Supplementary Material

Instability of circular RNAs in clinical tissue samples impairs their reliable expression analysis using RT-qPCR: from the myth of their advantage as biomarkers to reality

Hannah Rochow, Antonia Franz, Monika Jung, Sabine Weickmann, Bernhard Ralla, Ergin Kilic, Carsten Stephan, Annika Fendler, Klaus Jung

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1. Pre-analytical and analytical characteristics of published circRNA tissue expression studies in cancers

**Supplemental Table S1.** Analytical characteristics of 25 randomly selected studies of circRNA expression in different cancers between 2015 and 2020

| No. | Reference, Year | Cancer type | Tissue collection and storage | Analysis of RNA integrity | Applied references for normalization |
|-----|-----------------|-------------|--------------------------------|---------------------------|-------------------------------------|
| 1   | Li et al., 2015 [1] | Gastric cancer | 5                              | No                        | GAPDH                               |
| 2   | Ahmed et al., 2016 [2] | Ovarian cancer | 1                              | No                        | ACTB, GAPDH                         |
| 3   | Lu et al., 2017 [3] | Breast cancer | 5                              | Standard denaturing gel electrophoresis, no further comments | GAPDH                               |
| 4   | Zhu et al., 2017 [4] | Lung adenocarcinoma | 1 | No | GAPDH                               |
| 5   | Dang et al., 2017 [5] | Gastric cancer | 5                              | Denaturing gel electrophoresis, no further comments | GAPDH                               |
| 6   | Weng et al., 2017 [6] | Colorectal cancer | 1 | No | U6, GAPDH                           |
| 7   | Cao et al., 2017 [7] | Hypopharyngeal cancer | 4 | Denaturing gel electrophoresis, no further comments | ACTB                               |
| 8   | Chen et al, 2017 [8] | Gastric cancer | 5                              | No                        | GAPDH                               |
| 9   | Huang et al, 2017 [9] | Hepatocellular cancer | 4 | No | GAPDH                               |
| 10  | Zhang et al., 2018 [10] | Lung adenocarcinoma | 1 | No | ACTB                               |
| 11  | Sun et al., 2018 [11] | Oral squamous cancer | 2 | Denaturing gel electrophoresis, no further comments | ACTB                               |
| 12  | Huang et al., 2019 [12] | Kidney cancer | 4                              | No                        | GAPDH                               |
| 13  | Chen et al., 2019 [13] | Glioma | 0 | No | GAPDH                               |
| 14  | Lu et al., 2020 [14] | Colon cancer | 4                              | No                        | GAPDH                               |
| 15  | Jin et al., 2020 [15] | Melanoma | 3 | No | GAPDH                               |
| 16  | Xing et al., 2020 [16] | Esophageal cancer | 2 | No | Only Cq based                       |
| 17  | Sun et al., 2020 [17] | Thyroid cancer | 2 | No | GAPDH                               |
| 18  | Liu et al., 2020 [18] | Pancreatic cancer | 3 | No | GAPDH                               |
| 19  | Zhang et al., 2020 [19] | Laryngeal carcinoma | 3 | No | ACTB                               |
| 20  | Li et al, 2020 [20] | Osteosarcoma | 0 | No | GAPDH                               |
| No. | Reference, Year | Cancer type               | Tissue collection and storage | Analysis of RNA integrity | Applied references for normalization |
|-----|-----------------|---------------------------|--------------------------------|---------------------------|--------------------------------------|
| 21  | Zhou et al., 2020 [21] | Multiple myeloma          | 0                             | Agilent analysis, no further comments | GAPDH, after RNA treatment with RNase R |
| 22  | Kong et al., 2020 [22]    | Prostate cancer           | 0                             | No                         | ACTB                                 |
| 23  | Meng et al., 2020 [23]   | Cervical cancer           | 0                             | No                         | GAPDH                                |
| 24  | Wei et al., 2020 [24]    | Hepatocellular cancer     | 0                             | No                         | GAPDH                                |
| 25  | Yu et al., 2020 [25]     | Bladder cancer            | 0                             | No                         | GAPDH                                |

The studies were reviewed only with regard to the data of tissue collection and storage, RNA integrity, and reference standards for normalization.

Information on tissue collection and storage in the studies was categorized with following specifications: 0=no detailed information regarding collection and storage; 1=fresh frozen; 2=stored at 
-80 °C; 3=collected and stored in liquid nitrogen until use; 4=collected in liquid nitrogen and stored at 
-80 °C until use; 5=collected in RNA storage solution and stored at -80 °C until use.

ACTB: actin beta; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; U6: RNA, U6 small nuclear 1.
2. Clinicopathological characteristics of the study own cohorts in relation to the degraded RNA samples

Supplemental Table S2. Clinicopathological characteristics of patients suffering from clear cell renal cell carcinoma.

| Characteristics                          | Total   | Patient samples with RIN ≤6 | Patient samples with RIN >6 | P value a |
|------------------------------------------|---------|-----------------------------|-----------------------------|-----------|
| Patients, no. (%)                        | 61 (100)| 28 (46)                     | 33 (54)                     |           |
| Sex, female/male; no. (%)                | 20/41 (33/67) | 8/20 (29/71) | 12/21 (36/64) | 0.591     |
| Age, years, median (IQR)                 | 61 (56-69) | 60 (57–66)               | 64 (56–70)               | 0.311     |
| Pathological stage, no. (%)              |         |                            |                            |           |
| pT1a                                     | 8 (13)  | 3 (11)                      | 5 (15)                     | 0.329     |
| pT1b                                     | 14 (23) | 6 (21)                      | 8 (24)                     |           |
| pT2                                      | 5 (8)   | 1 (4)                       | 4 (12)                     |           |
| pT3a                                     | 22 (36) | 13 (46)                     | 9 (27)                     |           |
| pT3b                                     | 8 (13)  | 4 (14)                      | 4 (12)                     |           |
| pT3c                                     | 3 (5)   | 0                           | 3 (9)                      |           |
| unclassified                              | 1 (2)   | 1 (4)                       | 0 (0)                      |           |
| Metastatic status, no. (%)               |         |                            |                            |           |
| negative                                  | 47 (77) | 20 (72)                     | 27 (82)                    | 1.000     |
| positive                                  | 10 (16) | 4 (14)                      | 6 (18)                     |           |
| unclassified                              | 4 (7)   | 4 (14)                      | 0 (0)                      |           |
| TNM stage grouping, no. (%) b            |         |                            |                            |           |
| I                                        | 22 (36) | 9 (32)                      | 13 (39)                    | 0.361     |
| II                                       | 5 (8)   | 1 (4)                       | 4 (12)                     |           |
| III                                      | 24 (39) | 14 (50)                     | 10 (30)                    |           |
| IV                                       | 10 (16) | 4 (4)                       | 6 (18)                     |           |
| Tumor size, mm, median (IQR)             | 65 (49-87) | 75 (60-118)               | 60 (47-81)               | 0.309     |
| Surgical margin, no. (%)                 |         |                            |                            |           |
| negative                                  | 47 (77) | 19 (68)                     | 28 (85)                    | 0.336     |
| positive                                  | 12 (20) | 7 (25)                      | 5 (15)                     |           |
| unclassified                              | 2 (3)   | 2 (7)                       |                            |           |
| Fuhrman grade, no. (%)                   |         |                            |                            |           |
| G1                                       | 4 (7)   | 1 (4)                       | 3 (9)                      | 0.689     |
| G2                                       | 31 (51) | 13 (46)                     | 18 (55)                    |           |
| G3                                       | 17 (28) | 6 (21)                      | 11 (33)                    |           |
| G4                                       | 3 (5)   | 2 (7)                       | 1 (3)                      |           |
| unclassified                              | 6 (9)   | 6 (21)                      | 0 (0)                      |           |

a Calculated with Fisher's exact test, Chi-squared test or Mann-Whitney U test between the two RIN groups without considering unclassified data.

b TNM stage grouping according to UICC classification system.

G: histopathological grading according to Fuhrman; IQR: interquartile range; pT: pathological tumor classification; RIN: RNA integrity number.
**Supplemental Table S3.** Clinicopathological characteristics of patients suffering from prostate cancer.

|                       | Total         | Patient samples with RIN ≤6 | Patient samples with RIN >6 | P value<sup>a</sup> |
|-----------------------|---------------|-----------------------------|-----------------------------|---------------------|
| Patients, no. (%)     | 57 (100)      | 26 (46)                     | 31 (54)                     |                     |
| Age, years, median (IQR) | 67 (62–71)    | 71 (66–73)                  | 65 (61–69)                  | 0.004               |
| PSA, µg/L, median (IQR) | 8.3 (5.95–14.0) | 11.4 (6.7–17.8)            | 7.4 (5.7–12.2)              | 0.157               |
| Prostate volume, cm<sup>3</sup>, median (IQR) | 31 (25–38)    | 32 (28–36)                  | 30 (25–38)                  | 0.539               |
| DRE, no. (%)          |               |                             |                             |                     |
| non-suspicious        | 30 (53)       | 13 (50)                     | 17 (55)                     | 1.000               |
| suspicious            | 16 (28)       | 7 (27)                      | 9 (29)                      |                     |
| unclassified          | 11 (19)       | 6 (23)                      | 5 (16)                      |                     |
| pT status, no. (%)    |               |                             |                             |                     |
| pT2a                  | 1 (2)         | 1 (4)                       | 0 (0)                       | 0.726               |
| pT2c                  | 22 (39)       | 10 (38)                     | 12 (39)                     |                     |
| pT3a                  | 14 (25)       | 6 (23)                      | 8 (26)                      |                     |
| pT3b                  | 19 (33)       | 8 (31)                      | 11 (35)                     |                     |
| unclassified          | 1 (2)         | 1 (4)                       | 0 (0)                       |                     |
| ISUP Grade groups, no. (%) |           |                             |                             |                     |
| 1                     | 8 (14)        | 2 (7.5)                     | 6 (19)                      | 0.150               |
| 2                     | 19 (33)       | 9 (35)                      | 10 (32)                     |                     |
| 3                     | 15 (26)       | 4 (15)                      | 11 (35)                     |                     |
| 4                     | 5 (9)         | 3 (12)                      | 2 (7)                       |                     |
| 5                     | 8 (14)        | 6 (23)                      | 2 (7)                       |                     |
| unclassified          | 2 (4)         | 2 (7.5)                     | 0 (0)                       |                     |
| pN status, no. (%)    |               |                             |                             |                     |
| pN0/Nx                | 51 (89)       | 22 (85)                     | 29 (94)                     | 0.396               |
| pN1                   | 6 (11)        | 4 (15)                      | 2 (6)                       |                     |
| Surgical margin, no. (%) |             |                             |                             |                     |
| negative              | 28 (49)       | 12 (46)                     | 16 (52)                     | 1.000               |
| positive              | 28 (49)       | 13 (50)                     | 15 (48)                     |                     |
| unclassified          | 1 (2)         | 1 (0)                       | 0 (0)                       |                     |

<sup>a</sup>Calculated with Fisher's exact test, Chi-squared test or Mann-Whitney U test between the two RIN groups without considering unclassified data.

DRE: digital rectal examination; IQR: interquartile range; ISUP: histopathological grade system based on Gleason score according to the International Society of Urologic Pathology; pN: lymph node status; PSA: total prostate specific antigen; pT: pathological tumor classification; RIN: RNA integrity number.
3. RT-qPCR methodology

**Supplemental Table S4. MIQE checklist according to Bustin et al. [26].**

| ITEM TO CHECK | IMPORTANCE | CHECK-LIST | WHERE; COMMENT |
|---------------|------------|------------|----------------|
| **EXPERIMENTAL DESIGN** |            |            |                |
| Definition of experimental and control groups | E          | Yes        | Main text: Materials and Methods; Tables S2 and S3 |
| Number within each group | E          | Yes        | Main text: Materials and Methods; Results; Tables S2 and S3 |
| Assay carried out by core lab or investigator's lab? | D          | Yes        | Investigator's lab |
| Acknowledgement of authors' contributions | D          | Yes        | Section Acknowledgements |
| **SAMPLE** |            |            |                |
| Description | E          | Yes        | Main text: Results; Materials and Methods. |
| Volume/mass of sample processed | D          | Yes        | Main text: Results; Materials and Methods |
| Microdissection or macrodissection | E          | Yes        | Main text: Materials and Methods |
| Processing procedure | E          | Yes        | Main text: Materials and Methods |
| If frozen - how and how quickly? | E          | Yes        | Main text: Materials and Methods |
| If fixed - with what, how quickly? | E          | Yes        | Main text: Materials and Methods |
| Sample storage conditions and duration (esp. for FFPE samples) | E          | Yes        | Main text: Materials and Methods |
| **NUCLEIC ACID EXTRACTION** |            |            |                |
| Procedure and/or instrumentation | E          | Yes        | Main text: Materials and Methods |
| Name of kit and details of any modifications | E          | Yes        | Main text: Materials and Methods |
| Source of additional reagents used | D          | N/A        | Not used |
| Details of DNase or RNase treatment | E          | Yes        | Main text: Materials and Methods: RNA extraction, on-column DNase digestion |
| Contamination assessment (DNA or RNA) | E          | Yes        | Main text: Methods and Materials. Supplementary Material: Genomic DNA contamination was excluded by control experiments without reverse transcription of RNA for all target genes |
| Nucleic acid quantification | E          | Yes        | Main text: Materials and Methods, spectrophotometric |
| Instrument and method | E          | Yes        | Main text: Materials and Methods, Nanodrop |
| Purity (A260/A280) | D          | Yes        | Main text: Materials and Methods |
| Yield | D          | Yes        | Main text: Materials and Methods |
| RNA integrity method/instrument | E          | Yes        | Main text: Materials and Methods: RIN; Bioanalyzer 2100, Agilent RNA 6000 Nano Chip Kit |
| RIN/RQI or Cq of 3' and 5' transcripts | E          | Yes        | Main text: Materials and Methods: RIN; Bioanalyzer 2100, Agilent; see Figure S1 |
| Electrophoresis traces | D          | Yes        | see RNA integrity: Agilent electrophoresis |
| Inhibition testing (Cq dilutions, spike or other) | E          | Yes        | Supplementary Material: Cq dilution, see standard curve characteristics in Supplemental Table S10 |
| **REVERSE TRANSCRIPTION** |            |            |                |
| Complete reaction conditions | E          | Yes        | Main text: Materials and Methods; Supplementary Material: RT-qPCR methodology, cDNA synthesis in 3.1.1 |
| Amount of RNA and reaction volume | E          | Yes        | Main text: Materials and Methods; Supplementary Material: RT-qPCR methodology, cDNA synthesis in 3.1.1 |
### qPCR TARGET INFORMATION

|                         | E | Yes | Main text: Materials and Methods; Supplementary Material: RT-qPCR methodology, cDNA synthesis in 3.1.1 |
|-------------------------|---|-----|---------------------------------------------------------------------------------------------|
| Gene symbols            | E | Yes | Main text: Table 1; Supplementary Material: Table S6–S8                                      |
| If multiplex, efficiency and LOD of each assay | E | N/A | Only singleplex qPCR                                                                       |
| Sequence accession number | E | Yes | Main text: Table 1; Supplementary Material: Supplemental Table S6–S9.                         |
| Location of amplicon    | D | Yes | Supplementary Material: Supplemental Tables S6-S7                                            |
| Amplicon length         | E | Yes | Supplementary Material: Supplemental Tables S6-S7; Bioanalyzer 2100 DNA1000 expert series II Chip analysis: Figure S2 |
| In silico specificity screen (BLAST, etc) | E | Yes | Main text: Materials and Methods; Supplementary Material: RT-qPCR methodology. All primers and amplicons were checked by screens in different databases, see URL links in 3.2.1 |
| Pseudogenes, retropseudogenes or other homologs? | D | N/A |                                                                                              |
| Sequence alignment      | D | Yes | Supplementary Material: RT-qPCR methodology; see URL links as mentioned above in 3.2.1. Using NCBI-based Megablast against standard Nucleotide collection databases (nr/nt) and RefSeq, filtered Homo sapiens (taxid.9606). Analyses using databases circBase and CircInteractome |
| Secondary structure analysis of amplicon | D | No |                                                                                              |
| Location of each primer by exon or intron (if applicable) | E | Yes | Supplementary Material: Supplemental Tables S6-S7. Analysis of different databases: Ensembl NCBI nucleotide, circBASE and CircInteractome |
| What splice variants are targeted? | E | N/A |                                                                                              |

### qPCR OLIGONUCLEOTIDES

|                         | E | Yes | Supplementary Material: RT-qPCR methodology with Supplemental Tables S8                      |
|-------------------------|---|-----|---------------------------------------------------------------------------------------------|
| Primer sequences        | E | Yes |                                                                                              |
| RTPrimerDB Identification Number | D | N/A |                                                                                              |
| Probe sequences         | D | Yes | Supplementary Material: RT-qPCR methodology; UPL probes only for ALAS1 and HPRT1 in 3.2.1.  |
| Location and identity of any modifications | E | N/A | No modifications                                                                           |
| Manufacturer of oligonucleotides | D | Yes | TIB MolBiol (Berlin, Germany); Applied Biosystems; Probes from Roche                        |
| Purification method     | D | Yes | TIB MolBiol: GSF purification                                                               |
### qPCR PROTOCOL

| Parameter                                           | E/D   | Yes/No | Description                                                                 |
|-----------------------------------------------------|-------|--------|-----------------------------------------------------------------------------|
| Complete reaction conditions                        | E     | Yes    | Main text: Materials and Methods. Supplementary Material: RT- qPCR methodology |
| Reaction volume and amount of cDNA/DNA              | E     | Yes    | Main text: Materials and Methods. Supplementary Material: RT- qPCR methodology |
| Primer, (probe), Mg++ and dNTP concentrations        | E     | Yes    | Main text: Materials and Methods. Supplementary Material: RT- qPCR methodology |
| Polymerase identity and concentration               | E     | Yes    | Main text: Materials and Methods. Supplementary Material: RT- qPCR methodology |
| Buffer/kit identity and manufacturer                | E     | Yes    | Main text: Materials and Methods. Supplementary Material: RT- qPCR methodology |
| Exact chemical constitution of the buffer           | D     | No     | The manufacturer does not provide this information                         |
| Additives (SYBR Green I, DMSO, etc.)                | E     | Yes    | Main text: Materials and Methods. Supplementary Material: RT- qPCR methodology, SYBR Green in ready-to-use soft master |
| Manufacturer of plates/tubes and catalogue number   | D     | Yes    | Supplementary Material: RT- qPCR methodology in 3.1.1 and 3.2.1              |
| Complete thermocycling parameters                   | E     | Yes    | Main text: Materials and Methods. Supplementary Material: RT- qPCR methodology for all runs in 3.2.1 |
| Reaction setup (manual/robotic)                     | D     | Yes    | Manual setup                                                                |
| Manufacturer of qPCR instrument                     | E     | Yes    | Main text: Materials and Methods: LightCycler 480 (Roche)                    |

### qPCR VALIDATION

| Parameter                                           | E/D   | Yes/No | Description                                                                 |
|-----------------------------------------------------|-------|--------|-----------------------------------------------------------------------------|
| Evidence of optimisation                            | D     | Yes    | Supplementary Material: RT- qPCR methodology: all run conditions of qPCRs were optimized, for circEGLN3, linEGLN3, circRHOBTB3, and linRHOBTB3 see also ref. [27]; for reference genes [28, 29] with primers as indicated in Table S8; for circCSNK1G3 see Supplemental Figure S2 according to Chen et al. [30]. |
| Specificity (gel, sequence, melt, or digest)        | E     | Yes    | Supplementary Material: RT- qPCR methodology with Supplemental Figure S2 for circEGLN3, circRHOBTB3, and circCSNK1G3 (Agilent electropherogram, melting curves on LightCycler); other circRNAs also in ref. [27]) |
| For SYBR Green I, Cq of the NTC                      | E     | Yes    | Main text: Materials and Methods; Supplementary Material: RT- qPCR methodology, no Cqs <40 in reaction without RT |
| Standard curves with slope and y-intercept          | E     | Yes    | Supplementary Material: RT- qPCR methodology; 3.3. Performance data, Table S10 |
| PCR efficiency calculated from slope                | E     | Yes    | Supplementary Material: RT- qPCR methodology; 3.3. Performance data, Table S10 |
| Confidence interval for PCR efficiency or standard error | D     | Yes    | Supplementary Material: RT- qPCR methodology; 3.3. Performance data, Table S10 |
| r² of standard curve                                | E     | N/A    | Not provided by the LC480 software                                          |
| Linear dynamic range                                | E     | Yes    | Supplementary Material: RT- qPCR methodology; 3.3. Performance data, Table S10 with endpoints of standard curves |
| Cq variation at lower limit                          | E     | Yes    | Supplementary Material: RT- qPCR methodology; Supplemental Table S10 with Cq range of the measured samples, only two samples (see Table S10) with outside of the dynamic range |
| **Confidence intervals throughout range** | D | N/A |
|-----------------------------------------|---|-----|
| **Evidence for limit of detection**     | E | Yes |
| **If multiplex, efficiency and LOD of each assay.** | E | N/A |

### DATA ANALYSIS

| **qPCR analysis program (source, version)** | E | Yes |
|--------------------------------------------|---|-----|
| **Cq method determination**                | E | Yes |
| **Outer identification and disposition**   | E | N/A |
| **Results of NTCs**                        | E | Yes |
| **Justification of number and choice of reference genes** | E | Yes |
| **Description of normalisation method**    | E | Yes |
| **Number and concordance of biological replicates** | D | Yes |
| **Number and stage (RT or qPCR) of technical replicates** | E | Yes |
| **Repeatability (intra-assay variation)**  | E | Yes |
| **Reproducibility (inter-assay variation, %CV)** | D | Yes |
| **Power analysis**                         | D | Yes |
| **Statistical methods for result significance** | E | Yes |
| **Software (source, version)**             | E | Yes |
| **Cq or raw data submission using RDML**   | D | No |

E: essential information; D: desirable information if available; N/A: not applicable.
Supplementary Figure S1. Bioanalyzer 2100 results of RNA samples after heat incubation at 80 °C. The time-dependent RIN decay of samples is shown in Figure 1 of the main text.
3.1. cDNA synthesis

3.1.1. cDNA synthesis of circRNAs and mRNAs

Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, MA, USA; Cat.No. K1642) was used in final reaction volume of 20 µL according to the following protocol:

Supplemental Table S5A. cDNA synthesis using the Maxima First Strand cDNA Synthesis Kit.

| Volume (µL) | Reagent/Sample | Components |
|-------------|----------------|------------|
| 4           | 5X Reaction Mix | Reaction buffer, dNTPs, oligo(dT)<sub>18</sub> and random hexamer primers without specified concentration |
| 2           | Maxima Enzyme Mix | Maxima Reverse Transcriptase (M-MuLV RT) and Thermo Scientific™ RiboLock™ RNase Inhibitor |
| 2           | Total RNA (500 ng) | Diluted RNA (gDNA free); see RNA isolation |
| 12          | Water, nuclease free | |

The RT reaction was carried out in 0.2 mL PCR Soft Tubes (Biozym Scientific GmbH, Germany; Article No. 711080) in a thermal block cycler (Biometra GmbH, Göttingen, Germany) as follows: 10 min at 25 °C, followed by 15 min at 50 °C, and terminated by heating at 85 °C for 5 min; end 4 °C. All cDNA samples were stored at -20 °C until qPCR analysis (see the following Section 3.2.1).

As explained in the Main text: Materials and Methods, cDNA synthesis, we used the Transcriptor First Strand cDNA Synthesis Kit (Life Science Roche, Mannheim, Germany; Cat. No. 04379012001) for the cDNA synthesis of circRNAs for the comparative priming with random hexamer and oligo(dT)<sub>18</sub> primers according to the following protocol:

Supplemental Table S5B. cDNA synthesis using Transcriptor First Strand cDNA Synthesis Kit.

| Volume (µL) | Reagent/Sample | Components and final (1x) concentration |
|-------------|----------------|---------------------------------------|
| 2           | Total RNA (500 ng) | 1 µg |
| 2 or 10     | Random Hexamer Primer | 60 µM or: 2.5 µM |
|             | or: Anchored-oligo(dT)<sub>18</sub> Primer | |
| 4           | Transcriptor Reverse Transcriptase | 50 mM Tris/HCl, 30 mM KCl, 8 mM MgCl₂ |
| 0.5         | Protector RNase Inhibitor | 20 U |
| 2           | Deoxynucleotide Mix | 1 mM each |
| 0.5         | Transcriptor Reverse Transcriptase | 10 U |

The RT reaction conditions were primer dependent. Using random hexamer primers: 10 min at 25 °C, followed by 30 min at 55 °C and inactivation for 5 min at 85 °C; end 4 °C. Using anchored-oligo(dT)<sub>18</sub> primers, the initial incubation step was omitted, the other temperature steps were identical. Both cDNA samples were stored at -20 °C until qPCR analysis (see the following Section 3.2.1).

3.1.2. cDNA synthesis of microRNAs

The TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) was used according to the manufacturer’s instructions. Using a Biometra thermal block cycler as mentioned above, the cDNA synthesis was performed in a reaction mixture containing 10 ng of total RNA, 15 nmol of dNTP mix, 50 U AB Multiscribe Reverse Transcriptase, 1x microRNA specific stem-looped RT-primer (AB), 3.75 U AB RNase Inhibitor, and 1x RT buffer. The steps were the following: priming at 16 °C for 30 min, transcription at 42 °C for 30 min, and enzyme inactivation at 85 °C for 5 min. All cDNA samples were stored at -20 °C until qPCR analysis.

3.2. qPCR measurements

3.2.1. Quantification of circRNAs and mRNAs

All real-time qPCR runs were performed on the LightCycler 480 Instrument (Roche Molecular Systems, Mannheim, Germany) in white 96-well plates (Cat.No. 04729692001) using at least technical
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duplicates and resulting mean values were used for further calculations. Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific; Cat.No. K0252) was used. The determination of circRNAs is based on the measurement of their specific backsplice junctions using divergent primers [31]. The backsplice junction sequences of the three circRNAs measured in this study and the amplicon characteristics are listed in Supplemental Table S6; information regarding the linear counterparts of the circRNAs and the normalizers are given in Supplemental Table S7.

All primers of circRNAs (divergent) and mRNAs (convergent) were designed using the Primer3 tool (http://bioinfo.ut.ee/primer3/) or with Roche/UPL ProbeFinder web-based software (https://lifescience.roche.com/en_de/brands/universal-probe-library.html#assay-design-center) and are compiled in Supplemental Table S8 [32]. Primers were synthesized by TIB Molbiol GmbH (Berlin, Germany). Expression of mRNAs of peptidylprolyl isomerase (PPIA), TATA-box binding protein (TBP), 5'-aminolevulinate synthase 1 (ALAS1), and hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used for normalization as these genes are generally used as normalizers in expression studies of clear cell renal cell carcinoma and prostate cancer [28, 29]. PPIA was quantified by QuantiTect Primer assay from Qiagen (Supplemental Table S7) in SYBR Green assay format, ALAS1 and HPRT1 were measured with Universal ProbeLibrary-probes (ALAS1 Probe #40 Prod. No: 0468799001; HPRT1 Probe #22 Prod. No: 04686969001) and LightCycler 480 Probes Master from Roche (Prod. No. 04707494001) in hydrolysis probe assay format on the LightCycler 480 (Supplemental Table S8). Quantitative PCR data analysis was done using qbase` software, version 3.2 (Biogazelle, Zwijnaarde, Belgium; www.qbaseplus.com).

In general, genes and primer sequences were checked using the following database links: https://circinteractome.nia.nih.gov/; http://www.circbase.org/; http://www.ensembl.org (Ensembl release 99 - January 2020); https://www.ncbi.nlm.nih.gov/nucleotide; https://blast.ncbi.nlm.nih.gov/Blast.cgi; https://www.ncbi.nlm.nih.gov/tools/primer-blast/.
Supplemental Table S6. List of backsplice junctions of circRNAs and primers used for RT-qPCR detection of circEGLN3, circRHOBTB3, and circCSNK1G3.

| circRNA (circBase ID)          | Backsplice junction sequence of amplicon                       | Divergent primers               | Amplicon location (exon no.) | Amplicon length (nt) |
|--------------------------------|-----------------------------------------------------------------|---------------------------------|------------------------------|----------------------|
| circEGLN3 (hsa_circ_0101692)   | TCCCTGCAGACATCCTAC TCAGCCAGCAGTTAACGTG AGTATCCGGAATGCTG         | Forward: TCCTGCAG               | Ex5(F)/Ex2(R)              | 126 (129)            |
|                                | TGCTTCAGCTATCCGGAGAAAAGTTA                                      | ACATCTCTA CTCCGCTG              |                             |                      |
|                                | TGGAAATGAGTTATGTTGCGCCAGTCA                                       | Reverse: GATGCAG                |                             |                      |
|                                | GGTAGATGGTCGCTGCA                                                | CGACCACCAGCTACATTT              |                             |                      |
|                                | TC                                                              | GGAATACAGATTCTTAA               |                             |                      |
|                                | GCAATTCGAGATCGTTGAC                                              | GCAGTTAGGATCTGGAGC              |                             |                      |
|                                | TCTCTATCAATATCAGCT                                               |CACATCATGAAAAGATA               |                             |                      |
|                                | TTTGAAAGACATGTTTGTG                                              | GCTGAAAAGACACTAAG               |                             |                      |
|                                | AAAATTTTACGAATGGGCCTCC                                            |                                 |                             |                      |
|                                | Ex3(F)/Ex1(R)                                                    |                                 |                             |                      |
|                                | Numbers in brackets indicate the lengths of amplicons detected with Agilent Bioanalyzer 2100. |
**Supplemental Table S7.** QPCR target information of the linear counterparts of the circRNAs and the reference genes for normalization.

| RNA name in the manuscript | Official gene symbol of the host gene and its full name | NCBI Ref.Seq Accession nos. | Primer location (exon number in RefSeq) | Intron spanning size (nt) | Amplicon size (nt)* |
|---------------------------|----------------------------------------------------------|-----------------------------|-----------------------------------------|--------------------------|---------------------|
| linEGLN3                  | EGLN3, egl-9 family hypoxia inducible factor 3           | NM_022073.4                 | Ex4(F)/Ex5(R)                           | 1037                     | 88 (92)             |
| linRHOBTB3                | RHOBTB3, Rho related BTB domain containing 3             | NM_014899.4                 | Ex5(F)/Ex6(R)                           | 3045                     | 94 (96)             |
| linCSNK1G3                | CSNK1G3, casein kinase 1 gamma 3                        | NM_0010447 23.2             | Ex6(F)/Ex8(R)                           | 273/1901                 | 142 (144)           |
| PPIA                      | PPIA, peptidylprolyl isomerase                           | NM_021130.5                 | QuantiTect Primer Assay (Cat.No.: QT-00052311) in Ex 4/5 | 1412                     | 121 (123)           |
| TBP                       | TBP, TATA-box binding protein                           | NM_003194.5                 | Ex3(F)/Ex5+6 (R)                        | 2311/2285/2602           | 227 (226)           |
| ALAS1                     | ALAS1, 5'-aminolevulinate synthase                       | NM_000688.6                 | Ex4(F)/Ex5(R)                           | 1128                     | 77 (77)             |
| HPRT1                     | HPRT1, hypoxanthine phosphoribosyltransferase 1          | NM_000194.3                 | Ex3(F)/Ex4+5 (R)                        | 11100/3657               | 126 (119)           |

*Numbers in brackets indicate the lengths of amplicons detected with Agilent Bioanalyzer 2100.
Supplemental Table S8. List of primers.

| circRNA (circBase ID) | Divergent Primers | Primer sequences (5’… 3’) |
|-----------------------|------------------|-------------------------|
| circEGLN3 (hsa_circ_0101692) | circEGLN3-F<sup>a</sup> | TCCTGAGACATCCTACTCG |
| circEGLN3 (hsa_circ_0101692) | circEGLN3-R<sup>a</sup> | GATGCAAGCGACCACATCACC |
| circRHOBTB3 (hsa_circ_0007444) | circRHOBTB3-F<sup>a</sup> | TTCTGGGATGTCTAAAATG |
| circRHOBTB3 (hsa_circ_0007444) | circRHOBTB3-R<sup>a</sup> | ACACACTGGCAGCAGACAG |
| circCSNK1G3 (hsa_circ_0001522) | circCSNK1G3-F<sup>b</sup> | GCACCACAGCTACATTTGGA |
| circCSNK1G3 (hsa_circ_0001522) | circCSNK1G3-R<sup>b</sup> | GGAGCATGTTCCATCCATTC |
| circRNA4 (hsa_circ_0001900) | circRNA4-F<sup>c</sup> | TGTGCTCCTGTCTACACTGGTCAA |
| circRNA4 (hsa_circ_0001900) | circRNA4-R<sup>c</sup> | TCAGTGCCTCAGAGAACTTCCGT |
| circRNA9 (hsa_circ_0001423) | circRNA9-F<sup>c</sup> | GCTCTCAAAAAGGGGAATC |
| circRNA9 (hsa_circ_0001423) | circRNA9-R<sup>c</sup> | CCCCTGAACCTGAAACCACCTG |

| Transcript.version (NCBI mRNA-RefSequence) | Convergent Primers | Primer sequences (5’… 3’) |
|---------------------------------------------|------------------|-------------------------|
| NM_022073.4 linEGLN3-F<sup>a</sup> | CTGTGCGGTTATGCGTGA |
| NM_022073.4 linEGLN3-R<sup>a</sup> | TCAGTGAAGCGAGACATTTG |
| NM_014899.4 linRHOBTB3-F<sup>a</sup> | CCACCTCAATGGAAACCA |
| NM_014899.4 linRHOBTB3-R<sup>a</sup> | GGCAGCAGAACAGAAGTTA |
| NM_001044723.2 linCSNK1G3-F<sup>b</sup> | TGAGAGGGCATCTCTCTTTG |
| NM_001044723.2 linCSNK1G3-R<sup>b</sup> | ACATAACGAAGATATGGCAT |
| NM_021130.5 PPIA-F+R mix QuantiTect Primer Assay (QT00052311), Qiagen | | |
| NM_003194.5 TBP-F<sup>d</sup> | TTCGGGAGGATGCTGCTG |
| NM_003194.5 TBP-R<sup>d</sup> | TGGAGTAGCTGCTCTG |
| NM_000688.6 ALAS1-F<sup>e</sup> | GAATATGACAGG |
| NM_000688.6 ALAS1-R<sup>e</sup> | CCTCCATCGGTTTCACACT |
| NM_000194.3 HPRT1-F<sup>e</sup> | TGATAGATCCACTTGTAGTGA |
| NM_000194.3 HPRT1-R<sup>e</sup> | AAGACATCTTCCAGGAAAGTTG |

<sup>a</sup> According to Franz et al. [27].
<sup>b</sup> According to Chen et. [30].
<sup>c</sup> According to Memczak et al. [33].
<sup>d</sup> According to Jung et al. [28].
<sup>e</sup> According to Ohl et al. [29] with changed HPRT1-R primer sequence at the 3’- and 5’-ends.
Protocols for all LightCycler runs in SYBR Green and Probe assay format

qPCR reaction mix for all SYBR Green assays

| Volume (µL) | Reagent/Sample | Components |
|-------------|----------------|------------|
| 5           | Maxima SYBR Green qPCR Master Mix (2X) | Maxima Hot Start Taq DNA Polymerase, dNTPs (also dUTP) and SYBR Green I in an optimized PCR buffer |
| 2           | Primer Mix<sup>a</sup> | Forward and reverse primer mix, final concentration each 0.250 µM |
| 1           | cDNA<sup>b</sup> | Un- or prediluted |
| 2           | Water, nuclease free | |
| **Total volume 10 µL** | | |

<sup>a</sup> For PPIA qPCR, F+R primer mix included in QuantiTect Primer assay (Qiagen) was used; all other qPCR primers were synthesized by TIB Molbiol.

<sup>b</sup> Undiluted cDNA input for qPCR of circCSNK1G3 and linCSNK1G3 and 1:10 prediluted cDNA input for qPCR of circ- and linEGLN3, circ- and linRHOBTB3, PPIA and TBP.

LightCycler 480 SYBR Green assay run templates

| Setup | Reaction volume (µL) |
|-------|---------------------|
| Block type | 96 |
| Detection format | 10 |
| SYBR Green | 483 nm |
| Emission filter | 533 nm |

| Programs | Cycles | Analysis mode |
|----------|--------|---------------|
| Pre-incubation | 1 | None |
| Amplification | 45 | Quantification |
| Melting curve | 1 | Melting curve |
| Cooling | 1 | None |

The setup and programs are equal for all assays in SYBR Green detection format.

LightCycler 480 SYBR Green assay run template for circEGLN3

| Target (°C) | Acquisition mode | Hold time (s) | Ramp rate (°C/s) | Acquisitions (per °C) |
|-------------|-----------------|---------------|-----------------|----------------------|
| Pre-incubation | 95 | None | 600 | 4.4 | - |
| Amplification | 95 | None | 15 | 4.4 | - |
| | 60 | None | 30 | 2.2 | - |
| | 79 | Single | 2 | 4.4 | - |
| Melting curve | 95 | None | 5 | 4.4 | - |
| | 65 | None | 60 | 2.2 | - |
| | 95 | Continuous | - | 0.11 | 5 |
| Cooling | 40 | None | 30 | 1.5 | - |

LightCycler 480 SYBR Green assay run template for linEGLN3, linCSNK1G3, lin- and circRHOBTB3 qPCR

| Target (°C) | Acquisition mode | Hold time (s) | Ramp rate (°C/s) | Acquisitions (per °C) |
|-------------|-----------------|---------------|-----------------|----------------------|
| Pre-incubation | 95 | None | 600 | 4.4 | - |
| Amplification | 95 | None | 15 | 4.4 | - |
| | 60 | None | 15 | 2.2 | - |
| | 70 | Single | 15 | 4.4 | - |
| Melting curve | 95 | None | 5 | 4.4 | - |
| | 65 | None | 60 | 2.2 | - |
| | 95 | Continuous | - | 0.11 | 5 |
| Cooling | 40 | None | 30 | 1.5 | - |
### LightCycler 480 SYBR Green assay run template for circCSNK1G3

| Temperature targets | Target (°C) | Acquisition mode | Hold time (s) | Ramp rate (°C/s) | Acquisitions (per °C) |
|---------------------|-------------|------------------|---------------|-----------------|----------------------|
| Pre-incubation      | 95          | None             | 600           | 4.4             | -                    |
| Amplification       | 95          | None             | 10            | 4.4             | -                    |
|                     | 60          | None             | 30            | 2.2             | -                    |
|                     | 72          | Single           | 2             | 4.4             | -                    |
| Melting curve       | 95          | None             | 5             | 4.4             | -                    |
|                     | 65          | None             | 60            | 2.2             | -                    |
|                     | 95          | Continuous       | -             | 0.11            | 5                    |
| Cooling             | 40          | None             | 30            | 1.5             | -                    |

### LightCycler 480 SYBR Green assay run template for reference genes PPIA and TBP

| Temperature targets | Target (°C) | Acquisition mode | Hold time (s) | Ramp rate (°C/s) | Acquisitions (per °C) |
|---------------------|-------------|------------------|---------------|-----------------|----------------------|
| Pre-incubation      | 95          | None             | 900           | 4.4             | -                    |
| Amplification       | 95          | None             | 15            | 4.4             | -                    |
|                     | 58          | None             | 20            | 1.0             | -                    |
|                     | 72          | None             | 20            | 4.4             | -                    |
|                     | 79          | Single           | 2             | 4.4             | -                    |
| Melting curve       | 92          | None             | 5             | 4.4             | -                    |
|                     | 65          | None             | 60            | 2.2             | -                    |
|                     | 95          | Continuous       | -             | 0.11            | 5                    |
| Cooling             | 40          | None             | 30            | 1.5             | -                    |

### qPCR reaction mix for Probe assays for the reference genes ALAS1 and HPRT1

| Volume (µL) | Reagent/Sample | Components |
|-------------|---------------|------------|
| 5           | 2x LightCycler 480 Probes Master | Mix containing FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), and 6.4 mM MgCl₂ |
| 1           | Primer Mix    | Forward and reverse primer mix, final concentration each 0.250 µM |
| 1           | Probe         | Roche UPL Probe #40 for ALAS1 or #22 for HPRT1, final concentration 0.2 µM |
| 1           | cDNA          | Undiluted |
| 2           | Water, nuclease free | |

**Total volume 10 µL**

### LightCycler 480 Probe assay run template for the reference genes ALAS1 and HPRT1

| Setup | Reaction volume (µL) |
|-------|----------------------|
|       | 10                   |

| Detection format | Excitation filter | Emission filter |
|------------------|-------------------|-----------------|
| Mono Color Hydrolysis Probe | 483 nm | 533 nm |

| Programs | |
|----------|---|
| Program names | Cycles | Analysis mode |
| Pre-incubation | 1 | None |
| Amplification | 45 | Quantification |
| Cooling | 1 | None |

| Temperature targets | Target (°C) | Acquisition mode | Hold time (s) | Ramp rate (°C/s) | Acquisitions (per °C) |
|---------------------|-------------|------------------|---------------|-----------------|----------------------|
| Pre-incubation      | 95          | None             | 600           | 4.4             | -                    |
| Amplification       | 95          | None             | 10            | 4.4             | -                    |
|                     | 60          | None             | 30            | 2.2             | -                    |
|                     | 72          | Single           | 1             | 4.4             | -                    |
| Cooling             | 40          | None             | 30            | 1.5             | -                    |
3.2.2. Quantification of miRNAs
TaqMan MiRNA Assays (Applied Biosystems) were used for the detection of mature miRNAs let-7a-5p, miR-17-5p, and miR-210-3p (Supplemental Table 9). Technical details corresponded with analysis parameters given in our previous reports [34-37].

Supplemental Table S9. TaqMan MicroRNA Assays (Applied Biosystems; Assay name, Assay ID) for the measurement of mature miRNAs characterized by the miRBase accession number, the miRBase IDs, and the sequences in reference to the miRBase 22 version.

| Assay name | Assay ID | miRBase accession no. | miRBase ID | Sequence                  |
|------------|----------|------------------------|------------|---------------------------|
| hsa-let-7a | 000377   | MIMAT0000062           | hsa-let-7a-5p | UGAGGUAGUAGGUUGUAUAGUU   |
| hsa-miR-17 | 002308   | MIMAT0000070           | hsa-miR-17-5p | CAAAGUGCUUACAGUGCAGGUAG   |
| hsa-miR-210 | 000512  | MIMAT0000267           | hsa-miR-210-3p | CUGUGCGUGUGACAGCGGCUGA   |

The qPCR reaction mixture of 10 µL contained: 1 µL miRNA-specific cDNA, 5 µL TaqMan 2x Universal PCR Master Mix No AmpErase UNG, 0.5 µL gene-specific TaqMan MicroRNA real-time PCR-Assay solution (20x), and 3.5 µL nuclease-free water. Following cycling conditions were set: initial activation of Taq polymerase at 95 °C for 10 min, amplification steps: denaturation at 95 °C, 15 s, annealing/elongation at 60 °C for 1 min with fluorescence acquisition, and final cooling step at 40 °C for 1 min. All non-template controls were negative.

3.3. Performance data of the assays
Analytical specificity and characteristics of circEGLN3, circRHOBTB3, their linear counterparts, the reference genes, and the three miRNAs were already reported in our previous publication of circRNAs in ccRCC (Sanger sequencing, melting-point analysis, PCR-product specificity) or in other preceding publications [27-29, 35]. For the PCa-specific circRNA circCSNK1G3, analytical specificity data are given in the article by Chen et al. [30]. The analytical specificity of circCSNK1G3 was confirmed by the usual tests including Sanger sequencing. In Figure S2, its melting-point analysis and PCR-product specificity is shown together with those of circEGLN3 and circRHOBTB3. In Figure S3, the decreased cDNA synthesis of the circRNAs in the clinical samples with oligo(dT) primers is shown in comparison to the cDNA synthesis with random hexamer primers, indicating that the circRNAs have no poly(A) tail. For all assays used in this study, analytical PCR characteristics are presented in Table S10 and data of repeatability and reproducibility in Table S11.
Supplementary Figure S2. Amplicon analyses of circCSNK1G3, circEGLN3, and circRHOBTB3 with Agilent Bioanalyzer 2100 (gel view and electropherogram) and with Roche LightCycler 480 Instrument (melting curve analysis). For qPCR of circCSNK1G3, published primers from Chen et al. [30] were used.
Supplemental Figure S3. Random vs. oligo(dT) primers for cDNA synthesis of circEGLN3, circCSNK1G3, and circRHOBTB3. The results of qPCR measurements showed that the relative expression was markedly decreased in all circRNAs (at least n = 3 of tissue pools) when using oligo(dT) primers in comparison to random hexamer primers, indicating that the circRNAs lack a poly(A) tail.
**Supplemental Table S10.** Characteristics of the qPCR standard curves. Standard curves were generated either from diluted cDNAs or from diluted amplicons. Cq values were calculated by the LightCycler480 Software Version 1.5.1.62 using the "second derivative maximum" method. The efficiency, the slope, intercept, and error of the standard curve as well as the so-called dynamic range resulted from LightCycler480 software.

| Gene       | PCR efficiency | Slope | y-Intercept | Error | Dynamic range | Cq range of samples |
|------------|----------------|-------|-------------|-------|---------------|---------------------|
| circEGLN3  | 1.975          | -3.385| 19.75       | 0.0174| 19.73-35.24   | 23.12 – 36.51       |
| circRHOBTB3c | 2.190          | -2.938| 20.75       | 0.0466| 20.96 - 32.76 | 23.05 - 31.13       |
| circCSNK1G3 | 1.867          | -3.689| 21.39       | 0.0258| 20.61 - 32.64 | 19.73 - 25.57       |
| linEGLN3c  | 1.922          | -3.523| 14.39       | 0.0112| 14.40 - 34.90 | 17.82-31.88         |
| linRHOBTB3  | 1.929          | -3.504| 13.50       | 0.0134| 13.48-32.27   | 19.60 - 29.26       |
| linCSNK1G3  | 1.958          | -3.426| 20.57       | 0.0204| 20.68-34.02   | 20.30 – 24.55       |
| ALAS1      | 1.920          | -3.529| 18.89       | 0.0118| 18.89-34.18   | 21.08 - 25.50       |
| HPRT1      | 1.863          | -3.702| 8.881       | 0.0100| 9.01-31.34    | 23.82 - 28.42       |
| PPIA       | 1.928          | -3.509| 16.13       | 0.0472| 16.08-33.45   | 17.48 - 22.18       |
| TBPc       | 1.840          | -3.777| 22.51       | 0.000786 | 22.20 - 32.20 | 22.02 - 28.40       |
| let-7a-5p  | 1.864          | -3.698| 22.74       | 0.00602 | 21.60 - 33.76 | 21.53 - 26.19       |
| miR-17-5p  | 1.871          | -3.676| 12.35       | 0.00698 | 12.29 - 34.96 | 22.81 - 25.87       |
| miR-210-3p | 1.968          | -3.400| 22.97       | 0.0647 | 21.92 - 38.50 | 24.27 - 24.78       |

a PCR efficiency is calculated by the LightCycler480 software using the equation: Efficiency=$10^{-\text{slope}}$.
b The error value is the mean squared error of the single data points fit to the regression line according to the LightCycler 480 operator’s manual.
c Dynamic range represents the range of Cq values between the highest and the lowest Cq value of the generated standard curve.
d Cq range of the measured samples represents the lowest and highest Cq value measured in all samples of the degradation experiments and in the 118 clinical samples.
e The qPCR standard curves of circRHOBTB3, linEGLN3, and TBP were the same as previously reported [27].
### Supplemental Table S11. Repeatability and reproducibility of RT-qPCR measurements.

| RNA variables | Repeatability* | Reproducibilityb |
|---------------|----------------|-----------------|
|               | Cq values Mean (%RSD) | Relative quantities Mean (%RSD) | Cq values Mean ± SD (%RSD) | Relative quantities Mean ± SD (%RSD) |
| circEGLN3     | 20 26.80 (0.44) | 1.879 (8.83) | 6 30.56 ± 0.21 (0.68) | 0.957 ± 0.092 (9.61) |
| circRHOBTB3   | 20 24.90 (0.37) | 1.420 (6.35) | 4 24.73 ± 0.14 (0.56) | 1.018 ± 0.098 (9.63) |
| circCSNK1G3   | 20 22.12 (0.41) | 1.321 (6.56) | 5 21.53 ± 0.17 (0.89) | 1.291 ± 0.160 (12.4) |
| linEGLN3      | 20 22.83 (0.28) | 2.995 (6.16) | 6 23.83 ± 0.17 (0.71) | 1.006 ± 0.123 (12.2) |
| linRHOBTB3    | 20 21.08 (0.38) | 1.187 (5.61) | 5 20.51 ± 0.20 (0.97) | 1.438 ± 0.198 (13.8) |
| linCSNK1G3    | 20 22.17 (0.36) | 1.266 (5.31) | 5 21.04 ± 0.17 (0.82) | 1.979 ± 0.241 (12.2) |
| ALAS1         | 20 23.66 (0.26) | 1.154 (4.19) | 4 22.74 ± 0.11 (0.50) | 1.675 ± 0.147 (8.78) |
| HPRT1         | 20 25.88 (0.41) | 1.441 (7.30) | 4 23.86 ± 0.12 (0.50) | 3.404 ± 0.284 (8.34) |
| PPIA          | 20 19.36 (0.34) | 1.062 (4.78) | 7 19.16 ± 0.11 (0.57) | 1.003 ± 0.113 (7.88) |
| TBP           | 20 27.27 (0.39) | 1.360 (7.53) | 7 24.92 ± 0.16 (0.64) | 1.005 ± 0.113 (11.2) |
| let-7a-5p     | 20 23.22 (0.49) | 1.177 (7.92) |               |                         |
| miR-17-5p     | 15 24.31 (0.26) | 1.384 (4.54) |               |                         |
| miR-210-3p    | 20 23.87 (0.27) | 1.361 (4.56) |               |                         |

*a Using the root mean square method, %RSD values were calculated from duplicate measurements of the Cq values and relative quantities, respectively. Relative quantities were calculated using the 2^{-∆∆Cq} approach with qbase+ software.

b Interassay controls; %RSD of Cq values corresponds to the percent relative standard deviation of the interassay controls. %RSD of relative quantities corresponds to the percent relative standard deviation calculated on basis of relative quantities using the 2^{-∆∆Cq} approach.

Cq: quantitation cycle; %RSD: percent relative standard deviation; SD: standard deviation.
4. Statistical data regarding the relationship of expression results to RIN values

Table S12. *P*-values of the expression data calculated as relative quantities and normalized quantities in cancer samples classified by the RIN limits 7 or 6. Values for the groups of RIN 6 are indicated in Figure 4.

| Renal cell carcinoma |  |  |
|----------------------|------------------|------------------|
| **RNA**              | **Samples with RIN ≤7 and >7 (n=39/n=22)** | **Samples with RIN ≤6> and >6 (n=28/n=33)** |
| **P-values between the two RIN groups within the quantifications** | **P-values between the two RIN groups within the quantifications** |
|                      | Relative quantities | Normalized quantities | Relative quantities | Normalized quantities |
| circEGLNN3           | <0.0001            | 0.006             | <0.0001            | <0.0001             |
| linEGLN3             | <0.0001            | 0.0272            | <0.0001            | <0.0004             |
| circRHOBTB3          | 0.0003             | 0.3595            | <0.0001            | 0.2122             |
| linRHOBTB3           | 0.0252             | 0.5434            | 0.0052             | 0.7473             |

| Prostate cancer      |  |  |
|----------------------|------------------|------------------|
| **RNA**              | **Samples with RIN ≤7 and >7 (n=38/n=19)** | **Samples with RIN ≤6> and >6 RIN limit ≤6> (n=26/n=31)** |
| **P-values between the RIN groups within the quantifications** | **P-values between the RIN groups within the quantifications** |
|                      | Relative quantities | Normalized quantities | Relative quantities | Normalized quantities |
| circCSNK1G3          | 0.0104             | 0.8208            | 0.0021             | 0.6390             |
| linCSNK1G3           | <0.0001            | 0.1384            | <0.0001            | 0.3411             |
| circRHOBTB3          | <0.0001            | 0.2611            | <0.0001            | 0.0976             |
| linRHOBTB3           | <0.0001            | 0.1274            | <0.0001            | 0.1173             |
Table S13. Regression line analysis of circRNAs and their linear counterparts calculated as relative quantities (RQ) and normalized relative quantities (NRQs) in relation to the RIN values of total RNA samples from kidney cancer in Figure 5A.

| Quantification of RNA variable (RQ, NRQ) | Regression line equation | 95% CI of slopes | Is slope significantly non-zero? (P-value) | Are the slopes equal? (P-value) |
|----------------------------------------|--------------------------|------------------|------------------------------------------|-------------------------------|
| circEGLN3 RQ (2.3-9.4)                 | Y = 0.9455*X - 2.188    | 0.5431 to 1.348  | <0.0001                                   | <0.0001                       |
| NRQ (2.3-9.4)                          | Y = 0.4433*X + 0.1145   | 0.1120 to 0.7746 | 0.0096                                    |                               |
| RQ (6.1-9.4)                           | Y = -0.2766*X + 7.750   | -1.564 to 1.011  | 0.6644                                    | 0.7462                        |
| NRQ (6.1-9.4)                          | Y = -0.5372*X + 8.053   | -1.545 to 0.4703 | 0.2852                                    |                               |
| linEGLN3 RQ (2.3-9.4)                  | Y = 0.6381*X + 1.606    | 0.4427 to 0.8335 | <0.0001                                   | 0.0041                        |
| NRQ (2.3-9.4)                          | Y = 0.2615*X + 0.2944   | 0.09397 to 0.4291| 0.0028                                    |                               |
| RQ (6.1-9.4)                           | Y = 0.2046*X + 1.969    | -0.4299 to 0.8391| 0.5156                                    | 0.4644                        |
| NRQ (6.1-9.4)                          | Y = -0.08939*X + 3.176  | -0.6001 to 0.4214| 0.7236                                    |                               |
| circRHOBT3 RQ (2.3-9.4)                | Y = 0.3908*X - 0.7319   | 0.1925 to 0.5891 | 0.0002                                    | 0.0093                        |
| NRQ (2.3-9.4)                          | Y = 0.07253*X + 0.9296  | -0.06392 to 0.2090| 0.2919                                    |                               |
| RQ (6.1-9.4)                           | Y = 0.04727*X + 2.055   | -0.6825 to 0.7770| 0.8958                                    | 0.7929                        |
| NRQ (6.1-9.4)                          | Y = -0.06250*X + 2.025  | -0.4961 to 0.3711| 0.7708                                    |                               |
| linRHOBTB3 RQ (2.3-9.4)                | Y = 0.3033*X - 0.2363   | 0.1124 to 0.4941 | 0.0023                                    | 0.0159                        |
| NRQ (2.3-9.4)                          | Y = 0.000459*X + 1.426  | -0.158 to 0.159  | 0.9954                                    |                               |
| RQ (6.1-9.4)                           | Y = 0.02537*X + 1.964   | -0.6619 to 0.7126| 0.9405                                    | 0.7489                        |
| NRQ (6.1-9.4)                          | Y = -0.08375*X + 2.012  | -0.5157 to 0.3482| 0.6952                                    |                               |

RQ: relative quantification; NRQ: normalized relative quantification using the reference genes PPIA and TBP. The numbers in brackets (2.3-9.4) and (6.1-9.4) refer to the total RNA samples with the ranges of RIN values used for relative or normalized relative quantification.
### Supplemental Table S14

Regression line analysis of circRNAs and their linear counterparts calculated as relative and normalized quantities in relation to the RIN values of total RNA samples from prostate cancer in Figure 5B.

| RNA variables | Regression line equation | 95% CI of slopes | Is slope significantly non-zero? (P-value) | Are the slopes equal? (P-value) |
|---------------|--------------------------|------------------|------------------------------------------|-------------------------------|
| **circCSNK1G3** |                          |                  |                                          |                               |
| RQ (2.2-8.2)  | $Y = 0.1689X + 0.2721$   | 0.06446 to 0.2734 | 0.0020                                    | 0.0491                        |
| NRQ (2.2-8.2) | $Y = 0.04611X + 0.8797$  | -0.03305 to 0.1253 | 0.2481                                    |                               |
| RQ (6.3-8.2)  | $Y = 0.1673X + 0.2970$   | -0.4058 to 0.7404  | 0.5551                                    | 0.4206                        |
| NRQ (6.3-8.2) | $Y = -0.09948X + 1.871$  | -0.4519 to 0.2529  | 0.5682                                    |                               |
| **linCSNK1G3** |                          |                  |                                          |                               |
| RQ (2.2-8.2)  | $Y = 0.1583X + 0.2697$   | 0.08914 to 0.2274  | <0.0001                                   | 0.0015                        |
| NRQ (2.2-8.2) | $Y = 0.03401X + 0.8372$  | 0.000987 to 0.06704 | 0.0438                                   |                               |
| RQ (6.3-8.2)  | $Y = 0.4374X - 1.712$    | 0.1384 to 0.7364   | 0.0056                                    | 0.0702                        |
| NRQ (6.3-8.2) | $Y = -0.09948X + 1.871$  | -0.4519 to 0.2529  | 0.5682                                    |                               |
| **circRHOBT3** |                          |                  |                                          |                               |
| RQ (2.2-8.2)  | $Y = 0.2821X - 0.2899$   | 0.2084 to 0.3557   | <0.0001                                   | 0.0160                        |
| NRQ (2.2-8.2) | $Y = 0.1423X + 0.4346$   | 0.05470 to 0.2300  | 0.0019                                    |                               |
| RQ (6.3-8.2)  | $Y = 0.5899X - 2.537$    | 0.2227 to 0.9571   | 0.0027                                    | 0.0808                        |
| NRQ (6.3-8.2) | $Y = 0.1244X + 0.4702$   | -0.2659 to 0.5146  | 0.5196                                    |                               |
| **linRHOBTB3** |                          |                  |                                          |                               |
| RQ (2.2-8.2)  | $Y = 0.2200X + 0.01879$  | 0.1336 to 0.3063   | <0.0001                                   | 0.0089                        |
| NRQ (2.2-8.2) | $Y = 0.08193X + 0.6510$  | 0.02423 to 0.1396  | 0.0062                                    |                               |
| RQ (6.3-8.2)  | $Y = 0.5374X - 2.267$    | 0.1721 to 0.9027   | 0.0054                                    | 0.0691                        |
| NRQ (6.3-8.2) | $Y = 0.1514X + 0.1060$   | -0.06801 to 0.3709 | 0.1688                                    |                               |

RQ: relative quantification; NRQ: normalized relative quantification using the reference genes ALAS1 and HPRT1. The numbers in brackets (2.2-8.2) and (6.3-8.2) after RQ and NRQ refer to the total RNA samples with the ranges of RIN values used for relative or normalized relative quantification.

### 5. References

1. Li P, Chen S, Chen H, Mo X, Li T, Shao Y, et al. Using circular RNA as a novel type of biomarker in the screening of gastric cancer. Clin Chim Acta. 2015; 444: 132-6.
2. Ahmed I, Karedath T, Andrews SS, Al-Azwani IK, Mohamoud YA, Querleu D, et al. Altered expression pattern of circular RNAs in primary and metastatic sites of epithelial ovarian carcinoma. Oncotarget. 2016; 7: 36366-81.
3. Lu L, Sun J, Shi P, Kong W, Xu K, He B, et al. Identification of circular RNAs as a promising new class of diagnostic biomarkers for human breast cancer. Oncotarget. 2017; 8: 44096-107.
4. Zhu X, Wang X, Wei S, Chen Y, Chen Y, Fan X, et al. hsa_circ_0013958: a circular RNA and potential novel biomarker for lung adenocarcinoma. FEBS J. 2017; 284: 2170-82.
5. Dang Y, Ouyang X, Zhang F, Wang K, Lin Y, Sun B, et al. Circular RNAs expression profiles in human gastric cancer. Sci Rep. 2017; 7: 9060.
6. Weng W, Wei Q, Toden S, Yoshida K, Nagasaka T, Fujiwara T, et al. Circular RNA ciRS-7-A promising prognostic biomarker and a potential therapeutic target in colorectal cancer. Clin Cancer Res. 2017; 23: 3918-28.
7. Cao S, Wei D, Li X, Zhou J, Li W, Qian Y, et al. Novel circular RNA expression profiles reflect progression of patients with hypopharyngeal squamous cell carcinoma. Oncotarget. 2017; 8: 45367-79.
8. Chen S, Li T, Zhao Q, Xiao B, Guo J. Using circular RNA hsa_circ_0000190 as a new biomarker in the diagnosis of gastric cancer. Clin Chim Acta. 2017; 466: 167-71.
9. Huang XY, Huang ZL, Xu YH, Zheng Q, Chen Z, Song W, et al. Comprehensive circular RNA profiling reveals the regulatory role of the circRNA-100338/miR-141-3p pathway in hepatitis B-related hepatocellular carcinoma. Sci Rep. 2017; 7: 5428.
10. Zhang F, Zhao X, Dong H, Xu J. circRNA expression analysis in lung adenocarcinoma: comparison of paired fresh frozen and formalin-fixed paraffin-embedded specimens. Biochem Biophys Res Commun. 2018; 500: 738-43.
11. Sun S, Li B, Wang Y, Li X, Wang P, Wang F, et al. Clinical significance of the decreased expression of hsa_circ_001242 in oral squamous cell carcinoma. Dis Markers. 2018; 2018: 6514795.
12. Huang Y, Zhang Y, Jia L, Liu C, Xu F. Circular RNA ABCB10 promotes tumor progression and correlates with pejorative prognosis in clear cell renal cell carcinoma. Int J Biol Markers. 2019; 34: 176-83.
13. Chen J, Chen T, Zhu Y, Li Y, Zhang Y, Wang Y, et al. circPTN sponges miR-145-5p/miR-330-5p to promote proliferation and stemness in glioma. J Exp Clin Cancer Res. 2019; 38: 398.
14. Lu C, Jiang W, Hui B, Rong D, Fu K, Dong C, et al. The circ_0021977/miR-10b-5p/P21 and P53 regulatory axis suppresses proliferation, migration, and invasion in colorectal cancer. J Cell Physiol. 2020; 235: 2273-85.
15. Jin C, Dong D, Yang Z, Xia R, Tao S, Piao M. CircMYC regulates glycolysis and cell proliferation in melanoma. Cell Biochem Biophys. 2020; 78: 77-88.
16. Xing Y, Zha WJ, Li XM, Li H, Gao F, Ye T, et al. Circular RNA circ-Foxo3 inhibits esophageal squamous cell cancer progression via the miR-23a/PTEN axis. J Cell Biochem. 2020; 121: 2595-605.
17. Sun D, Chen L, Lv G, Ouyang Y, Liu X, Zhang X. Circ_0058124 upregulates MAPK1 expression to promote proliferation, metastasis and metabolic abilities in thyroid cancer through sponging miR-940a. OncoTargets Ther. 2020; 13: 1569-81.
18. Liu Y, Xia L, Dong L, Wang J, Xiao Q, Yu X, et al. CircHIPK3 promotes mictumab (GEM) resistance in pancreatic cancer cells by sponging miR-330-5p and targets RASSF1. Cancer Manag Res. 2020; 12: 921-9.
19. Zang Y, Li J, Wang J, Tai Y. CircRNA circ-CCND1 promotes the proliferation of laryngeal squamous cell carcinoma through elevating CCND1 expression via interacting with HuR and miR-646. J Cell Mol Med. 2020; 24: 2423-33.
20. Li S, Pei Y, Wang W, Liu F, Zheng K, Zhang X. Extracellular nanovesicles-transmitted circular RNA has_circ_0000190 suppresses osteosarcoma progression. J Cell Mol Med. 2020; 24: 2202-14.
21. Zhou F, Wang D, Wei W, Chen H, Shi H, Zhou N, et al. Comprehensive profiling of circular RNA expressions reveals potential diagnostic and prognostic biomarkers in multiple myeloma. BMC Cancer. 2020; 20: 40.
22. Kong Z, Wan X, Lu Y, Zhang Y, Huang Y, Xu Y, et al. Circular RNA circFOXO3 promotes prostate cancer progression through sponging miR-29a-3p. J Cell Mol Med. 2020; 24: 799-813.
23. Meng QH, Li Y, Kong C, Gao XM, Jiang XJ. Circ_000388 exerts oncogenic function in cervical cancer cells by regulating miR-337-3p/TCF12 axis. Cancer Biother Radiopharm. 2020; Epub ahead of print, March 2, 2020; doi: 10.1089/cbr.2019.3159.
24. Chen S, Huang Y, Xu X, Livingstone J, Soares F, Jeon J, et al. Widespread and functional RNA circularization in localized prostate cancer. Cell. 2019; 176: 831-43.
25. Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. Nat Biotechnol. 2014; 32: 453-61.
26. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3 - new capabilities and interfaces. Nucleic Acids Res. 2012; 40: e115.
34. Jung M, Mollenkopf H-J, Grimm C, Wagner I, Albrecht M, Waller T, et al. MicroRNA profiling of clear cell renal cell cancer identifies a robust signature to define renal malignancy. J Cell Mol Med. 2009; 13: 3918-28.

35. Jung M, Schaefer A, Steiner I, Kempkensteffen C, Stephan C, Erbersdobler A, et al. Robust microRNA stability in degraded RNA preparations from human tissue and cell samples. Clin Chem. 2010; 56: 998-1006.

36. Schaefer A, Jung M, Mollenkopf HJ, Wagner I, Stephan C, Jentzmik F, et al. Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma. Int J Cancer. 2010; 126: 1166-76.

37. Wotschofsky Z, Meyer H-A, Jung M, Fendler A, Wagner I, Stephan C, et al. Reference genes for the relative quantification of microRNAs in renal cell carcinomas and their metastases. Analyt Biochem. 2011; 417: 233-41.