Supplementary Information

Materials and Methods

Astrocytoma cell lines

The well-characterized permanent human astrocytoma cell lines A172 (CRL-1620^{TM}), U118 MG (HTB-15^{TM}) and U87 MG (HTB-14^{TM}), derived from patients with malignant glioblastoma, were obtained from American Type Culture Collection and kindly supplied by Dr. S. Galardi (University of Tor Vergata, Rome, Italy). All cell lines, including U118 stably transfected with EGFP vector, EGFP-ADAR2, and EGFP-ADAR2 E/A, were grown in DMEM supplemented with 10% FCS and antibiotics, at 37°C in 5% CO_2. Stably transfected U118 cells were polyclonal to avoid problems due to positional insertion effects. Both the active and inactive ADAR2 proteins were fused with EGFP at the amino-terminus to track correct cell localization as well as to measure and compare the average level of expression of ectopic proteins among cell lines (MIF= mean intensity of fluorescence). For *in vitro* assays cells were cultured for no more than 4-6 passages.

Proliferation assay

$5x10^4$ cells were seeded in 35mm dishes and viability (Trypan blue dye exclusion) was determined daily, from day 1 to day 4. Assays were repeated five times in duplicates.

Apoptosis

$5x10^4$ cells were seeded in 35mm dishes and apoptotic cells were evaluated daily, from day 1 to day 4. Cells were stained with Annexin V (BD Pharmingen^{TM}) and propidium iodide (PI) and analyzed on a FACSCanto II flow cytometer (Beckton Dickinson) using the DIVA software.

Cell-cycle analysis

$5x10^4$ cells were seeded in 35mm dishes and cell-cycle distribution was evaluated daily, from day 1 to day 4. Cells were pulsed with 10 μM BrdU (Sigma) for 30 min at 37°C. After BrdU incorporation,

cells were harvested and fixed in ice-cold 70% ethanol. DNA was denatured with HCl 2N/Triton 20% and labelled with an anti-BrdU antibody (BD Bioscience) for 1 h RT. Cells were labeled with anti-mouse APC-conjugated antibody (Beckton Dickinson). Labeled cells were resuspended in PBS containing 5 mg/ml propidium iodide and analyzed on a FACSCanto II flow cytometer (Beckton Dickinson) using the DIVA software. All flow cytometry experiments were performed at least three times. Additionally, the same number of cells were tested using the Click-iT^{TM} EDU Flow Cytometry Assay Kit (Invitrogen), in accordance with the manufacturer's instructions.

For the *in vivo* cell-cycle analysis, tumor mass was dissected from the mice at day 15 p.i. (at the exponential growth phase of the tumors). Xenograft tumors were washed twice in cold PBS plus antibiotics, cut by scalpel and incubated in 2 ml of DMEM supplemented with 200U/ml of Collagenase II and 200U/ml of Collagenase IV (Sigma) for three hours at 37°C. Single cell suspensions were obtained by filtering the supernatant through 200μM cell strainer (BD Biosciences). $5x10^5$ live cells (Trypan blue dye exclusion) were washed in PBS and plated, and five hours later the Click-iT EDU Flow Cytometry Assay Kit (Invitrogen) was performed according to the manufacturer's instructions. The cells were analysed by FACSCanto flow cytometer (Beckton Dickinson) using the DIVA software.

Analysis of RNA editing and bioinformatics

Total RNA was isolated from tumors, control brain tissues and cultured astrocytoma cell lines with TRIzol (Invitrogen) according to the manufacturer's instructions. Each RNA sample was DNase treated (Ambion, Recombinant DNase I) and quantified with the Agilent 2100 bioanalyzer (Agilent). cDNAs were generated by SuperScript II reverse transcriptase (Invitrogen) using random hexamers or specific primers. Direct sequencing was performed on cDNA pools and editing was calculated as described previously (Cenci *et al.*, 2008). For each sample, 2–3 independent RT-PCR reactions were performed. Primers and PCR conditions are shown in Table S1.

To identify potential A-to-I editing events in *p21*, *p27*, *Skp2* and *CDC14B*, we took advantage of a newly available database including all the editable substrates in humans (DARNED;

http://darned.ucc.ie) (Kiran and Baranov, 2010) and the ExpEdit web server (public domain, http://www.caspur.it/ExpEdit/) (Picardi et al., 2011) together with the brain Illumina short reads from SRA accessions SRP002274 and SRX027129. Briefly, short reads in SRA format were loaded on the ExpEdit web server including per each gene all genomic positions corresponding to adenosine residues. In detail, ExpEdit maps short reads to the reference human genome using the Tophat program (Trapnell *et al.*, 2009) and then returns the nucleotide distribution per each genomic position included in the DARNED database. No relevant editing patterns were found in *p21*, *p27* and *Skp2* mRNAs.

Analysis of mRNA and pre-mRNA expression levels

Gene-specific exon-exon boundary PCR products (TaqMan gene expression assays, Applied Biosystems) were measured by means of a PE Applied Biosystems PRISM 7700 sequence detection system during 40 cycles. *Actin* mRNA was used for normalization and relative quantification of gene expression was performed according to the $2^{-\Delta\Delta Ct}$ method. Real-time assays were repeated in triplicates from two independent RTs. The primers used were supplied by Applied Biosystems: p27, ID Hs01597588_m1; p21, ID Hs00355782_m1; Skp2, ID Hs00180634_m1; CDC14B, ID Hs00269351_m1; ADAR2, ID Hs00953730_m1, exons 5–6; β-actin, ID Hs99999903_m1. *CDC14B* pre-mRNA was tested using 2μg of total RNA and intron-exon specific primers shown in Table S1. Levels of expression are expressed in arbitrary units calculated as a relative-fold increase compared to the control sample arbitrarily set to 1 and normalized to β-actin.

Immunoblotting

30 μg of total protein extracts (RIPA lysis buffer, plus protease inhibitor mixture (Sigma) and phosphatase inhibitor cocktail) were separated by SDS–PAGE, transferred onto nitrocellulose membrane, and analyzed by immunoblotting with the appropriate antibodies and detected with the

ECL system (Amersham Pharmacia Biotech). Specific proteins were quantified by densitometric analysis.

Antibodies

The antibodies used for Western-blot analysis were: anti-ADAR2, anti-p27, anti-p21, anti-Skp2, anti-CDK4, anti-CDK2, anti-cyclin D1, anti-cyclin E and anti-β-actin (Santa Cruz Biotechnology); anti-ADAR1 polyclonal antibody (Cenci *et al.*, 2008) and anti-CDC14B (LifeSpan); anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The antibodies used for immunohistochemistry analysis were: anti-GFP (Abcam); anti-Ki67 (Novocastra Laboratories Ltd); anti-p27, anti-p21, anti-Skp2 and anti-CDC14B (Santa Cruz Biotechnology). *In situ* detection of apoptosis in formalin-fixed, paraffin-embedded samples was performed with the TUNEL assay (Apoptag Plus, Chemicon), according to the manufacturer's instructions.

Statistical analysis

Non parametric Sperman's Rho correlation was used. A p value less than 0.05 was set as the limit for statistical significance.

Supplementary Figure legends

Figure S1a and b

Sequence chromatograms of known ADAR substrates, isolated from normal white matter, ADAR2-U118 and U118 cells. Of note, identical editing levels were found in U118, ADAR2 E/A and vector cell lines (data not shown). The malignant astrocytoma cell line U118 showed low/undetectable editing levels for all the substrates tested, while the ADAR2 over-expressing astrocytoma cell line rescued editing levels at each specific site in all substrates. Moreover, recovered editing levels in the ADAR2-U118 cell line appeared similar to those found in white matter used as control. Editing sites in *ADAR2*, *GLUR5*, *GLUR-6*, *Kv1.1*, *CYFIP2*, *FLNA*, *GLUR-B*, *BLCAP* and *PDE8A1* transcripts are indicated by arrows. The new ADAR-editable substrate *PDE8A1* has been reported to undergo editing at 7 independent sites by both ADAR1 and ADAR2. Interestingly, we found that ADAR2 specifically edits nucleodite 42424 (arrow) but not the nt 42423 and nt 42421 (dots) in both control white matter and ADAR2-U118 cells, demonstrating that ectopic ADAR2 edits only specific adenosines in the target RNA. Similar results were obtained using additional astrocytoma cell lines (A172 and U87), also modified to over-express EGFP-ADAR2 three-fold over endogenous enzyme levels (data not shown). ADAR2 self editing positions -1 and +10 (indicated by arrows) are edited by ADAR2. Notably, the editing sites -2 (dot) within *ADAR2* pre-mRNA is not modified by ADAR2 in our cells, indeed this site is mainly modified by ADAR1 (Dawson et al., 2004).

Figure S2

ADAR1 immunoblotting of total protein extract from U118, ADAR2 and ADAR2 E/A cells. The over-expression of ADAR2 and ADAR2 E/A in U118 astrocytoma cells did not modify the levels of either the 110-kDa and 150-kDa ADAR1 isoforms.

Figure S3

in vivo cell-cycle analysis of U118 and ADAR2 tumor cells isolated from xenograft tumors (at day 15 p.i.). $5x10^5$ live cells were stained for cell-cycle analysis by Click-iT EDU Flow Cytometry Assay and analysed by FACSCanto flow cytometer. Mean ± sd, (n=4), *$p<0.05$ compared to the untreated.

Figure S4

Immunohistochemistry analysis of untreated U118, EGFP, ADAR2 and ADAR2 E/A tumors dissected at the exponential growth phase (15 days post-injection). Representative fields of tumor sections stained with anti-GFP, anti-Ki67 and TUNEL antibodies are shown. The related percentage of positive cells for GFP, Ki67 and TUNEL are shown in Figure 2c.

Figure S5

CDC14B as a novel ADAR substrate. (a) Diagram summarizing the investigated pathway. (b) A pre-mRNA portion of *CDC14B* undergoes editing events: AluJr (in gray) and AluSx (underlined) are shown. (c) dsRNA structure of AluJr and AluSx within intron 7-8 of the *CDC14B* RNA predicted by the Zuker algorithm (ΔG= -391). (d) Sequence chromatogram of the endogenous *CDC14B* transcript (AluJr) in human brain white matter. Similar editing levels were observed in the ADAR2 U118 cell line. Arrows indicate the 13 editing positions (named 1I to 13I). Percentages of editing are reported. (e) Particular of the dsRNA structure boxed in red in panel (c).

Figure S6

qRT-PCR of *CDC14B* in different astrocytoma cell lines (A172, U118, U87) upregulated with ADAR2 (black) or ADAR2 E/A (gray).

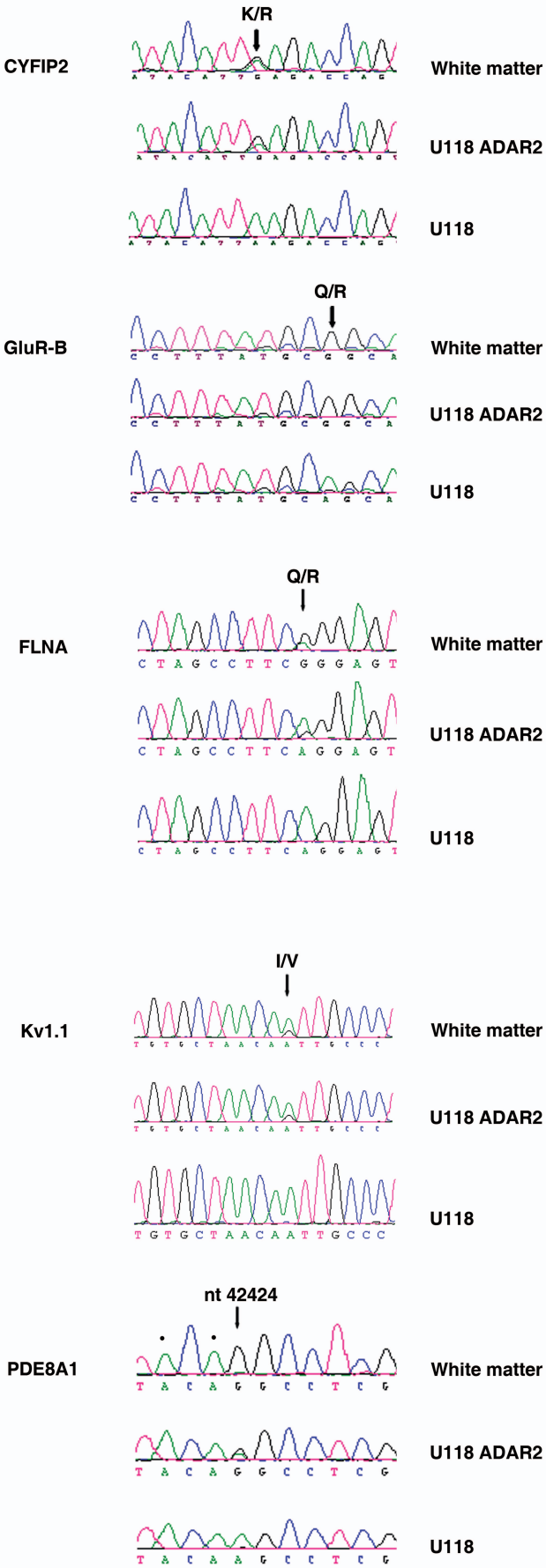
Figure S7

(a) Western blotting analysis of CDC14B at 48 and 72 hours after transfection of either CDC14B-flag or C314S-CDC14B-flag in U118 (left panel) and in U87 (right panel) cells. Dot indicates non-specific

band. (b) Experimental diagram and qRT-PCR of the *CDC14B* mRNA levels in U118-ADAR2 cells silenced for endogenous *CDC14B*. About 90% *CDC14B* silencing was maintained at 72 hours and 96 hours post transfection. *Error bars* indicate standard deviations of two independent experiments. The level of expression is represented in arbitrary units calculated as a relative-fold increase in expression when compared to the control sample (U118-ADAR2). Each sample was normalized to *β*-actin mRNA. (c) Experimental diagram and semiquantitative assay for ectopically over-expressed CDC14B-flag and inactive C314S CDC14B-flag using vector-specific primers.

Figure S8

Editing events within the *CDC14B* intron 7-8 and the recognition sequence for intronic splicing enhancers (ISE) and alternative conserved exons (ACEs), as identified in http://genes.mit.edu/acescan2/index.html.


CYFIP2
K/R
White matter
U118 ADAR2
U118
GluR-B
Q/R
White matter
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White matter
U118 ADAR2
U118
Kv1.1
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PDE8A1
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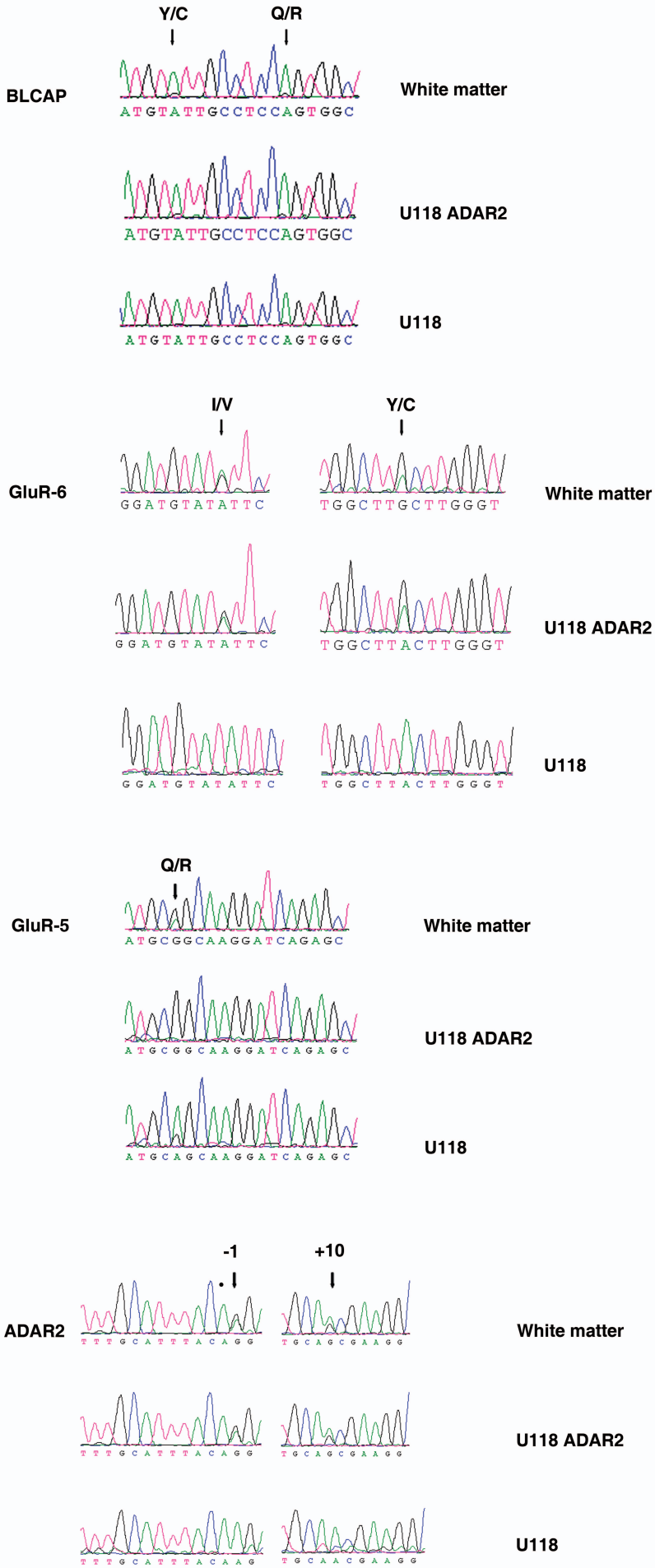
White matter
U118 ADAR2
U118

Figure S1a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Q/R
BLCAP
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I/V
Y/C
GluR-6
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TGGCTTGCTTGGGT
White matter
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TGGCTTACTTGGGT
U118 ADAR2
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TGGCTTACTTGGGT
U118
Q/R
GluR-5
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White matter
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U118 ADAR2
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U118
-1
+10
ADAR2
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U118

Figure S1b

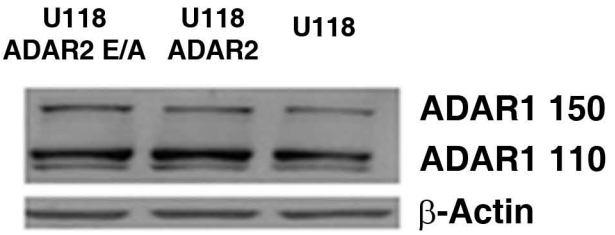


Figure S2

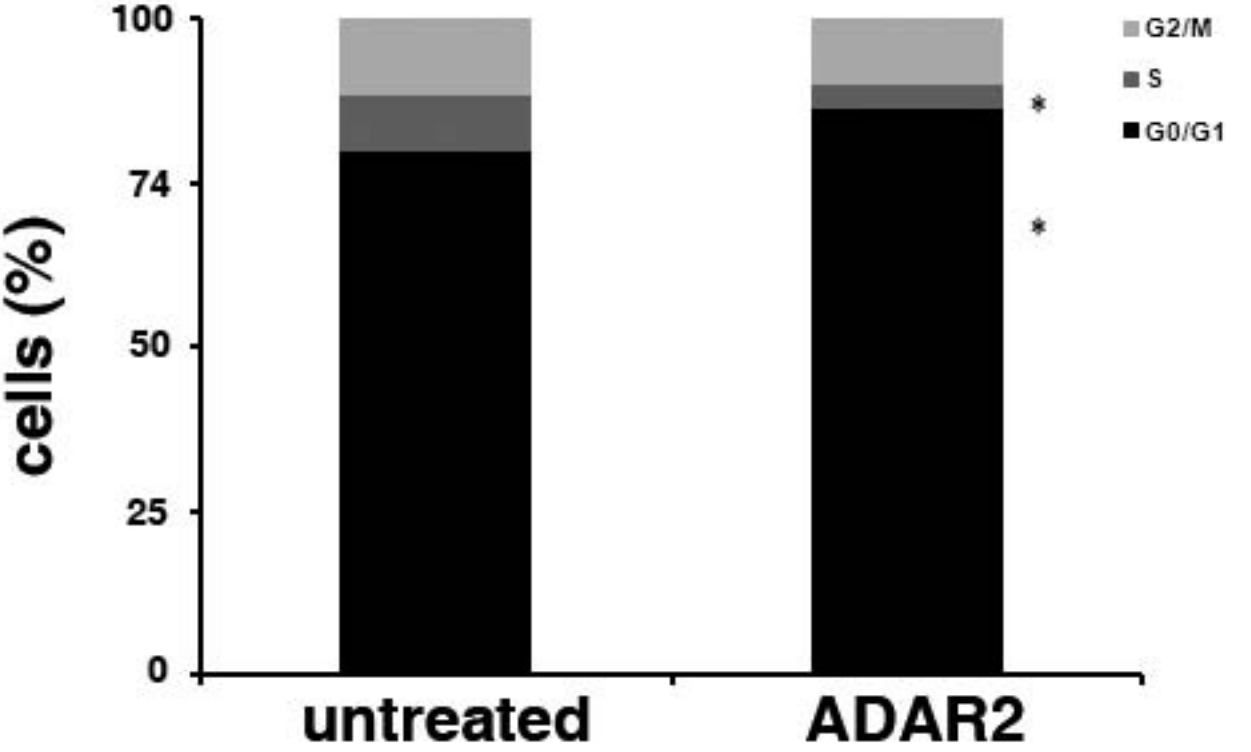


Figure S3

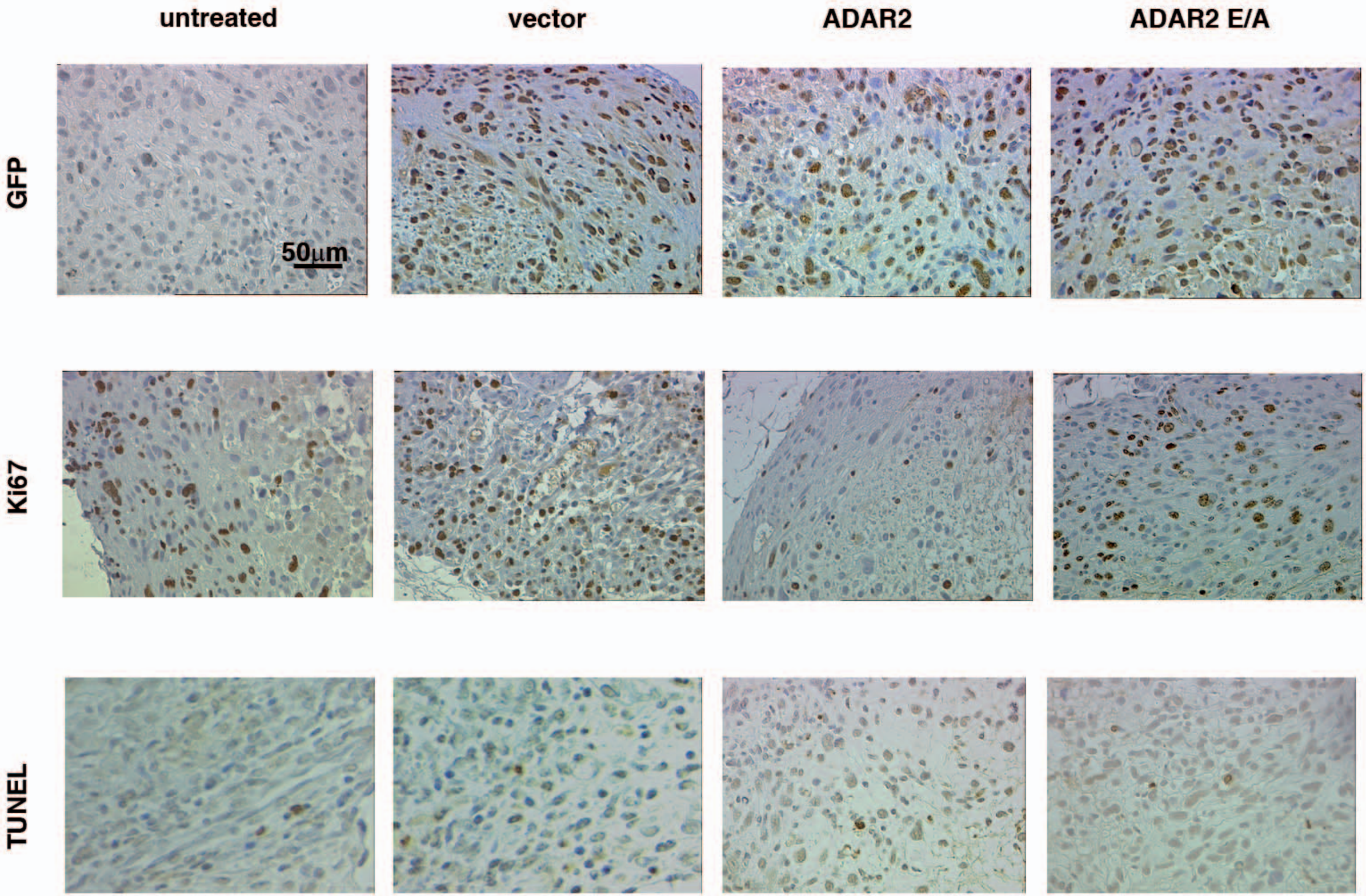
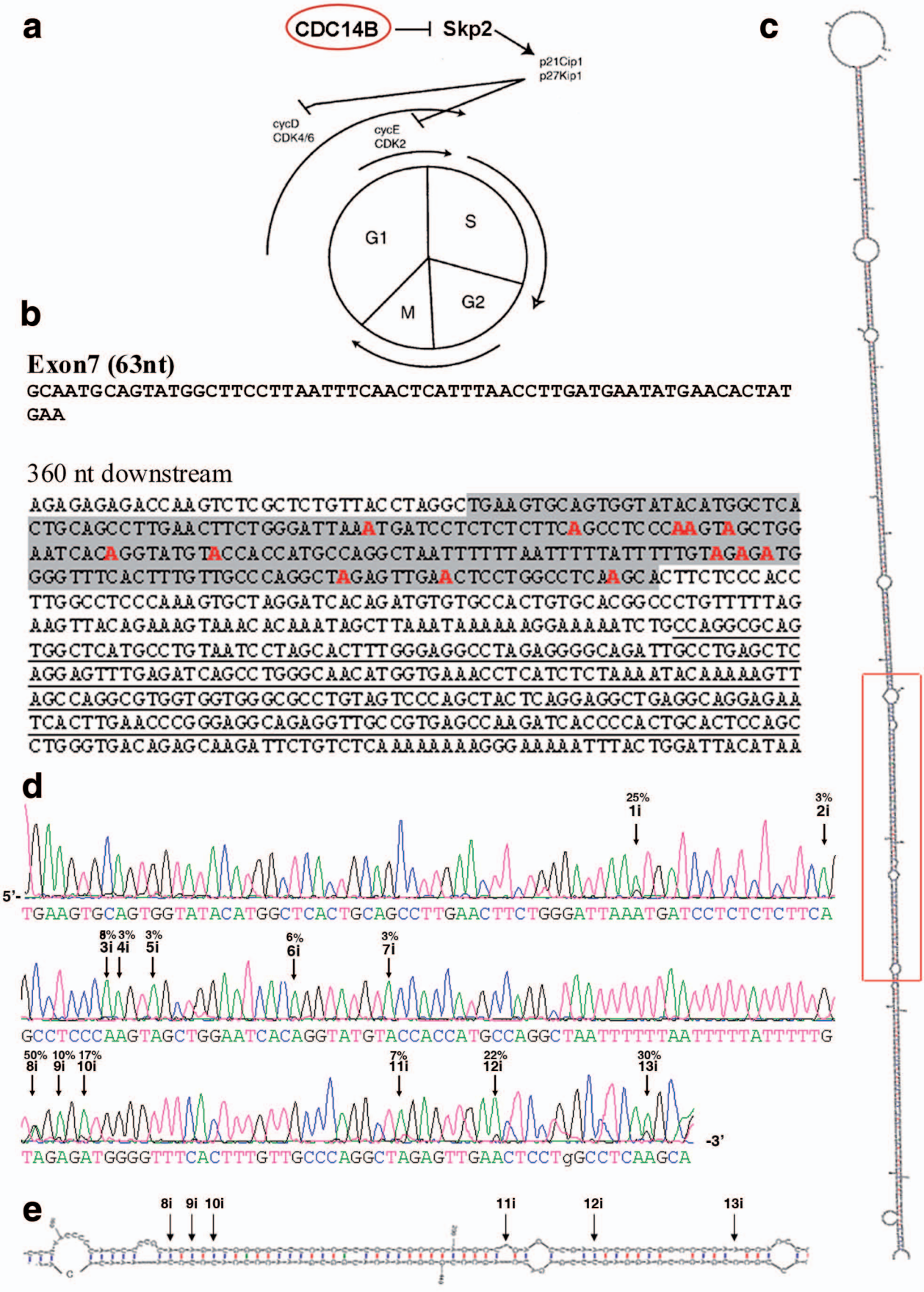


Figure S4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CDC14B
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p21Cip1
p27Kip1
cycD
CDK4/6
cycE
CDK2
G1
S
M
G2
b
Exon7 (63nt)
GCAATGCAGTATGGCTTCCTTAATTTCAACTCATTTAACCTTGATGAATATGAACACTAT
GAA
360 nt downstream
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Figure S5

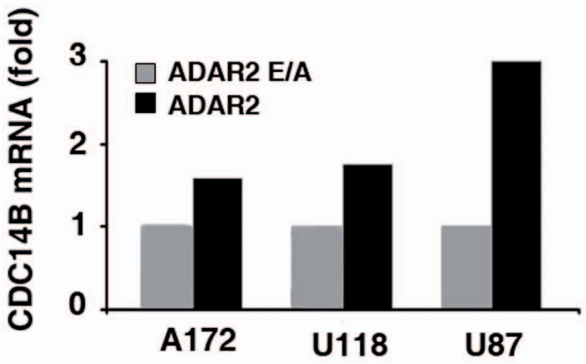
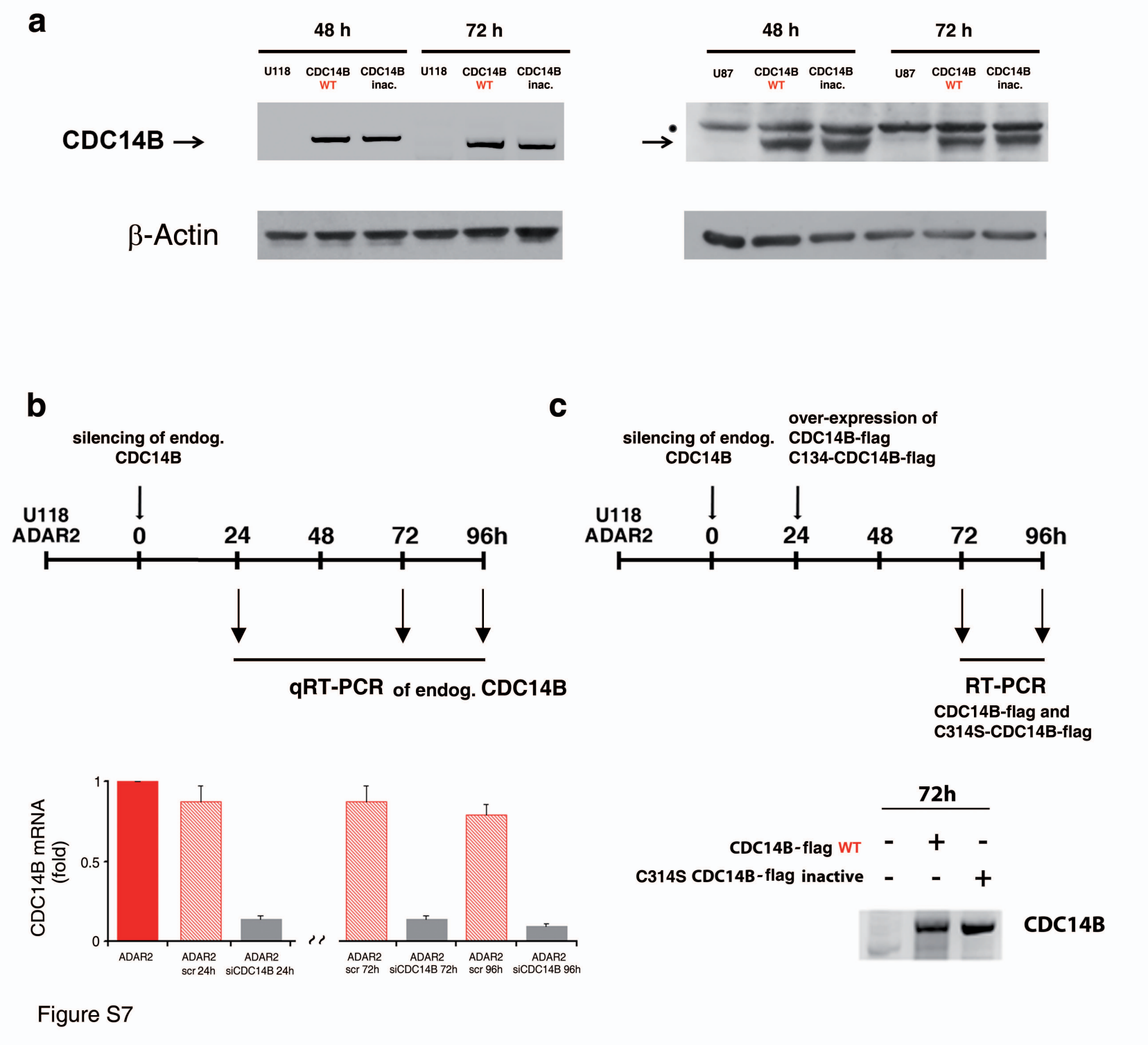


Figure S6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72 h
U118
CDC14B WT
CDC14B inac.
U118
CDC14B WT
CDC14B inac.
CDC14B →
β-Actin
48 h
72 h
U87
CDC14B WT
CDC14B inac.
U87
CDC14B WT
CDC14B inac.
b
silencing of endog. CDC14B
U118 ADAR2
0
24
48
72
96h
qRT-PCR of endog. CDC14B
c
silencing of endog. CDC14B
over-expression of CDC14B-flag C134-CDC14B-flag
U118 ADAR2
0
24
48
72
96h
RT-PCR
CDC14B-flag and C314S-CDC14B-flag
CDC14B mRNA (fold)
1
0.5
0
ADAR2
ADAR2 scr 24h
ADAR2 siCDC14B 24h
ADAR2 scr 72h
ADAR2 siCDC14B 72h
ADAR2 scr 96h
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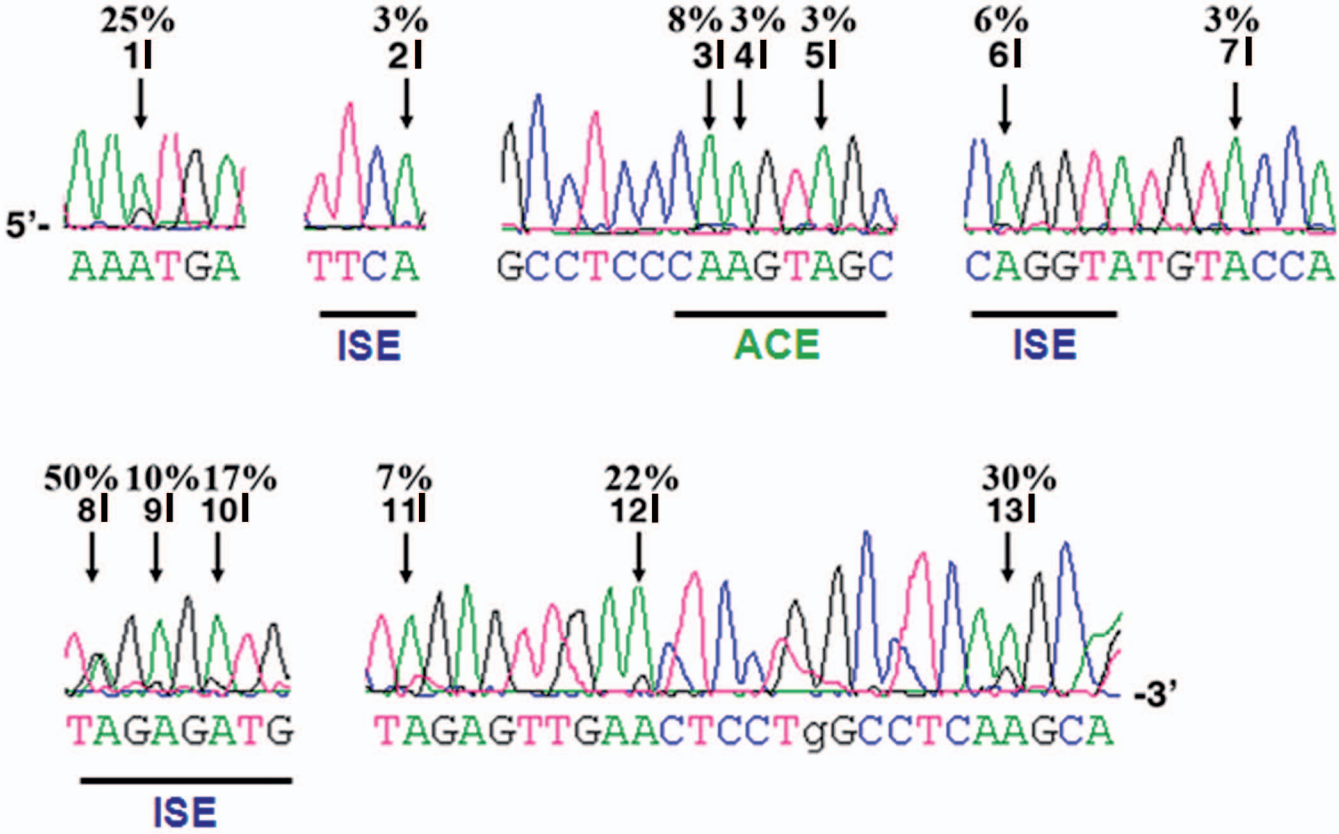
72h
CDC14B-flag WT - + -
C314S CDC14B-flag inactive - - +
CDC14B

Figure S7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TTCA
GCCTCCCAAGTAGC
CAGGTATGTACCA
ISE
ACE
ISE
50%
8I
10%
9I
17%
10I
7%
11I
22%
12I
30%
13I
-3'
TAGAGATG
TAGAGTTGAACTCCTgGCCTCAAGCA
ISE

Figure S8

Table S1

Target RNA	Primers	Oligonucleotides sequence 5'-3'	Annealing	PCR products length
BLCAP	BC10 Rev	TCTGCTTCCTTGGAAAGCTAACAG	60°C 30s	201bp
	BLCAP Fw	TCCCATTAGGTCGGTTCCTG		
	BLCAP Rev	AATTGTGCAAGGCTTCCGTT		
GluR6	hGluR6 Rt	ATACGAAGAAATGATGATAAGTG	60°C 40s	390 bp
	hGluR6 Fw	ACCTTGCAGTTGCTCCACTGG		
	hGluR6 Rev	CCAAATGCCTCCCACTATCC		
GluR5	hGluR5 Fw	GCTTGGGAGTCAGCTGTGTA	55°C 30s	290bp
	hGluR5 Rev	ATGGGGGATTCCATTCTCTC		
ADAR2	hADAR2-Intron1 Fw	GGTATTAGTCACTAAGCAAAGTGTCAG	62°C 30s	289bp
	hADAR2-Exon2 Rev	CCCAGGTGTTGCTGCCATCCTTGG		
CYFIP2	hCYFIP2 Fw	CTACCTAATGGATGGAAATGTCAGTAAC	60°C 30s	241bp
	hCYFIP2 Rev	CCCGGATCTGAACCATCTG		
GluRB Q/R	hGluRB R/G Rev	CAAGGATGTAGAATACTCCAGCAAC	60°C 40s	839 bp
	hGluRB Q/R Fw	TTTAGCCTATGAGATCTGGATGTGC		
	hGluRB Q/R Rev	GTGTAGGAGGAGATTATGATCAGG	60°C 40s	290bp
FLNA	hFLNA Fw	AGATCTCTTTTGAGGACCGCAAGG	60°C 30s	310bp
	hFLNA Rev	TGGTCAATTTCTGTGACATAGCACTCC		
KV1.1	hKv F1	TCGGGGTCATCCTGTTTTCTAGTG	66°C 30s	318bp
	hKv R1	GGTCACTGTCAGAGGCTAAGTTAGG		
	hKv F2	AGTCGCACTTCTCCAGTATCCC	68°C 30s	205bp
	hKv R2	CCCTCAGTTTCTCGGTGGTAGA		
PDE8A	hPDE8A Fw	GGTTCTTAGTATATTCACAGT	58°C 1m	432 bp
	hPDE8A Rev	TTCAGACTCCTCTGGGAAAGC		
CDC14B intron7	hCDC14B Rt	ATTGCTTCTGTAAGTGAAATGGG	62°C 30s	290bp
	hCDC14B Fw	AGTGCAGTGGTATACATGG		
	hCDC14B Rev	ATTAAGAAAGCTAAAAACAGGGCC		
CDC14B mRNA	hCDC14B 12-14 Rev	CTTGAAATGGAGAGAGTGAGAGG	63°C 30s	794 bp
	hCDC14B 7 Fw	GCAATGCAGTATGGCTTCCT		
	hCDC14B 4-5 Fw	AAATTGTTCATTTTATGGCTCTGA		1008 bp
CDC14B pre-mRNA	hCDC14B exon8 Fw	GCAGAAAATCCAGATTTAAATTGG	62°C 40s	393 bp
	hCDC14B intr-exon 8-9 Rev	TTGGTGGTAACCTACATAAAGAAATG		