 μ M CNO or vehicle for 48 hours. EdU (Red) positive α cells are indicated by arrows (scale bar, 10 μ m). e. Quantification of EdU positive α cell number at 5 dpf in control, *TgaGq;casr^{-/-};gcgrDKO* and *TgaGq;gcgrDKO* larvae treated with 20 μ M CNO or vehicle for 48 hours (Data represent means $\pm$ SEM, n = 8 for each group). ***P < 0.001, ****P < 0.0001, NS means no significant difference (One-way ANOVA, Tukey's multiple comparisons test).

Figure 4

Synergism of Gq and mTORC1 pathways is sufficient to induce α cell proliferation. a. Quantification of α cell number at 5 dpf in control, Tg α CA-Rheb, Tg α Gq and Tg α CA-Rheb;Tg α Gq larvae with the presence or absence of 1 μ M CNO (Data represent means $\pm$ SEM, n = 6-11 for each group). b. Representative images of α cells at 5 dpf in control, gcgrDKO and Tg α Gq larvae with the presence or absence of 20 μ M CNO. Scale bar, 10 μ m. c. Quantification of α cell number at 5 dpf in control, gcgrDKO and Tg α Gq larvae treated with 20 μ M CNO or vehicle for 48 hours (Data represent means $\pm$ SEM, n = 5/9 for each group). d. Representative images of EdU staining at 5 dpf in control, gcgrDKO and Tg α Gq larvae treated with 20 μ M CNO or vehicle for 48 hours. EdU (Red) positive α cells are indicated by arrows (scale bar, 10 μ m). e. Quantification of EdU positive α cell number at 5 dpf in control, gcgrDKO and Tg α Gq larvae treated with 20 μ M CNO or vehicle for 48 hours (Data represent means $\pm$ SEM, n = 5/10 for each group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS means no significant difference (One-way ANOVA, Tukey's multiple comparisons test).

Figure 5

CaSR-Gq induces α cell proliferation via ERK1/2 Pathway. a. Representative images of pERK1/2 and GFP immunofluorescence in islet sections of control, gcgr^{DKO} and casr^{-/-};gcgr^{DKO} larvae at 5 dpf. pERK1/2 signal in α cell is indicated by arrows (scale bar, 8 μ m). b. Quantification of the percentage of pERK1/2 positive α cells in control, gcgr^{DKO} and casr^{-/-};gcgr^{DKO} larvae at 5 dpf (Data represent means $\pm$ SEM, n = 10 for each group). c. Quantification of average pERK1/2 fluorescence intensity in α cells of control,

gcgrDKO and casr^{-/-};gcgrDKO larvae at 5 dpf (Data represent means $\pm$ SEM, n = 9 for each group). d. Representative images of pERK1/2 and GFP immunofluorescence in islet sections of control, TgaGq;casr^{-/-};gcgrDKO and TgaGq;gcgrDKO larvae at 5 dpf. The larvae were treated with or without 20 μ M CNO for 48 hours. pERK1/2 positive α cells are indicated by arrows (scale bar, 8 μ m). e. Quantification of the percentage of pERK1/2 positive α cells at 5 dpf in control, TgaGq; casr^{-/-};gcgrDKO and TgaGq;gcgrDKO larvae treated with or without 20 μ M CNO for 48 hours (Data represent means $\pm$ SEM, n = 9 for each group). f. Quantification of average pERK1/2 fluorescence intensity in α cells at 5 dpf of control, TgaGq;casr^{-/-};gcgrDKO and TgaGq;gcgrDKO larvae treated with or without 20 μ M CNO for 48 hours (Data represent means $\pm$ SEM, n = 10 for each group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS means no significant difference (One-way ANOVA, Tukey's multiple comparisons test).

Figure 6

α cell proliferation requires Mek1/2 activation. a. Diagram of 48h treatment with 10 μ M U0126. b. Representative images of α cells at 5 dpf in control, *gcgr*DKO and *casr*^{-/-}*gcgr*DKO larvae treated with or without 10 μ M U0126 for 48 hours (scale bar, 10 μ m). c. Quantification of α cell number at 5 dpf in control, *gcgr*DKO and *casr*^{-/-}*gcgr*DKO larvae treated with or without 10 μ M U0126 for 48 hours (Data represent means $\pm$ SEM, n = 18 for each group). d. Representative images of EdU staining at 5 dpf in

control, gcgrDKO and casr-/-;gcgrDKO larvae treated with or without 10 μ M U0126 for 48 hours. EdU (Red) positive α cells are indicated by arrows (scale bar, 10 μ m). e. Quantification of EdU positive α cell number at 5 dpf in control, gcgrDKO and casr-/-;gcgrDKO larvae treated with or without 10 μ M U0126 for 48 hours (Data represent means $\pm$ SEM, n = 15 for each group). f. Representative images of cells from primary mouse islets cultured in LAA and HAA medium with or without 10 μ M U0126. Ki67 (Red) positive α cells are indicated by arrows (scale bar, 50 μ m). g. Quantification of the percentage of Ki67 positive α cells in dispersed cells from cultured islets (Data represent means $\pm$ SEM, n = 3 for each group). ***P < 0.001, ****P < 0.0001, NS means no significant difference (One-way ANOVA, Tukey's multiple comparisons test).

Figure 7

Hyperaminoacidemia-induced mTORC1 activation in α cells requires CaSR and MEK1/2. **a**. Representative images of pS6(240/244) and GFP immunofluorescence in islet sections of control, *gcgr*^{DKO} and *casr*^{-/-}; *gcgr*^{DKO} larvae at 5 dpf. pS6(240/244) signal in α cells is indicated by arrows (scale bar, 7 μ m). **b**. Quantification of the percentage of pS6(240/244) positive α cells in control, *gcgr*^{DKO} and *casr*^{-/-}; *gcgr*^{DKO} larvae at 5 dpf (Data represent means $\pm$ SEM, n = 9 for each group). **c**. Quantification of

average pS6(240/244) fluorescence intensity in α cells of control, gcgrDKO and casr^{-/-};gcgrDKO larvae at 5 dpf (Data represent means $\pm$ SEM, n = 9 for each group). d. Representative images of pS6(240/244) and GFP immunofluorescence in islet sections of control and gcgrDKO larvae (5 dpf) treated with DMSO or 10 μ M U0126 for 48 h. pS6(240/244) signal in α cells is indicated by arrows (scale bar, 7 μ m). e. Quantification of the percentage of pS6(240/244) positive α cells in control and gcgrDKO larvae (5 dpf) treated with DMSO or 10 μ M U0126 for 48 h (Data represent means $\pm$ SEM, n = 9 for each group). f. Quantification of average pS6(240/244) fluorescence intensity in α cells of control and gcgrDKO larvae (5 dpf) treated with DMSO or 10 μ M U0126 for 48 h (Data represent means $\pm$ SEM, n = 9 for each group). *P < 0.05, ***P < 0.001, ****P < 0.0001, NS means no significant difference (One-way ANOVA, Tukey's multiple comparisons test).

Figure 8

Gq hyperactivation-induced α cell proliferation requires mTORC1. **a**. Representative images of α cells at 5 dpf in control, *gcgr*DKO and *Tga*Gq-CNO larvae treated with or without 2.5 μ M rapamycin for 48 hours (scale bar, 10 μ m). **b**. Quantification of α cell number at 5 dpf in control, *gcgr*DKO and *Tga*Gq-CNO larvae treated with or without 2.5 μ M rapamycin (Data represent means $\pm$ SEM, $n = 10/14$ for each group). **c**. Representative images of pS6(240/244) and GFP immunofluorescence in islet sections of 5 dpf control,

TgaGq;casr^{-/-};gcgrDKO and TgaGq;gcgrDKO larvae treated with or without 20 μ M CNO. pS6(240/244) signal in α cell is indicated by arrows (scale bar, 7 μ m). d. Quantification of pS6(240/244) positive α cell percentage in control, TgaGq;casr^{-/-};gcgrDKO and TgaGq;gcgrDKO larvae with 20 μ M CNO presence or absence at 5 dpf (Data represent means $\pm$ SEM, n = 9 for each group). e. Quantitative results of pS6(240/244) average fluorescence intensity in control, TgaGq;casr^{-/-};gcgrDKO and TgaGq;gcgrDKO larvae α cells with 20 μ M CNO presence or absence at 5 dpf (Data represent means $\pm$ SEM, n = 10 for each group). f. Representative pS6(240/244) and GFP immunofluorescence images of control, gcgrDKO and TgaGq with 20 μ M CNO larvae islet sections at 5 dpf. pS6(240/244) signal in α cell is indicated by arrows (scale bar, 7 μ m). g. Quantitative results of pS6(240/244) positive α cell percentage in control, gcgrDKO and TgaGq with 20 μ M CNO larvae islet sections at 5 dpf (Data represent means $\pm$ SEM, n = 6 for each group). h. Quantitative results of pS6(240/244) average fluorescence intensity in control, gcgrDKO and TgaGq with 20 μ M CNO larvae islet sections at 5 dpf (Data represent means $\pm$ SEM, n = 5, 7, 6 for each group). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, NS means no significant difference (One-way ANOVA, Tukey's multiple comparisons test).

Figure 9

Working model depicting the necessary and sufficient signaling pathways for hyperaminoacidemia-induced α cell proliferation. Interrupted glucagon signaling results in hyperaminoacidemia. Amino acid surges activate the CaSR-Gq-Mek1/2-Erk1/2 pathway. Concurrently, amino acids are transported into the cytoplasm and activate mTORC1. Activation of mTORC1 is likewise CaSR-Gq-Mek1/2 dependent. The synergism between the CaSR-Gq and mTORC1 pathways mediate hyperaminoacidemia-induced α cell proliferation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [GongetalSupplementaryinformation.docx](#)