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## ROLE OF GLUTAMINE METABOLISM IN VESSEL FORMATION

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Running title: Glutamine and asparagine team up in angiogenesis

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## ABSTRACT

Endothelial cell (EC) metabolism is emerging as a regulator of angiogenesis, but the role of glutamine metabolism in ECs is unknown. Here, we show that depriving ECs of glutamine or inhibiting glutaminase 1 (GLS1) caused vessel sprouting defects due to impaired proliferation and migration, and reduced pathological ocular angiogenesis. Inhibition of glutamine metabolism in ECs did not cause energy distress, but impaired tricarboxylic acid (TCA) cycle anaplerosis, macromolecule production and redox homeostasis. Only the combination of TCA cycle replenishment plus asparagine supplementation restored the metabolic aberrations and proliferation defect caused by glutamine deprivation. Mechanistically, glutamine provided nitrogen for asparagine synthesis to sustain cellular homeostasis. Silencing asparagine synthetase (ASNS, which converts glutamine-derived nitrogen and aspartate to asparagine) impaired EC proliferation and sprouting even in the presence of glutamine and asparagine. Asparagine further proved crucial in glutamine-deprived ECs to restore protein synthesis, suppress ER stress and reactivate mTOR signaling. These findings reveal a novel link between endothelial glutamine and asparagine metabolism in vessel sprouting.

## SIGNIFICANCE

Endothelial cells (ECs) abundantly consume glutamine, more than other amino acids. Glutamine sustained EC sprouting by serving multiple metabolic functions, but was not essential for energy homeostasis. Both TCA anaplerosis and asparagine metabolism were required for glutamine-driven EC proliferation. ECs relied on asparagine synthetase (ASNS), using glutamine to produce asparagine from aspartate, to sustain EC proliferation and vessel sprouting. Such a role for glutamine-dependent asparagine metabolism has not been established previously in ECs and vessel formation. Also, pharmacological blocking of GLS1 reduced pathological angiogenesis. Targeting GLS1 or ASNS may merit further consideration for therapeutic angiogenesis inhibition.

## HIGHLIGHTS

1. Glutamine is required for vessel sprouting in vitro and in vivo
2. Glutamine mediates multiple metabolic functions in ECs
3. Asparagine and TCA anaplerosis are both required for glutamine-restricted ECs
4. Glutamine-dependent asparagine synthesis is indispensable for EC growth

## INTRODUCTION

Recent studies documented an important role of EC glycolysis and fatty acid oxidation in angiogenesis <sup>1-4</sup>, and illustrated that targeting endothelial cell (EC) metabolism may represent a novel anti-angiogenic strategy <sup>5</sup>. In contrast to the well-established role of glutamine metabolism in cancer cells <sup>6,7</sup>, little is known about a possible role of glutamine metabolism in ECs during vessel sprouting. In fact, only two previous papers reported enzyme kinetics of glutaminase 1, the enzyme metabolizing glutamine to glutamate, in EC homogenates and lysates. However, nothing is known about a role of glutamine metabolism in vessel formation *in vivo*, or in underlying processes of endothelial tip versus stalk cell specification. Endothelial tip cells are located at the forefront (tip) of the vascular sprout and lead the sprout by migrating (they do not / rarely proliferate) towards the source of angiogenic signals, which are sensed by protruding filopodia <sup>8,9</sup>. Endothelial stalk cells following the tip cell elongate the vessel sprout through proliferation <sup>8,9</sup>. Tip and stalk cell phenotypes are not predetermined irreversible cell differentiation states, but rather can dynamically interchange, so that the EC with the greatest tip cell competitiveness always leads the vessel sprout at the tip <sup>10</sup>. Emerging evidence reveals that endothelial tip and stalk cells have characteristic metabolic signatures <sup>2,4</sup>. Given that glutamine is the most abundant non-essential amino acid <sup>6</sup> and critical for many other cell types, we hypothesized that glutamine metabolism might also be critical for vessel sprouting.

Glutamine is a key carbon/nitrogen source, and is metabolized for ATP generation and macro-molecule production in cancer cells <sup>6</sup>. Glutamine is first hydrolyzed by glutaminase (GLS) to ammonia and glutamate, which is then further metabolized to  $\alpha$ -ketoglutarate ( $\alpha$ KG) via glutamate dehydrogenase (GLUD1) <sup>11</sup>. Glutamine is also used for the production of nucleotides and proteins <sup>12</sup>, glutathione (GSH) for redox balance and for the synthesis of the pro-angiogenic factors nitric oxide (NO) and polyamines. While glutamine donates its amino group at the gamma position for nucleotide synthesis (thereby being converted to glutamate),

glutamate donates nitrogen at the alpha position for the synthesis of other non-essential amino acids in transamination reactions that convert glutamate to  $\alpha$ -ketoglutarate ( $\alpha$ KG). Cell type specific use of glutamine has been reported <sup>11-13</sup>. For instance, pancreatic ductal adenocarcinoma cells rely on glutamine metabolism to maintain cellular redox homeostasis <sup>11</sup>. Several phase I clinical trials are ongoing to evaluate the safety, pharmacokinetics and pharmacodynamics of the GLS1-specific inhibitor CB-839 in patients with hematological malignancies and solid tumors such as breast cancer, renal cancer and lung cancer ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). Whether and how ECs rely on glutamine metabolism is unknown.

Glutamine is also involved in mTORC1 activation and the ER stress response. mTORC1 is a master regulator of cell metabolism, controlling the synthesis of protein, lipids <sup>14</sup> and nucleotides <sup>15</sup>. mTORC1 activity is stimulated by growth factors and amino acids <sup>16</sup>. In parallel with mTORC1, the ER stress response pathway can also sense nutrient deficiency. Amino acid deprivation can activate the serine threonine kinase GCN2 (general control nonderepressible 2), which in turn phosphorylates the translation initiation factor eIF2a, resulting in increased translation of the transcription factor activating transcription factor 4 (ATF4), which induces expression of multiple ER stress response genes <sup>17</sup>. Several of these genes are involved in amino acid synthesis or transport, such as asparagine synthetase (ASNS), which catalyzes glutamine-dependent asparagine synthesis from aspartate<sup>17</sup>.

The conditionally essential amino acid asparagine can be de novo synthesized by ASNS in most cells. In normal conditions, its expression levels are low, but they can be rapidly induced in response to limitation of glucose, asparagine, but also leucine, isoleucine or glutamine, or even a single essential amino acid, as may occur during protein limitation or an imbalanced dietary amino acid composition <sup>18,19</sup>. Low serum asparagine levels have also been documented in certain cancers <sup>20</sup>, child stunting due to malnutrition <sup>21</sup>, food deprivation <sup>22</sup>, or dietary asparagine starvation <sup>23</sup>. In cancer cells, increased ASNS expression is a

component of solid tumor adaptation to nutrient deprivation and/or hypoxia <sup>18</sup>. Not a single report documented the expression or role of ASNS in ECs.

Serum asparagine levels are also therapeutically lowered to nearly undetectable levels as part of the treatment of various hematological cancers. Indeed, acute lymphoblastic leukemia (ALL) cells and some types of non-Hodgkin's lymphoma, including natural killer (NK)-cell lymphoma, are unable to produce sufficient asparagine for their growth, and therefore rely on the availability of extracellular asparagine <sup>24</sup>. This makes these cancer cells sensitive to low asparagine levels, as can be therapeutically achieved by treatment with asparaginase, the enzyme that converts asparagine into aspartate and ammonia <sup>25,26</sup>. Delivering asparaginase to the bloodstream rapidly depletes plasma asparagine, causing a rapid efflux of cellular asparagine, which is then also converted and thus, the cells of the entire body are challenged with asparagine depletion. Since asparagine is a downstream metabolite of glutamine, combined inhibition of GLS1 with asparagine deprivation is synthetically lethal for solid tumor cells <sup>27</sup>. Notably, asparaginase treatment is also effective for solid tumors, when combined with inhibition of autophagy <sup>27,28</sup>.

Recent studies uncovered important roles of asparagine in cancer cell proliferation <sup>29</sup> and cell adaptation to glutamine withdrawal <sup>30</sup>. It is also noteworthy that a defective ASNS gene blocks cells at the G1 step of the cell cycle <sup>31</sup> and asparagine deprivation induces apoptosis <sup>32</sup>. It has been postulated that asparagine limitation causes these effects, not simply through decreased availability for (glyco)protein synthesis, but possibly by serving another role, perhaps as a signal molecule. Since a link between glutamine and asparagine metabolism in ECs during vessel sprouting has not been studied, we characterized glutamine metabolism in ECs and provide proof-of-principle for blocking glutamine metabolism as a novel possible anti-angiogenic strategy.

## RESULTS

### GLUTAMINE METABOLISM IS ESSENTIAL FOR ENDOTHELIAL CELL SPROUTING IN VITRO

For an unbiased assessment of amino acid consumption/secretion, we cultured human umbilical vein endothelial cells (HUVECs, further abbreviated as “ECs”) in standard M199 medium plus 20% fetal bovine serum (FBS) (concentrations of the different amino acids in this medium at the start of the experiment are listed in Supplemental Fig. 1a). Subsequent measurements of consumption/secretion rates of various amino acids in the culture medium revealed that proliferating ECs consumed glutamine more than other amino acid (Fig. 1a). Given the dearth of published data on glutamine metabolism in ECs, we first characterized the consequences of glutamine deprivation (now utilizing glutamine-free M199 medium with dialyzed FBS to obtain zero glutamine levels). Notably, in the absence of glutamine, ECs failed to sprout (Fig. 1b-e), and glutamine deprivation dose-dependently reduced EC proliferation (Fig. 1f) and impaired migration (Fig. 1g). In agreement, compared to quiescent ECs, glutamine uptake and oxidation were higher in proliferating ECs (Fig. 1h,i).

We also analyzed the impact of inhibiting glutaminase (GLS) on EC behavior. Given the very low expression levels of GLS2 in ECs (Supplementary Fig. 1b), we focused on GLS1. We silenced GLS1 expression by lentiviral transduction with a shRNA against GLS1 (GLS1<sup>KD</sup>), which lowered GLS1 expression by more than 75% (Supplementary Fig. 1c,d). GLS1 knockdown (GLS1<sup>KD</sup>) impaired EC sprouting (Fig. 1j-m), proliferation (Fig. 1n) and migration (Fig. 1o). Treatment of ECs with the GLS1-specific blocker CB-839 (ref <sup>33</sup>) dose-dependently reduced EC proliferation (Fig. 1p). Of note, CB-839 did not affect EC proliferation upon overexpression of GLS2, indicating that it did not inhibit GLS2 (Supplementary Fig. 1e). Also, supplementation of exogenous glutamate (the end-product of the GLS reaction) overruled the anti-proliferative effect of CB-839, confirming that this blocker selectively inhibited GLS1 (Supplementary Fig. 1f). Interestingly, blocking GLS1 activity did not induce



apoptosis (Fig. 1q; Supplementary Fig. 1g,h) or cytotoxicity (Fig. 1r), arguing against a general cell demise as underlying cause for the above effects on EC behavior.

For confirmation, we isolated mouse endothelial cells (mECs) from the liver of GLS1<sup>ECKO</sup> mice, which were generated by intercrossing GLS1<sup>lox/lox</sup> mice<sup>34</sup> with Pdgfb-Cre<sup>ERT2</sup> mice (an EC-specific Cre-driver line)<sup>35</sup>. Prior to mEC isolation, GLS1 was deleted from the endothelium by 5 consecutive days of tamoxifen treatment. GLS1 mRNA expression levels were substantially reduced in GLS1<sup>KO</sup> mECs (Supplementary Fig. 1i), which proliferated less actively (Fig. 1s), confirming the data obtained with GLS1 silencing and pharmacological blockade.

## **GLUTAMINE METABOLISM IS ESSENTIAL FOR VESSEL SPROUTING IN VIVO**

In order to examine the role of endothelial glutamine metabolism in vivo, we analyzed vascular development in GLS1<sup>ECKO</sup> mice, using the postnatal retinal angiogenesis model<sup>36</sup>. Isolectin-B4 (IB4) staining of retinal vessels of GLS1<sup>ECKO</sup> pups at postnatal day 5 (P5) revealed that endothelial loss of GLS1 diminished the number of vascular branch points both at the front and the rear of the vascular plexus (Fig. 2a-d) and reduced radial expansion of the plexus (Fig. 2e). Confirming the above in vitro data, endothelial loss of GLS1 reduced EC proliferation as revealed by counting ECs, stained for IB4 and phospho-histone 3 (pH3) (Fig. 2f-h). Fewer distal sprouts with filopodia were observed in GLS1<sup>ECKO</sup> pups, suggestive of an EC migration defect (Fig. 2i). In addition, loss of GLS1 in ECs did not affect vessel maturation, determined by NG2 staining for mural cell pericyte coverage (Fig. 2j-l).

We also explored if pharmacological blockade of glutamine metabolism reduced pathological angiogenesis and therefore used the oxygen-induced model of retinopathy of prematurity (ROP). In this model, mouse pups are exposed to hyperoxia during postnatal

days P7–P12. This causes capillary depletion and vascular rarefaction, which induces retinal ischemia upon return to ambient oxygen levels, and secondary formation of proliferative vascular tufts between P12 and P17 (ref <sup>37</sup>). Treatment of pups with the GLS1 blocker CB-839 from P12 to P17 reduced the formation of vascular tufts at P17 (Fig. 2m-o). These findings underscore the importance of endothelial GLS1-driven glutamine metabolism in both developmental and pathological angiogenesis. However, given that we only studied retinal angiogenesis models, analysis of angiogenesis in other organs is warranted in future research.

### **GLUTAMINE METABOLISM REGULATES TIP/STALK CELL SPECIFICATION**

To substantiate the finding that blocking GLS1 inhibits vessel sprouting and obtain further underlying mechanistic insight, we studied EC tip cell competition, given the key role of tip / stalk cell dynamics in sprouting angiogenesis <sup>2,10</sup>. Using chimeric spheroids consisting of a 1/1 ratio of mCherry<sup>+</sup>-control (red) and GFP<sup>+</sup>-GLS1<sup>KD</sup> (green) ECs, we observed that GLS1<sup>KD</sup> ECs displayed reduced competitiveness to obtain the tip position in the spheroid sprout (Fig. 2p-r). These data argue for a prominent role for GLS1 in tip / stalk cell dynamics, revealing that glutamine metabolism is essential for ECs to reach the tip position in the vessel sprout.

### **GLUTAMINE METABOLISM IS REDUNDANT FOR OTHER VASCULAR FUNCTIONS**

In order to assess if GLS1 selectively regulated vessel formation, we also explored if glutamine metabolism by GLS1 affected other key features of the endothelium, such as vascular tone, EC activation (in conditions of vascular inflammation), and EC differentiation (arterial, venous, lymphatic). However, blocking GLS1 did not affect expression levels of endothelial nitric oxide synthase (eNOS) or endothelin 1 (ET-1), both major regulators of

vascular tone<sup>38,39</sup> (Fig. 2s). Likewise, ex vivo treatment of aortic rings with CB-839 did not affect their vasorelaxation (Fig. 2t,u). Second, we assessed the expression of the adhesion molecules VCAM and E-selectin upon IL-1 $\beta$  stimulation in order to explore if glutamine metabolism affected the activation of the endothelium in conditions of vascular inflammation<sup>40</sup>. Pharmacological GLS1 inhibition did however not alter the expression of these markers (Fig. 2v) or the adhesion of leukocytes (Fig. 2w). Finally, we checked the effect of GLS1 inhibition on arterial, venous and lymphatic specification. No effect was seen on the expression of Ephrin B2 (arterial marker), Ephrin B4 (venous marker) and Prox1 (lymphatic marker)<sup>41,42</sup> (Fig. 2x). Taken together, GLS1 inhibition affected tip cell competitiveness in vessel sprouting, yet had minimal to no effect on other key features of the endothelium.

## **GLUTAMINE CONTROLS MULTIPLE METABOLIC FUNCTIONS IN ECTS**

To explore how glutamine metabolism regulates EC sprouting, we studied various possible mechanisms.

ANAPLEROSIS & ENERGY HOMEOSTASIS: (i) Glutamine is a major anaplerotic carbon source for the TCA cycle in cancer cells<sup>7</sup>. [U-<sup>13</sup>C]-glutamine tracing in ECTS revealed that glutamine donated carbons to several TCA cycle intermediates, often even more than [U-<sup>13</sup>C]-glucose (Fig. 3a). Not surprisingly therefore, glutamine deprivation depleted the intracellular pool of TCA metabolites (Fig. 3b). (ii) Glutamine metabolism was however largely dispensable for ATP production in ECTS, as glutamine deprivation did not alter intracellular ATP levels (Fig. 3c) or the energy charge, defined as  $([ATP] + 1/2 [ADP]) / ([ATP] + [ADP] + [AMP])$  (Fig.3d). Thus, glutamine deprivation did not cause an energy crisis, likely because ECTS generate most of their energy via glycolysis<sup>2</sup>.

AMINO ACID SYNTHESIS: We analyzed the intracellular levels of non-essential amino acids (NEAAs), because glutamine is a precursor of several NEAAs. Glutamine starvation lowered the intracellular pool of glutamate (Glu), aspartate (Asp) and asparagine (Asn), while not affecting (or even slightly increasing) the levels of alanine (Ala), serine (Ser) and glycine (Gly) (Fig. 3e). Consistently, [U-<sup>13</sup>C]-glutamine and [<sup>15</sup>N<sub>2</sub>]-glutamine tracing experiments showed high label incorporation into glutamate, aspartate and asparagine, but much less (minimally) in alanine, serine and glycine (Fig. 3f).

MACROMOLECULE SYNTHESIS: We examined the effect of glutamine deprivation on macromolecule synthesis, in particular of proteins, nucleotides and lipids. (i) Protein synthesis: consistent with the fact that glutamine itself and glutamine-derived NEAAs are used as building blocks for proteins, glutamine starvation reduced protein synthesis in ECs (Fig. 3g). (ii) Nucleotide synthesis: [<sup>15</sup>N<sub>2</sub>]-glutamine tracing showed that glutamine donated its nitrogen for nucleotide synthesis in ECs under glutamine-replete conditions (Supplementary Fig. S2a). In agreement, glutamine starvation lowered the intracellular pool of deoxynucleotides (dNTPs) (Supplementary Fig. 2b), consistent with the proliferation defect. Glutamine starvation did however not affect the intracellular pool of ribonucleotides (rNTPs) (Supplementary Fig. 2c), possibly due to an increased activity of salvage pathways or lower net rNTP consumption. (iii) Lipid synthesis: we explored if glutamine contributes to lipogenesis in ECs through reductive carboxylation<sup>43,44</sup>, but found only marginal label incorporation in M+5 citrate upon [U-<sup>13</sup>C]-glutamine incubation (Supplementary Fig. 2d). In cells with high malic enzyme activity, glutamine-derived pyruvate can also serve as a lipogenic substrate<sup>45</sup>. However, in [U-<sup>13</sup>C]-glutamine labeled ECs, <sup>13</sup>C-pyruvate and <sup>13</sup>C-lactate were nearly undetectable (Supplementary Fig. 2d). In agreement, compared to [U-<sup>14</sup>C]-labeled glucose and acetate, label incorporation from [U-<sup>14</sup>C]-glutamine into the lipid fraction was much lower (Supplementary Fig. 2e). Although a driving force for glutamine-

mediated lipogenesis in cancer cells <sup>44,46</sup>, hypoxia did not increase the incorporation of [U-<sup>14</sup>C]-glutamine derived carbon into the lipid fraction in ECs (Supplementary Fig. 2f).

**MTOR SIGNALING:** Glutamine modulates mTOR signaling <sup>47,48</sup> and endoplasmic reticulum (ER) stress <sup>30</sup>. In agreement, glutamine starvation reduced the size of ECs (Fig. 3h-i), and decreased mTOR activity (Fig. 3j). The ER can also sense amino acid deficiencies, and trigger an ER stress response <sup>49</sup>. In agreement with previous studies <sup>50</sup>, we observed that glutamine starvation induced the expression of C/EBP homologous protein (Chop) and ATF4 (Fig. 3k) and of several other ER stress marker genes, such as asparagine synthetase (ASNS), thioredoxin-interacting protein (Txnip) and tribbles pseudokinase 3 (Trib3) (Fig. 3l).

**REDOX HOMEOSTASIS:** We also examined if glutamine is critical for redox homeostasis. Glutamine deprivation impairs proliferation, in part by elevating intracellular ROS levels <sup>11</sup>. In agreement, intracellular ROS levels were elevated in glutamine-depleted ECs (Fig. 3m). Moreover, glutamine-starved ECs were more sensitive to ROS-induced cell death (Fig. 3n). In agreement, glutamine depleted ECs showed a trend towards reduced total glutathione levels (Fig. 3o).

### **ASPARAGINE SUPPLEMENTATION AND TCA CYCLE FUELING RESTORE PROLIFERATION OF GLUTAMINE-DEPRIVED ECs**

We then, unbiasedly, explored how we could rescue the EC proliferation defect induced by glutamine deprivation. Supplementing dNTPs or a nucleoside mixture failed to restore proliferation of glutamine-deprived ECs (Supplementary Fig. 3a). Replenishing the TCA cycle by adding extra carbons with cell-permeable dimethyl- $\alpha$ -ketoglutarate (DM- $\alpha$ KG, further abbreviated to  $\alpha$ KG for readability), dimethyl-succinate (DM-succinate, further abbreviated to suc), oxaloacetate (OAA) or pyruvate recovered EC proliferation for only 20-25% (Supplementary Fig. 3b). A similar modest rescue was achieved by supplementing glutamine-

starved cells with a pool of NEAAs (Supplementary Fig. 3c). Supplementation of the antioxidant N-acetyl-cysteine (NAC) or the cell permeable glutathione ethyl ester (GSH-EE) also failed to recover EC proliferation upon glutamine depletion (Supplementary Fig. 3d,e).

The above results show that restoring individual parameters of the glutamine deprivation-induced cellular alterations was insufficient to yield a complete rescue. We therefore tested combination strategies. Surprisingly, the combination of  $\alpha$ KG plus NEAAs was able to fully rescue the proliferation defect of glutamine-deprived ECs (Fig. 4a). Further sub-analysis revealed that asparagine was the only NEAA critically needed for a full rescue, when given in combination with  $\alpha$ KG (Fig. 4b). In agreement with the proliferation rescue, the  $\alpha$ KG plus asparagine combination was able to restore intracellular dNTP pools (Fig. 4c). In fact, as long as asparagine was provided, all other carbon sources capable of TCA replenishment (pyruvate, OAA, DM-succinate) were able to largely restore proliferation of glutamine-deprived ECs (Supplementary Fig. 3f). The combination of asparagine plus  $\alpha$ KG also rescued all other glutamine-dependent phenotypes, including the intracellular pool of the TCA intermediates (Fig. 4d), cell size (Fig. 4e-f), mTORC1 signaling (Fig. 4g), protein synthesis (Fig. 4h), the ER stress response (Fig. 4i), and redox imbalance (Fig. 4j,k). These data underscore the importance of asparagine and TCA replenishment in rescuing the metabolic and cellular changes of glutamine-deprived ECs.

### **ASPARAGINE ALONE PARTIALLY RESCUES GLUTAMINE-RESTRICTED EC DEFECTS**

We studied the role of asparagine in rescuing the proliferation defect of glutamine-deprived ECs in more detail. In total absence of glutamine, supplementation of asparagine alone was unable to rescue the proliferation defect (Fig. 4b, 4l). However, in low glutamine conditions (0.1 mM), supplementation of asparagine alone was capable of partially restoring EC proliferation (Fig. 4l). These data further emphasize the pivotal role of asparagine in

glutamine metabolism-restricted ECs and suggest that maintaining cellular asparagine levels in ECs is necessary to ensure cellular homeostasis.

### **ECs USE GLUTAMINE TO SYNTHESIZE ASPARAGINE**

The above data indicate that asparagine (in combination with some TCA cycle anaplerosis) is critical to rescue the cellular defects of glutamine-deprived ECs, while glutamine deprivation lowered the levels of asparagine (Fig. 3e). This connection between glutamine and asparagine raised the question whether ECs need to maintain asparagine levels for proliferation and cellular homeostasis, and whether glutamine is necessary for asparagine production. We therefore hypothesized that ECs, in glutamine-replete conditions, might be able to de novo synthesize asparagine, a reaction catalyzed by asparagine synthetase (ASNS)<sup>51</sup>, an enzyme that uses glutamine as nitrogen donor to convert aspartate into asparagine. These experiments were performed in culture medium containing a final concentration of 100  $\mu$ M asparagine (M199 medium does not contain asparagine, but it was supplemented with 20% FBS), i.e. within the range of physiological asparagine plasma levels in adults (50 to 130  $\mu$ M)<sup>52,53</sup>. ECs indeed expressed detectable levels of ASNS, especially in conditions of hypoxia or ER stress (Fig. 5a; Supplementary Fig. 3h), consistent with previous reports in other cell types. Tracing studies further revealed a high contribution (54%) of [<sup>15</sup>N<sub>2</sub>]-glutamine into asparagine (Fig. 3f), confirming that glutamine was used for asparagine synthesis by ASNS. Furthermore, ASNS<sup>KD</sup>, lowering its expression by 73% (Fig. 5a; Supplementary Fig. 3g) in the presence of glutamine reduced EC proliferation and impaired vessel sprouting (Fig. 5b-f).

We then used medium without asparagine to explore whether re-addition of extracellular asparagine could rescue the proliferation defects induced by impaired asparagine synthesis in ASNS<sup>KD</sup> ECs. Indeed, supplementation of extracellular asparagine rescued the proliferation defect induced by ASNS<sup>KD</sup> (Fig. 5f), thus showing the importance of

asparagine, regardless of whether it is de novo synthesized or taken up from the extracellular milieu. These data support the notion that under glutamine-replete conditions, proliferating ECs rely on de novo asparagine synthesis or asparagine uptake.

### **MULTIPLE MECHANISMS OF THE ASPARAGINE-MEDIATED RESCUE**

In an attempt to explore how asparagine rescued the EC defects in glutamine-deprived conditions, we studied different reported biological functions of this amino acid. (i) Consistent with the fact that asparagine is used for protein synthesis<sup>54</sup>, we noted that asparagine supplementation recovered protein synthesis by glutamine-deprived ECs (Fig. 5g). (ii) Asparagine is also known to be essential for the adaptation of cancer cells to glutamine deprivation by suppressing the ER stress response<sup>30</sup>. Consistent with this, supplying asparagine to glutamine-deprived ECs inhibited the ER stress response (Fig. 5h). (iii) Asparagine was recently identified as mTOR complex 1 (mTORC1) regulator by virtue of its role as an amino acid exchange factor<sup>29</sup>. Interestingly, supplying asparagine (but not  $\alpha$ KG) indeed reactivated mTOR signaling in glutamine-deprived ECs (Fig. 5i). In summary, while unraveling the role of glutamine metabolism in ECs, we unexpectedly found a pivotal role for glutamine-dependent asparagine synthesis in vessel sprouting.



## DISCUSSION

This study provides the following key novelties: (i) It demonstrates for the first time the role and importance of glutamine metabolism for angiogenesis in vitro and (using GLS1<sup>ECKO</sup> mice) in vivo. (ii) It unravels a critical role of glutamine as both carbon and nitrogen donor to sustain EC growth. (iii) It identifies a new mechanism in ECs of how glutamine affects vessel growth, i.e. through a previously unknown reliance on asparagine, which ECs can de novo synthesize by asparagine synthetase (ASNS) or take up from the extracellular milieu; this study also documents for first time evidence a key role of ASNS in vessel sprouting. (iv) It uncovers biological functions of asparagine in ECs, i.e. for protein synthesis, mTOR activation and suppression of glutamine deprivation-induced ER stress. And, finally, (v) it provides unprecedented evidence that pharmacological blockade of GLS1 inhibits pathological angiogenesis as a potential novel anti-angiogenic strategy.

### **GLUTAMINE REGULATES ANGIOGENESIS VIA MULTIPLE MECHANISMS**

Earlier studies documented the expression of glutaminolysis genes in ECs <sup>55,56</sup>, but did not evaluate the role of glutamine metabolism in vessel sprouting in vivo. Here, we show that glutamine is indispensable for vessel sprouting both in vitro and in mouse models of developmental and pathological angiogenesis in vivo. Like many cancer cells <sup>57</sup>, ECs too consume high amounts of extracellular glutamine, and in fact consume more glutamine than any other amino acid. Glutamine metabolism is crucial for ECs, since glutamine deprivation severely impaired EC proliferation and vessel sprouting.

Mechanistically, lack of glutamine caused multiple types of cellular defects, including reduced protein synthesis, decreased TCA anaplerosis, redox imbalance and inactivation of mTOR signaling. Together, these defects suffice to explain the impaired angiogenesis upon

glutamine deprivation in vitro or following genetic inhibition or pharmacological blockade of GLS1 in vivo. Glutamine has been previously implicated in several of these mechanisms in other cell types (mostly malignant cells) <sup>6</sup>, though never previously in ECs. When considering these mechanisms, ECs thus seemingly resemble cancer cells.

However, in sharp contrast to cancer cells, for which single treatments like replenishment of the TCA cycle <sup>58</sup> or supplementation of anti-oxidants <sup>11</sup> sufficed to rescue the phenotypes, these and other single treatments failed to rescue glutamine deprivation-induced proliferation arrest in ECs. Possibly, the genetic instability of cancer cells <sup>59</sup> might perhaps enable them to cope more flexibly with glutamine deprivation-induced cellular changes. The stronger reliance of ECs on glutamine on the other hand might create an opportunity for selective targeting of ECs in the tumor microenvironment.

Notably, glutamine metabolism was dispensable for energy homeostasis, as glutamine starvation did not affect ATP levels or the energy charge. This should however not be surprising, as ECs rely primarily on glycolysis to generate >85% of their ATP <sup>2</sup>.

Interestingly, chimeric EC spheroid assays revealed that GLS1 promoted tip cell competitiveness. This is likely attributable to the role of GLS1 in EC migration, as high motility is essential for ECs to reach the tip position in the vascular sprout <sup>10,60</sup>. While the precise mechanisms of how glutamine metabolism regulates EC motility remain to be defined, circumstantial evidence provides a link to cellular migration. Indeed, glutamine metabolism regulates migration of non-EC types <sup>61</sup>, promotes cancer cell migration and dissemination by altering signaling pathways <sup>62-64</sup>, is essential for the tip cell motility machinery <sup>65</sup>, and regulates adhesion receptors <sup>66</sup>. Furthermore, through regulating redox homeostasis, glutamine metabolism can also affect cell migration <sup>11,67</sup>. Of note, glutamine metabolism was less important for other endothelial functions, such as regulation of vascular tone, inflammation or differentiation.

## **GLUTAMINE-DEPENDENT ANGIOGENESIS – NOVEL LINK TO ASPARAGINE**

The combination treatment of asparagine with an anaplerotic carbon donor rescued the defects of glutamine-deprived ECs. Under low glutamine conditions, asparagine mono-treatment could even partially rescue the EC defects, implying that maintaining asparagine levels is crucial for ECs to sustain growth and homeostasis. The finding that asparagine can rescue glutamine-low phenotypes suggests that asparagine is downstream of glutamine's activity in ECs. Indeed, tracing experiments showed that glutamine-derived carbon and nitrogen are incorporated into asparagine in ECs, consistent with previous studies in other cell types. To maintain asparagine levels, ECs can rely on endogenous de novo synthesis by ASNS, or alternatively, they can take up extracellular asparagine. Hence, ECs are equipped with mechanisms to take up asparagine when available, or to synthesize it themselves in nutrient-limited environments, such as occurs when ECs migrate into avascular regions or in the tumor setting.

ECs may additionally rely on ASNS in conditions, when ASNS expression levels are elevated (upon glucose or amino acid deprivation, or ER stress) or when asparagine levels in the extracellular milieu are low (in certain cancers, or upon protein limitation, unbalanced amino acid intake, asparaginase treatment, etc) <sup>20-23,68-70</sup>. In fact, asparaginase treatment of neonatal pups, which depleted asparagine without affecting glutamine levels in the blood (Supplemental Figure 4a), did not impair retinal angiogenesis (Supplemental Figure 4b-f), possibly suggesting that endothelial ASNS provided the necessary asparagine to mediate glutamine's role in angiogenesis. These findings provide the first evidence for a role of ASNS and asparagine downstream of glutamine metabolism in angiogenesis.

## **ASPARAGINE – COORDINATING EC HOMEOSTASIS WITH METABOLIC RESERVES?**

Mechanistically, upon glutamine deprivation, asparagine treatment restored protein synthesis, suppressed the ER stress response, and reactivated mTOR signaling. Of note, when cancer cells use asparagine to exchange amino acids for mTOR activation, they have a net asparagine efflux<sup>29</sup>. However, the finding that ECs consumed asparagine and thus lowered asparagine levels in the culture medium suggests that ECs did not activate mTOR signaling via this exchange mechanism.

Emerging evidence in cancer and transformed cells indicates that intracellular asparagine has a more important role in regulating cell survival than previously expected<sup>28,29</sup>, but it remained unknown whether this is also the case for healthy non-transformed cells. In cancer cells, ASNS levels correlated with c-Myc amplification and were the highest in dedifferentiated brain cancer cells, when progressing from adenoma to carcinoma. Given that: (i) cancer cells rewire their metabolism differently from healthy non-transformed cell types; (ii) the cancer cell genotype is mutated (unlike healthy non-transformed cells) and thus may rewire metabolism differently; and (iii) EC metabolism differs from that of cancer and immune cells<sup>4</sup>, metabolic concepts in cancer cells cannot be simply extrapolated to normal healthy cells. In addition, glutamine depletion of cancer cells increased apoptosis, while only modestly affecting proliferation and no effects on mTOR signaling, though this finding is contextual. In cancer cells, asparagine suppressed glutamine deprivation-induced cancer cell apoptosis, but not proliferation. In contrast, we show in our study that asparagine plus  $\alpha$ KG rescues the proliferation defect and all other glutamine-dependent phenotypes, including the intracellular pool of the TCA intermediates, cell size, mTORC1 signaling, protein synthesis, the ER stress response, and redox imbalance.

Even though asparagine has been only documented in protein synthesis<sup>54</sup>, unlike the other 19 common amino acids, increasing evidence suggests alternative roles for this amino acid as a metabolite that coordinates cellular homeostatic responses with metabolic fuel

reserves. Since intracellular asparagine levels are the lowest of the non-essential amino acids in proliferating cells <sup>30</sup>, and asparagine amination exclusively relies on glutamine, asparagine synthesis has been proposed to be a rheostat, sensing the availability of TCA cycle intermediates and supply of reduced nitrogen to maintain non-essential amino acid synthesis. In line, carbohydrate and amino acid starvation may trigger asparagine biosynthesis, because asparagine is an indicator of insufficient substrate for continued cell division. This notion is further supported by findings that other amino acid synthesis pathways are not as highly regulated and other amino acids do not appear to play as critical a role. Of note, it has been proposed that because the carbon to nitrogen ratio of asparagine is less than that for glutamine, plants switch to asparagine as the primary nitrogen transporter and storage molecule to spare carbon during carbohydrate starvation <sup>71</sup>. Whether mammalian ECs can also rely on asparagine for such purpose, or utilize this amino acid as a signaling metabolite, is an exciting but outstanding question that requires further study.

## **POSSIBLE THERAPEUTIC IMPLICATIONS**

Even though future research is warranted to assess effects on angiogenesis in other organs, pharmacological blockade of GLS1 reduced pathological ocular angiogenesis, suggesting an alternative therapeutic strategy for diseases caused by excess vessel growth. Furthermore, the different enzymes involved in the asparagine-glutamine interplay can potentially represent novel anti-angiogenic targets. As such, our in vitro data show how silencing ASNS inhibits EC sprouting. In this respect, inhibiting intracellular asparagine production by blocking ASNS in ECs in combination with depleting extracellular asparagine levels (by asparaginase treatment) may offer novel therapeutic opportunities for anti-angiogenesis in pathological conditions, as was suggested for cancer cells. This might be attractive for inhibiting tumor angiogenesis, given that an increasing number of studies documented that asparaginase treatment, in combination with GLS1 or autophagy inhibition, is also effective in slowing down

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solid tumor growth. Hence, blocking GLS1 or ASNS merit further attention as alternative strategies to inhibit pathological angiogenesis.

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**CONFLICT OF INTERESTS:** The authors declare that they have no conflict of interest.

## EXPERIMENTAL PROCEDURES

### CHEMICALS AND REAGENTS

Collagen type 1 (rat tail) and nucleoside mix (100 X) were from Merck-Millipore. The GLS1 inhibitor CB-839 was kindly provided by Calithera BioSciences (South San Francisco, CA, USA). dNTP mix, all amino acids, pyruvate, oxaloacetate, glutathione reduced ethyl ester, dimethyl- $\alpha$ -ketoglutarate (DM- $\alpha$ KG, this is the cell-permeable form, which was used for supplementation / rescue experiments and which we further abbreviated to  $\alpha$ KG in the text and figures for reasons of readability), dimethyl-succinate (DM-Succinate), thapsigargin, tamoxifen and asparaginase were purchased from Sigma-Aldrich. Non-Essential Amino Acids Solution (NEAA, 100X) was from ThermoFisher Scientific. [ $^3$ H]-Thymidine, [ $^3$ H]-tyrosine, [U- $^{14}$ C]-L-glutamine, [U- $^{14}$ C]-acetate, [U- $^{14}$ C]-L-glucose were from Perkin Elmer. [U- $^{13}$ C]-L-glutamine, [U- $^{13}$ C]-D-glucose and [ $^{15}$ N $_2$ ]-L-glutamine, were purchased from Cambridge Isotope Laboratories.

The following primary antibodies or dyes were used: isolectin Griffonia simplicifolia (GS)-IB4-Alexa 488, isolectin GS-IB4-Alexa 568, isolectin GS-IB4-Alexa 647 (Molecular Probes), anti-phH3 (Merck-Millipore), anti-NG2 (Chemicon), anti-4EBP1, anti-p-S6 (S235/236), anti-S6, anti-Chop, anti-ATF4, anti-caspase-3 (Cell Signaling Technology), anti- $\beta$ -Actin, secondary Alexa-488, -568 or -633 conjugated antibodies were from Molecular Probes.

### CELL CULTURE

Freshly isolated (protocol as described previously <sup>72</sup>) human umbilical vein endothelial cells (ECs) were cultured in M199 medium (Invitrogen) containing 20% FBS, 1 mg/ml D-glucose, heparin and Penicillin/Streptomycin (10 U/ml) and endothelial cell growth factor supplements (ECGS; 30 mg/l). As specified below under 'Glutamine deprivation assay and rescue', a



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specific set of experiments required the use of glutamine-free M199 supplemented with 20% dialyzed serum. The ECs were isolated and used with approval of the Medical ethical commission of KU Leuven/University hospital Leuven (file S57123), and with informed consent obtained from all subjects. ECs were regularly tested for mycoplasma and only used between passages 1 and 5. ECs were kept at 37°C and 5% CO<sub>2</sub> and the growth medium was changed at least every other day. Hypoxia incubation of ECs was at 1% oxygen for 18 hrs and thapsigargin-treatment was at 1 μM for 18 hrs. When needed, ECs were grown to quiescence following a previously established model of contact inhibition<sup>2</sup>. In brief, ECs were seeded at an initial density of 15,000 cells/cm<sup>2</sup> and were allowed to grow to a fully contact-inhibited, quiescent monolayer for 6-7 days with regular medium changes. To obtain a corresponding proliferative control, contact inhibited cells (at day 6) were trypsinized and cultured in growth medium for at least 36 hours to re-initiate proliferation. HEK293T cells (ATCC) were grown in DMEM, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. For stimulation with an inflammatory cue, ECs were treated with IL-1β (1ng/ml, ThermoFisher, 10139HNAE25) for 2 hrs (37°C, 5% CO<sub>2</sub>) prior to downstream experiments. Mouse liver endothelial cells (mECs) were isolated from healthy livers of 8 to 10 week-old control or GLS1<sup>ECKO</sup> mice. Prior to isolation, GLS1<sup>ECKO</sup> mice (see further below) and their control littermates were treated with tamoxifen (30 mg/kg) for 5 consecutive days to delete GLS1 from the endothelium. Mice were anesthetized with Nembutal (60 mg/kg) and were then perfused with 5 ml of a water based perfusion buffer containing 1.7 M NaCl, 84 mM KCl, 120 mM HEPES and 1 mM NaOH followed by 5 ml of a PBS-based digestion buffer containing 0.1% collagenase II, collagenase I, 2 mM CaCl<sub>2</sub>, 1% antibiotic-antimycotic (Thermo Fisher Scientific) and 10% FBS (Biochrome) at a perfusion rate of 1 ml/min. Perfusion was considered complete when the liver and mesenteric vessels

were blanched and the desired amount of digestion buffer ( $\geq 5$  ml) had passed through the circulatory system. Livers were dissected, placed into a 50 ml conical tube with 3 ml of digestion buffer and incubated at 37°C for approximately 30 min, with regular shaking. After digestion, the tissue was homogeneously dissociated and the reaction was stopped with 10 ml of isolation buffer containing PBS plus 0.1% BSA (Sigma-Aldrich). Subsequently, the cell suspension was filtered through a 100  $\mu$ m cell strainer and cells were washed twice with isolation buffer. Finally, the ECs were isolated by magnetic bead sorting with Dynabeads (CELLlection™ Biotin Binder Kit, Thermo Fisher Scientific) coated with anti-mouse CD31 (eBioscience, Anti-Mouse CD31 Clone 390), according to the manufacturer's instructions. Cells recovered from beads isolation were resuspended in M199 medium and plated at the desired density on cell culture plates pre-coated with 0.1% gelatin.

### **KNOCK-DOWN AND OVEREXPRESSION STRATEGIES**

Lentiviral overexpression constructs for GLS2 were created by cloning the cDNA into pRRLsinPPT.CMV.MCS MM WPRE vector <sup>73</sup>. shRNA vectors against GLS1 (TRCN0000051135, TRCN0000051136), ASNS (TRCN0000290113, TRCN0000290105), were purchased from Sigma. Lentiviruses were produced by transfecting 293T cells as previously described <sup>74</sup>. A nonsense scrambled shRNA sequence was used as a negative control. Cells were transduced overnight at a multiplicity of infection (MOI) of 10, which was kept for all shRNA and overexpression experiments. Transduced cells were used in functional assays at least 3 to 4 days post-transduction.

### **RNA ISOLATION AND GENE EXPRESSION ANALYSIS**

RNA was extracted from the lysate using the PureLink RNA Mini Kit (Ambion), according to the manufacturer's protocol. The concentration and purity of the extracted RNA was

measured using the Nanodrop 2000 (Thermo Scientific). RNA samples were stored at  $-80^{\circ}\text{C}$  or immediately converted to cDNA using the iScript cDNA Synthesis Kit (Biorad), according to the manufacturer's instructions. An Applied Biosystems 7500 Fast device with in house-designed primers and probes or premade primer sets (Applied Biosystems or Integrated DNA Technologies) (sequences and/or primer set ID numbers available upon request) was used for quantitative RT-PCR analyses. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a housekeeping gene. For comparison between GLS1 and GLS2, absolute expression levels were determined based on respective cDNA standard curves, and levels are expressed as copies mRNA per  $10^3$  copies HPRT mRNA.

## **IMMUNOBLOT ANALYSIS**

Extraction of protein was performed in RIPA buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), supplemented with protease and phosphatase inhibitor mixes (Roche Applied Science) and followed by shearing genomic DNA. Lysates were then separated by SDS-PAGE under reducing conditions, transferred to a nitrocellulose or PVDF membrane, and analyzed by immunoblotting. Signal was detected using the ECL system (Amersham Biosciences, GE Healthcare) or SuperSignal Femto Western blotting substrate (Thermo Fisher Scientific) according to the manufacturer's instructions.

## **IN VITRO ASSAYS**

Measurement of amino acid consumption or secretion rate: Cells were initially seeded in M199 full medium. After 24 hrs, cells were re-fed with fresh full medium (T0) and some of the T0 medium was kept for assessment of initial amino acid concentrations (Supplemental Fig. 1a). ECs were then allowed to grow for 48 hrs prior to medium sampling (T48). Medium from T0 and T48 were subjected to LC-MS analysis (see below) and the consumption or secretion

rates were calculated accordingly. Please note that although there is no asparagine in M199 medium, supplementation of 20% normal FBS brings asparagine levels to around 100 $\mu$ M.

[<sup>3</sup>H]-thymidine incorporation: Proliferation was measured by incubating cells with 1  $\mu$ Ci/ml [<sup>3</sup>H]-thymidine for 2 hrs. Thereafter, cells were fixed with 100% ethanol for 15 min at 4°C, precipitated with 10% trichloroacetic acid (TCA) and lysed with 0.1 N NaOH. The amount of [<sup>3</sup>H]-thymidine incorporated into DNA was measured by scintillation counting and corrected for cell number.

[<sup>3</sup>H]-tyrosine incorporation: to determine protein synthesis rate, cells were incubated with medium containing 1  $\mu$ Ci/ml [<sup>3</sup>H]-tyrosine for 6 hrs. Thereafter, medium was removed and cells were washed with ice cold PBS. After protein precipitation with 10% TCA for more than 1 hour, cells were transferred to an eppendorf tube. Subsequently, the protein pellet was collected by a centrifugation at 15,000 rpm for 15 min. Finally, the protein pellet was dissolved in 0.5 M NaOH and 0.1% Triton X. The amount of [<sup>3</sup>H]-tyrosine incorporated into protein was measured by scintillation counting and corrected for protein content.

Spheroid sprouting assay: ECs were incubated overnight in hanging drops in M199 medium containing 20% methylcellulose (Sigma-Aldrich) to form spheroids. Then, they were embedded in collagen gel as described <sup>75</sup> and cultured for 24 hrs to allow sprouting. Finally, spheroids were fixed with 4% PFA at room temperature and imaged under bright field using a Motic AE 31 microscope (Motic Electric Group Co Ltd.) or a Leica DMI6000 microscope (Leica Microsystems). Analysis of the number of sprouts per spheroid and the total sprout length (cumulative length of primary sprouts and branches per spheroid) was done on phase contrast images using the NIH Image J software package.

Mosaic spheroids for tip cell competition were generated as indicated above, and consisted of a 1/1 mixture of GFP<sup>+</sup>-GLS1<sup>KD</sup> ECs and mCherry<sup>+</sup>-wt ECs. Spheroids consisting of a 1/1 mixture of GFP<sup>+</sup>-wt ECs and mCherry<sup>+</sup>-wt ECs were used as controls in this set-up. Tip cell competition was assessed by manual counting of the number of red versus green ECs at the tip of individual sprouts per spheroid. Control spheroids yielded a near 50/50% distribution at the tip position, indicating

that overexpression of GFP versus mCherry by itself has no effect on tip cell competition.

Leukocyte adhesion assay: Whole blood from healthy human volunteers was collected and anticoagulated with K<sub>2</sub>EDTA (1.8 mg/ml, using plastic whole blood spray-coated K<sub>2</sub>EDTA tubes, Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation over Ficol-paque plus (GE healthcare). PBMCs were labeled with Calcein (cell-permeant dye, ThermoFisher Scientific). ECs (plated 5 days before ( $7.5 \times 10^4$  cells/well, 12-well plate) to reach a confluent, contact-inhibited, quiescent monolayer) were washed with PBS and incubated either with vehicle (sterile PBS) or CB-839 (100 nM), overnight (37°C, 5% CO<sub>2</sub>). After this period, medium was removed and ECs were washed with PBS. The mononuclear cells were added ( $5 \times 10^5$ /well) and incubated for 60 min (37°C, 5% CO<sub>2</sub>). Non-adherent cells were removed by washing 5 times with PBS and cells were fixed using 4% PFA. Five fields per well, randomly chosen, were analyzed and the number of adherent leukocytes per field was determined using a Leica DMI6000B microscope (magnification 20X).

Scratch wound migration assay: At T<sub>0</sub>, a scratch was made in confluent EC monolayers using a sterile 200 µl pipette tip and cells were further incubated for 8 hrs (T<sub>8</sub>). Wound closure (gap area at T<sub>0</sub> minus gap area at T<sub>8</sub>) was measured with NIH Image J software.

Glutamine deprivation assay and rescue: Cells were initially seeded in full medium for 1 day. Cells were re-fed with glutamine-deprived medium (Thermo Fisher Scientific) with 20% dialyzed FBS (Thermo Fisher Scientific), 1 mg/ml D-glucose, heparin and Penicillin/Streptomycin (10 U/ml) and endothelial cell growth factor supplements (ECGS; 30 mg/l) for 24 hours prior to the start of experiments. For all the rescue experiments, metabolites were added at the time of refeeding cells with glutamine-deprived medium; asparagine was added at 2 mM, DM- $\alpha$ KG at 1 mM, NEAA mix at 1 mM (final concentration for each amino acid), individual NEAAs at 5 mM (some of the NEAAs required heating the medium to 37°C for optimal solubility and required readjustment of the pH after adding), pyruvate at 5 mM, mono-Methyl hydrogen succinate at 5 mM, oxaloacetate at 4 mM, N-acetylcysteine at 10 mM and

glutathione reduced ethyl ester at 1 mM. Cell number counting was used as a measure of proliferation in glutamine-deprived cells when treated with dNTPs (500  $\mu$ M) or nucleoside mix (10X); [ $^3$ H]-thymidine incorporation can not be used as a measure for proliferation here as addition of dNTPs and NSX dilute the [ $^3$ H]-thymidine pool. dNTPs and nucleoside mix were added at the same time as glutamine deprivation was initiated. The absolute cell number was counted every 24 hrs using a haemocytometer. Bicinchoninic acid (BCA) assay (Pierce) was used to determine protein content following manufacturer's guidelines. Intracellular reactive oxygen species (ROS) detection: 10  $\mu$ M 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF) dye (Thermo Fisher Scientific) was added into cell culture medium for 30 min. After washing with PBS, cells were incubated in fresh culture medium for 30 min. Finally, CM-DCF fluorescence was measured with a plate reader and normalized to protein content. Energy charge assessment:  $5 \times 10^5$  cells were collected in 100  $\mu$ l ice cold 0.4 M perchloric acid containing 0.5 mM EDTA. The protein pellet was removed by centrifugation at 15,000 rpm for 15 min. The pH of the supernatant was adjusted with 100  $\mu$ l of 2 M  $K_2CO_3$ . 100  $\mu$ l of the mixture was subsequently injected onto an Agilent 1260 HPLC with a C18-Symmetry column (150 x 4.6 mm; 5 mm; Waters), thermostated at 22.5  $^{\circ}C$ . Flow rate was kept constant at 1 ml/min. A linear gradient using solvent A (50 mM  $NaH_2PO_4$ , 4 mM tetrabutylammonium, adjusted to pH 5.0 with  $H_2SO_4$ ) and solvent B (50 mM  $NaH_2PO_4$ , 4 mM tetrabutylammonium, 30%  $CH_3CN$ , adjusted to pH 5.0 with  $H_2SO_4$ ) was accomplished as follows: 95% A for 2 min, from 2 to 25 min linear increase to 100% B, from 25 to 27 min isocratic at 100% B, from 27 to 29 min linear gradient to 95% A and finally from 29 to 35 min at 95% A. ATP, ADP and AMP were detected at 259 nm. The energy charge is calculated as  $([ATP] + 1/2 [ADP]) / ([ATP] + [ADP] + [AMP])$ . Flow cytometry:  $5 \times 10^5$  cells were collected and resuspended in 500  $\mu$ l PBS prior to analysis by fluorescence-activated cell sorting (FACS) (BD FACS Verse). To measure cell size, the voltages were adjusted to have the mean forward light scatter (FSC) of the control population at a value of 100000. These

voltages were kept constant for all samples. FlowJo analysis software (FlowJo, LLC) was used to determine the mean FSC value as a measure for cell size. Annexin V/PI apoptosis measurement: Cells were collected by trypsinization and resuspended in binding buffer containing 10mM HEPES, 140 mM NaCl, 2.5M CaCl<sub>2</sub> (pH = 7.4). After counting, Annexin V antibody and PI were added at RT in the dark for 15 min. prior to acquisition on a BD FACS Verse. Data were analyzed with FlowJo analysis software. LDH release assay (cytotoxicity): Cell death was measured by determining LDH release in the medium with the Cytotoxicity Detection Kit (Roche Applied Sciences) following manufacturer's instructions. Nuclear DNA degradation: Cells were seeded in 24-well plates and treated with the indicated doses of CB-839 for 24 hrs. Treatment with doxorubicin at 1µg/ml (final concentration) for 24 hrs was used as a positive control. Then, cells were trypsinized, subjected to a centrifuge spin, and resuspended in PBS. From this suspension, aliquots were deposited on slides with a cytospin centrifuge. For morphological analysis, cells were fixed with ice-cold methanol for 10 min and washed twice with PBS. Cells were then stained with TUNEL (Roche Therapeutics) according to the manufacturer's conditions. Next, Hoechst 33258 (1/1000 in PBS) was put on the cells for 10 min at room temperature and mounted with Pro-Long Gold. Nuclei were imaged on a Leica fluorescence microscope. H<sub>2</sub>O<sub>2</sub> challenge assay: Cells were treated with the indicated amounts of H<sub>2</sub>O<sub>2</sub> for 2 hrs. Bright field pictures were taken with a Leica DMI6000 microscope (Leica Microsystems, Mannheim, Germany). Glutamine oxidation assay: Glutamine oxidation was measured as <sup>14</sup>CO<sub>2</sub> formation as previously described <sup>2</sup>. In brief, cells were grown for 6 hrs in medium containing [U-<sup>14</sup>C]-glutamine (0.5 µCi/ml) (Perkin Elmer). Perchloric acid (HClO<sub>4</sub>) (2M) was added to lyse the cells and to promote the release of CO<sub>2</sub>. Immediately after adding perchloric acid, the wells were covered with a hyamine hydroxide-saturated Whatman filter paper. After overnight absorption at room temperature, the paper was transferred to scintillation vials for liquid scintillation counting. Glutamine uptake assay: Cells were incubated with medium containing 0.5 µCi/ml [U-<sup>14</sup>C]-L-glutamine for 30 min after which

they were washed at least five times with ice-cold PBS. The last PBS wash was collected and checked for residual radioactivity. Cells were then lysed with 0.2 N NaOH and lysates were used for scintillation counting. <sup>14</sup>C-glutamine, glucose and acetate incorporation into lipid fraction: Cells were incubated with medium containing 0.5  $\mu\text{Ci/ml}$  [ $U\text{-}^{14}\text{C}$ ]-L-glutamine, 0.5  $\mu\text{Ci/ml}$  [ $U\text{-}^{14}\text{C}$ ]-L-glucose or 0.5  $\mu\text{Ci/ml}$  [ $U\text{-}^{14}\text{C}$ ]-L-acetate for 24 hrs after which lipid fractions were prepared by a two phase methanol-water-chloroform extraction method. In brief, cells were washed at least three times with ice-cold PBS followed by a protein precipitation step by adding 800  $\mu\text{l}$  methanol: water (5:3) solution. Cells were scratched with rubber policeman and the lysates were transferred to fresh eppendorf tubes; 500  $\mu\text{l}$  chloroform was added subsequently. After vortexing, the mixture was centrifuged at 15,000 rpm for 15 min to form an upper polar phase and a lower non-polar (lipid) phase. The percentage of <sup>14</sup>C-glucose, -glutamine or -acetate into lipid fraction was calculated as counts per minute (cpm) in non-polar fraction divided by cpm in whole cell extract. To compare the incorporation of glutamine carbon in normoxia versus hypoxia, a similar method was performed except that cells were cultured in 0.5% oxygen for 1 day during the phase of tracer incubation.

## LC-MS ANALYSIS

For all stable isotope labeling experiments, cells were incubated for 48 hrs with labeled substrates in a 100% labeling way (glucose: 5.5 mM; glutamine: 2 mM). Metabolites from  $5 \times 10^5$  cells were extracted in 150  $\mu\text{l}$  methanol-acetonitrile-water (50:30:20) solution. Next the extracts were centrifuged at 4°C for 15 min at 20,000 x g and the supernatants were used for LC-MS. Measurements were performed using a Dionex UltiMate 3000 LC System (Thermo Scientific) in-line connected to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific). 40  $\mu\text{l}$  of sample was injected and loaded onto a SeQuant ZIC/pHILIC Polymeric column (Merck Millipore). A linear gradient was carried out starting with 84% solvent A (95:5 acetonitrile- $\text{H}_2\text{O}$ , 2 mM ammoniumacetate pH 9.3) and 16% solvent B (2 mM



ammoniumacetate pH 9.3). From 2 to 29 min the gradient changed to 75% B and was kept at 75% until 34 min. Next a decrease to 16% B was carried out to 42 min and then 16% B was maintained until 58 min. The solvent was used at a flow rate of 100  $\mu$ l/min and the column temperature was kept constant at 25  $^{\circ}$ C. The mass spectrometer operated in negative ion mode; settings of the HESI probe were as follows: sheath gas flow rate at 25, auxiliary gas flow rate at 5 (at a temperature of 260  $^{\circ}$ C). Spray voltage was set at 4.8 kV, temperature of the capillary at 300  $^{\circ}$ C and S-lens RF level at 50. Normal full scan (resolution of 35,000 and scan range of m/z 50-1050) was applied. In case of low metabolite levels (such as dNTPs), a targeted Selected Ion Monitoring (SIM) method was applied. Data collection (total metabolite abundances as well as  $^{13}$ C or  $^{15}$ N isotopic incorporation) was performed with the Xcalibur software (Thermo Scientific). For calculation of the total carbon/nitrogen contribution, we corrected for naturally occurring isotopes using the method of Fernandez et al.<sup>76</sup> For relative metabolite levels, the total ion count was normalized to the protein content. The total contribution of carbon/nitrogen was calculated using the following equation<sup>77</sup>:

$$\text{total contribution of carbon/nitrogen} = \sum_{i=0}^n i \times m_i / \left( n \sum_{i=0}^n m_i \right)$$

where n is the number of C or N atoms in the metabolite, i represents the different mass-isotopomers and m refers to the abundance of a certain mass.

## GC-MS ANALYSIS

For GC-MS analyses  $5 \times 10^5$  cells were extracted in 800  $\mu$ l 80% methanol followed by centrifugation at 4  $^{\circ}$ C for 15 min at 20,000  $\times$  g; supernatants were dried in a vacuum centrifuge. 25  $\mu$ l of a 2% methoxyamine hydrochloride solution (20 mg dissolved in 1 ml pyridine) was added to the dried fractions which were then incubated at 37  $^{\circ}$ C for 90 min. Then 75  $\mu$ l of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% N-tert-

butyldimethyl-chlorosilane (Sigma-Aldrich) was added and the reaction was carried out for 30 min at 60 °C. Reaction mixtures were centrifuged for 15 min at 20,000 x g at 4 °C in order to remove insolubilities, the supernatant was transferred to a glass vial with conical insert (Agilent). GC-MS analyses were performed on an Agilent 7890A GC equipped with a HP-5 ms 5% Phenyl Methyl Silox (30 m - 0.25 mm i.d. - 0.25 µm; Agilent Technologies) capillary column, interfaced with a triple quadrupole tandem mass spectrometer (Agilent 7000B, Agilent Technologies) operating under ionization by electron impact at 70 eV. The injection port, interface and ion source temperatures were kept at 230 °C. Temperature of the quadrupoles was kept at 150 °C. The injection volume was 1 µl, and samples were injected at 1:10 split ratio. Helium flow was kept constant at 1 ml/min. The temperature of the column started at 100 °C for 5 min and increased to 260 °C at 2 °C/min. Next, a 40 °C/min gradient was carried out until temp reached 300 °C. After the gradient, the column was heated for another 3 min at 325 °C. The GC-MS analyses were performed in Selected Ion Monitoring (SIM) scanning for the isotopic pattern of metabolites.

## **EX VIVO MODELS**

Aortic ring relaxation: Vasomotor function was assessed in segments of thoracic aorta as previously described <sup>78</sup>. Segments were mounted in an organ bath (Panlab; ADInstruments Ltd., UK) containing Krebs Henseleit buffer and equilibrated at a resting tension of 3g for 2 hrs in the presence of CB-839 (1 µM) or vehicle control. Relaxation responses to cumulative addition of acetylcholine (1 nM – 10 µM) and then sodium nitroprusside (0.1 nM – 1 µM) were performed after pre-constriction with phenylephrine (which dose ??), and responses expressed as percentage decrement in pre-constricted tension.

## IN VIVO MODELS

GLS1<sup>ECKO</sup> MICE: To obtain inducible EC-specific GLS1 knock-out mice, GLS1<sup>lox/lox</sup> mice<sup>79</sup> were intercrossed with Pdgfb-Cre<sup>ERT2</sup> mice<sup>35</sup>. Neonatal retinal angiogenesis: EC-specific GLS1 deletion was achieved by intraperitoneal (i.p.) administration of tamoxifen (Sigma; 10 mg/kg; dissolved in 1:10 EtOH:oil solution) once at P2. At P5, eyes were enucleated, fixed with 2% PFA for 2 hrs. Isolectin-B4 (IB4), pH3 and NG2 stainings were performed as previously described<sup>2,4,80</sup>. Radial outgrowth, branching points, number of distal sprouts, pericyte coverage (NG2<sup>+</sup> area) and pH3<sup>+</sup> cell numbers per vessel area were analyzed using the NIH Image J software package and the AxioVision morphometric analysis software (Carl Zeiss, Munich, Germany) with in-house developed macros. Oxygen induced retinopathy: Oxygen induced retinopathy (ROP)<sup>37</sup> was induced by exposing C57BL/6 pups to 70% oxygen from P7-P12. At P13, pups were placed back in normoxia and injected daily with 40 mg/kg CB-839 or vehicle. At P17, pups were euthanized and eyes were enucleated, fixed in 4% PFA and retinal flat mounts were stained with isolectin-B4 as described<sup>2,4</sup>. Mosaic images were captured using the inverted Leica DMI6000B epifluorescence microscope (Leica, Mannheim, Germany) and analysis of the vascular tuft area was performed with NIH Image J software. Asparaginase treatment: Newborn pups were IP-injected with 2U/g/day asparaginase for 4 consecutive days (P1-P4). Retinas were isolated at P4 as described above. Prior randomization was not applicable for any of the above mouse models given that all animal treatments were done in baseline conditions. No statistical methods were used to predetermine the sample size. All animal procedures were approved by the Institutional Animal Care and Research Advisory Committee of the University of Leuven.

## STATISTICS

Data represent mean  $\pm$  SEM of pooled experiments, while “n” values represent the number of independent experiments performed or the number of individual mice phenotyped. All

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experiments with freshly isolated ECs were performed at least three times using different donors. Statistical significance between groups was calculated with two-tailed unpaired t-test. Sample size for each experiment was not pre-determined. For Fig 1a, a one-sample t-test versus a hypothetical value of 0 was used; for Fig. 2s,w, the same test was used versus a hypothetical value of 1. All statistical analyses were performed with GraphPad Prism. A p-value  $<0.05$  was considered significant.

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## FIGURE LEGENDS

### FIGURE 1: GLUTAMINE METABOLISM SUSTAINS EC PROLIFERATION

**a**, Quantification of amino acid consumption or secretion rate (values below zero indicate consumption, values above zero indicate secretion). **b-e**, Representative images of EC spheroids sprouting under control (b) and glutamine-free conditions (c), and corresponding quantification of total sprout length (d) and number of sprouts per spheroid (e). **f**, [<sup>3</sup>H]-Thymidine incorporation in DNA in ECs at different doses of extracellular glutamine. **g**, Percentage of wound closure in control and glutamine-deprived ECs in monolayer scratch assays. **h-i**, Quantification of glutamine uptake (h) and glutamine oxidation (i) in quiescent (Q) versus proliferating (P) ECs. **j-m**, Representative images of control (j) and GLS1<sup>KD</sup> (k) EC spheroids, and quantification of total sprout length (l) and number of sprouts per spheroid (m). **n**, [<sup>3</sup>H]-Thymidine incorporation in DNA in control and GLS1<sup>KD</sup> ECs. **o**, Percentage of wound closure in control and GLS1<sup>KD</sup> ECs in scratch assays. **p**, [<sup>3</sup>H]-Thymidine incorporation in ECs treated with increasing doses of the GLS1 inhibitor CB-839. **q**, Annexin V<sup>+</sup> ECs (relative to control) after treatment with different doses of CB-839. **r**, Percent cell death (as measured by LDH release) in ECs treated with different doses of CB-839. **s**, [<sup>3</sup>H]-Thymidine incorporation in DNA in mouse liver ECs isolated from GLS1<sup>ECKO</sup> mice and their corresponding wild type littermates (n=3 for both genotypes). All data are mean ± SEM from at least three independent experiments, each performed with ECs derived from a different individual donor. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001, n.s., not significant versus corresponding control. Ala, alanine; Asn, asparagine; Asp, aspartate; Arg, arginine; Gln, glutamine; Glu, glutamate; Gly, glycine; Lys, lysine; His, histidine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Q, quiescent; P, proliferating; WT, wild type. Scale bars in b, c, j and k are 100 μm.

**FIGURE 2: GLS1 INHIBITION CAUSES SPROUTING DEFECTS IN RETINAL ANGIOGENESIS**

**a,b**, Representative pictures from isolectin-B4 (IB4)-stained retinal vascular plexi obtained from wild type (a) or GLS1<sup>ECKO</sup> (b) mice at P5. **c-e**, Quantification of branch points at the front (c) or rear (d), and radial expansion (e) of the retinal vascular plexus in wild type and GLS1<sup>ECKO</sup> animals (n=9). **f,g**, Representative pictures of IB4 / phospho-histone 3 (phH3) double stained wild type (f) and GLS1<sup>ECKO</sup> (g) retinal vasculature at P5. **h**, Quantification of phH3<sup>+</sup> ECs in the retinal vascular plexus of wild type and GLS1<sup>ECKO</sup> mice (n=6). **i**, Quantification of distal sprouts with filopodia in the retinal vasculature of wild type and GLS1<sup>ECKO</sup> mice at P5 (n=7). **j-l**, Quantification of pericyte coverage (NG2<sup>+</sup> area in % of isolectin-B4<sup>+</sup> area) (n=6) (j), and corresponding representative pictures of IB4 / NG2 (pericyte marker) double staining of P5 wild type (k) and GLS1<sup>ECKO</sup> (l) retinal vascular plexi. **m-o**, Representative images of retinal flat-mounts of retinopathy of prematurity (ROP) mice treated with vehicle (m) or CB-839 (n) (red arrowheads indicate vascular tufts) and corresponding quantification of vascular tuft area (o) (n=8). **p-r**, Representative pictures of mosaic spheroids consisting of a 1/1 mixture of mCherry<sup>+</sup>-control (red) and GFP<sup>+</sup>-control (green) ECs (p) or a 1/1 mixture of mCherry<sup>+</sup>-control (red) and GFP<sup>+</sup>-GLS1<sup>KD</sup> (green) ECs (q) and per spheroid quantification of percentages of red versus green ECs occupying the tip cell position (r) (n=3). **s**, fold-changes in mRNA level of eNOS and endothelin-1 in GLS1<sup>KD</sup> and CB-839-treated ECs (n=4). **t,u**, Relaxation of aortic rings pretreated with vehicle or CB-839 in response to acetylcholine (t) and in response to the nitric oxide donor nitroprusside (u) (n=8). **v**, fold-changes in mRNA levels of VCAM and E-selectin in GLS1<sup>KD</sup> and CB-839-treated ECs under IL-1 $\beta$  stimulation (n=4). **w**, Quantification of number of leukocytes adhering to a vehicle- or CB-839-treated EC monolayer (n=4). **x**, fold-changes in mRNA level of arterial (Ephrin B2), venous (Ephrin B4) and lymphatic (Prox1) markers (n=4). All data are mean  $\pm$  SEM; n refers

to the number of individual animals per genotype or per treatment group, or to the number of individual EC donors used, or to the number of aortic rings analyzed. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  n.s., not significant versus corresponding control. Panels s,u,w represent data as fold-change versus control with a value equal to 1 meaning no change in mRNA level for a given condition in comparison to the control condition. In panels s,u, statistical differences indicate if the fold change differs from 1. In panel u, statistical differences are calculated versus the control IL-1 $\beta$ -treated condition. eNOS, endothelial nitric oxide synthase, ephB2; ephrin B2; ephB4, ephrin B4; ET-1, endothelin 1; IL-1 $\beta$ , interleukin 1 $\beta$ ; pH3, phospho-histone 3; PROX1, prospero homeobox 1; NG2, chondroitin sulfate proteoglycan 4; SELE, selectin-E; VCAM, vascular cell adhesion molecule; WT, wild type. Scale bars are 250  $\mu\text{m}$  in a, b; 50  $\mu\text{m}$  in f, g; 25  $\mu\text{m}$  in k, l and 100  $\mu\text{m}$  in m, n, p, q.

**FIGURE 3: GLUTAMINE CONTROLS MULTIPLE METABOLIC FUNCTIONS IN ECs**

**a**, Gas chromatography–mass spectrometry (GC/MS)-based analysis of total contribution of [U-<sup>13</sup>C]-glutamine and [U-<sup>13</sup>C]-glucose to tricarboxylic acid (TCA) intermediates in ECs. [U-<sup>13</sup>C]-Glutamine and [U-<sup>13</sup>C]-glucose were supplemented to the culture medium at 2 and 5.5 mM final concentration, respectively. All labelings were performed under steady state conditions. **b**, Quantification of intracellular TCA metabolites in control and glutamine-depleted (Gln free) ECs. **c**, Intracellular ATP levels measured by liquid chromatography–mass spectrometry (LC/MS) in control and glutamine-depleted ECs. **d**, Energy charge measurement in control and glutamine-depleted ECs. **e**, Intracellular levels of the non-essential amino acids glutamate, aspartate, asparagine, alanine, glycine and serine in control and glutamine-depleted ECs. Level in control cells is indicated by the dashed line. **f**, Total contribution of [U-<sup>13</sup>C]-glutamine carbon and [<sup>15</sup>N<sub>2</sub>]-glutamine nitrogen to non-essential

amino acids in ECs. **g**, Measurement of protein synthesis rate ( $[^3\text{H}]$ -tyrosine incorporation) in control and glutamine-deprived ECs. **h,i**, Representative cell size distribution graph (h) and corresponding quantification of cell sizes (i) measured by forward scatter value of flow cytometry in control and glutamine-deprived ECs. **j**, mTOR signaling activity in control and glutamine-deprived ECs, revealed by immunoblotting for phosphorylated ribosomal protein S6 (p-S6) and by 4EBP1 mobility shift.  $\beta$ -Actin was used as loading control. Representative blot of 3 independent experiments is shown. **k**, Measurement of ER stress in control and glutamine-deprived ECs, determined by accumulation of ATF4 and Chop protein. Results from 3 independent experiments are shown. Asterisk indicates a non-specific band. **l**, mRNA levels of ER stress marker genes in glutamine-deprived versus control ECs determined by qRT-PCR. **m**, Intracellular reactive oxygen species (ROS) levels in control and glutamine-deprived ECs measured by CM-DCFDA staining. **n**, Representative pictures of control and glutamine-deprived ECs at 2 hrs after treatment with increasing doses of  $\text{H}_2\text{O}_2$ . In glutamine starvation (but not control) conditions, treatment with 250  $\mu\text{M}$   $\text{H}_2\text{O}_2$  already resulted in cell death, revealed by loss of normal cell morphology. **o**, Total glutathione content in control and glutamine-deprived ECs. All data are mean  $\pm$  SEM of at least three independent experiments each performed with ECs from a different individual donor. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , n.s., not significant versus corresponding control.  $\alpha$ -KG,  $\alpha$ -ketoglutarate; Cit, citrate; Fum, fumarate; Mal, malate; Suc, succinate; NEAA, non-essential amino acids; Chop, C/EBP homologous protein; ATF4, activating transcription factor 4; Trib3, Tribbles Pseudokinase 3; Txnip, Thioredoxin-interacting protein; Asns, asparagine synthetase; CM-DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate. Scale bar in n is 100  $\mu\text{m}$ .

**FIGURE 4:** ASPARAGINE AND TCA ANAPLEROISIS ARE REQUIRED FOR EC PROLIFERATION UNDER GLUTAMINE-DEPLETED CONDITIONS

**a**, [<sup>3</sup>H]-Thymidine incorporation in DNA in glutamine-starved ECs with and without supplementation of exogenous cell-permeable dimethyl  $\alpha$ -ketoglutarate (hereafter referred to as  $\alpha$ -KG) or a non-essential amino acid mixture (NEAA). **b**, [<sup>3</sup>H]-Thymidine incorporation in DNA in glutamine-starved ECs with and without single or combined supplementation of exogenous  $\alpha$ -KG and individual NEAAs. **c**, Quantification of intracellular deoxynucleotide (dNTP) levels in control and glutamine free (without or with supplementation of asparagine and  $\alpha$ -KG) conditions. **d**, Quantification of intracellular TCA metabolites in control or glutamine-deprived ECs with and without combined supplementation of asparagine and  $\alpha$ -KG. **e-f**, Representative graph of cell size distribution (**e**) determined by flow cytometry and corresponding quantification of cell size (**f**) of control or glutamine-deprived ECs with and without combined supplementation of asparagine and  $\alpha$ -KG. Of note: The first two bars in this graph are the same as in Fig. 3i. The data in the two panels originate from the same set of experiments, only in panel 4f an additional condition is displayed. **g**, mTOR activation, revealed by immunoblotting for phosphorylated ribosomal protein S6 (p-S6) and by 4EBP1 mobility shift, in control or glutamine-deprived ECs with and without combined supplementation of asparagine and  $\alpha$ -KG.  $\beta$ -Actin was used as loading control. Immunoblots shown are representative of 3 independent experiments. **h**, Protein synthesis ([<sup>3</sup>H]-tyrosine incorporation assay) in control and glutamine-deprived ECs with and without combined supplementation of asparagine and  $\alpha$ -KG. **i**, mRNA levels of ER stress markers in control or glutamine-deprived ECs with and without combined supplementation of asparagine and  $\alpha$ -KG. **j**, Intracellular reactive oxygen species (ROS) levels (CM-DCFDA staining) in control and glutamine-deprived ECs with and without combined supplementation of asparagine and  $\alpha$ -

KG. **k**, Representative pictures of control and glutamine-deprived ECs with and without combined supplementation of asparagine and  $\alpha$ -KG at 2 hrs after treatment with  $H_2O_2$ . **l**, [ $^3H$ ]-Thymidine incorporation into DNA in ECs grown at different doses of glutamine, with or without supplementation of asparagine. All data are mean  $\pm$  SEM from at least three independent experiments each performed with ECs from a different individual donor. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , n.s., not significant versus corresponding control. Pro, proline; dATP, deoxyadenosine triphosphate; dTTP, deoxythymidine triphosphate; dCTP, deoxycytidine triphosphate; all other abbreviations as in previous figures. Scale bar in **k** is 100  $\mu m$ .

**FIGURE 5: GLUTAMINE-DEPENDENT ASPARAGINE SYNTHESIS IS REQUIRED FOR VESSEL SPROUTING**

**a**, mRNA level of ASNS in control and ASNS<sup>KD</sup> ECs and under hypoxia, glucose deprivation and ER stress. **b-e**, Quantification of number of sprouts (**b**) and total sprout length (**c**) in control and ASNS<sup>KD</sup> EC spheroids and corresponding representative images (**d,e**). **f**, [ $^3H$ ]-Thymidine incorporation into DNA in control and ASNS<sup>KD</sup> ECs with or without asparagine supplementation. **g**, Protein synthesis ([ $^3H$ ]-tyrosine incorporation assay) in control and glutamine-deprived ECs with and without supplementation of asparagine. Of note: The first two bars in this graph are the same as in Fig. 4h. The data in the two panels originate from the same set of experiments, only in panel 5g another third condition is displayed. **h**, mRNA and protein levels of ER stress markers in control and glutamine-deprived ECs with and without supplementation of asparagine. **i**, Representative immunoblots of mTOR activation, revealed by immunoblotting for phosphorylated ribosomal protein S6 (p-S6) and 4EBP1 mobility shift, in control and glutamine-deprived ECs with and without single or combination supplementation of asparagine and  $\alpha$ -KG. Of note: the first three lanes of the actin loading



control in panel i are the same as the actin loading controls in panel h because the Chop immunoblotting was done in the same experiment as in panel i but was found to fit more logically in panel h. All data are mean  $\pm$  SEM from at least three independent experiments each performed with ECs from a different individual donor. \*  $p < 0.05$ , \*\*  $p < 0.01$ , n.s., not significant versus corresponding control. ASNS, asparagine synthetase; other abbreviations as in previous figures. Scale bar in d, e is 100  $\mu\text{m}$ .

## SUPPLEMENTARY FIGURE LEGENDS

### **SUPPLEMENTARY FIGURE 1: GLUTAMINE METABOLISM IS ESSENTIAL FOR EC PROLIFERATION**

**a**, Amino acid concentration in standard M199 containing 20% FBS at the starting point (T0) of the consumption/excretion experiment (Fig. 1a). **b**, mRNA levels of GLS1 and GLS2 in ECs determined by qRT-PCR. cDNA from GLS2 over-expressing (GLS2-OE) ECs was used as a positive control for the specificity of the GLS2 primer/probe set. **c**, GLS1 mRNA level in control and GLS1<sup>KD</sup> ECs. **d**, Representative Western blot for GLS1 in control and GLS1<sup>KD</sup> ECs with  $\beta$ -actin as a loading control. **e,f**, [<sup>3</sup>H]-Thymidine incorporation into DNA in control and CB-839-treated ECs upon GLS2 over-expression (e) or glutamate supplementation (f). **g**, Representative Western blot and detection of caspase-3 (apoptosis marker) in ECs treated with the indicated doses of CB-839.  $\alpha$ -Tubulin was used as a loading control. Note that none of the concentrations of CB-839 induced activation of caspase-3, as can be judged from the lack of the typical 17 kDa cleavage product. **h**, Representative images of TUNEL (red)/Hoechst (blue)-stained ECs treated with the indicated doses of CB-839. Doxorubicin (1  $\mu\text{g/ml}$ ) was used as an apoptosis inducer and served as a positive control. **i**, mRNA level of

GLS1 in mouse ECs obtained from livers of GLS1<sup>ECKO</sup> mice and their wild type littermates (n=3). All data are mean  $\pm$  SEM from at least three independent experiments each performed with ECs from a different individual donor; n refers to the number of individual animals per genotype. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001. doxo, doxorubicin; GLS2-OE, GLS2 overexpression; other abbreviations as in main figures.

### **SUPPLEMENTARY FIGURE 2: ROLE OF GLUTAMINE METABOLISM IN MACROMOLECULE SYNTHESIS**

**a**, Total contribution of [<sup>15</sup>N<sub>2</sub>]-glutamine to ribonucleotide triphosphates (rNTPs) in ECs. **b**, Quantification of deoxynucleotides (dNTP) in glutamine-deprived ECs; the level in control ECs is indicated by the dashed line. **c**, Quantification of intracellular ribonucleotide content in glutamine-deprived ECs; the level in control ECs is indicated by the dashed line. **d**, Quantification of total contribution of [U-<sup>13</sup>C]-glutamine to m+4 citrate (Cit(m4)), m+5 citrate (Cit(m5)), pyruvate (Pyr) and lactate (Lac) in ECs. **e**, Quantification of percentage of [U-<sup>14</sup>C]-acetate (Ace), [U-<sup>14</sup>C]-glucose (Glc) and [U-<sup>14</sup>C]-glutamine (Gln) used for lipid production in ECs under normal culture conditions. **f**, Quantification of percentage of [U-<sup>14</sup>C]-glutamine (Gln) used for lipid production in ECs cultured in normoxia (Nor) or hypoxia (Hyp, 0.5% O<sub>2</sub>). All data are mean  $\pm$  SEM from at least three independent experiments each performed with ECs from a different individual donor. \* p<0.05, \*\* p<0.01, n.s., not significant versus corresponding control.

### **SUPPLEMENTARY FIGURE 3: ASPARAGINE IS REQUIRED FOR EC PROLIFERATION**

**a**, Quantification of cell numbers in glutamine-starved ECs during culture with or without nucleotide supplementation. dNTPs and NSX refer to deoxyribonucleotide triphosphates and nucleoside mixture, respectively. **b-e**, [<sup>3</sup>H]-Thymidine incorporation into DNA in glutamine-

starved ECs with or without supplementation with exogenous carbon sources (b), NEAAs (c) or the antioxidant NAC (d) or GSH-EE (e). Pyr, pyruvate; Suc, mono-Methyl hydrogen succinate; OAA, oxaloacetate; NEAA: pool of glycine, alanine, asparagine, aspartate, glutamate, proline, and serine. NAC, N-acetylcysteine. GSH-EE, glutathione reduced ethyl ester. **f**, [<sup>3</sup>H]-Thymidine incorporation into DNA in glutamine-starved ECs with or without asparagine supplementation combined with exogenous carbon sources. **g**, Representative Western blot for ASNS in control and ASNS<sup>KD</sup> ECs with  $\beta$ -actin as a loading control. Red asterisk indicates non-specific band. **h**, ASNS mRNA levels in control ECs, ECs grown under hypoxia and thapsigargin-treated ECs. All data are mean  $\pm$  SEM from at least three independent experiments each performed with ECs from a different individual donor (except stated otherwise). \*  $p < 0.05$ , n.s. not significant versus corresponding control.

**SUPPLEMENTARY FIGURE 4: ASPARAGINASE TREATMENT DOES NOT AFFECT RETINAL ANGIOGENESIS**

**a**, Quantification of asparagine, aspartate, glutamine and glutamate levels in serum of P4 mouse pups treated with saline or 2U/g/day asparaginase. Data are mean  $\pm$  SEM,  $n = 5$  pups for saline-treated and  $n = 4$  for asparaginase-treated; \*\*\* $p < 0.001$  versus saline treated; n.s. not significant according to Student's t test. **b,c**, Representative pictures of the isolectin B4-stained retinal microvasculature in vehicle- (b) and asparaginase-treated (c) P4 pups. **d**, Radial outgrowth of the vascular plexus in vehicle- and asparaginase-treated pups. **e,f**, Quantification of the number of branchpoints in the front (e) and the center (f) part of the microvascular plexus in vehicle- and asparaginase-treated pups. Data are mean  $\pm$  SEM of  $n = 5$  pups for vehicle-treated and  $n = 4$  for asparaginase-treated; n.s., not significant according to Student's t test.