The cytotoxic T cell proteome and its shaping by the kinase mTOR

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We used high-resolution mass spectrometry to map the cytotoxic T lymphocyte (CTL) proteome and the effect of the metabolic checkpoint kinase mTORC1 on CTLs. The CTL proteome was dominated by metabolic regulators and granzymes, and mTORC1 selectively repressed and promoted expression of a subset of CTL proteins (~10%). These included key CTL effector molecules, signaling proteins and a subset of metabolic enzymes. Proteomic data highlighted the potential for negative control of the production of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) by mTORC1 in CTLs. mTORC1 repressed PtdIns(3,4,5)P3 production and determined the requirement for mTORC2 in activation of the kinase Akt. Our unbiased proteomic analysis thus provides comprehensive understanding of CTL identity and the control of CTL function by mTORC1.

RESULTS

The CTL proteome

High-resolution MS characterized the proteome of P14 CTLs (which have transgenic expression of a T cell antigen receptor specific for an epitope of lymphocytic choriomeningitis virus glycoprotein) (Supplementary Fig. 1) and identified more than 93,000 peptides from 6,800 protein groups in these cells (Fig. 1a). Summed peptide intensities derived from peak areas of ion-extracted MS chromatograms measure relative protein abundance when divided by the theoretically observable numbers of peptides and yield iBAQ (‘intensity-based absolute quantification’) intensities2,5 and can be transformed into absolute quantification by proteomic ruler methodology17. Copy numbers for proteins from three biological replicates showed strong Pearson correlation coefficients (0.86–0.89), with very few outliers and with ~94% of all proteins detected in all three biological replicates (Fig. 1b); this indicated the robustness and reproducibility of our MS-based peptide quantitation methods.

Proteomic data revealed protein abundance and specific protein isoforms or orthologs, which created an objective description of cell identity. We ranked CTL proteins by estimated copy number and plotted this against cumulative protein copy number. Proteins showed a wide range of expression spanning over seven orders of magnitude (Fig. 1c). 12 proteins constituted 25% of the CTL protein mass; 249 proteins constituted 75% of the total CTL mass; and 6,579 proteins...
The proteomic data showed that much of the CTL protein mass was contributed to the remaining 25% of the CTL mass (Fig. 1c). The 20 most abundant CTL proteins constituted nearly a third of all proteins and included histones and the cytoskeleton components vimentin and cofilin, as well as translational machinery proteins, ribosomal proteins, initiation and elongation factors (Table 1). The CTL effector molecule granzyme B and multiple glycolytic enzymes were also in this ‘top 20’ list (Table 1), and the quartile of the CTL proteome with highest intensity showed enrichment for pathways involved in metabolism and macromolecular biosynthesis (Fig. 1c) compared with the abundance of these proteins in the whole data set. As CD8+ T cells differentiate into CTLs, they switch from metabolizing glucose mainly through oxidative phosphorylation to using the glycolytic pathway18. The proteomic data showed that much of the CTL protein mass was dedicated to glycolysis, although CTLs retained abundant amounts of the protein machinery for oxidative phosphorylation (Fig. 1c), which suggested that it might be important for them to retain flexibility in terms of their metabolic strategy for glucose metabolism.

The proteomic data revealed new insight into the expression of protein isoforms and orthologs in T cells. For example, CTLs expressed multiple nutrient transporters, but, in terms of abundance, the amino acid transporter SLC7A5 and its dimer partner SLC3A2 predominated (Fig. 2a). This finding would explain why deletion of SLC7A5 has such a severely deleterious effect on CTL function19. Glucose transport is important for CTLs20, and published studies have focused on the glucose transporter GLUT1 in T cells21. We found that GLUT3, which has a higher glucose transport capacity than that of GLUT1 (ref. 22), was expressed in amounts equivalent to those of GLUT1 in CTLs (Fig. 2a). Deletion of GLUT1 affects T cell function21, but the presence of GLUT3 explains why loss of GLUT1 is not catastrophic.

We also used proteomic ruler methodology27 to estimate absolute protein copy numbers to quantify the key cytokine receptors, transcription factors and effector molecules that define CTL identity. Granzymes A and B were present in high copy numbers in CTLs (4.9 × 10^6 and 2.2 × 10^7, respectively; Fig. 2b), which would explain how CTLs can rapidly kill multiple targets. We observed a wide range in the copy number of transcription factors: the copy numbers of STAT1, STAT3 and STAT5 were higher (1 × 10^5 to 1 × 10^6) than those of T-bet, Foxo1, Foxo3, EOMES, STAT4 or STAT6 (1 × 10^3 to 1 × 10^4) (Fig. 2c). The higher copy number of antigen receptor–coupled tyrosine kinases Lck and Zap70 than that of cytokine receptor–coupled kinases Jak1, Jak3 and Tyk2 was notable (Fig. 2d). The tyrosine phosphatases CD45 and

Table 1. Contribution of the 20 most abundant CTL proteins to the CTL protein pool

| Rank | Protein        | Gene symbol | Copies | % Cum |
|------|----------------|-------------|--------|-------|
| 1    | Histone H2B    | Hist1h2bb   | 7.9 × 10^7 | 5     |
| 2    | Histone H4     | Hist1h4a    | 6.1 × 10^7 | 4     |
| 3    | Actin          | Actb        | 5.0 × 10^7 | 3     |
| 4    | Thymosin β-4   | Tmsb4x      | 4.7 × 10^7 | 3     |
| 5    | Cofilin-1      | Cfl1        | 3.0 × 10^7 | 2     |
| 6    | Histone H2A    | Hist1h2ab   | 3.0 × 10^7 | 2     |
| 7    | Peptidyl-prolyl cis-trans isomerase A | Ppia | 2.8 × 10^7 | 19 |
| 8    | Alpha-enolase   | Eno1        | 2.5 × 10^7 | 1     |
| 9    | Vimentin       | Vim         | 2.4 × 10^7 | 1     |
| 10   | Granzyme B     | Gzmb        | 2.2 × 10^7 | 1     |
| 11   | Profilin-1     | Pfnl        | 2.1 × 10^7 | 1     |
| 12   | 60S acidic ribosomal protein P2 | Rplp2 | 2.1 × 10^7 | 1     |
| 13   | Histone H3.2   | Hist1h3b    | 1.8 × 10^7 | 1     |
| 14   | Histone H1.2   | Hist1h1c    | 1.7 × 10^7 | 1     |
| 15   | Phosphoglycerate kinase 1 | Pgtk1 | 1.7 × 10^7 | 1     |
| 16   | Elongation factor 1-α | Eef1a1 | 1.5 × 10^7 | 1     |
| 17   | L-lactate dehydrogenase A chain | Ldh a | 1.5 × 10^7 | 1     |
| 18   | Eukaryotic translation-initiation factor 3A-1 | Eif5a | 1.4 × 10^7 | 1     |
| 19   | Fructose-bisphosphate aldolase A | Aldoa | 1.4 × 10^7 | 1     |
| 20   | Heat-shock cognate 71-kDa protein | Hspa8 | 1.3 × 10^7 | 1     |

Ranking (by protein copies per cell; most to least) of the 20 most prevalent proteins in CTLs, including frequency relative to total cellular protein pool (%) and cumulative relative abundance of all proteins to that rank (Cum %). Gene symbols derived from the UniProtKB Mouse reference proteome.
**Figure 2** Abundance of key CTL proteins. Copy number (left) of key nutrient transporters (a), CTL effector molecules (b), transcription factors (c), tyrosine kinases and phosphatases involved in signaling via the T cell antigen receptor and IL-2 receptor (d), and IL-2 receptor subunits and associated tyrosine kinases (e), evaluated by proteomic ruler methodology and presented (as in Fig. 1c) as log-transformed mean estimated (est) values. Right, quantification of copies (mean estimated copy number per cell), coefficient of variation (CV) between replicates, and estimated quantification accuracy (QA) for selected proteins at left; quantification accuracy (key) based on number of detected peptides, fraction of unique and non-unique peptides assigned to the protein group (‘Razor’ peptides) to total number of peptides, and theoretically observable peptides. Copy numbers for all CTL proteins are in the Encyclopedia of Proteome Dynamics database. Data are from three independent experiments with one mouse in each (mean values).

SHP-1 was expressed at an abundance similar to that of these tyrosine kinases (Fig. 2d), indicative of the importance of negative regulators in intracellular signaling networks. The data also revealed the stoichiometry of cytokine receptor subunits. For example, interleukin 2 (IL-2) signals to T cells via a high-affinity receptor comprising CD25 (IL-2 receptor α-chain), CD122 (IL-2 receptor β-subunit (IL-2RB)) and CD132 (the common γ-chain (γc)). CTLs expressed approximately 100-fold more copies of CD25 than of IL-2RB or γc (Fig. 2e). Published studies have reported an excess of CD25 relative to the number of high-affinity IL-2 receptor complexes on CTL membranes, and an excess of CD25 over IL-2RB has been quantified by flow cytometry. Our data revealed that the abundance of IL-2RB and γc was limiting at approximately $1 \times 10^4$ copies of IL-2RB per cell and $2 \times 10^4$ to $3 \times 10^4$ copies of γc per cell (Fig. 2e) IL-2RB and γc bind to JAK1 and JAK3, respectively, and the copy number of these kinases was broadly equivalent to that of IL-2RB and γc (Fig. 2e), which indicated that formation of the high-affinity IL-2 receptor in CTLs would be limited by availability of IL-2RB and γc and their associated tyrosine kinases. These examples all illustrate how understanding protein copy number can afford new insight into cell identity and cellular control mechanisms.

**Comparison of the CTL transcriptome and proteome**

Systematic analysis of transcriptomes has yielded critical insight into how T cells direct adaptive immune responses. We assessed whether proteomic data provided additional insight by correlating estimated protein copy numbers in CTLs with the transcript intensities of corresponding mRNAs by using the probe intensities derived from a parallel Affymetrix microarray data set (Fig. 3a). The rather moderate positive correlation between mRNA abundance and protein abundance, with a coefficient of determination of 0.43 (Fig. 3a), indicated that post-transcriptional regulatory mechanisms substantially affected the CTL proteome. Examples of discordance between mRNA abundance and protein abundance included the finding that CTLs had comparable expression of T-bet mRNA and EOMES mRNA, whereas T-bet protein was much more abundant than EOMES protein (Fig. 3b). In a second example, the ratio for the IL-2 receptor subunits estimated from transcript intensities was 3:1:2 (α:β:γ), whereas the corresponding ratio for protein intensity was 92:1:2 (Fig. 3c). There was close correspondence between transcript abundance and protein abundance for some proteins, such as ribosomal proteins and granzymes (Fig. 3d,e). Nevertheless, these data highlighted the importance of direct measurement of the protein, rather than measurement of the mRNA as a surrogate, for estimation of protein expression.

**Selection of the proteome by mTORC1**

CTLs had high mTORC1 activity, as judged by phosphorylation of the mTORC1 substrates S6K1 (at Thr389) and 4E-BP1 (at Ser37 and Ser46 and at Ser65) (Fig. 4a). Treatment of CTLs with the mTORC1 inhibitor rapamycin caused dephosphorylation of these two substrates. mTOR exists in two protein complexes, mTORC1 and mTORC2, that are defined by their scaffolding and regulatory components. CTLs had high activity of mTORC2, as judged by phosphorylation of the mTORC2 substrate Akt at Ser473. Treatment with rapamycin did not cause dephosphorylation of Akt at Ser473. In contrast, the mTOR catalytic inhibitor KU-0063794, which blocks the activity of both mTORC1 and mTORC2 (ref. 26), caused dephosphorylation of Akt at Ser473 and of Ser61K at Thr389, as well as dephosphorylation of 4E-BP1.

Treatment with rapamycin decreased protein synthesis in CTLs over a 24- to 48-hour period and decreased the size and protein content CTLs (Fig. 4b,c). We next used quantitative MS to assess whether inhibition of mTORC1 caused a small reduction in the abundance of all proteins or targeted a protein subset. We treated CTLs with the vehicle dimethyl sulfoxide (DMSO) or rapamycin and analyzed the cells at a single time point of 48 h to assess the sustained effect of inhibiting...
mTORC1. Treatment with rapamycin controlled a small protein subset in CTLs and decreased the expression of 413 proteins and increased the expression of 427 proteins (Fig. 4d). Notably, inhibition of mTORC1 decreased the expression of various CTL effector molecules, including granzymes, perforin, tumor-necrosis factor and interferon-γ (IFN-γ) (Fig. 4e). The decrease in IFN-γ expression in rapamycin-treated CTLs was confirmed by enzyme-linked immunosorbent assay (ELISA) (Fig. 4f). Published studies have reported that mTORC1 controls IFN-γ by controlling expression of T-bet(27). We found no difference between untreated cells and rapamycin-treated cells in their T-bet expression, by MS or immunoblot analysis (Fig. 4g). Notably, expression of CD62L (L-selectin), an adhesion molecule that controls the trafficking of T cells into secondary lymphoid tissues, was upregulated substantially in rapamycin-treated CTLs (Fig. 4d), a result confirmed by ELISA (Fig. 4h). Studies of non-lymphoid cells have reported mTORC1-induced degradation of the adaptors GRB10 (refs. 14, 15) and IRS1 and IRS2 (ref. 16). GRB10 and IRS1 were not detected in CTLs, but there was accumulation of IRS2 in rapamycin-treated CTLs, a result confirmed by immunoblot analysis (Fig. 4i). In terms of signaling molecules, mTORC1 activity was needed to sustain expression of the transcription factor NFIL3 and the phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) phosphatase PTEN (reported below).

mTORC1 was also needed for the expression of glucose transporters and enzymes that control glycolysis (Fig. 5a), cholesterol-biosynthesis enzymes (Fig. 5b), and cytosolic aminoacyl-tRNA synthetases and cytosolic ribosomal subunits (Fig. 5c). Conversely, inhibition of mTORC1 increased the expression of CTL protein subsets, including

![Figure 3](image1.png) Comparison of the CTL transcriptome and CTL proteome. (a) Transcript intensity (mean value; Affymetrix microarray) plotted against the corresponding copy number for CTL proteins (estimated mean value). (b,c) Transcript intensity (as in a) and protein copy number for T-bet and EOMES (b) and the IL-2 receptor subunits IL-2RA (α-chain), IL-2RB (β-chain) and γc (c). NS, not significant (P = 0.8); *P = 0.02 (two-sided t-test) (transcript intensity) or equal variance t-test (protein copy number)). (d,e) Transcript intensity (as in a) and protein copy number for subunits of ribosome protein complexes (d) or granzyme isoforms (A–G) (e). Data are representative of three independent experiments with one mouse in each.

![Figure 4](image2.png) The mTORC1-regulated CTL proteome. (a) Immunoblot analysis of the mTORC1 substrates 4E-BP1 phosphorylated at Ser37 and Ser46 (p-4E-BP1(S37,S46)) or at Ser65 (p-4E-BP1(S65)) and S6K1 phosphorylated at Thr389 (p-S6K1(T389)), and of the mTORC2 substrate Akt phosphorylated at Ser473 (p-Akt(S473)), as well as total SMC1 (loading control throughout), in P14 CTLs cultured with IL-2 and IL-12 with or without (−) 48 h of treatment with rapamycin (RA) or KU-0063794 (KU). Right margin, molecular size, in kilodaltons (kDa). (b) Incorporation of 3H-methionine into nascent proteins in CTLs cultured with IL-2 and IL-12 and treated for 12, 24 or 48 h with rapamycin (horizontal axis); results are presented relative to those of cells treated for 48 h with DMSO (DM), set as 100%. (c) Protein content of CTLs treated for 48 h with DMSO or rapamycin. (d,e) MS analysis of the expression of total proteins, including the known rapamycin-sensitive proteins perforin (PERF) and L-selectin (CD62L) (d), and CTL effector molecules, including tumor-necrosis factor (TNF) and perforin, granzyme B (GZMB) and IFN-γ (e), in CTLs treated for 48 h with rapamycin, relative to that CTLs treated for 48 h with DMSO, plotted against log-transformed P values (two-tailed, unequal-variance t-test) in volcano plots. Numbers in plot (d) indicate total proteins upregulated (top right; red) or downregulated (top left; blue) in rapamycin-treated cells relative to their expression in DMSO-treated cells (proteins with a P value of <0.05 were considered regulated). (f) ELISA of the secretion of IFN-γ by CTLs treated as in d.e. (g) Immunoblot analysis of total T-bet and of S6K1 phosphorylated at Thr389 in CTLs treated as in d.e. (h) ELISA of L-selectin (CD62L) in supernatants of CTLs treated as in d.e. (i) Immunoblot analysis of total IRS2 and of S6K1 phosphorylated at Thr389 in CTLs treated as in d.e. Each symbol (b,c,f,h) represents an individual data point; small horizontal lines indicate the mean. *P < 0.05, **P = 0.01 and ***P < 0.001 (one-way analysis of variance (Holm-Sidak) of non-normalized data, versus DMSO-treated cells (b), or two-tailed Student’s t-test (c,f,h). Data are representative of at least three independent experiments with one mouse in each (a,g,i) or are from three (b,d,e,f,h) or four (c) independent experiments with one mouse in each (mean values).
oxidative phosphorylation enzymes (Fig. 5d), mitochondrial aminocytosine synthetases and ribosomes (Fig. 5e), and the guanine-exchange factor DOCK1 (data not shown). mTORC1 was thus able to both positively regulate and negatively regulate the expression of a subset of CTL proteins and its role was selective and cell specific.

Control of transcriptomes versus proteomes by mTORC1
mTORC1 controls expression of the transcription factors SREBP1, SREBP2 and HIF-1α. We thus used Affymetrix microarray analysis to assess the full extent of transcriptional changes caused by inhibition of mTORC1 and detected a total of 8,198 expressed transcripts (Fig. 6a). Treatment with rapamycin decreased the expression of 226 mRNA transcripts and increased the expression of 220 mRNA transcripts in CTLs (Fig. 6a). There was a strong correlation between the effect of mTORC1 inhibition on transcript abundance and its effect on protein abundance, for glucose transporters, glycolytic enzymes, cholesterol biosynthesis enzymes, granzymes, perforin and IFN-γ (Fig. 6b–d). However, of the 413 proteins whose expression was downregulated in the rapamycin-treated CTLs, only 95 showed a corresponding change in transcript abundance (Fig. 6b–d). Similarly, of the 427 proteins upregulated, only 83 showed an increased abundance of mRNA transcripts (data not shown). Inhibition of mTORC1 thus regulated the expression of cytoplasmic and mitochondrial subunits of ribosomal complexes, oxidative phosphorylation enzymes and proteins encoded by mRNA transcripts with a 5′-terminal oligopyrimidine motif at the protein level but not at the transcript level (Fig. 6e–g). Furthermore, treatment of CTLs with rapamycin regulated the expression of IRS2, DOCK1, and PTEN at the protein level but not at the mRNA level (data not shown), which highlighted the importance of direct proteomic analysis for cell phenotyping.

Figure 5 mTORC1 regulation of cellular pathways. MS analysis of the expression of total proteins in CTLs treated for 48 h with rapamycin (relative to that of CTLs treated for 48 h with DMSO), plotted against log-transformed \( P \) values (two-tailed, unequal-variance \( t \)-test), in volcano plots. Colored symbols indicate proteins assigned to KEGG pathways that are overrepresented among the 413 proteins downregulated (a–c) or 427 proteins upregulated (d,e) in CTLs (Fig. 4d); compared with the frequency of these proteins in the total data set (darker color intensity indicates the \( P \) value is below the threshold of 0.05). (a–c) Downregulated expression of proteins involved in glycolysis and glucose transporters (including GLUT1 and GLUT3) (a), terpenoid backbone and steroid biosynthesis (including the rate-limiting enzyme HMGCR) (b), and cytoplasmic subunits of ribosomes (cyto) ribosomal and aminoacyl-tRNA biosynthesis (c). (d,e) Upregulated expression of proteins involved in oxidative phosphorylation (d) and mitochondrial (mito) subunits of ribosomes and aminoacyl-tRNA biosynthesis (e). Data are from three independent experiments with one mouse in each (mean values).

Figure 6 Comparison of the mTORC1-controlled transcriptome and proteome in CTLs. (a) Microarray probe intensities of RNA isolated from CTLs cultured in IL-2 and IL-12, together with 48 h of treatment with DMSO or rapamycin, showing transcripts upregulated (top left) or downregulated (bottom right) in the rapamycin-treated cells. (b–g) Transcript expression versus protein expression, in rapamycin-treated cells relative to that in DMSO-treated cells, for glycolytic enzymes and glucose transporters (b), terpenoid backbone and steroid-biosynthesis pathways (targets of SREBP1 and SREBP2) (c), cytolitic effector molecules (single letters indicate granzyme isoforms) (d), axis scaling different from that of other plots), cytoplasmic and mitochondrial ribosomal subunits (e), factors involved in oxidative phosphorylation (f), and mRNA with a 5′-terminal oligopyrimidine (5′-TOP) motif and the proteins encoded (as reported10) (g). Numbers in parentheses (above plots) indicate transcript-protein pairs for each pathway. \( P \) values (horizontal (top left), transcriptomic analysis; vertical (right), proteomic analysis) derived from testing against the total transcriptomic data set \( n = 5,516 \) or proteomic data set \( n = 6,641 \) (Mann-Whitney \( U \)-test). Data are representative of three independent experiments with one mouse in each.
Selective programming of CTL metabolism by mTORC1

Our data showed that only a small subclass of metabolic pathways, notably steroid-biosynthesis and glycolytic pathways, were controlled by mTORC1 in CTLs. CTLs express at least 72 nutrient transporters, but only 6 of these were regulated by mTORC1 (Fig. 7a), which highlighted the selectivity of mTORC1’s control of T cell metabolism. In particular, inhibition of mTORC1 did not prevent the expression of SLC1A5 (ASCT2), the key glutamate transporter in T cells24 (Fig. 7a), or decrease the expression of enzymes that regulate glutamine metabolism (Supplementary Fig. 2). Indeed there was increased expression of some enzymes that control glutaminolytic reactions (for example, GLUD1) in CTLs in which mTORC1 was inhibited (Fig. 7b). We assessed the relevance of these changes by measuring glutaminolysis activity and found a higher glutaminolytic rate in CTLs in which mTORC1 was inhibited (Fig. 7c).

Another example of mTORC1’s selectivity was that rapamycin caused a loss of glycolytic enzymes but increased expression of oxidative phosphorylation enzymes (Fig. 7d). The ability of rapamycin to increase the expression of oxidative phosphorylation enzymes was consistent with the ability of rapamycin to promote the development of memory CD8+ T cells7 that are dependent on oxidative phosphorylation rather than glycolysis25. The changes in the expression of glycolytic enzymes were significant and systematic, albeit not large (Fig. 7d). Inhibition of mTORC1 thus decreased the expression of various glycolytic enzymes, but these enzymes were still abundant. 8% of the proteome of CTLs treated with the mTORC1 inhibitor thus consisted of glycolytic enzymes (Fig. 7e). Furthermore, the quartile of the CTL proteome with the greatest protein abundance still showed enrichment for glycolytic enzymes even after prolonged inhibition of mTORC1 (Fig. 7f).

We then measured glycolysis and oxygen-consumption rates of DMSO- and rapamycin-treated CTLs. We assessed the cells in the basal state and after the addition of oligomycin (to block ATP synthesis), DNP (to uncouple ATP synthesis from the electron-transport chain), and antimycin A plus rotenone (AA + rot) added at various time points (downward arrows), presented as normalized values. (i) Glucose uptake in DMSO- and rapamycin-treated CTLs, presented as 2-deoxy-o-glucose molecules/x cell). (j) Lactate output by CTLs in the presence of various concentrations of glucose (horizontal axis), presented as nmol lactate/(h x 10^6 cells). Each symbol (c,i,j) represents an individual data point; small horizontal lines indicate the mean. P values (a,b,d), two-tailed, unequal variance t-test; *P < 0.05 (paired t-test of non-normalized data (c,i,j). Data are from three (a-f,j), two (g,h) or five (i) independent experiments with one mouse in each (mean (a-f,j) or mean ± s.d. (g,h)).

Figure 7 Selective control of CTL metabolism by mTORC1. (a,b) Expression of members of the solute carrier (SLC) family (a) or glutaminolytic proteins (b) in rapamycin-treated CTLs (relative to their expression in DMSO-treated CTLs), plotted against P values (presented as in Fig. 4d,e). (c) Glutaminolysis rates in CTLs treated for 48 h with rapamycin, quantified by measurement of the release of 14CO2 from [U-14C]-glutamine and presented relative to that in DMSO-treated CTLs, set as 100%. (d) Contribution of the glycolytic pathways (light blue) and oxidative phosphorylation pathways (red) to increase the expression of oxidative phosphorylation enzymes was 62 × 10^3 molecules of GLUT1 and 73 × 10^3 molecules of GLUT3, and this abundance decreased to 36 × 10^3 and 48 × 10^3, respectively, in CTLs treated with the mTORC1 inhibitor, which correlated with a twofold difference in glucose uptake (Fig. 7i). In this context, the rate of lactate output in CTLs was very sensitive to a reduction in
the supply of glucose (Fig. 7j), which indicated that a diminished glucose supply, not the loss of glycolytic enzymes, limited glycolysis in the rapamycin-treated CTLs. The decreased glucose uptake in CTLs in which mTORC1 was inhibited would explain the lack of a detectable increase in oxidative phosphorylation associated with their increased expression of oxidative phosphorylation enzymes.

mTORC1 represses PI(3)K-Akt signaling in CTL

We consistently saw downregulation in the expression of PTEN protein in rapamycin-treated CTLs, and this finding was confirmed by immunoblot analysis (Fig. 8a). PTEN dephosphorylates PtdIns(3,4,5)P₃, and this loss of PTEN raised the possibility that mTORC1 signaling normally restrains cellular accumulation of this lipid. We explored this directly and found that CTLs had a density of approximately 30 × 10⁴ molecules of PtdIns(3,4,5)P₃ per cell (Fig. 8b). Inhibition of the phosphatidylinositol-3-OH kinase (PI(3)K) catalytic subunit p110δ resulted in depletion of PtdIns(3,4,5)P₃, but treatment with rapamycin increased the abundance of cellular PtdIns(3,4,5)P₃ (more than 60 × 10⁴ molecules of PtdIns(3,4,5)P₃ per CTL after sustained inhibition of mTORC1; Fig. 8b).

PtdIns(3,4,5)P₃ binds to the plextrin homology (PH) domain of Akt, which allows the kinase PDK1 to phosphorylate Akt at Thr308 and thereby activate the enzyme⁵⁰. Rapamycin-treated CTLs had more Akt phosphorylated at Thr308 than did untreated CTLs (Fig. 8c), and this phosphorylation was lost when the rapamycin-treated CTLs were treated with an inhibitor of p110δ (Fig. 8d) or with the inhibitor AKT1/2 (Fig. 8e), which prevents binding of PtdIns(3,4,5)P₃; the Akt PH domain. The increased abundance of PtdIns(3,4,5)P₃ in rapamycin-treated CTLs thus increased Akt activity, which showed that mTORC1 activity limited Akt function in CTLs.

Many signaling models position mTOR as a positive regulator of Akt. This is because mTORC2 can phosphorylate Akt at Ser473 (ref. 32) and thus create a docking site for the PDK1-interacting fragment (PIF) pocket of PDK1, which promotes efficient phosphorylation of Akt at Thr308 by PDK1 and activates the enzyme. In this context, Rictor-deficient T cells, which lack mTORC2, have less the phosphorylation of Akt at Ser473 and Thr308 than that of T cells with functional mTORC2 signaling, which indicates that the docking of Akt phosphorylated at Ser473 to the PIF pocket of PDK1 can control Akt activity in T cells. However, PDK1 contains a PtdIns(3,4,5)P₃-binding PH domain, and PtdIns(3,4,5)P₃-mediated co-localization of Akt and PDK1 can occur, which makes activation of Akt independent of its phosphorylation at Ser473 (ref. 35).

The mTOR catalytic inhibitor KU-0063794 was as effective as rapamycin in blocking mTORC1 activity and downregulating the expression of PTEN (Fig. 8f). KU-0063794 also caused CTLs to accumulate PtdIns(3,4,5)P₃ (Fig. 8g). High levels of PtdIns(3,4,5)P₃ might switch the balance between the regulation of Akt activity by the PIF pocket–dependent mechanism for the activation of Akt and the use of a PDK1 PH domain–dependent mechanism. In this context, integrity of the PDK1 PH domain is needed for optimal activation of Akt in CTLs. Therefore, to address the role of Akt activation dependent on mTORC2 and the PIF pocket in CTLs, we assessed the effect of KU-0063794 on Akt in CTLs over an 18-hour period. KU-0063794 caused a rapid and sustained loss of mTORC1 activity and mTORC2-mediated phosphorylation of Akt at Ser473 (Fig. 8h).

The effect of KU-0063794 on the phosphorylation of Akt at Thr308, however, was biphasic (Fig. 8h). The rapid de-phosphorylation of Akt at Ser473 in KU-0063794-treated cells was thus initially accompanied by de-phosphorylation of Akt at Thr308 and loss of Akt catalytic activity, as monitored by the loss of phosphorylation of the Akt substrates Thr24 in Foxo1 and Thr32 in Foxo3A (Fig. 8h). Hence, treatment of CTLs with the catalytic inhibitor of mTOR caused rapid...
loss of Akt activity. However, the loss of phosphorylation of Akt at Thr308 in KU-0063794-treated CTLs was transient, and this phosphorylation was restored after approximately 6 h of treatment with KU-0063794 (Fig. 8h). This re-phosphorylation was paralleled by restoration of Akt activity, as judged by corresponding re-phosphorylation of Foxo1 at Thr24 and of Foxo3A at Thr32 (Fig. 8h). At the 18-hour time point, the phosphorylation of Akt at Thr308 and activity of Akt were enhanced compared with that of untreated CTLs, despite the absence of any detectable phosphorylation of Akt in Ser473 (Fig. 8h).

Akt’s activity in CTLs was thus normally dependent on mTORC2-mediated phosphorylation of Akt at Ser473. However, in CTLs treated with the mTOR inhibitor KU-0063794, there was a reprogramming event, such that Akt’s activity became independent of mTOR-controlled phosphorylation of Akt at Ser473. The importance of phosphorylation of Akt at Ser473 in T cells thus depended on the cellular concentration of PtdIns(3,4,5)P3. If this was high, then phosphorylation of Akt at Ser473 was not required for its phosphorylation at Thr308 or its catalytic activity (Supplementary Fig. 3).

The results reported above indicated that mTORC1’s control of PtdIns(3,4,5)P3 dominated mTORC2’s control of Akt in CTLs, such that catalytic inhibitors of mTOR did not effectively disrupt Akt’s activity. In this context, inhibition of Akt in CTLs causes re-expression of Foxo-regulated genes37. However, there was no re-expression of Foxo-regulated genes in KU-0063794-treated CTLs (Table 2), in support of the conclusion that mTOR inhibitors did not disrupt Akt signaling in CTLs. Moreover, we found no difference in the transcriptional changes induced in CTLs by inhibition of mTORC1 with rapamycin versus those induced by catalytic inhibition of mTOR with KU-0063794 (Fig. 8i).

Comparison of the effects of rapamycin and those of KU-0063794 on the CTL proteome by quantitative MS also found no major difference between these effects (Supplementary Fig. 4). Hence, the catalytic inhibitor of mTOR blocked the activity of mTORC1 and mTORC2, but there was no discernable additional (on- or off-target) effect of this inhibitor on the T cells compared with the loss of mTORC1 activity alone. All proteomic data presented are available in the online, searchable Encyclopedia of Proteome Dynamics database to maximize accessibility to the scientific community (Supplementary Fig. 5).

### DISCUSSION

In this study we have characterized the CTL proteome, mapping the abundance and expression of isoforms or orthologs of more than 6,800 proteins. These proteomic data are available in the Encyclopedia of Proteome Dynamics database. The abundance of some CTL proteins was striking: granzymes constituted collectively 1–2% of the CTL proteome, and glycolytic enzymes constituted 9% of the CTL proteome. The threshold of molecules needed for function is often unknown, but there is undoubtedly a new perspective in considering the biological relevance of changes in protein expression when protein abundance is factored into the equation. For example, a 100-fold decrease in the expression of granzyme B and perforin would reduce the density of granzyme B to ~1 × 10^5 copies per CTL and would reduce the density of perforin to approximately ~1 × 10^2 copies per CTL, a case in which granzyme B would still be abundant, whereas perforin would seem limiting. In another example, CD25 (IL-2 receptor α-chain) is expressed at an excess of ~100-fold relative to the IL-2 receptor β-chain and γδ subunit. The expression of CD25 could thus decrease tenfold without affecting expression of the high-affinity IL-2 receptor.

Knowledge of protein copy number is thus invaluable information for full understanding of cell function. Information about the expression of protein isoforms can also provide new ideas about cellular control mechanisms. For example, CTLs express isoforms M1 and M2 of pyruvate kinase (PKM1 and PKM2). However, PKM2 dominates in terms of abundance, at >1 × 10^7 copies per CTL, versus <1 × 10^5 copies per CTL for PKM1. The PKM1 and PKM2 isoforms both use phosphoenolpyruvate as a substrate during glycolysis, but PKM2 can also function as a kinase for STAT3 and the kinase MEK5 (ref. 38) and is a co-activator of HIF-α-mediated transcription39. The quantity of PKM2 in CTLs (>1 × 10^7 molecules per cell) permits this enzyme to serve multiple roles as a transcriptional and metabolic regulator.

One key insight here was that mTORC1 was not a global regulator of protein output in CTLs but instead selectively shaped the CTL proteome by controlling the expression of a small (<10%) subset of metabolic, effector and adhesion molecules that define CTL identity: mTORC1 repressed and stimulated the expression of equal numbers of proteins, which indicated that it simultaneously controlled protein production and protein degradation. The selectivity of mTORC1’s control of the CTL proteome was notable, as was the finding that there was no considerable difference in CTLs in which mTORC1 alone was inhibited versus those with combined inhibition of mTORC1 and mTORC2. This suggested dominant role for mTORC1 in CTLs, compared with that of mTORC2. Genetic strategies that selectively delete either mTORC1 or mTORC2 have shown very different roles for these two complexes in T cells6. In particular, loss of mTORC2 complexes as a consequence of deletion of Rictor prevents phosphorylation of Akt at Ser473 and regulates Akt-mediated exclusion of Foxo transcription factors from the nucleus. We found that mTORC2 did control phosphorylation of Akt at Ser473 in T cells. However, we also discovered that mTORC1 repressed the accumulation of PtdIns(3,4,5)P3 in CTLs. Specific inhibitors of mTORC1 and mTOR thus cause CTLs to accumulate very high levels of PtdIns(3,4,5)P3 and reprogram their regulation of Akt such that activation of Akt becomes independent of the phosphorylation of Akt at Ser473 and thus becomes independent of mTORC2 activity. These results indicate that the biological effect of the combined catalytic inhibition of mTORC1 and mTORC2 cannot be predicted from genetic modifications that individually disrupt mTORC1 or mTORC2 complexes. In this context, although published studies have reported feedback control of Akt by mTORC1 in non-lymphoid cells14–16, the magnitude of the potentiation of PtdIns(3,4,5)P3 levels in T cells by rapamycin, an immunosuppressant, was notable and was not an intuitive result, because PtdIns(3,4,5)P3 is normally thought of as a positive regulator of T cells. However, constitutive activation of the PI(3)K catalytic subunit p110δ in humans results in an immunodeficiency syndrome (activated PI(3)Kδ syndrome). Hence, hyper-activation of PtdIns(3,4,5)P3 signaling pathways in T cells is effectively immunosuppressive40,41. The ‘hyper-production’ of PtdIns(3,4,5)P3 might be part of the mechanism whereby inhibitors of mTORC1 suppress T cell immunity. Our study has thus demonstrated the power of unbiased proteomic analysis in generating new understanding of the mechanisms of drug action.

Published studies have suggested that mTORC1 phosphorylates and targets for degradation GRB10, a negative regulator of PI(3)K14,15. We found that GRB10 was not expressed in CTLs. However, mTORC1 controls the expression of PTEN, another key negative regulator of PI(3)K pathways. In this context, it has been reported that

| Table 2 Effects of inhibitors on key Foxo-regulated genes in CTLs |
|------------------|------------------|------------------|
| Gene       | Rapamycin | KU-0063794 | AKTi1/2 |
| Kif2       | 0.93      | 1.3       | 5.8     |
| Il7r       | 0.88      | 0.95      | 2.2     |
| Gcr7       | 1.9       | 1.3       | 3.1     |
| Sis1p1     | 0.91      | 0.89      | 2.6     |

Microarray analysis of transcript intensity in CTLs treated with rapamycin, KU-0063794 or AKTi1/2 relative to that in untreated cells. AKTi1/2 data obtained from a published study37.
mTORC1-mediated phosphorylation and degradation of the adaptor IRS2 acts to restrain Akt activity in non-lymphoid cells\(^6\). We showed here that mTORC1 controlled the amount of IRS2 in T cells and controlled another key adaptor, DOCK1. In this context, the increased expression of DOCK1 and IRS2 in rapamycin-treated CTLs indicated that inhibition of mTORC1 would promote the signaling pathways controlled by these molecules. mTOR thus has cell type–specific actions, and full understanding of its role it will require analysis of its function in different leukocyte populations.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** ProteomeXchange Consortium, via the PRIDE archive: raw MS and MaxQuant search output data, PXD002928; GEO: microarray data, GSE70925.

**AUTHOR CONTRIBUTIONS**

J.L.H., design and performance of proteomic and transcriptomic experiments and most other experiments; K.E.A., measurement of PtdIns(3,4,5)P3; L.V.S., with peptide fractionation using hSAX; and D. Lamont and the team of the MS resource: raw MS and MaxQuant search output data, PXD002928; D.A.C., experimental design and manuscript authorship (with input from J.L.H.).

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ONLINE METHODS

Mice. All mice used were bred and maintained under specific pathogen-free conditions in the Biological Resource Unit at the University of Dundee. The procedures used were approved by the University Ethical Review Committee and were authorized by a project license under the UK Home Office Animals (Scientific Procedures) Act 1986. P14 mice have been described42.

Cell culture. CTLs were generated as described43. Lymphocytes isolated from spleens of P14 mice (for proteomics and microarray experiments: female mice only, 8–10 weeks old; other experiments: female and male mice, 7–18 weeks old) were activated for 48 h at 37 °C with 100 ng/ml of soluble antigenic peptide (glycoprotein amino acids 33–41; 20 ng/ml IL-2 (Proleukin) and 2 ng/ml IL-12 (R&D Systems). Cells were then cultured for another 96 h in 20 ng/ml IL-2 and 2 ng/ml IL-12, which resulted in CD8+ T cell populations that were >98% pure. Where needed, cells were treated with the following inhibitors: 20 nM rapamycin (EMD Millipore), 1 µM AKT1/2 (EMD Millipore), 10 µM IC-87114 (synthesized in house) or 1 µM KU-0063794 (Tocris). DMSO was used as a solvent and vehicle control for all experiments.

Immunoblot analysis. Cells (20 × 10^6) were lysed in RIPA buffer (100 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM TCEP (Pierce), and protease and phosphatase inhibitors (Roche)). Lysates were sonicated in a Branson Digital sonicator on ice and were centrifuged (4 °C at 16,000g for 10 min). 4x LDS sample buffer (life technologies) and tris(2-carboxyethyl)phosphine (Pierce) were added to the samples at final concentrations of 1× and 25 mM, respectively, before samples were boiled for 10 min. Each gel lane was loaded with the equivalent of 14,000 CTLs and samples were separated by SDS-PAGE (NuPAGE precast gels (life technologies) or Mini-PROTEAN Tetra cell system (Bio-Rad)), and were transferred to nitrocellulose membranes (Whatman). Blots were probed with the following antibodies: antibody to (anti-) 4E-BP1 phosphorylated at Ser37 and Ser46 (2855), antibody to 4E-BP1 phosphorylated at Ser65 (9451), anti-4E-BP1 (9452), antibody to S6K phosphorylated at Thr389 (9239), anti-S6K (9202), antibody to Akt phosphorylated at Thr308 (4056), antibody to Akt phosphorylated at Ser473 (4058), anti-IRS2 (4502), antibody to Foxo1 phosphorylated at Thr24 and to Foxo3A phosphorylated at Thr32 (9464), and anti-Foxo1 (9454) (all from Cell Signaling Technology); anti-SMC1 (A300-055A; Bethyl Laboratories); anti-T-bet (14-5825; E Bioscience); and anti-PTEN (sc-7974; Santa Cruz). X-ray film (Konica) was used to monitor chemiluminescence reactions catalyzed by horseradish peroxidase (HRP)- conjugated secondary antibodies (goat anti-rabbit (31460; Pierce) and goat anti-mouse (31431; Pierce)).

Glucose uptake. Glucose was measured as described13. 1 × 10^6 CTLs were suspended in 400 µl glucose-free medium containing 0.5 mM CI/ml 2-deoxy-d-[1-3H]glucose ([3H]2-DG; GE Healthcare), followed by incubation for 3 min. Cells were pelleted and washed, and were lysed overnight with 200 µl of 1 M NaOH, then the 3H radioactivity incorporated was quantified via liquid scintillation counting.

Lactate measurement. Lactate was measured as described13. 1 × 10^6 CTLs were suspended in 400 µl glucose-free medium containing 0.5 mM Cl/ml 2-deoxy-d-[1-3H]glucose ([3H]2-DG; GE Healthcare), followed by incubation for 3 min. Cells were pelleted and washed, and were lysed overnight with 200 µl of 1 M NaOH, then the 3H radioactivity incorporated was quantified via liquid scintillation counting.

Sample lysis and in-solution digestion for MS (label-free quantification). 25 × 10^6 CTLs treated with either DMSO or rapamycin were harvested into a 50-ml Falcon tube and were washed three times in cold Hank's balanced salt solution and transferred into a 2.0-ml Eppendorf Protein LoBind tube. Cells were lysed in 0.5 ml urea lysis buffer (8 M urea, 100 mM Tris, pH 8.0, and protease and phosphatase inhibitors), followed by vigorous mixture for 15 min at 22 °C. The samples were then sonicated with a Branson digital sonicator before vigorous mixture for another 15 min. The protein concentration was determined by BCA assay as per manufacturer's instructions (Pierce) before DTT was added at a working concentration of 10 mM. Lysates were incubated for 30 min at 30 °C. Iodoacetamide was added at a working concentration of 50 mM, and lysates were incubated for 45 min in the dark at 22 °C. Lysates were diluted with digest buffer to a concentration of 4 mM urea. Lysyl endopeptidase (Wako) was added to the samples at a ratio of 50:1 (protein/lysyl endopeptidase), and the samples were then incubated overnight at 30 °C. The samples were then split in half. One half was diluted to a concentration of 0.8 M urea with digest buffer, and trypsin (Promega) was added at a ratio of 50:1. The other half was kept as a lysyl endopeptidase fraction. The samples were then incubated for a further 8 h at 30 °C. Samples were adjusted to 1% trifluoroacetic acid before being desalted. C18 Sep-Pak cartridges were washed twice with 1 ml elution buffer and were equilibrated twice with 1 ml wash buffer before the acidified peptide samples were loaded. The flow-through was loaded again to ensure maximal peptide binding. The peptide-loaded cartridges were washed three times with 1 ml washing buffer. Peptides were eluted into 2 ml Eppendorf Protein LoBind tubes by two subsequent elutions with 600 µl elution buffer each. The eluted samples were reduced to dryness in a vacuum concentrator.
Strong anion-exchange chromatography. Samples were separated via hSAX chromatography as described. Samples were resuspended in 210 µL SAX sample buffer (10 mM sodium borate, pH 9.3, and 20% acetonitrile), and the pH was readjusted to 9.3 with 1 M NaOH, where necessary. Samples were injected into a Dionex Ultimate 3000 HPLC system equipped with an AS24 strong anion exchange column, and peptides were separated. The following buffers were used for the separation of peptides: 10 mM sodium borate, pH 9.3 (Buffer A), and 10 mM sodium borate, pH 9.3, and 500 mM NaCl (Buffer B). An exponential elution gradient starting with Buffer A was used for separation of the peptides into 12 fractions of 750 µL, which were desalted before analysis by liquid chromatography and tandem MS.

Sample lysis, size-exclusion chromatography and in-solution digestion for MS (SILAC-based quantification). CTLs were cultured in SILAC medium as described. 50 × 10^6 CTLs grown in ‘light’ SILAC medium and treated with either rapamycin or KU-0063794 were mixed with 50 × 10^6 CTLs grown in ‘heavy’ SILAC treated with DMSO and were washed twice with ice-cold Hank’s balanced-salt solution. Cells were lysed and fractionated into five different subcellular fractions (cytoplasmic, membrane, soluble nuclear, chromatin-bound nuclear and cytoskeletal) with a Subcellular Fractionation Kit for Cultured Cells (Pierce) following the manufacturer’s instructions for a 200-µL packed-cell volume. The protein content in each fraction was measured by BCA assay. 300 µg of each subcellular fraction were used for the chloroform-methanol precipitation. Samples were adjusted to a final concentration of 2% SDS, 10 mM TCEP and 20 mM NEM in a volume of 1 mL, followed by heating to 65 °C for 10 min for denaturation of proteins. Samples were precipitated by a chloroform-methanol method and were air-dried. The precipitated cytoplasmic, membrane, nuclear and chromatin-bound nuclear fraction were resuspended in 60 µL size-exclusion chromatography sample buffer and were separated with a mAbPacSEC column (Dionex) with 0.2% SDS, 100 mM NaCl and 10 mM sodium phosphate buffer, pH 6.0. The flow rate was 0.2 mL/min, and eight fractions of 200 µL were collected into Protein LoBind 1-ml 96-deep well plates (Eppendorf). Tetraethylammonium bicarbonate was added to the size-exclusion chromatography fractions to a final concentration of 0.1 M. Trypsin (Promega) was added at a ratio of 50:1 (protein/trypsin). The unseparated cytoskeletal fraction was diluted with digest buffer to a urea concentration of 1 M. Trypsin was added at a ratio of 50:1 (protein/trypsin). All samples were incubated overnight at 37 °C. Detergents were removed using detergent removal 96-well spin plates (Pierce). The detergent free flow through and the cytoskeletal fraction were then kept for desalting as described above and further sample processing.

Liquid chromatography and tandem MS. Samples from desalting were resuspended in 5% formic acid, and 1 µg of peptides was used for analysis. A Dionex RSLCnano HPLC was used for the peptide chromatography. A 5-µm PepMap-C18 pre-column with an inner diameter of 0.3 mm was used, and a 75-µm × 50-cm PepMap-C18 column was used for the subsequent chromatography. The mobile phase consisted of 2% acetonitrile plus 0.1% formic acid (solvent A) and 80% acetonitrile plus 0.1% formic acid (solvent B). A constant flow rate of 300 nL/min was used, and the linear gradient increased from 5% to 35% solvent B over a run time of 156 min. The eluted peptides were injected into a Velos Orbitrap mass spectrometer (Thermo) through a nanoelectrospray emitter. A typical ‘Top 15’ acquisition method was used. The primary MS scan was performed at a resolution of 60,000. The aforementioned top 15 most abundant m/z signals from the primary MS scan were selected for subjected to collision-induced dissociation and secondary MS scan in the Orbitrap mass analyzer at a resolution of 17,500.

Data analysis for MS data. The data were processed, searched and quantified with the MaxQuant software package, version 1.5.0.0, as described, with the default settings and the mouse Uniprot database (reviewed SwissProt database, accessed April 2014) and the contaminants database supplied by MaxQuant. The following settings were used: two miscleavages were allowed; fixed modification was carbamidomethylation on cysteine; the enzyme specificities of trypsin and/or lysyl endopeptidase were applicable; variable modifications included in the analysis were methionine oxidation, deamidation of glutamine or asparagine, amino-terminal pyroglutamic acid formation, and protein amino-terminal acetylation. The default MaxQuant settings included a mass tolerance of 7 p.p.m. for precursor ions, and a tolerance of 0.5 daltons for fragment ions. A reverse database was used for application of a maximum false-positive rate of 1% for both peptide identification and protein identification. This cut-off was applied to individual spectra and whole proteins in the MaxQuant output. The selection-of-probes feature was activated with an allowed time window of 2 min. All proteins were quantified on the basis of unique and Razor peptides with the quantification feature enabled. Razor peptides are defined by MaxQuant as peptides assigned to a specific protein group without being unique to that group. The MaxLFQ algorithm was used for assessment of changes between control and rapamycin-treated CTLs. Estimated abundance and changes were calculated separately for samples digested with lysyl endopeptidase only or with lysyl endopeptidase and trypsin. The abundance or change presented for each biological replicate are the mean of log2-transformed results obtained for samples digested with lysyl endopeptidase only and for samples digested with lysyl endopeptidase and trypsin. Further downstream analysis was performed with Microsoft Excel, Perseus 1.5.1.6 (developed by the Matthias Mann laboratory), SigmaPlot 12.5 and the language R (version 3.1.3, with R Studio 0.98.11.03). An initial pilot proteomics experiment was performed to determine the changes for the known rapamycin sensitive proteins perforin and L-selectin, and a total of three biological replicates was required to determine these changes with a P value of 0.05 (two-tailed, unequal variance t-test). The same two-tailed, unequal variance t-test without further adjustment was used for calculation of the significance of all changes in proteomic experiments. For SILAC-based proteomics, SILAC ratios obtained for each subcellular fraction were weighted by the contribution of the respective subcellular fraction to the overall cellular protein content and by the contribution of reported SILAC ratio counts for each subcellular fraction ratios to the number of total SILAC ratio counts for each experiment. Log-normalized SILAC ratios were then used to determine statistical significance (P ≤ 0.01, as determined by a two-tailed, unequal variance t-test). Pathway analyses were performed using the DAVID (‘database for annotation, visualization and integrated discovery’) bioinformatics tools based on KEGG. A plug-in for Perseus was used for calculation of protein copy number by the proteomics ruler, as follows: total histone copy numbers in a diploid mouse cell (2.2 × 10^6) were calculated from the size of the mouse genome and assigned to the summed peptide intensities of all histones in the control CTLs (1.3%). The correlation between summed peptide intensities and histone protein copy numbers was subsequently used for estimation of copy numbers for all proteins within the data set. Protein groups were assigned a quantification accuracy of ‘high’ (a minimum of eight peptides detected, a minimum of 75% all peptides (unique plus Razor), and a minimum of three observable peptides per 100 amino acid), ‘medium’ (a minimum of three peptides detected, a minimum of 50% all peptides (unique plus Razor), and a maximum of three observable peptides per 100 amino acid) or ‘low’ (all other) for both peptides derived from digestion with lysyl endopeptidase only and those derived from digestion with lysyl endopeptidase and trypsin, and the results were averaged, which generated five classifications, from ‘high’ and ‘high’ to ‘low’ and ‘low’.

Affymetrix GeneChip mouse genome array analysis. CTLs were treated for 48 h DMSO (control), rapamycin or KU-0063794 as described above. RNA was extracted with an RNeasy RNA purification minikit (QIAGEN) according to the manufacturer’s specifications. Microarray analysis was carried out by the Finnish DNA Microarray Centre at the Centre for Biotechnology (Turku, Finland) via 430_2.0 mouse expression arrays (Affymetrix) and the manufacturer’s recommended protocol. Affymetrix Expression Console v1.1 (Affymetrix) was used for normalization of data. Normalization with Microarray Suite 5 (MASS) was used for the selection of probes present in at least one sample, and robust multi-array averaging was used for normalization of data. Significant differences in gene expression were identified with Multiple Experiment Viewer version 4.3 (ref. 49) by the SAM (‘significance analysis of microarrays’) algorithm, with the 90th-percentile false-discovery rate set as 5%. Transcritp data were matched to proteomics data by matching of the gene symbol of the Affymetrix probe to the corresponding gene symbol reported by the Uniprot FASTA-headers.
Statistical methods. All statistical tests not involved in the analysis of the raw proteomic and microarray data were performed with SigmaPlot 12.5 (Systat Software) for Windows or Prism V6 (Graphpad Software) for Mac. A Shapiro-Wilk test for normality was performed to determine suitable tests for parametric or non-parametric populations. F-tests were performed to determine equal variance of populations. All tests were two-tailed and are named in the figure legends. Samples were considered biological replicates if CTLs were generated from separate spleens.

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