Circular RNAs of the nucleophosmin (NPM1) gene in acute myeloid leukemia

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SUPPLEMENTARY INFORMATION

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Supplementary Methods

Cell line models

Leukemia cell lines (KASUMI-1, NB-4, OCI-AML5, OCI-AML3, ME-1, MV4-11 and K-562) were purchased from the German cell line repository (DSMZ) and grown according to standard protocols.

Additional patient and healthy sample information

Patient samples were collected at first diagnosis and chosen from the larger AMLSG_07-04 study cohort by applying the following criteria: availability of RNA material, high RNA quality and availability of gene expression data. Mononuclear cells of AML samples were enriched via Ficoll-Hypaque density gradient, and the percentage of leukemic blasts was >85%. For the quantification of hsa_circ_0075001, 46 cytogenetically normal AML cases were selected of which 23 carried an *NPM1* mutation. For RNA-Seq, 10 cytogenetically normal AML cases were selected of which 5 carried an *NPM1* mutation.

For PCR experiments, the healthy cells were derived from the peripheral blood-derived mononuclear cell fraction of healthy donors.

RNA isolation, quality control and reverse transcription

RNA was isolated from cell lines and patient samples using the AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA integrity was assessed with the Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) and only samples with an RNA Integrity Number (RIN) of 7.5 or higher were included in the study. Using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA), 1 µg total cell line RNA or 700 ng total patient RNA was converted to single-stranded cDNA with the use of random primers.

TOPO cloning reaction and cycle sequencing reaction (CSR)

Polymerase chain reaction (PCR) products were cloned into a TOPO vector using the TOPO® TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) and transformed into TOP10 E.coli cells according to the manufacturer's instructions. Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (QIAGEN) and 300 ng were used in the cycle sequencing reaction (CSR). The CSR was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

PCR and quantitative real-time PCR (qPCR)

PCR primers were used at a final concentration of 200 nM, and 0.5 units of the HotStarTaq DNA polymerase were present per 40 cycle reaction. Primers for qPCR were used at a final concentration of 500 nM and primer sequences are listed in Supplementary Table 1. qPCR was performed on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and for data analysis the 7900HT SDS 2.3 software (Applied Biosystems) was used. All values are means of two replicates and results were normalized to β-Actin (*ACTB*). qPCR efficiencies were calculated by creating a standard curve with 10-fold serial dilutions with 6 points of DNA templates. For all qPCR reactions used in the analysis, the efficiency was >99% and R^2 was >0.99.

Gene expression profiling

Gene expression data of 46 bone marrow or peripheral blood samples from AML patients were obtained as previously reported using GeneChip® Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) [1-3].

Databases used for pathway analysis

Pathway analysis was performed using the iPathwayGuide web application (Advaita, Plymouth, MI, USA). Statistical thresholds were set at $p<0.05$ and $\log_2FC>|0.6|$. Databases used in this web application include the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Release 73.0+/03-16, Mar 15) [4] for pathway analysis and diseases, the Gene Ontology Consortium database (2014-Sep19) [5-7] for gene ontology analysis, and miRBase (Release 21) and TARGETSCAN [TargetScan Release 6.2 (updated March 2015)] databases [8-10] for miRNA analysis.

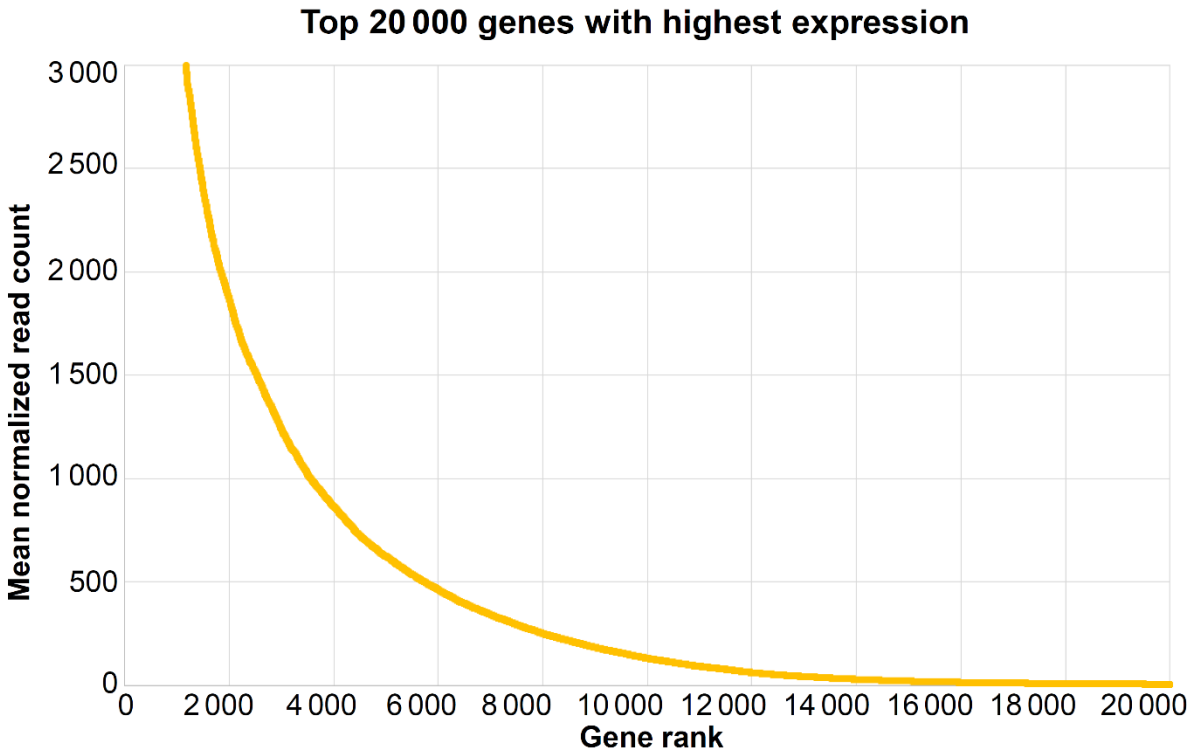
In-house computational pipeline for the identification of circRNAs in ribominus RNA sequencing data

Human hg19 reference genome and GENCODE annotation (version 19, Ensembl 74) served as a reference for STAR two-pass alignment (STAR version 2.4.2a, June 2015) and were downloaded from GATK (https://software.broadinstitute.org/gatk/download/bundle) and GENCODE (https://www.gencodegenes.org/releases/19.html), respectively. STAR output files *Chimeric.out.junction and *Chimeric.out.sam were used as input. Reads derived from circRNAs mapping to a backsplice junction were extracted applying the following criteria:

- Splice donor lies downstream of splice acceptor
- Acceptor and donor are mapped to the same strand on the same chromosome and lie within 1 million bp
- Backsplice junction overlaps known exon-exon borders of the same gene with a minimum of 15 bp in each exon of the junction (*chimSegmentMin* and *chimJunctionOverhangMin*=15)
- The paired mate lies within acceptor and donor position; circRNAs whose chimeric reads span beyond the genomic backsplice coordinates are filtered out

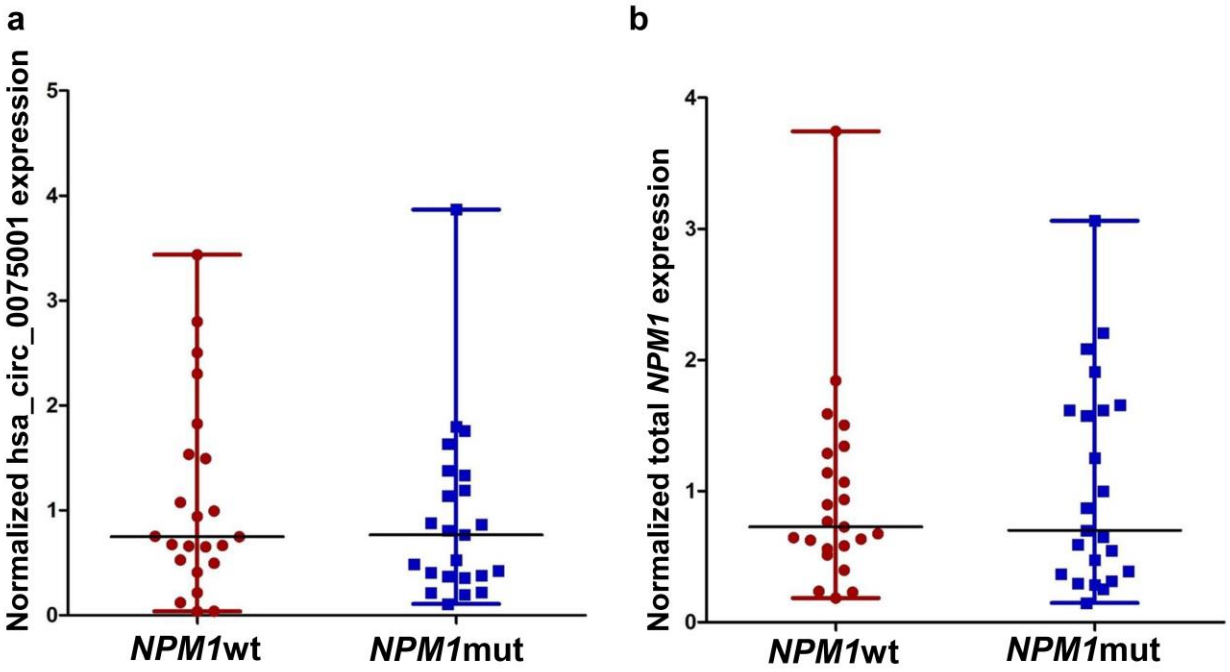
Circular reads of each junction were counted. Output files included a BED file listing all supported circular junctions and a file with information about the circular junction coordinates, strand, gene annotation, number of supporting reads and coordinates of start and end positions of donor, acceptor and paired-mate segments. In addition, another BED file was created listing the total number of each gene's circRNAs' supporting reads and used for gene-based analyses, for example PCA.

Supplementary Figures

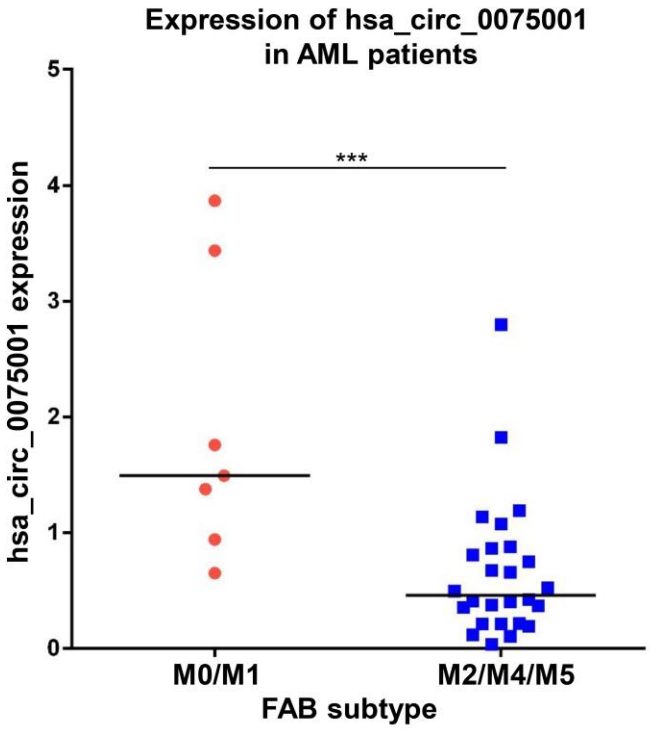


Mean normalized read count CUTOFF	100	50	20	10	5
No. of genes	10 794	12 601	15 217	17 755	20 847
No. of genes also producing circRNAs	5 173	5 403	5 561	5 635	5 658
Percentage of genes also producing circRNAs	47.9 %	42.9 %	36.5 %	31.7 %	27.1 %

Supplementary Figure 1: Mean normalized read counts in 20 RNA-Seq samples of 20000 genes with highest expression. Normalization was performed using DESeq2. The top 10794 genes with a mean read count > 100 were considered as “highly expressed”. The top 17755 genes with a mean read count > 10 were considered “markedly expressed”. Only values up to a mean of 3000 normalized reads are displayed.



Supplementary Figure 2: Expression of hsa_circ_0075001 and total *NPM1* in a cohort of 46 AML patients. Expression was measured by SYBR® Green based qPCR. All values were normalized to β-Actin (*ACTB*) and are relative to the respective mean expression, which was set to 1. The black line indicates the median expression level and whiskers illustrate the expression range. **(A)** Scatter plot comparing the normalized hsa_circ_75001 expression in *NPM1*wt (n=23) and *NPM1*mut (n=23) AML patients. **(B)** Scatter plot comparing the total *NPM1* expression in *NPM1*wt (n=23) and *NPM1*mut (n=23) AML patients.



Supplementary Figure 3: Expression of hsa_circ_0075001 in different AML subtypes based on FAB classification. Scatter plot comparing the normalized hsa_circ_75001 expression in AML subtypes based on the French-American-British (FAB) classification. Expression was measured by SYBR® Green based qPCR. All values were normalized to β-Actin (*ACTB*). The black line indicates the median expression level. The asterisks mark $p < 0.001$ (unpaired t test).

Supplementary Tables

Supplementary Table 1: Clinical data of AML patients with high or low hsa_circ_0075001 expression

Criterion	75001 high group	75001 low group	*p* value
Age (y)	45.3 ± 2.1	45.0 ± 1.8	0.71 #
Male	15	10	0.14 §
Response to therapy	18	16	0.49 §
Type AML	AML n=19 sAML n=2 tAML n=1	AML n=20 sAML n=3 tAML n=0	
ELN risk classification	Favorable n=4 Intermediate-I n=18	Favorable n=11 Intermediate-I n=12	0.06 §
BM blasts %	61.2 ± 5.5	67.4 ± 4.7	0.30 #
PB blasts %	34.0 ± 6.1	32.6 ± 6.5	0.70 #
*NPM1*mut	12	11	0.77 §
FLT3 ITD *FLT3* TKD	10 1	17 1	0.07 § 1.00 §
*CEBPA*mut	1	5	0.19 §
*NRAS*mut	2	5	0.41 §
MLL PTD	1	0	0.49 §
*DNMT3A*mut *IDH2*mut *TET2*mut	6 2 2	11 4 1	0.09 § 0.40 § 1.00 §
*SF3B1*mut *SRSF2*mut *U2AF1*mut *ZRSR2*mut	0 1 0 0	1 0 1 1	0.49 § 1.00 § 0.49 § 0.49 §

Mann Whitney test , § Fisher's exact test

sAML = secondary AML ; tAML = therapy-related AML ; ELN = European LeukemiaNet ; ITD = internal tandem duplication mutation; TKD = tyrosine kinase domain mutation ; PTD = partial tandem duplication mutation

Supplementary Table 2: Description of primers used in PCR, quantitative real-time PCR and Oxford Nanopore sequencing experiments

Target	Transcript ID	Sequence	Exon	Experiment
ACTB	β-Actin	5'AGAGCTACGAGCTGCCTGAC 3' sense	4	PCR, qPCR
		5'AGCACTGTGTTGGCGTACAG 3' antisense	5	
NPM1	total NPM1	5'AGCACTTAGTAGCTGTGGAGGA 3' sense	4/5	PCR, qPCR
		5'TGGAACCTTGCTACCACCTC 3' antisense	5	
NPM1	circNPM1	5'ATGTGAAGAATTGCTTCCGG 3' sense	11	PCR
		5'GGATTCTTGTCCTTTTGATCTTG 3' antisense	9/8	
NPM1	hsa_circ_0074995	5'CTCCTACCTAAGTGCGTGCC 3' sense	1	PCR
		5'CCACTCCTTTCTCGTTCTTAAAGA 3' antisense	1/2	
NPM1	hsa_circ_0074996	5'GGTTGTGAACTAAAGGCCGA 3' sense	1/2	PCR
		5'CACTCCTTTCTCGTTGGCTG 3' antisense	1/3	
NPM1	hsa_circ_0074997	5'GGTTGTGAACTAAAGGCCGA 3' sense	1/2	PCR
		5'TCCTTTCTCTGTGGAACCTTG 3' antisense	1/5	
NPM1	hsa_circ_0074998	5'GGTTGTGAACTAAAGGCCGA 3' sense	1/2	PCR
		5'CCACTCCTTTCTTCTTCATCATC 3' antisense	1/6	
NPM1	hsa_circ_0075000	5'TATCTGGAAAGCGGTCTGC 3' sense	5	PCR
		5'TTCACAACTCTTCATCATCATCC 3' antisense	2/6	
NPM1	hsa_circ_0075001	5'TTGCTGCTGATGAAGATGATG 3' sense	6	PCR
		5'CCTTTAGTTCACAACTTTCTTCACTG 3' antisense	2/7	
NPM1	hsa_circ_0075001	5'AGCGCCAGTGAAGAAAGTTG 3' sense	7/2	PCR, qPCR
		5'CCTCTGCTTCAACAATGTGC 3' antisense	3	
NPM1	hsa_circ_0075002	5'CACCAAGATCAAAAGGACAAGAA 3' sense	8/9	PCR
		5'GTTCACAACTTGATGCATTCAAA 3' antisense	2/10	
NPM1	hsa_circ_0075003	5'AACTTTGAAAATGTCTGTACAGCC 3' sense	3	PCR
		5'AACTGACCTACTAAGTGCTGTCCA 3' antisense	3/4	
NPM1	hsa_circ_0075004	5'GTTCAGGGCCAGTGCATATT 3' sense	4	PCR
		5'AAACTGACCTGTGGAACCTTG 3' antisense	3/5	
NPM1	hsa_circ_0075005	5'TTGCTGCTGATGAAGATGATG 3' sense	6	PCR
		5'AACTGACTTTCTTCACTGGCG 3' antisense	3/7	
NPM1	hsa_circ_0075012	5'AAAAAGCGCCAGTGAAGAAA 3' sense	7	PCR
		5'CTCCACAGTTTTGATCTTGGTG 3' antisense	5/8	
NPM1	hsa_circ_0075016	5'GACGATGATGATGAAGAGGATG 3' sense	6	PCR
		5'TTTTTTTCTTCACTGGCGC 3' antisense	6/7	
NPM1	hsa_circ_0005341	5'ACTGACCAAGAGTGATGATGATG 3' sense	11/7	PCR
		5'TGCATTTTTGGCTGGAGTATC 3' antisense	8	
NPM1	hsa_circ_0075019	5'AAAGCAAAAATGCAAGCAAGT 3' sense	9	PCR
		5'TCTCGTATAGACTCTTGGTCAGTCA 3' antisense	8/11	
NPM1	hsa_circ_0075022	5'GAGAACCACCCTCTTGGTCA 3' sense	11	PCR
		5'AGTGGAAGCCAAATTCATCAA 3' antisense	11	
NPM1	circNPM1	5'GGTGGATAATGATGAAAATGAGCACCAGT 3' sense	2	Ox.Nanopore
		5'TGATAATCTTTGTCGGCCTTTAGTTCACA 3' antisense	2	
NPM1	circNPM1	5'GAGGCAATGAATTACGAAGGCAGTCC 3' sense	3	Ox.Nanopore
		5'GCTTCAACAATGTGCAACTCATCCTTTG 3' antisense	3	
NPM1	circNPM1	5'GCCCCTGGAGGTGGTAGCA 3' sense	5	Ox.Nanopore
		5'TCATCTTCTGACTCTGCATCTTCCTCC 3' antisense	5	

Supplementary Table 3: Normalized circular read counts of 20 RNA-Seq samples

NormalizedCircularCounts.allGenes.allFiles.xlsx

Supplementary Table 4: Normalized linear read counts of 20 RNA-Seq samples

NormalizedLinearCounts.allGenes.allFiles.xlsx

Supplementary Table 5: Reverse complementary sequences in introns flanking backsplices of annotated *NPM1* circRNAs as determined by alignment with NCBI BLAST

circBase ID	Exons	Flanking intron	Flanking intron	Complementary sequences	Sequence identity
74995	1-2	Upstream	I 2	298 bp +	81%
74996	1-3	Upstream	I 3		
74997	1-5	Upstream	I 5		
74998	1-6	Upstream	I 6	247 bp +	85%
74999	2-3	I 1	I 3		
75000	2-6	I 1	I 6	23 bp +	91%
75001	2-7	I 1	I 7		
75002	2-10	I 1	I 10	12 bp	100%
75003	3-4	I 2	I 4		
75004	3-5	I 2	I 5		
75005	3-7	I 2	I 7		
75006	3-8	I 2	I 8	299 bp +	79%
75007	3-9	I 2	I 9	17 bp	94%
75008	3-11	I 2	I 11	180 bp +	77%
75009	4-6	I 3	I 6		
75010	4-10	I 3	I 10		
75011	5-6	I 4	I 6	292 bp +	74%
75012	5-8	I 4	I 8	175 bp +	69%
75013	5-10	I 4	I 10		
75014	5-12	I 4	I 12		
75015	6-6	I 5	I 6		
75016	6-7	I 5	I 7		
75017	7-8	I 6	I 8	660 bp +	69%
5341	7-11	I 6	I 11	257 bp +	79%
75018	8-10	I 7	I 10		
75019	8-11	I 7	I 11	177 bp +	79%
75020	9-10	I 8	I 10	12 bp	100%
75021	10-10	I 9	I 10		
75022	11-11	I 10	I 11		

+ additional alignments possible, I = Intron

Supplementary Table 6: Top 40 pathways hsa_circ_0075001 high vs low based on FDR-corrected *p* value

Pathway name	*p* value
Lysosome	4.05E-13
Endocytosis	1.08E-06
Tuberculosis	1.08E-06
Toll-like receptor signaling pathway	1.26E-06
Natural killer cell mediated cytotoxicity	1.26E-06
Fc gamma R-mediated phagocytosis	1.0675E-05
Chemokine signaling pathway	1.10E-05
Regulation of actin cytoskeleton	1.43E-05
Salmonella infection	1.4875E-05
Fc epsilon RI signaling pathway	1.4875E-05
Pertussis	2.33E-05
B cell receptor signaling pathway	3.40E-05
Platelet activation	3.96E-05
Leukocyte transendothelial migration	4.72E-05
Neurotrophin signaling pathway	4.72E-05
Phagosome	7.50E-05
Legionellosis	9.04E-05
Epstein-Barr virus infection	9.04E-05
Insulin signaling pathway	9.04E-05
Osteoclast differentiation	9.0568E-05
Non-small cell lung cancer	9.25E-05
Sphingolipid signaling pathway	9.76E-05
Leishmaniasis	0.00013166
Bacterial invasion of epithelial cells	0.00013166
Vibrio cholerae infection	0.00020694
Focal adhesion	0.0002143
FoxO signaling pathway	0.00022659
Metabolic pathways	0.0002324
Influenza A	0.00026067
Toxoplasmosis	0.0002678
Shigellosis	0.00028744
Apoptosis	0.00035859
Morphine addiction	0.00039993
Ribosome	0.00042885
HIF-1 signaling pathway	0.0004995
mTOR signaling pathway	0.00059807
Proteoglycans in cancer	0.00086768
Protein processing in endoplasmic reticulum	0.00087855
Hepatitis B	0.00090901
Viral carcinogenesis	0.001275

Supplementary Table 7: Top 40 pathways total *NPM1* high vs low based on FDR-corrected *p* value

Pathway name	*p* value
Ribosome	2.64E-08
Natural killer cell mediated cytotoxicity	1.0348E-05
Leukocyte transendothelial migration	1.4233E-05
Legionellosis	2.1718E-05
Estrogen signaling pathway	2.2556E-05
Endocytosis	2.2556E-05
Chemokine signaling pathway	2.2556E-05
Fc gamma R-mediated phagocytosis	2.358E-05
Lysosome	6.4079E-05
Amoebiasis	8.9326E-05
Rap1 signaling pathway	0.00010595
T cell receptor signaling pathway	0.00014899
Bacterial invasion of epithelial cells	0.00015672
Salmonella infection	0.00015672
Cell adhesion molecules (CAMs)	0.00018421
GnRH signaling pathway	0.00018421
Oxytocin signaling pathway	0.00021269
Non-small cell lung cancer	0.00024702
Adherens junction	0.00024702
Antigen processing and presentation	0.00031204
Osteoclast differentiation	0.00034316
VEGF signaling pathway	0.00040787
Tuberculosis	0.00040787
Focal adhesion	0.00046128
NF-kappa B signaling pathway	0.00050486
Acute myeloid leukemia	0.00056555
Glioma	0.00058259
Proteoglycans in cancer	0.00071989
Endometrial cancer	0.00071989
ErbB signaling pathway	0.00080798
Toxoplasmosis	0.00080798
Prolactin signaling pathway	0.00080798
Phagosome	0.00082025
Leishmaniasis	0.00084896
B cell receptor signaling pathway	0.00084896
Thyroid hormone signaling pathway	0.00088137
HTLV-I infection	0.00094627
Viral myocarditis	0.00116632
Regulation of actin cytoskeleton	0.00129737
Fc epsilon RI signaling pathway	0.00133788

Supplementary Table 8: Genes differentially expressing circRNAs between 10 AML and 10 healthy control RNA-Seq samples

Ensemble ID	Gene symbol	Log2 fold change	*p* value	*p* adj
ENSG00000131149	GSE1	-4.286	3.33E-12	5.37E-09
ENSG00000122025	FLT3	-3.429	8.72E-12	7.02E-09
ENSG00000196323	ZBTB44	-2.428	7.23E-07	0.000388
ENSG00000130584	ZBTB46	-4.666	1.13E-06	0.000455
ENSG00000155966	AFF2	2.918	2.30E-06	0.000742
ENSG00000120071	KANSL1	-2.751	5.63E-06	0.001513
ENSG00000153317	ASAP1	2.212	1.03E-05	0.002379
ENSG00000148154	UGCG	3.483	2.53E-05	0.004947
ENSG00000187147	RNF220	-3.519	2.76E-05	0.004947
ENSG00000146555	SDK1	4.139	3.83E-05	0.006163
ENSG00000174776	WDR49	-2.864	5.09E-05	0.007447
ENSG00000163960	UBXN7	1.587	0.00015594	0.020935
ENSG00000003402	CFLAR	2.397	0.000185321	0.022286
ENSG00000179869	ABCA13	3.524	0.000193668	0.022286
ENSG00000135749	PCNXL2	-2.958	0.000312913	0.033607
ENSG00000082512	TRAF5	-3.131	0.000589879	0.059393
ENSG00000112146	FBXO9	2.290	0.000644526	0.061078
ENSG00000136783	NIPSNAP3A	-3.603	0.000752047	0.065192
ENSG00000101152	DNAJC5	2.467	0.000769281	0.065192
ENSG00000151948	GLT1D1	3.211	0.000837059	0.065192
ENSG00000154188	ANGPT1	-3.345	0.000900161	0.065192
ENSG00000229358	DPY19L1P1	2.577	0.000904527	0.065192
ENSG00000155099	TMEM55A	2.969	0.00095581	0.065192
ENSG00000086598	TMED2	1.727	0.000971209	0.065192
ENSG00000112893	MAN2A1	-2.067	0.001474614	0.095024
ENSG00000117713	ARID1A	-2.467	0.001563261	0.096862
ENSG00000248049	UBA6-AS1	-3.146	0.001665133	0.099353

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