Inverse Data-Driven Modeling and Multiomics Analysis Reveals Phgdh as a Metabolic Checkpoint of Macrophage Polarization and Proliferation

Graphical Abstract

Highlights

- Metabolomics and inverse modeling reveal a Tsc2/mTORC1-dependent checkpoint in macrophages
- M2 macrophages have high Phgdh activity
- Phgdh activity promotes M2 polarization
- Phgdh activity supports macrophage proliferation

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In Brief

Wilson et al. show that Tsc2, a negative regulator of mTORC1 signaling, critically influences the metabolome of macrophages. Inverse data-driven modeling and multiomics data reveal that Phgdh is an mTORC1-dependent metabolic checkpoint of macrophage proliferation and polarization. Phgdh is required for the expression of key anti-inflammatory molecules and M2 proliferation.

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Inverse Data-Driven Modeling and Multiomics Analysis Reveals Phgdh as a Metabolic Checkpoint of Macrophage Polarization and Proliferation

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SUMMARY
Mechanistic or mammalian target of rapamycin complex 1 (mTORC1) is an important regulator of effector functions, proliferation, and cellular metabolism in macrophages. The biochemical processes that are controlled by mTORC1 are still being defined. Here, we demonstrate that integrative multiomics in conjunction with a data-driven inverse modeling approach, termed COVRECON, identifies a biochemical node that influences overall metabolic profiles and reactions of mTORC1-dependent macrophage metabolism. Using a combined approach of metabolomics, proteomics, mRNA expression analysis, and enzymatic activity measurements, we demonstrate that Tsc2, a negative regulator of mTORC1 signaling, critically influences the cellular activity of macrophages by regulating the enzyme phosphoglycerate dehydrogenase (Phgdh) in an mTORC1-dependent manner. More generally, while lipopolysaccharide (LPS)-stimulated macrophages repress Phgdh activity, IL-4-stimulated macrophages increase the activity of the enzyme required for the expression of key anti-inflammatory molecules and macrophage proliferation. Thus, we identify Phgdh as a metabolic checkpoint of M2 macrophages.

INTRODUCTION
Macrophage activation and differentiation are processes involved in several human diseases, including inflammatory and autoimmune diseases, as well as cancer (Murray and Wynn, 2011). Mechanistic or mammalian target of rapamycin (mTOR) complex 1 (mTORC1) is a conserved serine-threonine kinase that senses and integrates a range of environmental and nutrient signals to coordinate fundamental cellular processes (Ben-Sahra and Manning, 2017; Saxton and Sabatini, 2017). Tuberous sclerosis complex 2 (Tsc2) is a negative regulator of mTORC1 activity and its deletion leads to constitutive activation of mTORC1 (Saxton and Sabatini, 2017). In macrophages, mTORC1 is activated by cytokines and pathogen-associated molecular patterns (PAMPs), such as interleukin (IL)-4 and lipopolysaccharide (LPS), respectively, by inactivation of Tsc2 to coordinate innate effector functions, including inflammatory cytokine production, macrophage polarization, antigen presentation, and T cell activation (Weichhart et al., 2015). Considering the role of mTORC1 as an important regulator of cellular metabolism, it is probable that such effector functions are controlled by mTORC1-dependent metabolic networks. Metabolomics provides an endpoint of cellular dynamics by measuring concentrations of metabolites in different cellular activation states. Typically, the resultant high-dimensional data are analyzed by correlation/association or covariance network analysis (Fiehn et al., 2000; Jain et al., 2012; Price et al., 2017; Weckwerth, 2003; Leitner et al., 2017; Weckwerth et al., 2004a), which reveal novel and unexpected connections in the biochemical networks derived from omics data. However, these statistical analyses are not able to identify causal relationships. By drafting dynamic metabolic computer models, causal gene-protein-metabolite networks can be derived from metabolomics data, but most kinetics parameters, constants, and initial conditions will be estimated and therefore will not necessarily reflect the real network (Iglesias and Ingalls, 2010). In contrast, inverse data-driven modeling approaches may be suitable for deriving these causal relationships, thereby extending beyond association analysis or forward design of metabolic networks (Weckwerth, 2019). The reason for this is that by inverse modeling, we exploit the data for solving the underlying regulatory structure (Weckwerth, 2019). By knowing the trajectory of the system state variables measured as multiomics molecular
dynamics, we can extract information about the systems equations, which is in contrast to the forward approach relying on basic assumptions of the systems equations and delivering an indefinite number of solutions (Strogatz, 2015; Weckwerth, 2012). Accordingly, we have implemented an inverse modeling algorithm by combining the metabolite covariance matrix from metabolite profiles (COV) and the corresponding metabolic reconstruction from genome sequences (RECON), termed COVRECON (Figure 1). We applied the COVRECON strategy to the analysis of Tsc2/mTORC1-dependent macrophage differentiation.

**RESULTS**

**Tsc2 Controls Multiple Metabolic Processes in Macrophages and Regulates the α-Ketoglutarate:Glutamate Reaction Rate Elasticity in Macrophages**

We recently demonstrated that macrophage-specific deletion of Tsc2 in Tsc2\textsuperscript{fl/fl}Lyz2-Cre mice promotes M2 macrophage polarization and shifts cellular metabolism toward both increased glycolysis and mitochondrial respiration (Linke et al., 2017). However, the biochemical processes underpinning Tsc2-dependent macrophage differentiation remain ill-defined. To search for critical metabolic processes involved in the Tsc2/mTORC1-dependent function of macrophages, we applied an integrative protocol for the simultaneous extraction and analysis of metabolites and proteins (Figure 1; Weckwerth et al., 2004b) from Tsc2\textsuperscript{fl/fl} (control) and Tsc2\textsuperscript{fl/fl}Lyz2-Cre (knockout [KO]) bone marrow-derived macrophages (BMDMs). The metabolite data (Data S1) were then analyzed by principal components analysis (Figure 2A). These statistical analyses do not necessarily reveal causal relationships in the data (Weckwerth, 2011, 2019). Thus, we applied a hybrid mathematical-statistical algorithm called COVRECON, which is able to combine metabolic reconstruction and multivariate metabolomics data (Figure 1). COVRECON is capable of inferring biochemical checkpoints directly from metabolomics data (Weckwerth, 2019). First, we reconstructed a simplified metabolic interaction network (RECON) for macrophage metabolism (Figures 1 and 2B; Data S1). This metabolic interaction network is reconstructed in a way that the metabolic nodes correspond to the measured metabolites (Nägele et al., 2014). Therefore it represents an a priori simplification of the total genome-scale metabolic reconstruction where many reactions are lumped into overall reactions. The measured metabolites are then used to generate a metabolic covariance data matrix (COV) for Tsc2-deficient and control BMDMs. The covariance matrix of metabolite profiles and the reconstructed metabolic interaction network were subsequently combined using Equations 1, 2, 3, 4, and 5 below (Sun and Weckwerth, 2012):

\[
COV + JAC^T + JAC \cdot COV = -2D, \quad \text{(Equation 1)}
\]

\[
JAC = \begin{pmatrix}
\frac{\partial f_1}{\partial M_1} & \frac{\partial f_1}{\partial M_2} & \cdots & \frac{\partial f_1}{\partial M_n} \\
\frac{\partial f_2}{\partial M_1} & \frac{\partial f_2}{\partial M_2} & \cdots & \frac{\partial f_2}{\partial M_n} \\
\vdots & \vdots & \ddots & \vdots \\
\frac{\partial f_n}{\partial M_1} & \frac{\partial f_n}{\partial M_2} & \cdots & \frac{\partial f_n}{\partial M_n}
\end{pmatrix}_{n \times n}, \quad \text{(Equation 2)}
\]

\[
f_i(M_1, M_2, \ldots M_n) = \frac{\partial M_i}{\partial t}, \quad \text{(Equation 3)}
\]

\[
COV = \begin{pmatrix}
cov(M_1, M_1) & cov(M_1, M_2) & \cdots & cov(M_1, M_n) \\
cov(M_2, M_1) & cov(M_2, M_2) & \cdots & cov(M_2, M_n) \\
\vdots & \vdots & \ddots & \vdots \\
cov(M_n, M_1) & cov(M_n, M_2) & \cdots & cov(M_n, M_n)
\end{pmatrix}_{n \times n}, \quad \text{(Equation 4)}
\]
The metabolic interaction matrix (RECON) forms part of the Jacobian (JAC) in Equation 5:

\[
\text{JAC} = \text{RECON} \frac{df}{dM} \quad \text{(Equation 5)}
\]

The elements of JAC represent the reaction rate elasticities ($\epsilon$) of enzymes in the corresponding biochemical network (Steuer et al., 2003; Nägele et al., 2014). Matrix D in Equation 1 represents a stochastic fluctuation value that is added to the calculation. Equation 1 can be used to inversely calculate JAC and the corresponding reaction rate elasticities ($\epsilon$) from metabolite profiling data (Sun and Weckwerth, 2012). The correlation of metabolites reflected by the covariance matrix is thereby translated into the dynamic change of an existing biochemical pathway reflected by the Jacobian (Weckwerth, 2019).

The COVRECON strategy, which incorporates the covariance data matrix of the measured metabolite profiles from Tsc2<sup>fl/fl</sup> and Tsc2<sup>fl/fl</sup>Lyz2<sup>-Cre</sup> BMDMs in conjunction with Equation 1, was used to identify metabolic perturbation points. The largest perturbation in the differential Jacobian, when comparing Tsc2-deficient versus control macrophages, was detected for the reaction rate elasticity of $\alpha$-ketoglutarate to glutamate ($\frac{df_{\text{AKG}}}{dM_{\text{GLU}}}$) (Figure 2C), which points to three potential conversions: (1) pyruvate-alanine, (2) 3-phosphoglycerate-serine, and (3) oxaloacetate-aspartate (Figure 2B). The question remained which of these three perturbation points showed largest control. To answer this question, we compared the prediction with proteomic profiles from the same macrophage sample.

**Tsc2-mTORC1 Regulates Phosphoglycerate Dehydrogenase (Phgdh) at the Protein and Gene Expression Level**

The employed extraction protocol for metabolomics enables the simultaneous extraction and analysis of proteins from the same sample using a shotgun proteomics approach (Figure 1; Weckwerth et al., 2004b). We could therefore validate the predicted biochemical perturbation points with proteome data from the same macrophage samples. Out of 1,730 identified and quantified proteins, Phgdh, the first enzyme in the de novo serine/glycine biosynthesis pathway (Ducker and Rabinowitz, 2017), was one of the most significantly altered proteins in Tsc2-deficient macrophages, which correlated with the proposed biochemical perturbation (Figure 3A). Consequently, we further investigated the Tsc2-mTORC1-dependent regulation of Phgdh and observed higher levels of Phgdh protein expression in Tsc2-deficient macrophages compared with control Tsc2<sup>fl/fl</sup> cells (Figure 3B). Moreover, rapamycin partially reduced the expression of Phgdh in Tsc2<sup>fl/fl</sup>Lyz2<sup>-Cre</sup> macrophages, indicating a direct involvement of mTORC1 in the regulation of the enzyme (Figure 3B). Interestingly, mRNA expression of Phgdh was also diminished by Tsc2-deletion in an mTORC1-dependent manner, as well as phosphoserine aminotransferase 1 (Psat1), phosphoserine phosphatase (Psph), and phosphoglycerate mutase 1 (Pgam1), which control the metabolic flux from glycolysis into de novo serine/glycine biosynthesis (Figure 3C; Amelio et al., 2014; Hitosugi et al., 2012).

**IL-4 Stimulates Phgdh Activity in Macrophages**

Distinct functional states of macrophages play essential roles in the maintenance of tissue homeostasis and induction of inflammatory immune responses (Murray et al., 2014). These functional states are closely related to cellular metabolism, and it is now well-established that the effector responses of macrophages are shaped by metabolic reprogramming (Diskin and Palsson-McDermott, 2018; Galván-Peña and O’Neill, 2014; O’Neill et al., 2016). Thus, to investigate the regulation of Phgdh in differentially activated macrophages, we assessed the expression of key enzymes of the serine biosynthesis pathway. We observed high Phgdh mRNA expression in IL-4-polarized macrophages after 24 h, whereas macrophages polarized with LPS showed lower expression of the gene (Figure 4A). mRNA expression of Psat1 was also higher in M2- versus M1-polarized cells (Figure 4A). Furthermore, we observed a time-dependent induction of Phgdh mRNA expression in response to IL-4 that peaked at 8 h, whereas LPS gradually repressed Phgdh expression over time (Figure 4B). IL-4 also induced Phgdh protein expression in macrophages (Figure 4C) and enhanced Phgdh enzyme activity compared to LPS regardless of the presence of serine and glycine in the medium (Figure 4D). Taken together, these results suggest that IL-4-stimulated macrophages divert more of their imported glucose into de novo serine/glycine biosynthesis.

**Phgdh Activity, but Not Serine and Glycine, Is Required for M2 Macrophage Polarization**

Next, we assessed whether Phgdh contributes to IL-4-induced macrophage polarization. We confirmed the activity of two Phgdh inhibitors, CBR-5884 (Mullarky et al., 2016) and NCT-503 (Pacold et al., 2016), in IL-4-stimulated BMDMs from wild-type mice (Figure 5A), and showed that inhibition of Phgdh with either of these inhibitors reduced the expression of the M2 signature genes Arg1, Retnla, and Chil3 (Figures 5B and 5C). Moreover, secretion of the anti-inflammatory cytokine IL-10 was also reduced by the Phgdh inhibitor CBR-5884 in...
direct role of Phgdh in macrophage polarization (Figure 7A). To were reduced in the inhibitor-treated cells, thus corroborating a (Figure 7A). We found that the M2 marker genes Glutamate synthase (Glut) were reduced in the inhibitor-treated cells, thus corroborating a direct role of Phgdh in macrophage polarization (Figure 7A). To identify additional processes that were modified by CBR-5884, we performed Gene Ontology (GO) enrichment analysis and identified proliferation as a major pathway that is regulated by Phgdh in IL-4-stimulated macrophages (Figure 7B). To functionally evaluate this finding, we treated Tsc2−/− and Tsc2−/−/Ly22-Cre BMDMs with CBR-5884 and analyzed cell-cycle progression. Interestingly, in both genotypes we found that inhibition of Phgdh decreased cells in the S-phase while increasing cells in the G1-phase, indicating a block in cell-cycle progression (Figures 7C and 7D). Hence, the prediction of COVRECON using Tsc2-deficient macrophages identified a general mode for the regulation of macrophage proliferation and polarization by Phgdh.

**DISCUSSION**

We recently demonstrated that Tsc2 maintains macrophage quiescence, and its deletion in macrophages leads to chronic mTORC1 activation and spontaneous M2-like granuloma formation in mice (Linke et al., 2017). Because mTORC1 is a major regulator of cellular metabolism (Ben-Sahra and Manning, 2017), we applied metabolomics in conjunction with the COVRECON strategy to search for critical metabolic processes involved in the Tsc2-dependent function of macrophages. The analysis of metabolite covariance or correlation networks from metabolomics data is a powerful method for the description of...
systemic biochemical regulation (Nägele et al., 2014; Weckwerth et al., 2004a). Furthermore, it has been demonstrated that differential metabolite correlation or covariance networks reflect biochemical regulation depending on the genotype or the genotype-environment-phenotype interactions (Nägele et al., 2014; Sun and Weckwerth, 2012). In recent years, we established that correlation networks are a result of biochemical regulation (Steuer et al., 2003; Sun and Weckwerth, 2012; Weckwerth, 2003; Weckwerth et al., 2004a). This was also demonstrated by other groups as well (Camacho et al., 2005; Kügler and Yang, 2014; Öksüz et al., 2013). Based on these initial studies, it has been possible to underline the dynamics of these different metabolic signatures and especially reveal causal relationships between changed metabolite levels and enzymatic regulation by applying an inverse data-driven modeling approach (Nägele et al., 2014; Sun and Weckwerth, 2012; Doerrler et al., 2013; Nukainen et al., 2016).

In the present study, the application of COVRECON predicted the Tsc2-dependent perturbation of Phgdh, the rate-limiting enzyme in de novo serine biosynthesis from glycolysis. The serine generated via this pathway can then be converted to glycine and both provide essential precursors for the synthesis of proteins, nucleic acids, and lipids (Ducker and Rabinowitz, 2017). Currently, the serine/glycine pathway is known to regulate cell growth and proliferation in normal and tumor cells, and inhibitors of Phgdh are actively being developed as novel anti-tumor therapies (Amelio et al., 2014; Ducker and Rabinowitz, 2017; Locasale, 2013; Mattaini et al., 2016). Using proteomics and mRNA expression analysis, we were able to validate the role of Phgdh as a major hub controlled by the Tsc2-mTORC1 pathway in macrophages. As Tsc2-deficient macrophages are prone to differentiate toward M2 polarization (Linke et al., 2017), we hypothesized that Phgdh could represent a general metabolic signature of alternatively activated macrophages. Additional work revealed that IL-4 not only induced the expression of Phgdh in BMDMs but also enhanced Phgdh enzymatic activity compared to LPS; an effect that was observed regardless of the presence of serine and glycine in the medium. Furthermore, inhibition of Phgdh with CBR-5884 in IL-4-stimulated macrophages resulted in reduced expression of the M2 signature genes Arg1, Retnla, and Chil3 as well as IL-10 production, while enhancing the secretion of the pro-inflammatory cytokine IL-1β. In addition, we demonstrate a clear involvement of Phgdh activity in macrophage proliferation.

It should be noted that our findings contend with recent data revealing that Phgdh-induced serine metabolism promotes a pro-inflammatory macrophage response by driving IL-1β production (Rodriguez et al., 2019; Yu et al., 2019).
of serine and glycine in the media. In our analysis, the greatest perturbation in the differential Jacobian was found for the reaction rate elasticity of α-ketoglutarate to glutamate. Psat1, as part of the serine biosynthesis pathway, transfers nitrogen from glutamate to the Phgdh product 3-phosphohydroxy-pyrurate to produce phosphoserine and α-ketoglutarate. As α-ketoglutarate is important for M2 macrophage activation (Liu et al., 2017), our data suggest that the serine biosynthetic pathway could be a major contributor of α-ketoglutarate production and M2 polarization in macrophages. Thus, we propose that although Phgdh activity promotes an initial pro-inflammatory response driven by the generation of serine (Rodriguez et al., 2019; Yu et al., 2019), over time this may switch to a more anti-inflammatory profile due to the production of α-ketoglutarate, which may also explain the enhanced proliferation of Tsc2-deficient macrophages. Phgdh was shown to be important for cell survival and neovascularization in endothelial cells in a serine-independent manner (Vandekeere et al., 2018). However, the precise role of Phgdh as a regulatory enzyme in different cell types remains to be elucidated. In particular, subcellular compartmentalization requires further investigation, because there is no dependency of such processes on the presence or absence of serine in the medium (Vandekeere et al., 2019).

In conclusion, the presented concept of data-driven inverse modeling and multiomics analysis allows for the systematic integration of genome-scale metabolic reconstruction, prediction, and analysis of causal biochemical regulation. The application of this approach in our study enabled us to identify a functional role for the serine/glycine biosynthetic intermediate, Phgdh, in macrophage proliferation and polarization. Future studies should aim to expand the metabolic interaction network to incorporate a more diverse set of metabolites, including fatty acids and lipids. We envision COVRECON as useful strategy in the progression from correlation studies to the identification of causal relationships in metabolomics data.
STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2020.01.011.

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AUTHOR CONTRIBUTIONS

W.W. and T.W. conceived the concept of the study, analyzed the data, and wrote the manuscript. J.L.W., M.L., F.D., S.D.F., Z.C., H.K.M., K.K., L.F., A.M., and A.H. performed experiments and measurements, analyzed the data, and wrote parts of the manuscript. T.N., X.S., A.P., A.B., and M.H. analyzed the data and wrote parts of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interest.
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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Hsp90               | Cell Signaling | Cat#4877; RRID: AB_2233307 |
| Anti-Arginase-1 Antibody | Sigma-Aldrich | Cat# ABS535; RRID: |
| Anti-β-Tubulin Mouse mAb (DM1A) | Calbiochem | Cat#CP06; RRID:AB_2617116 |
| Goat anti-Rabbit IgG cross-adsorbed Antibody HRP Conjugated | Bethyl Laboratories | Cat# A120-209P, RRID:AB_10634086 |
| Anti-PHGDH antibody produced in rabbit | Sigma Aldrich Prestige Antibodies | Cat#HPA021241; RRID: AB_1855299 |
| **Biological Samples** |        |            |
| L-929-conditioned supernatant | This paper | N/A |
| Bone marrow-derived macrophages (BMDMs) | This paper: C57BL/6J, Tsc2fl/fl, Lyz2+/+ or Tsc2fl/fl, Lyz2cre/+ mice | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Recombinant Murine M-CSF | Peprotech | Cat#315-02 |
| Endoproteinase Lys-C | Biolabs | Cat# P8109S |
| Urea | Sigma-Aldrich | Cat# 57-13-6 |
| Ammonium bicarbonate | Sigma-Aldrich | Cat#1066-33-7 |
| Ammonium acetate | Sigma-Aldrich | Cat#631-61-8 |
| Dithiothreitol (DTT) | Sigma-Aldrich | Cat#16096-97-2 |
| IAA | Sigma-Aldrich | Cat#144-48-9 |
| Acetonitrile | Merck | Cat#75-05-8 |
| SPEC C18 | Varian | Cat#A59603 |
| Graphite | Thermo-Scientific- Pierce | Cat#88302 |
| Recombinant Murine IL-4 | Peprotech | Cat#214-14 |
| Lipo polysaccharides from Escherichia coli O111:B4 | Sigma Aldrich | Cat#L2630 |
| β-Nicotinamide adenine dinucleotide sodium salt (NAD) | Sigma Aldrich | Cat#N0632-1G |
| D(-)-3-Phosphoglyceric acid disodium salt | Sigma Aldrich | Cat#P8877 |
| CBR-5884 | Axon Medchem | Cat#Axon 2585 |
| NCT-503 | Sigma Aldrich | Cat#SML1659 |
| InSolution Rapamycin | Calbiochem | Cat#553211 |
| cOmplete, EDTA-free Protease Inhibitor Cocktail | Sigma-Aldrich | Cat# COEDTAF-RO Roche |
| Aprotinin Pro tease Inhibitor | Thermo Scientific | Cat# 78432 |
| Leupeptin Pro tease Inhibitor | Thermo Scientific | Cat# 78435 |
| Benzamidine | Sigma-Aldrich | Cat# B6506 |
| Trypsin inhibitor | Merck | Cat# 10109878001 |
| PMSF | Sigma Aldrich | Cat# P7626 |
| TRI Reagent® | Sigma Aldrich | Cat#T9424 |
| RNase A | Merck | Cat# R6513 |
| 7-Aminoactinomycin D | Sigma Aldrich | Cat#A9400 |
| **Critical Commercial Assays** |        |            |
| ELISA MAX Deluxe Set Mouse IL-10 | BioLegend | Cat#431414 |
| ELISA MAX Deluxe Set Mouse IL-1β | BioLegend | Cat#432604 |
| ELISA MAX Deluxe Set Mouse TNF-α | BioLegend | Cat#430904 |
| Click-IT EdU Alexa Fluor 647 Flow Cytometry Assay Kit | Invitrogen | Cat#C10419 |
| GoScript Reverse Transcription Mix, Oligo(dT) | Promega | Cat#A2791 |

(Continued on next page)
Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| GoTaq® qPCR Master Mix | Promega | Cat#A6001 |
| RNeasy Plus Micro Kit (50) | QIAGEN | Cat#74034 |
| RNA 6000 Nano Kit | Agilent Technologies | Cat#5067-1511 |
| NEBNext® Ultra Directional RNA Library Prep Kit for Illumina® | New England Biolabs | Cat#E7420 |
| Agilent High Sensitivity DNA Kit | Agilent Technologies | Cat#5067-4626 |
| Qubit dsDNA HS Assay Kit | Invitrogen | Cat#Q32851 |

Deposited Data

Mass spectrometry proteomics data
This paper
Proteomexchange (http://www.proteomexchange.org/)
Accession: PXD010657

RNA-Seq sequencing data
This paper
Gene Expression Omnibus: https://www.ncbi.nlm.nih.gov/geo/GEO
Accession: GSE118119

Experimental Models: Cell Lines

NCTC clone 929 cell line, CLS
NA
Cat# 400260/p757_L-929, RRID:CVCL_0462

Experimental Models: Organisms/Strains

Mouse: C57BL/6J
Department of Laboratory Animal Science & Genetics, Medical University of Vienna
N/A

Mouse: Tsc2^{fl/fl},Lyz2^{+/+}
Linke et al., 2017
N/A

Mouse: Tsc2^{fl/fl},Lyz2^{cre/+}
Linke et al., 2017
N/A

Oligonucleotides

Arg1 Forward primer AAGGACAGCCTCGAGGGAGGGGT
This paper
N/A

Arg1 Reverse primer AGGTCCCCGTGGCTCTCTACG
This paper
N/A

β-actin Forward primer CACACCCGCCAC CAGTTGC
This paper
N/A

β-actin Reverse primer TTGCACATGCCGGAGCCGTT
This paper
N/A

Chil3 Forward primer CCAGCAGAAGCTCTCCAGAAGCA
This paper
N/A

Chil3 Reverse primer TGGTAGGAAGATCCCAGCTGTACG
This paper
N/A

Igf1 Forward primer ATCTGCCTCTGTGACTTCTTGA
This paper
N/A

Igf1 Reverse primer GCCTGTGGGCTTGGTGAAGT
This paper
N/A

Pgaml Forward primer CATCAGCAAGGATCGACGAGG
This paper
N/A

Pgaml Reverse primer TGCTCTGGCAATGTCCTG
This paper
N/A

Phgdh Forward primer CAGTTGCTACACAGAAGGAAC
This paper
N/A

Phgdh Reverse primer GTCTGCTGCTTAGCTGCTT
This paper
N/A

Psat1 Forward primer AGAAGAATGGCAGGGCTG
This paper
N/A

Psat1 Reverse primer CCCATGAGTGATGCTGAA
This paper
N/A

Psat1 Reverse primer GGCTGAGGGAGCTGGTAAG
This paper
N/A

Software and Algorithms

MaxQuant
Max Planck Institute of Biochemistry, Germany; Tyanoa et al., 2016
RRID:SCR_014485; https://www.biochem.mpg.de/5111795/maxquant

Microsoft Excel
Microsoft
RRID:SCR_016137; https://www.microsoft.com/en-gb/

MATLAB (v8.4.0 R2014b)
MathWorks
RRID:SCR_001622; https://www.mathworks.com/products/matlab/
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Wolfram Weckwerth, wolfram.weckwerth@univie.ac.at

This study did not generate new unique reagents or mouse lines.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

BMDMs were generated from wild-type C57BL/6, Tsc2<sup>fl/fl</sup> or Tsc2<sup>fl/fl</sup>Lyz2-Cre mice according to Linke et al. (2017). All mouse studies were approved by the official Austrian ethics committee for animal experiments (GZ.BMWF-66.009/0304-II/3b/2013 and GZ.BMWF-66.009/0116/II/3b/2014). Male and female mice, typically 8 to 20 weeks old, were used randomly and no major sex-specific differences were observed (Linke et al., 2017).

METHOD DETAILS

Cell culture

Cells were cultured at 37°C in a humidified (5% CO₂) atmosphere. BMDMs were generated from wild-type C57BL/6, Tsc2<sup>fl/fl</sup> or Tsc2<sup>fl/fl</sup>Lyz2-Cre mice (Linke et al., 2017). Bone marrow, isolated from femur, tibia and humerus, was differentiated for 6 days in Petri dishes. Differentiation medium consisted of DMEM high glucose (GIBCO), 10% low endotoxin FBS (GIBCO), 2 mM L-glutamine (Lonza), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 50 μg/ml β-mercaptoethanol (GIBCO), supplemented with either 15 ng/ml CSF1 (Peprotech) or 10%–20% L929-conditioned supernatant. Three days after bone marrow isolation,
non-adherent cells were removed and cells were split 1:2 in fresh medium. If the BMDMs were differentiated using recombinant CSF1, fresh growth factor was provided the day before harvesting. On day 6, differentiated BMDMs (96% of the cells were positive for F4/80 and CD11b) were washed, harvested and seeded in the indicated medium.

**Simultaneous extraction and analysis of metabolites and proteins from the same sample**

Integrative extraction of metabolites and proteins was performed according to Weckwerth et al., 2004b. Frozen BMDMs (5 and 16 million cells, respectively) were resuspended and transferred from the falcon tube with three times 333 μL MCW extraction buffer (methanol: chloroform: water = 2.5: 1: 0.5) into a 2 mL “Precellys lysis kit” tube with a rubber seal. The samples were homogenized using 1 mm ceramic beads in a “Precellys 24 homogenizer” for 15 s at 5000 rpm twice and centrifuged for 5 min at 14000 x g and 4 °C before the supernatant was transferred to an Eppendorf tube. The pellet was washed with 400 μL MCW by vortexing and centrifuging for 5 min at 14000 x g and 4 °C, before the washed supernatant was combined with the first supernatant in the Eppendorf tube. Finally, the supernatant was centrifuged again and transferred to a new Eppendorf tube. The supernatant was dried in a “ScanSpeed MiniVac Beta” at 35 °C, 1100 rpm and 0.0001 mbar, then the pellets were frozen at –80 °C for subsequent analysis. To induce a phase separation, samples were dissolved in 500 μL MCW and 200 μL mQH2O, vortexed for 3 s and centrifuged for 5 min at 14000 x g and 4 °C. The upper polar phase was transferred to a new Eppendorf tube and both fractions were dried in a speedvac at 30 °C, 650 rpm and 0.0001 mbar. Measurement of metabolites was performed using gas chromatography-mass spectrometry standard protocols according to Linke et al. (2017) and Weckwerth et al., 2004b. The metabolomics data are presented in Data S1. Measurement of proteins was performed according to Weckwerth et al., 2004b. Pellets from the MCW extraction step were dried and dissolved in 500 μL protein extraction buffer (50 mM Tris, 1.5% SDS, 1% β-mercaptoethanol, pH7.6). Water saturated phenol (1 ml) was added to the protein solution and incubated on a thermoshaker at 37 °C and 700 rpm. After phase separation by centrifugation at 16000 x g for 20 min at room temperature, the upper polar phase was discarded and 600 μL centrifugation buffer (50 mM Tris, 1% β-mercaptoethanol, 600 mM sucrose, 8 M urea, 100 mM NH4+-bicarbonate) were added. After an additional centrifugation step at 16000 x g for 20 min at 30 °C, the upper apolar phase was transferred to a 15 mL falcon tube containing 5 mL ice cold 100 mM ammonium acetate in methanol to precipitate the proteins at –20 °C overnight. The following day, the tubes were centrifuged at 4000 rpm and 4 °C for 10 min, the supernatant discarded and the pellet washed with 1 mL ice cold 100 mM ammonium acetate in methanol. The pellet was resuspended by carefully pipetting up and down and ultrasonicating the Krainer tube for 5 min at room temperature. The solution was transferred to a new LoBind Eppendorf tube and the Krainer tube was washed with 1 mL ice-cold methanol to get the entire protein out of the tube and ultrasonicated for 5 minutes before the solution was combined with the first one in the LoBind Eppendorf tube. The Eppendorf tubes were centrifuged at 16000 g for 10 minutes at 4 °C, the supernatant was discarded and the pellet was resuspended in 1 ml ice cold acetone for the final washing step before it was centrifuged, the supernatant was discarded and the protein pellets were dried at room temperature until no liquid could be observed anymore and stored at ~80 °C over night. On the next day the amount of protein in the lung and BMDM samples respectively was determined with a Bradford assay. The BMDM protein pellets were solved in 60 μl lung samples in 300 μL solubilisation buffer (8M urea, 100mM NH4+-bicarbonate) by gently pipetting up and down and individually ultrasonicating them for 5 s.

60 μg of BMDM protein of each sample were taken and solubilisation buffer (8M urea, 100mM NH4+-bicarbonate) was added to a final volume of 60 μl for further proteomic analysis of the BMDM samples. To reduce disulfide bonds the samples were adjusted to 5mM DTT and incubated for 45 minutes at 37 °C and 700rpm on a thermoshaker and subsequently to 10mM IAA and incubated 60 minutes at 23 °C and 700 rpm in the dark for the alkylation of the received thiol groups. To inactivate spare IAA, DTT was added to a final concentration of 10mM and the Eppendorf tubes were incubated at 23 °C and 700rpm for another 15 minutes in the dark. For the digestion of the proteins the urea concentration in the samples was adapted from 8M to 4M with aqueous 100mM ammonium bicarbonate in 20% acetonitrile. 1 μL endoproteinase Lys-C (0.1 μg/μl) was added to the BMDM samples and 10 μL to the lung samples respectively before they were incubated on a thermoshaker for 5 hours at 30 °C and 500rpm in the dark. For the digestion with Trypsin the urea concentration was set to 2M with 10% acetonitrile, 25mM ammonium bicarbonate, 10mM CaCl2 in H2O before 3 μL of Trypsin beads were added to the BMDM samples and 20 μL to the lung samples respectively and incubated at 37 °C on a rotating incubator for 15.5 hours. The peptides were desalted with C18 and graphite according to Nukarinen et al. (2016). The measurements of the alkylated, reduced and LysC and trypsin digested BMDM peptides were conducted on a Thermo Scientific Dionex UltiMate 3000 equipped with a Thermo Scientific ES803 easy-spray C18 reversed phase column [50cm x 75μm ID / 2μm particles / 100Å pore size] coupled to a Thermo Scientific Orbitrap Elite. The proteomic “.raw” files obtained by the mass spectrometer by analyzing the BMDM samples were processed with MaxQuant (Tyanova et al., 2016) in order to match the calculated peptide sequences against the corresponding organism specific “.fasta” file obtained from https://www.uniprot.org and to identify the particular proteins. Statistics were performed with Excel, MATLAB and the COVAIN toolbox (Sun and Weckwerth, 2012). Only proteins were taken into account which occurred in all samples of one genotype and where at least two peptides led to one protein. The data was log2 transformed and missing values were replaced according to default settings (width: 0.3; downshift: 1.8; mode: total matrix). Subsequently a t test and a PCA were performed and proteins which showed a significant change (p value < 0.01) or contributed to a separation in the PCA plot were examined in more detail. The mass spectrometry proteomics data have been deposited in proteomexchange (http://www.proteomexchange.org/) with the accession number PXD010657.
Inverse modeling strategy
The functional integration of GC-MS metabolomics data into a biochemical metabolic network structure was performed by the inverse approximation of the biochemical Jacobian matrix, as described previously (Nägøle et al., 2014; Sun and Weckwerth, 2012). This approximation directly connects the covariance matrix (COV), which was built from the experimental metabolomics data, to the metabolic network structure of the primary metabolism. The COV matrix exploits the biological variance of independent replicate analysis of the same cell state (Weckwerth, 2019; Weckwerth et al., 2004a). The metabolic network model is provided in Data S1. The linkage of covariance data (COV) with the network structure is described by Equation 1:

\[ \text{COV} + JAC^T + JAC \times \text{COV} = -2D \]  

Equation 1

JAC represents the Jacobian matrix and D is a fluctuation matrix that integrates a Gaussian noise function simulating metabolic fluctuations around a steady state condition. In a biochemical context, entries of the Jacobian matrix (JAC) represent the elasticity of reaction rates to any change of metabolite concentrations, which are characterized by Equation 5:

\[ JAC = \text{RECON} \cdot f \]  

Equation 5

RECON is the metabolic interaction matrix, also referred to as stoichiometric matrix N, describing the interdependencies of metabolic fluxes and metabolites. f represents the rates for each reaction and M represents metabolite concentration changes. As stated before, the Jacobian approximation comprises the stochastic term D. The robustness of the calculation was tested according to Nägøle et al. (2014). Inverse approximations (10 × 10^3 for each population) were performed, resulting in 10 technical replicates of the Jacobian matrices. All calculations of Jacobian matrices were performed based on a modified script from COVAIN (Sun and Weckwerth, 2012).

Phgdh activity assay
Differentiated BMDMs from wild-type C57BL/6 mice were seeded in RPMI-1640 media without Glucose, Glycine and Serine (Teknova), containing 10% low endotoxin FBS (GIBCO), 2 mM L-glutamine (Lonza), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 50 µg/ml β-mercaptoethanol (GIBCO). When indicated, this medium was supplemented with 2 g/L D-Glucose (Sigma), 30 mg/L L-Serine (Sigma) and 10 mg/L Glycine (Sigma), mimicking the concentrations found in RPMI-1640 media. The cells were rested for 3-4 h in the new medium before they were stimulated for 24 h with either 100 ng/ml LPS (E. coli 0111.B4 from Sigma) or 10 ng/ml IL-4 (Peprotech). The Phgdh activity assay was based on previously published protocols (Willis and Sallach, 1964). Briefly, after stimulation macrophages were washed once in ice cold PBS, then immediately snap frozen in liquid nitrogen and stored at −80°C. The cells were homogenized in a Phgdh stabilizing buffer (0.5 M Tris pH 8.5, 1 mM EDTA, 0.02% Triton-X, 0.01 M NaCl) and the cell debris removed by centrifugation (16000 x g for 10 min at 4°C). The protein concentration in the supernatant was determined using a BCA protein assay kit. The Phgdh activity of 50-65 µg crude protein was assessed in a buffer containing 50 mM Tris pH 7.1, 10 mM NaCl and 20 mM of the substrate 3-phospho-D-glycerate. To analyze the activity of the Phgdh inhibitors, 30 µM CBR-5884, 25 µM CNT-503 or solvent control (DMSO) were added to the reaction buffer. The assay buffer was transferred to a quartz cuvette and the reaction was started by addition of the respective protein lysates. The subsequent formation of NADH was measured at 340 nm for 5 min at room temperature using a Hitachi U-2900 spectrophotometer. Phgdh activity is presented as the rate of NADH production per mg protein per minute (1 Unit = 1 µmol NADH min⁻¹) at saturating substrate conditions.

Immunoblotting
Differentiated Tsc2fl/fl and Tsc2fl/fl.Lyz2-Cre BMDMs were seeded in DMEM high glucose (GIBCO) containing 10% low endotoxin FBS (GIBCO), 2 mM L-glutamine (Lonza), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 50 µg/ml β-mercaptoethanol (GIBCO). The cells were rested for 3-4 h before they were treated for 18 h with rapamycin (100 nM; Calbiochem) or solvent control. The cells were then washed once and scraped into cold PBS and the cell pellet dissolved in lysis buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 25% (v/v) Glycerin, 1 mM EDTA, 0.5 mM Na3VO4, 0.5 mM DTT, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Roche) and 4 µg/ml aprotinin, 4 µg/ml leupeptin, 0.6 µg/ml benzamidinchlorid, 20 µg/ml trypsin inhibitor and 2 mM PMSF (Sigma). After 10 min incubation on ice the lysates were subjected to two freeze-thaw cycles in liquid N2 to ensure complete lysis. The pellet was discarded and the protein concentration in the supernatant was measured. Equal amounts of denatured lysate were resolved on 7.5%–12% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 4% low-fat milk for 1 h at 20°C and incubated with primary antibodies at 4°C overnight. Membranes were then incubated with HRP-conjugated secondary antibodies (Bethyl) at a dilution of 1:10,000 for 1 h at 20°C in 4% low-fat milk. Proteins were visualized using ECL substrate (Thermo Scientific).

mRNA expression analysis
BMDMs, incubated as indicated, were washed and suspended directly in TRI Reagent. RNA was isolated according to the manufacturer’s instructions. cDNA was synthesized from equal amounts of RNA using a GoScriptTM Reverse Transcription system (Promega). mRNA levels were determined using a GoTaq qPCR Master Mix (Promega) on a StepOnePlus Real-Time PCR System. Relative expression was normalized to β-actin. The following primer pairs were used: Arg1, AAGGACAGCCTCGAGGAGGGGT, Tsc2, AAGGACAGCCTCGAGGAGGGGT, and C3, AAGGACAGCCTCGAGGAGGGGT.
Differentiated BMDMs from wild-type C57BL/6 mice were seeded in RPMI-1640 media without Glucose, Glycine and Serine (Teknova), containing 10% low endotoxin FBS (GIBCO), 2 mM L-glutamine (Lonza), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 50 µg/ml β-mercaptoethanol (GIBCO). When indicated, this medium was supplemented with 2 g/L D-Glucose (Sigma), 30 mg/L L-Serine (Sigma) and 10 mg/L Glycine (Sigma). The cells were rested for 3-4 h in the new medium before they were pre-treated with the Phgdh inhibitor CBR-5884 (30 µM) or solvent for 24 h. Thereafter, BMDMs were incubated for 2 h with 10 µM Edu (Click-iT Edu Flow Cytometry Assay, Invitrogen) in fresh cell culture medium at 37°C in a humidified atmosphere (5% CO2). The cells were then harvested and washed in 1% BSA-PBS. Cell pellets were resuspended in cold (~20°C) MeOH and incubated for 10 min at ~20°C. MeOH was removed by centrifugation and discarded, and the cells washed again in 1% BSA-PBS. Click-iT reaction cocktail (125 µl) was added to each sample and the cells incubated for 30 min at room temperature in the dark. The pellets were then washed in 1% BSA-PBS and resuspended in 125 µl RNaseA7-AAD (50 µg/ml and 1 µg/ml, respectively), followed by an additional 30 min incubation at room temperature in the dark. The supernatant was discarded and the pellets resuspended in FACS buffer. Cells were analyzed on a CytoFLEX S Flow Cytometer (Beckman Coulter).

RNA Sequencing

BMDMs were pre-treated for 60 min with either the Phgdh inhibitor CBR-5884 (30 µM; Axon Medchem) or solvent. Thereafter, IL-4 (10 ng/ml; Peprotech) was added and cells were harvested after 24 h. Total RNA was isolated using an RNeasy Plus Micro Kit (QIAGEN), according to the manufacturer’s instructions. Quality control of RNA samples was performed using an RNA 6000 Nano Kit on a Bioanalyzer 2100 (Agilent). Sequencing libraries were prepared at the Core Facility Genomics of the Medical University of Vienna using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina, according to the manufacturer's instructions (New England Biolabs). Libraries were QC-checked on a Bioanalyzer 2100 (Agilent) using a High Sensitivity DNA Kit for correct insert size and quantitated using a Qubit dsDNA HS Assay (Invitrogen). Pooled libraries had an average length of 330-360 bp and were sequenced on a NextSeq500 instrument (Illumina) in 1 × 75 bp sequencing mode. One of the 6 samples was identified as being contaminated with DNA and was excluded from further analysis. The raw reads in fastq format were processed by the Biomedical Sequencing Facility (BSF) at CeMM (Vienna, Austria). Reads have been aligned to the mouse mm10 genome with Bowtie2/Tophat2 (Langmead and Salzberg, 2012; Kim et al., 2013) on the combined reference transcriptome from Ensembl 75 and UCSC mm10. The identified transcripts were further assembled and analyzed for differential analysis using the Cufflinks pipeline (Trapnell et al., 2013). Differentially expressed genes were selected based on the following criteria: minimum average expression of at least 1 FPKM, adjusted p-value smaller than 0.05 and absolute log fold-change superior to 0.7. RNA sequencing data have been deposited under GEO accession number GSE118119.

Gene ontology analysis

Enrichment analyses were performed on 68 annotated genes identified as differentially expressed using the Cytoscape 3.6.0 module ClueGO v2.3.3 (Bindea et al., 2009). Enrichments were performed using the GO, KEGG, REACTOME and Wiki Pathway databases. Only pathways with a p-value smaller than 0.05 and at least a 4 gene overlap were considered for grouping (kappa score 0.4).

QUANTIFICATION AND STATISTICAL ANALYSIS

The metabolomics data were normalized to BMDM cell number. Outliers were identified using R, and two-sample homoscedastic t tests performed using Microsoft Excel. PCAs were generated using MATLAB (V8.4.0 R2014b). Analysis of variance (ANOVA) and k-means clustering were also performed using the numerical software environment MATLAB (V8.4.0 R2014b). Significance levels are presented with lower case letters according to the results of Duncan’s test (p < 0.05). PCA and hierarchical clustering heatmaps were generated using COVAIN (Sun and Weckwerth, 2012). Replicate and error bar information is indicated in the figure legends.
DATA AND CODE AVAILABILITY

All codes are available as MATLAB scripts (Sun and Weckwerth, 2012). The complete model for inverse calculation is available as Data S1. The Metabolite data used for statistics and modeling are available in Data S1. RNA sequencing data have been deposited under GEO accession number GSE118119. The proteomics data are available in proteomexchange (http://www.proteomexchange.org/) with the accession number PXD010657. All other data are available upon request (wolfram.weckwerth@univie.ac.at).