Dynamics in protein translation sustaining T cell preparedness

Tobias Wolf1,2,12, Wenjie Jin1,12, Giada Zoppi1, Ian A. Vogel1, Murodzhon Akhmedov1, Christopher K. E. Bleck2,3, Tim Beltraminelli1, Jan C. Rieckmann4, Neftali J. Ramirez5,6, Marco Benevento1, Samuele Notarbartolo3, Dirk Bumann3, Felix Meissner4,7, Bodo Grimbacher5,8,9,10, Matthias Mann11, Antonio Lanzavecchia1, Federica Sallusto1,12, Ivo Kwee1 and Roger Geiger1,10

In response to pathogenic threats, naive T cells rapidly transition from a quiescent to an activated state, yet the underlying mechanisms are incompletely understood. Using a pulsed SILAC approach, we investigated the dynamics of mRNA translation kinetics and protein turnover in human naive and activated T cells. Our datasets uncovered that transcription factors maintaining T cell quiescence had constitutively high turnover, which facilitated their depletion following activation. Furthermore, naive T cells maintained a surprisingly large number of idling ribosomes as well as 242 repressed mRNA species and a reservoir of glycolytic enzymes. These components were rapidly engaged following stimulation, promoting an immediate translational and glycolytic switch to ramp up the T cell activation program. Our data elucidate new insights into how T cells maintain a prepared state to mount a rapid immune response, and provide a resource of protein turnover, absolute translation kinetics and protein synthesis rates in T cells (https://www.nature.com/natureimmunology).

R

est T cells patrol the body in a quiescent yet poised state, prepared to mount a robust immune response to pathogenic threats such as infectious diseases and cancers. Naive T cells may remain inactive for many years in a quiescent state1,2. While surveying for cognate antigen, quiescent T cells maintain a cellular program with minimal energy expenditure3,4. However, following activation T cells need to rapidly undergo a substantial reprogramming to mount an effective response5. Thus, T cells face a trade-off between minimizing their metabolic activity while sustaining a maximally prepared state for rapid execution of the activation program.

In response to an antigenic stimulus, T cells exit quiescence and rewire their transcriptional and metabolic programs. Activated T cells ramp up their translational activity6, increase nutrient uptake7 and rapidly engage glycolysis to provide energy and building blocks that support cell growth, proliferation and the acquisition of effector functions8–10. The extensive reprogramming of activated T cells is increasingly well understood owing to epigenomic, metabolomic, and transcriptomic and proteomic analyses11–14. However, translational dynamics and protein turnover in T cells have not been investigated.

In this study, we examined the dynamics of protein synthesis and turnover in human T cells using pulsed stable isotope labeling by amino acids in cell culture (SILAC)-based high-resolution mass spectrometry (MS)15–18. We identified several key proteins that were rapidly renewed in naive T cells, which maintained quiescence. Intrinsically high turnover in naive T cells facilitated their rapid depletion following stimulation to enable exit of quiescence. Our data also revealed that naive T cells contained large numbers of idling ribosomes that were rapidly engaged following stimulation to ramp up the activation program. In addition, naive T cells also maintained a reservoir of repressed messenger RNAs, which were translated following activation. Finally, although naive T cells exhibited very low glycolytic activity, 11% of their cytosolic proteins were glycolytic enzymes, which were immediately engaged following T cell activation to increase aerobic glycolysis. Together, these findings define molecular underpinnings of T cell preparedness, licensing T cells with the ability to rapidly undergo activation.

Results

Naive T cells rapidly renew a small set of proteins. To measure protein synthesis and turnover rates we employed a pulsed SILAC approach where T cells are cultured for increasing times in a medium containing stable isotope labeled amino acids Arg10 and Lys8 (hereafter referred to as heavy amino acids). Under these conditions, newly translated proteins incorporate heavy amino acids and can be distinguished from pre-existing proteins by a mass shift (Fig. 1a). Given that non-activated T cells remain quiescent and do not grow nor divide, the total proteome mass remains constant over time (Extended Data Fig. 1a). As such, newly synthesized proteins do not contribute to cell growth but replenish their degraded counterparts. Hence, protein synthesis in naive T cells is a proxy for protein renewal.

First, naive CD45RA+CCR7+ CD4+ T cells were isolated to high purity (>98%) from peripheral blood mononuclear cells of 4 healthy donors and cultured without a stimulus in a medium containing

1Institute for Research in Biomedicine, Università della Svizzera italiana, Bellinzona, Switzerland. 2Institute of Microbiology, ETH Zürich, Zurich, Switzerland.
3Institute for Immunodeficiency, Center for Chronic Immunodeficiency, Medical Center, Faculty of Medicine, Albert-Ludwigs-University of Freiburg, Freiburg, Germany. 4Integrated Research Training Group (IRTG) Medical Epigenetics, Collaborative Research Centre 992, Freiburg, Germany. 5Institute of Innate Immunity, Department of Systems Immunology and Proteomics, Medical Faculty, University of Bonn, Bonn, Germany. 6DZIF - German Center for Infection Research, Satellite Center Freiburg, Freiburg, Germany. 7CIBSS – Centre for Integrative Biological Signalling Studies, Albert-Ludwigs University, Freiburg, Germany. 8RESIST - Cluster of Excellence 2155 to Hanover Medical School, Satellite Center Freiburg, Freiburg, Germany. 9Institute for Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, Munich, Germany. 10Institute for Immunodeficiency, Center for Chronic Immunodeficiency, Medical Center, Faculty of Medicine, Albert-Ludwigs-University of Freiburg, Freiburg, Germany. 11Institute of Systems Immunology, Department of Systems Immunology and Proteomics, Medical Faculty, University of Bonn, Bonn, Germany. 12Institute for Innate Immunity, Department of Systems Immunology and Proteomics, Medical Faculty, University of Bonn, Bonn, Germany. 13These authors contributed equally: Tobias Wolf, Wenjie Jin. 14E-mail: roger.geiger@irb.usi.ch
Fig. 1 | A pulsed SILAC approach shows that a small set of important proteins is rapidly renewed in naive T cells. **a**, A schematic of the pulsed SILAC workflow. **b**, A plot of 205 protein species, which incorporated heavy amino acids after a 6 h pulse, ranked according to the fraction that was newly synthesized within 6 h. Average values from \( n = 3 \) are shown. The inset shows protein species that were reliably identified in 3 out of 4 donors increased by more than 90% after 24 h. The second inset shows components of the T cell receptor. **c**, A plot of 1,313 protein species, which incorporated heavy amino acids after a 24 h pulse, ranked according to the fraction that was newly synthesized within 24 h. Average values from \( n = 3 \) are shown. The first inset shows protein species that were renewed by more than 90% in 24 h. The second inset shows components of the T cell receptor. **d**, Renewal kinetics of selected proteins. \( t_{1/2} \) was calculated by fitting a cumulative Weibull distribution. \( n = 3 \) from 3 different donors.

Constitutive protein degradation in naive T cells. Protein turnover is the net result of protein synthesis and degradation. To further validate that proteins for which we found high turnover rates are also rapidly degraded, we quantified protein degradation rates using cycloheximide (CHX), a reversible inhibitor of the ribosome. Resting naive T cells were treated for 24 h with CHX and then analyzed by LC–MS. A comparison of their proteome to that of non-treated cells identified those proteins that were constitutively degraded as they largely disappeared in the absence of protein synthesis. Notably, the vast majority of proteins that decreased in abundance had a high turnover, as determined by pulsed SILAC (Fig. 2a; the color code of the dots shows the renewal rate). For example, heavy amino acids. The cell viability was higher than 90% after 48 h of culturing (Extended Data Fig. 1b). Samples were collected after 0, 6, 12, 24 and 48 h, and analyzed by liquid chromatography-coupled high-resolution MS (LC–MS). We quantified 7,029 proteins and calculated protein renewal rates based on the MS signal intensities of total and newly synthesized proteins.

After a 6 h pulse, 205 protein species were detected that incorporated heavy amino acids. Strikingly, at this early time point several components of major histocompatibility complex class 1 (MHC-I; HLA-A, HLA-B, HLA-C, HLA-E, HLA-β, and β, M) as well as the transcription factor ETS1 were already renewed by more than 80% as judged by the percentage of proteins that incorporated heavy amino acids (Fig. 1b). After a 24 h pulse, the number of renewed protein species that were reliably identified in 3 out of 4 donors increased to 1,313, which is 19% of all protein species in naive T cells (Fig. 1c and Supplementary Table 1). Among the 23 protein species with the fastest renewal rates (>90% after 24 h) were SORL1, SQSTM1 and transcriptional regulators, including ETS1, TCF-1, AES, FOXO1, FOXP1, LEF1, STK7/8 and LBH.

By fitting the renewal rates of different time points to a Weibull distribution, we estimated half-lives of 1,822 out of 7,029 identified proteins. The fastest renewed proteins had a half-life of less than 1 h; that is, 45 min for β, M and 53 min for ETS1 (Fig. 1d; https://www.immunomics.ch). Examples with an intermediate turnover rate were the T cell receptor subunits CD3δ, CD3ε, CD3γ, CD247, and CD247 (half-time \( t_{1/2} = 11–14 h \)), which displayed a similar renewal rate (Fig. 1c,d). Examples with slow turnover were the ribosomal protein RPL8 (\( t_{1/2} = 210 h \)) and the glycolytic enzyme GAPDH (\( t_{1/2} = 210 h \)). The slowest turnover rates were observed for several histones (\( t_{1/2} > 1,000h \)), suggesting that nucleosomes were stable in naive T cells. Taken together, these findings show that most protein species in naive T cells were stable, or only slowly renewed, while a small set of proteins was renewed within only a few hours.
ETS1 and SORL1, which were among the fastest renewed proteins in naive T cells, were both strongly reduced following inhibition of translation (Figs. 1c and 2a), validating our approach for quantifying protein turnover.

To identify which proteins were constitutively degraded by the proteasome, we simultaneously treated naive T cells for 24 h with CHX and bortezomib (PS-341), a specific inhibitor of the proteasome (Extended Data Fig. 1c). We then analyzed proteomes and estimated absolute protein copy numbers using a previously described method19. While the abundances of SQSTM1, a cargo receptor for selective autophagy29, as well as the plasma membrane proteins SORL1, HLA-A, HLA-B, β2M and CD62L, were reduced in the presence of CHX, simultaneous addition of CHX and bortezomib did not stabilize these proteins, suggesting that they were degraded through a proteasome-independent pathway (Fig. 2b). In contrast, the degradation of the transcription factors and regulatory proteins ETS1, FOXP1, TCF-1, LEF1, ELF1, LBH, AES, PBX1P1, FAM65B and STK17B was stabilized by bortezomib, indicating that they were constitutively degraded by the proteasome (Fig. 2c). This analysis further revealed that TXNIP, which suppresses glucose uptake30, and KLF2, a transcription factor that controls the expression of chemokine receptors and adhesion molecules that regulate T cell trafficking29, were constitutively degraded by the proteasome in naive T cells (Fig. 2c). Owing to their low abundance (200–2,000 copies), TXNIP and KLF2 were not reliably identified in the pulsed SILAC experiments. Taken together, these findings show that membrane proteins with high turnover were degraded through a proteasome-independent pathway, likely in endosomes/autophagosomes, whereas the rapidly renewed transcription factors and regulatory proteins were degraded by the proteasome.

As shown in the previous experiments, inhibition of protein synthesis in naive T cells with CHX for 24 h led to a decrease in the abundance of β2M and HLA-ABC proteins by about 50%, from ~500,000 to ~225,000 copies and from ~380,000 to ~190,000 copies, respectively (Fig. 2b). We next treated naive T cells with CHX and followed surface MHC-I protein abundance by flow cytometry. Consistent with the proteomics data, surface MHC-I decreased about 50% within 24 h (Fig. 2d). Notably, following wash-out of CHX, MHC-I surface abundance was completely restored in less than 6 h, confirming that naive T cells rapidly synthesize large amounts of MHC-I components.

To rule out possible artifacts caused by CHX, we used a CRISPR-Cas9 approach to disrupt the B2M gene in resting naive T cells. Freshly isolated naive CD4+ T cells were electroporated with ribonucleoprotein (RNP) complexes consisting of Atto 550-labeled trans-activating CRISPR (tracr) RNA, Cas9 protein and guide RNAs targeting two independent sites of B2M. The abundance of MHC-I at the cell surface was reduced already after 24 h in greater than 90% of naive T cells that had taken up the RNP (Atto 550+). By 72 h, MHC-I surface abundance further decreased as judged by a reduction in mean fluorescence intensity (Fig. 2e). Together, these results confirm that MHC-I is continuously degraded and replenished in naive T cells.

**Transcription factors display a high turnover.** Cell identity and function are largely determined by transcription factors. The half-lives of transcription factors in naive T cells ranged from 53 min to 126 h (Fig. 3a), raising the question whether transcription factors with high turnover play a defining role in the cell state. The fastest renewed transcription factor in naive T cells was ETS1, which plays an essential role in maintaining T cell homeostasis23,24. To corroborate ETS1 activity in naive human T cells, we generated a genome-wide map of chromatin accessibility in purified resting naive CD4+ T cells from three donors using the assay for transposase-accessible chromatin (ATAC-seq)29. We identified a total of 34,370 distinct chromatin accessibility peaks of which 13,592 were located in promoter regions (Extended Data Fig. 2a and Supplementary Table 2). Notably, the transcription-factor-binding motifs that were enriched the most in these regions were recognized by ETS1 (P=10^-44). To confirm broad DNA binding of ETS1, we performed chromatin immunoprecipitation following by sequencing (ChIP-seq) for genome-wide binding of ETS1 in purified T cells. ETS1 was bound at 11,222 sites in the genome that were associated with over 9,688 genes (Supplementary Table 3). Fifty percent of the ETS1-binding sites were in promoter regions suggesting direct ETS1-mediated transcriptional regulation (Extended Data Fig. 2b). Collectively, these findings show that ETS1, which has the highest turnover of any transcription factor in naive T cells, has broad binding activity and thus plays a defining role in the cell state.

**High protein turnover facilitates T cell reprogramming.** Perpetual degradation and re-synthesis of proteins is an energy-consuming process. However, high turnover of specific proteins allows for rapid fine-tuning of their abundance, facilitating an immediate adaptation of the cellular program in response to various stimuli. In general, a decrease in the abundance of mRNA transcripts encoding proteins with high turnover results in their immediate depletion, whereas proteins with low turnover are not as promptly responsive to decreased transcription. We therefore explored whether T cells rely on high protein turnover in steady-state conditions for rapid reprogramming in response to different stimuli.

We quantified absolute and relative protein abundances immediately following activation of naive T cells with plate-bound anti-CD3 and anti-CD28. This resulted in a comprehensive and dynamic data-set on proteomes of naive and activated T cells (7,772 quantified proteins). Interestingly, we observed that the rapidly renewed proteins FAM65B, KLF2, TCF-1, TXNIP, SORL1, PBX1P1 and CD247 were downregulated early after activation (Fig. 3b). For example, loss of KLF2 is necessary to acquire T cell effector function while down-regulation of FAM65B promotes exit of quiescence26. For comparison, examples with an opposite pattern were β2M and NF-κB1, the slowest renewed transcription factor in naive T cells (Fig. 3a,b).

---

**Fig. 2 | Constitutive protein degradation in naive T cells.** a. Naive CD4+ T cells were cultured in the presence or absence of 50 μg ml^-1 CHX for 24 h, after which their proteomes were analyzed by MS. The volcano plot shows the results of differential abundance analysis (two-tailed Welch's t-test) between control and CHX-treated cells. Each dot represents a protein; a negative log2[fold change] means that the protein is less abundant in CHX-treated T cells. b. Naive CD4+ T cells were treated with 50 μg ml^-1 CHX alone or together with 10 μM bortezomib (PS-341 (PS)). Only the 1,313 proteins shown in Fig. 1c, for which a renewal rate was also determined, are shown. The color code shows the renewal rate as determined by SILAC. n=4 from four different donors. b. Naive CD4+ T cells were treated with 50 μg ml^-1 CHX alone or together with 10 μM bortezomib (PS-341 (PS)). The boxplots show copy numbers of selected membrane proteins. n=5 foor CHX, n=6 for CHX + PS. The box plots denote the medians and the interquartile ranges (IQRs). The whiskers of each box plot are the lowest datum still within 1.5 IQR of the lower quartile and the highest datum still within 1.5 IQR of the upper quartile. c. The same as in b but selected transcription factors and regulatory proteins are shown. The box plot elements are defined as in b. d. Naive CD4+ T cells were treated with 50 μg ml^-1 CHX (blue points) and analyzed by flow cytometry at different time points. Shown is the fluorescence intensity relative to that of control cells that were not treated with CHX. n=3 from 3 different donors. The error bars indicate the s.d. of the mean. e. Naive CD4+ T cells were electroporated with Cas9, Atto 550-labeled tracrRNA and two single guide RNAs targeting the B2M locus. Cells were stained with an antibody to HLA-ABC and analyzed by flow cytometry 24 h and 72 h after electroporation. NT, non-targeting single guide RNA control. The experiment was repeated three times with similar results.
Taken together, these findings show that several proteins responsible for maintaining T cell quiescence had constitutively high turnover in naive T cells and were rapidly downregulated following activation.

To further explore whether some rapidly renewed proteins are downregulated in activated T cells in vivo, we analyzed tumor-infiltrating T cells (TILs), which respond to antigenic stimulation and other signals in the tumor microenvironment (Extended Data Fig. 2c). We compared transcriptomes of TILs and circulating T cells from patients with hepatocellular carcinoma. Interestingly, the vast majority of mRNAs that were downregulated were involved in immune responses and cell cycle regulation, such as SORL1, ETS1, SELL, HLA-E, LBH, and ELF1. Interestingly, the vast majority of mRNAs that were downregulated were involved in immune responses and cell cycle regulation, such as SORL1, ETS1, SELL, HLA-E, LBH, and ELF1.
in TILs encoded proteins that are rapidly renewed in resting T cells, including KLF2, LEF1, TCF-1, FOXP1, AES, TXNIP, FAM65B, SORL1 and CD62L (Fig. 3c). Thus, several key proteins depleted in the tumor microenvironment were maintained at high turnover in resting T cells, suggesting that protein turnover is an important factor in facilitating T cell responsiveness.

Pre-existing glycolytic enzymes in naive T cells. As metabolic reprogramming is a requirement for the exit of quiescence, we compared turnover of glycolytic enzymes in naive and activated T cells using the pulsed SILAC approach. Estimating protein turnover in activated T cells with the pulsed SILAC method is possible only for proteins whose abundances remain constant following activation. Therefore, we first sought to identify glycolytic enzymes in T cells whose abundance does not change within 12 h of activation. Surprisingly, although glycolysis markedly increased early after activation (Extended Data Fig. 3a), the only enzyme that was strongly upregulated was hexokinase-2 (HK2), while all other glycolytic proteins did not change in abundance during the first 12 h after activation (Fig. 4a). Although naive T cells have low glycolytic activity, they contained millions of copies of glycolytic enzymes (11% of all cytosolic proteins; Extended Data Fig. 3b). For each reaction step in the glycolytic pathway, naive T cells already had highly abundant enzymes (Fig. 4b, in blue). Thus, following activation naive T cells increase the expression of HK2, engage a large pool of pre-existing enzymes and concomitantly increase their glycolytic activity.

Having established that nearly all glycolytic enzymes remain constant in abundance early after activation, we determined protein turnover rates in T cells that were activated for 6h and 12h in the presence of heavy amino acids. Strikingly, the turnover of all measured glycolytic enzymes substantially increased 12 h after activation (Extended Data Fig. 3c). For example, naive T cells contained ~1.3 million copies of lactate dehydrogenase A (LDHA), of which 6.5% were renewed within 12 h in resting naive T cells (Fig. 4c). In contrast, 12 h after activation the fraction of renewed LDHA increased to 65% while the total amount of LDHA remained steady (~1.4 million copies) (Fig. 4c; examples are also shown for GAPDH, ALDOA and PGK1). In conclusion, naive T cells contain large pools of glycolytic enzymes whose activity and turnover markedly increase following activation.

Pre-existing, idle ribosomes in naïve T cells. Naïve T cells have only a thin layer of cytoplasm with a volume of 82.5±9 fl as determined by serial block-face scanning electron microscopy and subsequent three-dimensional (3D) reconstruction of images. Following
activation T cells expand their cytoplasm to 670 ± 74 fl (Fig. 5a–d and Extended Data Fig. 4), which requires a substantial increase in protein synthesis. We implemented an algorithm to compare the ribosomal output in naive and activated T cells by further analyzing the pulsed SILAC datasets. Absolute protein synthesis rates (copy numbers per minute) were calculated as total protein copy numbers multiplied by the fraction of heavily labeled proteins over time. We found that naive T cells synthesized in total ~60,000 proteins per minute, a result that was consistently found regardless of the duration of the pulse with heavy amino acids. After 6 h of activation, protein synthesis rates increased to ~300,000 proteins per minute and after 24 h to ~800,000 proteins per minute (Fig. 5e). Thus, the ribosomal output increased more than 13-fold following activation.

In naive T cells, the proteins with the highest synthesis rates were β, M with a median of ~4,300 copies per minute followed by β-actin, clusterin, ubiquitin and HLA-A (~4,000, ~2,900, ~2,300 and ~2,000 copies per minute, respectively) (Fig. 5f). In 24-h-activated T cells, β-actin had the highest median synthetic rate of 54,000 copies per minute, a result that was consistently found regardless of the duration of the pulse.

Repressed mRNAs are rapidly translated after activation. To investigate whether the rapid increase in the ribosomal output following T cell activation was regulated transcriptionally, we analyzed transcriptomes of naive resting and activated T cells by RNA sequencing (RNA-Seq) and established an algorithm to estimate absolute mRNA copy numbers. In contrast to the proteome consisting of ~410 million proteins, naive T cells contained only ~77,000 mRNA molecules (Supplementary Table 4; individual mRNA species in naive T cells ranged from <1 to 3,700 mRNA copies). On average, this corresponds to an mRNA to protein ratio of 1 to 5,400 (Extended Data Fig. 5d). At 6 h following activation, the total number of mRNAs increased only 1.4-fold to 105,000 transcripts, indicating that the 5-fold increase in the ribosomal output was predominately regulated at the post-transcriptional level (Extended Data Fig. 5e,f).

A comparison between mRNA copy numbers and protein synthetic rates revealed a Spearman’s rank correlation of 0.41 in naive T cells. At 6 h following activation, the correlation between transcript abundance and protein synthesis rates increased to 0.61 after 24 h to 0.65, confirming extensive post-transcriptional regulations (Fig. 6a–c). Most highly abundant mRNAs were translationally repressed and encoded ribosomal proteins (blue dots), and translation initiation (green) and elongation (light green) factors (Fig. 6a,b). In total, the repressed transcripts constituted 52% of all mRNA molecules in naive T cells (40,740/77,000). However, translation was not generally repressed in naive T cells, as illustrated by the two highly abundant mRNA species encoding ubiquitin and β,M, which were both frequently translated (Fig. 6a,b).

To further investigate the post-transcriptional regulations underlying rapid T cell activation, we calculated the rate by which individual mRNAs were processed by ribosomes. The efficiency of mRNA translation was heterogeneous, with a median rate of 1.6 times per minute in naive T cells. At 6 h following activation, the median rate by which a single mRNA molecule was read off slightly
Fig. 5 | Translational preparedness of naive T cells. a, A 3D reconstruction of a naive CD4^+ T cell based on scanning block-face electron microscopy images. The plasma membrane is drawn in purple, the nucleus in green and mitochondria in blue. Scale bar, 2 μm. b, A 3D reconstruction of a 72-h-activated CD4^+ T cell. Both cells in a and b are drawn at the same scale. c, Examples of electron microscopy images of naive (left) and 72-h-activated (right) T cells that were used for the 3D reconstructions. A total of seven naive and eight activated T cells were analyzed and quantified in d. All cells were fixed and embedded together. Scale bars, 2 μm. d, Quantification of cell and organellar volumes based on 3D reconstructions of naive CD4^+ and 72-h-activated T cells. n = 7 for naive T cells, n = 8 for 72-h-activated T cells. The error bars indicate the s.e.m. The P values were determined by a two-tailed unpaired Student’s t-test. e, The numbers of total proteins that are synthesized per minute in resting, naive and activated CD4^+ T cells are shown. For resting cells, data from 3 different pulse durations (6 h, 12 h and 24 h) are shown. n = 3 from 3 different donors. The box plot elements are defined as in Fig. 2b. f, A boxplot showing the synthesis rates of the five proteins with the highest synthetic rates in naive T cells. n = 3 from 3 different donors. The box plot elements are defined as in Fig. 2b. g, Comparison of the total protein synthesis in naive T cells to synthesis of actin B in 24-h-activated T cells. n = 7 for naive cells; n = 3 for memory cells. The box plot elements are defined as in Fig. 2b. h, Estimations of ribosomal protein numbers based on quantifications of ribosomal RNA and the median number of ribosomal proteins. Sample numbers are indicated on the graph. The error bars represent the s.e.m. i, Average translation rate per ribosome in resting, naive and activated CD4^+ T cells. n = 3 from 3 different donors.
increased to 2 times per minute (Extended Data Fig. 5g). In general, these numbers agree well with previous studies in cell culture systems, in which translation initiation was found to occur 1.5–2 times per minute based on imaging of nascent polypeptide chains being synthesized from single mRNA molecules.

Strikingly, we identified 242 mRNA species that were strongly repressed in naive T cells and more than 3 times faster translated 6 h after activation (Fig. 6d and Supplementary Table 5). For example, naive T cells contained 67 copies of mRNAs encoding the early activation marker CD69, yet in our pulsed SILAC experiments active synthesis of CD69 proteins was never detected in naive T cells (Fig. 6a). In contrast, 6 h after activation, the translation rate of CD69 increased from 0 to 598 proteins minute while the number of CD69 mRNAs increased only 1.6-fold, from 67 to 111 copies. Thus, CD69 transcripts were completely repressed in naive T cells, but 6 h after activation each mRNA was read off by ribosomes 5.4 times per minute (mRNA translation rate; Fig. 6b). Consistent with a rapid onset of translation, total numbers of CD69 proteins increased from 0 copies in naive T cells to 65,000 copies in 6 h-activated T cells (Fig. 6e). Similarly, mRNAs encoding CD40L and JUN-B were completely silenced in naive T cells but rapidly translated following activation, which led to a rapid increase in total protein copies (Fig. 6a,b and Extended Data Fig. 6a). Collectively, these findings illustrate a further example of preparedness whereby naive T cells maintain a reservoir of repressed mRNAs that are rapidly engaged to initiate the activation program.

**A majority of repressed mRNAs are regulated by mTOR.** We next asked whether the rapid translational switch of repressed mRNAs in naive T cells is regulated by mTOR signaling pathways, because the majority of repressed mRNAs contained a 5′-terminal oligopyrimidin (TOP) motif whose translation is regulated by mTORC1 in mouse embryonic fibroblasts. To address this, we performed pulsed SILAC experiments with resting and 6 h-activated T cells that were treated with Torin-1, which inhibits mTORC1 and mTORC2 signaling pathways. Consistent with low mTOR activity in naive T cells, Torin-1 had a minimal effect on translation in naive T cells (Fig. 6f). However, 6 h after activation Torin-1 strongly and specifically inhibited the translational switch of TOP mRNAs.

Interestingly, the activation-induced translational switch of 15 out of the 242 repressed mRNA species was not markedly affected by Torin-1 (log2[fold change] < 1). These included the mRNA binding proteins RBMX, RBM3 and RBP39, as well as the early activation proteins CD69, NRA1 (Nur77) and NRA2 (Fig. 6g and Extended Data Fig. 6b). Thus, mTOR serves as a metabolic checkpoint controlling the translational switch for the majority of repressed mRNAs, yet a small subset including the fastest upregulated proteins were engaged independently of metabolic control.

**Memory T cells have increased basal protein turnover.** We next assessed translational dynamics in memory T cells, which sustain a higher metabolic activity and are more prepared to respond faster to antigens than naive T cells. Freshly isolated CD45RO+ CD4+ memory T cells from 4 healthy donors were labeled for 0, 6, 12, 24 and 48 h with heavy amino acids and their proteomes were analyzed by LC–MS. We found that the 23 proteins with the highest turnover in naive T cells displayed comparable renewal kinetics in memory T cells (Fig. 7a and Extended Data Fig. 7a). However, the global protein turnover rate was higher in memory T cells, with 100 proteins having significantly increased renewal rates at each time point (P < 0.05, two-tailed Welch’s t-test) (Supplementary Table 6). Of those, the strongest enriched KEGG annotations were ribosome (P_{adj} = 2.916 × 10^{-17}), glycolysis (P_{adj} = 2.356 × 10^{-17}) and proteasome (P_{adj} = 4.997 × 10^{-17}) (Fisher’s exact test). For example, within 48 h the ribosomal proteins RPS6 and RPL21 were renewed by ~25% in naive T cells, which increased to ~50% in memory T cells (Fig. 7b). Similarly, renewal rates of the glycolytic enzymes GAPDH and LDHA as well as the proteasomal proteins PSMA1 and PSMA6 were approximately doubled in memory T cells (Fig. 7b). Thus, memory T cells are imprinted with high turnover of ribosomal, proteasomal and glycolytic proteins to support a higher state of preparedness.

With a total protein synthesis rate of ~110,000 proteins per minute, memory T cells had a translational output that was 80% higher than in naive T cells (Fig. 7c). The average ribosomal output in resting memory T cells was ~1.47 amino acids per second per ribosome, which was 93% higher than in naive T cells. At 6 h following activation, the ribosomal output increased to ~4.3 amino acids per second (Fig. 7d). Taken together, these findings show that memory T cells have a higher baseline translational activity than naive T cells and reach a higher ribosomal output early after initiation of the activation program, supporting a faster response.

**Discussion**

In this study we employed a pulsed SILAC approach to analyze the dynamics in protein synthesis and turnover in T cells. Our data revealed that naive T cells are not entirely inert since they continuously replenish a small set of proteins that are either unstable or actively degraded. Thus, our analysis defines a minimal maintenance program that supports homeostasis of naive T cells. For example, MHC-I-peptide complexes are unstable and T cells continuously replenish MHC-I molecules on their surface, which promotes the presentation of peptides. Another example of proteins that displayed high turnover were several transcription factors that actively maintain cell identity, quiescence and homeostasis. These transcription factors were likely rapidly renewed as a consequence of their usage. These findings suggested that differential turnover rates of proteins support the execution of key cellular processes.
Our study also provides novel insights into mechanisms of T cell preparedness, enabling the rapid execution of their activation program. High turnover of specific proteins in resting T cells, while having high energy costs, provides the advantage that protein abundance can be rapidly tuned down. Our analyses showed that a large number of proteins downregulated in response to different stimuli are of high turnover in the resting state, thus demonstrating that high protein turnover is a process T cells employ to rapidly adjust their proteome for transitioning cell states.

A second mechanism of T cell preparedness is the maintenance of a reservoir of glycolytic enzymes, allowing naive T cells to jump-start glycolysis. Curiously, this reservoir was maintained in naive T cells despite a lack of dependency on glycolysis as a metabolic program. However, T cells quickly engaged glycolysis following activation, and having a pre-existing reservoir of glycolytic enzymes facilitated this rapid switch. This finding also explains how naive T cells can increase their glycolytic rate threefold within just a few minutes following stimulation.*
A third mechanism of preparedness relies on a large pool of idling ribosomes, whose average translational output in naive T cells increased from 0.8 to 4 amino acids per second within 6 h following activation. Notably, the ribosomal output rates that we determined in activated T cells are in agreement with previously measured ribosome translocation rates of 3.1–5.6 amino acids per second based on translation imaging in live cells and ribosome profiling.\(^{11,12,20}\) Thus, our data suggest that ribosomes idle in naive T cells but operate at a high capacity in activated T cells.

Continuous transcription of untranslated mRNAs, which have a short half-life of about 7 h (ref. \(^{40}\)), comes at an energetic cost. Yet, sustaining the expression of repressed mRNAs has the advantage that they can be immediately engaged in protein synthesis. For example, naive T cells contain several untranslated copies of mRNAs encoding CD69, which are immediately translated following activation to retain activated T cells in secondary lymphoid organs.\(^{42,43}\) Globally, many of the mRNAs repressed in naive T cells contained a TOP motif and were regulated by mTOR, linking metabolism to translational regulation in the T cell activation program. Most of these mRNAs encoded ribosomal proteins as well as transcription initiation and elongation factors. Consistent with our observations, previous studies showed that mRNAs encoding the translational machinery, although highly abundant, were barely associated with polysomes.\(^{6,44,45}\) However, we also identified 15 repressed mRNAs that were rapidly engaged following activation independent of mTOR. Thus, there is a default, initial T cell activation program that is mTOR-independent and includes the upregulation of CD69 (to retain T cells in lymph nodes) and Nur77 (to regulate metabolism).\(^{44}\)

Previous studies also demonstrated that naive T cells are prepared to rapidly generate mRNAs as 90% of the promoters from genes to be expressed in activated T cells are loaded with polymerase.\(^{45}\) Thus, naive T cells also sustain a transcriptional preparedness level.

By combining absolute mRNA concentrations with quantifications of the transcriptome, we estimated mRNA copy numbers of each transcript in T cells. We found a total of ~77,000 and ~420,000 mRNA molecules in naive and 24-h-activated T cells, respectively. Previously, RNA-seq-based estimates suggested that an average mammalian cell contains about 200,000 mRNA molecules. Our data showed that in naive T cells proteins are about 5,000 times more abundant than transcripts and in 24-h-activated T cells about 3,000 times more abundant, indicating that translation is a crucial amplification step for the abundance of proteins in T cells.
On the basis of our pulsed SILAC data and protein quantifications, we estimated absolute synthesis rates for more than 1,300 proteins in T cells. Combining these with estimations on mRNA copy numbers, we calculated that transcripts are on average processed 1.6 times per minute in naive T cells and this increased to 2 times per minute following activation. These data are in excellent agreement with previous observations of translation initiation frequencies in live cells, which occurred every 30 to 40 s on actively translated mRNAs31–32. Thus, our global estimates of absolute protein synthesis rates and transcript abundances are both in accordance with previous studies in other cell types.

In conclusion, this study provides absolute numbers of mRNAs and protein copies as well as transcript processing and protein synthesis rates in T cells, representing an important resource for quantitative immunology. Our analysis of these datasets revealed a minimal maintenance and preparedness program in resting, human T cells.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-020-0714-5.

Received: 19 March 2018; Accepted: 19 May 2020; Published online: 6 July 2020

References
1. Crabtree, G. R. Contingent genetic regulatory events in T lymphocyte activation. Science 243, 355–361 (1989).
2. Vieira, S. et al. Sparse production but preferential incorporation of recent synthesized CD8 effector T cells in the human peripheral pool. Proc. Natl Acad. Sci. USA 105, 6115–6120 (2008).
3. Chapman, N. M., Boothby, M. R. & Chi, H. Metabolic coordination of T cell and macrophage immunity. Nat. Rev. Immunol. 14, 500–508 (2013).
4. Gubser, P. M. et al. Rapid effecter function of memory CD8+ T cells requires an immediate-early glycolytic switch. Nat. Immunol. 14, 1064–1072 (2013).
5. Buck, M. D., Sowell, R. T., Kaech, S. M. & Pearce, E. L. Metabolic instruction of immunity. Cell 160, 570–586 (2015).
6. Geiger, R. et al. L-Arginine modulates T cell metabolism and enhances survival and anti-tumor activity. Cell 167, 829–842.e13 (2016).
7. Howden, A. J. et al. Quantitative analysis of T cell proteomes and transcriptomes. J. Immunol. Methods 374, 101–115 (2012).
8. Ron-Hasel, N. et al. Mitochondrial biogenesis and proteome remodeling promote one-carbon metabolism for T cell activation. Cell Metab. 24, 104–117 (2016).
9. Kaech, S. M., Hemby, S., Kersh, E. & Ahmed, R. Molecular and functional profiling of memory CD8+ T cell differentiation. Cell 111, 837–851 (2002).
10. Phan, A. T., Goldrath, A. W. & Glass, C. K. Metabolic and epigenetic coordination of T cell and macrophage immunity. Immunity 46, 714–729 (2017).
11. Schwanzel-Huus, B. et al. Global quantification of mammalian gene expression control. Nature 473, 337–342 (2011).
12. Ong, S.-E. et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics 1, 376–386 (2002).
13. Jovanovic, M. et al. Dynamic profiling of the protein life cycle in response to pathogens. Science 347, 1259038 (2015).
14. Aebersold, R. & Mann, M. Mass-spectrometric exploration of proteome structure and function. Nature 537, 347–355 (2016).
15. Wiśniewski, J. R. et al. Extensive quantitative remodeling of the proteome between normal colon tissue and adenocarcinoma. Mol. Syst. Biol. 8, 611 (2012).
16. Buettner, G. et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J. Cell Biol. 171, 603–614 (2005).
17. Wu, N. et al. AMPK-dependent degradation of TXXNIP upon energy stress leads to enhanced glucose uptake via GLUT1. Mol. Cell 49, 1167–1175 (2013).
18. Weinrich, M. A. et al. KLF2 transcription-factor deficiency in T cells results in unrestrained cytokine production and upregulation of bystander chemokine receptors. Immunity 31, 122–130 (2009).
19. Muthusamy, N., Barton, K. & Leiden, J. M. Defective activation and survival of T cells lacking the Ets-1 transcription factor. Nature 377, 639–642 (1995).
20. Borjis, J.-C. et al. Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. Nature 377, 635–638 (1995).
21. Buenrostro, J. D., Giresi, P. G., Zaba, L. C., Chang, H. Y. & Greenleaf, W. J. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nuclease position. Nat. Methods 10, 1213–1218 (2013).
22. Freelon, J. I. et al. FAM65B controls the proliferation of transformed and primary T cells. Oncotarget 7, 63215 (2016).
23. Baines, A. J. et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. Nat. Med. 24, 541–550 (2018).
24. Zheng, C. et al. Imaging translatomes and ubiquitomes in live cancer revealed by single-cell sequencing. Cell 169, e1316 (2017).
25. Morisaki, T. et al. Real-time quantification of single RNA translation dynamics in living cells. Science 352, 1425–1429 (2016).
26. Wu, B., Elisovcivich, C., Yoon, Y. J. & Singer, R. H. Translation dynamics of single mRNAs in live cells and neurons. Science 352, 1430–1435 (2016).
27. Van, X., Hoek, T. A., Vale, R. D. & Tanenbaum, M. E. Dynamics of translation of single mRNA molecules in vivo. Cell 165, 976–989 (2016).
28. Wang, C., Han, B., Zhou, R. & Zhuang, X. Real-time imaging of translation on single mRNA transcripts in live cells. Cell 165, 990–1001 (2016).
29. Thoreen, C. C. et al. A unifying model for mTORC1-mediated regulation of mRNA translation. Nature 485, 109–113 (2012).
30. Thoreen, C. C. et al. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J. Biol. Chem. 284, 8023–8032 (2009).
31. Yang, K., Neale, G., Green, D. R., He, W. & Chi, H. The tumor suppressor Tsc1 enforces quiescence of naive T cells to promote immune homeostasis and function. Nat. Immunol. 12, 888–897 (2011).
32. Iezzi, G., Karjalainen, K. & Lanzavecchia, A. The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity 8, 89–95 (1998).
33. Harndahl, M., Rasmussen, M., Roder, G. & Buus, S. Real-time, high-throughput measurements of peptide–MHC-I dissociation using a scintillation proximity assay. J. Immunol. Methods 374, 5–12 (2011).
34. Lipford, J. R. & Deshaies, R. J. Diverse roles for ubiquitin-dependent proteolysis in transcriptional activation. Nat. Cell Biol. 5, 845–850 (2003).
35. Ingolia, N. T., Lareau, L. F. & Weissman, J. S. Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. Cell 147, 789–802 (2011).
36. Sharova, L. V. et al. Database for mRNA half-life of 19,977 genes obtained by DNA microarray analysis of pluripotent and differentiating mouse embryonic stem cells. DNA Res. 16, 45–58 (2008).
37. Show, L. R. et al. CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature 440, 540–544 (2006).
38. Riccardi, S. et al. The Translational Machinery of Human CD4+ T Cells Is Poisoned for Activation and Controls the Switch from Quiescence to Metabolic Remodeling. Cell Metab. 28, 895–906.e5 (2018).
39. Tan, T. C. et al. Suboptimal T-cell receptor signaling compromises protein translation, ribosome biogenesis, and proliferation of mouse CD8+ T cells. Proc. Natl Acad. Sci. USA 114, E6117–E6126 (2017).
40. LiebmAnn, M. et al. Nrf7 serves as a molecular brake of the metabolic switch during T cell activation to restrict autoimmunity. Proc. Natl Acad. Sci. USA 115, E8017–E8026 (2018).
41. Koz裁定, F. et al. Global regulation of promoter melting in naive lymphocytes. Cell 153, 988–999 (2013).
42. Shapiro, E., Biezuner, T. & Linnarsson, S. Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat. Rev. Genet. 14, 618–630 (2013).

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2020
Methods

Human primary T cells. Blood from healthy donors was obtained from the Swiss Blood Donation Center of Basel and Lugano, and used in compliance with the Federal Office of Public Health (authorization no. A000197/1 to EFS). Peripheral blood mononuclear cells were isolated by Ficoll gradient centrifugation. CD4+ T cells were enriched with magnetic microbeads (Miltenyi Biotec). Naïve CD4+ T cells were sorted as CD4+CD8−CCR7+CD45RA−CD25− on a FACS Aria III cell sorter (BD Biosciences). For cell staining, the following antibodies were used: anti-CD-4–APC (allophycocyanin), clone 13B8.2; anti-CD-4–FITC (fluorescein isothiocyanate), clone 13B8.2; anti-CD-8–APC, clone B9.11; anti-CD-8–FITC, clone B9.11; anti-CD45RA–PE (phycoerythrin), clone alb11; anti-CD25–FITC, clone B1.19.9 (all from Beckman Coulter); anti-CR7–Biotin Brilliant 421, clone G043HF (BioLegend).

Cell culture. Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, penicillin (50 μk g−1), streptomycin (50 μg ml−1), all from Invitrogen) and 10% (v/v) human serum (Swiss Blood Center, Basel). Human T cells were activated with plate-bound anti-CD3 (5 μg ml−1), clone TR66) and anti-CD28 (1 μg ml−1, clone CD28.2, BD Biosciences) for 48 h in 96-well Nunc Maxisorb plates. A total of 1.5 × 10^5 T cells were plated per well. After 48 h of activation, cells were transferred to 96-well U-bottom plates and cultured in IL-2-containing medium (500 U ml−1). In experiments, in which T cells that were cultured without a stimulus, 1.5 × 10^5 T cells were plated in 96-well U-bottom plates and 20 μl of medium culture was added per well. Drugs were added at the following concentrations: CHX, 50 μg ml−1; and zebromeb (PS-341), 10 μM. For SILAC experiments, SILAC RPMI-1640 (GBCO) was supplemented with 73 mg l−1 lys8 HCl and 42 mg l−1 Arg10 HCl, 2 mM glutamine, 1% (v/v) sodium pyruvate, penicillin (50 μg ml−1), streptomycin (50 μg ml−1; all from Invitrogen) and 5% (v/v) dialyzed human serum.

CRISPR-Cas9 mediated knockdown of B2M. Lyophilized crRNAs targeting B2M (5′-CGUGGAGAACCUGUUAUGUUGUUGGAGACCUGCU-3′, 5′-AAGCUACUUCUGAAGGGUGGUUGAGACCUGCU-3′, CRISPR-Cas9 Negative Control crRNA (Alt-R CRISPR-Cas9 Negative Control crRNA, catalog no. 1072544) and tracrRNAs were chemically synthesized (IDT) and resuspended in Nuclease-Free Duplex Buffer at a stock concentration of 200 μM. To assemble Cas9 RNPs, crRNAs and tracrRNAs were mixed at a 1:1 v/v ratio and incubated for 20 min at 21 °C. Freshly isolated naive CD4+ T cells were washed twice with PBS, resuspended in Buffer T (Neon Transfection System Kit, cat. no. MPK1025) and mixed with the Cas9 RNP complex. Then, Cas9 Electrotransfection Enhancer (Alt-R Cas9 Electrotransfection Enhancer, IDT, stock 10.8 μM) was added to the mix to a final concentration of 1.8 μM. A total of 1 × 10^5 cells were electroporated under the following conditions: voltage, 2,200 V; width, 20 ms; pulse, 1; 1000 V; and a column pressure of 0.32 torr. The RAW images had a pixel size of 6.6 μm × 6.6 μm and a resolution of 60,000 × 60,000 at m/z 200. Nonlinear retention time alignment of all measured samples was performed in MaxQuant. Peptide identifications were matched across different replicates within a time window of 1 min of the aligned retention times. For the ion injection time and ion target values were set to 20 ms and ×10^4 for the survey scans and 55 ms and 1 × 10^4 for the MS/MS scans, respectively. Data were further filtered for common contaminants and peptides identified only by side modification were excluded from further analysis. Copy numbers were estimated on the basis of the protein mass of cells. We set the protein mass of a naïve T cell to 25 pg and of an activated T cell to 75 pg.

Calculation of protein synthesis rates. Total protein copy numbers were estimated on the basis of total LFQ values (heavy and light proteins). The number of newly synthesized protein copies was calculated on the basis of the proportion of heavy and light proteins. For naïve T cells, the number of newly synthesized proteins within 6, 12, 24 and 48 h was analyzed and the highest rate (proteins per hour) was selected.

RNA-seq. Total RNA was isolated from flow-sorted naïve CD4+ T cells using the RNeasy Plus Mini Kit from Qiagen. RNA samples from two healthy donors were pooled and sent to IGA Technology Services. The TruSeq Stranded mRNA Sample Prep kit (Illumina) was used for library preparation starting with 1–2 μg of total RNA (RNA integrity number > 7) as input. The polyA mRNA was fragmented for 3 min at 94 °C and every purification step was performed by using 1X Agencourt AMPure XP beads. Both RNA samples and final libraries were quantified using the Qubit 2.0 Fluorometer (Invitrogen) and quality tested by Agilent 2100 Bioanalyzer RNA Nano assay (Agilent Technologies). Libraries were processed with Illumina cDNA on the flow cell and sequenced on single-end mode at the multiplexing level requested on HiSeq2500 (Illumina). The CASAVA 1.8.2 version of the Illumina pipeline was used to process raw data for format conversion and de-multiplexing.

Estimation of absolute transcript copy numbers. To determine the total mRNA content per T cell, total RNA was isolated from naïve and activated T cells by using the E.Z.N.A. total RNA Kit I (Omega BIO-TEK). From total RNA, polyA mRNA was isolated using polyT-coated Dynabeads (Dynabeads mRNA purification kit, Thermo Fischer Scientific). The purity of polyA mRNA was quantified on a Qubit fluorometer (Thermo Fischer Scientific). The total mRNA content (M) of each transcript was calculated using equation (1), where M_{total} is the mass of total polyA mRNA, FPKM is the FPKM value of each transcript determined by RNA-seq and l is the transcript length.

\[
M = M_{total} \times \sum_{l} FPKM \times l
\]
ATAC-seq. Accessible chromatin mapping was performed using the ATAC-seq method as previously described, with minor adaptations. In each experiment, 5 × 10⁶ cells were centrifuged for 10 min at 8 °C, resuspended in 50 ml ATAC-seq lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.1% NP-40) and centrifuged for 30 min at 500g and 4 °C. After centrifugation, the pellet was incubated in 25 μl transposase reaction mix (12.5 μl 2× TD buffer, 1 μl transposase (Illina) and 11.5 μl nuclease-free water) for 60 min at 37 °C. After DNA purification with the Clean and Concentrator kit (ZymoResearch), a 50 μl PCR reaction was set up (10 μl transposed DNA, 10 μl nuclease-free H₂O, 2.5 μl PCR primer 1, 2.5 μl barcoded PCR primer 2 and 25 μl kNEBNext Ultra H Q5 master mix (New England Biolabs)). After 5 cycles, 5 μl of the eluted DNA was used in a quantitative PCR (qPCR) reaction to estimate the optimum number of amplification cycles. The remaining 45 μl was amplified for the determined cycle number. Library amplification was followed by 2 rounds of AMPure XP bead (Agencourt) size selection to exclude fragments larger than 1,200 base pairs (bp). DNA concentration was measured with a Qubit fluorometer (Life Technologies). The libraries were sequenced by the Genomic Core Sequencing Facility at EMBL using the Illumina HiSeq2500 platform and the 50-bp single-end configuration.

Processing of the ATAC-seq data. Reads were aligned to the GRCh38/hg38 assembly of the human genome using Bowtie with the “−best and -m 1” parameters to eliminate strand bias and report only the best alignment in terms of quality values at mismatched positions and to allow only one (depending on quality value) alignment per read, respectively. All downstream analyses were performed on the filtered reads. Greater than 100 million reads were obtained for the library, and reads mapping to mitochondrial and nuclear DNA were excluded from the analysis. Between 22.4 million and 27.6 million high-quality reads per sample that mapped uniquely to genomic DNA were retained. Peaks were called for each sample using HOMER with the parameters ‘−style-factor and -L 20’, to analyze transcription factors and call peaks with 20-fold greater tag density than in the surrounding 10-kilobase region, respectively. Differential peaks were identified using getDifferentialFeatures.pl balanced (statistical program) −L 10 (10-fold greater tag density than background). Peaks were merged for the same cell type using mergePeaks and annotated with annotatePeaks.pl. One non-annotated file was generated with the option ‘−noannotate’, uploaded to Rstudo and analysed by principal component analysis with the package ggplot2. Motif analysis on peak regions was performed with the HOMER function findMotifGenome. p value parameters ‘−size 200 (peak size)’ and ‘−en 8,10,12,15 (motif length)’. With the ‘−size 200 −hist 400 (bin size in bp)’ −ghost (output genes profiles for each gene) parameters from HOMER, a data matrix file was generated and uploaded to Cluster 3.0 for clustering (parameters: normalize genes, cluster genes and array; similarity parameters from HOMER, a data matrix file was generated and uploaded to Cluster for analysis on peak regions was performed by the HOMER function findMotifsGenome.

Statistical analysis. Statistical analyses were performed in the R programming environment (version 3.3.3) or with GraphPad Prism 7 (GraphPad Software).

**Data availability**
The MS proteomics raw data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017159. RNA-seq and ChIP-seq data have been deposited to the NCBI Gene Expression Omnibus (GEO) with the accession numbers GSE147229 and GSE146787. The data that support the findings of this study are available at [https://www.immunomics.ch](https://www.immunomics.ch), attached as Supplementary Tables 1-6 or are available from the corresponding author upon request.

**References**

47. Akat, K. et al. Molecular characterization of desmosomes in meningiomas and archonoidal tissue. Acta Neuropathol. 106, 337–347 (2003).

48. Hohening, H., Mannweiler, K. & Müller, M. High-pressure freezing of cell suspensions in cellulose capillary tubes. J. Microsc. 175, 34–43 (1994).

49. Cordona, A. H. et al. An integrated micro-and macroarchitectural analysis of the Drosophila brain by computer-assisted serial section electron microscopy. PLoS Biol. 8, e1000502 (2010).

50. Kremer, J. R., Mastronarde, D. N. & McIntosh, J. R. Computer visualization of three-dimensional image data using IMOD. J. Struct. Biol. 116, 71–76 (1996).

51. Rappulser, J., Mann, M. & Ishiihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. 2, 1896–1906 (2007).

52. Schellema, R. A. et al. The Q Exactive HE, a benchtop mass spectrometer with a pre-filter, high-performance quadrupole and an ultra-high-field Orbitrap analyzer. Mol. Cell. Proteom. 13, 3698–3708 (2014).

53. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372 (2008).

54. Cox, J. et al. Andromeda: a peptide search engine integrated into the MaxQuant environment. J. Proteome Res. 10, 1794–1805 (2011).

55. Blecher-Gonen, R. et al. High-throughput chromatin immunoprecipitation for genome-wide mapping of in vivo protein–DNA interactions and epigenic states. Nat. Protoc. 8, 539–554 (2013).

56. Barozzi, I., Termanini, A., Minucci, S. & Napolitani, G. Fish the ChIPs: a pipeline for automated genomic annotation of ChIP-seq data. Biol. Direct 6, 51 (2011).

57. Machanick, P. & Bailey, T. L. MEME-ChIP: motif analysis of large DNA datasets. Bioinformat. 27, 1696–1697 (2011).

58. McLean, C. Y. et al. GREAT improves functional interpretation of cis-regulatory regions. Nat. Biotechnol. 28, 495–501 (2010).

**Acknowledgements**

We thank D. Jarrossay for cell sorting. This work was supported by a grant from the Swiss SystemsX.ch initiative, evaluated by the Swiss National Science Foundation (grant no. 142033 to R.G.), the European Research Council (grant no. 803150 to R.G.), the Swiss National Science Foundation (grant no. 170213 to F.S.), the Federal Ministry of Education and Research (GAIN_01GM1910A to B.G.) and the German Research Foundation (SFB1160/2_85, RESIST–EXC 2155–Project ID 39087428 to R.G.). The Institute for Research in Biomedicine is supported by the Helmut Horten Foundation.

**Author contributions**

R.G. conceived the study, designed experiments, analyzed data and wrote the manuscript. T.W., W.I., G.Z., A.V. and J.C.R. designed and performed experiments with human T cells. M.A. and J.K. wrote the R shiny code for the online platform. M.B. helped with MS. C.K.E.B. and T.B. performed the electron microscopy and analysis. N.J.R. carried out ATAC-seq, and S.N. carried out ChIP-seq analysis. D.B., F.M., B.G., M.M., A.L., F.S., I.K. and R.G. supervised the work.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Extended data is available for this paper at [https://doi.org/10.1038/s41590-020-0714-5](https://doi.org/10.1038/s41590-020-0714-5).

Supplementary information is available for this paper at [https://doi.org/10.1038/s41590-020-0714-5](https://doi.org/10.1038/s41590-020-0714-5).

Correspondence and requests for materials should be addressed to R.G.

Peer review information L. A. Dempsey was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.

Reprints and permissions information are available at [www.nature.com/reprints](http://www.nature.com/reprints).
Extended Data Fig. 1 | Viability of resting T cells in culture. (a) Protein mass of naïve T cells does not change after 72 h of culturing. Proteomes of T cells that were either analyzed immediately after isolation or after 72 h of culturing were analyzed by LC-MS. Protein content was estimated using the proteome ruler approach\(^{59}\). \(n=5\) for freshly isolated and \(n=2\) for 72 h-cultured T cells from different donors. Bars represent the S.E.M. (b) FACS-purified naïve and memory T cells were cultured in complete medium without the addition of growth factors. To measure cell viability, T cells were stained with Annexin-V-FITC directly after sorting or after 24 h and 96 h of culturing and then analyzed by flow cytometry. \(n=4\), Four independent experiments with T cells from two different donors. Bars represent the S.E.M. (c) FACS-purified naïve T cells were cultured for 24 h in complete medium containing either DMSO, 50 µg/ml CHX or 50 µg/ml CHX together with 10 µM bortezomib (CHX + PS). \(n=8\) from three different donors. Bars represent the S.E.M.

59. Wiśniewski, J. R., Hein, M. Y., Cox, J. & Mann, M. A "proteomic ruler" for protein copy number and concentration estimation without spike-in standards. Mol. Cell. Proteom. 13, 3497–3506 (2014).
Extended Data Fig. 2 | Analysis of hallmark transcription factors. (a) Frequency of ATAC-Seq peaks annotated to different genomic regions. (b) Frequency of ETS1 ChIP-Seq peaks annotated to different genomic regions. (c) Examples of activation markers that were up-regulated in TILs (CD69 and PD1) and proteins that were downregulated (KLF2, CD62L, and FAM65B). n = 1627 for T cells from blood, n = 2170 for T cells from tumors. Violin plot width is based on a Gaussian kernel density estimate of the data (estimated by the density function with standard parameters), scaled to have maximum width = 1. Data are from Zheng et al. 2016.
Extended Data Fig. 3 | Increased turnover of glycolytic enzymes following activation. (a) Naïve or 6h-activated T cells were analyzed on a Seahorse analyzer. Shown is the Extra Cellular Acidification Rate (ECAR), which is a measure of the glycolytic rate. n = 6 from three donors. Bars represent the S.E.M. (b) Gene ontology analysis of the proteome of naïve T cells. Glycolytic proteins (blue) contributed 11% to the cytosolic protein mass. (c) Fraction of newly synthetized (heavily labeled) glycolytic proteins after a 12-hours pulse in naïve or 12h-activated T cells. n = 3 from three different donors. Box plot elements are defined as in Fig. 2b.
Extended Data Fig. 4 | 3D reconstructions of seven naïve and eight 72 h-activated T cells. (a) Reconstructions of naïve CD4+ T cells. For the first two cells every layer of the plasma membrane (purple) was drawn, while for the other cells only every third layer was drawn. Scale bar = 2 μm (b) Reconstructions of 72 h-activated CD4+ T cells. For the first four cells every layer of the plasma membrane (purple) was drawn, while for the other cells only every third layer was drawn. Scale bar = 2 μm.
Extended Data Fig. 5 | Estimation of the number of ribosomes. (a) Copy numbers of 82 ribosomal proteins in naïve T cells. n = 7 from seven different donors. Box plot elements are defined as in Fig. 2b. (b) Distribution of the copy numbers of ribosomal proteins in naïve T cells. Average values from n = 7 are shown. Dashed line shows the median, which was used as an approximation of total ribosomes. (c) Total RNA in naïve and activated T cells. To estimate the number of ribosomes, it was assumed that 83% of total RNA was ribosomal RNA. n = 7 for naïve, 6 h and 12 h-activated T cells. n = 4 for 24 h, 48 h and 72 h activated T cells from different donors. Bars represent the S.E.M. (d) mRNA to protein ratio. n = 7 for naïve, n = 3 for 6 h and n = 4 for 24 h activated T cells from different donors. Bars represent the S.E.M. (e) Total amount of mRNA per T cell. n = 9 for naïve and 24 h activated T cells, n = 7 for 6 h and 12 h activated T cells from different donors. Bars represent the S.E.M. (f) Average mRNA copy numbers per T cell. (g) mRNA processing rate in naïve and 6 h-activated T cells. Average values from n = 3 from different donors are shown.
Extended Data Fig. 6 | Posttranscriptional regulations. (a) Absolute copy numbers of CD40LG and JUNB proteins in resting and activated T cells. n = 7 for naïve T cells, n = 3 for 6 h, 12 h, 48 h, 120 h-activated T cells, n = 4 for 24 h, 72 h, 96 h activated T cells. Box plot elements are defined as in Fig. 2b. (b) Absolute protein synthesis rates in resting and 6 h-activated T cells that were untreated or treated with Torin-1. n = 3 from three donors. Bars represent the S.E.M.
Extended Data Fig. 7 | Rapidly turned over proteins in naïve and memory T cells. (a) Comparison of protein turnover kinetics in resting naïve and memory CD4⁺ T cells of selected proteins. n = 3 for naïve 6 h and naïve 12 h; n = 4 for naïve 24 h, memory 6 h, memory 12 h and memory 24 h from different donors.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Mass spectrometry data were collected on a QExactive HF instrument. Data were acquired using the Xcalibur software (Thermo Scientific, version 4.0.27.13). RNA Sequencing was done at IGA technology.

Data analysis
Electron microscopy image stacks were aligned using the TrackEM2 plugin for ImageJ. Image analysis and quantification were performed using the IMOD software package. Mass spectrometry data were analyzed using the MaxQuant Software (version 1.5.3.54). Data was visualized using R (version 3.3.3.).
ChIP-seq data analysis was performed using the Fish the ChiPs software [DOI: 10.1186/1745-6150-6-51].

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All proteomics data is accessible through www.immunomics.ch

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD017159.

RNA-seq and ChIP-seq have been deposited to the NCBI Gene Expression Omnibus (GEO) with the accession number GSE147229 and GSE146787.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: No sample-size calculation was performed. All experiments were performed at least in triplicates. Sample sizes are sufficient as orthogonal experiments were performed to support the claims.

Data exclusions: Mass spec sample were excluded when they did not pass quality control (number of identified protein groups was too low).

Replication: Replicates were made from T cells of different donors (biological replicates). The attempts at replication were successful.

Randomization: Cell culture work and mass spec analysis was performed by different researchers.

Blinding: Investigators were not blinded but cell culture work and mass spectrometry analysis was performed by different researchers.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | n/a |
| ☐ ☑ Antibodies | ☑ ChiP-seq |
| ☑ Eukaryotic cell lines | ☐ Flow cytometry |
| ☑ Palaeontology | ☑ MRI-based neuroimaging |
| ☑ Animals and other organisms | |
| ☑ Human research participants | |
| ☑ Clinical data | |

Antibodies

Antibodies used: anti-CD4-APC [allophycocyanin], clone 13B8.2; anti-CD4-FITC [fluorescein isothiocyanate], clone 13B8.2; anti-CD8-APC, clone B9.11; anti-CD8-FITC, clone B9.11; anti-CD45RA-PE [phycoerythrin], clone alb11; anti-CD25- FITC, clone B1.49.9 (all from Beckman Coulter); anti-CCR7-Brilliant Violet 421, clone G043H7 [Biolegend]; HLA-ABC-FITC, clone W6/32 [eBioscience]; ETS-1 [C-20] [Santa Cruz]

Validation: All antibodies for T cell sorting as well as the HLA-ABC and ETS1 antibody have been previously used in other studies.

Human research participants

Policy information about studies involving human research participants

Population characteristics: Buffy-coats were from anonymous, random gender, random blood group adult donors from the Swiss Blood Donation Centers of Basel and Lugano (Switzerland)

Recruitment: Blood from healthy anonymized donors was obtained from the Swiss Blood Donation Centers of Basel and Lugano

Ethics oversight: Swiss Ethics Committees on research involving humans
ChIP-seq

Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g., BED files) for the called peaks.

Data access links

May remain private before publication.

Files in database submission

- 1_CD4+_ETS1_ChIP.fastq
- 2_CD4+_Input.fastq
- CD4+_ETS1_peaks_pc-10_FC>5_FDR<5.merge.xlsx
- CD4+_ETS1_ChIP.tdf
- CD4+_Input.tdf

Genome browser session

Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

Methodology

Replicates

CD4+ T cells were pooled from three different healthy donors and then subjected to the ChIP-seq protocol as described in the Methods section.

Sequencing depth

ChIP-seq data are from 50 bp single-end sequencing reads. The number of unique reads after filtering is 26,356,014 for the ETS-1 ChIP and 48,771,186 for the Input sample.

Antibodies

ETS-1 antibody was from Santa Cruz Biotechnology, ETS-1 [C-20], cat. num. sc-350 X. Ets-1 [C-20] is an affinity purified rabbit polyclonal antibody raised against a peptide mapping to the C-terminus of Ets-1 of human origin. This antibody has been already used in published ChIP-seq studies from the ENCODE consortium (doi: 10.1038/nature13986).

Peak calling parameters

Reads were aligned to reference genome (hg19) using Bowtie and peak calling was performed using MACS 1.4 with a p-value threshold of 1e-10, using the Input sample as control sample.

Data quality

Low quality reads were filtered out. Quality control reports were generated using FastQC. The number of peaks called by MACS with p-value ≤ 1e-10, FDR ≤ 5% and fold enrichment ≥ 5 is 11,220.

Software

ChIP-seq data analysis was performed using the Fish the ChIPs software (DOI: 10.1186/1745-6150-6-51). Default parameters were used except for "Band width" = 150.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

PBMCs were isolated from buffy coats by Ficoll-Paque Plus (GE Healthcare Life Sciences) gradient centrifugation and CD4+ T cells were enriched by positive selection with CD4 magnetic microbeads (Miltenyi Biotec). Then naive T cells were sorted.

Instrument

Cell sorting was performed on a FACS Aria III (BD Biosciences). MHC-I levels were analyzed on a LSRII Fortessa™ (BD Biosciences).

Software

Flow cytometry raw data were collected using the BD FACSDiva software version 6.2 and analysed with FlowJo software (Tree Star), version 9.6 or later.

Cell population abundance

After sorting CD4+ T cells populations had a purity of >98% (verified by flow cytometry).

Gating strategy

Lymphocytes were identified by cell size in a FSC-A vs. SSC-A plot, and single cells discriminated in a SSC-H vs. SSC-W plot. Naive (CCR7+ CD45RA+) and memory T cells (CD45RA-) were FACS sorted after exclusion of CD314+ and CD25+ cells.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.