siRNA-mediated epigenetic control of alternative splicing

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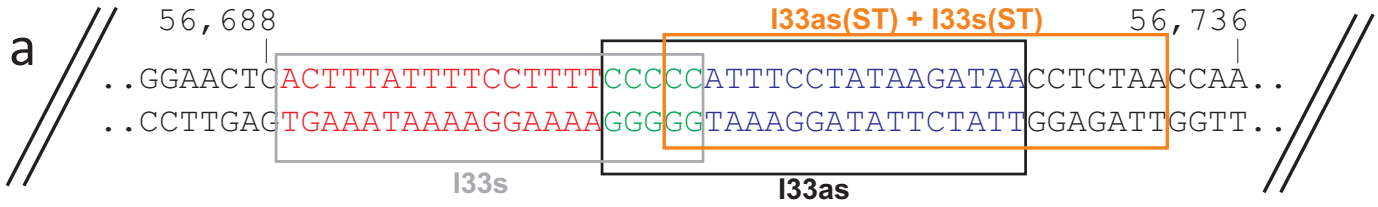
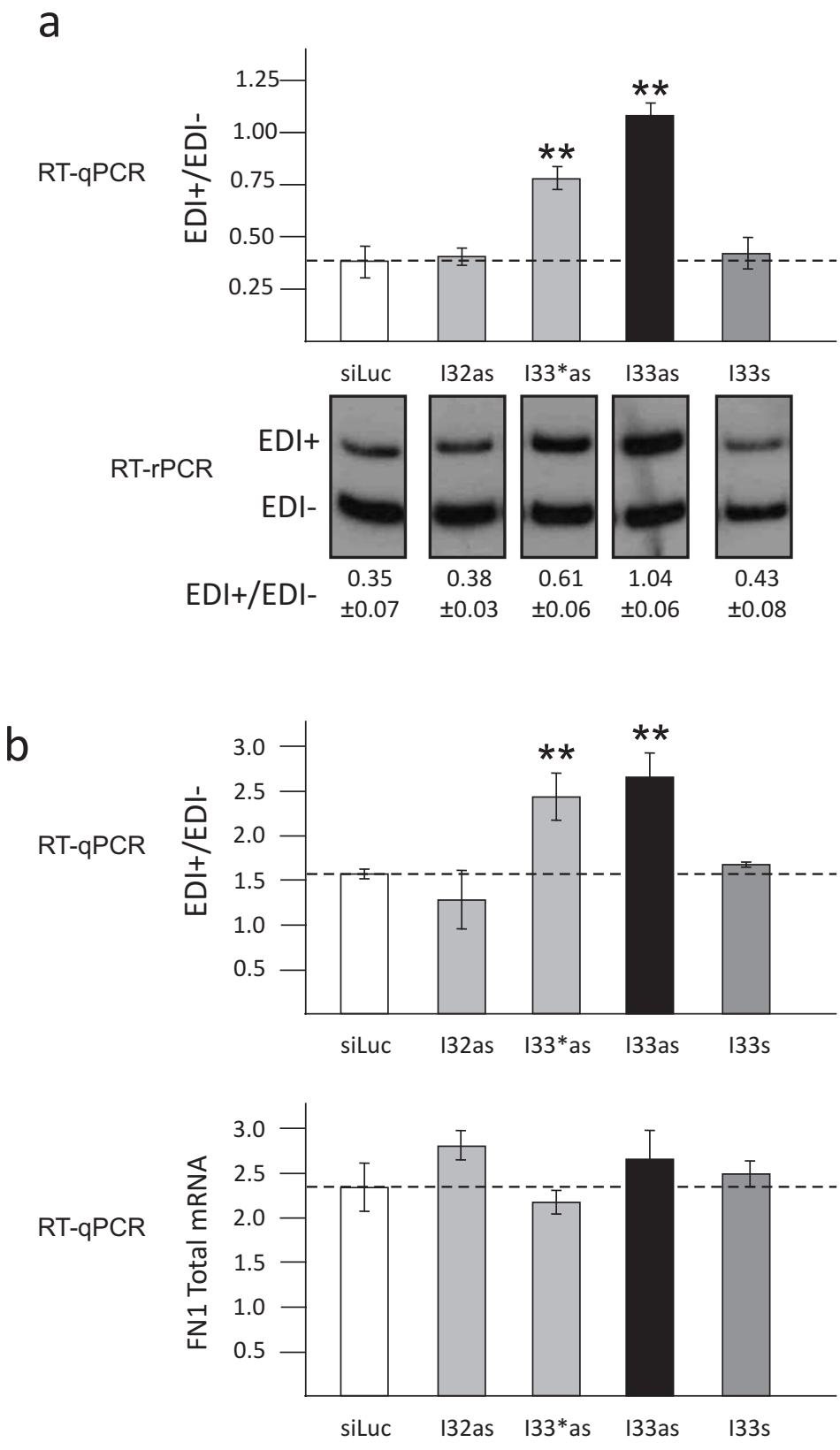
Sup. Fig. 1



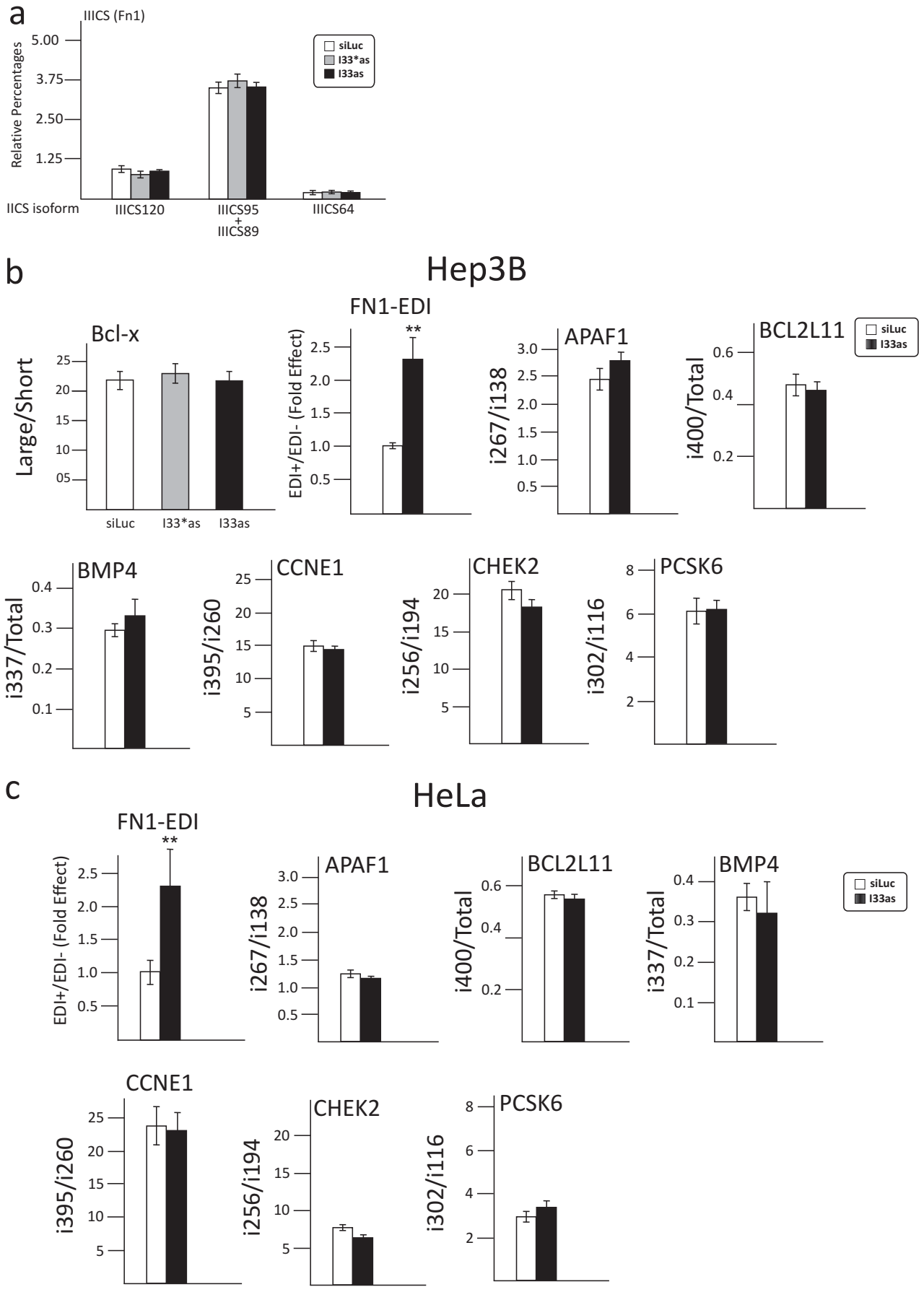
b

Supplementary Figure 1
a, Target sequence of intronic siRNAs I33s (gray frame), I33as (black frame), I33as(ST) and I33s(ST) (orange frame). Numbers indicate base position from transcription start site of the FN1 gene. **b**, Intronic and exonic siRNAs sequences are shown. Free energies (DG) were calculated from the first four positions of each 5' end (highlighted).

Sup. Fig. 2



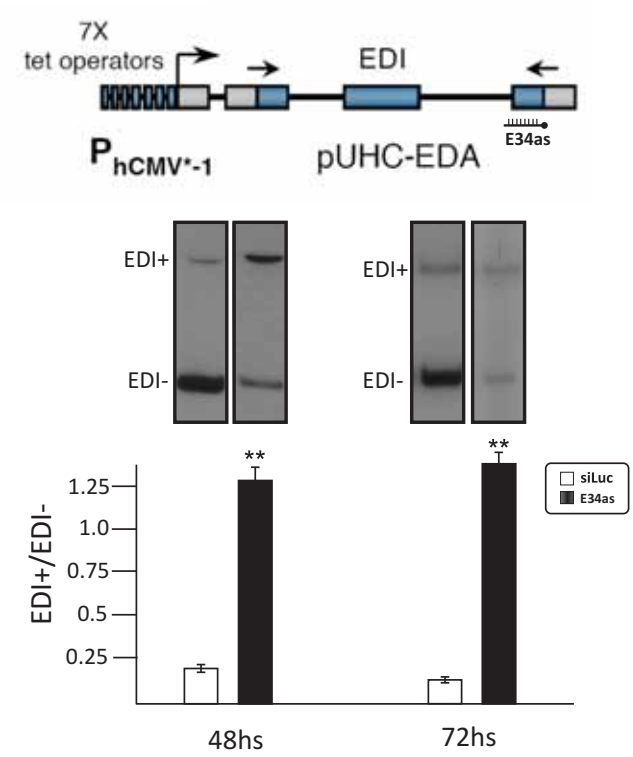
Supplementary Figure 2
a, Validation of RT-qPCR by comparison with conventional radioactive RT-rPCR, followed by gel electrophoresis analysis. The same RNA samples were used showing similar effects of siRNAs to introns 32 and 33 on EDI alternative splicing of the endogenous FN gene in Hep 3B cells. **b**, Intronic siRNAs do not affect total FN1 mRNA levels. RT-qPCR was used to measure EDI+/EDI- ratio and mature Fibronectin mRNA levels in cells transfected with different intronic siRNAs.



Supplementary Figure3

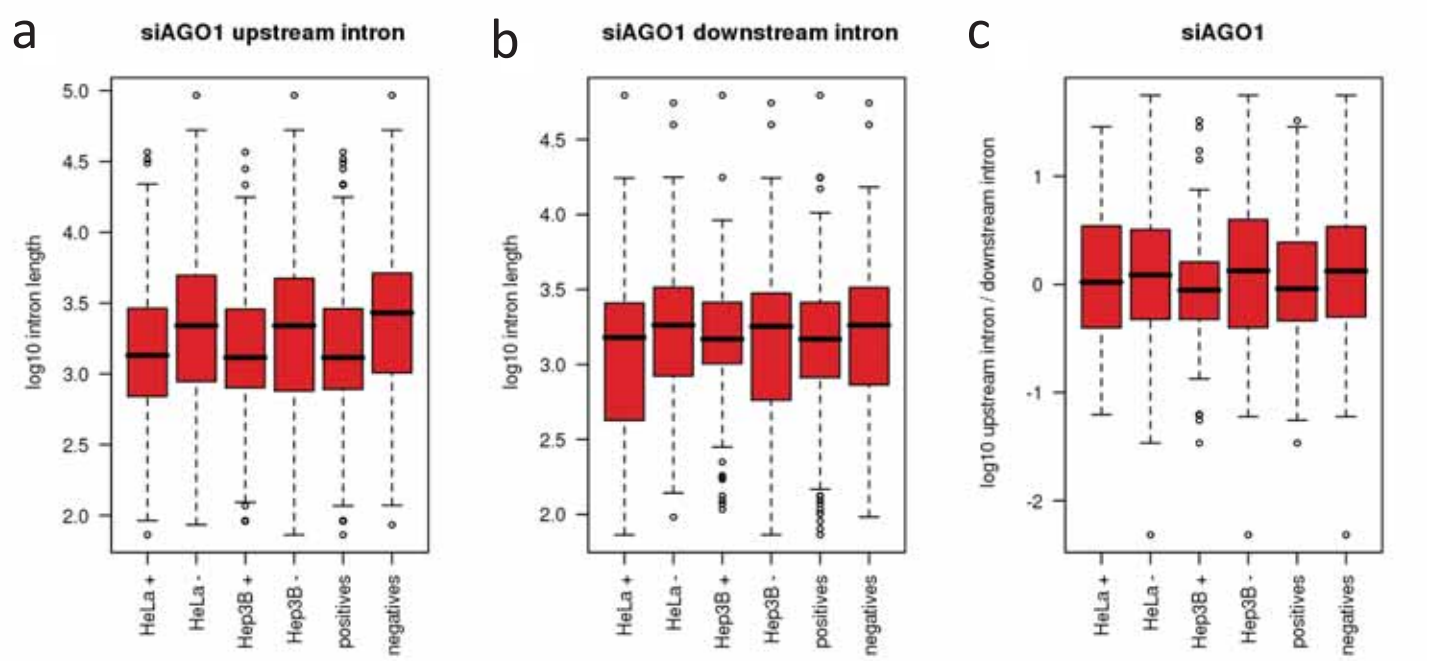
a, FN1 specificity gene internal control for I33as and I33*as. Both siRNAs were transfected in Hep 3B cells, and the relative abundance of three main isoforms of IIICS alternative exon were measured by RT-rPCR showing no significative differencies. **b**, Bcl-X isoform levels were not altered by transfection of I33as or I33*as. On the other hand, the specificity of I33as was tested in more detail by analyzing the alternative splicing patterns of other 6 genes, namely APAF1 (NM_001160), BCL2L11 (NM_006538), BMP4 (NM_130851), CCNE1 (NM_057182), CHEK2 (NM_007194) and PCSK6 (NM_002570) in Hep 3B cells. No significant change was observed for these genes, however, EDI inclusion was effectively upregulated. **c**, Similar results were observed in HeLa cells after transfection with I33as.

Suppl. Fig. 4



Supplementary Figure 4
Effect of siRNA to exon 34 (E34as) on alternative splicing of the EDI exon transcribed from minigene pUHC-EDA. Transcription was activated with tTA-Sp1. Alternative splicing was assessed by RT-rPCR followed by gel electrophoresis at 48 and 72 hours after transfection of E34as.

Suppl. Fig. 5



Supplementary Figure 5
a, Distributions of the lengths of introns flanking ASEs that were affected (positive) or not affected (negative) under the AGO1 knockdown experiment. a, Upstream intron lengths in log10 scale. **b**, Downstream introns lengths in log10 scale. **c**, Distributions of the ratio between the upstream and downstream introns of positive and negative events in log10 scale. Each panel shows the distributions for the events tested in HeLa cells (first two box-plots), Hep3B cells (second and third box plots) and for both sets together (last two box-plots).

Hep3B

	siAGO1	
	p value	ΔΨ
ECT2	2.E-05	41.40
DNMT3B	6.E-05	39.56
INSR	1.E-04	15.38
CHEK2	2.E-04	7.39
AKAP13	3.E-04	18.35
NUP98	4.E-04	4.62
FN1 (IIICS ↓)	5.E-04	34.61
DRF1	7.E-04	18.59
MYO18A	9.E-04	60.91
UTRN	1.E-03	1.54
TLK1	2.E-03	3.7
CASP9	3.E-03	16.76
FANCL	4.E-03	3.10
SYNE2	4.E-03	74.81
LIG4	5.E-03	3.10
OPA1	5.E-03	21.00
FN1 (EDII)	8.E-03	0.97
FGFR2	8.E-03	21.48
TOPBP1	9.E-03	13.89
POLB	1.E-02	12.25
BCL2L11	1.E-02	8.14
APC	2.E-02	46.01
CCNE1	2.E-02	1.10
C11orf17	2.E-02	10.47
C1orf16	2.E-02	32.89
SHC1	3.E-02	2.09
PTPRB	3.E-02	8.49
C11ORF4	3.E-02	3.93
MCL1	4.E-02	5.44
SDCCAG8	4.E-02	4.55
LRDD	5.E-02	21.34
FN1 (IIICS ‡)	5.E-02	3.36
PCSK6	5.E-02	12.65
FN1 (EDI)	5.E-02	19.98

HeLa		
	siAGO1	
	p value	ΔΨ
TIAF1	4.E-04	11.67
KITLG	6.E-04	17.11
SHC1	7.E-04	6.31
IGSF4	8.E-04	31.39
SHMT1	9.E-04	11.63
DDR1	1.E-03	24.99
RUNX2	1.E-03	29.09
DRF1	1.E-03	13.22
BCL2L11	2.E-03	32.34
C11ORF4	3.E-03	9.43
HSC20	3.E-03	6.69
TOPBP1	3.E-03	11.74
APP	4.E-03	11.10
BCL2L12	4.E-03	27.77
STIM1	5.E-03	2.03
CASC4	6.E-03	15.45
AKAP13	6.E-03	8.40
PAXIP1	6.E-03	50.33
CHEK2	7.E-03	11.18
PPP3CB	7.E-03	4.83
HNRPAB	1.E-02	8.89
LGALS9	1.E-02	27.77
C11orf17	1.E-02	17.92
FN1 (IIICS ↓)	1.E-02	27.93
HMMR	1.E-02	9.33
LIG3	2.E-02	4.93
MAPT	2.E-02	15.35
CCNE1	2.E-02	0.75
PCSK6	2.E-02	13.94
FGFR4	2.E-02	34.32
BTC	2.E-02	19.83
DSC3	3.E-02	38.20
PTPN13	4.E-02	7.78
SDCCAG8	4.E-02	5.80
CAPN3	4.E-02	36.21
FGFR1OP	5.E-02	10.94

Supplementary Table 1
AGO1 depletion mediated changes in splicing ratio for 36 detected cancer related alternative splicing events in HeLa and 34 events in Hep3B cells. For each splicing event, the ratio of long isoform versus total isoform concentrations, expressed as a percentage and termed the percent splicing index, Ψ, was determined. The absolute change in Ψ between control and AGO1 depleted samples, termed ΔΨ , is reported for RNA extracted from HeLa and Hep3B cell lines. Biological triplicates of both control and siRNA knockdowns were analyzed, and the differences in splicing ratio

between the two groups was subjected to a t-test, for which the p-values are reported.

HeLa				
	siAGO1		siDCR	
	p value	ΔΨ	p value	ΔΨ
BCL2L12	4.E-03	27.77	4.E-02	32.73
CHEK2	7.E-03	11.18	5.E-03	7.96
MAPT	2.E-02	15.35	2.E-02	47.07
PCSK6	2.E-02	13.94	2.E-03	18.63
SHMT1	9.E-04	11.63	2.E-02	8.89
MYO18A	4.E-04	11.67	3.E-02	23.54

Hep3B				
	siAGO1		siDCR	
	p value	ΔΨ	p value	ΔΨ
C11ORF4	3.E-02	3.93	8.E-04	3.04
CHEK2	2.E-04	7.39	1.E-02	6.96
DRF1	7.E-04	18.59	3.E-02	5.48
FGFR2	8.E-03	21.48	3.E-02	24.13
INSR	1.E-04	15.38	4.E-02	4.59
NUP98	4.E-04	4.62	2.E-02	4.26
PCSK6	5.E-02	12.65	3.E-02	12.15
POLB	1.E-02	12.25	3.E-02	4.89
SYNE2	4.E-03	74.81	1.E-02	64.44
MYO18A	9.E-04	60.91	2.E-03	46.53

Supplementary Table 2
Alternative splicing events showing similar changes in the percent splicing index ΔΨ following AGO1 or DCR depletion. For each splicing event, the ratio of long isoform versus total isoform concentrations, expressed as a percentage and termed the percent splicing index, Ψ, was determined. The absolute change in Ψ between control and AGO1 depleted samples, termed ΔΨ ,is reported for RNA extracted from HeLa and Hep3B cell lines. Biological triplicates of both control and siRNA knockdowns were analyzed, and the differences in splicing ratio between the two groups was subjected to a t-test, for which the p-values are reported.

SUPPLEMENTARY METHODS

siRNA sequences:

Intronic siRNAs:

I32as 5′-CUGCUUUGCAUGUUAUAAAAAdTdT-3′

I33*as 5′-CCGCAUUACAGAGAGAACGUCAAAUdTdT-3′

I33as 5′-CCCCCAUUUCCUAUAAGAUAAdTdT-3′

I33s 5′-ACUUUAUUUUCCUUUUCCCCCdTdT-3′

Exonic siRNAs:

E21as 5′- GCCUGCUCCAAGAAUUGGUUUdTdT-3′

E34as 5′-GCCCAGUGGACACCACCCAAUdTdT-3′

Control siRNAs:

I33as(ST) 5′- CCAUUUCCUAUAAGAUAACCUCUAA-3′ (S code)

I33as(ST) 5′- UUAGAGGUUAUCUUAUAGGAAAUGG-3′ (AS code)

I33s(ST) 5′- CCAUUUCCUAUAAGAUAACCUCUAA-3′ (AS code)

I33s(ST) 5′- UUAGAGGUUAUCUUAUAGGAAAUGG-3′ (S code)

siSCR 5′- GUCAAACCCUACACUAUCUUAdTdT-3′

Other used siRNAs:

siAGO1 5′-GAGAAGAGGUGCUCAAGAAUU-3′

3′-UUCUUGAGCACCUCUUCUCUU-5′P

siAGO2 (stealth) 5′-UGGAACACAUGGUCCAGCACUUUAA-3′

siHP1 5′-CCUGAGAAAAACUUGGAUUdTdT-3′

siLuc 5′-CUUACGCUGAGUACUUCGAdTdT-3′

Primers:

Total FN mRNA: GCTCATCCGTGGTTGTATCAGG Forward **(P1)** TGGTCTGCTTGTCAAAGTGTCC Reverse **(P2)**

Human endogenous EDI Exclusion: TGACTATTGAAGGCTTGCAG (For) AGTTGGTGCAGGAATAGTGG (Rev)

Human endogenous EDI Inclusion: CCGGGTTCTGAGTACACAGTC (For) AGTTGGTGCAGGAATAGCTG (Rev)

I33as target sequence: CCTAGCCCAACAAGAACAATCC **(P3)** GGTGTGACCTGAGTGAACTTC **(P4)**

AGO1: GCCAGATCCCTGTTCCCTTG (For) GTGAAAGCCGAACCAGACCTC (Rev)

AGO2: GCGAGACCACCAAGCACTG (For) GCTGGCTGTCACGGAAGG (Rev)

HSPCB: CCAAAAAGCACCTGGAGATCA (For) TGTCGGCCTCAGCCTTCT (Rev)

Human endogenous EDI radioactive: AGCCCCGCAAGCAGCAAGCC (For) GTAGCATCTGTCACACGAG (Rev)

Human EDI minigene: CACTGCCTGCTGGTGACTCGA (For) GCGGCCAGGGGTCACGAT (Rev)

Human endogenous IIICS: GGCTACTATTACTGGCCTGG (For) CTGAGAGAGAGCTTCTTGTCC (Rev)

Human Bcl-X: AGCTGGAGTCAGTTTAGTGATGTG (For) TGAAGAGTGAGCCCAGCAGAAC (Rev)

APAF1: GTGAAGTGTTGTTCGTGGTCTG (For) CATCACACCATGAACCCAAC (Rev)

BCL2L11: TACCTCCCTACAGACAGAG (For) CCTCCTTGCATAGTAAGCGT (Rev)

BMP4: CGAGAAGGCAGAGGAGGAG (For)

CAAACTTGCTGGAAAGGCTC (Rev)

CCNE1: CACAGGGAGACCTTTTACTTGG (For)

TCAAGGCAGTCAACATCCAG (Rev)

CHEK2: CAGCTCTCAATGTTGAAACAGAA (For)

TCTGGCTTTAAGTCACGGTGT (Rev)

PCSK6: GCCCTTTACTTCAACGACCC (For)

TCATTGCTGGCATCATATCG (Rev)

FN1senseantisense: ACTGCTTACTTGTTAAGACCACTG **(P7)**

AGGAGAGGCACCACGAGAAG **(P8)**

RTasFN1: TTCTTCTTACGCCGCATTACAG **(P5)**

RTFN1sense: CTGTCAGGAGCAAGGTTGATTTC **(P6)**

HP1: GGAGCACAATACTTGGGA(For)

CGGCACTGTTTGAGAAAT(Rev)

RT Proximal: ACCAACAAGTTCACTTTCCCTATG

RT Distal: CACCTTAACGGTATTTGGAGGCT

Proximal: CAGGGCAGAGGATCAACG (For)

GAGGCTGACTCTCTCCGCTTGGAT (Rev)

Distal: TTGGAACTACGTTTATTTTCC (For)

GCGGCCAGGGGTCACGAT (Rev)

nChIP regions:

A: AAGAGGCAGGCTCAGCAAATG (For)

TCGGCTTTAGGGTCCCATCC (Rev)

B: CACTCAGGTCACACCCACAAG (For)

CTGTCAGGAGCAAGGTTGATTTC (Rev)

C: TGGTCTGCTTGTCAAAGTGTCC (Rev)

ATGCTGTAGTGAAACTCCTTGGG (For)

D: AATGAGTTCCCTGACCTGTGATG (Rev)

GCTTCCAAGTTGATGCCGTTC (For)

Bioinformatics search

We extracted all 185,500 introns from the 19,339 RefSeq genes at UCSC. We classified the introns according to the splicing properties of the flanking exons. We used the available EST data from dbEST to classify the exons from the annotation. We considered only exons with more than 10 ESTs. Exons with more than 90% of inclusion level were classified as constitutive, and the rest were classified as alternative. From the latter set, we separated cassette exons from the other forms of alternative splicing (AS). In this way, we obtained 91,847 introns flanking constitutive exons, 56,937 introns flanking exons with AS, and 17,117 introns next to cassette exon events.

We downloaded the dataset of 294,058 transfrags from HeLa cells from (7) and calculated the overlap of the genomic coordinates with our intron data set using fjoin[1]. We found that 43% of the transfrags were completely included in introns.

All the cancer-related events tested in the PCR platform were located in the RefSeq annotation using the information on the primer location. Using ESTs we determined the inclusion levels of the alternative exon. For 91 out of the 95 events tested we found evidence of skipping in the EST set. The length of the flanking introns was obtained from the RefSeq exon annotation. For all distributions we performed the normality test (Shapiro-Wilk test) and p-values were calculated for each comparison using the Wilcoxon rank-sum test.

High-throughput RT-PCR and capillary electrophoresis

Design, execution and analysis of selected AS events was performed using the LISA platform, described previously[2]. Briefly, primer pairs flanking AS events

were designed to detect both long and short amplicons generated from a panel of cancer associated AS events. Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA USA), sequences available upon request. Reverse transcription (Omniscript reverse transcriptase, Qiagen Inc, Canada) was performed on 2 μg total RNA samples in the presence of an RNase inhibitor (Porcine RNAguard RNase inhibitor, Amersham Biosciences, Canada) according to the manufacturers' protocols. Reactions were primed with both (dT) and random hexamers at final concentrations of 1 μM and 0.9 μM respectively. End-point PCR reactions were performed on 20 ng cDNA in 10 μl final volume containing 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.6 μM each primer and 0.2 units of Taq DNA polymerase (Platinum Taq DNA polymerase, Invitrogen, Canada). An initial incubation of 2 minutes at 95°C was followed by 35 cycles at 94°C 30s, 55°C 30s, and 72°C 60s. The amplification was completed by a 2-minute incubation at 72°C. PCR amplified products were analyzed by automated chip-based microcapillary electrophoresis on Caliper LC-90 instruments (Caliper LifeSciences, Hopkinton, MA). Amplicon sizing and relative quantitation was performed by the manufacturer's software, prior to being uploaded to the LISA database. For each RNA source, the ratio of the concentration of the long amplicon to the total amplicon concentrations is calculated and expressed as a percentage, termed the percent splicing index, Ψ. The absolute change of Ψ values for control and siRNA knockdown samples is referred to as $\Delta\Psi$. Mean Ψ values of biological triplicates of control and AGO1 siRNA knockdown experiments were used in statistical t-tests.

Supplementary References

1. Richardson, J. E. *J Comput Biol* **13**, 1457 (2006).

2. Klinck, R. *et al.*, *Cancer Res* **68**, 657 (2008).