SUPPLEMENTAL MATERIAL

circRAB3IP modulates cell proliferation by reorganizing gene expression and mRNA processing in a paracrine manner

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- This supplement contains Supplemental Figs S1-S5 and Tables S1-S4.

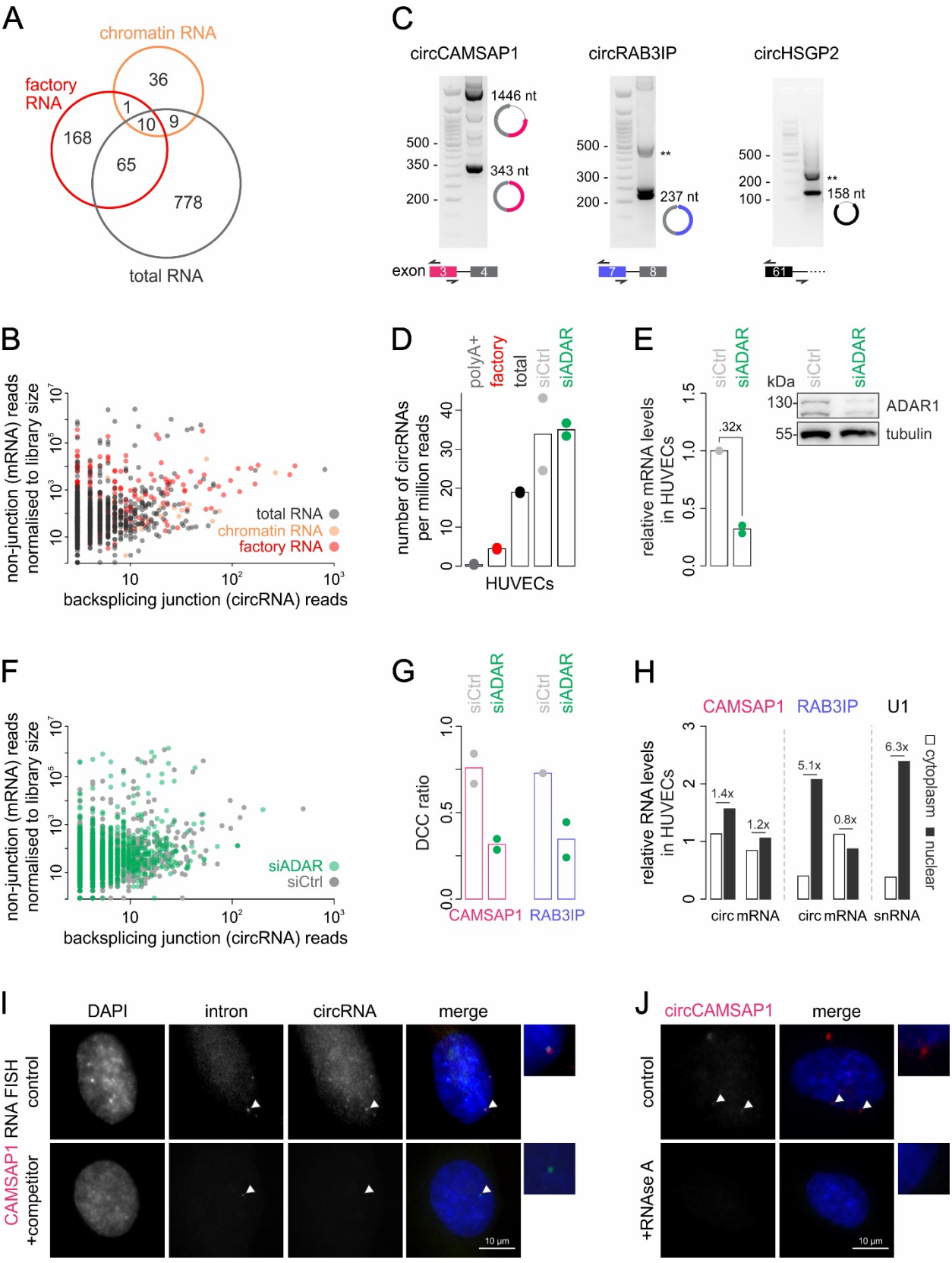
A
chromatin RNA
factory RNA
total RNA
36
1
10
9
168
65
778
C
circCAMSAP1
circRAB3IP
circHSGP2
1446 nt
343 nt
237 nt
158 nt
500
350
200
300
100
exon
3
4
7
8
61
B
non-junction (mRNA) reads normalised to library size
backsplicing junction (circRNA) reads
total RNA
chromatin RNA
factory RNA
D
polyA+
factory
total
siCtrl
siADAR
number of circRNAs per million reads
HUVECs
E
siCtrl
siADAR
relative mRNA levels in HUVECs
.32x
kDa
130
55
ADAR1
tubulin
F
non-junction (mRNA) reads normalised to library size
backsplicing junction (circRNA) reads
siADAR
siCtrl
G
siCtrl
siADAR
DCC ratio
CAMSAP1
RAB3IP
H
CAMSAP1
RAB3IP
U1
relative RNA levels in HUVECs
1.4x
1.2x
5.1x
0.8x
6.3x
cytoplasm
nuclear
circ mRNA
circ mRNA
snRNA
I
DAPI
intron
circRNA
merge
RNA FISH
control
CAMSAP1
+competitor
10 µm
J
circCAMSAP1
merge
control
+RNAse A
10 µm

FIGURE S1. Verification of circRNA identities and ADAR1 sensitivity. (***A***) Venn diagram showing detection of circRNAs in factory (*red*), chromatin-associated (*orange*) or total RNA fraction from HUVECs (*dark grey*). (***B***) Scatter plot of the number of reads mapping to circRNA backsplicing junctions plotted against reads from the same exons mapping to non-junction reads in the three RNA fractions from panel A. (***C***) Electrophoresis profiles showing circCAMSAP1 (*left*), circRAB3IP (*middle*), and circHSPG2 amplification (*right*) by PCR using divergent primers (*bottom*) with sizes indicated (*cartoons*). Size ladders are run in the first lane of each gel. **: PCR artefacts. (***D***) Bar plot showing the number of circRNAs detected in polyA-selected (*grey*), nascent "factory" (*red*), total RNA fractions (*black*), and in control (*light grey*) and ADAR-KD libraries (*green*) from HUVECs. (***E***) Bar plots (*left*) showing fold-reduction in *ADAR1* levels upon knockdown from two replicates in HUVECs. Western blotting (*right*) confirms ADAR1 knockdown and β-tubulin levels provide a control. (***F***) As in panel B, but for control (*light grey*) and ADAR1-KD libraries (*green*) from HUVECs. (***G***) Bar plots showing changes in DCC ratios for circCAMSAP1 and circRAB3IP upon ADAR-KD (two replicates). (***H***) Bar plot showing relative enrichment of circCAMSAP1, circRBA3IP or their mRNA counterparts in subcellular fractions as determined by RT-qPCR (from two replicates). U1 snRNA levels provide a control. (***I***) Representative RNA FISH images using probes targeting circCAMSAP1 (*red*) and intron 1 in *CAMSAP1* RNA (*green*) in the absence (control) or presence of 100x excess of unlabeled circRNA probe. (***J***) As in panel I, but for circCAMSAP1 in cells pretreated or not (control) with RNase A.

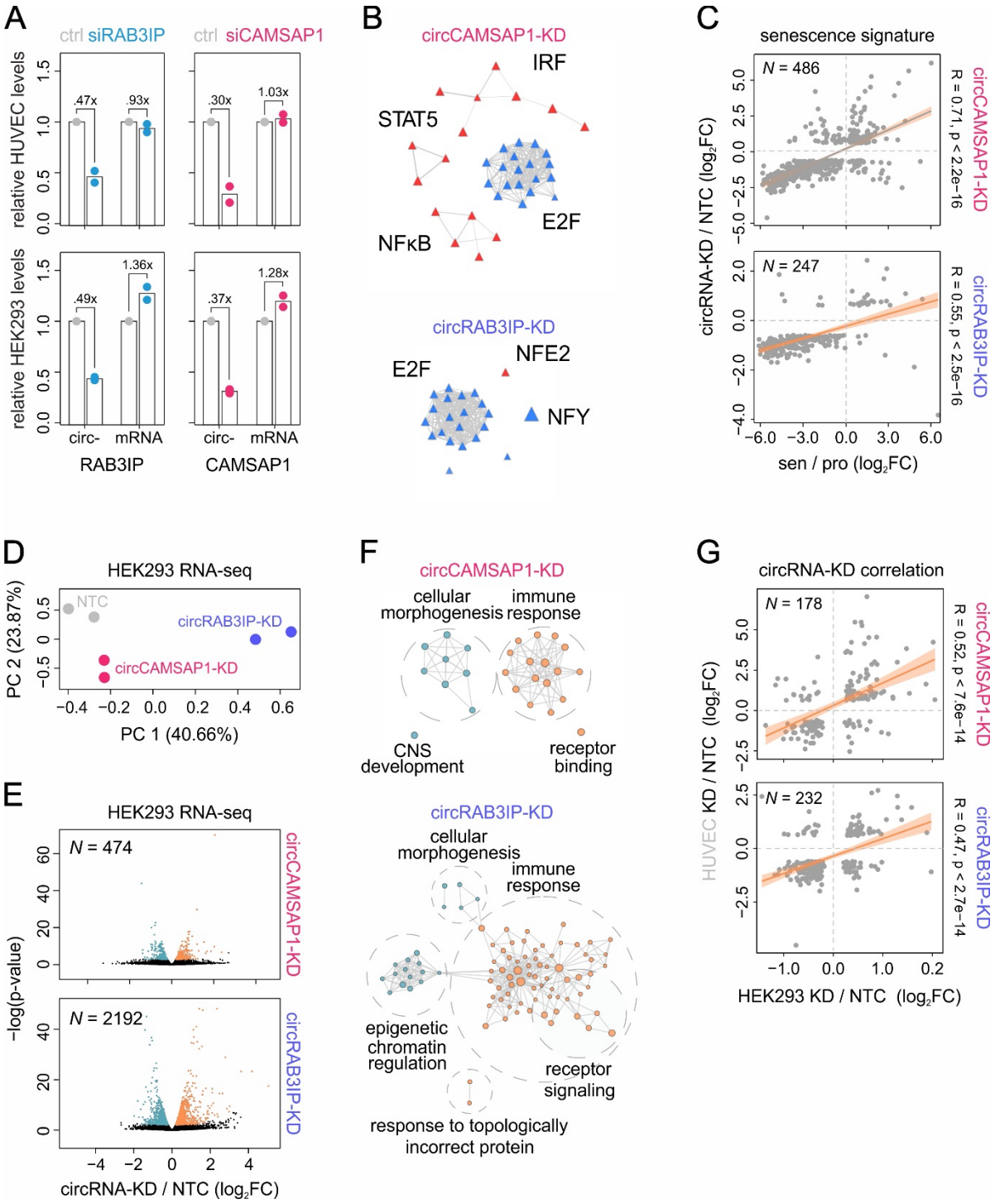

FIGURE S2. circCAMSAP1- and circRAB3IP-KD trigger transcriptional changes in HEK293 cells. (***A***) Bar plots showing fold-reduction in circRAB3IP, circCAMSAP1, and their respective mRNA levels upon knockdown in HUVECs (*top*) or HEK293 (*down*; from two replicates). (***B***) Network plot of regulatory transcription factor enrichment for genes differentially expressed upon circCAMSAP- (*top*) or circRAB3IP-KD (*bottom*) inferred

via overrepresentation analysis. (***C***) Scatter plot correlating circCAMSAP1- (*top*) and circRAB3IP-KD RNA-seq (*bottom*) to the gene expression signature of IMR90 replicative senescence. Pearson correlation coefficients (R) and their associated p-values for overlapping DEGs (*N*) are indicated. (***D***) PCA plot of RNA-seq replicates of circCAMSAP1- and circRAB3IP-KD in HEK293 cells. (***E***) Volcano plots showing fold changes (log_2) for up-/downregulated genes (*orange*/*blue*) following HEK293 circCAMSAP1- (*top*) or circRAB3IP-KD (*bottom*) at a significance cut-off set of $-log_{10}(p\text{-}value) \geq 1.3$. (***F***) Gene set enrichment analysis for genes differentially expressed in circCAMSAP1- (*top*) and circRAB3IP-KD (*bottom*) in HEK293 (absolute log_2 fold change ≥0.6 and $p_{adj} \leq 0.05$). Clusters of related terms were merged and labelled by the group-dominant gene ontology terms. (***G***) As in panel C, but correlating HEK293 to HUVEC circRNA-KD DEGs.

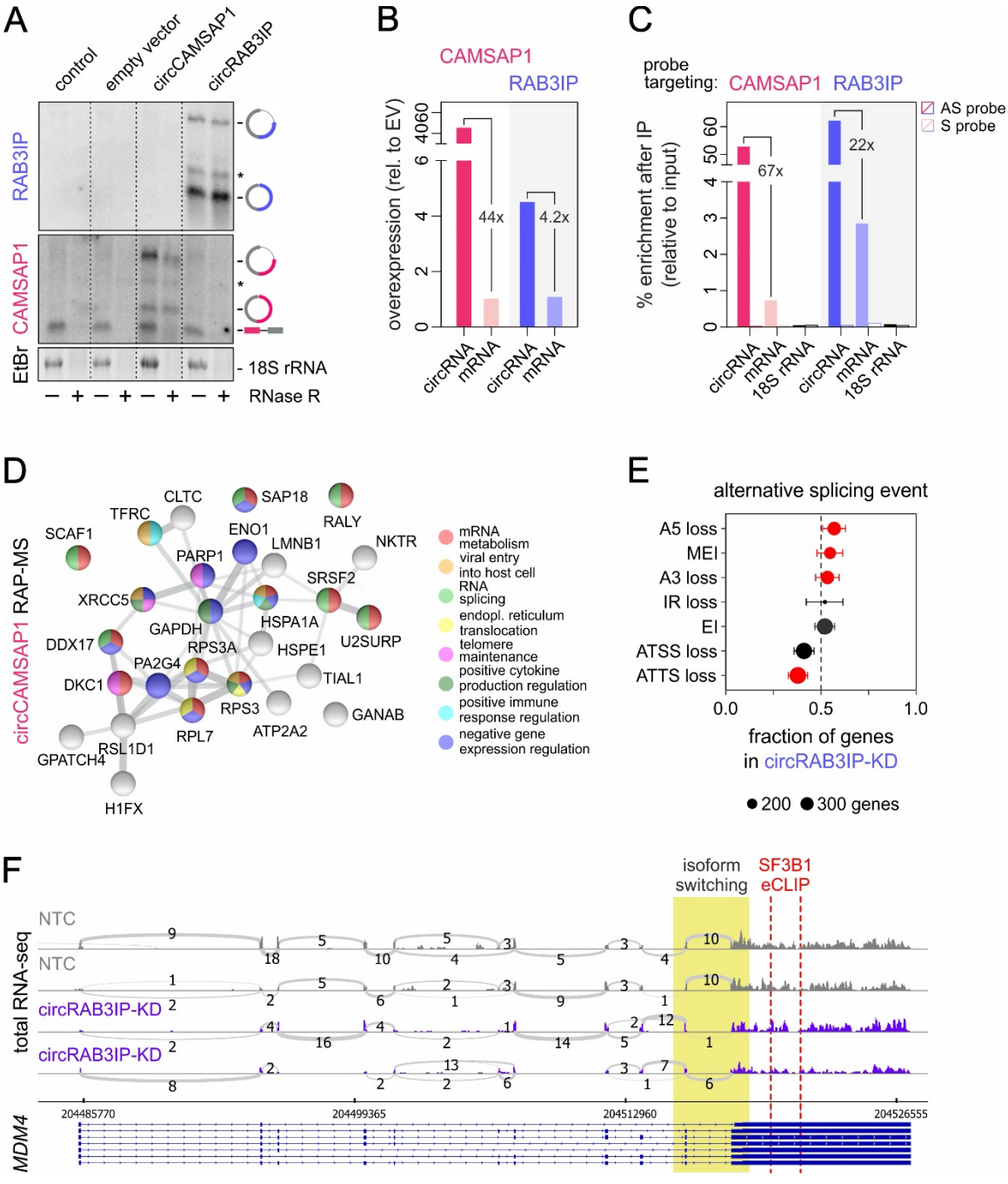

FIGURE S3. circRNA RAP-MS and circRAB3IP-KD effects on transcript isoform usage. (***A***) Northern blot for untransfected (control), empty vector (EV), and pcDNA3-circRNA-transfected HEK293 before or after RNase R treatment. 18S rRNA levels provide a control. *: artefactual concatamers. (***B***) Bar plots of circCAMSAP1 and circRAB3IP overexpression levels in HEK293 (from two replicates) normalized relative to *YWHAZ* and represented as fold-change over empty vector (EV) cells. mRNA levels of the respective host genes provide a baseline. (***C***) Bar plots showing per cent circCAMSAP1 and circRAB3IP enrichment after RAP-MS in HEK293 (from two replicates) normalized relative to input. Fold-enrichments over *CAMSAP1* or *RAB3IP* mRNA are

shown. 18S enrichment as well as enrichments following the use of sense probes (S) provide a control. (***D***) STRING interaction network of proteins co-purifying with circCAMSAP1 in RAP-MS. Proteins enriching for a particular GO term (*key on the right*) are color-coded. (***E***) Genome-wide changes of alternative splicing events (with ≥ 10% change) in circRAB3IP- KD data. Dot size indicates the number of genes and significant changes (*red*) are supported by an FDR < 0.05. A5, alternative 5' donor site; A3, alternative 3' donor site; MEI, multiple exon inclusion; ES, exon inclusion; IR, intron retention; ATSS and ATTS, alternative transcription start and transcription termination sites, respectively. (***F***) Sashimi plot for *MDM4* in control (NTC) and circRAB3IP-KD HUVECs. The number of normalized read counts for each exon-exon junction are indicated. The exons involved in isoform switching (*yellow rectangle*) and positions bound by SF3B1 (*red dashed line*) are highlighted.

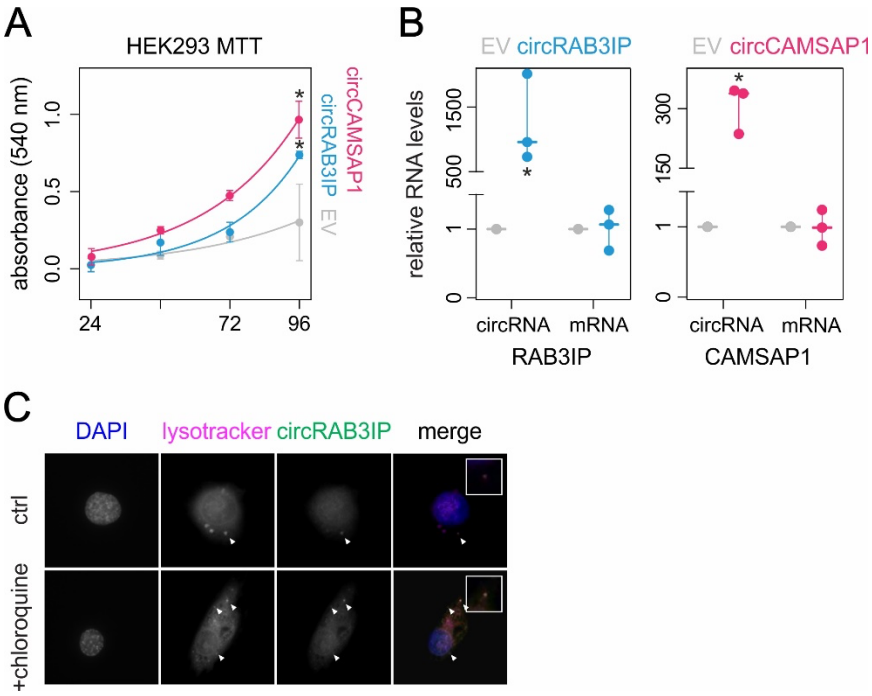

FIGURE S4. Effects of circRNA overexpression in human cells. (***A***) MTT assays (±SD from three replicates) under stable circRAB3IP (*blue*), circCAMSAP1 (*magenta*) or empty vector overexpression (*grey*). Relative absorbance was plotted relative to the 24-h time point. Doubling times were 17.4 h for circCAMSAP1-, 22.7 h for circRAB3IP- and 28.1 h for EV-overexpression. *: significantly different to EV control; $p < 0.05$, unpaired two-tailed Student's t-test. (***B***) Plot showing circRAB3IP (*left*) and circCAMSAP1 overexpression levels (*right*) in the cells used in panel A (three replicates) normalized to *YWHAZ* and represented as fold-change over empty vector (EV) levels. *: significantly different to EV control; $p < 0.05$, unpaired two-tailed Student's t-test. (***C***) Representative circRAB3IP FISH images of HUVECs pretreated or not (ctrl) with 10 µM chloroquine for 2 h and counterstained with DAPI. circRAB3IP signal foci are indicated (*arrowheads*).

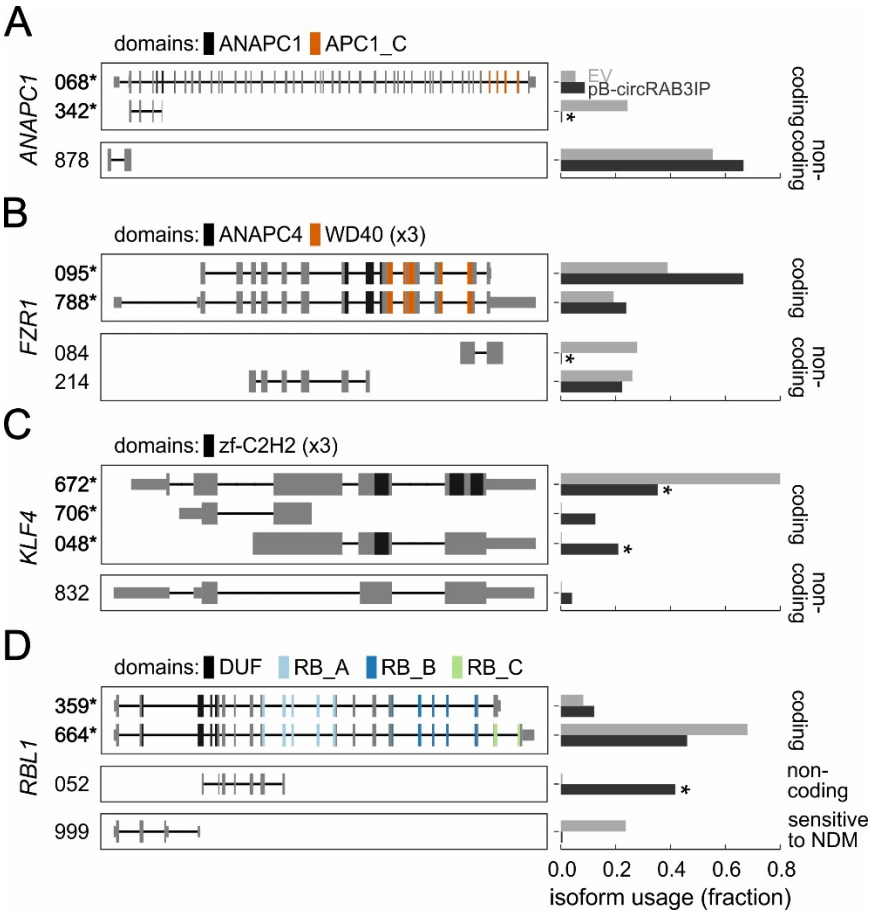

FIGURE S5. Isoform switching following paracrine pB-circRAB3IP treatment of HUVECs. Illustrations (*left*) showing detected (***A***) *ANAPC1*, (***B***) *FZR1*, (***C***) *KLF4*, and (***D***) *RBL1* isoforms. Functional domains in each isoform (color code, *top*) and coding isoforms (*) are indicated. Bar plots (*right*) showing usage changes between pB-circRAB3IP-treated (*black*) and control HUVEC RNA-seq data (*grey*) for each isoform. *significantly different to control: FDR ≤ 0.001.

TABLE S1. List of circRNA junctions, their respective read counts and DCC ratios from factory, chromatin and total RNA-seq experiments, as well as from ADAR-KD in HUVECs (provided as an *.xlsx* file).

TABLE S2. List of peptides identified via RAP-MS experiments in HEK293 cells overexpressing circCAMSAP1 or circRAB3IP. Usual MS contaminants are marked in grey (provided as an *.xlsx* file).

TABLE S3. Oligonucleotides used as PCR primers, siRNAs or cloning primers

PCR targets	forward primer (5'-3')	reverse primer (5'-3')
circRAB3IP	GTTCTGGAGGCTGTGGAAAA	GACACGTCCTGTCCATTGTG
RAB3IP mRNA	GAGGCTTTCAAAAGTGCGAG	TGTTTTTCTGCTGTTGCCTG
circCAMSAP1	CCCTGATGATGGCCTACACT	CTCAGGGATGTTATCTTGTTGAT
CAMSAP1 mRNA	ATTGCCCAGAGCAGATGAAA	ATACAGACTGTCGGCCATCG
NFKBIA mRNA	AGACCTGGCCTTCCTCAACTTCC	AGTCTCGGAGCTCAGGATCACAG
IL32 mRNA	CGAATGCACCAGGCCATAGA	GACATCACCTGTCCACGTCC
CCL20 mRNA	TATTGTGGGCTTCACACGGC	GATTTGCGCACACAGACAAC
TNFAIP3 mRNA	TCGACAGAAACATCCAGGCCAC	TGTCCTGAACGCCCCACATGT
U1 snRNA	CAGGGGAGATACCATGATCACGAAG	GGTCAGCACATCCGGAGTGCAATGG
cloning targets	**Sequence (5'-3')**	
circCAMSAP1-F-pcDNA (*Bam*HI)	GATCGGATCCCATGGGTGTTCACCCTCTGT	
circCAMSAP1-F-pB (*Mlu*I)	GATCACGCGTCATGGGTGTTCACCCTCTGT	
circCAMSAP1-R-pcDNA (*Not*I)	GATCGCGGCCGCTATTCACTGGCCAGGTGTGG	
circCAMSAP1-R-pB (*Pac*I)	GATCTTAATTAAGACTGGTCCCAAATGAAGGG	
circCAMSAP1-InvF-pcDNA (*Apa*I)	GATCGGGCCCATGGGTGTTCACCCTCTGT	
circcircCAMSAP1-InvF-pB (*Pac*I)	GATCTTAATTAAGAGCAACTAACAACGTGAAAAGG	
circCAMSAP1-InvR-pcDNA (*Not*I)	GATCCCGCGGCCGCGAGCAACTAACAACGTGAAAAGG	
circCAMSAP1-InvR-pB (*Spe*I)	GATCACTAGTCATGGGTGTTCACCCTCTGT	
circRAB3IP-F-pcDNA (*Not*I)	ATCGGCGGCCGCACAGTTCAGCCAGTCTCAGT	
circRAB3IP-F-pB (*Mlu*I)	ATCGACGCGTACAGTTCAGCCAGTCTCAGT	
circRAB3IP-R-pcDNA (*Xho*I)	ATCGCTCGAGCTGCAACCATAACATTTCAA	
circRab3IP-R-pB (*Pac*I)	ATCGTTAATTAACTGCAACCATAACATTTCAA	
circRAB3IP-InvF-pcDNA (*Apa*I)	TCGAGGGCCCACAGTTCAGCCAGTCTCAGT	
circRAB3IP-InvF-pB (*Pac*I)	ATCGTTAATTAACGCTTTACAAAACCAAAACT	
circRAB3IP-InvR-pcDNA (*Xho*I)	TCGACTCGAGAAATTGAACCACTCTTGGTG	
circRAB3IP-InvR-pB (*Spe*I)	ATCGACTAGTACAGTTCAGCCAGTCTCAGT	
siRNA targets	**Sequence (5'-3')**	
circCAMSAP1	GATCAACAAGATAACATCCCT	
circRAB3IP	GGAGGACCAAAGCTGACTTAT	
ADAR1-1	CGCAGAGUUCCUCACCUGUTT	
ADAR1-2	GGAUGCAAAUCAAGAGAAATT	

TABLE S4. Oligonucleotides used as probes in circRNA FISH, RAP-MS, and Northern blots.

circRNA FISH	sequence (5'-3')
circCAMSAP1 ex.3-2	TCTCTGAGGTCCTCAGGGATGTTATCTTGTTGATCCAGAACACCATGGCATCCTC
circCAMSAP1 intron 1	AAAGCCAGTCTCAGAAAGAGACATCATTGGCAAAGGTGTAAAAACTAGTTCGTGGT TCAGATTGTGAATTCATCATCCGATGAGTCAACAAGGATTAAAGAGTGACCTGGT CCTGTAAATTTAAAGAGAGTCTCAATTTTTACACAGTTTTACACAGGAGGTCTAA
circRAB3IP ex.8-7	GAATTCATTATACAAGGATAAGTCAGCTTTGGTCCTCCGCATTCAACTGCAGAAG
circHSGP2 ex.61-intron	CGCTGCTGTGGCTCCACTCTGTACCTGGGACGCTGGGGTCACCAGCTGGCTCAGG
RAP-MS	**sequence (5'-3')**
circCAMSAP1-Sense	TCTCTGAGGTCCTCAGGGATGTTATCTTGTTGATCCAGAACACCATGGCATCCTC
circCAMSAP1-AS	GAGGATGCCATGGTGTTCTGGATCAACAAGATAACATCCCTGAGGACCTCAGAGA
circRAB3IP-Sense	GAATTCATTATACAAGGATAAGTCAGCTTTGGTCCTCCGCATTCAACTGCAGAAG
circRAB3IP-AS	CTTCTGCAGTTGAATGCGGAGGACCAAAGCTGACTTATCCTTGTATAATGAATT
Northern blot	**sequence (5'-3')**
circRAB3IP	AAAGGACACGTCCTGTCCATTGTGGGCTCATCCTTCCACAATCGGAATTCATTATAC AAG
circCAMSAP1	TGCGCGACTGAGGTCGGACTCTGTCACGGGGGTGTCATCACTCTCCATC