

## **Increased expression of miR-23a mediates a loss of expression in the RAF kinase inhibitor protein RKIP**

Stefan Hatzl<sup>1</sup>, Olivia Geiger<sup>1</sup>, Maja Kim Kuepper<sup>1</sup>, Veronica Caraffini<sup>1</sup>, Till Seime<sup>2</sup>, Tobias Furlan<sup>2</sup>, Erika Nussbaumer<sup>1</sup>, Rotraud Wieser<sup>3</sup>, Martin Pichler<sup>4,5</sup>, Marcel Scheideler<sup>6,7,8,9</sup>, Katarzyna Nowek<sup>10</sup>, Mojca Jongen-Lavrencic<sup>10</sup>, Franz Quehenberger<sup>11</sup>, Albert Wölfler<sup>1</sup>, Jakob Troppmair<sup>2</sup>, Heinz Sill<sup>1</sup>, and Armin Zebisch<sup>1</sup>

<sup>1</sup>*Division of Hematology, Medical University of Graz, Graz, Austria;* <sup>2</sup>*Daniel Swarovski Research Laboratory, Department of Visceral, Transplant and Thoracic Surgery, Innsbruck Medical University, Innsbruck, Austria;* <sup>3</sup>*Department of Medicine I, Medical University of Vienna, Vienna, Austria and Comprehensive Cancer Center of the Medical University of Vienna, Vienna, Austria;* <sup>4</sup>*Division of Oncology, Medical University of Graz, Graz, Austria;* <sup>5</sup>*Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, USA;* <sup>6</sup>*Institute for Diabetes and Cancer, Helmholtz Center Munich, Munich, Germany;* <sup>7</sup>*University Hospital, Heidelberg University, Heidelberg, Germany;* <sup>8</sup>*German Center for Diabetes Research (DZD), Neuherberg, Germany;* <sup>9</sup>*Technical University of Munich, Munich, Germany;* <sup>10</sup>*Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands;* <sup>11</sup>*Institute of Medical Informatics, Statistics and Documentation, Medical University of Graz, Graz, Austria*

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**Corresponding Author:** Dr. Armin Zebisch, Medical University of Graz, Auenbruggerplatz 38, 8036 Graz, Austria; T: +43.316.385.80259; F: +43.316.385.14087; E: [armin.zebisch@medunigraz.at](mailto:armin.zebisch@medunigraz.at)

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## **Abstract**

RAF kinase inhibitor protein (RKIP) is a seminal regulator of intracellular signaling and exhibits both anti-metastatic and anti-tumorigenic properties. Decreased expression of RKIP has been described in several human malignancies, including acute myeloid leukemia (AML). As the mechanisms leading to RKIP loss in AML are still unclear, we aimed to analyze the potential involvement of micro-RNAs (miRNAs) within this study. miRNA microarray and qPCR data of more than 400 AML patient specimens revealed correlation between decreased expression of RKIP and increased expression of miR-23a, a member of the miR-23a/27a/24-2 cluster. In functional experiments, overexpression of miR-23a decreased RKIP mRNA and protein expression, whereas miR-23a inhibition caused the opposite effect. By employing an RKIP 3'UTR luciferase reporter construct with and without mutation or deletion of the putative miR-23a binding site, we could show that RKIP modulation by miR-23a is mediated via direct binding to this region. Importantly, miR-23a overexpression induced a significant increase of proliferation in hematopoietic cells. Simultaneous transfection of an RKIP expression construct lacking the miR-23a binding sites reversed this phenotype, indicating that this effect is truly mediated via downregulation of RKIP. Finally, by analyzing more than 4300 primary patient specimens via database retrieval from The Cancer Genome Atlas (TCGA), we could highlight the importance of the miR-23a/RKIP axis in a broad range of human cancer entities. In conclusion, we have identified miR-23a as a negative regulator of RKIP expression in AML and have provided data that suggest the importance of our observation beyond this tumor entity.

## Introduction

RAF Kinase Inhibitor Protein (RKIP) has been described as a negative regulator of various intracellular signaling cascades, most notably the RAS-mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) pathway and the nuclear factor- $\kappa$ B-Snail circuitry (1-3). The seminal role of RKIP as a gatekeeper in these signaling processes is further highlighted by the fact that complete or partial loss of this protein has been observed in a variety of human malignancies, including carcinomas of the prostate, colon and breast (1, 4-8). Noteworthy, decreased expression of RKIP correlated with a higher level of metastasis formation on the one hand, and with impaired sensitivity to chemo- and radiotherapy on the other hand (1, 4). Indeed, the functional involvement of RKIP within these processes could be corroborated in a broad range of *in vitro* and *in vivo* models (1, 9, 10). Recently, we described loss of RKIP in up to 20% of primary specimens of acute myeloid leukemia (AML), an aggressive malignancy of the hematopoietic system (11, 12). Most importantly, functional *in vitro* studies demonstrated that RKIP inhibits the proliferation and oncogenic transformation of hematopoietic cells (11, 12), which suggests an additional tumor suppressor function within the hematopoietic system.

Although much progress has been made in evaluating the consequences of RKIP loss, relatively little is known about the reasons behind its downregulation. Clarification of the causative mechanisms will provide further insight into the processes of oncogenesis and may form the basis for the development of specific therapeutic approaches, aiming to restore its expression. This is of particular relevance for AML, as overall survival with current treatment strategies is still dismal and new therapeutic options are desperately needed (13, 14). However, we and others have previously screened for mutations and deletions within RKIP but could not identify any abnormalities (1, 11, 12). Even though methylation of CpG rich islands within the RKIP promoter (15) as well as the overexpression of specific transcriptional

repressors (16, 17) have been linked to RKIP downregulation in specific tumor entities, we failed to observe these mechanisms in AML.

Micro-RNAs (miRNAs) are small non-coding RNAs with a length of approximately 21-23bp. They typically inhibit the translation and/or stability of messenger RNAs (mRNAs), thereby fulfilling a central role in the regulation of gene expression (18, 19). Indeed, aberrant miRNA expression levels can be detected in most human malignancies and their functional relevance for carcinogenesis could be proven in numerous *in vitro* and *in vivo* studies (18-21). Recently, aberrant miRNA expression profiles have been linked to the modulation of RKIP, as increased expression of miR-224 and miR-27a caused the downregulation of RKIP in breast and gastric cancer cell lines (22-24). Here, we aimed to evaluate whether miRNAs are involved in the development of RKIP loss in AML.

## **Materials and Methods**

### **Patient samples and cell lines**

AML patient samples for miRNA qPCR as well as for RKIP qPCR and Immunoblot analyses were collected at the Division of Hematology, Medical University of Graz, Graz, Austria, and processed as previously described (25-29). For *RKIP* and miRNA array analysis in the Dutch-Belgian AML cohort II, a total of 214 AML patients were collected at the Erasmus University of Rotterdam, The Netherlands, as previously reported (11, 30-32). Classification of AML was performed according to French-American-British (FAB) and World Health Organization (WHO) guidelines (13, 33). THP-1, U937 and NB4 cell lines were obtained in 2007 from the German National Resource Center for Biological Material (DSMZ, Braunschweig, Germany), where they were characterized by variable number of tandem repeat DNA profiling (VNTR). Low passage stocks were frozen and cells were always passaged for fewer than 6 months after resuscitation. Additionally, stocks were regularly re-authenticated in our laboratory by VNTR as previously described (11, 25, 4).

HEK-293 were obtained from the Center for Medical Research at the Medical University of Graz in 2015, where they were tested by VNTR and passaged for fewer than 6 months after resuscitation in our laboratory. The study was approved by the institutional review board of the Medical University of Graz (24-036 ex 11/12) and informed consent was obtained from all individuals.

### **Cell culture, lentiviral transduction and transfection**

Cell lines were maintained at 37°C/5%CO<sub>2</sub> in RPMI-1640 (for U937, NB4 and THP-1) and DMEM (for HEK-293 and 293T packaging cells), respectively, supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1X Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA, USA; comprising 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B). U937 with stable expression of pMSCV-FLAG-hRKIP or empty vector (as previously described in (11)) were additionally maintained with 2.5 µg/ml puromycin. For RKIP knockdown, U937 were lentivirally transduced with either *RKIP* shRNA or empty control (both psi-LVRU6GP, obtained from Genecopeia, Rockville, MD, USA) as previously described (34) and stable selection was performed using 2.5 µg/ml puromycin. miR-23a-3p mimics, hairpin inhibitors and scrambled controls (all obtained from Dharmacon, Lafayette, CO, USA and Qiagen, Hilden, Germany) were transfected at a concentration of 20 nmol/L using DharmaFECT2 (Dharmacon; for U937 and THP-1) or Lipofectamine RNAiMAX (Thermo Fisher Scientific; for HEK-293) in 6-well plates according to the manufacturer's instructions. Cells were harvested after 24 to 48 hours for further analyses. For luciferase reporter assays, HEK-293 were transfected with 0.25 ng/µl pCS2-red fluorescent protein (RFP), together with 0.125 ng/µl RKIP-3'UTR-pMirTarget (Origene, Rockville, MD, USA) as well as with miR-23a mimics or scrambled control (both at a concentration of 20 nM). The RFP-construct was included to enable the compensation of differences in transfection efficiency. Mutation and deletion of binding site 1, respectively,

were performed as previously described (35). DNA and Mimics were mixed with 4  $\mu$ l jetPrime transfection reagent (Polyplus, Illkirch-Graffenstaden, France) in jetPrime buffer (Polyplus) to reach a final volume of 200  $\mu$ l transfection mix per well. After briefly vortexing and incubating for 10 minutes at room temperature, the mix was added dropwise to each well. Transfection was carried out in complete DMEM medium.

### **qPCR expression analysis**

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 1  $\mu$ g of total RNA with TaqMan Reverse Transcription (RT) Reagents (Applied Biosystems, Waltham, MA, USA) for mRNA and miScript II RT Kit (Qiagen) for miRNA, respectively. Random hexamers were used for RT of mRNA whereas stem-loop RT primers were employed for RT of miRNA. Real time quantitative PCR (qPCR) for miRNA expression analysis was performed on a LightCycler 480 Instrument II (Roche Life Sciences, Penzberg, Germany) using the miScript SYBR Green PCR Kit (Qiagen). qPCR for mRNA expression analysis was performed on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) using the SYBR Green method (Invitrogen). Expression levels were evaluated using the ddCT method as previously described (11, 36). *B2M* and *GUSB* served as control genes for analyses performed with mRNA, *RNU6* and *SNORD44* for those performed with miRNA. Primer sequences are displayed in Supplementary Table S1. NB4 AML cells (for miRNA qPCR of the Austrian AML cohort I) as well as cells transfected with the empty vector/scrambled control (for all other experiments) served as calibrators.

### **Immunoblot analysis**

Cells were lysed in 100  $\mu$ l ice-cold lysis RIPA-Buffer (Sigma-Aldrich, St. Louis, MO, USA), supplemented with Protease and Phosphatase inhibitor cocktails (Sigma-Aldrich and

Thermo Fisher Scientific). Immunoblots were then performed as previously described (11, 12, 35) using Mini-PROTEAN TGX gels (BioRad, Hercules, CA, USA) for electrophoresis and the Bio-Rad Trans Blot Turbo Blotting System for transfer. Polyvinylidene difluoride membranes (BioRad) were incubated with anti-RKIP (#07-137, Merck Millipore, Billerica, MA, USA), anti-I $\kappa$ B (#9242S, Cell Signaling Technology, Danvers, MA, USA), anti-Beta-Actin (#A5441, Sigma-Aldrich) and anti-Vinculin (#Ab129002, Abcam, Cambridge, UK) as previously reported (11, 12, 35). Band intensities were compared using Image J (37).

### **Luciferase reporter assays**

Twenty-four hours after transfection, cells were harvested and lysis was performed using 1X cell culture lysis reagent (CCLR, Promega, Madison, WI, USA) according to the manufacturer's instructions. Subsequently, luciferase activity and RFP fluorescence values were determined utilizing a Plate chameleon reader (Hidex, Turku, Finland). The reader injected automatically 100  $\mu$ l of luciferase Assay System Reagent (Promega) into each well of a 96 well plate containing 20  $\mu$ l of cell lysate, CCLR was used as background control. Luminescence was measured for 10 s after a delay of 2 s. Fluorescence was detected with a 544/616 nm filter set.

### **Analysis of cell growth and proliferation**

For growth curves, U937 were seeded at a density of  $1 \times 10^4$  cells/ml in reduced serum conditions (5%). The amount of viable cells was measured five days later using a BioRad TC20 automated cell counter (BioRad) via trypan blue exclusion assay. Proliferation was additionally assessed via BrdU/7-AAD staining using the APC BrdU Flow Kit (BD Pharmingen, Franklin Lakes, New Jersey, USA) according to the manufacturer's instructions and as previously described (11). 50  $\mu$ M BrdU was added for 30 min and cells were stained

with APC-anti-BrdU (1:50) for 20 minutes. 10,000 stained cells were measured on a BD-LSRII Flow Cytometer (BD Biosciences) and analyzed with Kaluza Flow Cytometry Analysis Software v1.2 (Beckman Coulter, Brea, CA, U.S.A).

### **Database retrieval and statistical analyses**

Expression data for *RKIP* (as obtained by RNA Sequencing V2 RSEM as well as by Affymetrix U133 and Agilent microarrays) and miRNAs (as obtained by miRNA arrays and miRNA Sequencing) were downloaded and analyzed from The Cancer Genome Atlas (TCGA, [www.cancergenome.nih.gov](http://www.cancergenome.nih.gov); Supplementary Table S2). In case of availability, data were downloaded and analyzed using the cBioPortal for Cancer Genomics ([www.cbioportal.org/public-portal/index.do](http://www.cbioportal.org/public-portal/index.do)) (38, 39). Additionally, *RKIP* mRNA and miRNA expression values were analyzed in previously published array and qPCR datasets (data available at <http://www.ncbi.nlm.nih.gov/geo> under accession numbers GSE49665, GSE1159 and GSE6891) (30-32, 40). A comparison of miRNA expression levels between primary patient samples with and without *RKIP* loss was performed using the Mann-Whitney-Wilcoxon test. The same test was applied for comparison of *RKIP* and miRNA expression, respectively, as well as between AML patients with and without monocytic phenotypes (AML M4/M5 vs other AML subgroups). To test for a correlation between *RKIP* and miRNA expression levels, Spearman-Rho correlation coefficients were calculated. miR-23a expression was related to overall and relapse free survival in AML cohort II and to overall survival in AML cohort III by Cox Regression analysis. For analysis of *in vitro* experiments, Student's t-test was calculated from at least three independent experiments. SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and R 3.2.2 ([www.r-project.org](http://www.r-project.org)) were used for analysis. All tests were performed two-sided and a *P*-value of < 0.050 was considered statistically significant.

### **Results**

### **Increased expression of miR-23a in AML correlates with RKIP loss**

In a first step, we aimed to identify miRNAs, which are associated with decreased expression of RKIP in AML. Therefore, we analyzed 33 AML specimens, which had been evaluated for RKIP protein expression by Immunoblot, as well as for the expression of more than 600 miRNAs by microarray analyses previously (11, 40). By comparing miRNA expression profiles between patients defined as harboring either normal (n = 27) or decreased (n = 6) RKIP expression (for definition of RKIP loss see Supplementary Fig. S1 and ref. (11)), we identified a set of seven miRNAs with statistically significant differential expression between these groups (miR-23a, miR-23b, miR-24, miR-15a, miR-320a, miR-518b and miR-519d, data not shown). We then sought to validate these results by the means of qPCR in an additional cohort of 20 AML patient specimens (cohort I). Out of these, ten demonstrated normal RKIP expression, both at mRNA and protein level, whereas the other ten exhibited loss of RKIP. Importantly, a set of three miRNAs could be validated and demonstrated increased expression in samples with RKIP loss (miR-23a, miR-24 and miR-15a; Fig. 1A). To further corroborate these findings, we analyzed *RKIP* mRNA as well as miR-23a, miR-24 and miR-15a expression in two additional and independent data sets comprising almost 400 AML patient specimens. Cohort II consisted of 214 Dutch-Belgian AML patients and was previously analyzed by mRNA arrays and miRNA qPCRs (30-32), whereas cohort III consisted of 173 specimens and was acquired via TCGA database retrieval. Although the techniques of expression analysis differed between these cohorts, correlation of decreased *RKIP* expression with increased levels of miR-23a could be validated in all of them (Fig. 1B and C). While cohort II revealed an additional correlation of decreased *RKIP* expression with increased levels of miR-24 and miR-15a (Fig. 1B), no additional correlations with statistical significance could be observed in cohort III (Fig. 1C). Of note, miR-224 and miR-27a, which have been linked to the modulation of RKIP in solid cancer cell lines previously (22-24), failed to correlate with RKIP expression (data not shown).

### **RKIP loss and increased expression of miR-23a correlate to myelomonocytic and monocytic AML phenotypes**

We previously have shown that decreased expression of RKIP correlates with AML subgroups with myelomonocytic and monocytic differentiation (FAB subgroups M4/M5) (11). Therefore, we were interested, whether this applies for increased expression of miR-23a as well. When looking at the 20 AML specimens of cohort I, AML M4/M5 phenotypes were significantly enriched in the ten samples defined as RKIP loss (Supplementary Fig. S2). In agreement with our hypothesis, miR-23a expression was significantly increased in AML specimens characterized as AML M4/M5 (Fig. 2A). We then tried to corroborate these findings in the two additional AML cohorts comprising almost 400 patients. In line with our previous studies, *RKIP* expression levels were significantly decreased in AML M4/M5 as compared to all other subtypes in both cohorts (Supplementary Fig. S3 and (11)). Importantly, the expression of miR-23a was indeed increased in AML M4/M5 phenotypes (Fig. 2B and C). Of note, miR-23a expression failed to correlate with both overall and relapse free survival in these AML cohorts (data not shown).

### **RKIP expression is regulated by miR-23a**

As outlined above, we observed inverse correlation between RKIP and miR-23a expression in AML. To further evaluate a potential functional involvement of miR-23a in the downregulation of RKIP in AML, we employed a series of AML and non-hematopoietic cell lines, including U937, THP1 and HEK293. Initially, we aimed to test the possibility that RKIP loss is the primary event, which in turn causes the miRNA upregulation, as shown for miR-98 and miR-200 (41, 42). Therefore, we performed a lentiviral shRNA based knockdown of RKIP in U937 AML cells and subsequently monitored miR-23a expression levels. No significant differences could be observed between cells with and without RKIP knockdown

(Fig. 3 and Supplementary Fig. S4), indicating that increased expression of miR-23a in AML is no effect of RKIP loss. In a next step, we aimed to clarify the role of miR-23a in the downregulation of RKIP. HEK-293 cells were transiently transfected with miR-23a mimics and RKIP expression was monitored by qPCR and Immunoblot. Importantly, miR-23a overexpression caused a statistically significant downregulation of RKIP expression, both at the mRNA and protein level (Fig. 4A-C, left). To corroborate these findings in the context of AML, we repeated these experiments in U937, a cell line exhibiting strong expression of endogenous RKIP (11). Again, miR-23a transfection induced a statistically significant downregulation of RKIP (Fig. 4A-C, middle). To further prove the mechanistic involvement of miR-23a in RKIP regulation, we transfected a miR-23a hairpin inhibitor in THP-1 AML cells, which has been shown to express RKIP at a low level previously (11). Indeed, miR-23 knockdown caused the statistically significant upregulation of RKIP (Fig. 4A-C, right). These results were confirmed with different miR-23a mimics and hairpin inhibitors that were obtained from different companies (for details see Materials and Methods; data not shown). Taken together, these data indicate that miR-23a suppresses the expression of RKIP in AML.

### **miR-23a modulates RKIP expression by direct binding to the RKIP 3'UTR**

Having proven that miR-23a regulates RKIP expression, we next sought to identify, whether this regulation is mediated via direct binding of miR-23a within the *RKIP* 3'UTR. Therefore, we initially performed an *in-silico* analysis using ten miRNA target prediction algorithms and identified *RKIP* as a putative direct target of miR-23a in five out of ten (Supplementary Fig. S5). In more detail, we identified two potential binding sites for miR-23a within the 3'UTR region with binding site 1 displaying the strongest match within the seed region (Fig. 5A). In a next step, we continued to work with an expression construct, where the 3'UTR of *RKIP* was attached to the coding region of luciferase. Importantly, transfection of this construct together with miR-23a mimics resulted in a significant downregulation of

luciferase activity, whereas scrambled miR-controls failed to do so (Fig. 5B). To further prove that regulation of RKIP by miR-23a is caused by direct interaction, we altered the 3'UTR of *RKIP* either by mutation (*RKIP* Mut) or by deletion (*RKIP* Del) of the putative binding site 1 (Fig. 5A). Indeed, both approaches inhibited the miR-23a mediated downregulation of luciferase activity (Fig. 5B) indicating that miR-23a regulates the expression of RKIP by direct interaction with binding site 1 within the 3'UTR.

### **miR-23a increases proliferation of hematopoietic cells via downregulation of RKIP**

We previously demonstrated that stable overexpression of FLAG-RKIP in AML cell lines caused a significant decrease of cellular growth. This was mediated via decreased proliferation as assessed in BrdU/PI cell cycle assays (11). To delineate the role of miR-23a within these processes, we now performed additional transfection of miR-23a mimics within these cells. Interestingly, when vector transfected controls without RKIP overexpression were studied, miR-23a overexpression caused a significant increase in cellular growth (Fig. 6A), corresponding to an increased rate of proliferating cells in BrdU/7-AAD assays (Fig. 6B). To further prove that these biological effects are indeed mediated via downregulation of RKIP, we repeated these experiments in U937 with stable overexpression of FLAG-RKIP. Without a 3'UTR, this construct is resistant to miR-23a induced decay and should therefore rescue the biological effects caused by miR-23a. Indeed, transfection of miR-23a failed to induce a phenotype in this cellular system (Fig. 6). In agreement with these data, lentiviral shRNA knockdown of RKIP in parental U937 caused increased cellular growth (Fig. 6A) as well as increased proliferation in BrdU/7-AAD assays (Fig. 6D).

### **The miR-23a/27a/24-2/RKIP axis is of general relevance for other cancer entities as well**

Up to this point, we could show that increased expression of miR-23a causes downregulation of RKIP within AML. Of note, miR-23a is part of the miR-23a/27a/24-2

cluster, and the members within this network have been shown to share many common targets (43). Indeed, miR-27a has been identified as RKIP modulator in lung cancer previously (24). We thus were interested whether a miR-23a/27a/24-2 mediated RKIP downregulation is of relevance for other malignancies as well, and to possibly identify the responsible cluster member. Therefore, we performed a database retrieval via TCGA and analyzed tumor entities, where i) decreased expression of RKIP has been previously reported, and ii) data for RKIP and miR-23a/27a/24-2 expression levels were available. All together, we were able to analyze 14 additional cancer entities comprising 4342 primary patient specimens. A statistically significant inverse correlation between the expression of *RKIP* and at least one of the cluster miRNAs could be shown in ten out of fourteen cancers. miR-23a proved to be the predominant cluster member with significant correlations in nine of these entities (Table 1).

## Discussion

Loss of RKIP has been described in a significant subset of patients with AML and has shown to be functionally relevant for the pathogenesis of this aggressive malignancy (11, 12). In this study, we aimed at delineating the underlying mechanisms behind the downregulation of RKIP in this disease and therefore studied the role of miRNA involvement. By analyzing more than 400 primary AML patient specimens, we identified a strong correlation of RKIP loss with increased expression of miR-23a. In subsequent *in vitro* studies, we could show that enforced expression of miR-23a indeed causes a downregulation of RKIP, and that this downregulation is mediated via direct binding to the 3'UTR of *RKIP*. In agreement with our data, increased expression of miR-23a has been reported in acute leukemias previously (43, 44). Moreover, the pro-leukemogenic effects of increased miR-23a expression were recently demonstrated in a murine *in vivo* model (45). In agreement with this publication, we now demonstrate that overexpression of miR-23a – just like knockdown of RKIP - induces increased proliferation of hematopoietic cells. This phenotype could be rescued by additional

overexpression of an RKIP expression construct without the miR-23a binding region, indicating that at least some of the leukemogenic effects of miR-23a are truly mediated via downregulation of RKIP. Noteworthy, increased expression of miR-23a failed to prove as a prognostic marker within this study. This is unexpected, as we previously demonstrated a link between decreased expression of RKIP and favorable prognosis in AML (11). While the reasons behind this phenomenon remain to be elucidated, one might speculate that miR-23a additionally regulates other, RKIP independent targets as well. Irrespective of its effects on RKIP, miR-23a could thereby influence other properties of leukemic cells with prognostic relevance, such as therapeutic resistance. Hence, analysis of the genetic associations of RKIP loss and increased miR-23a expression within larger, preferably prospective and randomized patient cohorts with well-documented clinical and molecular data will be of great interest.

Previous work further suggested that increased expression of miR-23a is specific for acute leukemias with myeloid differentiation, as evidenced by the fact that its expression levels were significantly lower in specimens of acute lymphatic leukemia (43, 44). Our results, showing a significantly increased miR-23a expression and a decrease of RKIP in AMLs with monocytic and myelomonocytic phenotypes are in line with these observations and suggest a possible relation of the miR-23a/RKIP axis to the myelomonocytic differentiation of acute leukemias. In particular, increased expression of miR-23a might serve as a biomarker with diagnostic potential in this setting, which is based on the fact that the small size of miRNAs offers protection during tissue processing thereby guaranteeing stability and reproducibility of the expression analyses (46). Indeed, miRNA expression profiling has been shown to be feasible from formalin-fixed paraffin-embedded tissues including decalcified bone marrow biopsies (46). Whether the miR-23a/RKIP axis is indeed functionally involved in the myeloid lineage commitment of hematopoietic stem- and progenitor cells, thereby facilitating a myeloid and/or monocytic differentiation of the leukemic clone, is currently unclear.

miR-23a is part of the miR-23a/27a/24-2 cluster, a network of closely related miRNAs showing a variety of similar functions and targets. Deregulation and overexpression of the whole cluster or its single miRNAs has been shown in a variety of human cancers and proved to be functionally involved in malignant transformation (43). Of particular interest, this cluster has been related to RKIP modulation previously. Li and coworkers demonstrated, that enforced expression of miR-27a causes downregulation of RKIP, thereby conferring cisplatin resistance in lung cancer cell lines (24). Although the members of this cluster share many common targets, these miRNAs itself might underlie a completely different regulation and processing, which might vary between different disease states. Indeed, only miR-23a correlated to the expression of RKIP in AML, whereas the other cluster members failed to do so. Therefore, although our data further corroborate the functional involvement of the miR-23a/27a/24-2 cluster in the pathogenesis of RKIP loss, they suggest that miR-23a is the causative miRNA in AML. The recent description of pro-leukemogenic effects of miR-23a overexpression in a murine *in vivo* model further strengthens this conclusion (45).

In consideration of the relevance of the miR-23a/RKIP axis in AML and the importance of the miR-23a/27a/24-2 cluster in RKIP regulation, we were interested, whether such an association exists in other cancers with decreased RKIP expression as well. Therefore, we performed a database retrieval via TCGA and analyzed expression levels of RKIP and the miR-23a/27a/24-2 cluster members. Altogether, we were able to extract 14 additional cancer entities, comprising more than 4300 primary patient specimens. Importantly, an inverse correlation between RKIP and one or more members of the miR-23a/27a/24-2 cluster could be observed in ten out of fourteen cancer entities, suggesting that the miR-23a/27a/24-2/RKIP cascade is of general relevance for human carcinogenesis. With the exception of squamous cell lung carcinoma, where statistical significance could only be reached for miR-27a, increased expression of miR-23a correlated to decreased RKIP levels in all of these cancers making it the predominant cluster member. The relevance of this finding

is further highlighted by the fact that aberrant miR-23a expression levels have been described in most of these cancer entities previously. Moreover, its functional relevance for the pathogenesis of these diseases as well as a role in the development of chemoresistance have been suggested (43, 47-50).

In summary, we have identified that increased expression of miR-23a, a member of the miR-23a/27a/24-2 cluster, is correlated with a loss of RKIP in AML and that both events are linked to myelomonocytic and monocytic AML phenotypes. We could further show that miR-23a overexpression is indeed causative for RKIP decrease in AML by directly binding to its 3'UTR. Finally, we could highlight the importance of the miR-23a/27a//24-2/RKIP axis in a broad range of human cancer entities and thereby provide a mechanism for RKIP downregulation with general relevance for the field of cancer research. Therefore, our results might provide the basis to develop specific therapies with the potential to restore the expression of RKIP.

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## Tables

Cancer entity (n = 14)	No. of patient samples (total n = 4342)	miR-23a		miR-24-2		miR-27a	
		Coefficient	P - value	Coefficient	P - value	Coefficient	P - value
Lung Squamous Cell Carcinoma (LUSC)	104	-0.131	0.186	0.176	0.074	-0.291	0.003**
Lung Adenocarcinoma (LUAD)	447	-0.201	<0.001**	-0.021	0.661	0.172	<0.001**
Prostate Adenocarcinoma (PRAD)	493	-0.190	<0.001**	-0.174	<0.001**	-0.095	0.060
Kidney Renal Clear Cell Carcinoma (KIRC)	162	-0.280	<0.001**	-0.224	0.004**	-0.272	<0.001**
Bladder Urothelial Carcinoma (BLCA)	405	-0.306	<0.001**	-0.187	<0.001**	-0.354	<0.001**
Invasive Breast Carcinoma (BRCA)	299	-0.188	0.001**	-0.141	0.015*	-0.158	0.006**
Ovarian Serous Cystadenocarcinoma (OV)	513	-0.137	0.002**	-0.084	0.058	-0.051	0.253
Uterine Corpus Endometrial Carcinoma (UCEC)	174	-0.089	0.242	0.025	0.747	-0.098	0.196
Pancreatic Adenocarcinoma (PAAD)	179	-0.492	<0.001**	-0.368	<0.001**	-0.297	<0.001**
Liver Hepatocellular Carcinoma (LIHC)	369	-0.425	<0.001**	-0.277	<0.001**	-0.407	<0.001**
Colon and Rectal Adenocarcinoma (COAD)	78	0.1	0.382	0.001	0.997	0.073	0.526
Head and Neck Squamous Cell Carcinoma (HNSC)	476	-0.104	0.023*	-0.126	0.006**	-0.07	0.087
Skin Cutaneous Melanoma (SKCM)	447	0.044	0.354	0.039	0.412	0.069	0.145
Glioblastoma Multiforme (GBM)	196	0.048	0.506	0.002	0.975	0.044	0.542

**Table 1.**

Correlation between *RKIP* and miRNA expression in human cancer. Expression values of *RKIP* mRNA (as obtained by RNA Sequencing V2 RSEM, Affymetrix U133 or Agilent

microarrays) and miRNAs (as obtained by miRNA arrays) were correlated using Spearman-Rho. Negative Coefficients equal to inverse correlation. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

## Figure legends

### Figure 1.

Increased expression of miR-23a correlates with decreased expression of *RKIP* in AML. A, box plots showing a significant increase of miR-23a, miR-24 and miR-15a expression in AML patient specimens defined as *RKIP* loss in AML cohort I ( $n = 20$ ). miRNA expression values were assessed by qPCR and are displayed as x-fold expression of the calibrator (NB4 cells). *RKIP* expression was evaluated by qPCR and Immunoblot, *RKIP* loss was defined as described in Supplementary Fig. S1 and as previously reported (11). B, scatter plots showing a significant and inverse correlation between the expression of *RKIP* mRNA (displayed as microarray expression values at the y-axis) and the miRNAs miR-23a, miR-24 and miR-15a (displayed as miRNA array expression values at the x-axis) in 214 patient specimens of the Dutch-Belgian AML cohort II (30-32). C, scatter plots showing a significant and inverse correlation between the expression of *RKIP* mRNA (displayed as RNA Sequencing V2 RSEM expression values at the y-axis) and miR-23a (displayed as miRNA Sequencing expression values at the x-axis) in the TCGA AML cohort III ( $n = 173$ ).

### Figure 2.

miR-23a demonstrates increased expression in AML with myelomonocytic and monocytic differentiation. A, box plots showing a significant increase of miR-23a expression (measured by qPCR and displayed as x-fold expression of a calibrator [NB4 cells]) in AML phenotypes with myelomonocytic and monocytic phenotypes (M4/M5) in the Austrian AML cohort I ( $n = 20$ ). B and C, these results could be corroborated in the Dutch-Belgian AML cohort II (B;  $n = 214$ ; miR-23a expression displayed as miRNA array expression values (30-32)) and in TCGA

cohort III (C; n = 173; miR-23a expression displayed as miRNA Sequencing expression values).

**Figure 3.**

Increased expression of miR-23a is no effect of RKIP loss. A, U937 cells were lentivirally transduced with either *RKIP*-shRNA or an empty vector control (Cntrl). Successful knockdown was confirmed by Immunoblot, Beta-Actin was used as a loading control. B, qPCR expression analysis demonstrated that miR-23a expression is not affected by the knockdown of RKIP. Graphs represent the mean of three independent experiments  $\pm$  SD, expression values are given as x-fold expression of Cntrl.

**Figure 4.**

miR-23a modulates the expression of RKIP. A, HEK-293 and U937 (both exhibiting strong RKIP expression (11)) were transfected with either miR-23a mimic or unspecific control (Cntrl). THP-1 (exhibiting decreased expression of RKIP (11)) was transfected with either miR-23a hairpin inhibitor (miR-23a\_hi) or Cntrl. miR-23a expression was assessed by qPCR and is displayed as x-fold expression of the Cntrl transfected cells. B, *RKIP* mRNA expression analysis by qPCR of the conditions mentioned above demonstrated significant downregulation of *RKIP* after transfection with miR-23a mimic and significant upregulation after transfection with miR-23a\_hi. *RKIP* expression is displayed as x-fold expression of the Cntrl transfected cells. C, Immunoblot analyses demonstrating decreased expression of RKIP protein in cells transfected with miR-23a mimic and increased expression in conditions where miR-23a\_hi has been used. Beta-Actin was used as a loading control. D, Densitometric quantification of the Immunoblot results, displayed as x-fold expression of the Cntrl transfected cells. Graphs represent the mean of three independent experiments  $\pm$  SD, expression values are given as x-fold expression of Cntrl.

**Figure 5.**

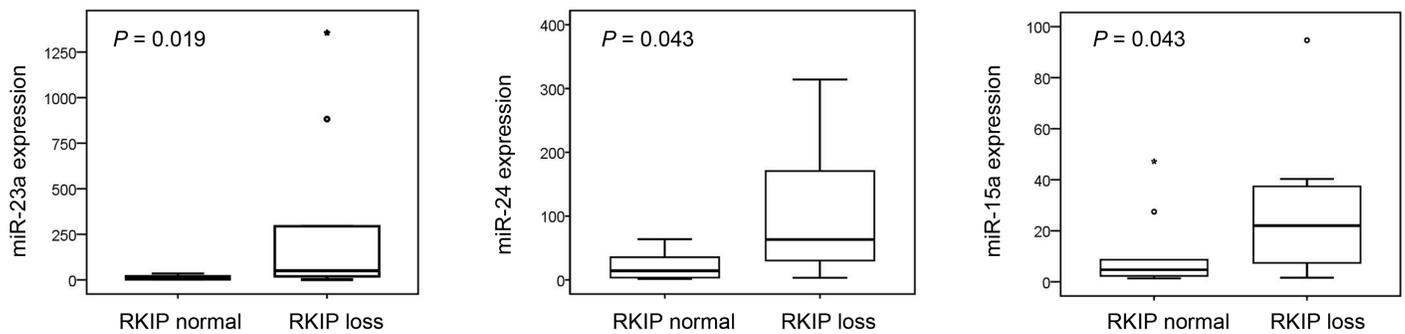
miR-23a modulates the expression of RKIP via direct interaction with the 3'UTR. A, sequence of the human *RKIP* 3'UTR (hsa *RKIP*) showing the two miR-23a binding sites (bs1-2). Matching regions are highlighted by lines. Alteration of bs1 by either deletion (*RKIP* Del) or mutation (*RKIP* Mut) is displayed. B, HEK-293 were transfected with either miR-23a mimic (white bars) or unspecific control (Cntrl, black bars) together with luciferase reporter clones, containing i) wildtype *RKIP* 3'UTR (*RKIP* 3'UTR), ii) *RKIP* Del or iii) *RKIP* Mut. pCS2-RFP was included in all transfections to compensate for different transfection efficiencies. Graphs represent the mean RFP-compensated luciferase activity of three independent experiments  $\pm$  SD, values are given as x-fold expression of the respective Cntrl transfected setting.

**Figure 6.**

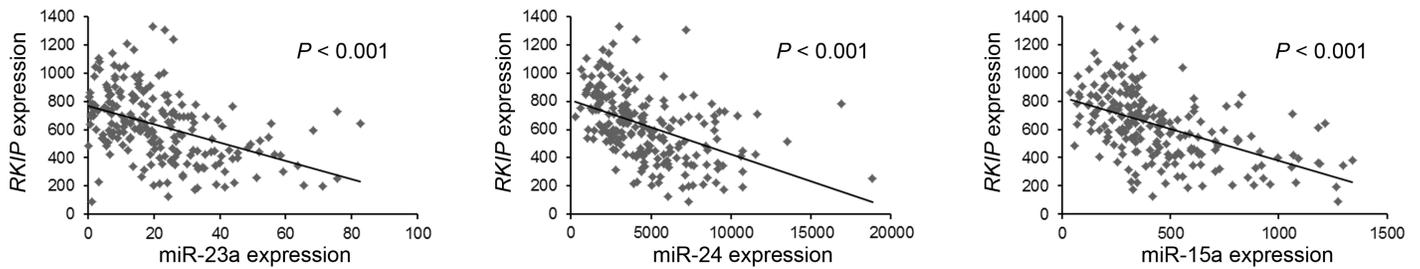
miR-23a induces the proliferation of hematopoietic cells via downregulation of RKIP. A, U937 with stable expression of FLAG-RKIP and empty vector, respectively, were transfected with either miR-23a mimic or unspecific control (Cntrl) as indicated. Additionally, parental U937 were lentivirally transduced with either *RKIP* shRNA or Cntrl. Cells were seeded and maintained as described in Materials and Methods and viable cells were counted after 5 days. For comparison of the different conditions, respective Cntrl situations have been set at a value of 1 and the relative increase of viable cells in the miR-23a/*RKIP* shRNA conditions have been calculated using the ratio viable cells miR-23a/*RKIP* shRNA to viable cells Cntrl. B-D, BrdU/7-AAD cell cycle/proliferation assays were performed in all the settings described above to evaluate the percentage of cells in S-phase (upper gate), G0/G1-phase (left lower gate) and G2/M-phase (right lower gate), respectively. Black bars, Cntrl; white bars, miR-23a/*RKIP* shRNA. The graphs summarize the results of at least three independent experiments. Data are expressed as mean values  $\pm$  SD. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

**Figure 1**

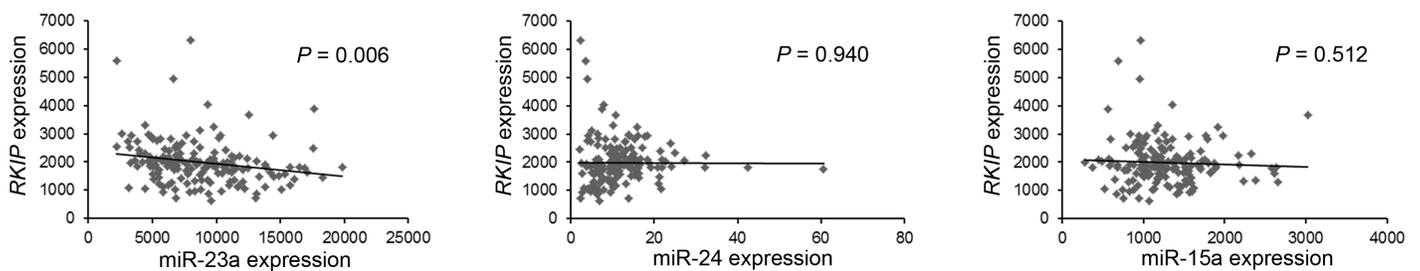
**A**



**B**

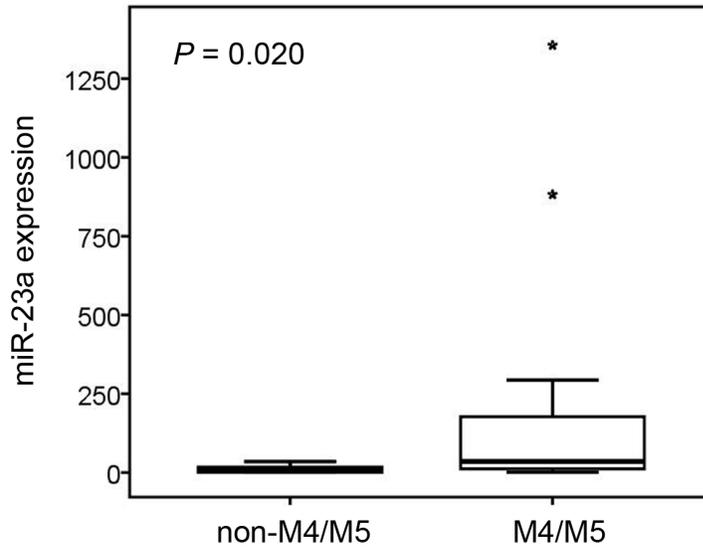


**C**

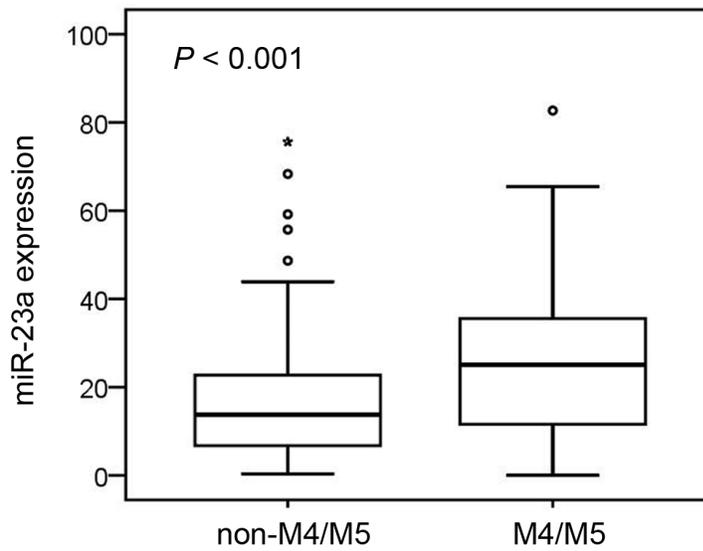


## Figure 2

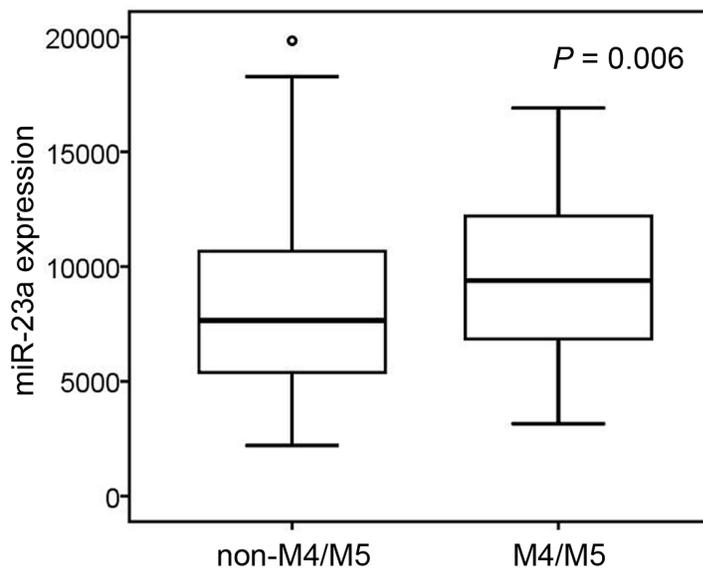
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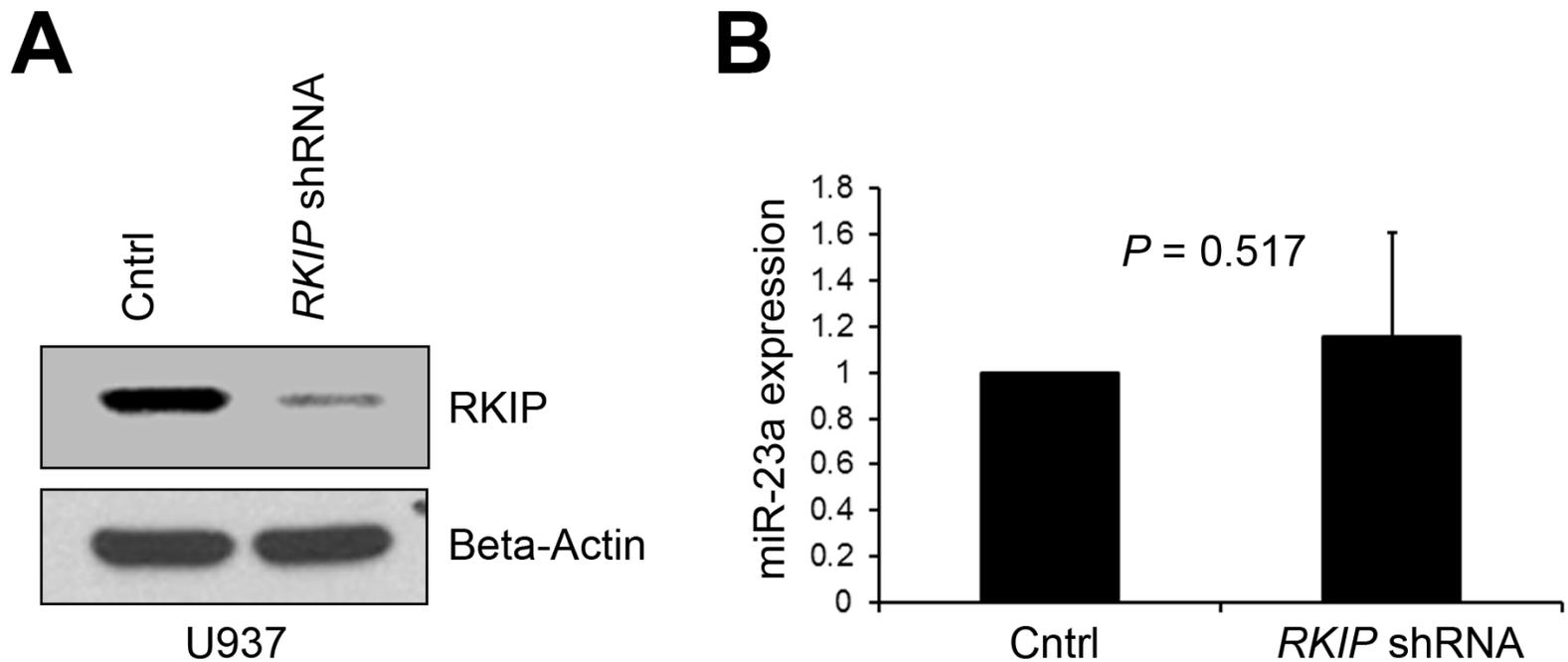
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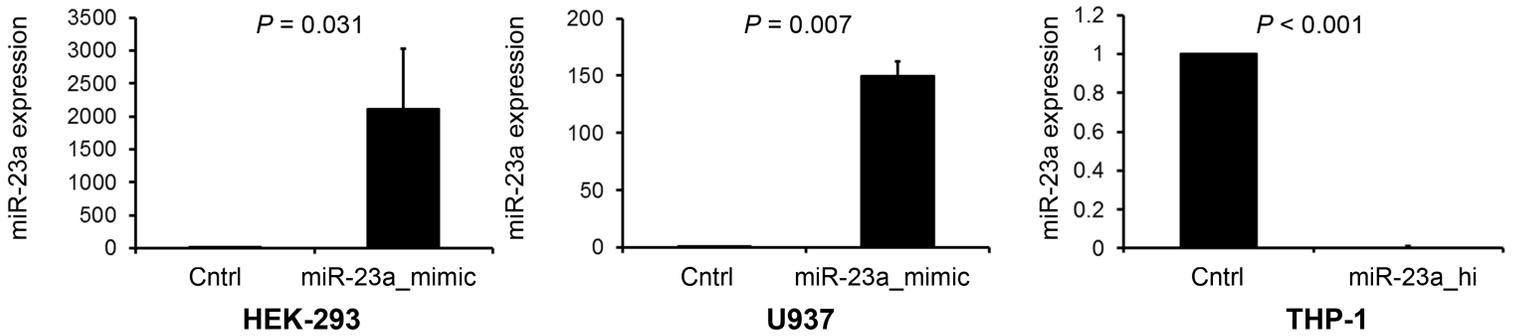


## Figure 3

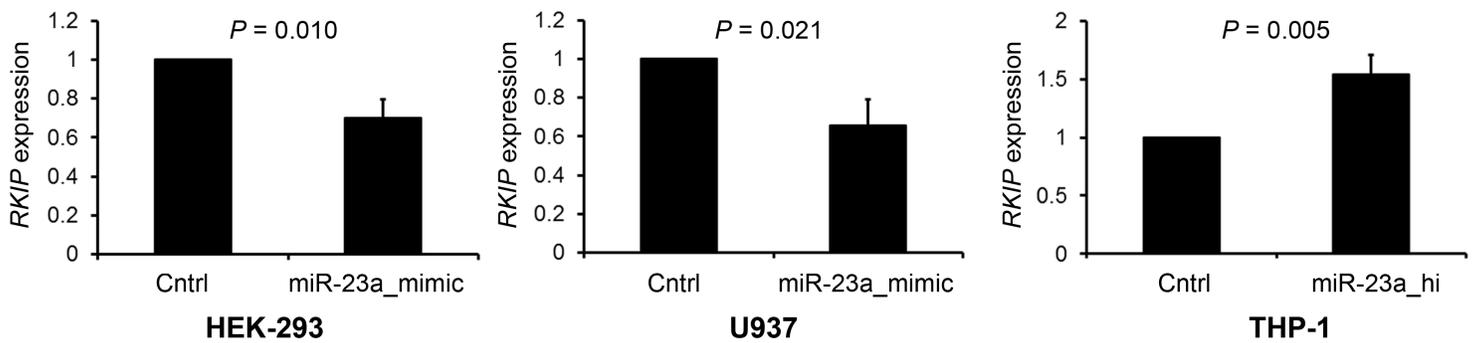


## Figure 4

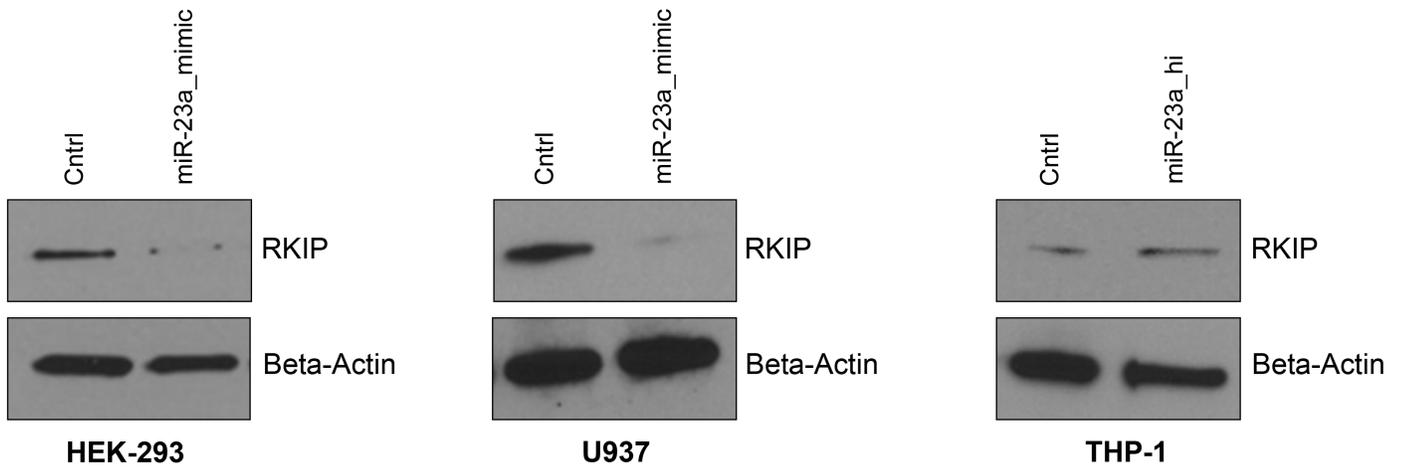
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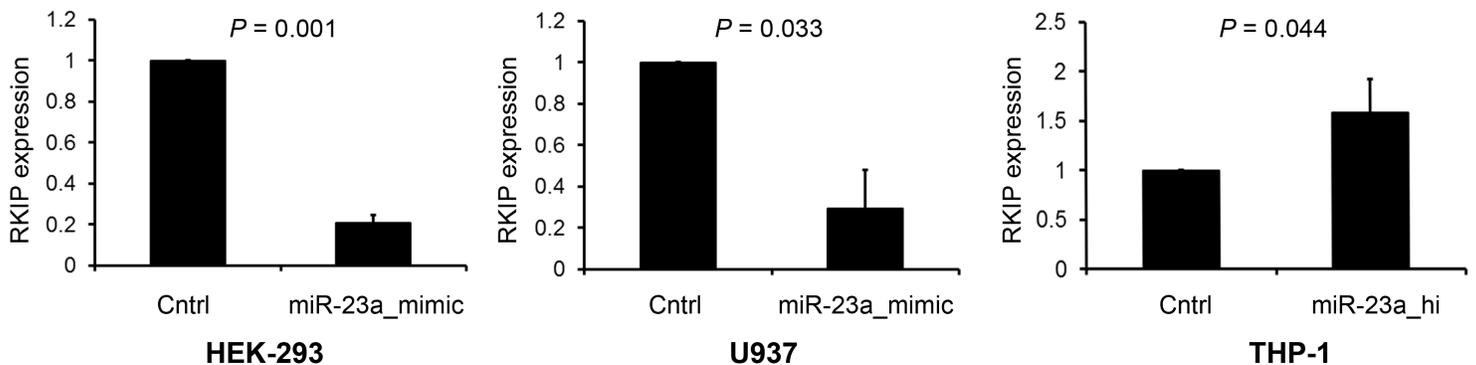
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**C**

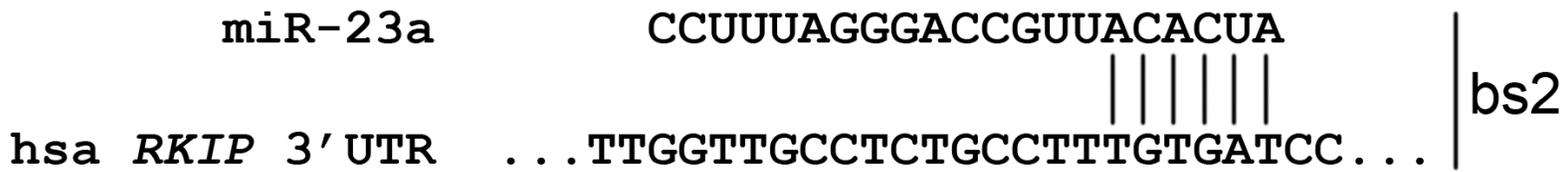
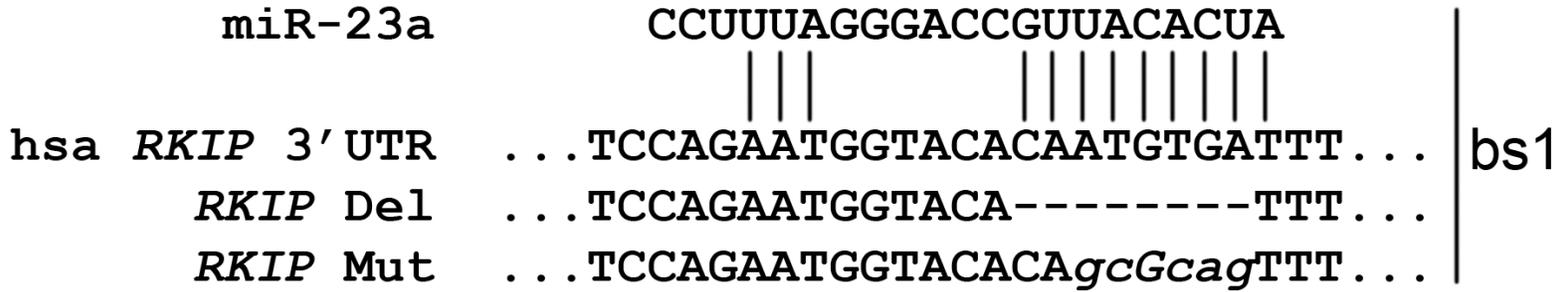


**D**

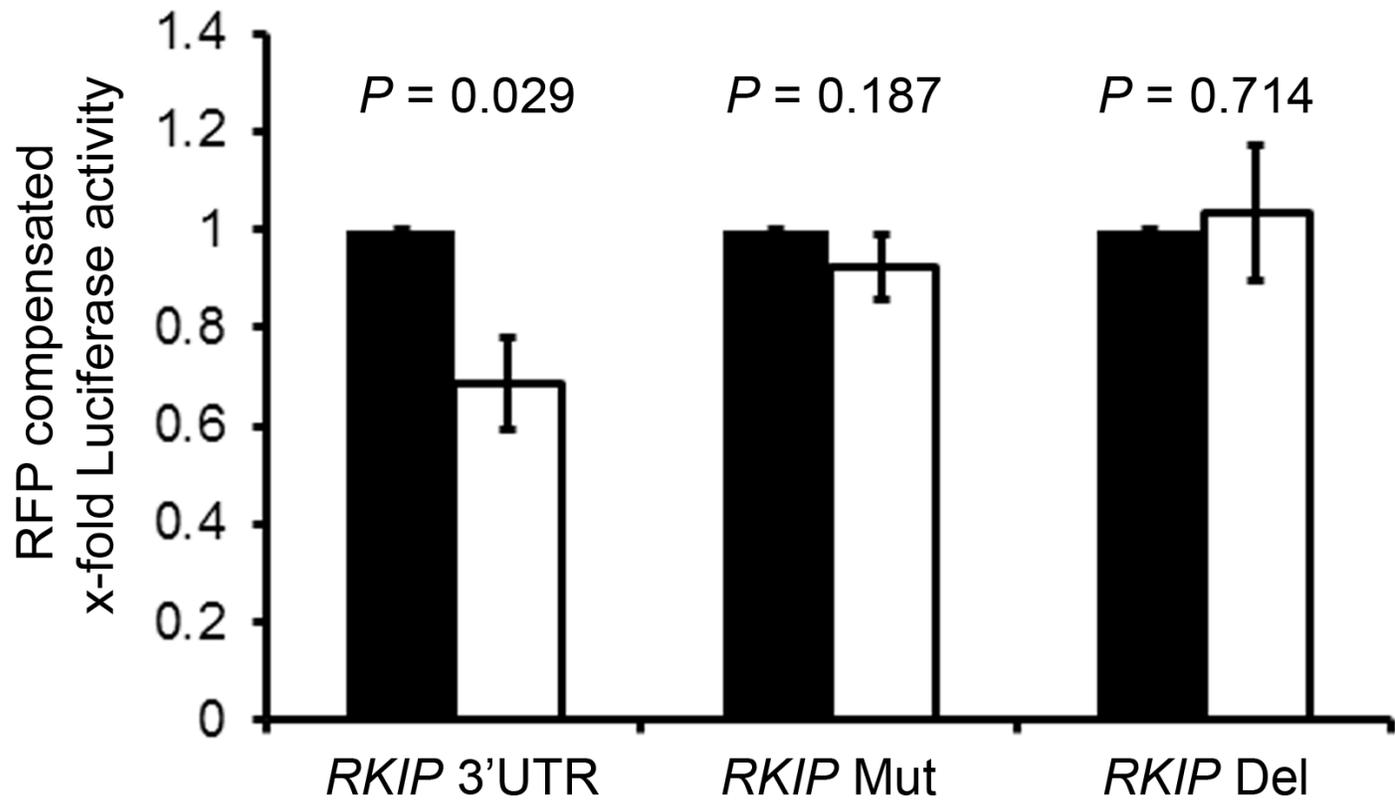


# Figure 5

## A

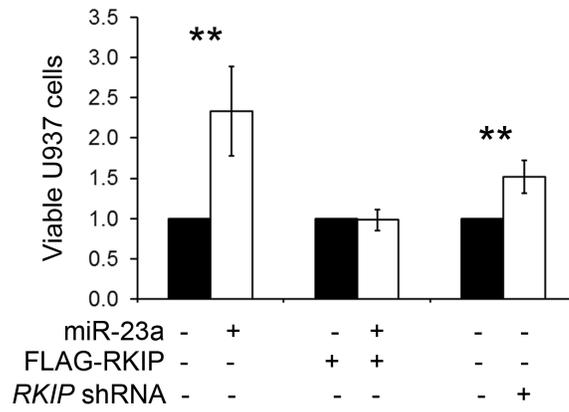


## B

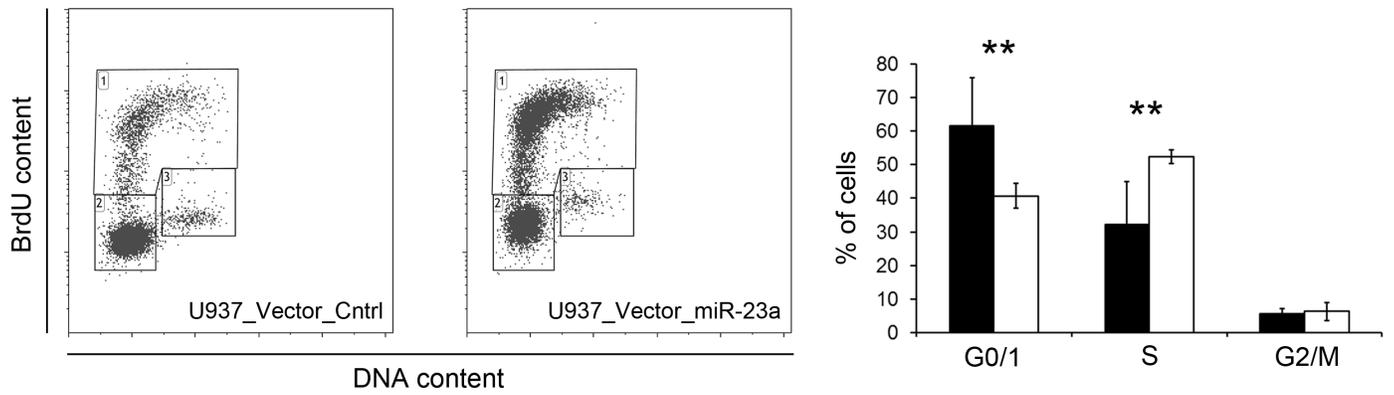


## Figure 6

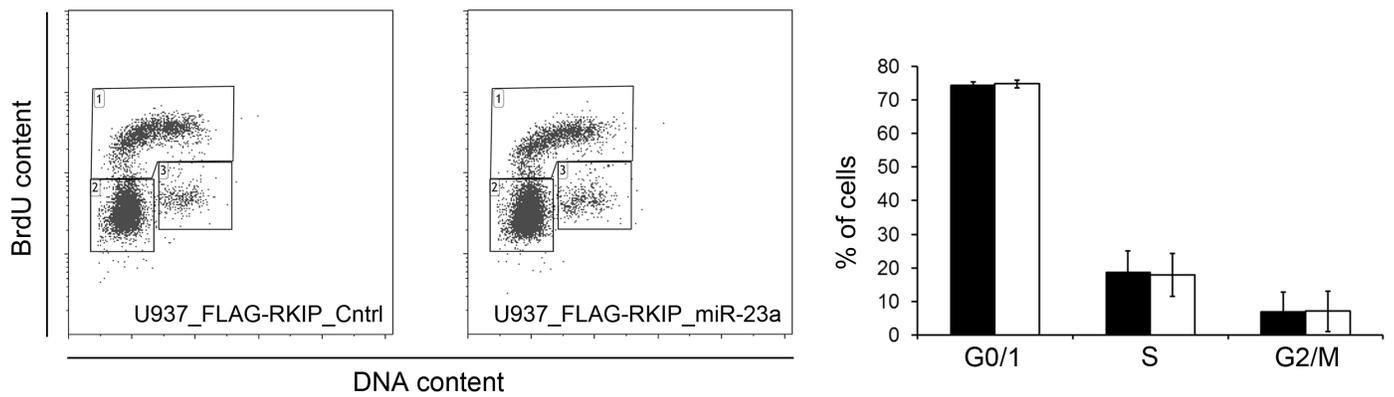
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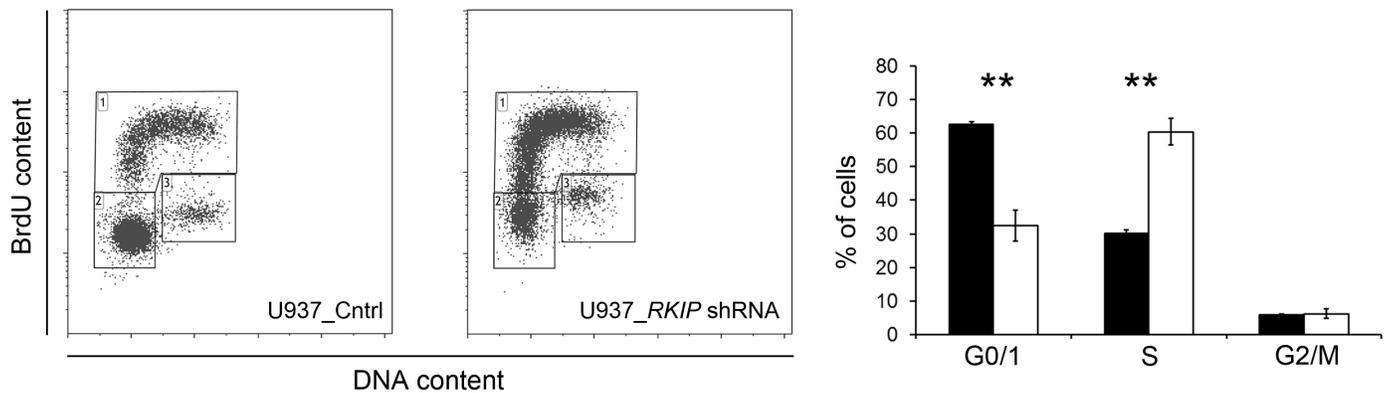
**B**



**C**



**D**



**Table 1.** Correlation between *RKIP* and miRNA expression in human cancer

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# Cancer Research

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## Increased expression of miR-23a mediates a loss of expression in the RAF kinase inhibitor protein RKIP

Stefan Hatzl, Olivia Geiger, Maja Kim Kuepper, et al.

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