mTORC1 Activator SLC38A9 Is Required to Efflux Essential Amino Acids from Lysosomes and Use Protein as a Nutrient

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mTORC1 activator SLC38A9 is required to efflux essential amino acids from lysosomes and use protein as a nutrient

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Summary

The mTORC1 kinase is a master growth regulator that senses many environmental cues, including amino acids. Activation of mTORC1 by arginine requires SLC38A9, a poorly understood lysosomal membrane protein with homology to amino acid transporters. Here, we validate that SLC38A9 is an arginine sensor for the mTORC1 pathway, and uncover an unexpectedly central role for SLC38A9 in amino acid homeostasis. SLC38A9 mediates the transport, in an arginine-regulated fashion, of many essential amino acids out of lysosomes, including leucine, which mTORC1 senses through the cytosolic Sestrin proteins. SLC38A9 is necessary for leucine generated via lysosomal proteolysis to exit lysosomes and activate mTORC1. Pancreatic cancer cells, which use macropinocytosed protein as a nutrient source, require SLC38A9 to form tumors. Thus, through SLC38A9, arginine serves as a lysosomal messenger that couples mTORC1 activation to the release from lysosomes of the essential amino acids needed to drive cell growth.

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Author Contributions: G.A.W. and D.M.S. initiated the project and designed the research plan. G.A.W. performed the experiments and analyzed the data with input from M.A.-R, E.F., and W.W.C. R.L.W. assisted in experimental design and immunohistochemical analyses with G.A.W. E.F. operated the LC/MS equipment. L.V.D. and M.V.G.H. performed the pancreatic cancer cell orthotopic injections. G.A.W and D.M.S. wrote the manuscript and all authors edited it.

Introduction

The mTORC1 protein kinase controls growth by balancing anabolic processes, such as protein, lipid, and nucleotide synthesis, with catabolic ones like autophagy and proteasomal activity. mTORC1 is deregulated in many diseases, including cancer and epilepsy, and modulates the aging process in multiple model organisms. Diverse environmental cues regulate mTORC1, including growth factors, nutrients, and stress, presenting the challenge of understanding how mTORC1 senses and integrates these various inputs to coordinate a coherent growth program (reviewed in (Saxton and Sabatini, 2017)).

Amino acids promote the interaction of mTORC1 with the Rag GTPases, which function as heterodimers of RagA or RagB bound to RagC or RagD and localize to the lysosomal surface (Sancak et al., 2010; Sancak et al., 2008; Sekiguchi et al., 2001). In response to amino acids, the Rag GTPases recruit mTORC1 to the lysosome, where Rheb, a distinct GTPase that directly stimulates the kinase activity of mTORC1, also resides. Growth factors activate Rheb by inducing its inhibitor, the TSC tumor suppressor, to leave the lysosomal surface (Menon et al., 2014). Thus, the Rheb and Rag GTPases form two arms of a co-incident detector that ensures that mTORC1 activation occurs only when nutrients and growth factors are both present (Kim et al., 2008; Sancak et al., 2010; Sancak et al., 2008).

The amino acid sensing pathway is complicated, with several multi-protein complexes regulating the Rag GTPases, each likely serving as the effector of a distinct sensing branch of the pathway. Two such complexes are GATOR1, which is a GTPase activating protein (GAP) for Rag A/B and inhibits the mTORC1 pathway, and GATOR2, a positive component of the pathway of unknown molecular function that acts upstream of or in parallel to GATOR1 (Bar-Peled et al., 2013). The best-characterized amino acid sensors, the Sestrin and CASTOR families of proteins bind cytosolic leucine and arginine, respectively, and interact with and suppress GATOR2 in the absence of their cognate amino acids (Chantranupong et al., 2016; Chantranupong et al., 2014; Kim et al., 2015; Parmigiani et al., 2014; Saxton et al., 2016a; Saxton et al., 2016b; Wolfson et al., 2016). A third is the FLCN-FNIP complex, a GAP for Rag C/D, which translocates to the lysosomal surface in the absence of amino acids, but for which the amino acid sensing mechanism is unknown (Petit et al., 2013; Tsun et al., 2013). Lastly, a fourth is Ragulator, which anchors the Rag GTPase heterodimer to the lysosomal surface and also regulates its nucleotide state (Bar-Peled et al., 2012). SLC38A9, a lysosomal 11-transmembrane segment protein with homology to amino acid transporters, binds the Rag GTPase-Ragulator complex and is necessary for the full activation of mTORC1 (Jung et al., 2015; Rebsamen et al., 2015; Wang et al., 2015).

Here, we validate our previous proposal that SLC38A9 is an arginine sensor for the mTORC1 pathway (Wang et al., 2015), and also uncover, using lysosomal metabolite profiling, a surprisingly important role for SLC38A9 in amino acid homeostasis. SLC38A9 is needed to transport, in an arginine-regulated fashion, most essential amino acids out of lysosomes, including leucine, which mTORC1 senses through the Sestrin1 and Sestrin2 proteins in the cytosol (Saxton et al., 2016b; Wolfson et al., 2016). In vitro, arginine promotes the leucine transport capacity of SLC38A9 as well as its interaction with the Rag GTPase-Ragulator complex. The transport function of SLC38A9 is required for leucine

produced by lysosomal proteolysis to exit lysosomes and activate mTORC1, and for the growth of pancreatic tumors that obtain amino acids from macropinocytosed extracellular protein. Thus, arginine stimulates SLC38A9 to activate mTORC1 and efflux essential amino acids from lysosomes to the cytosol, where they serve as the building blocks of mass.

Results

A mutant of SLC38A9 that does not interact with arginine cannot signal arginine sufficiency to mTORC1

Two main findings led us to propose that SLC38A9 is a lysosomal arginine sensor for the mTORC1 pathway: the activation of mTORC1 by arginine requires SLC38A9, and SLC38A9 can transport and thus interact with arginine (Wang et al., 2015). To further support this possibility, two predictions should be met: (1) the capacity of SLC38A9 to bind arginine should be necessary for the mTORC1 pathway to sense arginine, and (2) the arginine concentration in lysosomes should be sufficient for SLC38A9 to interact with arginine.

To test the first prediction it was necessary to identify a mutant of SLC38A9 that cannot interact with arginine. SLC38A9 has two distinct regions: an 11-transmembrane segment domain that likely mediates amino acid transport, and an ~110 amino acid cytosolic N-terminal domain that is necessary and sufficient to interact with the Rag GTPase-Ragulator complex (Rag-Ragulator, for short) (Jung et al., 2015; Rebsamen et al., 2015; Wang et al., 2015). We sought to identify a mutant that does not transport arginine *in vitro* because at the time it was the only known measure of the arginine-SLC38A9 interaction we had. Mutations in residues conserved amongst members of the SLC38 family of transporters did not strongly affect transport by SLC38A9, so we searched for sequence elements in SLC38A9 shared with other classes of transporters. We noted that a GTS amino acid sequence in transmembrane segment 1 of SLC38A9 aligns with the GSG motif in members of the APC superfamily of Na^+-coupled symporters (e.g., AdiC, CadB, and PotE), which is known to be critical for binding amino acids (Figure 1A) (Gao et al., 2009; Jack et al., 2000). Mutation of the threonine in the GTS to tryptophan (T133W) eliminated *in vitro* arginine transport by SLC38A9 without interfering with its capacity to traffic to lysosomes or associate with Ragulator (as detected by its p14 and p18 components) or RagA or RagC in cells (Figure 1B, 1C, and S1). In contrast, the previously identified I68A mutant that does not bind Rag-Ragulator (Wang et al., 2015) transported arginine similarly to wild-type SLC38A9 (Figure 1B and 1C).

To test the capacity of these mutants to activate mTORC1, we took advantage of HEK-293T cells engineered to lack SLC38A9. In these cells activation of mTORC1 in response to arginine, but not leucine, is suppressed, as detected by the phosphorylation of its substrate S6K1 (Figure 1D). In contrast to wild-type SLC38A9, re-constitution of the null cells with either the I68A or T133W mutant did not rescue the arginine-sensing defect (Figure 1E). Thus, to transmit arginine sufficiency to mTORC1, SLC38A9 must interact with Rag-Ragulator as well as arginine, fulfilling the first prediction set forth above.

Arginine, at concentrations found in lysosomes, promotes the interaction of SLC38A9 with Rag-Ragulator

To test the second prediction we measured the lysosomal concentration of arginine using a method we recently developed to rapidly purify lysosomes and profile their metabolites (Figure 2A). In HEK-293T cells starved of arginine for 50 minutes, lysosomal arginine drops to ~50 μM, and rises to ~250 μM after a 10 minute restimulation with arginine (Figure 2A). Across multiple cell lines, the concentration of arginine in lysosomes ranges from 150-535 μM under nutrient replete conditions (Figure 2A and S2A). These values are much less than the Michaelis constant (K_m) of ~39 mM for arginine in the SLC38A9 transport assay (Wang et al., 2015), suggesting that at the concentrations present in lysosomes arginine does not trigger the transport cycle of SLC38A9, but rather communicates with it through a different mechanism. Indeed, using purified proteins we found that arginine strongly promotes the interaction of full-length SLC38A9 with Rag-Ragulator at a half-maximal concentration of 100-200 μM, which encompasses the arginine concentrations in lysosomes (Figure 2B). At concentrations of 1 mM, no other amino acid besides lysine mimicked the effect of arginine on the interaction of SLC38A9 with Rag-Ragulator (Figure 2C). Consistent with arginine acting directly on SLC38A9, arginine did not promote the interaction of the T133W mutant of SLC38A9 with Rag-Ragulator (Figure 2D). Lastly, the soluble N-terminal domain of SLC38A9 interacted well with Rag-Ragulator (Figure S2C), suggesting that the transmembrane domain of SLC38A9 normally suppresses this interaction in the absence of arginine.

Thus, the arginine-sensitive interaction of SLC38A9 with Rag-Ragulator reveals that SLC38A9 has an affinity for arginine that is compatible with lysosomal arginine concentrations and much better than that indicated by the transport assay. This finding, along with previous data, supports the conclusion that SLC38A9 can serve as a direct sensor of lysosomal arginine levels for the mTORC1 pathway, and suggests that arginine transport is not required for arginine sensing. That a small molecule binds and regulates a transporter, but is not transported well, is an established concept in transporter biology, perhaps best exemplified by certain ligands of the GAP1 amino acid transporter (Hundal and Taylor, 2009; Popova et al., 2010; Van Zeebroeck et al., 2009; Van Zeebroeck et al., 2014). Future work, likely requiring high-resolution structures, will be needed to understand how arginine promotes the binding of SLC38A9 to Rag-Ragulator and how this interaction impacts Rag-Ragulator activity.

While lysine also promoted the interaction of SLC38A9 with Rag-Ragulator (Figure 2C), it did so less potently than arginine, acting at a half maximal concentration of 600-700 uM (Figure S2D), which is higher than that of lysine in lysosomes (Figure S2B). Lysine starvation did not appreciably change lysosomal lysine levels but did moderately inhibit mTORC1 signaling and loss of SLC38A9 partially suppressed its re-activation by lysine re-addition (Figure S2B and S2E). These data suggest that lysine is a less physiologically relevant ligand for SLC38A9 than arginine but that SLC38A9 does contribute to the sensing of lysine by the mTORC1 pathway.

Many essential amino acids accumulate in lysosomes lacking SLC38A9

Given its homology to amino acid transporters, it seemed reasonable that, in addition to signaling to mTORC1, SLC38A9 might also regulate lysosomal amino acid levels. To explore this possibility, we measured amino acid concentrations in lysosomes of wild-type and SLC38A9-null HEK-293T cells (Figure 3A). While loss of SLC38A9 had no effect on whole-cell amino acid levels, it strongly boosted the lysosomal concentrations of several amino acids, including leucine, while having minor effects on arginine levels (Figure 3B). Over-expression of wild-type SLC38A9, but not a control protein, had the opposite effect, reducing the lysosomal concentrations of many of the same amino acids that most increased in lysosomes lacking SLC38A9 (Figure 3C). In subsequent experiments we focused on the set of amino acids affected in both the loss and gain of function experiments, which includes most non-polar, essential amino acids (phenylalanine, leucine, isoleucine, tryptophan, and methionine) as well as tyrosine.

To rule out that SLC38A9 loss increased the lysosomal levels of these amino acids by partially inhibiting mTORC1, we expressed the I68A mutant of SLC38A9 or a variant lacking the N-terminal domain (Δ110) in the SLC38A9-null cells. Neither binds Rag-Ragulator or rescues mTORC1 signaling (Figure 1C, 1E, S3C, and S3D), but both reversed the increase in lysosomal amino acid concentrations to the same degree as wild-type SLC38A9 (3D and S3B). In contrast, expression of the transport mutant of SLC38A9 (T133W) or just the soluble N-terminal Rag-Ragulator-binding domain of SLC38A9, did not decrease the high concentrations of the amino acid in the lysosomes lacking SLC38A9 (Figure 3D and S3E). Thus, SLC38A9 controls the lysosomal concentrations of most non-polar essential amino acids independently of its effects on mTORC1 signaling.

SLC38A9 is a high affinity transporter for leucine

The simplest explanation for why loss of SLC38A9 causes leucine and the other amino acids to accumulate in lysosomes is that SLC38A9 normally transports them out of lysosomes. In previous work we were unable to assess the capacity of SLC38A9 to transport leucine because the radiolabelled leucine bound non-specifically to the SLC38A9-containing liposomes used in the *in vitro* transport assay (Wang et al., 2015). Using a different lipid composition for the liposomes and a purer preparation of SLC38A9 we developed an improved transport assay for SLC38A9 (see methods). In this new assay the K_m for arginine is ~4 mM, which is substantially lower than the ~39 mM we obtained previously, but still much higher than the ~100-200 μM arginine concentration in lysosomes (Figure 4A and 2A). In contrast, the K_m for leucine in the transport assay is only ~90 μM, which is much lower than that for arginine and compatible with the lysosomal leucine concentrations of 60-80 μM (Figure 4B, 2A, and S4A). The T133W mutant of SLC38A9, which we originally identified because of its inability to transport arginine, was also unable to transport leucine in vitro (Figure S4B), indicating that it is generally defective in amino acid transport. SLC38A9 also transported leucine when the assay was run in the efflux format by placing leucine inside the proteoliposomes (Figure S4C). Lastly, tyrosine, along with isoleucine, valine, and phenylalanine, competed well with the transport of leucine, and tyrosine itself was transported by SLC38A9 as detected using a radiolabelled version (Figure S4D and S4E).

These findings—in intact cells and with purified SLC38A9—suggest that SLC38A9 is a major lysosomal effluxer for leucine, and likely other amino acids such as tyrosine. In contrast, SLC38A9 loss had only minor effects on lysosomal arginine concentrations, suggesting that SLC38A9 is less important for transporting arginine out of lysosomes. This conclusion is consistent with the high K_m of SLC38A9 for arginine and with previous work identifying a different transporter, PQLC2, as the major effluxer of arginine from lysosomes (Liu et al., 2012). Interestingly, while it has been appreciated that a lysosomal protein must exist for leucine efflux (Milkereit et al., 2015; Taylor, 2014), no such transporter had been identified. Recent work identified SLC38A7 as an effluxer of glutamine from lysosomes (Verdon et al., 2017).

Arginine regulates the lysosomal concentrations of many essential amino acids via SLC38A9

Because arginine regulates the interaction of SLC38A9 with Rag-Ragulator, we asked if it also impacts the capacity of SLC38A9 to transport leucine. Indeed, the addition of 200 μM unlabeled arginine, but not glycine, to the transport assay boosted leucine transport by SLC38A9, increasing its V_{max} for leucine from ~220 to ~470 pmol min^{-1} without significantly affecting its K_m (Figure 4C). Thus, at a concentration that is much below its transport K_m, arginine has two effects on SLC38A9: it stimulates its capacity to transport leucine and to interact with Rag-Ragulator. In the same assay, 200 μM lysine had no effect and it only mildly boosted leucine transport when added at a higher concentration (Figure S4F).

To understand if arginine regulates the transport function of SLC38A9 in cells, we deprived cells of arginine, which lowered its levels in whole cells and in lysosomes and measured the concentrations of the amino acids that were most affected by loss and gain of SLC38A9 (Figure 5A and S5A). As a control, we starved cells for leucine, which suppressed mTORC1 signaling and induced autophagy (as assessed by LC3B lipidation) to similar extents as arginine deprivation (Figure 5B). Arginine, but not leucine, deprivation increased the lysosomal concentrations of the SLC38A9-regulated amino acids, and 15 minutes of arginine re-addition restored them to normal levels (Figure 5C). Consistent with arginine deprivation not acting through the modulation of mTORC1, it still increased lysosomal amino acid levels in cells lacking GATOR1, which have amino acid-insensitive mTORC1 signaling (Figure S5B and S5C). In contrast, arginine deprivation did not further boost the already high lysosomal levels of the amino acids in cells lacking SLC38A9 or expressing the transport-defective T133W mutant, indicating that arginine acts through the transport function of SLC38A9 (Figure 5D).

As arginine starvation might have effects in intact cells that are hard to control for, we sought to validate the effects of arginine on lysosomal amino acid transport in a cell-free system. We purified lysosomes from wild-type and SLC38A9-null cells and loaded them in vitro with radiolabeled leucine. Stimulation of the lysosomes with arginine, but not several other amino acids, caused the release of ~60% of the radiolabelled leucine in the lysosomes in a fashion that completely depended on SLC38A9 and its transport capacity (Figure 5E

and 5F). Stimulation of lysosomes with lysine also promoted the release of radiolabeled leucine, but it did so less potently than arginine (Figure S5D).

Collectively, these data reveal that arginine regulates SLC38A9-mediated transport at the level of the purified protein, in a cell free system, and in intact cells. Thus, arginine signals through SLC38A9 to link two processes: the activation of mTORC1 and the efflux of many essential amino acids from lysosomes. Consistent with arginine being a "lysosomal messenger," in arginine-stimulated cells arginine accumulates in lysosomes before the SLC38A9-regulated amino acids drop in concentration (Figure 5C). Furthermore, arginine is well-established to readily enter lysosomes (Pisoni et al., 1985; Pisoni et al., 1987), which we verified in intact cells using ^{15}N-arginine and in purified lysosomes using ^{3}H-arginine (Figure S5E). Loss of SLC38A9 does not affect arginine entry into lysosomes (Figure S5F).

SLC38A9 is required for leucine produced via autophagy to activate mTORC1

Because SLC38A9 is important for many essential amino acids to exit lysosomes, we asked if it has a special role in cells that obtain amino acids by degrading proteins in lysosomes, such as cells deprived of free amino acids. Leucine starvation acutely inhibits mTORC1 signaling, but with time the pathway reactivates because cells release endogenous leucine by activating autophagy to increase protein degradation in lysosomes (Yu et al., 2010). In HEK-293T cells, the removal of leucine from the media acutely inhibited mTORC1 signaling, but within 8 hours the pathway reactivated in a fashion that depended on the key autophagy component ATG7 (Figure 6A). Importantly, in the ATG7-null cells, the addition of free leucine to the cell medium reactivated mTORC1 signaling even after the 8 hours of leucine starvation, indicating that the leucine sensing mechanism remains functional even after long-term leucine deprivation (Figure S6A). Remarkably, loss of SLC38A9, or just its transport activity or ability to interact with Rag-Ragulator, mimicked that of ATG7, and prevented the reactivation of mTORC1 that normally occurs after 8 hours of leucine starvation in the wild-type cells (Figure 6B, 6C, and S6B). As in cells defective for autophagy, free leucine reactivated mTORC1 in the SLC38A9-null cells starved of leucine for 8 hours, showing that the leucine sensing mechanism is also intact in these cells. Importantly, the SLC38A9-null cells do not have a defect in autophagy activation, as assessed by LC3B lipidation (Figure S6C).

An explanation for these findings is that in cells lacking SLC38A9 the leucine produced by lysosomal proteolysis remains trapped in lysosomes. Indeed, while in wild-type cells long-term leucine starvation depletes leucine in whole cells and lysosomes, in cells without SLC38A9, lysosomal leucine levels do not drop despite its significant depletion in whole cells (Figure 6D). Collectively, these results indicate that under long-term leucine starvation, SLC38A9 transports the leucine generated through lysosomal proteolysis into the cytosol, where it reactivates mTORC1 through cytosolic mechanisms.

SLC38A9 is required for macropinocytosed albumin to activate mTORC1 and support cell proliferation and for tumor growth

A faster way than autophagy induction to reactivate mTORC1 in leucine-starved cells is to feed them extracellular proteins, such as albumin, which are engulfed via macropinocytosis

and degraded into amino acids in lysosomes for use in growth-promoting processes (Palm et al., 2015; Yoshida et al., 2015). In leucine-starved HEK-293T cells, which do not have high levels of macropinocytosis (Wang et al., 2014), the addition of 3% albumin to the medium took about 4 hours to moderately restore mTORC1 signaling, an effect that depended on SLC38A9 (Figure S7A).

Because oncogenic Kras signaling activates macropinocytosis, particularly in pancreatic cancer cells (Bar-Sagi and Feramisco, 1986; Commisso et al., 2013; Davidson et al., 2017; Palm et al., 2015), we undertook similar experiments in murine pancreatic $KRAS^{G12D/+}P53^{-/-}$ tumor cells, and found that albumin fully reactivates mTORC1 in 4 hours in these cells (Figure 7A). As in HEK-293T cells, loss of SLC38A9 suppressed the arginine-induced activation of mTORC1 and prevented albumin from reactivating mTORC1 in leucine deprived cells, an effect that was fully rescued by expression of wild-type SLC38A9, but not the T133W mutant (Figure 7A, 7B, S7B).

Cells with activated Ras can proliferate using albumin as the sole extracellular source of leucine, albeit at a slower rate than cells cultured in media with free leucine (Commisso et al., 2013; Palm et al., 2015). While the murine pancreatic cancer cells lacking SLC38A9 proliferated normally when cultured in traditional media, they proliferated much more slowly than control cells in media containing albumin as the leucine source (Figure 7C and S7C). We obtained very similar results in two human pancreatic cancer cell lines that also carry oncogenic KRAS (Figure S7D and S7E). In vivo, loss of SLC38A9 or its transport function, strongly inhibited tumor formation by the murine $KRAS^{G12D/+}P53^{-/-}$ cells in an orthotopic allograft model of pancreatic cancer (Figure 7D and S7F). Immunohistochemical detection of phospho-S6 in tumor sections showed decreased mTORC1 signaling in the absence of SLC38A9 or its transport function (Figure 7E). Thus, under conditions in which cells must obtain amino acids by degrading proteins delivered to lysosomes by macropinocytosis, SLC38A9 plays a key role in the activation of mTORC1 and in cell proliferation and tumor formation.

Discussion

Amino acid sensing by mTORC1 is complicated, with both cytosolic and lysosomal signaling branches transmitting amino acid sufficiency to the Rag GTPases. Here, we used similar criteria as we have for the Sestrin2 and CASTOR1 sensors of cytosolic leucine and arginine, respectively, to establish the lysosomal membrane protein SLC38A9 as a sensor of arginine. While SLC38A9 almost certainly binds arginine in its transmembrane domain, it is very difficult to determine if the sensed arginine comes from the luminal or cytosolic side of the lysosomal membrane and likely both are possible. We favor that the primary function of SLC38A9 is to sense luminal arginine produced in lysosomes through the digestion of proteins, but it is clear that arginine itself can freely enter lysosomes through a transporter (Pisoni et al., 1985; Pisoni et al., 1987) or via macropinocytosis (see model in Figure 7F). In our previous work, we found that SLC38A9 and the cytosolic sensor CASTOR1 both contribute to arginine sensing by mTORC1 (Chantranupong et al., 2016; Wang et al., 2015), and we hypothesize that different cell types use these sensing branches to varying extents, depending on whether they obtain amino acids mostly in the free form or through the

digestion of proteins. Recent work shows that SLC38A9 also has an important role in cholesterol sensing upstream of mTORC1 (Castellano et al., 2017).

In addition to its role as an arginine sensor in the mTORC1 pathway, we used a new method for lysosomal metabolomics to make the surprising finding that SLC38A9 is also a major effluxer from lysosomes of leucine and most likely several other non-polar, essential amino acids, as well as tyrosine. Mutants in SLC38A9 can decouple the signaling and amino acid efflux functions of SLC38A9, but normally they are linked and stimulated by arginine. Thus, at the same time that arginine triggers mTORC1 activation it also stimulates the efflux of most essential amino acids from lysosomes, where mTORC1-driven processes, such as protein synthesis, can consume them. Why arginine but not other amino acids would have a "lysosomal messenger" function is unclear. Perhaps it reflects that many of the proteins degraded in lysosomes are ribosomal proteins, which are highly basic because of their RNA binding capacity (Ashford and Porter, 1962; Cohn, 1967; Huang et al., 2015; Kraft et al., 2008; Kristensen et al., 2008; Takeshige et al., 1992). In this regard it is interesting that lysine, albeit less potently, also promoted the interaction of SLC38A9 with the Rag-Ragulator complex, and we find that SLC38A9 has a role in signaling lysine levels to mTORC1. Also, perhaps the sensing of arginine at the lysosome evolved from the capacity of its guanidinium group to serve as a storage form of nitrogen in the vacuoles of lower organisms. SLC38A9 loss has minor effects on the concentration of arginine in lysosomes and considering its low affinity for arginine transport, it is unlikely that SLC38A9 has a major role in controlling lysosomal arginine levels, at least in the HEK-293T cells we have studied the most.

The fact that arginine promotes the transport of leucine by SLC38A9 raises the question of how many amino acid binding sites SLC38A9 may have. Previous work suggests that the LeuT, SERT, NET, and DAT transporters, to which SLC38A9 is distantly related, have two small molecule binding sites, one for the transported metabolite and another for a molecule that allosterically regulates transport (Li et al., 2015; Neubauer et al., 2006; Quick et al., 2012; Shi et al., 2008; Singh et al., 2007; Zhou et al., 2007). Our in vitro data suggest that arginine increases the V_{max} of SLC38A9 for leucine transport without affecting its K_m for leucine. The T133W mutant eliminates the capacity of SLC38A9 to transport leucine *in vitro* and of arginine to induce the conformational change that promotes its interaction with Rag-Ragulator. Thus, this mutant does not have a selective effect on the arginine- or leucine-binding site, suggesting that these sites are either close to each other so that the mutation disrupts both or that the mutation puts SLC38A9 into a "frozen" unresponsive state. To distinguish between these and other possibilities it will be necessary to solve the structure of SLC38A9 in its various conformations. Our attempts to measure the direct binding of amino acids to purified SLC38A9 (rather than using transport into liposomes as a surrogate) have been unsuccessful, likely because its affinity for amino acids is relatively poor compared to that of transporters like LeuT, which has nanomolar affinity for its transport substrates (Piscitelli et al., 2010; Shi et al., 2008). It is also important to keep in mind that because we produce SLC38A9 in human cells, it is always possible that proteins that might co-purify with it can impact the activities we measure in vitro.

Pharmacologic inhibitors of mTORC1 have a variety of clinical uses, particularly in diseases with clear hyperactivation of the pathway (reviewed in (Saxton and Sabatini, 2017)). Because SLC38A9 has an amino acid binding pocket(s), it may be possible to develop small molecule inhibitors that specifically target the lysosomal amino acid sensing arm of the mTORC1 pathway. These may be of particular value in Ras-driven pancreatic cancers, which depend heavily on macropinocytosis to sustain their amino acid levels, and for cell survival and proliferation (Commisso et al., 2013; Davidson et al., 2017) and which we find require SLC38A9 to form tumors in vivo. In this regard, it is remarkable that SLC38A9 is as necessary as autophagy for protein-derived amino acids to activate mTORC1, revealing SLC38A9 as a key node between lysosomal amino acids and growth control.

Star Methods

Quantification and statistical analyses

Contact for Reagent and Resource Sharing—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David M. Sabatini (Sabatini@wi.mit.edu)

Experimental Model and Subject detail

Cell Lines: The following cell lines were kindly provided by: Mia-PaCa, KP4, and 8988T, Dr. Rushika Perera (UCSF); Kras G12D/+ P53-/- mouse PaCa cells, Dr. Matt Vander Heiden (MIT). Remaining cell lines (HEK-293T, HeLa) were purchased from the ATCC. Cell lines were verified to be free of mycoplasma contamination and the identities of all were authenticated by STR profiling.

Cell Culture Conditions: HEK-293T, HeLa, MIA-PaCa, 8988T, KP4, $P53^{-/-}$ MEFs, and $KRAS^{G12D/+}$ $P53^{-/-}$ mouse PaCa cells and their derivatives were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% inactivated fetal calf serum, 2 mM glutamine, penicillin, and streptomycin. For experiments involving amino acid starvation or restimulation, cells were treated as previously described (Wang et al., 2015). For experiments involving amino acid stimulation, wells were incubated in RPMI base media lacking the indicated amino acid for 50 min and then stimulated with indicated amino acid at RPMI concentrations for 10 min. For all experiments involving lysosomal purifications, cells were changed to fresh RPMI base media 1 hr prior to the start of the experiment.

Method Detail

Cell lysis, immunoprecipitations, and cDNA transfections: Cells were first rinsed with chilled PBS and lysed immediately on ice with Triton X-100 lysis buffer (1% Triton, 10 mM B-glycerol phosphate, 10 mM pyrophosphate, 40 mM HEPES pH 7.4, 2.5 mM $MgCl_2$) supplemented with 1 tablet of EDTA-free protease inhibitor (Roche) per 25 mL buffer. Lysates were kept at 4°C for 15 min and then clarified by centrifugation in a microcentrifuge at 13,000 rpm at 4°C for 10 min. For anti-FLAG immunoprecipitations, the FLAG-M2 affinity gel was washed with 1 mL lysis buffer three times and 30 uL of a 50% slurry of the affinity gel was then added to the clarified lysate and incubated with rotation at 4°C for 90 min.

For transfection-based experiments in HEK-293T cells, 2 million cells were plated in 10 cm culture plates. After twenty-four hours, cells were transfected using the polyethylenimine method using pRK5-based cDNA expression vectors as indicated (Boussif et al., 1995). The total amount of transfected plasmid DNA in each transfection was normalized to 5 μg using the empty pRK5 plasmid. After thirty-six hours, cells were lysed and analyzed as described above.

Generation of cells lacking SLC38A9: HEK-293T SLC38A9-null cells are clonal populations generated previously (Wang et al., 2015). sgSLC38A9 8988T and Mia-PaCa cell lines were made using the pLenticrispr system utilizing the same guides as described previously (Wang et al., 2015). For the generation of the SLC38A9-null murine $KRAS^{G12D/+}P53^{-/-}$ PaCa cells, the following guide sequence targeting the first exon of SLC38A9 were designed and cloned into the px459 crispr vector.

Sense: ATGCTATGTGTATAGTCCAT

Antisense: ATGGACTATACACATAGCAT

Generation of cells stably expressing cDNAs: The following lentiviral expression plasmids were used: pLJM1-FLAG-metap2, pLJM60-FLAG-SLC38A9 and subsequent mutants, pLJC5-FLAG-SLC38A9 and subsequent mutants. For lysosomal purifications, pLJC5-3XHA-TMEM192 and pLJC5-2XFLAG-TMEM192 or pLJC6-3XHA-TMEM192 and pLJC6-2XFLAG-TMEM192. Lentiviruses were produced by transfection of HEK-293T cells with plasmids indicated above in combination with the VSV-G and CMV ΔVPR packaging plasmids. Twelve hours post transfection, the media was changed to DMEM supplemented with 30% IFS. Thirty-six hours later, the virus containing supernatant was collected and frozen at -80°C for 30 min. Cells to be infected were plated in 12-well plates containing DMEM supplemented with 10% IFS with 8 μg ml^{-1} polybrene and infected with the virus containing medium. Twenty-four hours later, the cell culture medium was changed to media containing puromycin or blasticidin for selection.

Purification of wild-type and mutant FLAG-SLC38A9: For isolation of active SLC38A9, it was important to isolate FLAG-SLC38A9 from stably expressing cells rather than from those transiently expressing it. We also developed an optimized purification strategy to enrich for properly folded and membrane inserted SLC38A9. In brief, HEK-293T cells stably expressing FLAG-SLC38A9 were harvested from 15 cm cell culture plates by gentle scraping in Buffer A (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM DTT) containing protease inhibitors. Harvested cells were collected and disrupted by douncing 30 times on ice with douncers that were pre-chilled. Unbroken cells and the nuclear fraction were removed by centrifugation at 10,000 g for 20 min. The supernatant from the first centrifugation was then centrifuged at 150,000 g for 90 min to 2 hr to pellet the membrane fraction. The pellet was solubilized using Buffer B (20 mM HEPES pH 7.4, 500 mM NaCl, 2 mM DTT, and 1% DDM) for at least 4 hours to overnight with rotation at 4°C. Following solubilization of the membrane fraction, a supernatant was prepared by centrifuging at 20,000 g to pellet any insoluble material. FLAG-affinity beads (equilibrated in Buffer B) were added to the supernatant followed by a 3 hr immunoprecipitation at 4°C. FLAG-

affinity purified SLC38A9 protein was then washed 3 times in Buffer C (20 mM MES, 500 mM NaCl, 2 mM DTT, and 0.1% DDM) and eluted in Buffer C without NaCl containing 1 mg/ml FLAG peptide with rotation at 4°C for 1 hr. The eluted protein was concentrated using Amicon centrifuge filters to ~1 mg/ml, and if necessary, purified SLC38A9 was snap-frozen in Buffer C without NaCl supplemented with glycerol in liquid nitrogen and stored at -80°C.

In vitro binding of SLC38A9 to the Rag-Ragulator Complex: Purifications of GST-tagged Rag GTPases and Ragulator for in vitro binding assays were performed as previously described (Bar-Peled et al., 2012) with the following modifications. In brief, 4 million HEK-293T cells were plated in 15 cm culture dishes. For proteins produced via transient expression, after 48 hr cells were transfected with following amounts of cDNAs in the pRK5 expression vector using the PEI method (Boussif et al., 1995): 5 μg HA-GST-Rap2a; or 2 μg HA-GST-RagC, 8 μg of HA-RagB, 8 μg of HA-p18, 8 μg of HA-p14, μg of HA-MP1, 8 μg of HA-HBXIP, and 8 μg of HA-c7orf59. For isolation of Ragulator/Rag GTPase complexes, it was critical to purify them using HA-GST-tagged Rag components rather than HA-GST-tagged Ragulator components. Thirty-six hours post transfection, cells were lysed as indicated above. After clearing of cell lysates, 200 μL of 50% slurry of immobilized glutathione affinity resin equilibrated in lysis buffer was added to lysates expressing GST-tagged proteins. Recombinant proteins were incubated with the affinity resin for 2 hr at 4°C with rotation. Each sample was washed 3 times in binding buffer consisting of 0.1% TX-100, 2.5 mM $MgCl_2$, 20 mM HEPES pH 7.4, and 150 mM NaCl.

The purification of SLC38A9 is described above. For the purification of the FLAG-SLC38A9 1-119 fragment, HEK-293T cells were prepared as above and transfected with 10 μg of FLAG-SLC38A9 1-119 in the pRK5 expression vector. Following cell lysis as described above and clearing of the lysate, 100 μL of a 50% slurry of immobilized FLAG affinity resin equilibrated in lysis buffer was added and the immunoprecipitation proceeded for 2 hr at 4°C with rotation. The immunoprecipitated protein was washed 3 times in binding buffer and eluted in same buffer containing 1 mg/ml FLAG peptide by rotation at 4°C for 1 hr.

For in vitro protein-protein interaction studies, GST-purified complexes were first resuspended in 160 μL binding buffer and 20 μL of this resuspension was incubated with 5 μg of FLAG-SLC38A9 or FLAG-SLC38A9 1-119 and supplemented with 2 mM DTT and 1 mg/mL BSA in a final volume of 50 μL for 2 hours at 4°C with rotation. When required, arginine or specified amino acids was added to the binding reactions at the indicated concentrations. To terminate the binding reactions, samples were washed three times in 1 mL of chilled binding buffer followed by the addition of 50 μL of SDS-containing sample buffer.

Proteoliposome reconstitution and transport assay: The reconstitution and transport assay was previously described in (Wang et al., 2015) and used here with the following modifications. Chloroform-dissolved phosphatidylcholine or E. coli lipids (PC or EC, 50 mg) were evaporated dry under nitrogen in a round bottom flask and desiccated at least 2 hr to overnight under vacuum. Lipids were hydrated in inside buffer (20 mM MES pH 5, 90

mM KCl, and 10 mM NaCl) at 50 mg/ml with light sonication in a chilled water bath. Clarified lipids were aliquoted into 100 μL aliquots in Eppendorf tubes and then extruded through a 100 nm membrane with 15-20 passes (Avanti 6100).

Because the [^{3}H]labeled leucine bound non-specifically to the PC proteoliposomes we used previously, E.coli lipids were utilized for transport assays utilizing [^{3}H]labeled leucine or tyrosine as they achieve low background binding, while [^{3}H]labeled arginine transport assays were performed using PC lipids. In brief, the reconstitution reaction was set up containing 15 μg FLAG-SLC38A9, 80-60:1 ratio of lipid to protein (w/w), 6:1 ratio of detergent (DDM) to lipid, and 1 mM DDT in inside buffer in a volume of 700 μL. The reconstitution was initiated by rotation at 4°C for 60 min. After, in order to remove the detergent, the proteoliposomes were incubated 3 times in succession with bio-beads. First, bio-beads (50 mg/reaction) were activated by washes of 1 mL once in methanol, 5 times in dH_2O, and twice in inside buffer. After activation, proteoliposome reactions were incubated with 50 mg bio-beads as follows: 1 hr at 4°C with rotation, followed by transfer to fresh bio-beads for overnight incubation, and then once more for 1 hr at 4°C with rotation the following morning. In all transport assay experiments, a liposome only control was also prepared where glycerol-supplemented inside buffer was used in lieu of SLC38A9 and the background binding to these liposomes was subtracted from those containing SLC38A9. All transport experiments were preformed using a pH gradient across the proteoliposome membrane to mimic the pH gradient across the lysosomal membrane. The lumen of the proteoliposome was buffered to pH 5.0 and the external buffer was at pH 7.4.

Method for the rapid purification (LysoIP) and metabolite profiling of lysosomes: The method is described in Abu-Remaileh et al. (In press). LysoIP is a method for the immunoprecipitation-based isolation of TMEM192-3XHA expressing lysosomes, which we developed using insights from the recently reported method for the rapid isolation of mitochondria (Chen et al., 2016). The LysoIP method utilizes anti-HA magnetic beads to immunopurify lysosomes from HEK-293T cells expressing the transmembrane protein 192 (TMEM192) fused to 3XHA epitopes. Starting from live cells, isolation of intact lysosomes takes ~10 min and results in highly pure and intact lysosomes. The method uses buffers compatible with metabolite profiling and metabolites are extracted at the end of the procedure and analyzed via LC/MS.

On average, ~35 million HEK-293T cells are used for each LysoIP with each sample processed individually in order to ensure rapid isolation. Cells are initially washed with chilled PBS and then scraped in 1 mL of KPBS (136 mM KCl, 10 mM KH_2PO_4, pH 7.25 was adjusted with KOH) and centrifuged at 1000 × g for 2 min at 4°C. The supernatant is aspirated and the pelleted cells are resuspended in 950 uL and 25 uL of resuspension is saved for processing of whole-cell fraction. The remaining cell suspension is then homogenized with 20 strokes of a 2 mL dounce homogenizer. The homogenized sample is then centrifuged at 1000 × g for 2 mins at 4°C and the supernatant incubated with 100 uL of KPBS prewashed anti-HA magnetic beads on rotation at 4°C for 3 min. Immunocaptured lysosomes are then gently washed three times with 1 mL KPBS with a DynaMag spin magnet. Metabolites are then extracted from captured lysosomes after the third wash; beads were resuspended in 50 uL ice-cold metabolite extraction buffer (80% methanol, 20% water

containing internal standards). The metabolite extract is then centrifuged at 1000 × g for 2 min at 4°C. The supernatant is then collected and analyzed by LC/MS to determine the total moles of each metabolite. In order to control for the non-specific background binding of metabolites to the anti-HA magnetic beads, TMEM192-2XFlag expressing HEK-293T cells are processed similarly to those expressing TMEM192-3XHA.

Radiolabeled amino acid uptake by purified lysosomes: The amino acid uptake assay was adapted from previously published work (Pisoni et al., 1985). Lysosomes were isolated from ~35 million HEK-293T cells using the LysoIP method. Lysosomes were incubated with 20 μM [^{3}H]leucine or [^{3}H]arginine in 700 μL KPBS-based buffer consisting of 0.125 M sucrose, 2 mM $MgCl_2$, and 2 mM ATP at 4°C (Lyso Buffer). Lysosomes were then transferred to a 37°C warm bath for 30 min. Collected lysosomes bound to the magnet were washed three times with ice-cold KPBS and resuspended in 100 μL KPBS and mixed with standard scintillation counting fluid. Control-Lyso IP scintillation counts were subtracted and lysosomes lysed in dH_2O were used to determine the maximal amount of labeled leucine that could be released. Where lysosomes were stimulated with the indicated amino acid, [^{3}H]leucine-loaded lysosomes were washed 3 times in the Lyso buffer and then incubated in Lyso buffer containing the indicated amino acid for 10 min prior to collection of supernatant and scintillation counting.

Immunofluorescence assays: HEK-293T cells were plated on fibronectin-coated glass coverslips in 6-well cell culture dishes at 300,000 cells/well. After 12 hr, the coverslips were washed once in PBS and subsequently fixed and permeabilized in a single step using 1 mL of ice-cold methanol at -20°C for 15 min. The coverslips were then washed twice in 1 mL PBS and then incubated with primary antibody (FLAG CST 1:300 dilution, LAMP2 SCBT 1:400 dilution) in 5% normal donkey serum for 1 hr at room temperature. After incubation with the primary antibody, the cover slips were rinsed 4 times in PBS and incubated with secondary antibodies (1:400 dilution in 5% normal donkey serum) for 45 min at room temperature in the dark. The coverslips were then washed 4 times with PBS and once in dH_2O. Coverslips were mounted on slides using Vectashield containing DAPI (Vector Laboratories) and imaged on a spinning disc confocal system (Perkin Elmer).

Orthotopic implantation of cells in the mouse pancreas and tumor analyses: Male C57BL/6J mice aged 6-8 weeks at the start of the study were used for all pancreatic tumor studies. Recipient mice were first anesthetized with inhaled 2% isoflurane-oxygen mixture; an incision was then made in the abdomen at the left mid-clavicular line, and 50 μL of PBS containing 50,000 cells was injected into the tail of the pancreas. The tumors were grown for 2 weeks before the mice were sacrificed; the resulting tumors were dissected and measured using a caliper for length, width, and height. Tumor weight (mg) was also measured. Tumor volume was calculated for a modified ellipsoid shaped tumor using the following equation:

$$\text{Tumor volume} = (4/3)\pi(\text{length}/2)(\text{width}/2)^2$$

Tumors were fixed in formalin and processed for S6 pS235/S236 immunohistochemistry as described (Yilmaz et al., 2012).

Quantification and statistical analyses

A two-tailed t-test was used for comparison between two groups. All comparisons were two-sided, and P values are indicated in figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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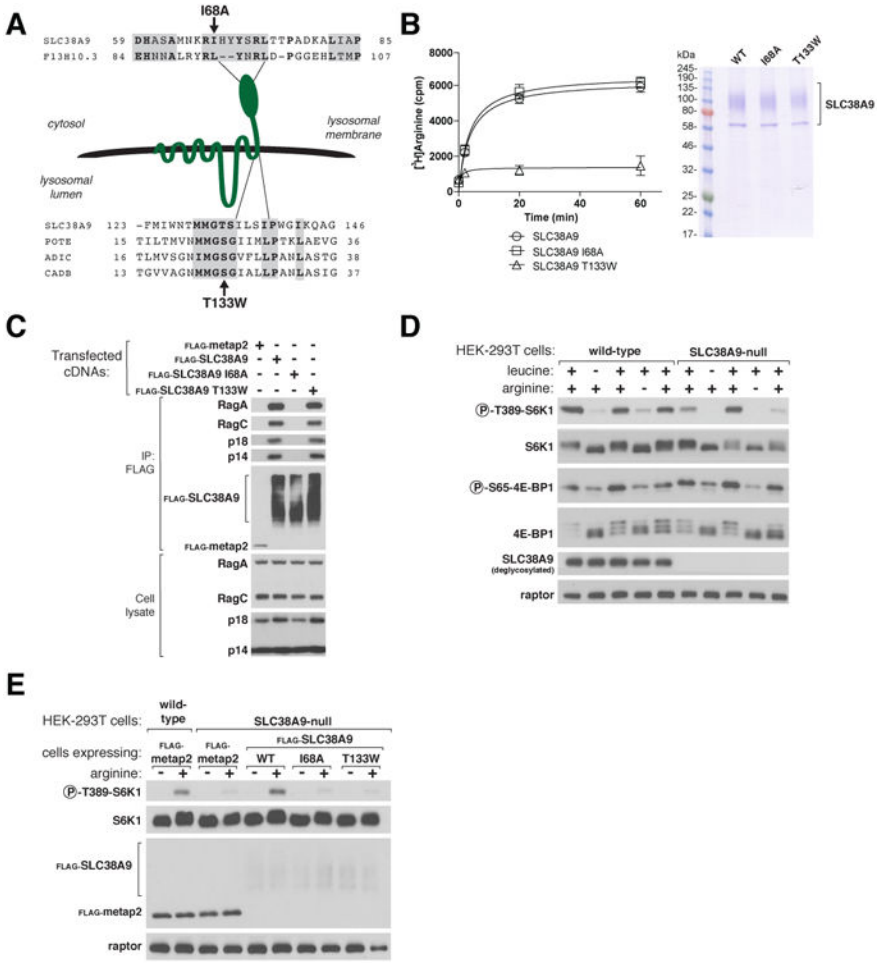

Figure 1. see also Figure S1: A mutant of SLC38A9 that does not interact with arginine cannot signal arginine sufficiency to mTORC1

(A) Schematic depicting domains of SLC38A9 and the location of the I68A and T133W point mutations. Transmembrane segment 1 of SLC38A9 shares sequence similarity with members of the APC superfamily of transporters. F13H10.3 is likely the C. elegans homolog of SLC38A9.

(B) The T133W, but not the I68A, mutant of SLC38A9 is deficient in arginine transport in vitro. SDS-PAGE and Coomassie-blue staining was used to analyze recombinant proteins purified from HEK-293T cells.

(C) Interaction of wild-type SLC38A9 and the T133W mutant, but not the Ragulator-Rag binding mutant I68A or the control protein metap2, with endogenous Ragulator (p18 and p14) and Rag GTPases (RagA and RagC). HEK-293T cells were transfected with the indicated cDNAs and lysates prepared and subjected to anti-FLAG immunoprecipitation and analyzed by immunoblotting.

(D) Loss of SLC38A9 inhibits activation of mTORC1 by arginine, but not leucine. Cells starved of the indicated amino acid for 50 minutes were stimulated for 10 minutes with leucine or arginine and cell lysates analyzed for the specified proteins and phosphorylation states.

(E) For arginine to activate mTORC1 signaling, SLC38A9 must be able to interact with both arginine and Rag-Ragulator. Wild-type and SLC38A9-null cells stably expressing the indicated proteins were analyzed as in (D).

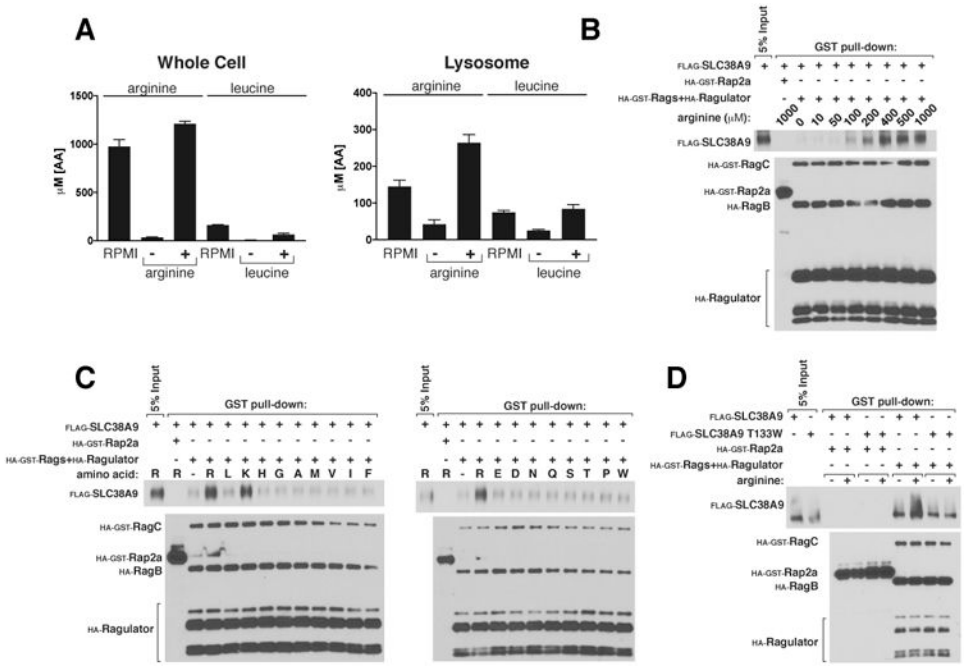

Figure 2. see also Figure S2: Arginine, at concentrations found in lysosomes, promotes the interaction of SLC38A9 with Rag-Ragulator

(A) Whole-cell and lysosomal arginine and leucine concentrations. HEK-293T cells were starved of the indicated amino acid for 50 minutes and re-stimulated with it for 10 minutes. The RPMI condition represents the non-starved state. Whole-cell and lysosomal arginine and leucine concentrations (μM) were measured using the LysoIP method described in methods. Bar graphs show mean ± SEM (n=3).

(B) In vitro, arginine promotes the interaction of SLC38A9 with the Rag-Ragulator complex in a dose-dependent manner. Purified HA-GST-RagC/HA-RagB and HA-Ragulator were immobilized on glutathione affinity resin and incubated with FLAG-SLC38A9 in the presence of the indicated concentrations of arginine. HA-GST-Rap2A was used as a control. Proteins captured in the glutathione resin pull-down were analyzed by immunoblotting for the indicated proteins using anti-epitope tag antibodies.

(C) Arginine and lysine, but not other amino acids, promote the interaction of SLC38A9 with Rag-Ragulator in vitro. Experiment was performed as in (B), except that all amino acids were at 1 mM.

(D) Arginine does not promote the interaction of SLC38A9 T133W with Rag-Ragulator. The experiment was performed as in (B) except that arginine was used at 500 μM.

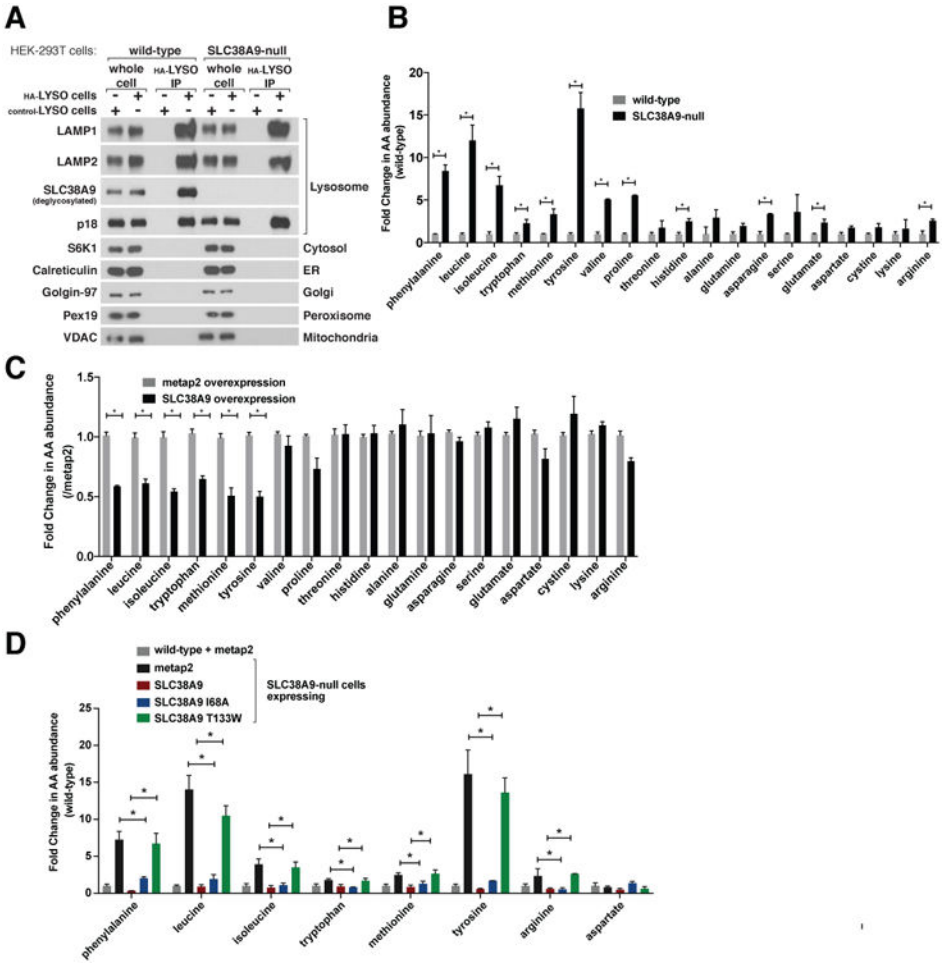

Figure 3. see also Figure S3: Many essential amino acids accumulate in lysosomes lacking SLC38A9

(A) The rapid immuno-isolation method for lysosomes (LysoIP) yields pure lysosomes from wild-type and SLC38A9-null HEK-293T cells as monitored by immunoblotting for protein markers of various subcellular compartments. Lysates and immunoprecipitates were prepared from HEK-293T cells expressing 2xFLAG-TMEM192 (Control-Lyso cells) or 3XHA-TMEM192 (HA-Lyso cells) as described in the methods.

(B) Many essential amino acids accumulate in the lysosomes of SLC38A9-null HEK-293T cells. Fold changes are relative to concentrations in wild-type HEK-293T cells and bar graphs show mean ± SEM (n=3; *p<0.05).

(C) Overexpression of SLC38A9, but not the control protein metap2, reduces the lysosomal concentrations of most non-polar, essential amino acids (phenylalanine, leucine, isoleucine, tryptophan, and methionine) as well as tyrosine. Fold changes are relative to concentrations in the control metap2-overexpressing HEK-293T cells and bar graphs show mean ± SEM (n= 3; *p<0.05).

(D) Expression of wild-type SLC38A9 or its Rag-Ragulator-binding mutant I68A, but not the transport-deficient T133W mutant, reverses the increase in lysosomal amino acid concentrations caused by loss of SLC38A9. Aspartate was used as a control amino acid as it

is unaffected by SLC38A9 loss. Lysosomes were analyzed as in (B) and bar graphs are mean ± SEM (n=3; *p<0.05).

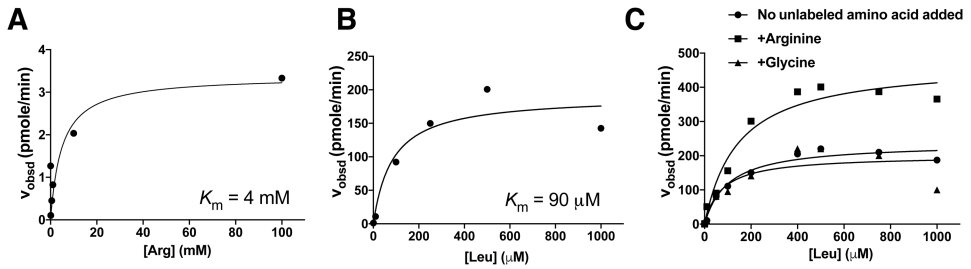

Figure 4. see also Figure S4: SLC38A9 is an arginine-regulated high affinity transporter for leucine

(A) In vitro SLC38A9 transports arginine with a K_m of ~4 mM in the improved transport assay described in the methods. Experiment was repeated more than three times with similar results, and a representative example is shown.

(B) In vitro SLC38A9 transports leucine with a K_m of ~90 μM. Experiment was repeated more than three times with similar results, and a representative example is shown.

(C) Steady-state kinetic analysis of SLC38A9-mediated leucine transport in the presence of 200 μM arginine, but not glycine, reveals an arginine-induced increase in V_{max} from ~220 to ~470 pmol min^{-1}. Velocity, as shown, was calculated as a function of the leucine concentration. The experiment was repeated three times, with a representative example shown.

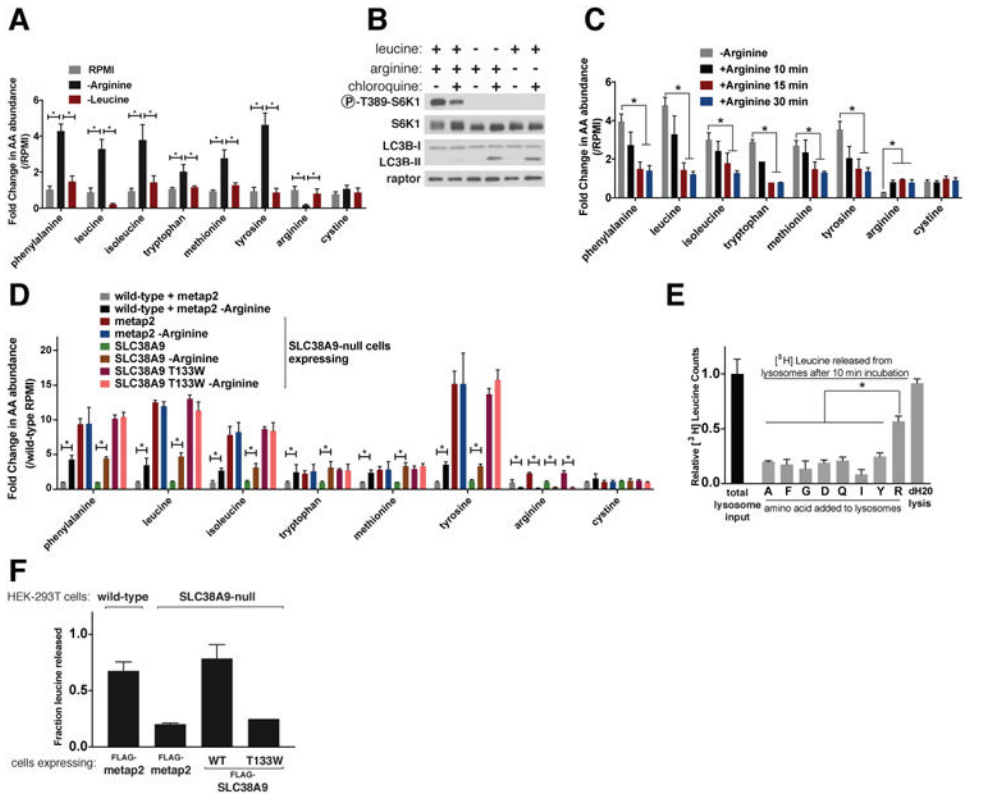

Figure 5. see also Figure S5: Arginine regulates the lysosomal concentrations of many essential amino acids via SLC38A9

(A) Arginine, but not leucine, deprivation increases the lysosomal concentrations of many of the same amino acids that are affected by loss of SLC38A9. Fold changes are relative to concentrations in cells cultured in RPMI and bar graphs show mean ± SEM (n=3, *p<0.05). HEK-293T cells were incubated in full RPMI media or in RPMI lacking the indicated amino acid for 60 minutes and lysosomes were purified and analyzed as described in methods. Cystine was used as a control metabolite.

(B) Deprivation of arginine or leucine activates autophagy to similar extents. HEK-293T cells were treated as in (A) in the absence or presence of chloroquine and lysates were analyzed for LC3B processing.

(C) Arginine re-addition time-dependently reverses the increase caused by arginine starvation in the lysosomal concentrations of the indicated amino acids. HEK-293T cells deprived of arginine for 50 minutes were re-stimulated with arginine for the indicated times. Fold changes are relative to concentrations in cells cultured in RPMI and bar graphs show mean ± SEM (n=3, *p<0.05). Cystine served as a control metabolite.

(D) In cells lacking SLC38A9 or expressing the transport-deficient T133W mutant, arginine deprivation does not further increase the already high lysosomal concentrations of the SLC38A9-regulated amino acids. Wild-type and SLC38A9-null HEK-293T cells were analyzed as in (A) and fold changes are relative to cells cultured in RPMI. Bar graphs show mean ± SEM (n=3, *p<0.05). Cystine served as a control metabolite.

(E-F) In vitro, arginine, but not several other amino acids, promotes the release of leucine from lysosomes in a fashion that requires SLC38A9 and its transport function. (E) Purified

lysosomes still attached to beads were loaded with [^{3}H]Leucine in vitro for 15 minutes and then stimulated with 500 μM of the indicated amino acid for 10 minutes. The amount of [^{3}H]Leucine released was quantified and normalized to the total amount of [^{3}H]leucine in lysosomes. This amount was obtained in two ways that gave the same value: by measuring the [^{3}H]Leucine in the bead-bound lysosomes not simulated with an amino acid after the 15 minute loading period or in the supernatant of lysosomes lysed with distilled water (dH_20 lysis). (F) Arginine does not induce leucine release in lysosomes lacking SLC38A9 or containing the T133W mutant. The experiment was performed as in (E) using lysosomes from the appropriate cell lines.

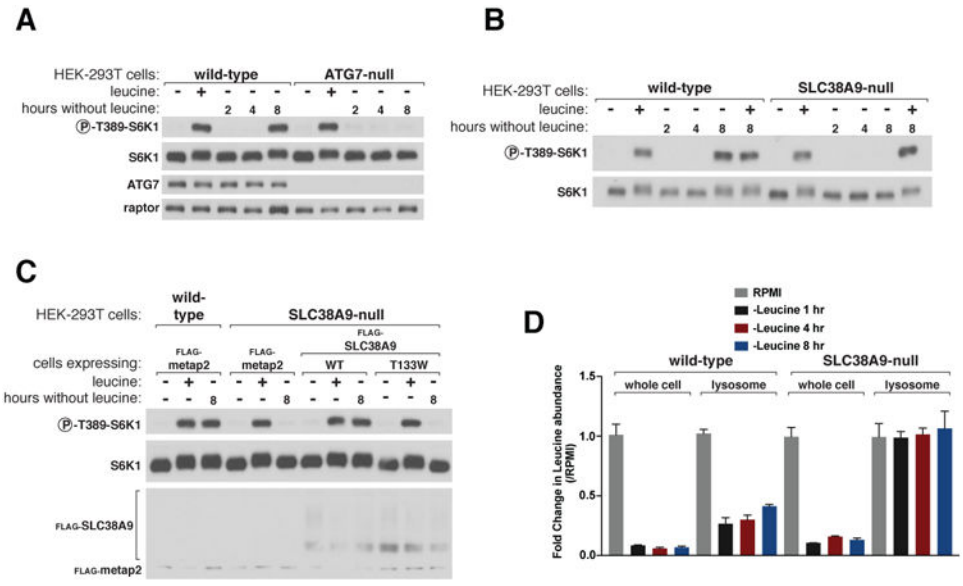

Figure 6. see also Figure S6: SLC38A9 is required for amino acids produced via autophagy to activate mTORC1 and to support cell proliferation

(A) Loss of ATG7 prevents the autophagy-mediated reactivation of mTORC1 that occurs after long-term leucine deprivation. Wild-type and ATG7-null HEK-293T cells were deprived of leucine for either 50 minutes or the indicated time points, and where specified, restimulated for 10 minutes with leucine. Cell lysates were analyzed by immunoblotting for the total levels and phosphorylation states of the indicated proteins.

(B) Loss of SLC38A9 prevents the autophagy-mediated reactivation of mTORC1 that occurs after long-term leucine deprivation. Wild-type or SLC38A9-null HEK-293T cells were deprived of leucine for 50 minutes or the indicated time points and, where indicated, re-stimulated with leucine for 10 minutes. Cell lysates were analyzed by immunoblotting for the levels and phosphorylation states of indicated proteins.

(C) mTORC1 signaling does not reactivate after long-term leucine deprivation in cells expressing the T133W SLC38A9 mutant. Wild-type or SLC38A9-null HEK-293T cells stably expressing the indicated proteins were starved for leucine for 50 minutes or 8 hours and, where indicated, re-stimulated with leucine for 10 minutes. Lysates were analyzed as in (A).

(D) In cells lacking SLC38A9, lysosomal leucine concentrations do not drop upon starvation for leucine despite its depletion at the whole-cell level. Metabolite profiling of lysosomes from wild-type and SLC38A9-null cells deprived of leucine for the indicated times. Fold changes are relative to concentrations of cells in cultured in RPMI. Bar graphs show mean ± SEM (n=3).

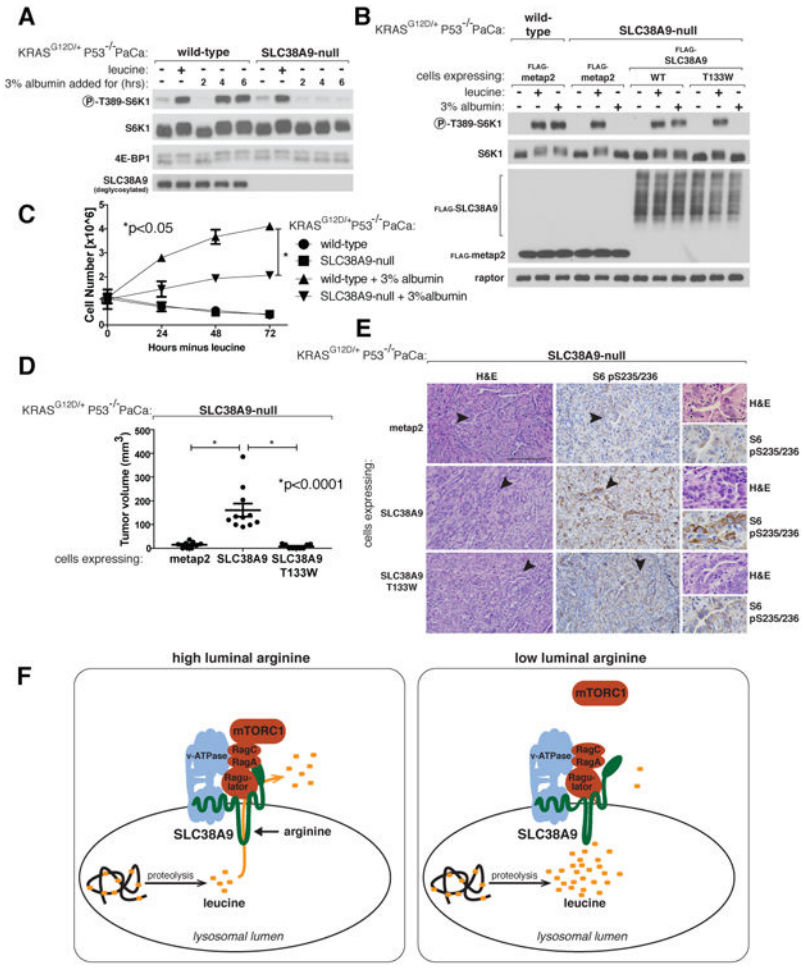

Figure 7. SLC38A9 and its transport function are required for albumin to activate mTORC1 and support cell proliferation and for pancreatic tumor growth

(A-B) Loss of SLC38A9 or just its transport capacity prevents the activation of mTORC1 induced by extracellular protein. Murine KRAS$^{G12D/+}$P53$^{-/-}$ pancreatic cancer cells that are wild-type, null for SLC38A9, or SLC38A9-null and expressing T133W SLC38A9, were deprived of leucine for 50 minutes and re-stimulated with leucine for 10 minutes or 3% albumin for the times indicated. Cell lysates were analyzed by immunoblotting for the levels or phosphorylation states of indicated proteins.

(C) Loss of SLC38A9 inhibits the proliferation of pancreatic cancer cells cultured in 3% albumin as the leucine source. Wild-type and SLC38A9-null murine KRAS$^{G12D/+}$P53$^{-/-}$ pancreatic cancer cells were cultured for 3 days in media lacking leucine, and supplemented, where indicated, with 3% albumin. Cells were counted every 24 hours and bar graphs show mean ± SD (n=3; *p<0.05).

(D) KRAS$^{G12D/+}$P53$^{-/-}$ pancreatic cancer cells lacking SLC38A9 or expressing its transport deficient T133W mutant have a severe defect in forming tumors in an orthotropic allograft model of pancreatic cancer. SLC38A9-null KRAS$^{G12D/+}$P53$^{-/-}$ PaCa cells expressing the control protein metap2, SLC38A9, or SLC38A9 T133W were used to generate tumors. Each dot represents the calculated tumor volume (mm^3) of an individual tumor. The mean ± SEM (n= 9-11, *p<0.0001) is shown.

(E) Tumors formed by $KRAS^{G12D/+}P53^{-/-}$ pancreatic cancer cells lacking SLC38A9 or expressing its transport deficient T133W mutant have decreased mTORC1 signaling. Tumors were analyzed by immunohistochemistry for S6 pS235/S236 levels and stained with hematoxyline and eosin (H&E) (10× and 40× magnifications are shown, arrow defines pancreatic cancer cells shown in 40× insets). Scale bars represent 100 μM (10×) and 20 μM (40×).

(F) A model depicting how arginine signals through SLC38A9 to promote mTORC1 activation as well as the lysosomal efflux of essential amino acids like leucine.

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-FLAG (clone D6W5B)	Cell Signaling Technology	14793hhk
Rabbit polyclonal anti-Raptor	EMD millipore	09-217
Rabbti polyclonal RagC	Cell Signaling Technology	3360
Goat anti-Rabbit IgG-HRP	Santa Cruz	Sc-2054
Goat anti-mouse IgG-HRP	Santa Cruz	Sc-205
Rabbit monoclonal anti-Pex19	Abcam	ab137072
Mouse monoclonal anti-LAMP2	Santa Cruz	sc-18822
Rabbit monoclonal anti-phospho-p70 S6 Kinase (Thr389)	Cell Signaling Technology	9234
Rabbit monoclonal anti- p70 S6 Kinase	Cell Signaling Technology	2708
Rabbit monoclonal anti-4E-BP1	Cell Signaling Technology	9644
Rabbit monoclonal anti-phospho-4E-BP1 (Ser65)	Cell Signaling Technology	9451
Polyclonal anti-rabbit LC3B	Cell Signaling Technology	2775
Rabbit monoclonal Anti-mTOR	Cell Signaling Technology	2983
Rabbit monoclonal Anti-LAMTOR1	Cell Signaling Technology	8975
Rabbit monoclonal Anti-LAMTOR2	Cell Signaling Technology	8145
Rabbit monoclonal Anti-RagA	Cell Signaling Technology	4375
Rabbit Anti-VDAC	Cell Signaling Technology	4866
Rabbit monoclonal Anti-Careticulin	Cell Signaling Technology	12238
Rabbit monoclonal Anti-Golgin97	Cell Signaling Technology	13192
Rabbit polyclonal Anti-SLC38A9	Novus Biologics	NBP1-69235
Mouse monoclonal anti-Rabbit IgG (conformation specific) (clone L27A9) HRP conjugate	Cell Signaling Technology	5127
Donkey anti-mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate	Thermo Fisher Scientific	A-21202
Donkey anti-Rabbit IgG (H+L) Secondary Antibody Alexa Fluor 568	Thermo Fisher Scientific	A-10042
Bio-beads	Bio Rad	**1528920**
Bacterial and Virus Strains		
N/A		
Biological Samples		
N/A		
Chemicals, Peptides, and Recombinant Proteins		
Anti-FLAG M2 Affinity Gel	Sigma Aldrich	A2220

REAGENT or RESOURCE	SOURCE	IDENTIFIER
X-tremeGENE 9 DNA Transfection Reagent	Roche	06365787001
FLAG Peptide (sequence DYDDDK)	Biopolymers Core, Koch Institute	N/A
RPMI	Sigma Aldrich	R0883
[^{3}H]labeled arginine	American Radiolabeled Chemicals	**ART 0841 5 mCi**
[^{3}H]labeled leucine	American Radiolabeled Chemicals	**ART 140D**
[^{3}H]labeled tyrosine	American Radiolabeled Chemicals	**ART 195a**
15N-labeled arginine	Cambridge Isotope Laboratories	**CNLM-539-H-0.25**
LysoTracker Red DND-99	Thermo FIsher	**L7528**
anti-HA magnetic beads	Thermo FIsher	**88836**
Phosphatidylcholine	Avanti Polar Lipids	**840051C500MG**
E.Coli Lipids	Avanti Polar Lipids	100600
Glutathione Agarose	Pierce	16100
Cholorquine	Sigma	C6628-25G
Critical Commercial Assays		
N/A		
Deposited Data		
N/A		
Experimental Models: Cell Lines		
HEK-293T	ATCC	CRL-3216
Mia-PaCa	Gift from RushikaPerera	N/A
8988T	Gift from RushikaPerera	N/A
KP4	Gift from RushikaPerera	N/A
KRAS G12D/+ P53 -/- mouse PaCa cells	Gift from Matthew G. Vander Heiden	N/A
HeLa	ATCC	ATCC CCL-2
Experimental Models: Organisms/Strains		
Male C57BL/6J mice 6-8 weeks	Charles River	027
Oligonucleotides		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primer sgSLC38A9 mouse Fwd: ATGCTATGTGTATAGTCCAT	This paper	N/A
Primer sgSLC38A9 mouse Rev: ATGGACTATACACATAGCAT	This paper	N/A
Recombinant DNA		
pLJM1-FLAG-metap2	This Paper	N/A
pLJM60-FLAG-SLC38A9	Wang et al., 2015	Addgene71858
pLJM60-FLAG-SLC38A9 I68A	Wang et al., 2015	Addgene 71864
pLJM60-FLAG-SLC38A9 T133W	This Paper	N/A
pLJM60-FLAG-SLC38A9 delta110	Wang et al., 2015	Addgene 71861
pLJC5-FLAG-SLC38A9	This Paper	N/A
pLJC5-3XHA-TMEM192	This Paper	#102930
pLJC5-2XFLAG-TMEM192	This Paper	#102929
pLJC6-3XHA-TMEM192	This Paper	N/A
pLJC6-2XFLAG-TMEM192	This Paper	N/A
Software and Algorithms		
Prism version 6.0.1	GraphPad	www.graphpad.com
Other		
PD10 gel filtration Column	General Electric	17085101
Extruder Set with Holder/Heating Block	Avanti	610000