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Zcchc11-dependent uridylation of microRNA directs cytokine expression

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Mounting an effective host immune response without incurring inflammatory injury requires the precise regulation of cytokine expression 1, 2. To achieve this, cytokine mRNAs are post-transcriptionally regulated by diverse RNA-binding proteins and microRNAs targeting their 3' untranslated regions 3, 4. The Zcchc11 protein contains RNA-interacting motifs⁵, and it has been implicated in signaling pathways involved in cytokine expression 6. The nature of the Zcchc11 protein and how it influences cytokine expression are unknown. Here we show that Zcchc11 directs cytokine expression by uridylating cytokine-targeting microRNAs. Zcchc11 is a ribonucleotidyltransferase with a preference for uridine and is essential to maintaining the poly(A) tail length and stability of transcripts for interleukin-6 (IL-6) and select other cytokines. The mir-26 family of miRNAs targets IL-6, and the addition of terminal uridines to the mir-26 3' end abrogates IL-6 repression. While 78% of mir-26a sequences in control cells contained 1–3 uridines on their 3' ends, less than 0.1% did so in Zcchc11 knock down cells. Thus, Zcchc11 fine tunes IL-6 production by uridylating mir-26a, which we propose to be an enzymatic modification of the terminal nucleotide sequence of mature miRNA as a means for regulating gene expression.

Zcchc11 is a member of the DNA polymerase β -like (pol β) nucleotidyltransferase superfamily 5 and it interacts with adaptor proteins in the Toll-like receptor (TLR) signaling pathway 6. Overexpression of a truncated form of Zcchc11 (containing amino acids 191 to 625 of the 1644 amino acid protein, including only the first 2 of the 7 domains of interest identified in Fig. 1a) decreased expression of the cytokines IL-6 and TNF- α 6. Because the

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degrees to which this truncation mutant conferred gain or loss of function were unclear, such an approach indicates that Zcchc11 mediates cytokine expression without illuminating the mechanism(s) by which it performed this function. The Zcchc11 protein contains domains capable of interacting with RNA, suggesting that the mechanism(s) for regulating cytokine expression may extend beyond its interaction with adaptors. As depicted in Figure 1a, these domains include a CCHH zinc finger, typically interacting with RNA or DNA; two poly(A) polymerase/2'-5' oligoadenylate synthetase (PAP/25A) associated domains, common in RNA modifying enzymes 7; three CCHC zinc knuckles, often RNA-binding domains 8, 9; and a pol β domain, usually mediating nucleotidyltransferase activity 5. The putative pol β catalytic domain of Zcchc11 is highly conserved across genomes from a wide spectrum of eukaryotes (Fig. 1b). Altogether, this primary structure suggested that Zcchc11 might be a ribonucleotidyltransferase.

Several proteins with homology to Zcchc11 are categorized as non-canonical poly(A) polymerases, including the *Caenorhabditis elegans* protein defective in germ line development 2 (GLD-2) 10 and the *Schizosaccharomyces pombe* proteins caffeine-induced death protein 1 (Cid1) and Cid13 11, 12. To determine whether Zcchc11 possessed such activity, we generated recombinant epitope-tagged wildtype Zcchc11 as well as a mutant form (Zcchc11 DADA) in which two aspartate residues predicted to be essential to catalysis 13 were mutated to alanines (Fig. 1b). Recombinant epitope-tagged Zcchc11 exhibited nucleotidyltransferase activity for all RNA substrates examined (Fig. 1c). In contrast, the DADA mutant exhibited no transfer of adenosine to RNA, implicating the pol β domain as responsible (Fig. 1c). Endogenous Zcchc11 immunoprecipitated from a human lung epithelial cell line (A549) also exhibited nucleotidyltransferase activity (Supplementary Fig. S1a). The radiolabeled product was sensitive to RNase digestion, confirming RNA as the substrate of the enzyme (Supplementary Fig. S1b). These data show that Zcchc11 is a ribonucleotidyltransferase enzyme whose activity is dependent on a conserved catalytic site.

Some pol β family members were recently identified as poly(U) polymerases 14, 15. Given this, we examined nucleotides other than ATP in Zcchc11 assays. Both endogenous (Fig. 1d) and recombinant epitope-tagged (Supplementary Fig. S1c) forms of Zcchc11 displayed the greatest nucleotidyltransferase activity with UTP, followed by CTP, and then ATP and GTP. The inclusion of an ATP regenerating system, designed to circumvent ATP loss from potentially co-precipitating ATPases, did not enhance adenosine incorporation (data not shown). As with adenylation, the uridylation activity of Zcchc11 did not display RNA substrate specificity, evidenced by the labeling of diverse RNA moieties including even an RNA ladder (Fig. 1e). These results indicate that Zcchc11 catalyzes the non-selective, template-independent uridylation of RNA.

Although Zcchc11 mRNA was ubiquitous among mouse tissues (data not shown), Zcchc11 protein was tissue-specific, most prominently expressed in the thymus, spleen, testes, and lung (Supplementary Fig. S1d). Zcchc11 was also expressed in human and mouse lung alveolar epithelial cell lines (A549 and MLE-15, respectively: Supplementary Fig. S1e). Expression of Zcchc11 in immune and mucosal cells pointed again to potential roles in cytokine regulation. Effective knockdown in A549 and MLE-15 cells by Zcchc11-targeted siRNA compared to control, non-targeting siRNA was confirmed by immunoblotting using

different siRNAs specific to human or mouse *Zcchc11* (Fig. 2a). Cell supernatants were collected from control or *Zcchc11* siRNA-treated A549 cells stimulated with TNF- α , and cytokine expression was analyzed. A subset of cytokines, namely IL-6, VEGF, TGF- α , and RANTES, was substantially decreased by loss of *Zcchc11* (Fig. 2b). In contrast, fractalkine, IP-10, MCP-1, G-CSF, and GM-CSF levels were not significantly affected, and IL-8 concentrations were increased (Fig. 2b). These cytokine-specific effects were not unique to A549 cells, but were also observed in HEK 293T cells treated with control non-targeting or *Zcchc11* targeting siRNA prior to TNF- α treatment (data not shown). The effects of *Zcchc11* knockdown on IL-6 elaboration by A549 cells was examined over a time-course after TNF- α . *Zcchc11* depletion led to decreased IL-6 at all time points examined, with differences between groups increasing over time (Fig. 2c). *Zcchc11*-dependent effects on IL-6 expression were not unique to TNF- α stimulation, as *Zcchc11* depletion similarly diminished IL-6 production elicited by *Escherichia coli* (Supplementary Fig. S2a). As with results obtained in human A549 cells, IL-6 expression was decreased in murine MLE-15 cells by knockdown using a different siRNA targeting mouse *Zcchc11* (Fig. 2d). Thus, these results were not specific to a given siRNA sequence and were consistent across species and stimuli.

To determine whether *Zcchc11* influenced cytokine expression at the mRNA level, we first measured steady-state transcripts. IL-6 mRNA was decreased by *Zcchc11* knockdown (Fig. 2e). Cytokine transcript stability was subsequently assessed, showing mRNA half-life for IL-6 to be decreased by *Zcchc11* knockdown (Fig. 2f). The effects of *Zcchc11* knockdown on the stability of transcripts for other cytokines generally mirrored the effects of *Zcchc11* knockdown on cytokine protein content (Supplementary Fig. S2b). These data suggest that *Zcchc11* mediates cytokine expression at the posttranscriptional level by influencing mRNA stability.

The poly(A) tail length of eukaryotic mRNAs is a critical determinant of transcript stability and translational efficiency 16, 17. By any of three independent measurement methods 18, the poly(A) tails on IL-6 mRNA were shortened due to *Zcchc11* knockdown (Figs. 2g and Supplementary Fig. S2c,d). Other *Zcchc11*-dependent cytokines examined also demonstrated shorter poly(A) tails due to *Zcchc11* knockdown (Supplementary Fig. S2e) whereas tail lengths for cytokines not decreased at the protein or mRNA stability levels were not shortened by *Zcchc11* knockdown (Supplementary Fig. S2f). In summary, decreasing *Zcchc11* results in shorter poly(A) tails on only those cytokines whose transcript stability and maximal protein expression require *Zcchc11*. Poly(A) tail length therefore appears to be the step at which *Zcchc11* directly or indirectly mediates cytokine expression.

Because of its uridylyltransferase activity, we considered whether *Zcchc11* might uridylylate the 3' polyadenylate terminus of mRNAs, as was observed for the *Zcchc6* homolog of *Zcchc11* 14. IL-6 transcripts were isolated, cloned, and sequenced from control and *Zcchc11* knockdown A549 cells, but no terminal uridines were observed (Supplementary Fig. S3). Given these results and the enzymatic activity of *Zcchc11*, we considered alternative RNA substrates that could influence transcript poly(A) tail length and stability.

mRNA deadenylation and degradation can be stimulated by miRNAs 19, 20, leading us to reason that the RNA-modifying Zcchc11 enzyme may alter cytokine expression through an miRNA-dependent mechanism. Support for this hypothesis was preceded by observations that miRNAs often contain untemplated nucleotides on their 3' ends, primarily adenosines or uridines 21–23. The cytoplasmic poly(A) polymerase GLD-2 24, a relative of Zcchc11, is capable of adenylating the 3' end of mature miRNA, but no enzyme has yet been identified as responsible for the uridylation of mature miRNA. Analyses using TargetScan 25–27 revealed that the 3' untranslated region (UTR) of IL-6 mRNA contains a highly conserved region potentially targeted by the mir-26 family of microRNAs (Fig. 3a), lying in close proximity to an AU-rich region and accessible within the predicted secondary structure of IL-6 mRNA, characteristics positively associating with miRNA-mediated repression 28. Prior studies indicated that mir-26b in naïve T cells contains non-genomic uridines on the 3' end 29, supporting the postulate that miRNAs in this family including mir-26b in particular may be modified in the proposed fashion. Northern analyses demonstrated that mir-26b is expressed in A549 cells, with no effect of Zcchc11 knockdown on its content (Fig. 3b). Transfection of A549 cells with mir-26b mimetics decreased TNF-induced IL-6 expression (Fig. 3c), demonstrating that this miRNA can repress IL-6. Complementarily, blocking endogenous mir-26b using a mir-26b-antisense inhibitor 30, 31 increased IL-6 expression (Fig. 3d). Blockade of endogenous mir-26a also increased IL-6 expression (Supplementary Fig. S4a). Taken together, these gain-of-function and loss-of-function approaches demonstrate that this family of miRNAs targets IL-6. Because the mir-26 mimetics showed less of a repressive effect on IL-6 than did Zcchc11 knockdown, mir-26 may be but one of multiple miRNAs potentially modified by Zcchc11 to regulate IL-6 expression.

Zcchc11 was capable of uridylating mir-26b *in vitro* (Figure 3e). The enzyme showed a preference for the single-stranded RNA in comparison with the duplex, and this activity was abolished by point mutations in the nucleotidyltransferase domain (Figure 3e). Thus, Zcchc11 directly uridylates mature, single-stranded microRNAs including mir-26b. To our knowledge, there is no prior evidence that sequence variations in the 3' ends of miRNAs impact their capability to repress targeted transcripts. Interestingly, we observed that 3' end uridylation of mir-26b mimetics abrogated repression of IL-6 by this miRNA (Figure 3f). Uridylation of mir-26a mimetics also exhibited a decreased ability to repress IL-6 expression (Supplementary Fig. S4b). To test whether this was unique to the mir-26 targeting of IL-6, we transfected cells with uridylated mir-16 mimetics and assessed VEGF concentrations in the supernatant after TNF- α stimulation. We observed strong repression of VEGF by mir-16, as previously reported³², but this repression was not attenuated by mir-16 uridylation (Supplementary Fig. S4c). Additionally we sought to determine whether oligouridine tracts at miRNA 3' termini are capable of increasing IL-6 expression independent of sequence specificity, such as by acting as ligands for TLR7 and TLR8 ³³. We found no differences in IL-6 production in the supernatants of TNF-stimulated cells treated with uridylated species of mir-16 (Supplementary Fig. S4d). Taken together, these data suggest that uridylation of IL-6 mRNA-targeting miRNAs may be the mechanism by which Zcchc11 influences IL-6 expression.

We next determined whether the influences of this enzyme and miRNA on IL-6 expression involved direct interactions with the 3'-UTR of the IL-6 transcript. The IL-6 3'-UTR was sufficient to reduce protein expression in a luciferase reporter assay (Figure 4a). Importantly, this repressive effect of the IL-6 3'-UTR was exacerbated by Zcchc11 knockdown (Figure 4a). Further, the overexpression of Zcchc11 increased expression of the IL-6 3'-UTR-associated gene (Figure 4b). Mutating the nucleotidyltransferase domain of Zcchc11 significantly diminished these effects (Figure 4b). Thus, the ability of Zcchc11 to enhance IL-6 expression is mediated by the 3'-UTR and is dependent upon its nucleotidyltransferase activity.

mir-26b gain of function further diminished expression associated with the IL-6 3'UTR (Figure 4c). The addition of terminal uridines to this miRNA decreased its repressive activity for the IL-6 3' UTR (Figure 4c). Importantly, both the decreased expression driven by mir-26b and the counteracting increased expression resulting from its uridylation were abrogated by mutation of the predicted miRNA target site within the 3'-UTR (Figure 4d). Overall these results demonstrate that the effects observed are direct results of miRNA interactions with the 3'UTR.

Altogether, our data strongly suggest that Zcchc11 influences IL-6 expression by the untemplated uridylation of mir-26b or related IL-6 3'-UTR-targeting miRNAs. We thus deep sequenced small RNAs isolated from control and Zcchc11 knockdown cells to assess 3' terminal modifications of mir-26 family members. In A549 cells, mir-26a sequences were far more abundant than mir-26b (7,068 and 235 sequences recovered, respectively). Of the mir-26a sequences collected from control cells, the majority (74%) were the 22-nucleotide sequence reported in Figure 3a, ending in a single uridine which could be genomically encoded. An additional 4% of sequences contained 2 or 3 uridines rather than the single uridine at the end, which must necessarily represent untemplated uridylation (Fig. 4e and Supplementary Fig. S5).

The Zcchc11 knockdown cells demonstrated remarkably different results in that out of 10,306 mir-26a sequences collected, none (0%) were the sequence reported in Figure 3a. Instead, the majority (67%) were 21 nucleotides in length and missing the terminal uridine (Fig. 4e), suggesting dicer cleavage of the mir-26a pre-miRNA at this nucleotide followed by Zcchc11-mediated uridylation in the control cells. For mir-26a sequences with definitively non-genomic uridines in the Zcchc11 knockdown cells, only 2 (0.02%) sequences ended in UU and none (0%) in UUU. These data indicate that Zcchc11 mediates the addition of 1 to 3 uridines to the 3' terminus of mir-26a, defining a role for Zcchc11 in miRNA end modification.

Most (33%) of the remaining mir-26a sequences in the knockdown cells ended in UA (Fig. 4e), also observed in the control cells albeit with lesser frequency (8%). One possible explanation for such a pattern could be an enzyme which adds UA residues to microRNAs, potentially competing with Zcchc11 for mir-26a modification. Alternatively, since adenylation of miRNAs increases miRNA stability ²⁴, it is conceivable that mir-26a that had been uridylated by Zcchc11 and then adenylated by GLD-2 or some other enzyme, but

not non-adenylated forms of uridylated Zcchc11, may persist from before Zcchc11 was knocked down.

Not only were mir-26b sequences less common than mir-26a, but the former also showed a distinct pattern of non-genomic end modification, with UA ends in 99% and 96% of mir-26b sequences from control and knockdown cells, respectively (Supplementary Fig. S6). Based on prior studies showing non-genomic uridines but not UA additions on mir-26b in T cells 29, we suggest that the modifications of miRNAs is context-dependent. The identity of putative UA-adding enzyme or adenylating enzymes in A549 cells and the significance of these nucleotide additions for mir-26b function remain to be determined.

The addition of non-templated nucleotides, most prominently uridine and adenine, at the 3' terminal ends of miRNAs has been observed in multiple species 34, 21, 35, 22, 23, 36, 37. The mechanisms determining which miRNAs are modified by Zcchc11, and under which circumstances, remain important avenues for future study. To our knowledge, these studies provide the first identification of an enzyme responsible for the untemplated uridylation of mature miRNA. Such miRNA modifications are not simply by-products of sloppy processing, but rather they impart functional differences that attenuate miRNA-targeted repression. We further demonstrate this to be a means of post-transcriptionally regulating cytokine expression, potentially relevant to inflammation and immunity.

Methods

Cell Culture and Transfection

Human lung epithelial A549 cells, human embryonic kidney 293T (HEK 293T), and murine lung epithelial (MLE-15) cells were transiently transfected with expression plasmids for 24 hours. Cells were grown in DMEM (if HEK 293T or MLE-15) or Ham's F12 (if A549) supplemented with 10% heat inactivated fetal bovine serum, penicillin, and streptomycin. siRNA knockdown of Zcchc11 was performed in epithelial cells using DharmaFECT 1 reagent (Dharmacon). HEK 293T cells were transfected with expression plasmids using SuperFect reagent (Qiagen). To target human Zcchc11, the annealed sense CAACAGACAUGUACAGAUUUU and antisense UAUCUGUACAUGUCUGUUGUU siRNAs were used for 48 hours. To target mouse Zcchc11, the annealed sense GCAAUUCUCCUUUACAUUUUU and antisense AAUGUUAAAGGAGAUUUGCUU siRNAs were used for 48 hours. Transfection with siCONTROL non-targeting siRNA #1 or #2 (Dharmacon) were used as negative controls in siRNA experiments and all siRNAs were used at a final 100 nM concentration. For luciferase experiments, IL-6 3' UTR reporter plasmids were cotransfected with either siRNA or miRNA mimetics for 48 hours using Lipofectamine 2000 as per manufacturers instruction (Invitrogen). phRL-TK (Promega) was used as a transfection efficiency control and both firefly and Renilla luciferase activity was determined with the Dual Luciferase Reporter Assay System (Promega). For cell infections, A549 cells treated with either nontargeting or Zcchc11 siRNA for 48 hours, were cultured in the presence of 5×10^8 CFU of *E. coli* for 24 hours in complete media. The supernatants were collected and subsequently assayed for IL-6 protein by ELISA.

Plasmid Construction and Mutagenesis

Zcchc11 (accession # NM_175472) was RT-PCR amplified from RNA isolated from C57BL/6 mouse lungs. Full length sequences were Flag-tagged and mutagenized using the QuikChange site-directed mutagenesis kit (Stratagene). For luciferase assays, the entire human IL-6 3' UTR was PCR amplified from genomic DNA and cloned into the pMIR-REPORT™ Luciferase reporter vector (Ambion) 38. The IL-6 3'UTR mutant contains two adenosines that were changed from uracil by site-directed mutagenesis to disrupt the first two mir-26 seed pairing with the target IL-6 sequence.

Nucleotidyltransferase Assays

Flag-tagged WT and DADA mutant Zcchc11 (immunoprecipitated from transfected HEK 293T cells 39) or endogenous Zcchc11 (immunoprecipitated from A549 cells) were incubated with RNA substrates and then electrophoresed in 6% TBE-Urea polyacrylamide gels. Endogenous Zcchc11 was immunoprecipitated after lysis (50 mM Tris HCl, pH 8.0, 150 mM NaCl, and 1% NP-40) using a goat polyclonal anti-Zcchc11 antibody (Imgenex). Prewashed protein G Dynabeads (Invitrogen) were used for magnetic immunoprecipitations. Immunoprecipitates (10 µL, estimated as 50–150 ng Zcchc11) were incubated for 30 minutes at 37 °C in a total volume of 50 µl with 0.13 mM [α -³²P] NTP (final concentration) and 1 µg yeast tRNA (Ambion), A₁₅ oligoribonucleotide (Invitrogen), or human RNA as substrates, or vehicle, together with 40 units RNAsin (Promega) in poly(A) polymerase buffer containing 25 mM Tris HCl pH 7.0, 50 mM KCl, 0.7 mM MnCl₂, 1 mM MgCl₂, 0.2 mM EDTA, 100 µg/ml acetylated BSA, and 10% glycerol (USB). Radiolabeled RNA was purified using Sephadex G-25 Quick Spin Columns (Roche). Samples were electrophoresed in 6% TBE-Urea polyacrylamide gels. Gels were dried under vacuum and exposed to radiographic film. Human non-coding RNA and poly(A) mRNA was fractionated using oligo(dT) cellulose from A549 cell total RNA using the Poly(A) Purist mRNA purification kit (Ambion).

Measuring mRNA and Protein

Human and murine cytokine and Zcchc11 mRNA contents were measured using real-time RT-PCR. Total RNA was extracted with Trizol (Life Technologies), and real-time RT-PCR was performed using the iScript One-Step RT-PCR Kit for Probes (Biorad) and the iCycler iQ Real-Time PCR detection system (Biorad). Probes contained the reporter dye 6-FAM at the 5' end and Black Hole Quencher-1 at the 3' end. For each sample, fold induction (vs. control siRNA) was normalized to the content of 18S rRNA 40. For immunoblotting, 20–45 µg protein in lysates from C57BL/6 mouse organs or cell cultures in a buffer supplemented with 1X complete protease inhibitors (Roche) were subjected to 3–8% tris-acetate SDS-PAGE and electroblotted onto Immobilon P transfer membranes. Antibodies used were against Flag M2 (Sigma), β -actin or pan actin (Cell Signaling), or a peptide from the C terminus of human and mouse Zcchc11 (Imgenex).

mRNA Decay, Northern, and Poly(A) Tail Length Analysis

Prior to RNA collection, A549 cells were transfected with siRNA for 48 hours and stimulated with TNF- α (10 ng/ml) for 1 hour. Transcription was inhibited when indicated

using 5 µg/ml actinomycin D following the 1 hour TNF-α exposure. mRNA was quantified using real-time RT-PCR. mRNA poly(A) tail length was assessed by H-northern, RACE-PAT, and LM-PAT as described 18. For H-northern assays, 20µg of total RNA were digested with RNase H after hybridization with an IL-6-targeting anti-sense oligonucleotide (5' GTGTCCTAACGCTCATACTTTT 3'). Select samples also included oligo(dT₁₂₋₁₈) for digestion of the poly(A) tails, as indicated. After digestion, RNA was electrophoresed on 6% polyacrylamide urea gels and transferred to nylon membranes. Membranes were hybridized to a ³²P-labeled IL-6 3'UTR-targeting probe and exposed to radiographic film and densitometry (NIH ImageJ). For amplifying this 3'UTR probe, primers were sense 5' AAATATGTGAAGCTGAGTTAATTTATGTAAG 3' and antisense 5' AAAATGCCATTTATTGGTATAAAAACC 3'. For RACE-PAT, 1 µg of total RNA was used for RT with an adaptor primer oligo(dT) anchor. PCR amplification products with the adaptor reverse primer and a forward primer specific to IL-6 mRNA (5' CTTTGTTCAGAGCCAGATCATTCTTG 3') were visualized on 2% agarose gels stained with ethidium bromide. For LM-PAT, 300 ng total RNA was saturated with phosphorylated oligo(dT₁₂₋₁₈) and ligated with 20 Weiss units T4 DNA ligase (Promega) while annealed to mRNA poly(A) tails. A second ligation was sequentially performed with the addition of an adaptor primer oligo(dT₁₂) anchor in five fold abundance over oligo(dT₁₂₋₁₈) at 14 °C for 2 hours. The oligo-primed mRNA was then reverse-transcribed with 40 units of AMV RT (Promega). All enzymes were heat inactivated and RT cDNA was used for PCR amplification using an mRNA specific sense primer and the adaptor primer oligo(dT₁₂).

Primer sequences

For qRT-PCR analysis, primer and probe sequences were as follows: IL-6, sense 5'TGCAATAACCACCCCTGACC 3', antisense 5'ATGCCATTAACAACAAC AATCTG 3', and probe 5'ACCACAAATGCCAGCCTGCTGACG 3'. IL-8 sense 5'CGGAAGGAACCATCTCACTCTG 3', antisense 5'AGAAATCAGGAAGGC TGCCAAG 3', and probe 5' TGACTTCCAAGCTGGCCGTGGCTC 3'. VEGF-A sense 5'TGAGATCGAGTACATCTTCAAGCC 3', antisense 5' CTGAGGTTT GATCCGCATAATCTG 3', and probe 5' CCTGTGTGCCCTGATGCGATGCG 3'. G-CSF sense 5' TTCCTGGAGGTGTCGTACCG 3', antisense 5' TCCGTT CTGCTCTCCCTGTC 3', and probe 5' TTCTACGCCACCTTGCCAGCCCT 3'. GM-CSF sense 5' ATCTCAGAAATGTTTGACCTCCAG 3', antisense 5' TTC AAAGGTGATAATCTGGGTTGC 3', and probe 5' AGCCGACCTGCCTACAGA CCCGCC 3'. TGF-α sense 5' TTCGCTCTGGGTATTGTGTTG G 3', antisense 5' GGCAGTCATTAATAATGGGACACC 3', and probe 5' CCACGGGCGGGTCTG CACTCAGCG 3'. Murine Zcchc11 sense 5' AGAGTGAACCTCGTCTCTTCC 3', antisense 5' AACCATACAACCTCAGTGAACATTC 3', and probe 5' TCCTTC CTCTGCTCACTTGGCTGC 3'. For H-northern, the IL-6 mRNA probe used for RNase H digestion was 5' GTGTCCTAACGCTCATACTTTT 3'. For amplifying an IL-6 3' UTR specific probe used in H-northern, primers were sense 5'AAATAT GTGAAGCTGAGTTAATTTATGTAAG 3' and antisense 5' AAAATGCCATTTATT GGTATAAAAACC 3'. For IL-6 RACE-PAT analysis IL-6 sense primer 5' CTT

TGTTTCAGAGCCAGATCATTTCTTG 3' and antisense adaptor primer 5' GCGAGCTCCGCGGCCGCG-T₁₂ 3' were used. The same antisense adaptor primer was used for LM-PAT and for mRNA specific sense primers we used 5' AATGGAAAGTGGCTATGCAGTTTGAATATC 3' (IL-6), 5' GTTATCATT TATTTATTGGTGCTACTGTTTATCCG 3' (VEGF-A), 5' CTGTAATGTGTTAAT GTGATTAGCAGTTATTTTCC 3' (TGF- α), 5' GGGAGCTGCTCTCATGAAA CAAG 3' (GM-CSF), and 5' TTAGAACTATTAACAGCCAAAACACTCCACAG 3' (IL-8).

MicroRNA Isolation, Cloning, and Northern Analysis

Total RNA was extracted with Trizol (Invitrogen) and subsequently used to further purification using the mirVana™ miRNA isolation kit as per manufacturers instruction (Ambion) to yield small RNAs < 200 nt in length. Small RNAs were pooled from 4 independent Control and Zcchc11 knockdown A549 cells treated with TNF- α for 3 hours for the library. Detailed methods on library cloning are available upon request. For northern blotting, 1–2 μ g of small RNAs were loaded per well of 15% PAGE urea gels and transferred to Brightstar-Plus Nylon membranes. Specific small RNAs were detected with polynucleotide kinase ³²P-labeled antisense oligonucleotides: 5' GCCATGCTAATCTTCTCTGTATC 3' (U6) and 5' ACCTATCCTGAATTACTTGAA 3' (mir-26b).

Deep sequencing

Short RNA cDNA libraries were run on an Illumina genome analyzer, and sequence was produced from IPAR data using Illumina pipeline 1.3.2. Individual reads were extracted from linker sequence, identical reads were collapsed to sequences, and the sequences were queried against the human mature miRNA database from miRBase (release 13.0) using the BLAST algorithm. Analyses of mir-26a end modification included all sequences which demonstrated complete fidelity across the first 21 mir-26a nucleotides and were free of contamination by sequences from the ligated adaptors.

MiRNA Mimetics and Inhibitors

Transient transfections of A549 cells were performed as described above with the annealed mir-26b sense (miRBase accession MIMAT0000083) UUCAAGUAAUUCAGGAUAGGU and antisense CCUAUCCUGAAUACUUGAAUU, with mir-26bU sense UUCAAGUAAUUCAGGAUAGGUU and antisense CCUAUCCUGAAUACUUGAAUU, or with mir-26bUUU sense UUCAAGUAAUUCAGGAUAGGUUUU and antisense AACCUAUCUGAAUACUUGAAUU. For mir-26a, annealed sense (miRBase accession MIMAT0000082) UUCAAGUAAUCCAGGAUAGGC and antisense CUAUCCUGGAUUACUUGAAGC, with mir-26aU sense UUCAAGUAAUCCAGGAUAGGCU and antisense CCUAUCCUGGAUUACUUGAACU, with mir-26aUUU sense UUCAAGUAAUCCAGGAUAGGCUUU and antisense AGCCUAUCCUGGAUUACUUGAAUU. Duplexes and siCONTROL non-targeting siRNA #1 were purchased from Dharmacon. For mir-26b inhibition, miRIDIAN inhibitors

specific to mir-26b and mir-26a2 were used to transfect A549 cells as described above along with the miRIDIAN microRNA inhibitor negative control #1 (Dharmacon).

Additional Methods and Reagents

Recombinant human TNF- α and matched antibody sets for ELISAs were from R&D Systems. The human cytokine/chemokines 30-plex immunoassay (Linco) was performed using Luminex100 on supernatants collected from A549 cells treated with nonspecific and Zcchc11 targeting siRNA and subsequently stimulated with TNF- α (10 ng/ml) for 24 hours. Primary human samples were provided by Drs. Stephanie Shore (HSPH) and Lester Kobzik (HSPH).

Statistics

Data were presented as means and standard errors. Statistical analyses were performed on data collected over at least 3 independent experiments. For comparisons across more than 2 groups (e.g., multiple cytokines or time-points), analyses were performed using ANOVA. Data that failed Levene's test for homogeneity of variance were log-transformed prior to analyses. For comparisons of 2 groups, analyses were performed using *t* tests. Differences were considered statistically significant when $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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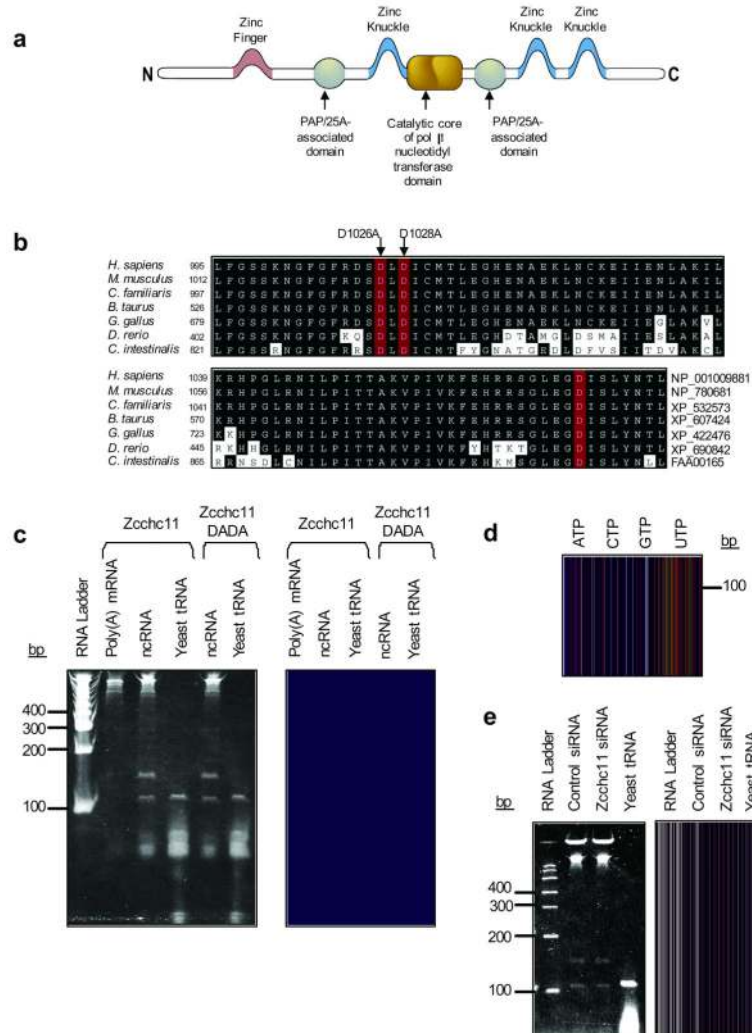


Figure 1. Zcchc11 is a uridylyltransferase
a, Key structural and catalytic domains of Zcchc11. **b**, The catalytic core of Zcchc11 is highly conserved among mammals and lower organisms. Three highlighted aspartates (two of which were mutated to alanines in the DADA construct, as indicated) are essential for pol β nucleotidyltransferase activity. **c**, Zcchc11 but not the DADA mutant (immunopurified using a Flag tag) transferred adenosines onto RNA substrates. Total RNA was visualized by SYPRO Green II staining (left) and labeled RNA was visualized using autoradiographic film (right). nc, non-coding. **d**, Nucleotide specificity, showing ability of endogenous Zcchc11 (immunoprecipitated from A549 cells) to transfer ^{32}P -labeled ATP, CTP, GTP, or UTP to yeast tRNA substrate. **e**, Flag-tagged Zcchc11 transferred ^{32}P -uridine to diverse substrates including a 100 bp RNA ladder, total RNA isolated from control or Zcchc11 siRNA treated A549 cells, or yeast tRNA. Total and radiolabeled RNA were visualized as in 1c.

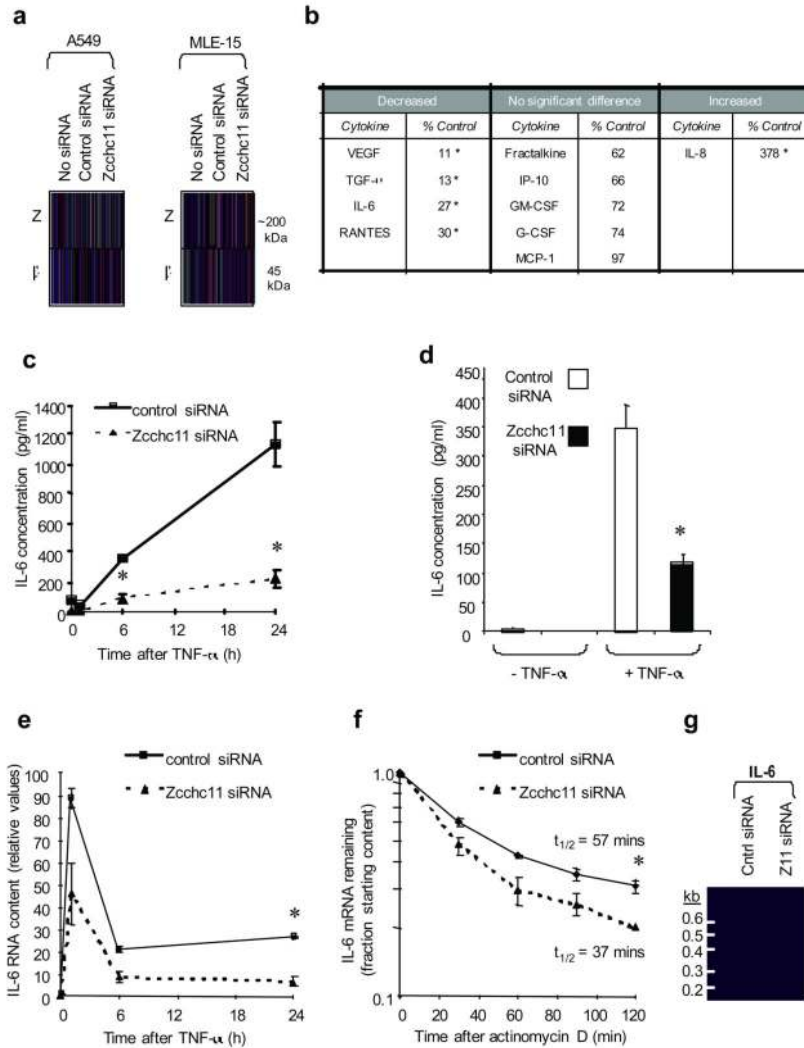


Figure 2. Zcchc11 knockdown alters cytokine mRNA stability and expression

a, siRNA-targeted depletion of Zcchc11 (Z) but not β -actin (β) in A549 and MLE-15 cells was confirmed by immunoblot. **b**, A549 cells transfected with nonspecific or Zcchc11-targeting siRNA were stimulated with TNF- α and supernatants were analyzed using a multiplex bead assay. The effect of Zcchc11 knockdown was indicated as “% Control,” with 100% equal to that observed after nontargeting transfection. “ns,” not significantly different ($P > 0.50$ for each; $N = 5$) from control (100%). *, significantly different from control (Factorial ANOVA and Bonferroni *post hoc* test, $P < 0.000001$ for each; $N = 5$). **c**, IL-6 protein concentrations in the supernatant of A549 cells treated with nonspecific and Zcchc11 siRNA was assessed by ELISA at 0, 1, 6, and 24 hours after stimulation with 10 ng/ml TNF- α . *significant effects of Zcchc11 siRNA on IL-6 at both 6 ($P = 0.01$) and 24 ($P = 0.0002$) hours (Factorial ANOVA and Bonferroni *post hoc* test; $N = 3-4$). **d**, IL-6 protein concentrations were also assessed in control and Zcchc11 knockdown groups in MLE-15 cells by ELISA after 24 hour incubations with or without mouse TNF- α . *, significant effect of Zcchc11 siRNA ($P = 0.001$; t test; $N = 3$). **e**, IL-6 mRNA levels were assessed by real-time RT-PCR at the indicated time points in TNF- α (10 ng/ml) stimulated A549 cells treated with

nonspecific and Zcchc11 targeting siRNA. Each time point represents the mean and SEM from $N=3$ independent experiments. Asterisks indicate statistical significance of $P < 0.05$. **f**, IL-6 mRNA stability in A549 cells stimulated with TNF- α for 1 hour, using actinomycin D to halt transcription. *, $P=0.0007$ (Factorial ANOVA; $N=3$). **g**, IL-6 poly(A) tail length was determined by LM-PAT to be decreased in Zcchc11 knockdown A549 cells stimulated with TNF- α for 1 hour. PCR products were electrophoresed in an agarose gel and visualized with ethidium bromide staining.

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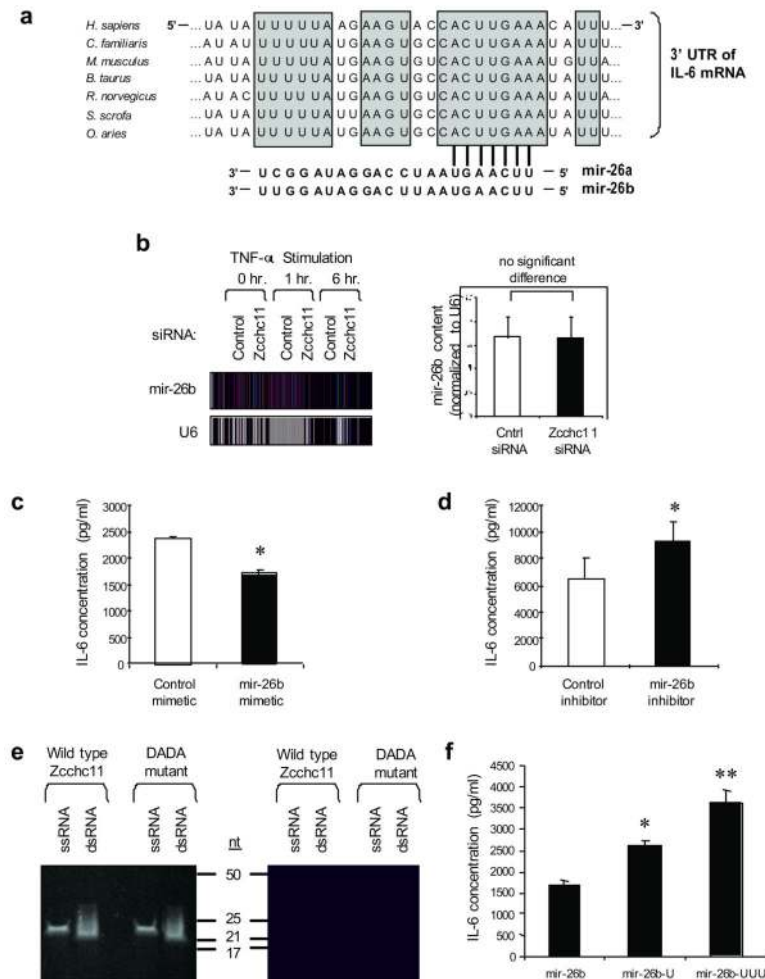


Figure 3. Mir-26 family regulates IL-6 expression

a, The mir-26 target sequence in the 3' UTR of IL-6 is conserved across multiple mammalian species. The human mir-26a and mir-26b indicated was consistent across clones from A549 cells (i.e., with no non-genomic modifications shown). Seed sequence pairing is indicated by lines. **b**, mir-26b content was unaffected by Zcchc11 siRNA. RNA was collected 0, 1, and 6 hours after TNF- α stimulation of A549 cells, and mir-26b was detected using Northern hybridization and quantified by densitometry ($P=0.95$, t test; $N=3$). **c**, Transfection with mir-26b mimetics decreased IL-6 from A549 cells stimulated with TNF- α for 24 hours. *, $P=0.005$ (paired t test; $N=3$). **d**, Oligonucleotide inhibition of endogenous mir-26b increased TNF-induced IL-6 expression from A549 cells. *, $P=0.004$ (paired t test; $N=3$). **e**, Zcchc11 but not the DADA mutant (immunopurified using a Flag tag) preferred to uridylate small single stranded RNA versus double stranded RNA. Total RNA was visualized by SYPRO Green II staining (left) and labeled RNA was visualized using autoradiographic film (right). **f**, Increasing uridylation of mir-26 caused enhanced IL-6 production from TNF-stimulated A549 cells. * $P=0.042$ vs. mir-26b, and ** $P=0.025$ vs. mir-26b-U and $P=0.001$ vs. mir-26b (Factorial ANOVA and Bonferroni *post hoc* test; $N=3$).

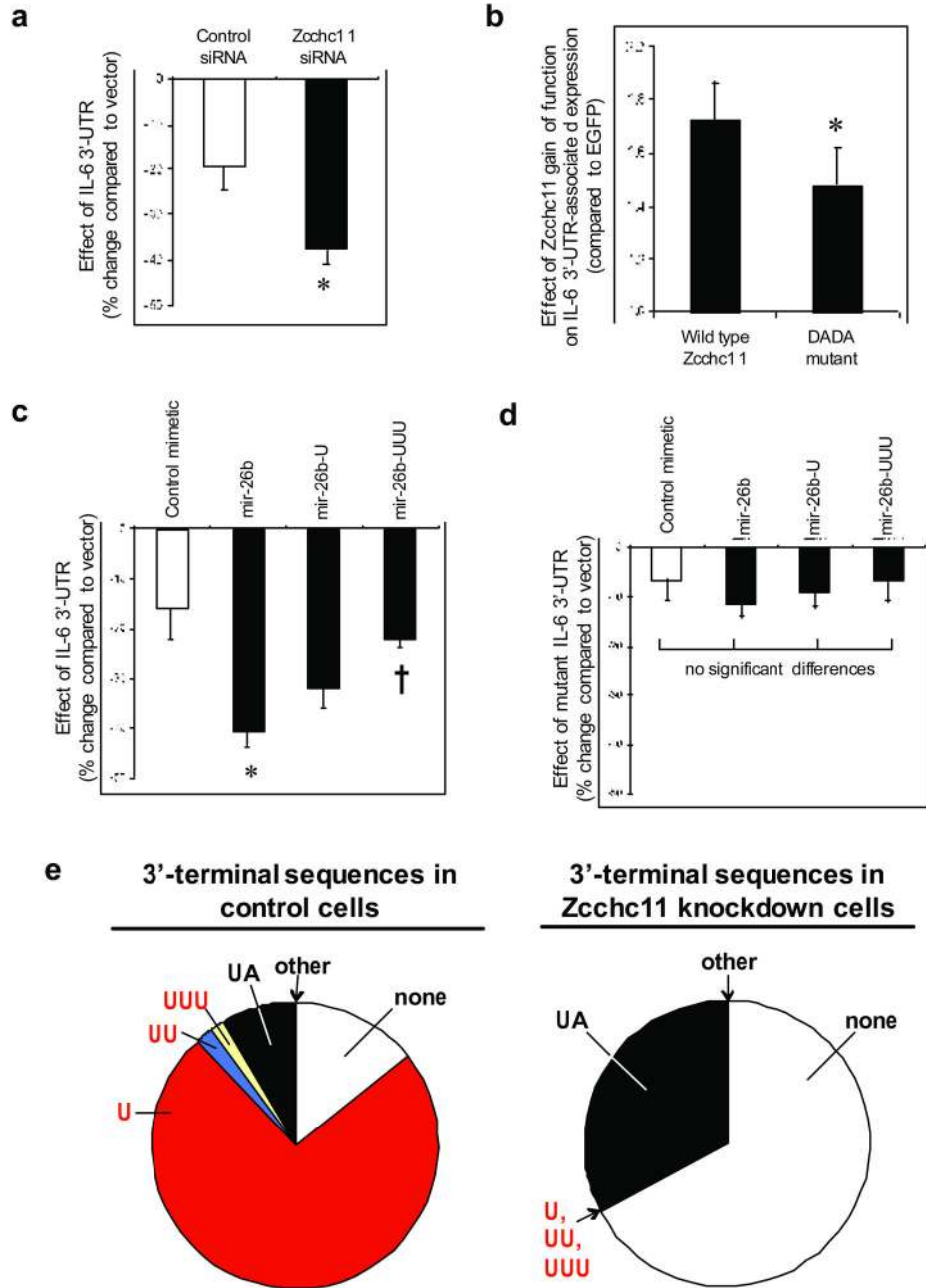


Figure 4. Zchc11-dependent uridylation of mir-26 abrogates IL-6 mRNA silencing
a, Zchc11 knockdown decreased luciferase expression from a reporter construct containing the IL-6 3'UTR. * $P=0.037$ (t test; $N=3$). **b**, Overexpression of Zchc11 increased luciferase expression driven by the IL-6 3'UTR reporter vector and this increase was attenuated by the nucleotidyltransferase null Zchc11 mutant (DADA). * $P=0.0019$ (paired t test; $N=5$). **c**, mir-26 drove down IL-6 3'UTR-dependent luciferase expression, which was inhibited by mir-26 uridylation. * $P=0.006$ vs. control mimetic, † $P=0.043$ vs. mir-26 (one-way ANOVA and Bonferroni *post hoc* test; $N=4$). **d**, mir-26 did not affect expression from a construct containing a version of the IL-6 3'UTR in which 2 nucleotides targeted by the mir-26 seed

sequence were changed. Neither the addition of mir-26b nor its uridylation had any significant effect ($P=0.73$, one-way ANOVA; $N=4$). **e**, *Zcchc11* knockdown resulted in a loss of uridines at the 3' termini of mir-26a. All sequences included in mir-26a analyses share 100% fidelity through the first 21 nucleotide mature miRNA sequence.

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