Molecular Cell, Volume *66*

Supplemental Information

Circ-ZNF609 Is a Circular RNA that Can Be Translated and Functions in Myogenesis

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Supplemental information

Figure S1

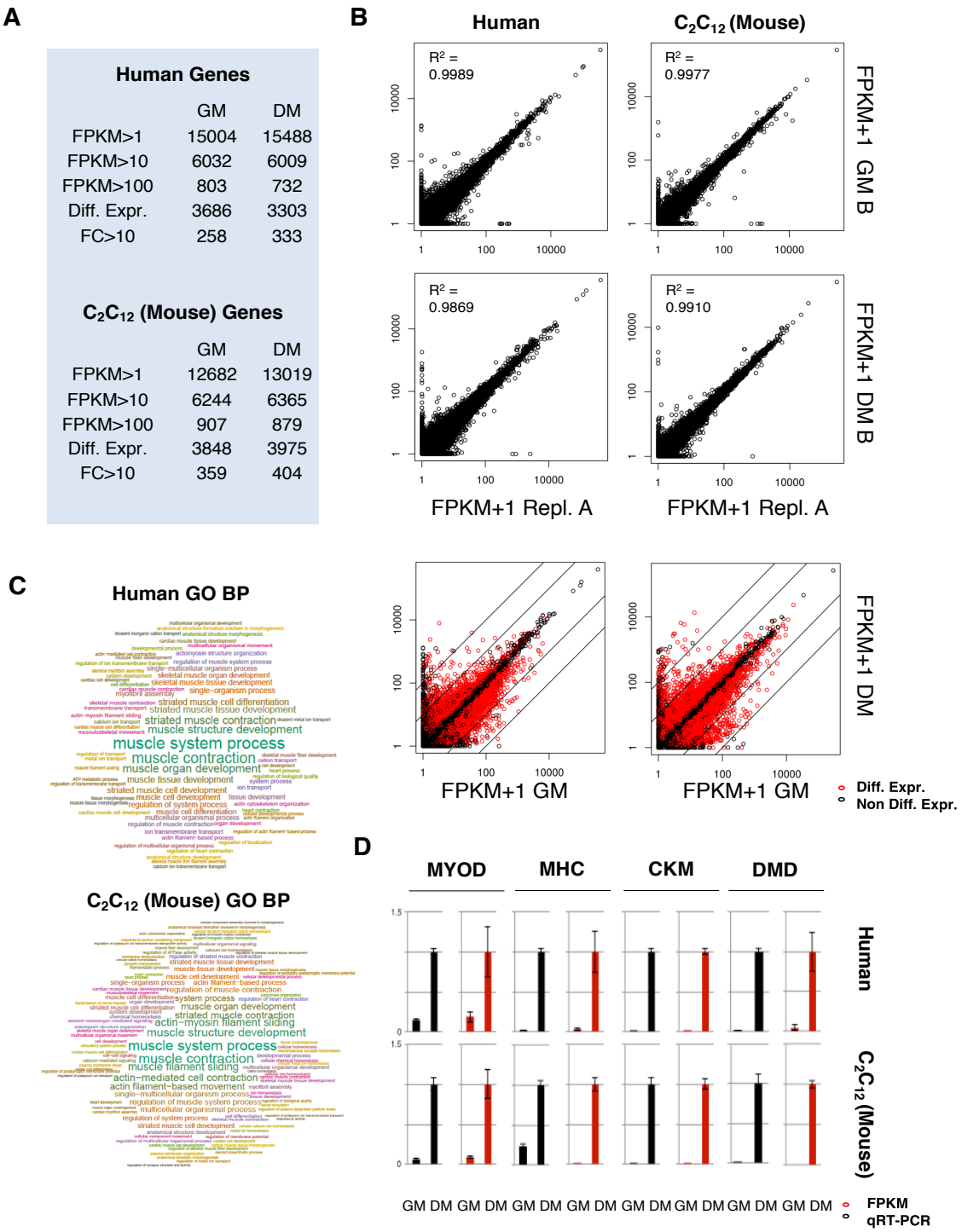
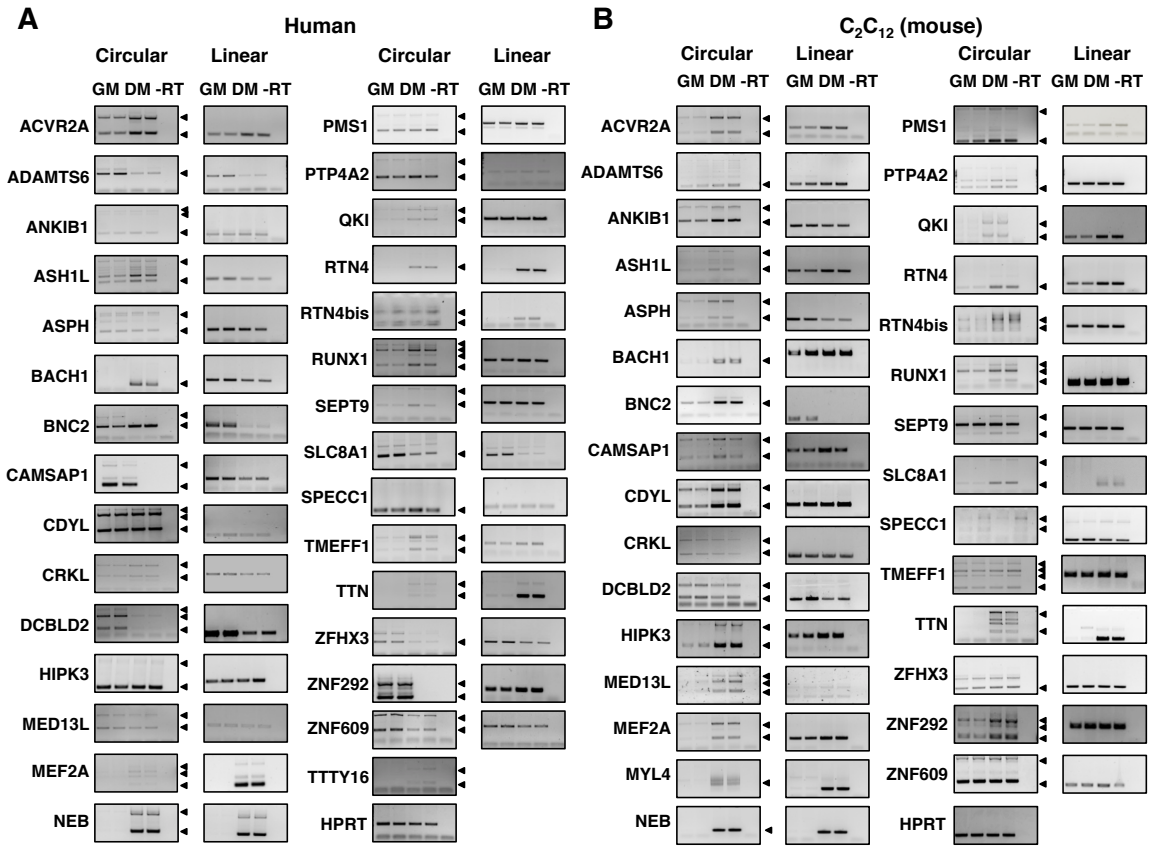
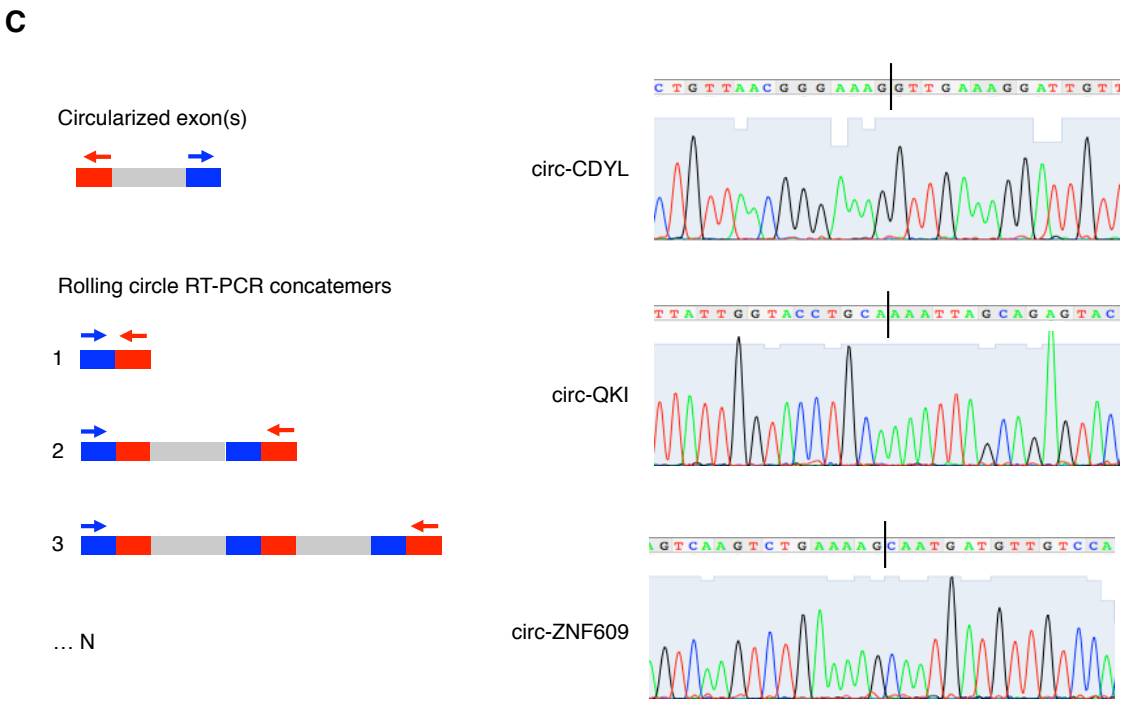
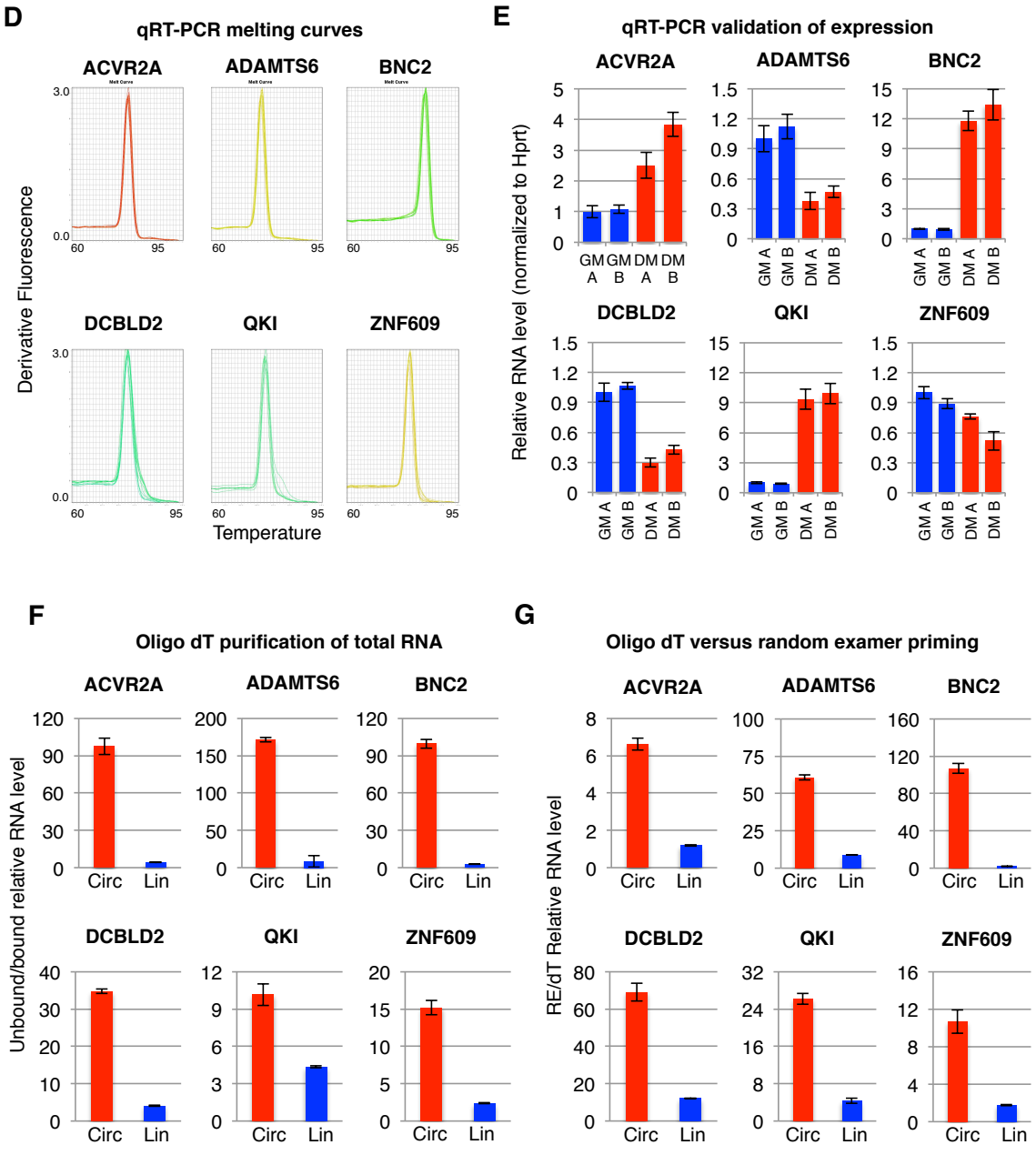


Figure S1. Related to Figure 1. RNAseq analysis of human and mouse myoblast differentiation.
(**A**) Number of genes expressed at various FPKM thresholds in human and mouse myoblasts (GM) and myotubes (DM), followed by number of genes differentially expressed according to Cuffdiff, and genes differentially expressed with a fold change >10. (**B**) Scatterplots showing correlation of RNAseq replicates (A and B) for human (left panels) and mouse (right panels) myoblasts (GM, upper panels) and myotubes (DM, middle panels); the correlation coefficient is shown on top left of each plot. Lower panels show FPKM of human and mouse genes in DM versus GM; genes differentially expressed according to Cuffdiff are highlighted in red. Fold change expression is indicated by 2 black lines at thresholds of 10 and 100. (**C**) Word clouds generated after gene ontology enrichment analysis of genes induced upon differentiation of myoblasts into myotubes (upper panel, human; lower panel, mouse). Non-differentially expressed genes were used as background. Word size is function of the enrichment of words found in gene ontology biological process. (**D**) qRT-PCR validation of myogenic markers in human (upper bar plots) and mouse (lower bar plots) myoblasts (GM) and myotubes (DM). FPKM values of the 4 markers MyoD (MYOD), Myosin Heavy Chain (MHC), Muscular Creatine Kinase (MCK) and Dystrophin (DMD), are shown in red. RNA levels measured by qRT-PCR, and normalized to HPRT, are shown in black. Bars show mean and standard error.

Figure S2


A
Human
Circular
Linear
GM DM -RT
ACVR2A
ADAMTS6
ANKIB1
ASH1L
ASPH
BACH1
BNC2
CAMSAP1
CDYL
CRKL
DCBLD2
HIPK3
MED13L
MEF2A
NEB
PMS1
PTP4A2
QKI
RTN4
RTN4bis
RUNX1
SEPT9
SLC8A1
SPECC1
TMEFF1
TTN
ZFHX3
ZNF292
ZNF609
TTTY16
HPRT
B
C2C12 (mouse)
MYL4


C
Circularized exon(s)
Rolling circle RT-PCR concatemers
1
2
3
… N
circ-CDYL
circ-QKI
circ-ZNF609


D
qRT-PCR melting curves
ACVR2A
ADAMTS6
BNC2
DCBLD2
QKI
ZNF609
Derivative Fluorescence
Temperature
E
qRT-PCR validation of expression
Relative RNA level (normalized to Hprt)
GM A
GM B
DM A
DM B
F
Oligo dT purification of total RNA
Unbound/bound relative RNA level
Circ
Lin
G
Oligo dT versus random examer priming
RE/dT Relative RNA level

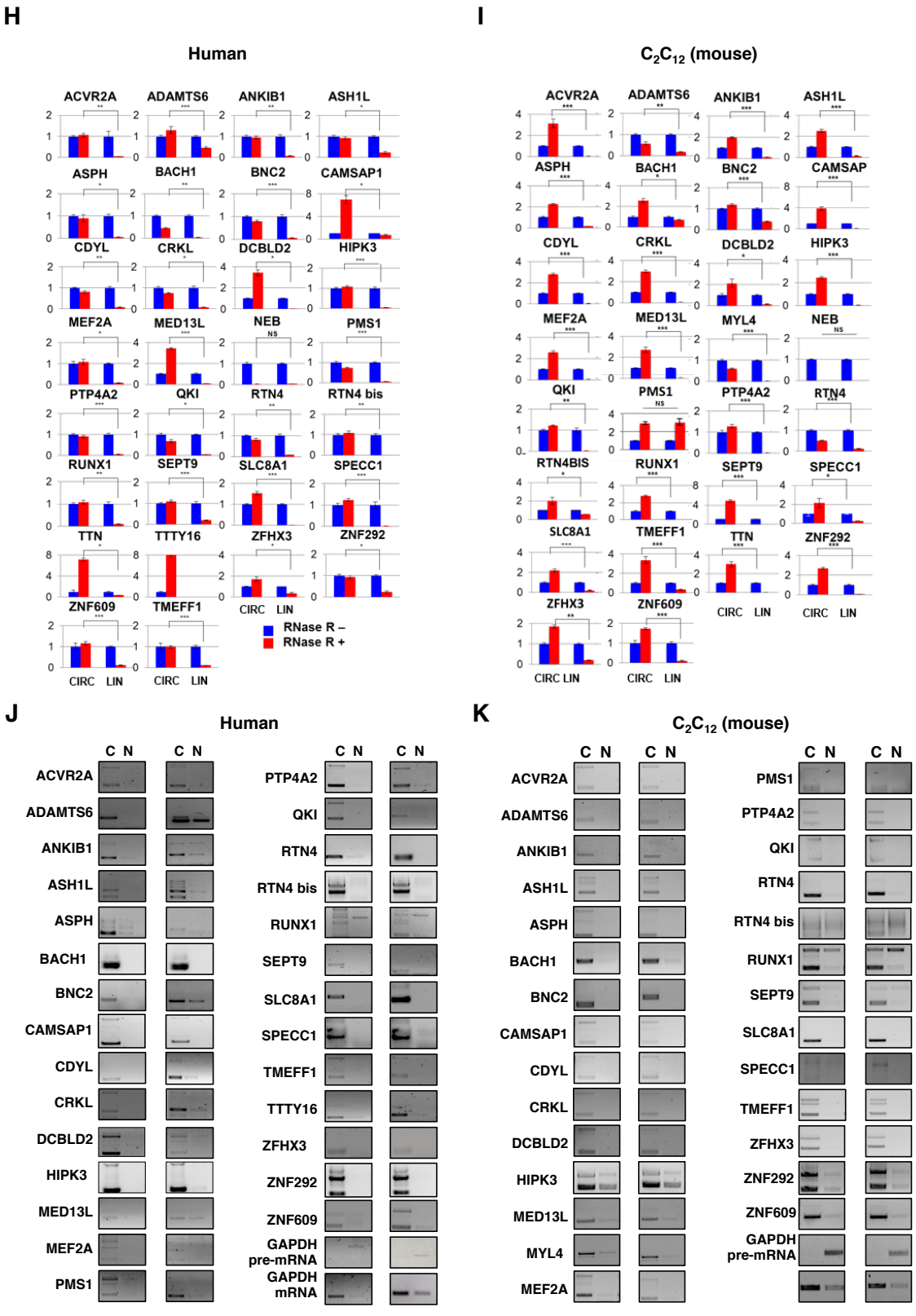

H
Human
ACVR2A
ADAMTS6
ANKIB1
ASH1L
ASPH
BACH1
BNC2
CAMSAP1
CDYL
CRKL
DCBLD2
HIPK3
MEF2A
MED13L
NEB
PMS1
PTP4A2
QKI
RTN4
RTN4 bis
RUNX1
SEPT9
SLC8A1
SPECC1
TTN
TTTY16
ZFHX3
ZNF292
ZNF609
TMEFF1
CIRC
LIN
RNase R –
RNase R +
I
C2C12 (mouse)
ACVR2A
ADAMTS6
ANKIB1
ASH1L
ASPH
BACH1
BNC2
CAMSAP
CDYL
CRKL
DCBLD2
HIPK3
MEF2A
MED13L
MYL4
NEB
QKI
PMS1
PTP4A2
RTN4
RTN4BIS
RUNX1
SEPT9
SPECC1
SLC8A1
TMEFF1
TTN
ZNF292
ZFHX3
ZNF609
CIRC
LIN
J
Human
C N
ACVR2A
ADAMTS6
ANKIB1
ASH1L
ASPH
BACH1
BNC2
CAMSAP1
CDYL
CRKL
DCBLD2
HIPK3
MED13L
MEF2A
PMS1
PTP4A2
QKI
RTN4
RTN4 bis
RUNX1
SEPT9
SLC8A1
SPECC1
TMEFF1
TTTY16
ZFHX3
ZNF292
ZNF609
GAPDH pre-mRNA
GAPDH mRNA
K
C2C12 (mouse)
C N
ACVR2A
ADAMTS6
ANKIB1
ASH1L
ASPH
BACH1
BNC2
CAMSAP1
CDYL
CRKL
DCBLD2
HIPK3
MED13L
MYL4
MEF2A
PMS1
PTP4A2
QKI
RTN4
RTN4 bis
RUNX1
SEPT9
SLC8A1
SPECC1
TMEFF1
ZFHX3
ZNF292
ZNF609
GAPDH pre-mRNA

Figure S2. Related to Figure 2. Experimental validation and subcellular localization of selected circRNAs.

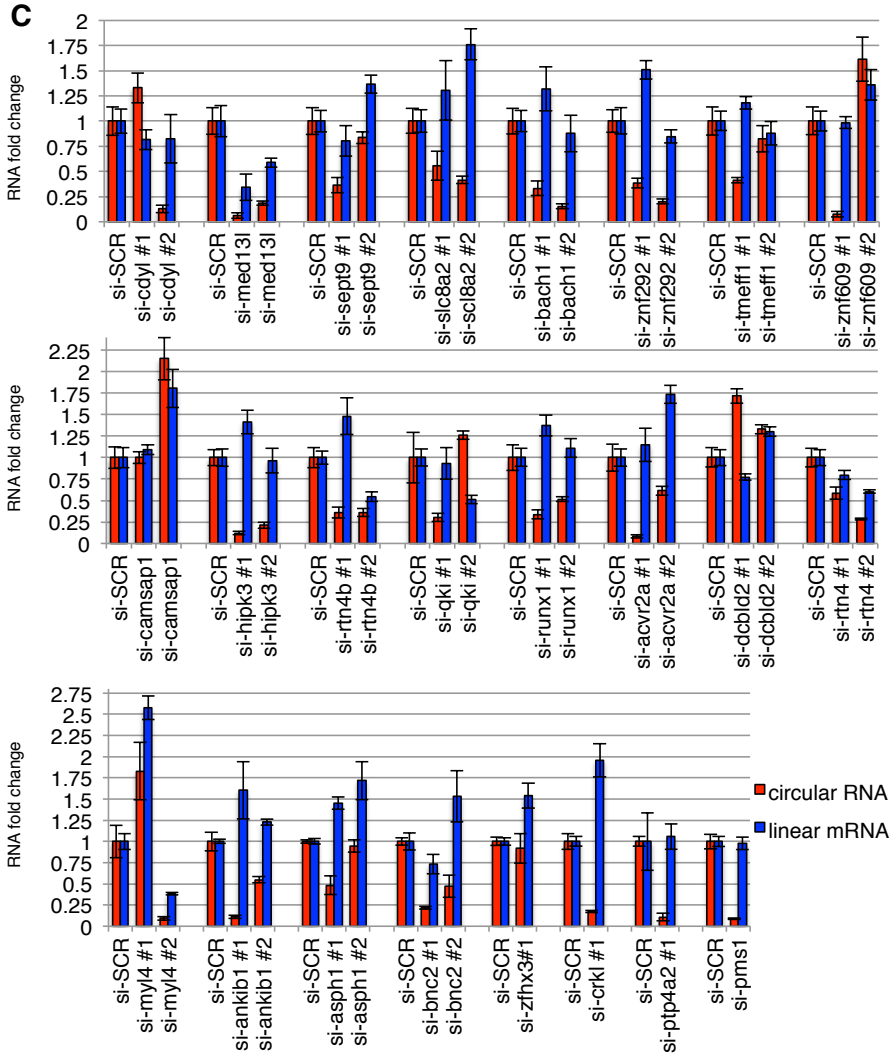
(**A**) RT-PCR validation of human circRNAs: circRNAs and the corresponding linear transcripts were amplified from cDNA obtained from myoblasts (GM) and myotubes (DM) in biological duplicates with divergent and convergent primers (see Table S2). Products were run on agarose gels together with RT-minus controls. On the right side of the gels for circRNAs, black arrows indicate the circRNA amplicon and amplicons of the expected size derived by rolling-circle retrotranscription. (**B**) Same as in A for mouse samples. (**C**) Left panel: schematic representation of the possible concatemers generated by PCR after multiple rounds of retrotranscription. In red and blue the two parts of the PCR product generated by the indicated primers (arrows). Right panel: electropherograms showing the circular RNA-specific splice junction detected in RT-PCR products for human circ-CDYL, QKI and ZNF609. The splice junction is indicated by a black bar. Larger bands sequencing resulted in the pattern shown in the left panel. (**D**) Melting curve analysis post-qPCR for 6 selected circRNAs. (**E**) qRT-PCR validation of expression of 6 selected circRNAs in human myoblasts (GM) and myotubes (DM) in biological duplicates ("A" and "B"). (**F**) RNA recovery after oligo dT purification of total RNA: barplots show the ratio of the RNA recovered in the unbound on the bound fraction for the circular (circ) and linear (lin) isoform of 6 candidates. qRT-PCR data are normalized for a DNA spike-in. (**G**) RNA level after oligo dT and random examers (RE) retrotranscription: barplots show the ratio of the RNA amplified with RE on dT for the circular (circ) and linear (lin) isoform of 6 candidates. qRT-PCR data are normalized for a DNA spike-in. (**H**) RNase R validation of human circRNAs. circRNAs that were amplified by qRT-PCR from cDNA prepared from RNA treated or non-treated with RNase R, together with their corresponding linear RNAs. The ratio of treated versus non-treated, of circRNA versus corresponding linear RNAs, was tested by two tailed Student's t test. Red bars: RNase R +; Blue bars: RNase R -. (**I**). Mouse samples were processed as in D. (**J**) Human circRNAs that passed the previous validation step were screened for subcellular localization by RT-PCR amplification from cDNA obtained by retrotranscribing nuclear and cytoplasmic RNA separately. Fractionation was performed in duplicate. (**K**) Mouse samples processed as in F. All bars show mean and standard error.

Figure S3

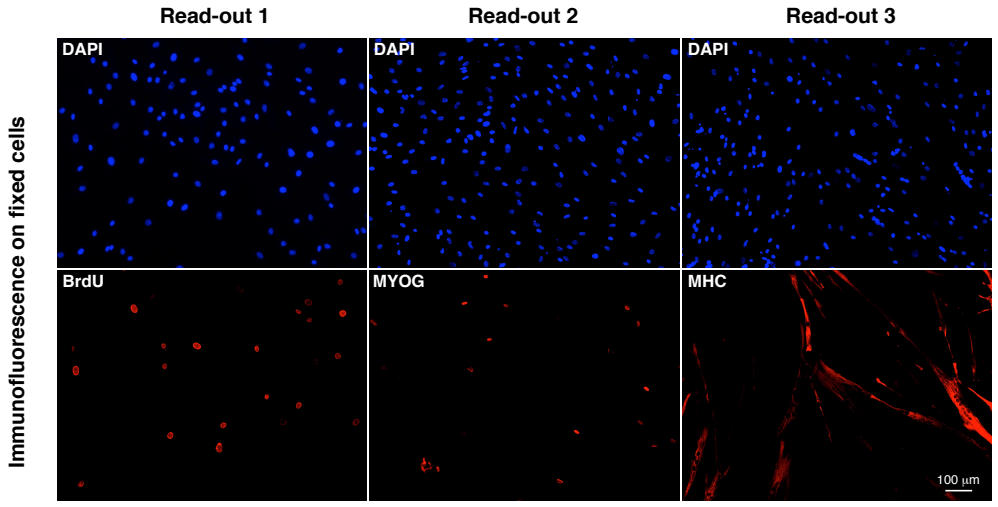
A

circRNA	siRNAs
circ-CDYL	2
circ-MED13L	2
circ-SEPT9	2
circ-Slc8a1	2
circ-BACH1	2
circ-ZNF292	2
circ-Tmeff1	2
circ-ZNF609	2
circ-CAMSAP1	2
circ-HIPK3	2
circ-RTN4bis	2
circ-QKI	2
circ-Runx1	2
circ-Acvr2a	2
circ-DCBLD2	2
circ-RTN4	2
circ-ZFHX3	1
circ-MYL4	2
circ-Ankib1	2
circ-ASPH	2
circ-BNC2	2
circ-CRKL	1
circ-PMS1	1
circ-PTP4A2	1
circ-TTN	1
Circ-ADAMTS6	NA
circ-ASH1L	NA
circ-MEF2A	NA
circ-SPECC1	NA
Circ-TYYY16	NA

C



B



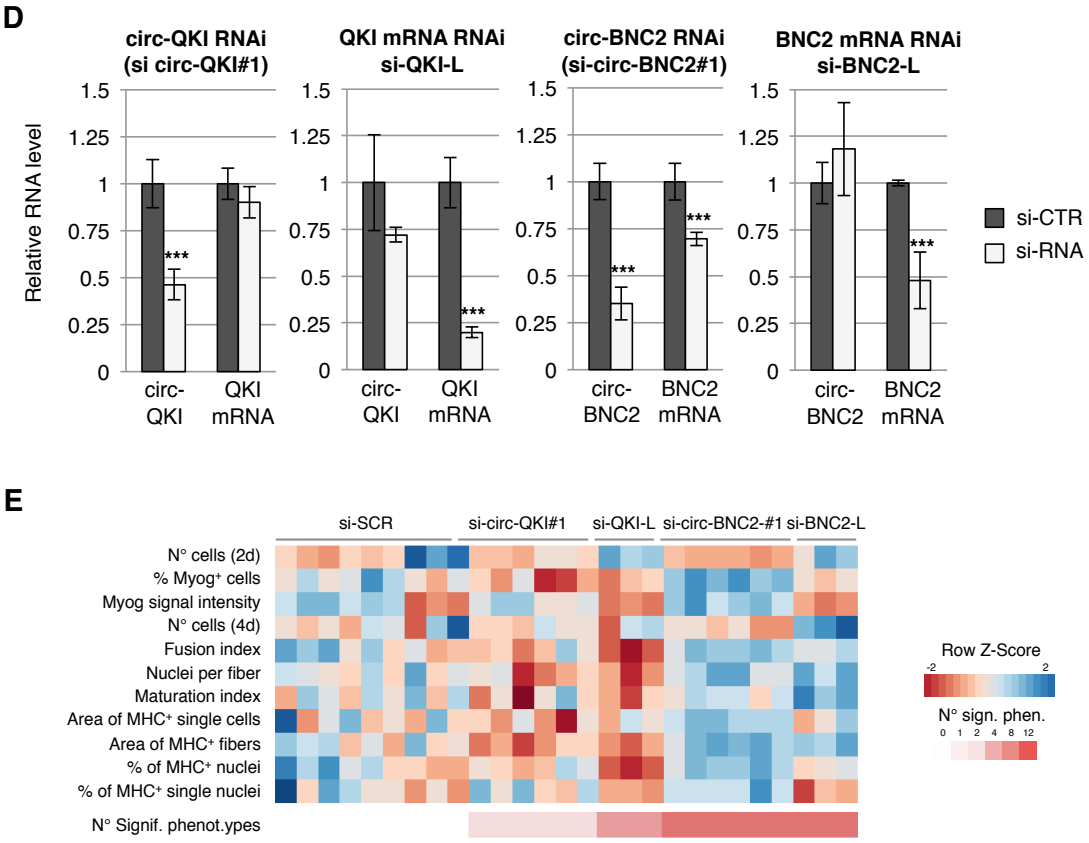


Figure S3. Related to Figure 3. CircRNA knock-down screening and validation.
(**A**) List of siRNAs designed to target human circular RNAs (NA – not applicable). (**B**) Knock-down efficiency of circRNAs and linear mRNAs for each siRNA, as measured in the high-throughput screening pilot experiment. All circRNAs/linear mRNAs level in control scramble siRNA (si-ctr) are set at 1. RNA levels in siRNA-treated cells are shown as fold change with respect to control and normalized to HPRT mRNA, measured by qRT-PCR. Levels of circRNA and linear mRNA are shown in red and blue, respectively. (**C**) A representative BrdU, Myogenin, Myosin and DAPI staining obtained in a pilot experiment in non-treated cells. (**D**) Knock-down of QKI and BNC2 circular and linear isoforms with the indicated siRNAs. Dark grey bars represent RNA level in negative control (si-CTR) and are set at 1. Light grey bars represent RNA level after siRNA treatment. All RNA levels are shown as mean and standard error and were measured by qRT-PCR in two independent experiments and normalized to Hprt mRNA. (**E**) Heat map showing selected "phenotypes" in color scale after Z-score normalization. High values are shown in blue, while low values in red. Each column indicates one phenotype, each row one sample, indicated on the right. The number of detected significant phenotypes is shown in red scale on the left.

Figure S4

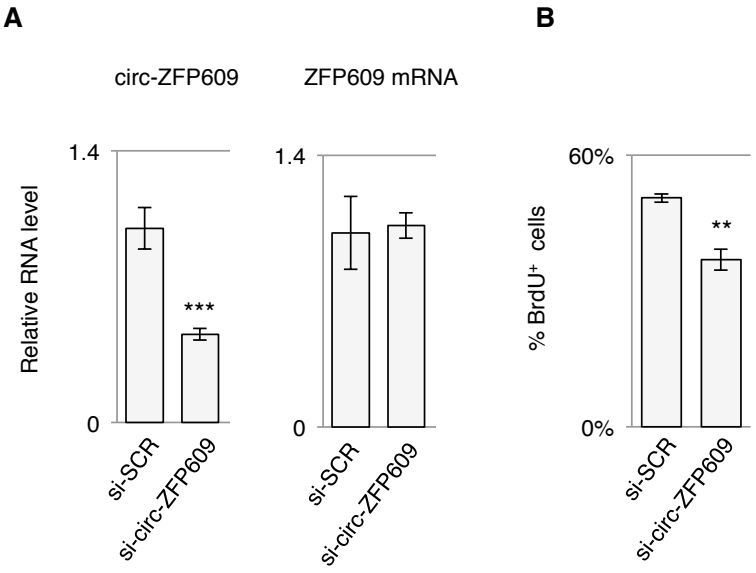


Figure S4. Related to Figure 4. Circ-ZNF609 phenotype validation in mouse.
(**A**) RNA level of circ-ZFP609 and ZFP609 mRNA measured by qRT-PCR in C2C12 cells treated with si-SCR and si-circ-ZFP609. (**B**) Percentage of the same cells positive for BrdU incorporation. Bars represent mean and standard error.

Figure S5

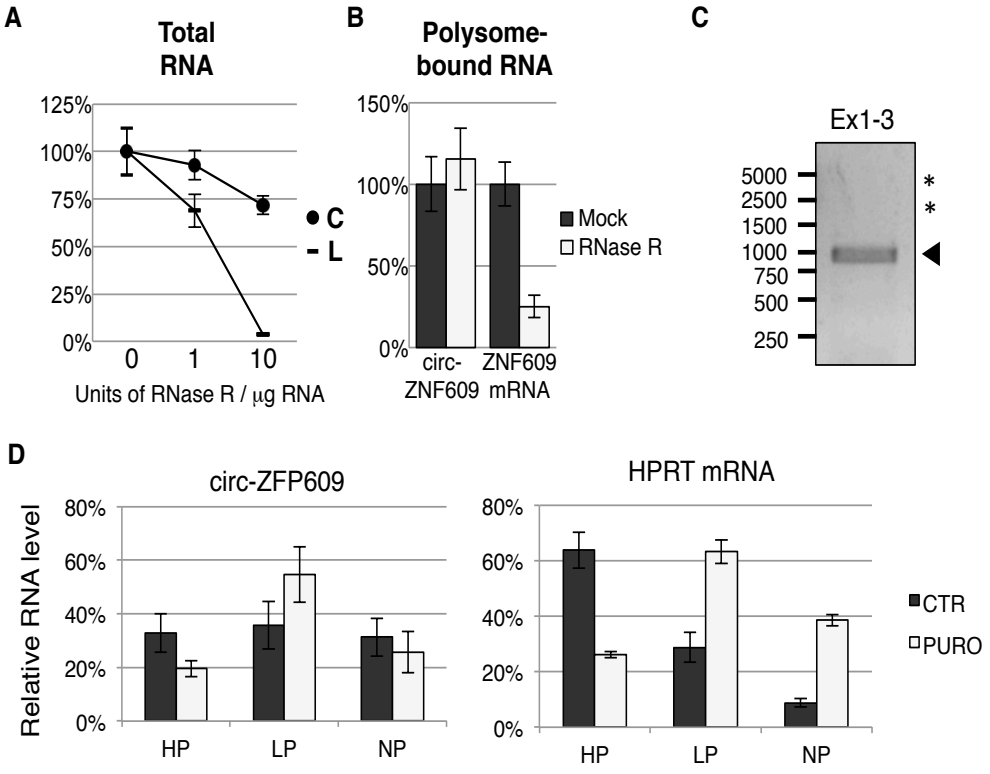
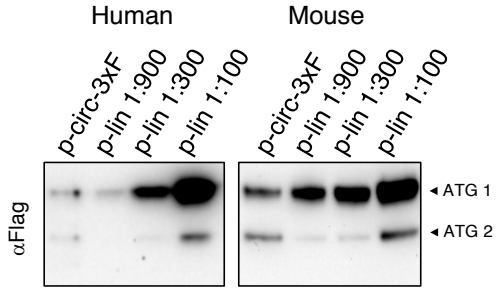


Figure S5. Related to Figure 5. Circ-ZNF609 associates with polysomes.
(**A**) RNaseR treatment of total RNA with different amounts of enzyme. The percentage of the RNA, recovered after treatment and measured by qRT-PCR, is shown. (**B**) RNase R treatment of polysome-bound RNA (obtained by sucrose fractionation): the percentage of recovery of circular and linear ZNF609 in control and RNase R treated samples is shown. (**C**) RT-PCR performed on total RNA with primers designed on exons 1 and 3 of ZNF609. A black arrow indicates the expected size of the PCR product, while asterisks highlight the size of possible concatemers. (**D**) Fraction of circ-ZFP609 or HPRT mRNA (positive control) associated with heavy polysomes (HP), light polysomes (LP) and non-polysomes (NP) as measured by qRT-PCR after sucrose fractionation of C2C12 lysates without (CTR, dark grey bars) or with Puromycin treatment (PURO, light grey bars). The values are represented as mean and standard error of three independent fractionations.

Figure S6

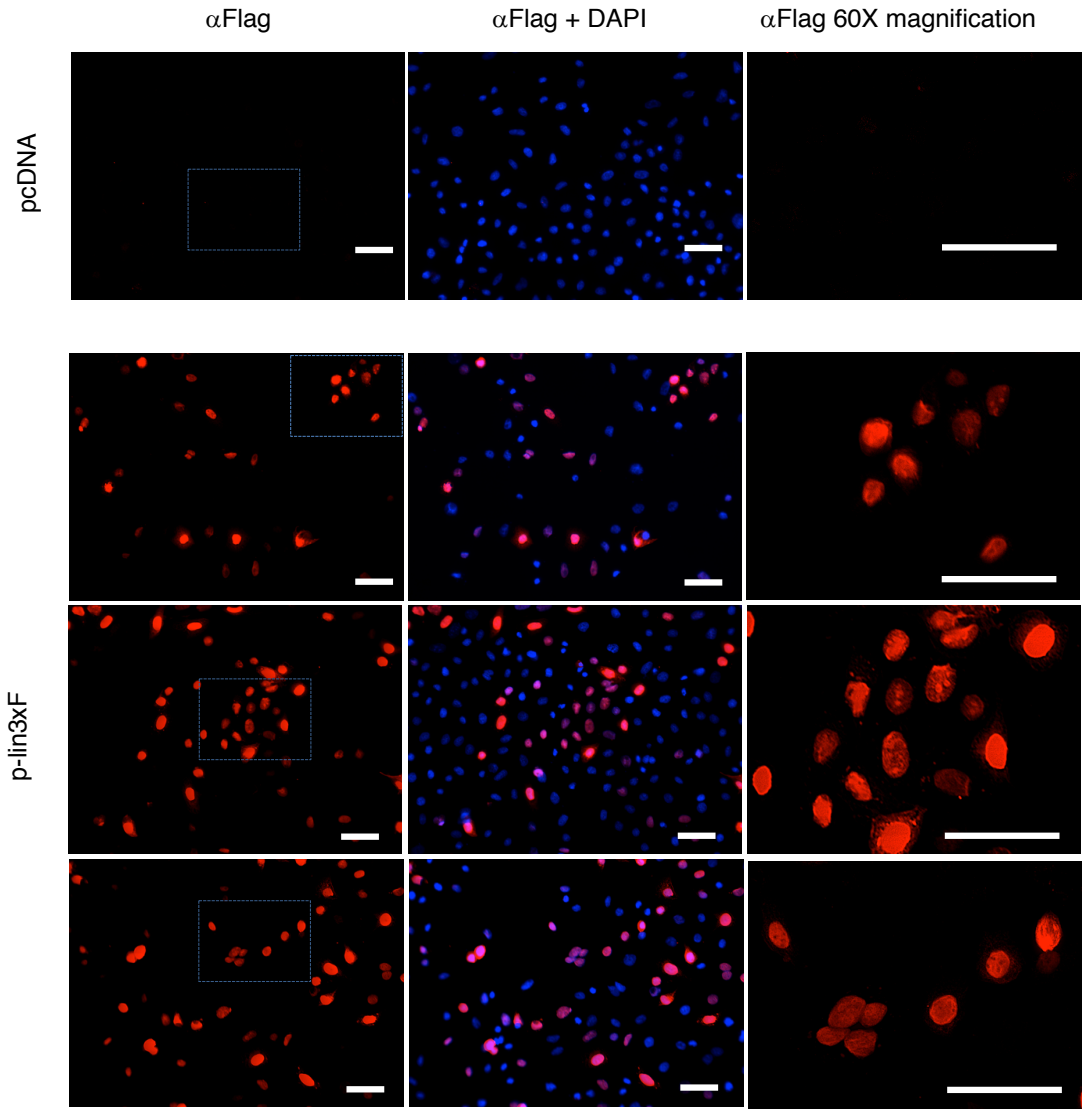
A

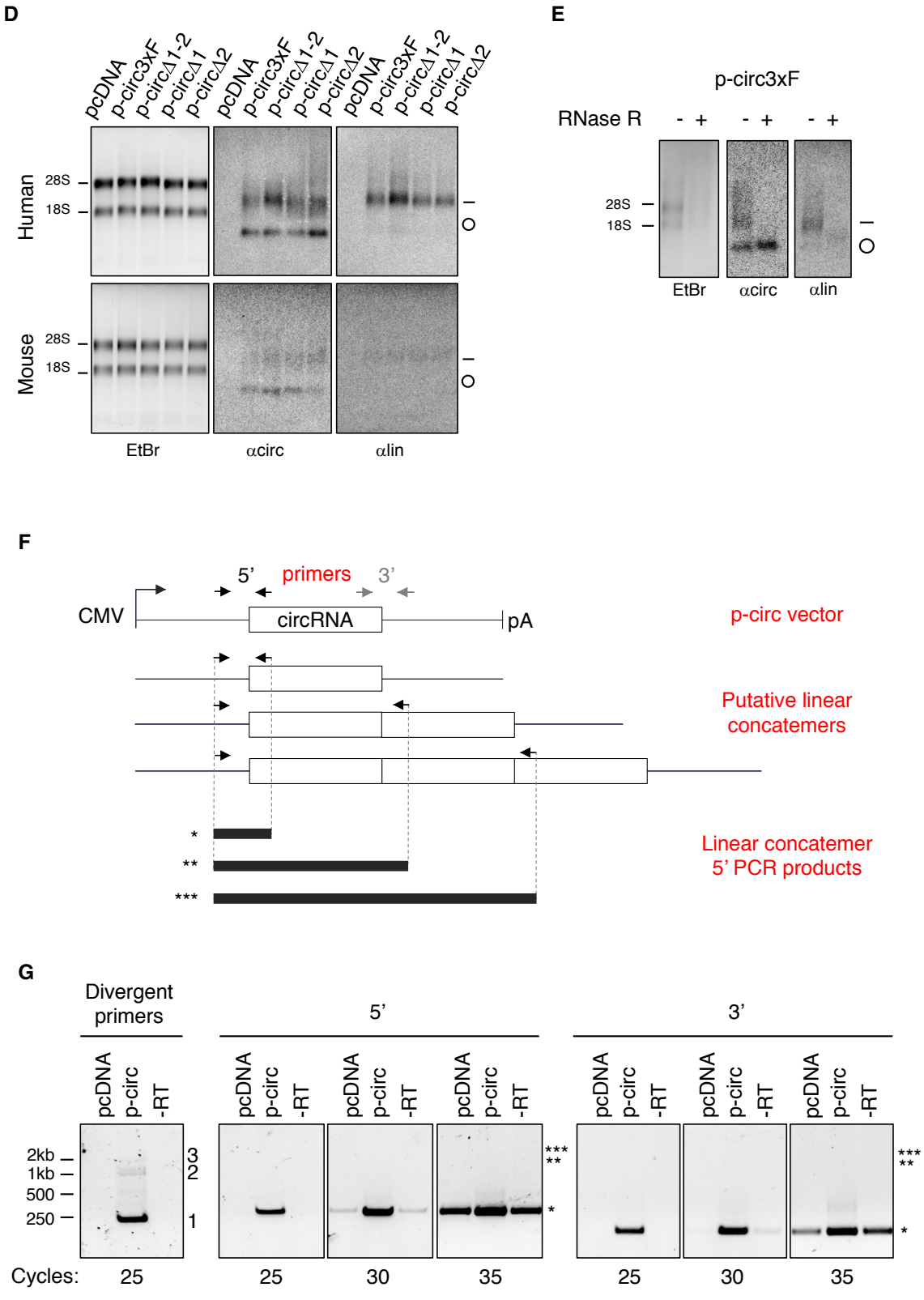


B

	Human	Mouse
C/L protein ratio	0.0014	0.0010
C/L RNA ratio	0.1020	0.0672
Norm. C/L transl. eff.	0.0134	0.0152

C




D
pcDNA
p-circ3xF
p-circΔ1-2
p-circΔ1
p-circΔ2
Human
Mouse
28S
18S
O
I
EtBr
αcirc
αlin
E
p-circ3xF
RNase R
- + - + - +
28S
18S
O
I
EtBr
αcirc
αlin
F
5'
primers
3'
CMV
circRNA
pA
p-circ vector
Putative linear concatemers
Linear concatemer 5' PCR products
*
**
***
G
Divergent primers
5'
3'
pcDNA
p-circ
-RT
2kb
1kb
500
250
3
2
1
***
**
*
Cycles:
25
25
30
35
25
30
35

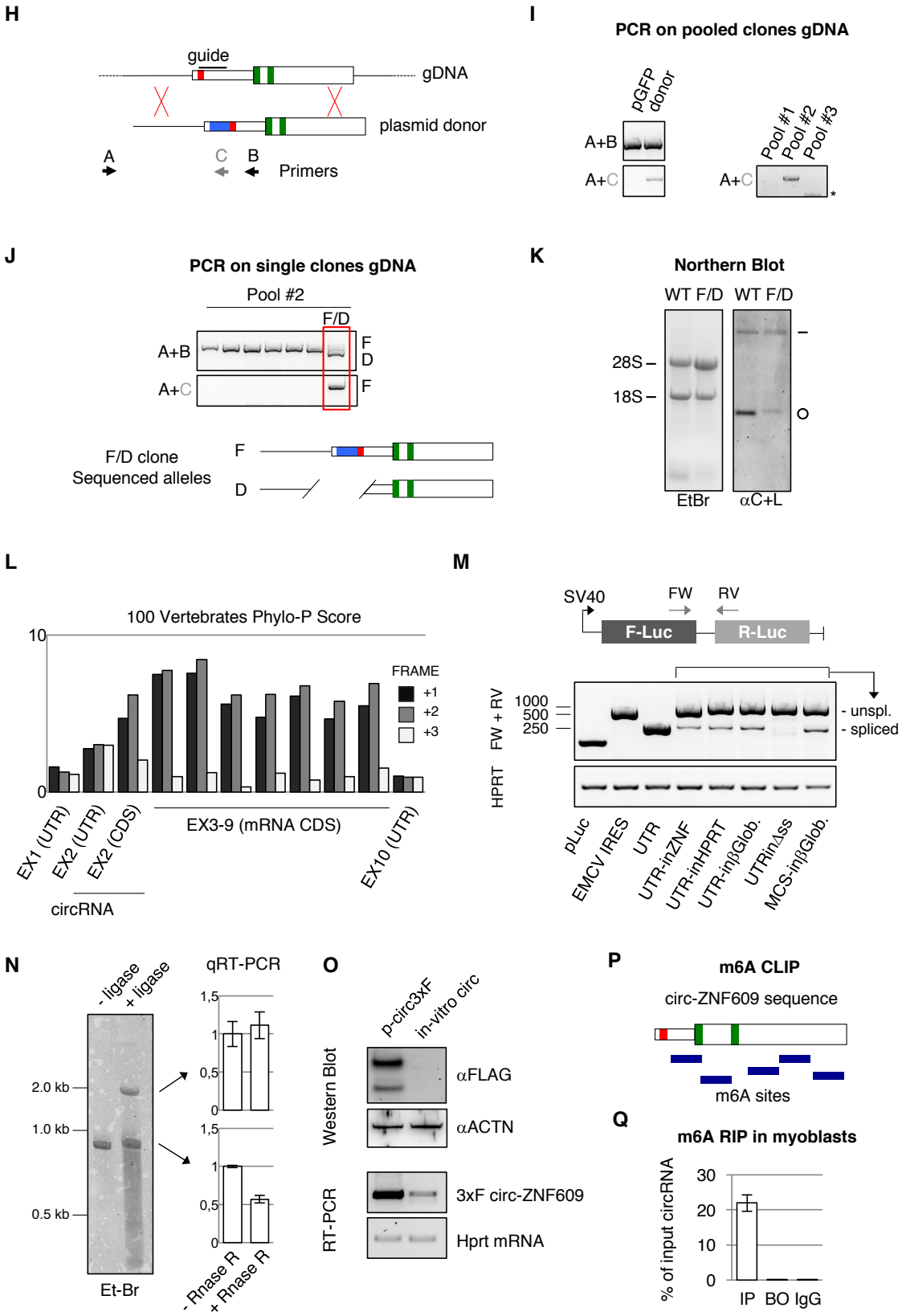

H
guide
gDNA
plasmid donor
A
C
B
Primers
I
PCR on pooled clones gDNA
pGFP donor
A+B
A+C
Pool #1
Pool #2
Pool #3
J
PCR on single clones gDNA
Pool #2
F/D
A+B
A+C
F
D
F/D clone
Sequenced alleles
K
Northern Blot
WT F/D
WT F/D
28S
18S
O
EtBr
αC+L
L
100 Vertebrates Phylo-P Score
10
0
FRAME
+1
+2
+3
EX1 (UTR)
EX2 (UTR)
EX2 (CDS)
EX3-9 (mRNA CDS)
EX10 (UTR)
circRNA
M
SV40
FW
RV
F-Luc
R-Luc
1000
500
250
FW + RV
HPRT
- unspl.
- spliced
pLuc
EMCV IRES
UTR
UTR-inZNF
UTR-inHPRT
UTR-inβGlob.
UTRinΔss
MCS-inβGlob.
N
- ligase
+ ligase
2.0 kb
1.0 kb
0.5 kb
Et-Br
qRT-PCR
1,5
1
0,5
0
- Rnase R
+ Rnase R
O
p-circ3xF
in-vitro circ
Western Blot
αFLAG
αACTN
RT-PCR
3xF circ-ZNF609
Hprt mRNA
P
m6A CLIP
circ-ZNF609 sequence
m6A sites
Q
m6A RIP in myoblasts
% of input circRNA
30
20
10
0
IP
BO
IgG

Figure S6. Related to Figure 6. Translation of circ-ZNF609.

(**A**) Western blot analysis of proteins produced by p-circ-3xF and p-lin-3xF (serial dilutions) in HeLa (left) and N2A (right) cells. (**B**) Table showing the ratio between the protein produced by p-circ-3xF and p-lin-3xF vectors ("C/L protein ratio", measured by densitometric analysis on blots shown in the left panel), the ratio of the RNA produced by p-circ-3xF and p-lin-3xF measured by qRT-PCR with divergent and convergent primers ("C/L RNA ratio", optimized for having amplicons of similar length in order to perform direct comparison), and the translation efficiency ratio obtained by dividing the amount of protein produced by the amount of RNA produced by the two vectors ("Norm. transl. eff."). (**C**) Immunofluorescence of pcDNA and p-lin3xF-transfected HeLa cells with an αFLAG antibody. Left panels: αFLAG staining (red). Middle panels: αFLAG (red) and DAPI staining (blue). Right panels: αFLAG staining (red) of a 60X magnification of the area indicated by a dashed blue line in the corresponding left panels. (**D**) 1 μg of total RNA from HeLa (Human) and N2a (Mouse) cells transfected with the indicated constructs was analysed by Northern blot with a ^{32}P-labelled probe against the circ-ZNF609 sequence (α circ) and the precursor sequence (α lin). EtBr staining of the gels is shown aside. (**E**) Northern blot showing RNase R resistance of the circRNA produced by p-circ3xF in HeLa cells. (**F**) Schematic representation of the possible linear concatemers generated by trans-splicing between different molecules of primary transcript and primers used for their amplification. Such concatemers would contain a functional ORF giving rise to the proteins observed by p-circ-3xF overexpression. However, in addition to Northern blot (figure 6C), RT-PCR proves that such aberrant splicing products are not present. (**G**) RT-PCR for concatemers. While rolling-circle RT-derived concatemers of circZNF609 are observed with divergent primers (left panel) even after 25 cycles of PCR, linear concatemers generated by trans-splicing in the cell are not observed with two distinct couples of primers after 25, 30 and even 35 cycles of PCR (middle and right panels). The expected size of such concatemers is indicated by asterisks. (**H**) Schematic representation of the ZNF609 locus and the plasmid donor used for Crispr/Cas9-mediated genome editing of mES cells. A 3xFLAG tag (in blue) is added to the endogenous circRNA sequence immediately before its stop codon (in red). Start codons are indicated in green. Primers for genotyping are indicated with arrows. (**I**) Genotyping of mES cells 48 hours after transfection with Crispr/Cas9 and donor DNA. Primers A and B amplify the WT locus, while A and C the recombinant locus, which is observed both in samples transfected with ssDNA and with plasmid DNA donors (left). Cells were then split and seeded at single cell density in 96 wells, and 34 clones where grouped in 5 pools, which were genotyped (right). (**J**) The clones from the two positive pools in (E) were genotyped separately: only one clone, named "F/D", was positive for the 3xFLAG insertion in one allele. The second allele contained a deletion around the 3' splice site (representative scheme on the bottom). Both alleles were characterized by Sanger sequencing. (**K**) Northern blot showing the presence of the tagged circRNA in "R/D" cells induced to neural differentiation. A probe recognizing both the circRNA and the mRNA was used (αC+L). EtBr staining of the gel is shown on the left. (**L**) Average of Phylo-P conservation score of the exons of the ZNF609 gene, divided by its 3 reading frames (in three grey shadows). (**M**) RT-PCR of the mature bicistronic mRNAs produced by the constructs shown in figure 6H, amplified by the primers FW and RV represented in the top panel. For the constructs carrying an intron, the two lines on the right indicate the expected sizes of the unspliced and spliced products. (**N**) Left panel: the polyacrylamide gel stained with EtBr shows the product of in vitro transcription before (- ligase) and after in vitro circularization obtained with T4 RNA ligase (+ ligase). The two bands where then eluted and tested for RNase R resistance (right panels). Bars represent mean and standard error of three technical replicates. (**O**) Top panel: Western blot analysis of HeLa cells transfected either with p-circ3xF (producing a FLAG-tagged circ-ZNF609 by back-splicing) or the synthetic FLAG-tagged circ-ZNF609 from panel (K) (in-vitro circ, produced by in vitro transcription and ligation). Hybridization with an anti-FLAG antibody reveals protein production only from the plasmid. ACTN was used as loading control. Bottom panel: RT-PCR showing expression of the FLAG-tagged circRNA in HeLa cells transfected with either p-circ3xF or in vitro circ. Hprt mRNA is used as loading control. (**P**) In blue m6A CLIP sites identified in circ-ZNF609 sequence (top) in Zhao et al., 2014. (**Q**) qRT-PCR analysis of circ-ZNF609 after m6A immunoprecipitation in C_2C_{12} myoblasts. Data are shown as % of input of RNA recovered after IP with anti-m6A antibodies (IP), isotypic IgGs (IgG) and beads only control (BO).

Table S1: list of circRNAs detected by RNAseq (provided as a separate Excel file), related to Figure 1.

Table S2: list of oligonucleotides used in this work (provided as a separate Excel file), related to STAR Methods.

Table S3: list of siRNAs used in this work (provided as separate Excel file), related to Figure 3.