Defining the RBPome of primary T helper cells to elucidate higher-order Roquin-mediated mRNA regulation

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Post-transcriptional gene regulation in T cells is dynamic and complex as targeted transcripts respond to various factors. This is evident for the Icos mRNA encoding an essential costimulatory receptor that is regulated by several RNA-binding proteins (RBP), including Roquin-1 and Roquin-2. Here, we identify a core RBPome of 798 mouse and 801 human T cell proteins by utilizing global RNA interactome capture (RNA-IC) and orthogonal organic phase separation (OOPS). The RBPome includes Stat1, Stat4 and Vav1 proteins suggesting unexpected functions for these transcription factors and signal transducers. Based on proximity to Roquin-1, we select ~50 RBPs for testing coregulation of Roquin-1/2 targets by induced expression in wild-type or Roquin-1/2-deficient T cells. Besides Roquin-independent contributions from Rbms1 and Cpeb4 we also show Roquin-1/2-dependent and target-specific coregulation of Icos by Celf1 and Igf2bp3. Connecting the cellular RBPome in a post-transcriptional context, we find contributions from multiple RBPs to the prototypic regulation of mRNA targets by individual trans-acting factors.
lymphocytes as central entities of the adaptive immune system must be able to make critical cell fate decisions fast. To exit quiescence, commit to proliferation, and exert effector functions or form memory they strongly depend on programs of gene regulation. Accordingly, they employ extensive post-transcriptional regulation through RBPs or miRNAs and 3′ end oligo-uridylation or m6A RNA modifications. These RBPs, or RBPs that recognize modifications, directly affect the expression of genes by controlling mRNA stability or translation efficiency. Previous studies of T helper cells have focused on a small number of RNA-binding proteins, including HuR and TTP/Zfp36l1/Zfp36l2 1–14, Roquin-1/2 15,16 and Regnase-1/4 17,18, as well as some miRNAs like miR-17–92, miR-155, miR-181, miR-125 or miR-146a 19. Moreover, the first evidence for m6A RNA methylation in this cell type has been provided 20. Underscoring the relevance for the immune system, loss-of-function of these factors has often been associated with profound alterations in T cell development or functions which caused immune-related diseases. Intriguingly, many key proteins of the immune system have acquired long 3′-UTRs enabling their regulation by multiple, and often overlapping sets of post-transcriptional regulators. RBPs can also recruit additional co-factors as for example Roquin-1 binds together with Nufip2 to RNA 21, and some of them have antagonistic RBPs like HuR and TTP25 or Regnase-1 and Arid5a 26. Such functional or physical interactions together with interdependent binding to the transcriptome create enormous regulatory potential. The major challenge is therefore to integrate our current knowledge about individual RBPs into concepts of higher-order gene regulation that reflect the interplay of different, and ideally of all cellular RBPs.

A prerequisite for studying higher-order post-transcriptional networks is to know the cell type-specific RBPomes that account for differential and dynamic expression of RBPs and miRN plasticity. To this end several global methods have been developed over the last decade, revealing a growing number of RBPs that may even exceed recent estimates of ~7.5% of the human proteome27. RNA interactome capture (RNA-IC) is one widely used, unbiased technique, however, it is constrained by design, intending to identify proteins binding to polyadenylated RNAs. In contrast, orthogonal organic phase separation (OOPS) analyzes all UV-crosslinked protein–RNA adducts from interphas after organic phase separation.

The interactions of RBPs with RNA typically involve charge-, sequence- or structure-dependent interactions, and to date over 600 structurally different RNA-binding domains (RBD) have been identified in canonical RBPs of the human proteome27. However, global methods also identified hundreds of non-canonical RBPs, which oftentimes contained intrinsically disordered regions (IDRs). Surprisingly, as many as 71 human proteins with well-defined metabolic functions were found to interact with RNA30 introducing the concept of "moonlighting". Depending on availability from their "day job" in metabolism such proteins also bear the potential to impact RNA regulation. Recent large-scale approaches have increased the number of EuRBPDB-listed human RBPs to currently 294931, suggesting that numerous RNA/RBP interactions and cell-type-specific gene regulations have gone unnoticed so far.

In this work, as the first step towards a global understanding of post-transcriptional gene regulation, we experimentally define all proteins that can be crosslinked to RNA in T helper cells. RNA-IC or OOPS identify ~310 or ~1200 proteins in primary CD4+ T cells interacting with polyadenylated transcripts or all RNA species, respectively. Importantly, this dataset now enhances the study of higher-order gene regulation. Testing how the cellular RBPs participate or intervene with post-transcriptional control of target mRNAs like Icos by specific trans-acting factors like Roquin-1/2, we show additional inputs from several other RBPs. These results not only exemplify a previously unrecognized complexity but also imply that post-transcriptional targets integrate simultaneous inputs from all RBPs able to interact with binding sites encoded in their mRNAs.

Results

Simultaneous and temporal regulation of Icos through several RBPs. A prominent example for complex post-transcriptional gene regulation is the inducible T-cell costimulator (Icos), which is essential for humoral immune responses23–25. Its mRNA has a long 3′-UTR, which responds in a redundant manner to Roquin-1 and Roquin-2 proteins26,38–40. Icos expression is also repressed by Regnase-115,18 and by microRNAs41,42. In addition, sites of TTP binding in the Icos mRNA have been determined by crosslinking and immunoprecipitation12. Moreover, the Icos 3′ UTR was proposed to be modified by m6A methylation43, which could either attract m6A-specific RBPs with YTH domains44, recruit or repel other RBPs45, or interfere with base-pairing and secondary structure or mRNA/miRNA-duplex formation46. Because of transcriptional and post-transcriptional regulation, Icos expression exhibits a hundred-fold upregulation on the protein level during T cell activation (d1–2), which quickly declines after removal of the TCR stimulus (d3–5) (Fig. 1). To investigate the temporal impact on Icos expression by Roquin-1/2, Regnase-1, m6A, and miRNA regulation we analyzed inducible, CD4-specific inactivation of Roquin-1 together with Roquin-2 (Rc3h1–2) or of Regnase-1 (Zc3h12a). We also analyzed the inactivation of Wtap, an essential component of the m6A methyltransferase complex47, or of Dgcr8, which is required for pre-miRNA biogenesis48. To this end, we performed tamoxifen gavage on mice expressing a Cre-ERT2 knockin allele from the CD4 locus49 together with the floxed, Roquin-1/2 paralogs encoding, Rc3h1 and Rc3h2 alleles (Fig. 1a–c) or Regnase-1 encoding Zc3h12a (Fig. 1d–l) or Wtap (Fig. 1g–i) or Dgcr8 alleles (Fig. 1j–l). We isolated CD4+ T cells from these mice and expanded them for 5 days. Confirming target deletion on the protein level (Fig. 1c, f, i, l and Supplementary Fig. 1a) we determined a strong negative effect on Icos expression by Roquin-1/2 and Regnase-1 on days 2–5 (Fig. 1a, b, and d, e), a moderate positive effect of Wtap on days 2–5 (Fig. 1g, h), and only a small effect of Dgcr8, with an initial tendency of negative (day 1) and later positive effects (days 4–5) (Fig. 1j, k). We next asked whether T cell activation affects the expression levels of known regulators of Icos, as well as other RBPs to establish temporal compartmentalization. To do so, we monitored the expression of a panel of RBPs in mouse CD4+ T cells over the same time course (Fig. 1m–q). Indeed, we revealed or confirmed fast upregulation of RBPs as determined with pan-Roquin, Nufip24, Fmrp, Fxr1, Fxr2, TTP/Zfp3612, pan-Ythdf (Supplementary Fig. 1b), or Celfl-specific antibodies, but also slower accumulation as demonstrated using Regnase-1 or Rbm1-specific antibodies (Fig. 1m, o). There was also downregulation of RBPs as shown with pan-Ago50, Regnase-1/417,18, as well as some miRNAs like miR-17–92, miR-155, miR-181, miR-125 or miR-146a19. Moreover, the first evidence for m6A RNA methylation in this cell type has been provided 20. Underscoring the relevance for the immune system, loss-of-function of these factors has often been associated with profound alterations in T cell development or functions which caused immune-related diseases. Intriguingly, many key proteins of the immune system have acquired long 3′-UTRs enabling their regulation by multiple, and often overlapping sets of post-transcriptional regulators. RBPs can also recruit additional co-factors as for example Roquin-1 binds together with Nufip2 to RNA 21, and some of them have antagonistic RBPs like HuR and TTP25 or Regnase-1 and Arid5a 26. Such functional or physical interactions together with interdependent binding to the transcriptome create enormous regulatory potential. The major challenge is therefore to integrate our current knowledge about individual RBPs into concepts of higher-order gene regulation that reflect the interplay of different, and ideally of all cellular RBPs.

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**Fig. 1 Icos responds to simultaneous inputs from several post-transcriptional regulators.** Naive CD4⁺ T cells (d0) from tamoxifen-gavaged mice of the indicated genotypes were activated by anti-CD3/anti-CD28 (day1–2) and subsequently cultivated in IL-2 containing media (d4–5). A, D, G, J Bar diagrams show the average of daily flow cytometric measurements of Icos expression with quantified mean fluorescence intensities (MFI) over a 5-day period to analyze changes due to inducible inactivation of Roquin-1 and Roquin-2 (Rc3h1 and Rc3h2), Regnase-1 (Zc3h12a) Wt and gcr8. Significance was calculated using the unpaired t-test (two-tailed) for data from three independent experiments using one mouse per genotype (n = 3). Error bars, mean ± s.d. B, E, H, K Representative histograms of Icos expression at the specified days. C, F, I, L Histograms confirming depletion of the respective target proteins. M–Q Western blots showing patterns of dynamic RBP regulation after anti-CD3/CD28 mediated T cell activation (n = 2). Significance *p value = 0.01-0.05; **p value = 0.001-0.01; ***p value = <0.001. Calculated p values: (A) 0d p = 0.005, 2d p = 0.016, 3d p = 0.012, 4d p = 0.0005, 5d p = 0.0017, (G) 5d p = 0.009, (J) 4d p = 0.022, 5d p = 0.009. Source data are provided as a Source Data file.
orchestrate redundant, cooperative, and antagonistic effects into a coordinated higher-order regulation.

The RNA-IC-identified proteins of mouse and human T helper cells. To analyze the post-transcriptional network, we set out to investigate the RNA-binding protein signature in primary mouse and human CD4+ T cells. To identify mRNA-binding proteins we first performed mRNA capture experiments on 2 x 10^7 CD4 + T effector cells (Teff) expanded under Th0 culture conditions, which omit cytokines and antibodies that skew them into specific subsets (Fig. 2a). Pull-down with oligo-dT beads enabled the enrichment of mRNA-bound proteins, which were increased in response to preceding UV irradiation of the cells, as determined by silver staining (Supplementary Fig. 1c). Reverse transcription-quantitative PCR (RT-qPCR) confirmed the recovery of specific mRNAs, such as for the housekeeping genes Hprt and β-actin.

Both mRNAs were enriched at least 2-3 fold after UV crosslink, but there was no detection of non-polyadenylated 18S rRNA.
Fig. 2 The T helper cell RBPome of polyadenylated RNAs. T6,0 cultures from three mice or three human donors were used as biologic replicates (n = 3) to investigate proteins interacting with mRNA. a Schematic illustration of the RNA-interactome capture (RNA-IC) method that was carried out to identify RBPs from mouse and human CD4+ T cells, b, c Volcano plots from two-sided Student’s T-test analysis using a permutation-based FDR method for multiple hypothesis corrections showing the −log10 p-value plotted against the log2 fold-change comparing the RNA-capture from crosslinked (CL) mouse CD4+ T cells (b) or human CD4+ T cells (c) versus the non-crosslinked (nCL) control. Red dots represent proteins significant at a 5% FDR cut-off level in both mouse and human RNA-capture experiments and blue dots were proteins that were significant only in mice or humans, respectively. d Enrichment analysis of GO molecular function terms of significant proteins in mouse or human RNA capture data. The 10 most enriched terms in mouse (dark blue) and the respective terms in human (light blue) are shown. The y-axis represents the number of proteins matching the respective GO term. p-values were calculated using the hypergeometric distribution and were adjusted by Benjamini–Hochberg multiple testing correction. Numbers above each term depict the adjusted p-value. e Distribution of IDRs in all Uniprot reviewed protein sequences (black line), in proteins of the mouse EuRBPDB database (green line), and in proteins significant in the mouse RNA-IC experiment (red line). The same plot is shown for human data at the bottom. According to two-sided Kolmogorov-Smirnov testing, the IDR distribution differences between RNA-IC (red lines) and all proteins (black lines) are highly significant in mice and man and reach the smallest possible p-value (p < 2.2 × 10−16).

(Supplementary Fig. 1d). Focusing on protein recovery, we determined greatly enriched polypyrimidine tract-binding protein 1 (Ptbp1) RBP compared to the negative control β-tubulin in mRNA capture experiments using the EL-4 thymoma cell line (Supplementary Fig. 1e). Next, we performed mass spectrometry (MS) on captured proteins from murine and human T cells (Fig. 2b, c). Quantifying proteins bound to mRNA in crosslinked (CL) versus non-crosslinked (nCL) samples we defined a total of 312 mouse (Fig. 2b) and 308 human mRNA-binding proteins (mRBPs) (Fig. 2c) with an overlap of ~70% (Supplementary Data 1), which is in concordance with the overlap of all listed RBPs for these two species in the eukaryotic RBP database (http://EuRBPDB.sysihospital.org). Gene ontology (GO) analysis identified the term ‘mRNA binding’ as most significantly enriched (Fig. 2d). The top 10 GO terms in the mouse were also strongly enriched in the human dataset with comparable numbers of proteins assigned to the individual GO terms in both species (Fig. 2d). RBPs not only bind RNA through classical RBDs, but RNA-interactions can also map to IDRs.32 Furthermore, low complexity regions (LCRs) have also been reported to be over-represented in RBPs.28 Indeed, IDRs (Fig. 2e) and LCRs (Supplementary Fig. 1f) were strongly enriched protein characteristics of mouse and human RNA-IC-identified RBPomes. We wondered how much variation existed in the composition of RBPs between different T helper cell subsets. Performing RNA-IC experiments using in vitro generated and phenotypically characterized mouse and human iTreg cells (Supplementary Fig. 2a–d), we found an overlap of ~96% or ~90% with the respective mouse or human iTreg with Teff RBPomes, suggesting that the same RBPs bind to the transcriptome in different T helper cell subsets (Supplementary Data 1 and Supplementary Fig. 2e). Nevertheless, 47 or 48 proteins were exclusively identified in mouse or human effector T cells, respectively, and 10 or 28 proteins were only found in mouse or human iTreg cells, respectively (Supplementary Fig. 2f). Taken together, these findings suggest that iTreg cells, as an example for T helper cell subset specialization, differentially express eight mouse and 20 human RBPs that do not overlap between the two species (Supplementary Fig. 2f).

The OOPS-identified proteins of mouse and human T helper cells. We then attempted to extend and confirm the T cell RBPome with a second approach that utilizes a different biochemical principle to experimentally enrich proteins crosslinked to RNA by employing orthogonal organic phase separation (OOPS).29 Similar to interactome capture, OOPS preserves cellular protein/RNA interactions by UV crosslinking of intact cells. The physicochemical properties of the resulting adducts direct them towards the interphase in the organic and aqueous phase partitioning procedure (Fig. 3a). Following several cycles of interphase transfer and phase partitioning, RNase treatment releases RNA-bound proteins into the organic phase, making them amenable to mass spectrometry29,33. Evaluating the method, we selected the UV dose that removes 75% of the total RNA from the aqueous phase29 (Supplementary Fig. 3a) and investigated selected RNAs and proteins from purified interphases derived from CL and nCL MEF cell samples (Supplementary Fig. 3b, c). RNAs with crosslinked proteins purified from interphases hardly migrated into agarose gels, but regained normal migration behavior after protease digest, as judged from the typical 18S and 28S rRNA pattern (Supplementary Fig. 3b, c). Conversely, known RBPs like Roquin-1 and Gapdh appeared after crosslinking in the interphase and could be recovered after RNase treatment from the organic phase (Supplementary Fig. 3c). Utilizing the same cell numbers and culture conditions of T cells, this method identified in total 1255 and 1159 significantly enriched RBPs for mouse or human T cells, respectively, when comparing CL and nCL samples (Supplementary Data 1). The overlap between both organisms was 55% (Fig. 3b) and 60% (Fig. 3c) in relation to the individual mouse and human RBPomes. Although glycosylated proteins are known to also accumulate in the interphase29, we experimentally verified that they did not migrate into the organic phase after RNase treatment (Supplementary Fig. 3d, e). Analyzing OOPS-derived RBPomes for gene ontology enrichment using the same approach as for RNA-IC the GO term ‘mRNA binding’ was again most significantly enriched in mice and humans (Fig. 3d). The top 10 GO terms were RNA related and six of them overlapped with those identified for RNA-IC-derived RBPomes. High similarity between mouse and human RBPomes becomes apparent by the similarity in all GO categories, including ‘molecular function’ (Fig. 3d), ‘biological process’, and ‘cellular component’ (Supplementary Fig. 4). Although our OOPS approach exceeded by far the quantity of RNA-IC identified RBPs, the number of ~1200 RBPs well-matched published RBPomes of HEK293 (1410 RBPs), U2OS (1267 RBPs), and MCF10A (1165 RBPs) cell lines29.

Defining the core T helper cell RBPome. To define a T helper cell RBPome we first made sure that neither RNA-IC nor OOPS preferentially identified high abundance proteins (Fig. 4a, b). In comparison to total proteome measurements, OOPS-identified RBPs spanned the whole range of protein expression without apparent bias. In general, this was also true for RNA-IC, with a tendency to more abundantly expressed proteins. This however might be a true effect since messenger RNA-binding RBPs have been reported to be higher expressed compared to other RBPs.27 We used the recently established comprehensive eukaryotic RBP database as a reference to compare OOPS- and RNA-IC-identified canonical and non-canonical RBPs from mouse and human CD4+ T cells. The numbers of proteins in the mouse T helper cell RBPomes created by RNA-IC and OOPS ranging from...
312 to 1255 made up 10–40% of all listed EuRBPDDB proteins, respectively (Fig. 4c). OOPS-identified T cell RBPs outnumbered those from RNA-IC experiments by a factor of four, which was predominantly due to the eight times higher number of non-canonical RBPs. Interestingly though, there were also twice as many canonical RBPs significantly enriched by OOPS (Fig. 4c).

Analyzing the 10 most abundantly annotated mouse RBDs (comprising 26–224 RBP family members) showed that RNA-IC and OOPS often identified the same canonical RBPs (Supplementary Tables 1 and 2), however at least equal, higher, or much higher numbers were detected in OOPS samples depending on the specific RBD (Fig. 4d). These findings underscored that the OOPS method is likely more sensitive and by design recovered RBPs from additional, non-polyadenylated RNAs. The data also
show that our RNA-IC-derived RBPomes are mostly specific but incomplete. These conclusions are also supported by highly similar results obtained for the human CD4+ T cell RBPome (Fig. 4e, f). In a four-way comparison of mouse and human RBPs identified by OOPS and RNA-IC (Fig. 4g), we conservatively defined all proteins that were identified by at least two datasets as ‘core CD4+ T cell RBPomes’ discovering 798 mice and 801 human RBPs in this category (Supplementary Data 1). A sizable number of 519 mouse and 424 human proteins were exclusively enriched by the OOPS method, of which more than 50% of the proteins of both subsets matched to mouse or human EuRPDB-listed annotations (Supplementary Data 1). These findings suggested that genuine RBPs are found even outside of the intersecting set of OOPS and RNA-IC identified proteins and that the definition of RBPomes profits from employing different biochemical approaches. We further compared published human OOPS data sets from the embryonic kidney (HEK293), osteosarcoma (U2OS), and mammary epithelial (MCF10a) cell lines79 with our dataset from primary human CD4+ T cells (Fig. 4h). The four-way comparison shows that although similar numbers of RBPs were identified overall, the number of uniquely identified RBPs was almost three times higher in CD4+ T cells than in each of the cell lines (Fig. 4h, left panel). Of the 439 CD4+ T cell unique RBPs 294 were newly discovered and 145 were previously annotated (Fig. 4h, right panel and Supplementary Data 1). The annotated RBPs can be further divided into 92 canonical and 53 non-canonical RBPs (Supplementary Data 1). We interpret the result such that RBPomes are strongly affected by tissue-specific expression of RBPs and/or RBP activity in the presence or absence of post-translational modifications, substrates, and cofactors.

**T cell signaling proteins with unexpected RNA-binding function.** Some of the identified RBPs of the core proteome including Stat1, Stat4, and Crip1 are not expected to be associated with mRNA in cells. We, therefore, established assays to confirm the RNA-binding of these candidates. To do so, GFP-tagged candidate proteins were overexpressed in HEK293T cells, which were UV cross-linked, and extracts were used for immunoprecipitation with GFP-specific antibodies. Using SDS–PAGE and protein blotting and detection with either anti-GFP antibodies or oligo(dT) probes verified the pull-down of GFP-tagged proteins (Fig. 5a, left panel) and the association with mRNA (Fig. 5a, right panel) for the RBPs Roquin-1 and Rbms1 as well as for the lactate dehydrogenase (Ldhα) protein, a metabolic enzyme with known ability to also bind RNA28 (Fig. 5a). Via this approach, the determined RNA association of Stat1, Stat4, and Crip1 was indeed confirmed (Fig. 5b). It appeared less pronounced as compared to prototypic RBPs but was similar with regard to Ldhα (Fig. 5a, b). We then tested human and mouse STAT1 and STAT4 proteins as purified recombinant proteins in RNA-EMSA with in vitro transcribed TSU lncRNA. This lncRNA is expressed in human cells and early work revealed a sequence-specific recognition by STAT1 using extracts of STAT1-transfected cell extracts34,55. Performing RNA-EMSA without and with competitor RNA we showed binary interaction of mouse and human STAT1 and STAT4 that was at least partially resistant to unspecific competition (Fig. 5c). Our results thereby excluded a requirement for additional factors or signal-induced STAT1 or STAT4 protein modification in eukaryotic cells or any indirect contribution from cell extracts in these RBP/RNA interactions. These findings support a potential moonlighting function of these signaling proteins. To address a regulatory function for STAT1 or STAT4 protein binding to RNA, we established a dual luciferase assay to investigate the impact of the different proteins on the expression of the renilla luciferase when they were tethered to its mRNA via an artificial 3'-UTR (Fig. 5d). We utilized the λN/5xboxB system56 and confirmed the expression of fusion proteins with a newly established λN-specific antibody (Fig. 5e, f). Importantly, Stat1 and Stat4 repressed luciferase function almost to the same extent as the known negative regulators Pat1b and Roquin-1, or other known RBPs, such as Celf1, Rbms1, and Cpeb4 (Fig. 5g), and this repression reduced the abundance of the boxB containing renilla luciferase mRNA (Fig. 5h). λN-Crip1 and λN-Vav1 expression did neither exert a positive nor a negative effect, since their relative luciferase expression appeared unchanged compared to cells transfected to express only the λN polypeptide (Fig. 5g). These data suggest that the transcription factors Stat1 and Stat4, which we defined here as part of the T helper cell RBPome, not only have the capacity to bind mRNA but can also exert RNA regulatory functions. While Vav1 was identified in mouse and human T cells by OOPS and RNA-IC, the tethering assay did not reveal obvious regulation, indicating a more specialized feature of this new RBP.

**Analyzing higher-order post-transcriptional regulation.** Since large-scale parallel screens with primary T cells are highly challenging, we devised an experimental strategy to reduce the RBPome to RBPs that are likely to antagonize or cooperate with the Roquin-1 RBP in the repression of its target mRNAs. To this end, we performed BioID experiments to define the cellular proteins that are physically close to Roquin-1 (Fig. 6a). In this proximity-based labeling method, we expressed a Roquin-1 BirA* fusion protein to identify proteins that reside within a short distance of ~10nm57 in T cells (Fig. 6a). In this dataset we sought for matches with the T cell RBPome (Fig. 4g) to identify proteins that shared the features, ‘RNA-binding’ and ‘Roquin-1 proximity’. We first verified that the mutated version of the biotin ligase derived from E. coli (BirA*) which was N-terminally fused to Roquin-1 or GFP was able to biotinylate lysine residues in Roquin-1 or other cellular proteins (Fig. 6b) but did not interfere with the ability of Roquin-1 to downregulate Icos (Fig. 6c). Doxycycline-induced BirA*-Roquin-1 compared to BirA*-GFP expression in CD4+ T cells significantly enriched biotin labeling
of 64 proteins (Supplementary Data 2), including Roquin-1 (Rc3h1) itself or Roquin-2 (Rc3h2) (Fig. 6d) as well as previously identified Roquin-1 interactors and downstream effectors, such as Ddx6 and Edc438, components of the Ccr4/Not complex58,59 and Nufip224 (Fig. 6d). More than half of all proteins in proximity to Roquin-1 were also part of the defined RBPome (Fig. 6e and Supplementary Table 3). Roquin-1 may come close to other RBPs while being bound to RNA, however, proximity labeling will take place in all different parts of the cell independent of RNA binding. Increasing the BioID list above with additional proteins that we found in proximity to Roquin-1 when establishing and validating the BioID method in fibroblasts (Supplementary Fig. 5a–d), we arrived at 143 proteins (Supplementary Data 2) of which 96 (67%) were part of the RBPome (Supplementary Figs. 5e, 6a, and Supplementary Table 4). From these, we cloned 46 candidate genes of interest (GOI) in the context of N-terminal GFP fusions and determined GFP expression in HEK293T cells (Supplementary Fig. 6b). We then transduced CD4$^+$ T cells and
analyzed the effects of GFP-GO1 expression on endogenous Roquin-1 targets (Supplementary Fig. 7a). CD4+ T cells were used from mice with Rc3h1fl/fl, Rc3h2fl/fl, Rcr3h1rtTA alleles in combination with (iDKO) or without the Cd4-Cre-ERT2 allele (WT) allowing induced inactivation of Roquin-1 and -2 by 4’-OH-tamoxifen treatment. The Roquin-1 targets Icos, Oxl0, Ctl4a, I1K8N, and Regnase-1 became strongly derepressed in induced double-knockout (iDKO) T cells (Supplementary Fig. 7b). This elevated expression was corrected to wild-type levels in iDKO T cells that were retrovirally transduced and doxycycline-treated to express GFP-Roquin-1 (Supplementary Fig. 7b). The target expression in WT T cells was only moderately reduced through ectopic expression of GFP-Roquin-1 (Supplementary Fig. 7b). For the majority of the 46 candidate genes, induced expression in WT or iDKO CD4+ T cells did not alter the expression of the five analyzed Roquin-1 targets, exemplified here by the results obtained for Vav1 (Supplementary Fig. 7c, d). Interestingly, we identified a new function for Rbms1 (transcript variant 2), specifically upregulating Ctl4a (Supplementary Fig. 7e, f). Furthermore, we demonstrated that Cpeb4 strongly upregulates Oxl0 and, most strikingly, in the same cells Cpeb4 repressed Ctl4a levels (Supplementary Fig. 7g, h). While these findings are noteworthy, they occurred in a Roquin-1-independent manner. In contrast to these effects, we discovered a higher-order regulation of Icos by Igf2bp3 (Fig. 7a, b). Interestingly, Igf2bp3, an unconventional reader of RNA methylation60, was consistently identified in mice but not in human CD4+ T cell RBPMes by RNA-IC and OOPS and hence may have divergent functions in these two species. An even stronger Roquin-1 dependent increase of Icos occurred upon induced expression of Celf1 (Fig. 7c, d). In wild-type T cells Celf1 clearly upregulated Icos, Ctl4a, and Oxl0 expression but not the Nfkbid mRNA encoded I1K8N protein expression and this function was obliterated in Roquin-1-deficient iDKO cells (Fig. 7c, d). While Igf2bp3 and Roquin-1 shared a strictly cytoplasmic localization and enrichment in BFP-Ddx6-labeled P-bodies (Supplementary Fig. 8a), the majority of GFP-Celf1 was nuclear. Only a small fraction of the protein was cytoplasmic, where it colocalized with Roquin-1 and Ddx6 in P-bodies (Fig. 7e). The antagonistic effect could not be explained by Celf1-mediated repression of Roquin-1 on the protein or mRNA level (Supplementary Fig. 8b, c). Vice versa, Roquin-1 KO also did not affect Celf1 mRNA levels (Supplementary Fig. 8d). Instead, the observed antagonistic effect likely involved simultaneous or mutually exclusive binding of Celf1 and Roquin-1 to the same mRNAs, since we determined strong interaction of Celf1 with Icos and Ctl4a mRNAs in RNA-IP experiments, but not with the Nfkbid mRNA (Fig. 7f). In conclusion, the combination of protein-centric and RNA-centric global approaches enabled us to discover higher-order functional interactions as shown for the Roquin-1/2-dependent regulation of the costimulatory receptor Icos by Igf2bp3 or Celf1.

Discussion

The work on post-transcriptional gene regulation in T helper cells has focused on some miRNAs and several RNA-binding proteins, and few reports described m6A RNA methylation in this cell type. Although arriving at a more or less detailed understanding of individual molecular relationships and regulatory circuits, this isolated knowledge assembles into a very incomplete picture. Defining the human and mouse T helper cell RBPMes has now opened the stage, allowing to work towards understanding connections, deciphering complexity and principles of post-transcriptional regulatory networks in these cells.

RNA-IC and OOPS are two complementary methods to define RBPs on a global scale. While RNA-IC mostly queries for proteins bound to polyadenylated RNAs, OOPS captures the RNA-bound proteome in its whole. Applying both methods to T helper cells of two different organisms allowed us to cross-validate the results from both methods and solidify our description of the core and human T helper cell RBPMes. While the vast majority of RNA-IC-identified CD4+ T cell RBPs were previously known RNA binders, OOPS typically confirmed and profoundly expanded these results (Supplementary Tables 1 and 2), and more than half of OOPS identified proteins that were exclusively found in mouse or man were EuRBPD-Listed.

Strikingly, the signaling proteins Stat1 and Stat4 were identified by mouse RNA-IC and human OOPS and were just below the cut-off (0.05 FDR, >2fold enrichment) in the mouse OOPS dataset, and we could support their RBP function by additional RNA-binding assays. Undoubtedly, the defined human and mouse T cell RBPMes contain many more unusual RBPs that would warrant further investigations. We assume that even RNA interactions of proteins without prototypic RBDS, like the Vav1 and Stat proteins, will have consequences for both binding partners. As the identity of the interacting mRNA(s) is currently unknown, we could only speculate about the post-transcriptional impact. Nevertheless, Stat1 and Stat4, but not Vav1, showed regulatory capacity in our tethering assays. Intriguingly, RNA-binding may impact the function of Stat proteins as transcription factors. Supporting this notion, early results found Stat1 bound to the non-coding, polyadenylated RNA ‘TSU’, derived from a trophoblast cDNA library, and translocation of Stat1 into the nucleus was reduced after TSU RNA microinjection into HeLa cells54,55. In line with this, Vav1 signal-transduction could be altered in an unknown way through its engagement with RNA.

Many 3’-UTRs, which effectively instruct post-transcriptional control, exhibit little sequence conservation between species, and the exact modules which specify regulation are not known. This is for example true for the Icos mRNA24,38. On the side of the transcriptional factors, we find a high similarity between the RBPMes of T helper cells of mouse and human origin, actually reflecting the general overlap of so far determined RBPMes from many cell lines of these species.
We define the first RBPsomes of human and mouse T helper cells and explore avenues of how to make use of this information. Screening a set of candidates from the T cell RBPsome for effects on Roquin-1/2 targets, our findings support a concept in which mRNA targets of one trans-acting factor are further separated into different "RNA operons" i.e. subsets of RNAs responding to the same post-transcriptional inputs. Such "RNAs operons" can then form "RNA regulons" comprising coordinately regulated mRNA subsets that are aligned to function in the same biological process. Therefore, complex and differential binding of targets by RBPs of the cellular RBPsome specify the possible operons and regulons and enable differential functions of the cell. Roquin-1/2 cooperated, coregulated, or antagonized in the regulation of Icos with Regnase-1 and m6A or miRNA functions. Our screening approach added Rbms1 or Cpeb4 as Roquin-1/2-independent regulators of Cta-4 and/or Ox40. It also revealed that different targets of Roquin-1/2 responded very differently to the expression of specific coregulators, as for example Ctla4 and Ox40 were inhibited or induced by the same RBP, Cpeb4, respectively. Moreover, Celf1 and Igf2bp3 were identified as Roquin-1/2-dependent coregulators in the Icos containing RNA operons. Intriguingly, Celf1 only interacted with those target mRNAs of Roquin-1/2 for which it antagonized repression. This suggests that Celf1 may either prevent binding of Roquin-1/2 to some
binding sites or Celf1 may be part of mRNPs with Roquin-1/2 and indicate an unexpected wealth of possible inputs originating from individual components to enable higher-order post-transcriptional gene regulation.

To solve the seemingly simple question of which RBPs regulate which mRNAs in T helper cells, we will require further knowledge about individual contributions, binding sites, and composite cis-elements, temporal and interdependent occupancies, interactions among RBPs and with downstream effector molecules. In this endeavor global protein and RNA-centric approaches make fundamental contributions.

Methods
Isolation, in vitro cultivation, and transduction of mouse primary CD4+ T cells. For in vitro cultivation of primary murine CD4+ T cells, mice were sacrificed and spleen, as well as cervical, axillary, brachial, inguinal, and mesenteric lymph nodes, were dissected and pooled. Single-cell suspensions were generated from lymphoid organs and passed through a 100 µm filter under rinsing with T cell isolation buffer (PBS supplemented with 2% FCS and 1 mM EDTA). Erythrocytes were eliminated by incubating cells with TAC–lysis buffer (13 mM Tris, 140 mM NaCl, pH 7.2) for 5 min at room temperature. CD4+ T cells were isolated by negative selection using EasySep Mouse CD4+ T cell isolation Kit (18352A, Stem Cell) according to the manufacturer’s protocol. CD4+ T cells were cultured in DMEM (41966-029, Invitrogen) T cell culture medium supplemented with 10% FCS (AC-0184, Anprotec), 1% Pen–Strep (15140-122, 10,000 U/ml Penicillin, 100,000 U/ml Streptomycin, Thermo Fisher), 10 mM HEPES-buffer (15630-056, Invitrogen), 1% non-essential amino acids (13-114E, 100x NEAA mixture; Invitrogen), and 50 µM β-mercaptoethanol (31350-010, Invitrogen) without antibodies or cytokines that skew their differentiation into specific T helper cell subsets (Th0 conditions). For activation and differentiation under Th1 conditions the T cells were stimulated with α-CD3 (0.5 µg/ml; cl. 145-2C11, in house production), α-CD28 (2.5 µg/ml; cl. OX-19, in house production), 10 µg/ml α-Ile-4 (cl. 1B11, in house production), and 10 ng/ml IL-12 (554592, BD Pharmingen) and seeded T cells on goat α-hamster IgG (0.05 mg/ml in PBS, 56894, MP Biologicals) pre-coated six-well (5 Mio cells/ml for 40 h for translations with retroviruses) or 12-well (1.5 Mio cells/ml for 48 h for expression analyses) plates. The cells were resuspended and cultured in a medium supplemented with 200 IE/ml recombinant hIL-2 (Proleukin S, Novartis) in a 10% CO2 incubator and expanded for 2–4 days, as indicated. Subsequently, cells were fed with fresh IL-2-containing medium every 24 h and cultured at a density of 0.5–1 × 10^6 cells/ml. In vitro deletion of floxed alleles of Rch3fl/fl;Rch2fl/fl;Cd4-Cre-ERT2fl/fl, Regnase-1(fl;3Hz12fl;Cd4-Cre-ERT2fl), Wtap(Wtapfl;Cd4-Cre-ERT2fl) and Dgr8(Dgr8fl;Cd4-Cre-ERT2fl) encoding alleles in Cd4-Cre-ERT2 mice was induced in vivo by oral transfer of 5 mg tamoxifen (Sigma) in corn oil. Details of tamoxifen each day were given on 2 consecutive days (total of 20 mg tamoxifen per mouse). Mice with the genotype Cd4-Cre-ERT2 (without floxed alleles) were used for wild-type controls. Mice were sacrificed 3 days after the last gavage and total CD4+ T cells were isolated using the EasySep Mouse T cell Isolation Kit (18352A, Stem Cell) and activated under Th1 conditions as described above. Animal breeding and experimentation followed the legal approval of the Government of Upper Bavaria (Regierung von Oberbayern, reference numbers 55.2-2532-Ver02-19-122 and 55.2-2532-Ver.02-19-68). The work was compliant with the relevant ethical regulations for animal testing and research. All animals were housed in a pathogen-free barrier facility in accordance with the Helmholtz Center Munich, the Ludwig Maximilians University Munich institutional, state, and federal guidelines.

Flow cytometry. Following in vivo deletion and Cd4+ T cell isolation (above) cells were cultured and used under Th1 conditions. Cells were obtained daily for FACS analysis. The single-cell suspensions were stained with fixable violet dead cell stain (L34955, Thermo Fisher) for 20 min at 4 °C. For the detection of surface proteins, cells were stained with the appropriate antibodies in FACS buffer for 20 min at 4 °C. After staining, cells were acquired on a FACS Canto II (3-laser). The data were further processed with the software FlowJo 10 software (BD Bioscience). The following antibodies were used at a dilution of 1:200: anti-CD4 (cl. GK1.5), anti-CD44 (cl. IM7), anti-CD62L (cl. MEL-14), anti-T-bet (cl. C98H.11, 5C7), anti-Ox40 (cl. OX-86), anti-CD25 (cl. PC61, Biolegend). Two doses of doxycycline-induced expression of 46 GFP-GFP fusion proteins in 2 × 10^6 wild-type and Roquin-1/2 iDKO CD4+ T cells were analyzed on day 6 after isolation (compare Supplementary Fig. 7b). First, proteins were treated with a fixable dead blue cell stain (L31305, Invitrogen) and after washing, stained in three panels to interrogate the surface expression of Icos and Ovaltin (Icos-PE, clone 7E.17G9/Ox40-APC, clone OX-86, both eBioscience) and to intracellularly measure Cx4, IkBa, Cx4-PE, UC10-489; ebioscience/cd 4C1 rat monoclonal; in house production) as well as Regnase-1 (cl. 15D11 rat monoclonal; in house production). For intracellular staining, cells were fixed in 2% formaldehyde for 15 min at RT, permeabilized by washing in Saponin buffer, and stained with the appropriate antibodies for 1 h at 4 °C. After washing, an anti-rat antibody (cl. polycl054; Biolegend) was added for 30 min. All in-house monoclonal antibody supernatants generated at the Helmholtz Center were used at a dilution of 1:10 and all commercial antibodies were diluted 1:200. After additional rounds of Saponin- and FACS buffer washing, the acquisition was performed using an LSR Fortessa device.

Isolation and differentiation of mouse effector and regulatory T cells for RNA-ic. naïve CD4+ T cells were isolated by using Dyna- and Detachabeads (11485D and 12406D, Invitrogen) from spleens and mesenteric lymph nodes of 8–12 weeks old C57BL/6J mice. For iTreg culture, cells were additionally selected for CD62L+ with anti-CD62L (clone: Mel14)-coated beads. All cells were then activated with plate-bound anti-CD3 (using first anti-hamster, 0.5 mg/ml in PBS, 55397, Novartis, then anti-CD3 in solution: clone: 1µg/ml), anti-IL-4 (cl. ME-14), anti-ICOS (cl. C98H.11, 5C7), anti-OX40 (cl. OX-86), all from eBioscience, anti-CD25 (cl. PC61, Biolegend). Two doses of doxycycline-induced expression of 46 GFP-GFP fusion proteins in 2 × 10^6 wild-type and Roquin-1/2 iDKO CD4+ T cells were analyzed on day 6 after isolation (compare Supplementary Fig. 7b). First, proteins were treated with a fixable dead blue cell stain (L31305, Invitrogen) and after washing, stained in three panels to interrogate the surface expression of Icos and Ovaltin (Icos-PE, clone 7E.17G9/Ox40-APC, clone OX-86, both eBioscience) and to intracellularly measure Cx4, IkBa, Cx4-PE, UC10-489; ebioscience/cd 4C1 rat monoclonal; in house production) as well as Regnase-1 (cl. 15D11 rat monoclonal; in house production). For intracellular staining, cells were fixed in 2% formaldehyde for 15 min at RT, permeabilized by washing in Saponin buffer, and stained with the appropriate antibodies for 1 h at 4 °C. After washing, an anti-rat antibody (cl. polycl054; Biolegend) was added for 30 min. All in-house monoclonal antibody supernatants generated at the Helmholtz Center were used at a dilution of 1:10 and all commercial antibodies were diluted 1:200. After additional rounds of Saponin- and FACS buffer washing, the acquisition was performed using an LSR Fortessa device.
produced antibodies were obtained in collaboration with and from Regina Feederle (Helmholtz Center Munich). After differentiation for 36–48 h cells were expanded for 2–3 days. iTreg cells were cultured in RPMI and 2000 units Proleukin S (02238131, MP Biomedicals) and Teff cells with 200 units Proleukin S. We only used iTreg cells for experiments if samples achieved at least 80% Foxp3 positive cells (00552300, Foxp3 Staining Kit, BD Bioscience). EL-4 T cells were cultured in the same medium as primary T cells. HEK293T cells were cultured in DMEM (supplemented with 10% (vol/vol) FCS, penicillin–streptomycin (100 U/ml, Gibco) and Hepes, pH 7.2 (10 mM, Gibco)).

**Fig. 6 Identification of proteins in proximity to Roquin-1 in CD4+ T cells.** Expression of BirA*-Roquin-1 and biotin tagging define a subset of the T cell RBPome in close proximity to Roquin-1. a Schematic overview of the BioID method showing how the addition of biotin to the medium leads to the activation of biotin, diffusion of biotinoyl-5’AMP and the biotinylation of the bait (Roquin-1) and all preys in the circumference. b Equimolar amounts of protein were loaded onto a PAGE gel for Western blotting applying an anti-biotin antibody. Efficient biotinylation of both baits BirA*-Roquin-1 and BirA*-GFP (control) could be demonstrated (n = 2). c Histogram showing that transduction of CD4+ T cells with retrovirus to inducibly express BirA*-Roquin-1 lead to the efficient downregulation of endogenous Icos. d Identified preys from Roquin-1 BioIDs (n = 5) in CD4+ T cells. Depicted are all significantly enriched proteins with the exception of highly abundant ribosomal and histone proteins. A two-sided heteroscedastic Student’s T-test analysis was performed. Dot sizes equal p-values and positioning towards the center implies increased x-fold enrichment over BirA*-GFP BioID results. e Venn diagram showing the overlap of RBPs from the CD4+ T cell RBPome with the proteins identified by Roquin-1 BioID in T cells. Source data are provided as a Source Data file.

**Culture preparation of human CD4+ T cell blasts.** The use of the material of human origin in this work was approved by the ethics committee of the Technical University of Munich (approvals 934/03 and 1872/07) and was in accordance with

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**Legend:**
- **Fig. 6 a** Dox-inducible BirA* fusion:
  - Roquin-1-binding proteins
  - Peripheral proteins
  - Biotinoyl-5’AMP
  - Covalently bound biotin

**Fig. 6 b**
- BirA*-GFP
- BirA*-Roquin-1
- BirA*-Roquin-1
- BirA*-GFP

**Fig. 6 c**
- Transduced T cells
- BirA*-Roquin-1
- BirA*-GFP

**Fig. 6 d**
- Rc3h1 = bait
  - p < 0.001
  - p < 0.002
  - p < 0.004
  - p < 0.02
  - p < 0.05

**Fig. 6 e**
- Expanded T cell RBPome (1352)
- T cell Roquin-1 BioID (64)
- 1314
- 3826
Fig. 7 Higher-order Icos regulation by Roquin-1 and Celf1. Inducible overexpression of candidate RBPs in CD4+ T cells was used to investigate cooperative or antagonistic effects on Roquin-1/2-regulated targets. CD4+ T cells with the genotypes \( \text{Rc3h1}^{fl}\text/\text{fl};\text{Rc3h2}^{fl}\text/\text{fl};\text{rtTA3} \) without (WT) or with the \( \text{Cd4-Cre-ERT2} \) allele (iDKO) were used for transduction with retroviruses after treatment with 4'-OH-tamoxifen. Expression levels of Icos and four additional Roquin-1 targets in WT and iDKO cells were analyzed 16 h after doxycycline-induced arrayed expression of 46 individual GFP-GOI fusion genes. a, c Each geometric mean of the Roquin-1 target in the GOI-GFP sample was divided by the geometric mean of the Roquin-1 target in the GFP sample. Summarized ratios are shown as bar diagrams for (a) Igf2bp3 and (b) Celf1. b, d Representative flow cytometry data are depicted as histograms and contour plots. Experiments were repeated at least three times. e Using confocal microscopy, co-localization of Roquin-1 and Celf1 in P bodies was demonstrated by co-transfecting Hela cells using plasmids coding for Cherry-Roquin-1, GFP-Celf1, and BFP-Ddx6, the latter of which served as a P body marker (n = 2). The standard bars equal 10 µm. f For RNA-immunoprecipitation CD4+ T cells were isolated and cultured as for (a, c). Anti-Roquin-1, anti-Celf1, and isotype control antibodies were coupled to magnetic beads and used to immunoprecipitate the respective proteins and their bound RNAs from T cell lysates. At the end, Trizol was added to the magnetic beads to isolate RNA, reverse-transcribed cDNA, and measure target mRNA levels by qPCR and relative to input. Isotype levels were set to 1. Depicted is one representative experiment (n = 2) and data points indicate technical replicates of qPCR measurements. Source data are provided as a Source Data file.
the Declaration of Helsinki of the World Medical Association (last amended in 2013). Blood (120 ml) was collected by venepuncture from two times four donors for RNA-capture and OOPS experiments. Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll gradient (P04-601000, Pancoll) centrifugation and CD4+ T cells were isolated from 1 x 10^8 cells using CD4+ Microbeads (130-045-101, Miltenyi) to arrive at 2-3 x 10^6 cells. The purity was >95% CD4+ T cells with >90 viable cells. These were resuspended at 2 x 10^6/ml in T cell medium (AIM-V (12055-091, Invitrogen), 10% fetal bovine serum (FBS), 2 mM glutamine, 10 mM HEPES and 1.25 µg/ml Fungizone), supplemented with 500 ng/ml PHA (MHC-1A6GB, Murex), and 100 µM IL-2 (Prolifor S, Novartis), 500 nM Rapamycin (S1039, Selleckchem), anti-Roquin-1/2, cl. 3F12; anti-Regnase-1, cl. 15D11; anti-pan-Ago, MAGO3-5, procedures were performed according to standard protocols. For silver staining we used a red dye containing silver (P02932SR, ab165402, ab150354, ab165403, ab165408, ab154535, ab165405), for Western blotting or silver staining.

**Functional analysis of human CD4+ T cells.** To test for T cell cytokine production, aliquots of effector CD4+ T cells (Teff) were cultivated for additional 2-3 weeks. Subsequently, 1 x 10^6 of these resting Teff cells were washed, resuspended in 200 µl T cell medium and left untreated or treated with 1.5 µg/ml of the anti-CD3 antibody OKT3 (Jansen-Cilag). Next day, the IFNγ content in the culture supernatant was measured by ELISA (3420-H2-20, Mabtech). Inhibitory activity of human iTreg cells was assessed in co-culture experiments by adding iTreg cells at different ratios to the co-cultures of 5 x 10^4 Epstein-Barr virus-specific CD4+ T cells. For RNA-capture, eluates were diluted 1:5 with 62.5 mM Tris, pH 8.1, and proteins digested with 0.5 µg Lys-C and 0.5 µg Trypsin at room temperature overnight. The resulting peptides were desalted using stage tips containing three layers of C18 material (Empore).

For OOPS experiments, 100 µl of the buffer (P.O.O.00027, PreOnts iSt kit) were added and samples were incubated at 100 °C for 10 min at 400 rpm. Samples were sonicated for 15 cycles (30 s on/30 s off) on a bioruptor (Diagonode). Protein concentration was determined using the BCA assay and about 30 µg of proteins were digested. To this end, trypsin and Lys-C were added in a 1:100 ratio, samples were digested for 16 h, and samples were vortexed for 15 s. Samples were transferred on SDB-RPS (Empore) stage tips (3 layers), washed twice with 100 µl isopropanol/1% TFA, and twice with 100 µl 0.2% TFA. Peptides were eluted with 80% of 2% ammonia/80% acetonitrile, dried on a centrifugal evaporator, and resuspended with 10 µl of buffer A* (2% ACN, 0.1% TFA).

**LC-MS/MS analysis.** Peptides were separated on a reverse-phase column (50 cm length, 75 µm inner diameter) packed in-house with ReproSil-Pur C18-AQ 1.9 µm resin (Dr. Maisch GmbH). Reverse-phase chromatography was performed with an EASY-nLC 1000 ultra-high pressure system, coupled to a Q-Exactive HF Mass Spectrometer (Thermo Scientific) for mouse RNA-capture experiments or a Q-Exactive HF Mass Spectrometer (Thermo Scientific) for human RNA-capture, OOPS experiments, and single-shot proteomes in combination with Thermo Q-Exactive HF Tune software (v4.0.23.0082). Chromatography was performed with buffer A (0.1% (v/v) formic acid) and eluted with a nonlinear 120-min (10-min gradient for human RNA-capture and OOPS experiments) gradient of 5–60% buffer B (0.1% (v/v) formic acid, 80% (v/v) acetonitrile) at a flow rate of 250 nl/min (300 nl/min for human RNA-capture and OOPS). After each gradient, the column was washed with 95% buffer B and re-equilibrated with buffer A. Column temperature was kept at 60 °C by an in-house designed oven with a Peltier element, and operational parameters were monitored in real-time by the SprayQc software. MS data were acquired using a data-dependent top 15 (10 for human RNA-capture and OOPS experiments) method in positive mode. Target value for the full scan confident protein and peptide identifications. False discovery rate was 1% for both proteins and peptides (minimum length of 7 amino acids). The maximum number of missed cleavages allowed was 2. Maximal allowed precursor mass deviation for peptide identification was 4 ppm for time-dependent modifications and maximal fragment mass deviation was 20 ppm. Protein intensities were calculated using the MaxLFQ algorithm, which is based on the pairwise calculation of peptide ratios. "Match between runs" was activated with a retention time alignment window of 20 min and a match time window of 0.5 min for RNA-capture experiments, while matching between runs was disabled for OOPS experiments. The maximum ratio count was set to 2 for label-free quantification.

**Data analysis.** Statistical analysis of MS data was performed using Perseus (version 1.6.0.28). Human RNA-capture, mouse RNA-capture, human OOPS, and mouse OOPS data were processed separately. For all experiments, MaxQuant (v1.5.1.6/5.1.1.6.0) was used. The raw data were analyzed by the MaxQuant software. The mass spectrometer data were processed according to standard protocols. For silver staining we used the SilverQuest kit (LC6070, Invitrogen). The following in-house generated monoclonal antibody supernatants were used in Western blots at a 1:10 dilution: anti-Roquin-1/2, cl. 3F12; anti-Regnase-1, cl. 15D11; anti-pam-Ago, cl. MAGO3-5, anti-Nucleolin, 2F6/5-9, YTHDF, cl. 17F2; anti-GFP, 3E5-111. Commercial antibodies used for Western blots were: anti-Fxrl, polyclonal, 4173, 1:1000, (Cell Signaling); anti-Fxr2, cl. D85D6, 7098, 1:1000 (Cell Signaling); anti-TTP, cl. T538, 1:10000 (Sigma); anti-Grp94, cl. 6C5, CB1001, 1:10000 (Calbiochem), anti-Cellf1, cl. 850717, MAB9388, 0.5 µg/ml (R&D SYSTEMS); anti-RBM5, cl. EP08295R, ab165402, ab150354, ab165403, ab165405, ab165408, ab165409, ab165410, ab165412, 1:AP, 1:750 (Proteintech); anti-Ptp1b, 1:1000, 8776 (Cell Signaling); anti-tubulin, 1:1000, 86298 (Cell Signaling); goat anti-rat antibody, cl. Poly4054, 1:200 (Biolegend); goat anti-mouse antibody, polyclonal, 554001, 1:400 (BD Bioscience) and anti-Ptp1b, 1:1000, 8776, Cell Signaling) were visualized by staining with anti-rabbit (1:4000, Cell Signaling) or anti-mouse antibody, polyclonal (1:2000, 7076, Cell Signaling) secondary antibodies conjugated to HRP.

**Sample preparation for mass spectrometry.** For RNA-capture, eluates were incubated with 10 µg/ml RNase A in 100 mM Tris, 50 mM NaCl, 1 mM EDTA at 37 °C for 30 min. RNase-treated eluates were acetone precipitated and resuspended in denaturant buffer (6 M urea, 2 M thiourea, 10 mM Heps, 8 µl), reduced with 10% β-mercaptoethanol and alkylated with iodoacetate, and samples were precipitated. For silver staining we used a red dye containing silver (P02932SR, ab165402, ab150354, ab165403, ab165405, ab165408, ab165409, ab165410, ab165412, 1:AP, 1:750 (Proteintech); anti-Ptp1b, 1:1000, 8776 (Cell Signaling); anti-tubulin, 1:1000, 86298 (Cell Signaling); goat anti-rat antibody, cl. Poly4054, 1:200 (Biolegend); goat anti-mouse antibody, polyclonal, 554001, 1:400 (BD Bioscience) and anti-Ptp1b, 1:1000, 8776, Cell Signaling) were visualized by staining with anti-rabbit (1:4000, Cell Signaling) or anti-mouse antibody, polyclonal (1:2000, 7076, Cell Signaling) secondary antibodies conjugated to HRP.
input RNA and oligo(dT)-isolated RNA with the Quant iT Reverse Transcription Kit (205311, Qiagen). The respective qRT-PCRs for Hprt, β-actin, and 18S rRNA were used as reference genes. For qRT-PCR, reverse transcription, and quantitative RT PCR for Renilla and Firefly luciferases, Re3h1, Icos, Cil4a, Nkbid, Cel1, and Cel2 were performed as published7 using the universal probes systems (Roche). For Primer sequences see Supplementary Table 5.

Expression and purification of Stat proteins. The pGEX-6P-2/Stat constructs (human as well as mouse Stat4 and Stat7) were transformed into E. coli Rosetta2 (DE3) or Rosetta2 (DE3) pl3YS and expressed overnight at 20°C in ZYM 5052 auto-induction medium (352000, Teknova). The cells were harvested, lysed by sonication, and clarified by centrifugation. Non-specifically bound bacterial nucleotides were precipitated by the addition of 0.5% polyethyleneimine (PEI) and the excess PEI was removed by ammonium sulfate pre- dicted at 95% saturation. The ammonium sulfate pellet was re-suspended in GSTTrap-binding buffer, dialyzed overnight against the same buffer and the Stat proteins were then purified on a Hi-R 3C protein (1.25 mM l-mer) and the Stat proteins further purified by size exclusion chromatography over a Superdex 200 column.

In vitro transcription. In vitro transcription of the non-coding RNA ‘TSU’ (AF800902) was performed using the HiScriber T7 High Yield RNA Synthesis Kit (E20505, NEB) according to the manufacturer’s instructions. Subsequently, the template DNA was used as template in a Dual-Luciferase Reporter Assay System (E1910, Promega). Renilla luciferase activity was normalized to Firefly luciferase activity in each well to control in transfection efficiency. psiCHECK2 lacking boxII sites served as a negative control, and each transfection was analyzed in triplicates.

Biod. The proximity-dependent biotin identification assay was performed according to Roux73 with modifications. For each sample 2 x 10⁶ MEF cells were grown on ten 15-cm culture dishes for 24 h before Biara-ROQ-1 or BirA expression was induced by doxycycline treatment (1 μg/ml). For T cells, transduction with the same BirA-fusions cloned into the plasmid pETxtox was performed as described above but with the same number of cells used for the experiment. Six hours after the addition of doxycycline, biotin was added for 16 h to arrive at an end concentration of 50 μM. Approximately 8 x 10⁶ cells per sample were trypsinized, washed twice with PBS, and lysed in 5 ml lysis buffer (50 mM Tris–HCl, pH 7.4; 500 mM NaCl, 0.2% SDS; 1× protease inhibitors (04693159001, Roche), 20 mM DTT, 25 U/ml Benzonase (1.01654.0001, Merck)) for 30 min at 4°C using an end-over-end mixer. After adding 50 μl of 20% Triton X-100 the samples were sonicated for two sessions of 30 pulses at 30% duty cycle and output level 2, using a Branson Sonifier 450 device. Keep on ice for 2 min in between sessions. Pipetting of 4.5 ml pre-chilled 50 mM Tris–HCl, pH 7.4 was followed by an additional round of sonicating. After centrifugation, the samples were mixed at 4°C, 500 μl Dynabeads MyOne Streptavidin C1 (65002, Invitrogen) or each sample was equilibrated in a 1:1 mixture of lysis buffer and 50 mM Tris–HCl pH 7.4. After overnight binding on a rotator at 4°C Streptavidin beads were stringently washed with wash buffers 1, 2, and 3 (78) and prepared for mass spectrometry by three additional washes with buffer 4 (1× lysis EDTA, 20 mM NaCl, 50 mM Tris–HCl pH 7.4). Proteins were eluted from streptavidin beads with 50 μl of biotin-saturated 1× sample buffer (50 mM Tris–HCl pH 6.8, 12% sucrose, 2% SDS, 20 mM DTT, 0.004% Bromphenol blue, 3 mM Biotin (B4501, Sigma) by incubation for 7 min at 98°C. For identification and quantification of proteins, samples were performed by LC-MSMS on a QExactive HF mass spectrometer (ThermoFisher Scientific) coupled directly to a Ultimate 3000 RSLC nano-HPLC ( Dionex). Label-free quantification was based on peptide intensities from extracted ion chromatograms and performed with the Progenesis QI for proteomics v3.0 software (Nonlinear Dynamics). Raw files were imported and after alignment, filtering, and normalization, all MSMS spectra were exported and searched against the Swissprot mouse database (16772 sequences, Release 2016_02) using the Mascot search
Confocal microscopy. One day prior to analysis, HeLa cells were transfected via calcium-phosphate precipitation, and cells were seeded 6 h prior to microscopic analysis on eight-well μ-slides (Glass bottom, Ibidi) in Leibovitz’s L-15 media (no phenol red, 21083027, Thermo Fisher). Confocal images were performed with a TCS SP8 X FALCON confocal head (Leica Microsystems, Wetzlar, Germany) mounted on an inverted microscope (DMi8; Leica Microsystems). For confocal imaging, a 405 nm diode and a white light laser were used as excitation sources (405 nm for BFP, 488 nm for GFP, and 594 nm for mCherry). Single photons were collected through a ×93/1.3 NA glycerin-immersion objective and detected on a ×100/1.34 NA oil immersion objective. For confocal imaging, a pixel size of 0.098 μm was applied, giving a pixel size of 0.098 μm and an image size of 50 × 50 μm. Scanning speed was 600 Hz.

Co-immunoprecipitation of Roquin or Celf5 bound RNAs. CD4 T cells (3 × 10^7) were lysed on ice in 500 μl cold, RNAse-free lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.25% (vol/vol) Nonidet-P40, 1.5 mM MgCl₂, protease inhibitor mix without EDTA (04693159001, Roche) and 1 mM dithiothreitol). Lysates were shock frozen, thawed, and cleared by centrifugation (10 min, 10,000 × g, 4 °C). In parallel, the protein lysate was pre-cleared using 20 µl of uncoupled magnetic (10004D, Invitrogen) magnetic beads, respectively, and incubated for 1 h at 4 °C. In this paper.

Antibodies. To generate monoclonal antibodies against pan-Ythdf proteins or λN-peptide, Wistar rats were immunized with purified GST-tagged full-length mouse Ythdf3 protein or an ovalbumin-coupled λN peptide (MNARTRRRE-RAEKQWKAAN) using standard procedures as described 78. The hybridoma clones of Ythdf- or λN-reactive supernatants were cloned at least twice by limiting dilution. Experiments in this study were performed with anti-pan-Ythdf clone D3F 17F2 (rat IgG2a/κ) and anti-λN clone LAN 4F10 (rat IgG2b/κ).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The mass spectrometry proteomics data that support the BioID results have been deposited to the ProteomeXchange Consortium via the PRIDE 80 partner repository with the dataset identifier PXD026716. The mass spectrometry data that support the identification of the CD11b+ cell RBPome have likewise been deposited to PRIDE with the accession codes PXD008830 (mouse RNA-IC), PXD021164 (human RNA-IC), PXD022795 (mouse OOPS), PXD021169 (human OOPS). Source data are provided with this paper.

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**Author contributions**

E.G. and V.H. conceived the idea for the project and together with M.W. and M.M. supervised the experimental work. K.P.H. and V.H. wrote the manuscript with contributions from E.G., M.W., and A.R. K.P.H. performed OOPS, BioID, and T cell transductions with help from G.B., K.D., S.M.H., J.M. and C.C. C.G. conducted RNA-IC experiments and RNA-binding assays. A.R. and S.M.H. analyzed the BioID experiments and RNA-binding assays. A.R. and S.M.H. performed mass spectrometry and A.R. analyzed RBPome data. E.G. and V.H. conceived the idea for the project. M.W., E.G. and V.H. conceived and designed the experiments, performed mass spectrometry and A.R. analyzed RBPome data. S.M.H. and K.P.H. analyzed the BioID experiment data. E.G. and V.H. conceived the idea for the project and together with M.W. and M.M. supervised the experimental work.

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**Competing interests**

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