Supporting Information

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SI Materials and Methods

Culture of HEK 293 Cells. HEK 293 cells (293-H cells; Invitrogen) were cultivated in DMEM supplemented with 10% heat-inactivated FBS and penicillin (100 U/mL)/streptomycin (100 μg/mL) at 37 °C in a 5% CO_2 humidified incubator.

Cell Transfection. ***Plasmids.*** *Pfkfb3v5* (GenBank accession no. BC040482) and *Cdh1* (GenBank accession no. BC013413) were subcloned by PCR in pcDNA3.3 (Invitrogen) using ORF clones IMAGE: 5271907 and IMAGE: 3160334, respectively, as templates (Geneservices). Site-directed mutagenesis of the KEN box in *Pfkfb3* was carried out according to the manufacturer's instructions (QuikChangeII; Stratagene) using the primer ATCCTTCATTTTGCCGCAGCAGCTGACTTTAAGGCG (and its reverse and complementary primer; Invitrogen). Underscored nucleotides indicate the mutated amino acids (KEN→AAA). A *Gls1* expression plasmid was obtained from OriGene Technologies (pCMV6XL4-*Gls1*). Δ*Gls1* was generated by subcloning in pcDNA3.3 a PCR product encoding for the GLS1 protein in which the last 14 amino acids (including the KEN box; Fig. 5*F*) were removed. *Pfkfb3, Gls1*, and scrambled shRNAs were obtained from Open Biosystems Human GIPZ shRNAmir lentiviral library (RHS4531, RHS4430, and RHS4346, respectively). All plasmid DNAs used for transfection (except for commercial pmaxGFP) were prepared using an EndoFree Plasmid Maxi Kit (Qiagen) following the manufacturer's protocol and confirmed by sequencing.

Nucleofection. T lymphocytes that had been activated to proliferate 24 h earlier were nucleofected with 4 μg of plasmid DNA at a cell density of 5×10^6 cells per 100 μL of nucleofector solution (human T cell nucleofector kit VPA-1002; Lonza) with the Amaxa Nucleofector II device using program T-23 (Lonza). After nucleofection, cells were immediately transferred into prewarmed culture medium (DMEM; Invitrogen) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated FBS (or autologous serum). Cells were cultured in six-well plates at 37 °C in a 5% CO_2 humidified incubator. Six hours after nucleofection cells were restimulated with anti-CD3/anti-CD28 antibodies for a further 42 h and then analyzed accordingly.

Transfection of HEK 293 cells. HEK 293 cells (18 h after seeding at 8×10^4 cells/cm^2) were transfected using Lipofectamine LTX reagent (Invitrogen) following the manufacturer's instructions. The total amount of DNA transfected in each experiment was 1.6 μg, comprising 0.4 μg *Gls1* (or β-gal) plasmid DNA (or empty vector) and 1.2 μg *Cdh1* plasmid DNA (or empty vector). Cells were harvested for protein extraction 24 h later.

Western Blotting. Aliquots (20–30 μg of protein) of purified T-lymphocyte (or HEK 293 cell) lysates, obtained with Cytobuster (Novagen) plus protease inhibitor mixture (Roche), were cleared by centrifugation (14,000 × *g*, 10 min). Supernatants were heated for 5 min at 95 °C under reducing conditions and then electrophoresed in a 4% to 15% gradient SDS acrylamide gel (Bio-Rad). Proteins were transferred electrophoretically to nitrocellulose membranes (Amersham), which were blocked in 5% (wt/vol) low-fat milk in 50 mM Tris-HCl pH 7.5, 150 mM sodium chloride, and 0.05% (wt/vol) Tween 20 for 30 min, and further incubated with antibodies against immunopurified PFKFB3 (custom antibody), Cdh1 (CC43; Calbiochem), geminin, (gift from Kai Stoeber, University College London, London, United Kingdom), GLS1 [12855–1-AP (Proteintech) and ab60709 (Abcam)], β-gal (ab616; Abcam), or GAPDH (ab9485; Abcam) overnight at 4 °C. After incubation with horseradish peroxidase-coupled secondary antibody (DAKO) for 1 h at room temperature, signal detection was performed by enhanced chemiluminescence (ECL plus; Amersham). A polyclonal antibody against PFKFB3 was obtained by rabbit immunization with 100 ng of the Keyhole Limpet hemocyanin-coupled synthetic peptide NMKGSRSSADSSRKH (Thermo Fisher Scientific), which is common to the translational products of the *Pfkfb3* mRNA splice variants K5 and K6 (1). Antisera were purified by affinity chromatography using columns of cyanogen bromide-activated Sepharose coupled to the peptide. For Western blotting after immunoprecipitation the antibodies used were PFKFB3 (H00005209-M08; Tebu-Bio) and Protein A HRP (GE Healthcare).

RT-qPCR. Total RNA was isolated using Qiagen RNeasy kit. First-strand cDNA was synthesized using the SuperScript III reverse transcriptase enzyme and oligo dT as primer (Invitrogen). Control PCR reactions were also performed on total RNA that had not been reverse-transcribed to test for the presence of genomic contamination. RT-qPCR reactions were performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in triplicate (Mastercycler ep realplex; Eppendorf). Gene-specific primers were designed by Primer Express 2.0 (Applied Biosystems) and then subjected to blast analysis to ensure the primer specificity. The primers used were the following: *Gls1*, forward, AGTGACTTGTGAATCAGCCAG; *Gls1*, reverse, GTTGCCCATCTTATCCAGAGG; *Gls2*, forward, CCATCTCCATAAGCACCCTAG; *Gls2*, reverse, AATTCCGATCCCCTGTTTCC; *Pfkfb3*, forward, AGTGATCATGGAGCTGGA; *Pfkfb3*, reverse, GGTGTGAAGAGGGCATTT; 18S, forward, CGCCGCTAGAGGTGAAATTC; and 18S, reverse, TTGGCAAATGCTTTCGCTC. Relative expression of *Pfkfb3* or *Gls1* to 18S was normalized to that observed in nonactivated cells. The specificity of the products of the RT-qPCR was verified by PCR melting curves and/or on 2% agarose gels.

Identification of the *Pfkfb3* Alternative Splice Variants in Human T Lymphocytes. Total RNA samples (1 μg) of these cells were reverse-transcribed as described earlier. cDNA was then subjected to PCR using the following forward and reverse oligonucleotides, respectively: TGAAACTGACGCCTGTCG and CCCACGGCTAAAGGCAGG. These primers were designed to span all reported variants of *Pfkfb3*. PCR conditions were as follows: 2 min at 95 °C; 35 cycles of 20 s at 95 °C; 20 s at 58 °C and 30 s at 68 °C; and then a final extension was carried out for 15 min at 68 °C. Amplicon products were resolved in agarose gel (0.8%) and visualized using SybrGreen Safe (Invitrogen). Bands obtained were cut from the gel, melted at 55 °C in the presence of 4M KI, purified using Qiagen columns, and finally identified by sequencing.

Immunoprecipitation. T lymphocytes were activated and, after 48 h, were incubated in the presence or absence of the proteasome inhibitor MG132 (10 μM) for 2 h. Purified T-lymphocyte lysates were prepared as described for Western blotting. Protein A Sepharose magnetic beads (Immunoprecipitation Kit–Dynabeads Protein A; Invitrogen) were prepared following the manufacturer's guidelines, and then incubated with anti-ubiquitin (10 μg, raised in rabbits; DAKO) for 10 min at room temperature. To immunoprecipitate PFKFB3 or GLS1, cell lysates (approximately 0.4 mg) were incubated with the bead/Ab complex for 2 h at room temperature on a rotating wheel. Beads were then washed four

times in 200 μL washing buffer. Immunoprecipitated proteins were eluted by heating at 70 °C for 10 min in 20 μL elution buffer and 10 μL SDS loading buffer. Proteins were detected using antibodies raised in a different species to avoid cross-reactivity with the immunoprecipitated complexes (e.g., anti-PFKFB3 raised in mouse for PFKFB3 immunoprecipitation) or with a Protein A HRP secondary antibody, which detected only native immunoglobulins (e.g., GLS1 immunoprecipitation).

DNA Replication Analysis by Flow Cytometry. The proportion of T lymphocytes in S phase of the cell cycle was assessed by flow cytometric analysis of BrdU incorporation into DNA. One hour before each endpoint, 10 μM BrdU was added to the proliferating cells and an APC-BrdU flow kit was used according to the manufacturer's instructions (BD Pharmingen). Cell acquisition and analysis were carried out in the CyAn ADP flow cytometer (DAKO/Becton Dickinson) by quantifying the populations in G0/1, S, and G2/M in a dot plot distribution of cells synthesizing DNA (determined by BrdU incorporation using allophycocyanin-conjugated anti-BrdU antibodies) versus total DNA content (measured by 7-amino-actinomycin fluorescence).

Statistical Analysis. Data obtained under different conditions were compared using an ANOVA test. Differences were evaluated using a Student t test. Values stated are means ± SEM. Results were considered to be significantly different at $P < 0.05$.

1. Kessler R, Eschrich K (2001) Splice isoforms of ubiquitous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in human brain. *Brain Res Mol Brain Res* 87: 190–195.

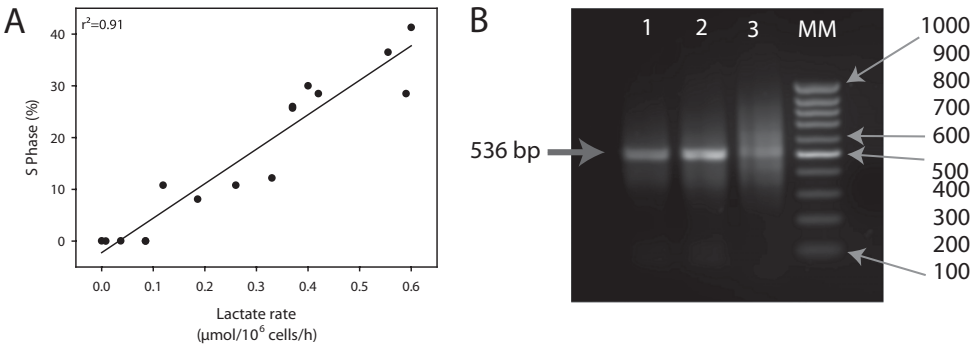

Fig. S1. (*A*) Correlation between S phase and rate of lactate generation in T lymphocytes at different stages of proliferation (n = 3). In noninduced cells there was no generation of lactate and no cells were in S phase. (*B*) Alternative splicing of *Pfkfb3* in T lymphocytes. Three independent samples of total RNA (numbered 1–3) from activated T lymphocytes (72 h) were subjected to cDNA synthesis following PCR using primers designed to span all of the known splice variants of *Pfkfb3* (for details see *SI Materials and Methods*). After sequencing these products, the splice variant most abundant in T lymphocytes was found to be variant 5.

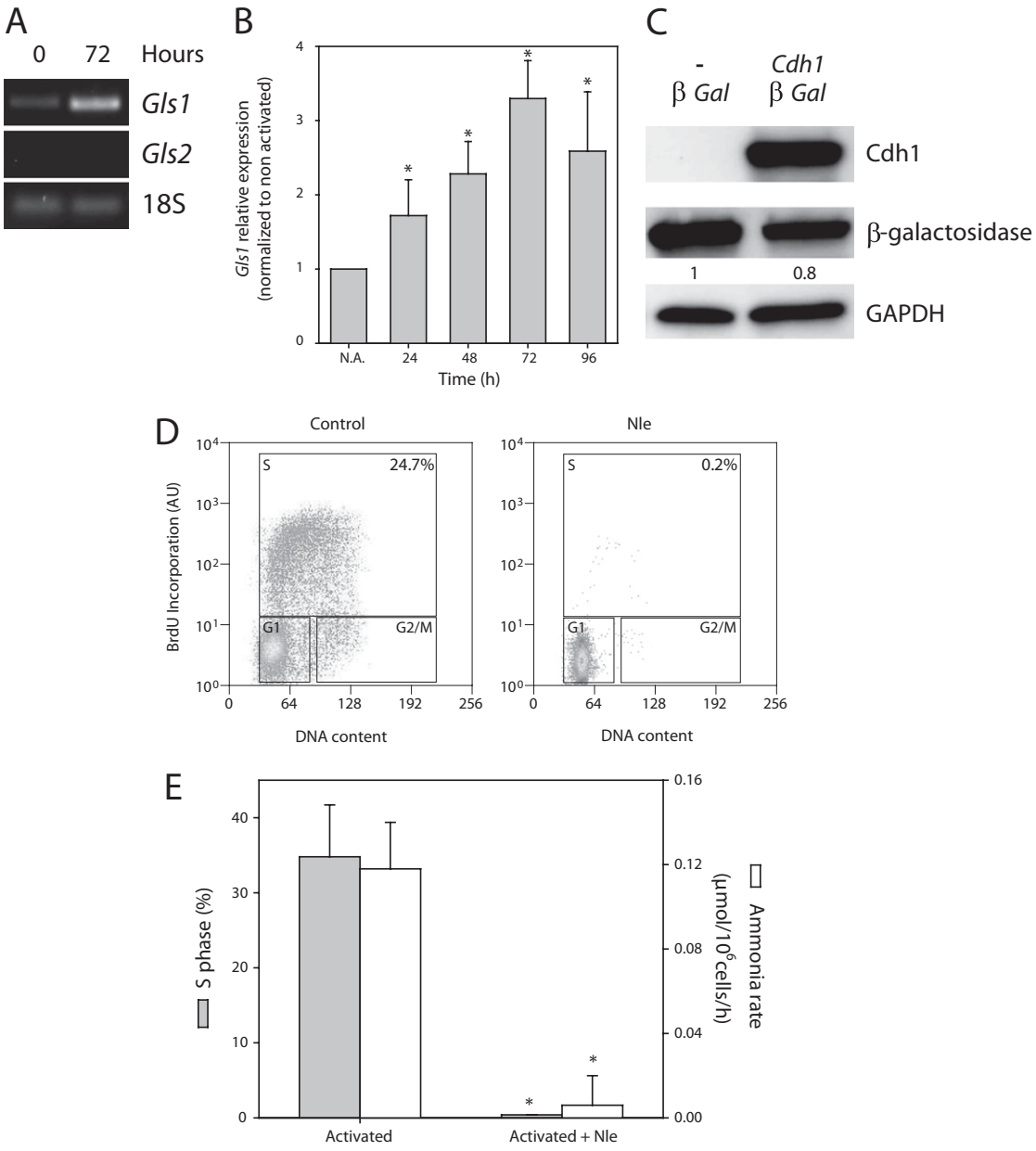

Fig. S2. (*A*) *Gls1* and *Gls2* mRNA expression in T lymphocytes. Agarose gel (2%) showing *Gls1* and *Gls2* RT-PCR products after 30 cycles from noninduced T lymphocytes or cells induced to proliferate (72 h). 18S was used as a control. (*B*) *Gls1* expression in proliferating T lymphocytes. The expression of *Gls1* mRNA in T lymphocytes increased with time after activation up to 72 h. The relative expression of *Gls1* was determined by RT-qPCR, as described in *SI Materials and Methods*. Results are the mean ± SEM of three independent experiments; $*P < 0.05$ versus nonactivated (N.A.) T lymphocytes. (*C*) Cdh1 did not affect the overexpression of a protein that does not contain a KEN box motif. β-Gal was overexpressed in HEK 293 cells in the absence or presence of overexpressed Cdh1 and identified by Western blotting. Proteins from a Western blot representative of three independent experiments were quantified by densitometry. (*D*) Effect of inhibition of glutaminase on proliferation in activated T lymphocytes. The transition of activated T lymphocytes into S phase at 72 h was virtually abolished by treatment with 6-diazo-5-oxo-L-norleucine (Nle, 250 μM). Result representative of three experiments. AU, arbitrary units. (*E*) Effect of inhibition of glutaminase on proliferation and ammonia production in activated T lymphocytes. Proliferation and the rate of ammonia production in activated T lymphocytes were significantly reduced by treatment with Nle; $n = 3$, mean ± SEM; $*P < 0.05$ versus untreated cells.