Argonaute 2 immunoprecipitation revealed large tumor suppressor kinase 1 as a novel proapoptotic target of miR-21 in T cells

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MicroRNA (miR)-21 is an important suppressor of T-cell apoptosis that is also overexpressed in many types of cancers. The exact mechanisms underlying the antiapoptotic effects of miR-21 are not well understood. In this study, we used the Jurkat T-cell line as a model to identify apoptosis-associated miR-21 target genes. We showed that expression of miR-21 rapidly increases upon αCD3/αCD28 activation of Jurkat cells. Inhibition of miR-21 reduced cell growth which could be explained by an increase in apoptosis. MicroRNA target gene identification by AGO2 RNA-immunoprecipitation followed by gene expression microarray (RIP-Chip) resulted in the identification of 72 predicted miR-21 target genes that were at least twofold enriched in the AGO2-IP fraction of miR-21 overexpressing cells. Of these, 71 were at least twofold more enriched in the AGO2-IP fraction of miR-21 overexpressing cells as compared to AGO2-IP fraction of control cells. The target gene for which the AGO2-IP enrichment was most prominently increased upon miR-21 overexpression was the proapoptotic protein LATS1. Luciferase reporter assays and western blot analysis confirmed targeting of LATS1 by miR-21. qRT-PCR analysis in primary T cells showed an inverse expression pattern between LATS1 transcript levels and miR-21 upon T-cell stimulation. Finally, LATS1 knockdown partially rescued the miR-21 inhibition-induced impaired cell growth. Collectively, these data identify LATS1 as a miR-21 target important for the antiapoptotic function of miR-21 in T cells and likely also in many types of cancer.

Introduction

Increased miR-21 levels have been shown in various types of solid tumors as well as in hematological malignancies [1–6]. Consistent with the marked overexpression in cancer, several studies showed an

Abbreviations
LATS1, large tumor suppressor kinase 1; miRNA, microRNA; RIP-Chip, RNA-binding protein immunoprecipitation–microarray profiling.
antiapoptotic effect of miR-21. Knockdown of miR-21 correlates with increased apoptosis and reduced proliferation of breast cancer cells [2,3]. Multiple cancer-relevant miR-21 target genes, such as tropomyosin 1 (TPM1), programmed cell death 4 (PDCD4), phosphatase and tensin homolog (PTEN), Maspin, and SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 4 (SMARCA4), have been identified [4–9]. More recent studies also showed effects of miR-21 on T-cell activation and survival [10–13]. We previously showed that memory T cells are characterized by a high expression of miR-21. In addition, we showed that activation-induced miR-21 provides critical antiapoptotic signals in memory T cells allowing long-term survival [11,14]. However, the miR-21 target genes responsible for the protection against activation-induced apoptosis of T cells remain unknown.

In this study, we set out to investigate miR-21 target genes related to its antiapoptotic effects on T cells. We employed an experimental RNA-immunoprecipitation followed by gene expression microarray (RIP-Chip)-based approach [15] in Jurkat cells. These cells are a commonly used model to study regulatory pathways involved in T-cell activation and apoptosis. We identified the proapoptotic large tumor suppressor kinase 1 (LATS1) as the miR-21 target gene whose enrichment in the argonaute 2 immunoprecipitated (AGO2-IP) increased the most upon miR-21 overexpression and showed its role in the antiapoptotic effect of miR-21.

Results and discussion

Jurkat is a suitable model to study the antiapoptotic role of miR-21

To assess if the Jurkat cell line is a suitable model to study the function of miR-21 in relation to apoptosis, we determined miR-21 expression levels in unstimulated cells and after stimulation with zCD3/zCD28. In comparison to other miRNAs known to be expressed in T cells at high (miR-17) or low (miR-146a) levels, miR-21 levels were moderate in unstimulated cells (Fig. 1A). Activation of Jurkat cells with zCD3/zCD28 for 3 days revealed a marked induction of miR-21 expression (~25-fold, P ≤ 0.001; Fig. 1B) consistent with previous studies on zCD3/zCD28-stimulated primary T cells [11,13]. To determine whether loss of miR-21 resulted in a growth defect, we infected Jurkat cells with a miR-21 inhibitor vector which coexpresses GFP. GFP analysis over time of a mixture of transduced and nontransduced cells (GFP competition assay) revealed a significant decrease of miR-21 inhibitor-transduced (GFP-positive) cells when compared to nontransduced (GFP-negative) cells (Fig. 1C). Cells transduced with three nontargeting (NT) control inhibitors showed no effect on cell growth in the GFP competition assay (Fig. 1C and data not shown). Jurkat cells transduced with miR-21 inhibitor showed a significant decrease of viable cells starting at day 4, which was not observed with control transduced cells (Fig. 1D). This effect was paralleled by an increase of apoptotic cells reaching > 80% at day 6 (Fig. 1E,F).

These findings are consistent with our reported findings in primary T cells [11] and indicate that endogenous levels of miR-21 in Jurkat cells provide an essential antiapoptotic signal. Together, these data show that the Jurkat cell line is a suitable model to study the antiapoptotic properties of miR-21 in T cells.

miR-21 target genes involved in apoptosis

To identify the antiapoptotic miR-21 target genes, we performed AGO2-RIP-Chip on Jurkat cells overexpressing miR-21 (Jurkat-miR-21) and used cells transduced with an empty vector construct (Jurkat-EV) as a control. The miR-21 levels showed an increase of ~22-fold in Jurkat-miR-21 compared to Jurkat-EV (Fig. 2A). Overexpression of miR-21 in Jurkat cells did not cause any obvious effects on the percentage of live cells (data not shown). The efficiency of the AGO2 immunoprecipitation (AGO2-IP) as determined by western blot was comparable between Jurkat-EV and Jurkat-miR-21 cells (Fig. 2B). As expected, miR-21 was strongly enriched in the Jurkat-miR-21 AGO2-IP fraction in comparison to the Jurkat-miR-21 total fraction and the Jurkat-miR-21 and Jurkat-EV IgG1 control IP fractions. Some miR-21 enrichment could also be observed in the Jurkat-EV AGO2-IP fraction as Jurkat cells endogenously express moderate miR-21 levels (Figs. 2C and 1A).

Gene set enrichment analysis (GSEA) [16] revealed a strong enrichment of multiple microRNA (miRNA)-binding motifs in both Jurkat-miR-21 (8 of the top 10) and Jurkat-EV (9 of the top 10, Table 1) further validating the efficiency of the AGO2-RIP. The miR-21-binding motif increased from the 46th position of most enriched gene sets in Jurkat-EV (false discovery rate (FDR) = 0.0013) to the 28th position in Jurkat-miR-21 cells (FDR ≤ 0.001). Comparison of both top-10 enriched gene sets revealed a marked overlap between Jurkat-miR-21 and Jurkat-EV cells with eight shared gene sets (Table 1). A marked difference was observed for two apoptosis-related gene sets. Genes regulated upon treatment with the growth and survival factor, IL-6, were among the top-10 most enriched gene sets in Jurkat-EV but not in Jurkat-miR-21 (position 258).
Genes involved in sensitivity to TRAIL-induced apoptosis were found among the top-10 most enriched in Jurkat-miR-21 but not in Jurkat-EV cells (position 1237). The expression levels of genes represented by the latter gene set showed an overall decrease in the total fraction of Jurkat-miR-21 as compared to Jurkat-EV (not shown). These differences can be explained by either direct or indirect effects of miR-21 and fit with the observed antiapoptotic role of miR-21 in Jurkat cells. Comparison of the expression levels of all predicted miR-21 target genes (209 of 14 514 unique genes) between the total fractions of Jurkat-EV and Jurkat-miR-21 revealed a systematic decrease in transcript levels in Jurkat-miR-21 cells (Fig. 3A). This indicates that transcript levels of predicted miR-21 genes were decreased upon miR-21 overexpression. As a control, we also analyzed differences in the expression levels of predicted miR-146a targets (162 of 14 514) and predicted miR-17 targets (929 of 14 514). No difference was observed for the predicted miR-146a target genes, while a mild decrease was observed for predicted miR-17 target genes (Fig. 3A). The latter observation can be explained by the marked overlap between the miR-21 and miR-17 predicted target genes, i.e., 62 shared predicted target genes. In line with these observations, we noted a specific enrichment
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Fig. 2. Efficiency of AGO2-RISC IP in Jurkat-EV and Jurkat-miR-21 cells. (A) qRT-PCR analysis of miR-21 in Jurkat cells transduced with empty vector (EV) or miR-21. miR-21 expression was normalized to RNU48 and the level detected in Jurkat-EV was set to 1 (n = 3, median with interquartile range is shown). (B) Western blot analysis for AGO2 to determine the efficiency of the AGO2-IP using total (T), Flowthrough (FT) and immunoprecipitated (IP) fractions of Jurkat-EV and Jurkat-miR-21. IP with mouse IgG1 served as a negative control. (C) qRT-PCR analysis of miR-21 levels in RNA isolated from T, FT, and IP fractions of AGO2- and control IgG1-IP experiments of Jurkat-EV and Jurkat-miR-21 cells. miR-21 expression was normalized to RNU48 and the level detected in the T of Jurkat-EV was set to 1. miR-21 was enriched in the AGO2-IP fractions of Jurkat-EV and Jurkat-miR-21, and was 86-fold higher in the IP fraction of Jurkat-miR-21 as compared to the IP fraction of Jurkat-EV.

Table 1. Gene set enrichment analysis.

| Gene set                                      | Position in GSEA | EV  | miR-21 |
|-----------------------------------------------|------------------|-----|--------|
| AGC A C T T, miR-93, miR-302A, miR-302B, miR-302C, miR-302D, miR-372, miR-373, miR-520E, miR-520A, miR-526B, miR-520B, miR-520C, miR-520D | 2                | 1   |
| T G A A T G T, miR-181A, miR-181B, miR-181C, miR-181D | 9                | 2   |
| G C A C T T T, miR-17-5P, miR-20A, miR-106A, miR-106B, miR-20B, miR-519D | 1                | 3   |
| T T T G C A C, miR-19A, miR-19B | 8                | 4   |
| A C A C T A C, miR-142-3P | 4                | 5   |
| G T G C A A T, miR-25, miR-32, miR-92, miR-363, miR-367 | 6                | 6   |
| T G C A C T T, miR-519C, miR-519B, miR-519A | 7                | 7   |
| T T G C A C T, miR-130A, miR-301, miR-130B | 10               | 8   |
| H A M A I_APOPTOSIS_VIA_TRAIL_UP | 1237             | 9   |
| S E N G U P T A_NASOPHARYNGEAL_CARCINOMA_WITH_LMP1_UP | 106              | 10  |
| A C T T T T A T, miR-142-6P | 5                | 17  |
| A T A A G C T, miR-21 | 46               | 28  |
| B R O C K E_APOPTOSIS_REVERSED_BY_IL6 | 3                | 258 |

Seventy-two predicted miR-21 target genes were enriched at least twofold in Jurkat-miR-21 IP as compared to the total fraction. Seventy-one of these 72 targets were
enriched at least twofold more in the AGO2-IP fraction of Jurkat-miR-21 as compared to the AGO2-IP fraction of Jurkat-EV. These 71 targets and their gene ontologies are listed in Table 2 and include 11 previously confirmed miR-21 target genes (at least by reporter assay). Of these 11, we validated the regulation of PIK3R1 by miR-21 using luciferase assays and the upregulation of PDCD4 protein expression upon miR-21 inhibition in Jurkat cells (data not shown). Within the 71 target genes identified in this study, six were related to regulation of apoptosis, i.e., PDCD4, Ras homology family member B (RHOB), mitogen-activated protein kinase 1 (MAP3K1), protein kinase C (PRKCE), RAS P21 protein activator (GTPase-activating protein) 1 (RASA1), and LATS1.

We selected LATS1 for further studies as it showed the strongest increase in AGO2-IP enrichment upon miR-21 overexpression (Table 2) and had a known function related to apoptosis [17,18]. In addition, it was recently shown that miR-21 can target LATS1 in cervical cancer [19]. We identified one 8-mer and two 7-mer miR-21-binding sites consistently showed significant increases in luciferase activity, thereby demonstrating that LATS1 is a bona fide miR-21 target (Fig. 4B). In agreement with this finding, inhibition of miR-21 in Jurkat cells increased the expression of LATS1 protein (Fig. 4C).

To determine the relevance of the increase of LATS1 for the miR-21 inhibition-induced phenotype (Fig. 1C), we studied the effect of miR-21 inhibition in LATS1-knockdown (KD) cells. Stable LATS1-KD cells were generated by infection of Jurkat cells with lentiviral LATS1-shRNA constructs or NT control shRNA vectors. Western blotting for LATS1 in sorted LATS1-KD cells showed that the efficiency of the shRNAs ranged between 70% and 90% (Fig. 5A). Next, we infected the LATS1-KD and control cells with miR-21 inhibitor and control inhibitor virus, containing GFP and monitored the GFP percentage over time within the LATS1-KD cells. As expected, miR-21 inhibition caused a strong reduction in the percentage of GFP+ cells in the NT shRNA-infected and wild-type cells with on average 15% of GFP+ cells left after

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**Fig. 3.** Transcript levels of predicted miR-21 targets are more enriched in the AGO2-IP fraction and depleted in total fraction upon miR-21 overexpression. (A) Bland–Altman plots comparing the expression levels of miR-21, miR-146a, and miR-17 predicted target genes in the total fractions of Jurkat-EV and Jurkat-miR-21. The expression difference of each gene is plotted against its mean expression. Red lines represent the best fit to the data. (B) Percentages of predicted miR-21, miR-146a, and miR-17 target genes in all 14 514 expressed genes and in the top-1500 and top-250 most enriched genes in the AGO2-IP fractions of Jurkat-EV and Jurkat-miR-21. Filled bars represent miR-21 IP, open bars represent EV IP.
Table 2. Functional annotation of 71 predicted miR-21 target genes with ≥2-fold increase in IP enrichment upon miR-21 overexpression.

| Gene symbol | EV IP/T | miR-21 IP/T | miR-21/EV | Proven target | Annotation |
|-------------|---------|-------------|-----------|---------------|------------|
| LATS1       | 4.8     | 126.9       | 26.6      | [19]          | Apoptosis induction, cell cycle inhibition, serine/threonine kinase activity |
| FAM63B      | 1.2     | 19.3        | 18.5      |               | Phosphoprotein |
| MBNL1       | 2.2     | 34.7        | 15.8      |               | RNA splicing regulation |
| TRPM7       | 1.9     | 21.8        | 11.3      |               | Transmembrane protein, cation channel activity |
| CDK6        | 0.8     | 7.8         | 9.7       | [1]           | Cell cycle regulation, cyclin-dependent protein kinase activity, p53 signaling pathway |
| RAB22A      | 0.8     | 6.4         | 7.9       |               | Intracellular signaling cascade, GTPase activity |
| PAG1        | 0.9     | 7           | 7.8       |               | Immune system, inhibition of lymphocyte activation, molecular adaptor activity |
| TET1        | 1.7     | 13.4        | 7.8       |               | Oxidoreductase activity |
| BMPR2       | 2.5     | 19.8        | 7.8       | [27]          | Phosphorylation induction, serine/threonine kinase activity |
| PLAG1       | 7.8     | 61.2        | 7.8       |               | Transcription regulation, transcription factor activity |
| STAG2       | 0.9     | 6.5         | 7.4       |               | Cell cycle regulation, chromosome segregation, mitosis, meiosis |
| FAM126B     | 2.4     | 17.2        | 7.2       |               | Phosphoprotein |
| UBN2        | 1.7     | 11.8        | 7         |               | Phosphoprotein |
| PIK3R1      | 1.3     | 9.3         | 6.9       | [28]          | Immune system development, lymphocyte activation, TCR signaling |
| YOD1        | 2.8     | 19.6        | 6.9       |               | Proteolysis, peptidase activity |
| RPS6KA3     | 0.6     | 4.1         | 6.5       |               | Serine/threonine kinase activity, MAPK signaling pathway |
| NFA5        | 2       | 12.7        | 6.4       |               | Immune system, transcription induction, transcription factor activity, TCR signaling |
| FBXO28      | 3       | 18.1        | 6.1       |               | Proteolysis |
| LCORL       | 1.3     | 7.7         | 6.1       |               | Transcription regulation |
| PDCD4       | 0.9     | 5.3         | 5.7       | [1,29,30]     | Apoptosis induction, cell cycle inhibition, inhibition of kinase activity |
| PAN3        | 4.1     | 23.5        | 5.7       |               | mRNA catabolism process, protein kinase activity |
| MSL1        | 0.9     | 5.3         | 5.6       |               | Chromatin organization |
| PLEKHA1     | 6       | 31.3        | 5.3       |               | Enzyme-linked receptor protein signaling pathway |
| AP4E1       | 1       | 5.1         | 5.3       |               | Intracellular protein transport |
| KHL15       | 3.4     | 17.8        | 5.3       |               | Na |
| PBRM1       | 0.7     | 3.7         | 5.2       |               | Cell cycle regulation, chromatin regulator |
| ZYG11B      | 0.8     | 4.1         | 5.1       |               | Proteolysis |
| CPEB3       | 4.2     | 20.9        | 5         |               | RNA binding |
| PRPF4B      | 1       | 4.8         | 5         |               | RNA processing, serine/threonine kinase activity |
| PIKfyVE     | 0.8     | 3.9         | 4.9       |               | Intracellular signaling cascade, phosphatidylinositol signaling system |
| SATB1       | 1.5     | 7.3         | 4.8       |               | Transcription inhibition, chromatin organization |
| RASGRP1     | 1       | 4.6         | 4.7       | [31]          | Immune system, intracellular signaling cascade, TCR signaling |
| FBXO11      | 1.2     | 5.6         | 4.6       | [32]          | Proteolysis, ubiquitin ligase complex |
| PURB        | 1.5     | 6.9         | 4.6       |               | Transcription inhibition, transcription factor activity |
| WWP1        | 2       | 9           | 4.4       |               | Proteolysis, ubiquitin ligase complex |
| TOPORS      | 0.9     | 3.8         | 4.4       | [33]          | Proteolysis, response to DNA damage stimulus |
| FAM3C       | 4.7     | 20.3        | 4.3       |               | Cytokine activity |
| BCL11A      | 2.8     | 12.2        | 4.3       |               | Immune system development, lymphocyte activation, transcription repressor activity |
| C10orf12    | 0.8     | 3.5         | 4.3       |               | Phosphoprotein |
| KLF12       | 1       | 4.1         | 4.3       |               | Transcription regulation, transcription factor activity |
| CHIC1       | 3.2     | 13.5        | 4.2       |               | Cytoplasmic membrane-bounded vesicle |
| SLC10A7     | 2.5     | 10.2        | 4.1       |               | Ion transport, organic acid: sodium symporter activity |
| RASA1       | 1.6     | 6.2         | 3.9       | [31,34]       | Apoptosis inhibition, GTPase activity, MAPK signaling pathway |
| SECISBP2L   | 1.7     | 6.4         | 3.9       |               | Phosphoprotein |
| FRS2        | 5.4     | 20.6        | 3.8       |               | Cell–cell signaling, phosphorylation regulation, phosphatase regulator activity |
| TRIM33      | 1       | 3.9         | 3.7       |               | Transcription inhibition |
| EIF2C4      | 1.1     | 4.2         | 3.7       |               | Translation inhibition, ribonucleoprotein complex |
| ZNF217      | 3.3     | 12          | 3.6       |               | Transcription regulation, transcription factor activity |
| CHD7        | 1       | 3.6         | 3.5       |               | Immune system development, lymphocyte activation, helicase activity |
| NFI A       | 1.1     | 3.9         | 3.4       |               | Transcription regulation, transcription factor activity |
Table 2. (Continued).

| Gene symbol | EV IP/T | miR-21 IP/T | miR-21/EV | Proven target | Annotation                                                                 |
|-------------|---------|-------------|-----------|---------------|---------------------------------------------------------------------------|
| SKI         | 2.5     | 8.1         | 3.3       |                | Transcription inhibition, transcription factor activity                   |
| C5orf41     | 7.5     | 24.5        | 3.3       |                | Transcription regulation, transcription factor activity                    |
| ZNF367      | 13.7    | 38.5        | 2.8       |                | Transcription regulation, transcription factor activity                    |
| EIF4EBP2    | 3.3     | 9.3         | 2.8       |                | Translation inhibition                                                    |
| RECK        | 2.7     | 7.5         | 2.8       | [35]           | Vasculature development, peptidase inhibitor activity                      |
| MARCH5      | 2.9     | 7.5         | 2.6       |                | Proteolysis                                                               |
| TNRC6B      | 1.4     | 3.6         | 2.6       |                | Translation inhibition, ribonucleoprotein complex                         |
| RHOB        | 1.9     | 4.8         | 2.5       | [36]           | Apoptosis induction, cell cycle inhibition, GTPase activity                |
| AP3M1       | 1.4     | 3.6         | 2.5       |                | Intracellular protein transport                                            |
| ATPAF1      | 2.1     | 5.2         | 2.5       |                | Protein complex assembly, mitochondrion                                    |
| KBTBD6      | 1.9     | 4.7         | 2.5       |                | Proteolysis                                                               |
| PPP1R3B     | 2       | 4.8         | 2.4       |                | Insulin signaling pathway, glucose metabolism process                      |
| ZFP36L2     | 2.5     | 6           | 2.4       |                | mRNA stability regulation, transcription factor activity                   |
| BCL7A       | 2.7     | 6.5         | 2.4       |                | Translation inhibition                                                    |
| MAP3K1      | 1.5     | 3.4         | 2.3       |                | Apoptosis induction, stress-activated protein kinase signaling, MAPK signaling |
| C17orf39    | 2.7     | 6.3         | 2.3       |                | Na                                                                         |
| CNOT6       | 1.8     | 4.2         | 2.3       |                | Transcription regulation, nuclease activity                                |
| CD69        | 31.9    | 72.6        | 2.3       |                | Transmembrane protein                                                     |
| KLF3        | 2.1     | 4.4         | 2.1       |                | Transcription regulation, transcription factor activity                    |
| PRKCE       | 2.1     | 4.1         | 2         |                | Apoptosis inhibition, calcium-independent protein kinase C activity        |
| SLC7A6      | 1.8     | 3.7         | 2         |                | Organic acid transport, amino acid transmembrane transporter activity       |

Fig. 4. Luciferase reporter assay for LATS1 3′UTR. (A) Schematic presentation of the location of the miR-21-binding sites in the 3′UTR of LATS1 and the regions cloned into the reporter vector. Binding site sequences are shown in the lower part. (B) Luciferase reporter assay shows that miR-21 can bind to the miR-21-binding sites in LATS1. Plotted is the ratio of Renilla luciferase (RL) to firefly luciferase (FL) signal detected in lysates of Cos-7 cells transfected with psiCHECK-2 construct and cotransfected with miR-21 inhibitor (closed bars) or control inhibitor (open bars). Shown per condition is the mean with SD (n = 5, unpaired t-test) (C) Quantification of LATS1 protein levels in Jurkat cells transduced with control or miR-21 inhibitor. Results from two independent experiments are shown, analyzed by western blot. A representative image of each experiment is shown. GAPDH was used as internal control. LATS1 to GAPDH ratio in control inhibitor-infected cells was set to 1. *P ≤ 0.05, **P ≤ 0.01.
Knockdown of LATS1 was found to partially rescue this effect as a more than 2.5-fold higher percentage of GFP\(^+\) cells was left (average of the three shRNAs, 38%) after 22 days of miR-21 inhibition (\(P\)-value \(\leq 0.05\), Fig. 5B). These results show that the proapoptotic effect observed upon miR-21 inhibition is at least in part mediated by downregulation of LATS1.

To study the relevance of the miR-21–LATS1 axis in primary T cells, we analyzed LATS1 and miR-21 levels of primary sorted naïve T cells (CD4\(^+\)CD45RO\(^-\)) stimulated for 3 days with \(\alpha\)-CD3 and \(\alpha\)-CD28. This revealed an inverse pattern, i.e., increased levels of miR-21 and decreased levels of LATS1 upon stimulation (Fig. 6A, B). The increase of miR-21 upon stimulation supports our previous findings and those of others [11–13,20]. The inverse expression pattern of LATS1 suggests that targeting of LATS1 by miR-21 is highly relevant for survival of primary T cells. We also studied miR-21 and LATS1 expression in sorted naïve (CD4\(^+\)CD45RO\(^-\)) and memory (CD4\(^+\)CD45RO\(^+\)) T cells. In line with what we and others have previously shown, we observed higher levels of miR-21 in memory T cells compared to naïve T cells (Fig. 6C) [11,12,20]. However, we did not observe decreased LATS1 transcript levels in memory T cells as compared to naïve T cells (Fig. 6D). This suggests that miR-21 does not lower the LATS1 RNA levels in memory cells, but regulates LATS1 protein level by post-transcriptional repression of protein translation. To confirm regulation of LATS1 protein levels by miR-21 specifically in memory T cells, LATS1 protein analysis in naïve and memory T cells as well as AGO2-RIP experiments should be performed.

In summary, we showed that the Jurkat cell line is a suitable model to study the role of miR-21 in the regulation of T-cell apoptosis. We experimentally identified multiple miR-21 target genes via employing AGO2-RIP-Chip, including the proapoptotic LATS1 gene. We showed that LATS1 is a bona fide miR-21 target whose knockdown can at least partially rescue the proapoptotic effect of miR-21 inhibition. Thus, LATS1 is likely to be an important target for the antiapoptotic role of miR-21 in (activated) T cells, possibly in combination with other targets such as PDCD4. As miR-21 is widely overexpressed in a variety of cancers, it is also of interest to further study the relevance of the miR-21 target LATS1 in relation to cancer.
Materials and methods

Cell lines

The Jurkat, human acute leukemic T-cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 200 mM l-glutamine, 100 mM Na-pyruvate, 10 mg/mL-1 gentamycin (Lonza, Breda, The Netherlands), 0.05 mM β-mercapto-ethanol (Merek, Darmstadt, Germany) at 37°C in 5% CO2. The SV40 large T-antigen-transformed human embryonic kidney cell line, HEK293T, and Phoenix-Ampho were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 200 mM l-glutamine, and 10 mg/mL-1 gentamycin sulfate (Lonza) at 37°C in 5% CO2. African Green Monkey SV40-transformed kidney fibroblast cell line COS-7 was cultured in DMEM supplemented with 10% FBS (Thermo Scientific, Breda, The Netherlands), 200 mM l-glutamine, and 10 mg/mL-1 gentamycin sulfate at 37°C in 5% CO2. Cell lines were obtained from ATCC. We routinely confirmed the identity of our cell lines using the PowerPlex 16 HS System (Promega, Leiden, The Netherlands).

Viral constructs

To generate the lentiviral miR-21 overexpression construct, pre-miR-21 with ~150 nucleotides of the flanking sequence was amplified from genomic DNA using forward 5'-gtcgaagtatgatgtgg-3' and reverse 5'-gtgcgtattgagccaaag-3' primers. NheI and XhoI restriction sites were added to the forward primer and an EcoRI site was added to the reverse primer to allow directional cloning into the retroviral MXW-PGK-ires-GFP vector [22] using standard laboratory procedures. To stably inhibit miR-21 function, we used a lentiviral miR-21 inhibition vector (pmiRZip-21; Cat. Nr: MZIP21-PA-1) and three NT lentiviral inhibitor vectors (control inhibitor, Cat. Nr: MZIP000-PA-1, both from Systems Biosciences, Mountain View, USA and shNT1 and shNT2, see below) as controls. The sequences of shRNAs against LATS1 and controls used for cloning to the BambH1 and EcoRI sites of the lentiviral pDsREDPuro vector (MZIP/pGreenPuro vector with the copGFP replaced by dsRED, Systems Biosciences) are as follows: LATS1-sh1-S: 5’GATCCCGCTGCTCCTTCGTCATATACATTCAAGAGATGTATATGACGAAGG
AGCAGCTTTTTG₃, LATS1-sh1-AS: 5’AATTCAAAAA
AGCTGCTCCCTTGCTATATACATCTCTCTTTGTAATGTA
TATGACGAGGGACGCGGC₃, LATS1-sh2-S: 5’GATC
CGAAATCTCGTCCTGTATATTCAAGGAGATAAC
ATGAGCGACCTTATTTCTTTTG₃, LATS1-sh3-AS:
5’AATTCAAAAAGAAATCAAGTCGCTCATATCTCTTAATCA
CTGGAATACACTGAGCGACCTTATTTTG₃, LATS1-
sh3-S: 5’GATCCGCTGCTGTATACATCTCTTTG₃
AGCTGCTCCCTTGCTTCATATACATCTCTTGAG₃, TA
TS1-sh3-S: 5’AATTCAAAAAGCTCTCCTACATCT
CTTGAATACACTGAGCGACCTTATTTTG₃, LATS1-
sh2-AS: 5’AATTCAAAAAAGAAATCAAG
TCTTGAATACACTGAGCGACCTTATTTTG₃, LATS1-
sh2-S: 5’GATCCGCTGCTTCATACATCTCTTTG₃
AGCTGCTCCCTTGCTTCATACATCTCTTGAG₃
CCTAATCTCTTGAATGTA

**Virus production and viral transduction**

Lentiviral particles were produced with a third-generation lentiviral system in 293T cells by CaPO₄ transfection as described previously [23]. Lentiviral transduction of Jurkat cells was carried out for 24 h in the presence of 4 μg·mL⁻¹ polybrene (Sigma-Aldrich, St. Louis, USA).

Retroviral particles were produced by calcium phosphate (CaPO₄)-mediated transfection of Phoenix-Ampho packaging cells with 10 μg pMXW-PGK-RES-GFP-miR-21 (miR-21 overexpression) or pMXW-PGK-RES-GFP (control) and 0.63 μg of pSuper-DGCR8 in T25 flask. Retroviral particles were collected 48 h after transfection, passed through a 0.45 μm Millex-HV filter (Millipore, Amsterdam, The Netherlands) and concentrated with Retro-X concentrator (Clontech, Saint-Germain-en-Laye, France) according to the manufacturer’s protocol. Jurkat cells were transduced with the virus by spinning at 1200 g for 2 h.

**GFP competition assay**

GFP percentage of pmiRZip-21- or pmiRZip-scrambled-infected Jurkat cells was followed over a period of 22 days. The starting GFP percentage varied between 30% and 40%. Data were acquired on FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FLOWJO software (version 7.6, Treestar, Ashland, OR, USA). The GFP percentage analyzed at the first day of measurement (day 4) was set to 1. The GFP competition assay was performed as described previously [23].

**Apoptosis measurement**

Percentages of apoptotic cells were assessed in Jurkat cells transduced with miR-21 or control inhibitor in > 95% of the cells on day 4, 6, and 8 following viral transduction by FACS-based measurement of mitochondrial transmembrane potential loss. Briefly, cells were stained for 20 min at 37 °C in cell culture medium containing 50 nM DiLC1 (Enzo Life Sciences, NY, USA), which was followed by a washing step with PBS. Cells were kept on ice and DiLC1 staining was measured at the FACS Calibur flow cytometer using Cell Quest software (BD Biosciences). Data were analyzed using Kaluza Flow Analysis Software (Beckman Coulter).

**Quantitative RT-PCR**

Total cellular RNA was extracted using the miRNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. The RNA quantity was measured on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).

MicroRNA expression levels were determined by quantitative RT-PCR. RNA was reverse transcribed using the Taqman miRNA Reverse Master Mix Plus (Eurogentec, Liege, Belgium).

cDNA synthesis for mRNA was performed using Superscript III RTase (Thermo Scientific). The qPCR reaction was performed using qPCR MasterMix Plus (Eurogentec, Liege, Belgium).

**AGO2-RIP-Chip procedure**

Immunoprecipitation of AGO2-containing RISC complexes was performed as described previously by Tan et al. [15] and SlezaK-Prochazka et al. [25]. Briefly, cleared lysates of 40 million cells were incubated with protein G Sepharose beads (GE Healthcare, Eindhoven, The Netherlands) coated with anti-AGO2 antibody (Clone 2E12-1C9, Abnova, Taiwan) at 4 °C overnight. IP with anti-IgG antibody was used as a negative control (Millipore BV, Amsterdam, The Netherlands). After washing the beads, RNA was harvested for microarray and qRT-PCR analysis and protein lysates were prepared for western blot. RNA from total (sample taken before start of the IP procedure), flow...
Western blotting

Cells were lysed in Cell Lysis buffer (Cat. # 9803, Cell Signaling Technologies, Leiden, The Netherlands) and incubated on ice for 45 min, centrifuged at 14 000 g, 4 °C and supernatant was collected. Protein concentration was determined using DC Protein Assay following the manufacturer’s instructions. For each transfection, luciferase activity was measured in duplicate with the Luminoscan Ascent Microplate Luminometer (Thermo Scientific). The renilla (RL) over firefly (FF) luciferase ratio for miR-21 inhibitor was calculated. The RL/FF ratio of negative control was set to a value of 1. Transfections were performed in triplicate.

Cloning of 3’-UTRs in reporter constructs, transient transfection, and luciferase assays

3’UTR sequences of \textit{LATS1} (\textit{LATS1.1}/\textit{LATS1.2}) harboring miR-21-binding sites were PCR amplified from genomic DNA using primers with an \textit{XhoI} (forward) or \textit{NotI} (reverse) restriction site, for \textit{LATS1.1} forward: 5’-AAGGAGA AACCTGTTACTCT-3’, reverse 5’-GAAACTAAGGAA TACAGG-3’ and \textit{LATS1.2} forward: 5’-AAATGCTGA TACCAAAGG-3’, reverse 5’-GGAGGGACTGAAATG TTAGG-3’ and cloned into psiCHECK2 vector (Promega, Madison, USA), as previously described [26]. The inserts were sequence verified (BaseClear, Leiden, The Netherlands). About 1.2 × 10^4 Cos-7 cells were transfected with 125 ng of the psiCHECK2 construct and 50 nM miR-21 inhibitor, molecule ID: 4102261-101 or Negative Control #1 inhibitor, (Exiqon, Vedbaek, Denmark), using the Saint-MIX compound (Synvolux Therapeutics B.V., Groningen, The Netherlands), in 250 µl serum-free medium. Four hours following the transfection, 500 µl of medium supplemented with 10% FBS was added. Cells were lysed 24 h after transfection, and Renilla and firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Leiden, The Netherlands) according to the manufacturer’s instructions. For each transfection, luciferase activity was measured in duplicate with the Luminoscan Ascent Microplate Luminometer (Thermo Scientific). The renilla (RL) over firefly (FF) luciferase ratio for miR-21 inhibitor was calculated. The RL/FF ratio of negative control was set to a value of 1. Transfections were performed in triplicate.

Prediction of miRNA target genes

The miRNA target prediction program \texttt{TARGETSCAN 6.2} (http://www.targetscan.org/) was used to determine predicted target genes of miR-21 (307 conserved genes), miR-146a (224 conserved genes), and miR-17 (1.220 conserved genes).

Functional annotation analysis

The functional annotation of genes was performed using the DAVID BIOINFORMATIC RESOURCES 6.7 (https://david.ncifcrf. org/) through (FT; sample taken of the supernatant after collection of the IP fraction), and IP fractions was isolated with miRNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. RNA from total and AGO2-IP fractions of Jurkat-miR-21 and Jurkat-EV cells was used for microarray analysis. Labeling and hybridization were performed using two-color Low Input Quick Amp Labeling Kit, according to the manufacturer’s protocol (Agilent, Santa Clara, USA). Briefly, 40–100 ng of RNA from T and AGO2-IP samples was used for cDNA synthesis, followed by cRNA amplification and Cy-3 and Cy-5 labeling. cRNA was purified with RNeasy Kit (Qiagen) and quantified on NanoDrop™ ND-1000 Spectrophotometer (Thermo Scientific). Equal amounts of cRNA Cy-3 and Cy-5-labeled samples were combined and hybridized at 65 °C for 17 h on the 60k SurePrint G3Human Whole Genome Oligo Microarray (Agilent). Next, slides were washed and scanned with SureScan Dx Microarray Scanner (Agilent). Scanned images were used for Agilent Feature Extraction software version 10.5, converted into Linear and Lowess normalized data. Quality control report was generated for each array. Using GeneSpring gX version 12.5 (Agilent), quantile normalization of the signals was performed. Next, probes not detected in more than half of the samples and probes that are inconsistent (more than twofold different) in Cy-3 and Cy-5 replicates of the same sample were filtered out. The averaged signals for Cy-3 and Cy-5 replicates were used to calculate the IP/T ratio for each sample. The microarray data have been deposited in NCBI’s Gene Expression Omnibus (GSE85116).
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gov/), based on the following GO categories: GOTERM_BP_FAT, GOTERM_CC_FAT, GOTERM_MF_FAT, KEGG_PATHWAY, and SP_PIR_KEYWORDS. About 1–4 GO terms were considered for description of each gene.

Gene set enrichment analysis
Gene sets significantly enriched in the AGO2-IP in comparison to T fraction of Jurkat-EV and Jurkat-miR-21 were determined by the GSEA using the Molecular Signatures Database (GSEA; http://software.broadinstitute.org/gsea/index.jsp) [16]. Lists containing the expression values of 14 415 genes detected in IP and total fractions of Jurkat-EV or Jurkat-miR-21 were uploaded for the analysis.

Statistical analysis
For comparison of qRT-PCR data of nonstimulated and stimulated Jurkat cells, we applied the Friedman repeated measurements nonparametric test. Data from day 0 were compared to other days of stimulation (days 1, 2, 3). For comparison of viable and apoptotic Jurkat cells upon transduction with control or miR-21 inhibitor, we applied two-way repeated measures ANOVA with a Bonferroni post-test. To determine whether miR-21-inhibited cells have a significant impaired cell growth as compared to control inhibitor-infected cells, we performed mixed model analysis as described previously [25]. Significance for (RL/FL) luciferase ratios between control and miR-21 inhibitor was calculated using unpaired t-test. The same test was used to determine whether the remaining percentages of GFP+ cells in LATS1-KD group (LATS1 sh1-3) infected with a miR-21 inhibitor were significantly different from the remaining GFP percentages within the controls group (wild-type, NT1 and NT2) infected with a miR-21 inhibitor. For comparisons of LATS1 and miR-21 in nonstimulated and stimulated CD4+ T cells, we applied the Mann–Whitney test. Statistical analysis were performed with GRAPHPAD Prism version 5.0 (GRAPHPAD Software, San Diego, CA, USA) or spss Statistics version 22.0 (IBM Corp. Armonk, NY, USA).

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Author contributions
NT, KSC, BJK, AB, and JK planned experiments. NT, KSC, YY, AS, DJ, BR, PJ, and RKL performed experiments. NT, KSC, YY, ISP, BR, PJ, AMB, BJK, AB, and JK analyzed data. NT, KSC, EB, AMB, BJK, AB, and JK wrote the manuscript.

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