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Cellular and computational models reveal environmental and metabolic interactions in MMUT-type methylmalonic aciduria

Charlotte Ramon1 | Florian Traversi2 | Céline Bürer2 | D. Sean Froese2 | Jörg Stelling1

1Department of Biosystems Science and Engineering and SIB Swiss Institute of Bioinformatics, ETH Zurich, Basel, Switzerland
2Division of Metabolism and Children’s Research Center, University Children’s Hospital Zurich, University of Zurich, Zurich, Switzerland

Correspondence
D. Sean Froese, Division of Metabolism and Children’s Research Center, University Children’s Hospital Zurich, University of Zurich, 8032 Zurich, Switzerland.
Email: sean.froese@kispi.uzh.ch
Jörg Stelling, Department of Biosystems Science and Engineering and SIB Swiss Institute of Bioinformatics, ETH Zurich, 4058 Basel, Switzerland.
Email: joerg.stelling@bsse.ethz.ch

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Abstract
Methylmalonyl-coenzyme A (CoA) mutase (MMUT)-type methylmalonic aciduria is a rare inherited metabolic disease caused by the loss of function of the MMUT enzyme. Patients develop symptoms resembling those of primary mitochondrial disorders, but the underlying causes of mitochondrial dysfunction remain unclear. Here, we examined environmental and genetic interactions in MMUT deficiency using a combination of computational modeling and cellular models to decipher pathways interacting with MMUT. Immortalized fibroblast (hTERT BJ5ta) MMUT-KO (MUTKO) clones displayed a mild mitochondrial impairment in standard glucose-based medium, but they did not show increased reliance on respiratory metabolism nor reduced growth or viability. Consistently, our modeling predicted MUTKO specific growth phenotypes only for lower extracellular glutamine concentrations. Indeed, two of three MMUT-deficient BJ5ta cell lines showed a reduced viability in glutamine-free medium. Further, growth on 183 different carbon and nitrogen substrates identified increased NADH (nicotinamide adenine dinucleotide) metabolism of BJ5ta and HEK293 MUTKO cells compared with controls on purine- and glutamine-based substrates. With this knowledge, our modeling predicted 13 reactions interacting with MMUT that potentiate an effect on growth, primarily those of secondary oxidation of propionyl-CoA, oxidative phosphorylation and oxygen diffusion. Of these, we validated 3-hydroxyisobutytyl-CoA hydrolase (HIBCH) in the secondary propionyl-CoA oxidation pathway. Altogether, these results suggest compensation for the loss of MMUT function by increasing anaplerosis through glutamine or by diverting flux away from MMUT through the secondary propionyl-CoA oxidation pathway, which may have therapeutic relevance.

KEYWORDS
constraint-based modeling, CRISPR-Cas9, metabolism, methylmalonic aciduria, rare disease

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1 | INTRODUCTION

Isolated methylmalonic aciduria (MMAuria) is an inborn error of metabolism caused by the loss of function of the enzyme methylmalonyl coenzyme A (CoA) mutase (MMUT), which transforms L-methylmalonyl-CoA into succinyl-CoA (metabolic reaction MMMm; Figure 1). MMAuria may result from mutations in the MMUT gene or in genes involved in the production of adenosylcobalamin, the cofactor of MMUT. Upon loss-of-function of the MMUT enzyme, upstream metabolites such as methylmalonic acid (MMA), propionyl-CoA, propionyl-carnitine and 2-methylcitrate accumulate. Individuals affected by MMAuria may have life-threatening episodes of metabolic crises and metabolic acidosis. Despite therapies including a low protein diet and supplemental carnitine or even liver transplantation, patients typically develop neurological complications and kidney failure.

Previous work suggested neurological damage is associated with mitochondrial energy impairment. This is supported by elevated levels of lactic acid in the globus pallidus, cerebrospinal fluid and plasma of patients. Further, lower activities of cytochrome c oxidase in the liver, kidney and muscle of patients and in the liver of mutant mice have been reported. However, other respiratory chain complex activities showed inconsistent results between different patients and studies for reasons that remain unclear.

Two mutually nonexclusive hypotheses aim to explain the clinical and biochemical observations of MMAuria. The “toxic metabolite” hypothesis attributes the reduced activity of the tricarboxylic acid (TCA) cycle enzymes and respiratory complexes to inhibition by organic acids (e.g., MMA) and CoA metabolites (e.g., propionyl-CoA) that accumulate in disease (Figure 1). Indeed, MMA induces cell damage in cultured neuronal cells. MMA inhibits TCA cycle and oxidative phosphorylation associated enzymes (e.g., pyruvate carboxylase) and mitochondrial transporters (e.g., malate shuttle, succinate transport). However, MMA failed to directly inhibit respiratory complex II in submitochondrial particles from bovine heart. Therefore, other MMAuria associated metabolites might play a role and explain neuronal cell death, such as 2-methylcitrate or propionyl-CoA, both of which accumulate in neurons exposed to MMA and in patient plasma. Consistently, 2-methylcitrate inhibits enzymes of the TCA cycle, and propionyl-CoA inhibits pyruvate dehydrogenase. The second, “TCA cycle depletion,” hypothesis suggests that the loss of MMUT enzyme function, which catalyzes an anaplerotic reaction replenishing the TCA cycle, leads to reduced TCA cycle flux and possible depletion of its metabolites.

![Metabolic pathways](image)

**FIGURE 1** Metabolic pathways surrounding the methylmalonyl-coenzyme A (CoA) mutase (MMUT) reaction. MMUT catalyzes the reversible transformation of succinyl-CoA into methylmalonyl-CoA (green) in the mitochondria. Metabolites known to accumulate in patients affected by MMUT deficiency are colored in red. OAA, oxaloacetate; akg, alpha-ketoglutarate; MMMm, reaction catalyzed by MMUT
with succinate partially rescuing MMA-exposed neurons from death.\textsuperscript{19}

Here, we ask how environmental and genetic perturbations could contribute to severe metabolic outcomes of MMUT-deficiency at the cellular level. A combination of cellular and computational models enables us to generate and validate detailed causal hypotheses. Our main findings are: (a) Despite showing mild mitochondrial impairment in standard conditions, MMUT-deficiency in cells does not lead to reduced growth or viability. (b) Growth-related phenotypes can be induced by glutamine limitation. (c) Metabolically, these phenotypes can be explained by increased nicotinamide adenine dinucleotide (NADH) metabolism, and exacerbated by perturbations in the secondary propionyl-CoA oxidation pathway. (4) Augmenting the influential “toxic metabolite” hypothesis for MMUT-type MMA, we postulate compensatory mechanisms for MMUT deficiency via anaplerotic and secondary propionyl-CoA oxidation pathways that may present novel therapeutic targets.
2 | RESULTS

2.1 Establishment and characterization of a MMUT-deficient cell-line model

To avoid variation typical for cell lines obtained from different individuals such as patient fibroblasts, we created three MMUT deficient (MUTKO) cell lines from wild-type (WT) immortalized fibroblasts (BJ5tA) using CRISPR-Cas9. We isolated the mutant cell lines as single clones, each of which contained a homozygous frameshift mutation in Exon 3 of the MMUT gene. Two separate clones harbored a deletion of two base pairs (c.613-614del, [Glu205Thrfs*5]; MUTKO2 and MUTKO3) and one clone harbored an addition of one base pair (c.417dup, [Leu140Serfs*8]; MUTKO7; Figure S1). In all three mutated cell lines, we detected no full-length MMUT protein (Figure 2A), and no MMUT activity (Figure 2B). We checked for potential off-target effects of the CRISPR-Cas9 procedure by whole-genome sequencing. MUTKO-2, 3, and, 7 showed 6962, 5952, and 7098 novel variants compared with the parental cell line, respectively (Table S9). However, only 10–13 variants per clone potentially affected genes, mostly pseudogenes or genes of unknown function, and all variants were found on a single allele and were noncoding (intronic, upstream, or downstream regions). Hence, the mutant cell lines have the desired metabolic perturbation and no other variants with a plausible effect on the metabolic phenotype.

2.2 MMUT-deficient cells show a reduced mitochondrial potential, but no growth phenotype

In a high glucose, high glutamax (glutamine-alanine dipeptide) medium (DMEM mixed with M199, see Section 4), we found no significant difference between MUTKO and WT growth rates (Figure 2C). However, tetramethylrhodamine methyl ester (TMRM) fluorescence, which accumulates in healthy mitochondria and measures mitochondrial potential,23 indicated that BJ5ta-MUTKO cells had significantly (≥20%) lower mitochondrial potential than BJ5ta-WT fibroblasts (Figure 2D). Similarly, in confocal microscopy characterization, although mitochondria of MUTKO cells displayed a similar aspect ratio (ratio of the major and short axis of an ellipse fitted to an object) as controls cells, they were slightly more circular (measured by the form factor, the inverse of circularity; its minimum value is one for a perfect circle24, Figure 2E and Figure S2). Although MMUT-deficiency reduces mitochondrial potential, it does not induce a growth phenotype in standard medium.

2.3 Changing medium conditions does not disrupt growth in MMUT-deficient cells

To induce growth disruption, we forced MMUT-deficient cells to rely more on respiratory metabolism, specifically by substituting glucose with galactose to increase reliance on oxidative phosphorylation.25–27 This reduced the growth rates of BJ5ta-MUTKO cells to a similar extent as controls (Figure 3A), suggesting that the respiratory defect of MMUT-deficient cells is not severe.

Because dramatic response to addition of precursor amino acids (threonine, valine, and isoleucine) were observed in MMUT-deficient mice,28 we next attempted increasing these pools (and of leucine) in the medium. Supplementation with all four amino acids jointly (Figure 3B) and individually (Figure S3A and Table S1) reduced the growth rates of all BJ5ta cell lines to a similar extent. Finally, to assess whether high concentrations of disease-related metabolites strongly affect viability in this cell type, we used medium with up to 100 mM MMA. However, we did not observe differences in viability between mutant and control cells (Figure 3C and Figure S3B). Hence, in our cell models, MMUT deficiency does not affect growth in these previously tested conditions, and the elucidation of growth phenotypes requires quantitative modeling.

2.4 Metabolic modeling predicts that MMUT-deficient cells compensate through anaplerotic pathways

We used structural sensitivity analysis29 to predict which fluxes change when MMUT is perturbed (see Section 4). This identified 380 out of the 5957 reactions featured in the model to be changed significantly (Figure 4 and Table S10). Specifically, for the MMUT deficient background, we predicted that: (a) fluxes in valine and threonine catabolism decrease, thereby increasing the extracellular concentrations of noncatabolizable pathway intermediates such as propionate and 2-methylcitrate; (b) anaplerotic TCA cycle fluxes, such as the flux from glutamate to succinyl-CoA through alpha-ketoglutarate dehydrogenase, the pyruvate dehydrogenase flux, and the citrate synthase flux increase to compensate for the reduced MMUT flux; and (c) extracellular concentrations of alpha-ketoglutarate and glutamate are reduced because of an increased intracellular need, for example, to feed in the TCA cycle. Such compensatory changes could explain the mild phenotypes observed in BJ5ta-MUTKO fibroblasts.
**FIGURE 3** Growth and viability in nonstandard media. (A) Growth rates of the BJ5ta fibroblasts were measured in normal DMEM with glucose, and when the carbon source was replaced with galactose. The circles are the raw data points, the error bars are the mean and standard deviations of the raw data. 
(B) Growth rates of the fibroblasts were measured in DMEM after adding leucine, isoleucine, valine and threonine each at a final concentration of 4 mM. c Viability as a function of the dose of methylmalonate. The viability was assessed after 72 h and the experiment was performed in a pH controlled manner (i.e., the acidity was neutralized with NaOH).

**FIGURE 4** Prediction of the effect of methylmalonyl-coenzyme A (CoA) mutase (MMUT) deficiency on the rest of the metabolic network. Selected metabolic fluxes and extracellular metabolites predicted to be increased (red) or decreased (blue) upon perturbation of MMUT using sensitivity analysis. akg: alpha-ketoglutarate; MMMm, reaction catalyzed by MMUT; OAA, oxaloacetate; red ellipse, postulated compensatory pathway.
FIGURE 5  Legend on next page.
2.5 | MMUT-deficient cells increase purine/pyrimidine and glutamine metabolism

To test the model predictions, we used a modified version of the tetrazolium-based redox assay, called Biolog phenotype microarrays. These assays measure NADH production when cells grow on various metabolites; absorbance is proportional to the NADH reductase activity and the number of living cells. For most metabolites, we found an increased signal in BJ5ta-MUTKO fibroblasts, in particular for MUTKO2, compared with WT (Figure 5A). Lenth analysis of unreplicated factorials (see Section 4) identified all metabolites that were significant for at least one cell line (Table S2 and Figure 5A).

Only inosine, D-salicin and alpha-D-glucose-1-phosphate were common to all MUTKO cell lines, with inosine and D-salicin showing the biggest increase. Overall, however, upregulated metabolites cluster in four categories: L-glutamine, glutamate and related dipeptides (in MUTKO2 and MUTKO7); purines and pyrimidines (adenosine, which is one step away from inosine, and to a lesser extent uridine and thymidine); organic acids (alpha-ketoglutarate,
succinamic acid, butyrate and mono-methyl succinate, mostly in MUTKO2); and sugars.

We performed the same experiment on CRISPR-Cas9 modified HEK293 cells deficient in MMUT, MMAB (an enzyme involved in MMUT cofactor synthesis), or both. We observed similar trends as in fibroblasts, in particular, upregulation with glutamine dipeptides (Gln-Gln, Gln-Gly) and adenosine (Figure S4).

Our modeling and Biolog results suggested that MUTKO cell lines have (a) an increased inosine metabolism and

| Reaction | Reaction description | Metabolic subsystem | FBA ratio | MoMA ratio |
|----------|----------------------|---------------------|-----------|------------|
| PRPNCOAHYDm | Propenoyl-CoA hydrolase (m) | Beta-Alanine metabolism | 0.95 | 0.79 |
| SUCD1m | succinate dehydrogenase | Citric acid cycle | 0.99 | 0.92 |
| r0596 | 3-hydroxypropionyl-CoA hydrolase | CoA synthesis | 0.95 | 0.79 |
| ATPS4m | ATP synthase (four protons for one ATP) | Oxidative phosphorylation | 0.97 | 0.91 |
| CYOR_u10m | ubiquinol-6 cytochrome c reductase, Complex III | Oxidative phosphorylation | 0.98 | 0.93 |
| NADH2_u10m | NADH dehydrogenase, mitochondrial | Oxidative phosphorylation | 0.99 | 0.98 |
| CYOOm3 | cytochrome c oxidase, mitochondrial | Oxidative phosphorylation | 0.99 | 0.98 |
| MMSAD3m | methylmalonate-semialdehyde dehydrogenase, mitochondrial | Propanoate metabolism | 0.95 | 0.79 |
| r0365 | 3-hydroxypropionate:NAD+ oxidoreductase | Propanoate metabolism | 0.95 | 0.79 |
| O2t | o2 transport (diffusion) | Transport, extracellular | 0.88 | 0.72 |
| O2tm | O2 transport (diffusion) | Transport, mitochondrial | 0.99 | 0.97 |
| r0838 | Free diffusion of ammonia | Transport, mitochondrial | 0.99 | 0.98 |
| r0193 | L-Cysteine L-homocysteine-lyase (deaminating) | Cysteine metabolism | 0.99 | 0.98 |

FIGURE 7  Genetic interactions with MMUT. (A) Single-reaction deletion in silico experiment. Reactions that create a growth defect in mutant versus wild-type (WT) simulated in a DMEM medium and corresponding growth ratio (see Section 4). (B) Growth of WT (blue) and MMUT-deficient (red) HEK293 cells when an additional gene is knocked-out: carnitine palmitoyltransferase II (CPT2), 3-hydroxyisobutyryl-coenzyme A (CoA) hydrolase (HIBCH), succinate dehydrogenase complex flavoprotein subunit A (SDHa) or when no other gene is knocked-out (None). Cell growth was measured using the Crystal Violet assay; absorbance is proportional to the number of cells. Dots: wells; curve: fit of a nonlinear mixed effect model based on a logistic curve (see Section 4). (C) MoMA (minimization of metabolic adjustment33) predictions of growth ratio of MMUT-deficient cells to WT when another gene is knocked-out (horizontal axis) or when the flux through reaction r0596 (3-hydroxypropionylCoA hydrolase) is blocked.
(b) an increased L-glutamine/glutamate metabolism. To pursue these hypotheses, we measured secretion and uptake rates in the spent medium of BJ5ta cell lines. The extracellular concentration of hypoxanthine (derived from inosine) was increased for MUTKO cell lines compared with WT at 24 h, followed by a decay (Figure 5B). When compared with WT, all MUTKO cell lines significantly increased glutamate secretion (Figure S5A), and MUTKO2/3 cells also significantly increased glutamine consumption (Tables S6 and S7).

We hypothesized that MUTKO cells depend more on glutamine to compensate for the reduced flux through the TCA cycle. Indeed, MUTKO2 and MUTKO3 died faster in a glutamine-free medium, while growth rates for increasing doses of glutammax were not significantly different from WT (Figure 5D,E and Tables S3 and S8). In contrast, MUTKO7 grows well in the absence of glutamine at early time points (Figure 5D) and its growth rate is not strongly stimulated in increasing concentrations of glutammax (Figure 5E), suggesting it does not rely on glutamine for growth. Overall, the results are consistent across experiments per cell line, but they illustrate clonal variability resulting from modifying cells with CRISPR-Cas9.

2.6 | In silico prediction of MMUT function

To interpret these results, we analyzed under which conditions the reversible reaction catalyzed by MMUT (MMMm) transforms methylmalonyl-CoA into succinyl-CoA (positive flux in the model), thereby fulfilling its anaplerotic function. Flux variability analysis predicted a positive flux through MMMm either with ATP or biomass flux at their maximum, or a linear combination of both (Figure 6A and Figure S5A). Also, for limiting uptake of glucose and glutamine from the medium, maximal growth of MUTKO cells is predicted to decrease compared with WT, but the reduction to 99% of WT is small (Figure 6B). Importantly, this differential growth arises when the MMMm flux becomes positive (Figure S5B). This is consistent with a decreasing maximum growth rate when there is an increasing fixed positive flux through MMMm (Figure 6C). To explain the upper limit to the MMMm flux for maximal growth, we identified which components of the biomass reaction limit growth (see Section 4). We predicted growth with and without each component i, yielding a positive difference of growth rates (δi) when compound i limits growth. As the MMMm flux increases, δi for direct (isoleucine, valine, and threonine) and more distant (deoxyctydine triphosphate (dCTP), deoxythymidine triphosphate (dTTP), cytidine triphosphate (CTP), and uridine triphosphate (UTP)) precursors of MMMm increases (Figure S6). Hence, a high flux through MMMm may be disadvantageous for the cell, explaining why MMMm will function in the forward direction only under stringent conditions.

2.7 | Metabolic interactions with MMUT

Finally, to investigate metabolic interactions, we predicted single-reaction deletions that decrease the growth ratio (see Section 4). We found only 13 out of the ~5400 model reactions to interact with the MUTKO phenotype (Figure 7A). They mostly relate to the secondary oxidation of propionyl-CoA and to respiration (respiratory chain complexes and oxygen transport), resulting in the smallest growth ratio of 88%. Because there can be many-to-many relations between genes and reactions, we repeated the analysis by knocking out individual genes, yielding consistent results (Table S11).

Based on the predictions, we created CRISPR-Cas knock-outs (KO) of HIBCH, CPT2, and SDHa as they belong to very different metabolic pathways in the WT and MUTKO background of HEK293 cells. To control for potential side effects of the CRISPR procedure we used the parental cell lines as well as control cells that went through one unsuccessful round of CRISPR-Cas9 (see Section 4). Growth rates of each cell line were measured (Figure 7B) and we assessed the significance of genetic interactions between MMUT and CRISPR-Cas KO with a nonlinear mixed effect model for cell growth measured by the Crystal Violet assay (see Section 4 and Table S12). We found a significant interaction between HIBCH and MMUT (Table S13). Importantly, the HIBCH enzyme catalyzes multiple reactions: the hydrolysis of 3-hydroisobutyryl-CoA in valine catabolism, as well as the hydrolysis of 3-hydroxypropionyl-CoA (reaction r0596) in the secondary propion acid oxidation pathway. Looking back in the model, we found the growth difference to be explained by reaction r0596 (Figure 7), suggesting that the secondary oxidation pathway of propionyl-CoA interacts with loss of MMUT function, consistent with our previous results.

3 | DISCUSSION

The exact pathomechanisms of MMAuria remain largely unknown, because of the interconnected nature of the human metabolic network. Our approach is distinct from earlier studies of individual enzymes in analyzing the network effects of perturbations with cellular and computational models. Further, we focused on growth and viability that integrate multiple signals in the cell. Assays specifically measuring mitochondrial function
Specifically, MMUT is expressed ubiquitously and in MMUT-deficient fibroblasts. Interestingly, we predict a global reduced oxidative phosphorylation and the pathway is functional in fibroblasts. This illustrates how the medium can be crucial when studying a metabolic disease.

Regarding glutamate metabolism, we observed increased glutamate secretion in BJ5ta-MUTKO fibroblasts. In liver cancer cells, which also display increased reliance on glutamine metabolism, increased glutamate secretion was hypothesized to be a consequence of increased flux through the de novo purine synthesis pathway, which also transforms glutamine into glutamate besides producing purines. This hypothesis would be consistent with our findings.

To interpret these results, we used constraint-based modeling. We predicted increased anaplerotic fluxes towards the TCA cycle and reduced oxidative flux in the TCA cycle. This is consistent with glutamate dehydrogenases being upregulated in the hepatic proteome of MMAPeria patients and in MMUT-deficient fibroblasts. Interestingly, we predict a global reduced oxidative TCA cycle flux consistent with a recent study, except for alpha-ketoglutarate dehydrogenase for which we predict increased flux, while the protein amount was decreased in MMUT-deficient primary fibroblasts. This discrepancy could be due to protein–protein interactions between OGDH and MMUT or kinetics that are not captured by the model.

In predicting which metabolic reactions would interact with MMUT to create a growth phenotype, we found that reactions involved in oxidative phosphorylation and the secondary propionyl-CoA oxidation pathway interact with MMUT deficiency. In one experimental test of the predictions, HIBCH, part of the secondary-propionyl-CoA secondary oxidation pathway, indeed interacted with MMUT by diverting flux to this secondary pathway. This pathway was transcriptionally activated in C. elegans on vitamin B₁₂ deficient diets. This illustrates how the combination of modeling and experiments yields interpretable hypotheses. However, our modeling analyses predict fluxes, which we did not experimentally validate and which could be the starting point of future studies. The increase of flux through the TCA cycle could be validated in cell line models using 13C flux analysis. Further, the activity of HIBCH could be measured and perturbed in different cell types of a MMUT-deficient mouse model to study its consequence in vivo.

Finally, we observed clonal variability of the BJ5ta-MUTKO cells that was not apparently caused by genetic modifications due to CRISPR-Cas9 offtarget effects. It could be the result of the clonal selection of single cells.
This variability complicated the interpretation of our experiments, but evidenced the need to test multiple mutants to avoid over-interpreting results. Although we cannot exclude that the effects we report are due to the CRISPR-Cas9 modification and not the MMUT deficiency, the findings are consistent with other models of the disease, as discussed above.

Overall, MMUT-type MMAuria has complex and mild effects in fibroblasts, which could be linked to the reduced TCA cycle flux. This work suggests novel candidate metabolites and pathways to test in the future.

4 | METHODS

4.1 | Cells

The human foreskin fibroblasts immortalized with hTERT were bought from ATCC (BJ5ta, CRL 4001) and were cultured as recommended in medium A: DMEM with Glutamax (31966047, Thermofisher), mixed in a 4:1 ratio with M199 (Thermofisher, 41150020), 10% (vol/vol) fetal bovine serum (FBS) and 0.01 mg/ml hygromycin. Human Embryonic Kidney (HEK293) cells were bought from ATCC (HEK293 CRL 3216) and cultured in DMEM with Glutamax, 10% FBS and 1× Antibiotic Antimycotic (15240062, Thermofisher). Cells were kept at 37°C under 5% CO2 atmosphere in 100 mm dishes. The cells were tested negative for mycoplasma contamination using Mycoplasmacheck service (Eurofins).

4.2 | CRISPR-Cas9 generation of KO

To generate KO in BJ5ta and HEK293 cells, we used CRISPR/Cas9 technology.45 BJ5ta cells and HEK293 cells were transfected using the 4D nucleofector (Lonza) and Neon transfection system (Thermo Fisher Scientific), respectively. Single-guide RNAs (sgRNAs) were provided either as gBlocks (IDT technologies)46 with a Cas9 plasmid (Addgene: 62988) or directly as a ribonucleoprotein complex (Cas9 Nuclease: A36496; IVT gRNA: A29377). After 48 hours, cells were transferred to a 96-well plate to obtain an average of 1 cell/well and cultured for clonal expansion. Sanger sequencing was performed for selection on genomic DNA. sgRNAs targeting MMUT in BJ5ta cells were designed to target Exon 3 of MMUT: ATAGTAACCTGGAAGAACA (sgRNA1) and ACGATGTGCAGCAGTCAA (sgRNA 2). In HEK293 cells, the sgRNA targeting MMUT was ATTTCTTATAATATATATTGGC. KO of HIBCH, CPT2, and SDHa was performed both on WT HEK293 cells and on successful MUT-KO HEK293 pre-generated cells. GRNAs were designed as follow: HIBCH: GCAGATTTATCCACAGCTAAAGG, CPT2: TGTGTTGTTGCGTGGAGCCTGG, SDHa: ACCTGTGCCACCCGACGACTGG. Control cells were WT HEK293 or MUT-KO HEK293 cells which have gone through CRISPR/Cas9 editing but did not show any KO of our targeted genes.

4.3 | Validation of KO using western blotting

Lysates from a confluent T75 were obtained using RIPA buffer and were mixed with RIPA lysis buffer and 2X Lammli buffer (containing 5% (vol/vol) β-mercaptoethanol) to obtain a protein concentration of 1.25 μg/μl. Electrophoresis was performed on a sodium dodecyl sulfate (SDS) page gel (10% polyacrylamide) in Tris-glycine buffer using 20 μl of protein. Proteins were transferred using the semidyed method onto a nitrocellulose membrane (Whatman, GE Healthcare). The membrane was blocked in the blocking buffer (5% skimmed milk, 20 mM Tris base, 150 mM NaCl, 0.2% Tween 20) for 1 h at RT, incubated with primary antibodies (anti-MMUT: ab67869, Abcam/ anti-ACTIN, Sigma, A1978) overnight at 4°C diluted 1:500 in blocking buffer and incubated with secondary antibodies (anti-mouse HRP, Sc516102-CM, Santa Cruz) diluted 1:5000 in blocking buffer for 1 h at RT. Signal was detected using the Clarity ECL Substrate (Biorad, 1705060 S) and ChemiDoc™ Touch Imaging System.

4.4 | Whole-genome sequencing and analysis

DNA was extracted using a blood and cell culture DNA mini kit (13 323, Qiagen) from low passage cell lines, as recommended. For the sequencing, library preparation was performed using a KAPA HyperPrep Kit PCR-free (Roche) with a target insert size of 500 bp. Paired-end libraries were sequenced using a GFB Novaseq 6000 sequencer (Illumina, RTA Version: v3.4.4). Base calls were converted into FASTQ files using bcl2fastq v2.20.0.422 and further analyzed to find the germline variants.

Raw reads are quality controlled. Sequencing adapters are removed from the reads and trimmed reads are aligned to the reference genome (GRCh38). Aligned reads are post-processed (removal of secondary alignments and PCR duplicates) and recalibration of quality scores is performed. Germline variant calling and filtering is performed for each cell line. Three separate variant callers are combined: Strelka, v2.9.2, GATK HaplotypeCaller, v4.1.6.0, and Varscan v2.4.3. Only calls
which are detected by at least two out of three tools are kept. Variant annotation is performed by VEP (v103.1). Only variants with a frequency higher than 30% are used since we expect 50% and 100% variant frequency for germline variants. Variants are compared across cell lines and only variants for which not a single read can be found in the WT cell line are considered novel in the mutant cell line.

4.5 | MMUT activity assay

MMUT enzyme activity assay was performed using crude cell lysates as described.43

4.6 | Cell proliferation and viability experiment in different media

These experiments were performed in DMEM without glucose, glutamine and phenol red (Thermofisher, A143001). Unless otherwise stated, this medium was supplemented with 25 mM glucose (Sigma, G5388), 1 mM Glutamax (Thermofisher, 35 050 061), 0.04 mM phenol red (ATCC, PCS-999-001) and 10% (vol/vol) dialyzed FBS (Thermofisher, 26400044).

A total of 2500 cells were seeded per well of a 96-well plate (IBIDI, 89626) in medium A. The following day, the wells were rinsed and replaced with the desired medium. Cell proliferation was assessed by imaging cells using microscopy (Figure S7A) The cells were imaged using a Nikon Ti2 microscope and an Andor Sona CSC-002200 camera at +150 μm above the focal plane using bright field imaging and using a magnification of 6× (4× plan Apo λ objective na = 0.2, and 1.5× zoom). Three or four days later, the cell nuclei were stained using the Nucblue stain (Thermofisher, R37605) as recommended and imaged. Cells were counted by segmenting the bright-field and fluorescent images using Fogbank algorithm47 (segmentation parameters: Table S14). The same number of cells are counted using bright-field imaging and nuclear staining when the number of cells in the field-of-view is below 1500 cells (Figure S7B) at the beginning of the experiment. The resulting growth rate (gr) is $\text{gr} = \ln(N(t) / N(t_0)) / \Delta t$ and has correspondingly unit (1/h).

To assess the viability of the cells upon treatment with a compound or a nutrient, cells were stained with a membrane impermeable dye Sytox (Thermofisher, S7020, diluted 1:5000), on top of the membrane permeable dye (Nucblue). A cell is then considered viable if it stained by the membrane permeant dye but not by the membrane impermeant dye.

4.7 | TMRM experiment and confocal imaging

Cells were seeded in a 96 well-plate. One day later, cells were stained with 50 nM Tetracythylrhodamine, Methyl Ester, Perchlorate (TMRM; Thermofisher, T668) diluted in medium A devoid of phenol red for 30 min at 37°C in the dark. After rinsing, fluorescent (20× Plan Fluor objective, excitation: 551 nm, Cy3 light filter) and bright-field images were acquired. A mask of individual cells was obtained by segmenting the bright-field image. The total fluorescence of a cell was computed by summing the blanked fluorescence of each pixel inside the cell, where the blank is the fluorescence of the background.

Mitochondria were also imaged using a Leica confocal microscope, using a HC PL APO 63×/1.40 oil objective. The difference in their shapes were assessed using descriptors such as the form factor and the aspect ratio of the mitochondria, which were computed using an existing script.44

4.8 | Biolog phenotype microarray

A total of 20 000 cells per well were resuspended in the assay medium (IF-M1[Biolog Inc., BL-72301], 0.3 mM L-glutamine, 5% FBS) and seeded in Biolog plates PMM1 (BL-13101) and PMM2 (BL-13102). After 46 h of incubation at 37°C, 5% CO2, the dye MB (Biolog Inc., BL-74352) was added. After 24 h, the absorbance of the plate was measured at 590 nm and 750 nm. Where $a_{m,d,g}$ denotes the absorbance at 590 nm subtracted with the absorbance at 750 nm, where $m$ is the metabolite considered, $g$ is the cell line, and $d$ is the day the experiment.

For details on the Lenth statistical analysis of Biolog data, see Methods S1.

4.9 | Metabolite detection experiment

A total of $10^6$ cells were seeded per 100 mm dish in duplicates. One day later, dishes were rinsed and 11 ml of a medium containing a DMEM free of glucose and glutamine, with 6.25 mM glucose, 1 mM glutamine, 50 μM inosine, 10% dialysed FBS and hygromycin B. In each dish, two times 1 ml of spent medium samples were taken at 24 and 48 h. Immediately after retrieval, samples were counted and were flash-frozen in liquid nitrogen and stored at −80°C for further analysis.

Hypoxanthine concentration was measured using the inosine fluorometric assay kit (Abcam, ab126286) without the converter enzyme. Glutamine and glutamate were assayed using a glutamine assay kit (Abcam,
ab197011), as recommended for glutamine and without the hydrolysis enzyme mix for glutamate.

More details on the parameter estimation for glutamate and glutamine can be found in Methods S1.

### 4.10 Crystal violet assay

A total of 12,500 cells were seeded on Poly-L-Lysine well of a 24-well plate in duplicates. Cells were counted using the crystal violet assay (Sigma, C6158) at Days 0, 1, 2, 3, 4, and 5. For each measurement, the well was washed using Dulbecco’s Phosphate Buffered Saline (DPBS), fixed in 200 μl of 4% paraformaldehyde (PFA) for 10 min, incubated with crystal violet (0.2%) for 20 min. The plate wasinked three times under water to remove the extra crystal violet. 1000 μl of 1% SDS was then added to each well followed by 30 min of shaking at RT. Absorbance was measured at 540 nm in a Tecan microplate reader.

A nonlinear mixed effect model was then fitted to the data. For a cell line  \(i\), the following nonlinear mixed effect model was estimated:

\[
y_{Li} = \frac{\text{asym}}{1 + \exp \left(\text{thresh} - f \cdot \text{scal}_i\right)} + e_{Li}, \quad e_{Li} \sim \mathcal{N}(0, \sigma^2)
\]

\[
\text{scal}_i = \text{background} \cdot \text{KO} + r_{\text{scal}}, \quad r_{\text{scal}} \sim \mathcal{N}(0, r_{\text{scal}}^2)
\]

where background and KO describe whether the cell line is MMUT deficient and if another gene was knocked-out, respectively.

### 4.11 In silico modeling

We performed constraint-based modeling with human reconstruction Recon 2.2 \(^{48}\) and simulated DMEM medium (see Table S15). To predict maximum growth and maximum ATP generation, we used standard flux balance analysis \(^{49}\) or minimization of metabolic adjustment. \(^{33}\) Varying amounts of glucose and glutamine were modeled by fixing the uptake fluxes as indicated. Reaction deletions were modeled by fixing the respective fluxes to zero.

To understand which compounds limit growth, we added artificial uptake reactions for components \(C_i\) with upper bound fixed to 1. If only \(C_i\) limits growth, biomass flux increases by \(1/c_i\), where \(c_i\) is \(C_i\)’s stoichiometric coefficient in the biomass reaction. We computed the resulting biomass increase as \(\delta_i = b_i - b_0\), where \(b_i\) (\(b_0\)) is the biomass flux after (before) adding \(C_i\).

To predict the spreading of the MMUT perturbation in the network, we used structural sensitivity analysis. \(^{29}\) Specifically, we computed the adjustments \(d_{k,e}\) of exchange fluxes \(e\) to a perturbation \(\delta_k\) of the MMMm reaction \(k\), yielding sensitivities

\[
s(k, e) = \frac{d_{k,e}}{|\delta_k|}
\]

To decide whether these sensitivities are significant, we compared them with the distribution of the sensitivities to all internal reactions \(i\) being perturbed \(s(i, e)\). With the median \(\text{med}_e\) and median absolute deviation (MAD) \(\text{mad}_e\) of this distribution, we classified metabolite \(e\) as a biomarker when

\[
|s(k, e) - \text{med}_e| > t_{\text{mad}} \text{mad}_e
\]

with \(t_{\text{mad}} = 30\), similar to previous methods for outlier detection. \(^{50}\)

### AUTHOR CONTRIBUTIONS

D. Sean Froese and Jörg Stelling conceived of and supervised the work. Charlotte Ramon performed the experiments and did the modeling. Charlotte Ramon and Céline Bürer supported CRISPR-Cas9-mediated creation of BJ5ta MMUT deficient cell lines, Céline Bürer and Florian Traversi generated the HEK293 CRISPR-Cas9 cells. Charlotte Ramon, Céline Bürer, and Florian Traversi performed the crystal violet assays. The Lenth statistical analysis was performed by Hans Michael Kaltenbach. Charlotte Ramon wrote the article with support from D. Sean Froese and Jörg Stelling.

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### CONFLICT OF INTEREST

Charlotte Ramon, Florian Traversi, Céline Bürer, D. Sean Froese, and Jörg Stelling declare that they have no conflict of interest.

### DATA AVAILABILITY STATEMENT

The data and custom code that support the findings of this study are available at https://doi.org/10.3929/ethz-b-000578898.
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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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