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Supplemental Information

Development and Mechanism of Small Activating RNA Targeting CEBPA, a Novel Therapeutic in Clinical Trials for Liver Cancer

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Supplemental Methods

Nucleotide walk and dose response curves for CEBPA saRNAs

Transfections were performed as described in the main text. The branched DNA assay (Panomics) was used for mRNA quantification, in the version Quantigene 2.0 for target genes, and in the version Quantigene 1.0 for hsGAPDH. This hybridization-based assay system provides a chemo-luminescence readout. Probe sets were custom designed by Panomics. The assay was performed according to the manufacturer's instructions: Briefly, lysates were hybridized overnight with the respective probe set and subsequently processed for signal development. Signals were measured on a Victor Light luminescence reader (Perkin Elmer). For analysis of transfection experiments, luminescence units obtained for target mRNAs were normalized to the housekeeper mRNA GAPDH. Relative expression values obtained for transfection reagent only ("mock") treated cells were set to 1.

Isolation of human PBMCs from buffy coat of healthy donors

Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation. Briefly, human buffy coat blood (obtained from Institute of Transfusion Medicine, Suhl, Germany) of three donors was fractionated by a Ficoll gradient (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The layer of white blood cells was aspirated, purified by a second gradient centrifugation and finally washed twice with cell culture medium (RPMI1640 without supplements). Viability and morphology of huPBMCs from all three donors were assessed by microscopy and PBMCs of two donors were nominated and used in subsequent experiments.

Assaying TNF-α stimulation in PBMCs

For monitoring a potential TNF-α stimulation, freshly isolated PBMCs from two healthy donors were seeded in regular 96-well tissue culture plates at a density of 100000 cells/well in 100µl complete medium (RPMI1640 supplemented with standard concentrations of L-Glutamine and 10% FCS). Cells were transfected in triplicate with 133 nM CEBPA-51 or control sequences RD-01010 (positive control) and RD-01011 (negative control) using Dotap (Roche Diagnostics, Mannheim, Germany) as a transfection reagent according to the manufacturer's protocol. Transfection reagent alone was used as mock control. In addition, controls ODN2216 (CpG-oligonucleotide) and RD-01002 (cholesterol-conjugated siRNA) were added directly at a concentration of 500nM without transfection. Cells were incubated for 20 h. Supernatants from triplicate transfections were pooled and TNF-α secretion was measured using the "Human TNF-α Instant ELISA" system (eBioscience, Frankfurt, Germany, #BMS223INSTCE), according to the manufacturer's protocol. Each sample was measured in duplicate.

Assaying IFN-α stimulation in PBMCs

For monitoring IFN-α stimulation, freshly isolated PBMCs of two healthy donors were seeded in regular 96-well tissue culture plates at a density of 100000 cells/well in 100µl complete medium (RPMI1640 supplemented with standard concentrations of L-Glutamine and 10% FCS plus

PHA-P (Phytohemagglutinin, 5µg/ml) and Interleukin-3 (10ng/ml)). Cells were transfected in triplicate with 133 nM CEBPA-51 or control sequences RD-01010 (positive control) and RD-01011 (negative control) using Geneporter-2 (Genlantis, San Diego, USA) as a transfection reagent according to the manufacturer's protocol. Transfection reagent alone was used as mock control. In addition, controls ODN2216 (CpG-oligonucleotide) and RD-01002 (cholesterol-conjugated siRNA) were added directly at a concentration of 500nM without transfection. Cells were incubated for 20 h. Supernatants from triplicate transfections were pooled and IFN-α secretion was measured using the "Human IFN-α Instant ELISA" system (eBioscience, Frankfurt, Germany, BMS216INSTCE), according to the manufacturer's protocol. Each sample was measured in duplicate.

Off-target analysis

At first potential off-target sites with full or partial complementarity to the sense and antisense strand of saRNA CEBPA51, respectively, were predicted in human, rhesus monkey, cynomolgus monkey, mouse, and rat transcriptomes (NCBI Reference Database release 69, January 2015) using a proprietary algorithm. Because positions 1 and 19 as well as the UU 3'-overhang of a siRNA are not essential for the siRNA activity only the 17mer sequence from position 2 through 18 was considered for the prediction of potential off-target sites with up to 4 mismatches to the examined saRNA strand. Based on the number and the position of the mismatches a specificity score was calculated for each predicted off-target site. The specificity score for the most likely off-target site was assigned to the corresponding saRNA strand. In addition the number of predicted off-target genes with 0, 1, 2, 3 or 4 mismatches (off-target frequency) was separately calculated for each saRNA strand.

At next potential seed-dependent, microRNA-like off-target effects were analyszed. siRNAs can function in a miRNA like manner via base-pairing of the seed-region (typically bases 2 through 7) with complementary sequences within the 3'-UTR of any mRNA molecule. In silico prediction of functional miRNA-target sites is still not well established and usually results in the prediction of thousands of potentially miRNA-regulated transcripts, which is inappropriate for the evaluation of the risk of potential microRNA-like off-target effects. Therefore we focused on seed-region sequences of known miRNAs for which it is highly likely that functional miRNA target-sites exist. This was accomplished by comparison of the seed-region (positions 2 through 7) of each saRNA strand with the seed-regions (positions 2 through 7) of known mature miRNAs from human, rhesus-monkey, rat, and mouse (miRBase release 21, June 2014). If applicable the seed-region identity and the name of the corresponding miRNA were tabulated for the sense and the antisense strand. Results are summarized in tab. 1 A and B. After that, listings with all predicted off-targets for all examined species and for both saRNA strands were created. Features of the predicted off-target sites were described in detail: strand orientation, accession number, gene ID, gene symbol, transcript description, sequence of off-target site, number and position of mismatches, location of target site (coordinates and region), indication of perfect seed match (6mer seed for position 2-7, and 7mer seed for positions 2-8). In order to allow a more refined ranking the predicted off-target sites were then further classified based on the number of mismatches, the position of the mismatches and the location of the predicted target-sites in the 5'-UTR and CDS or the 3'-UTR. The classification ranges from class 1 (most likely off-targets) to class 11 (least likely off-targets), with the most likely off-targets having no or few mismatches

and having a perfect match of the saRNA seed region with the 3‘-UTR of the predicted off-target. At next a representative transcript was defined for each off-target site in order to reduce redundancy of the potential presence of the same target-site sequence in multiple transcripts or within the same transcript. Finally the predicted off-targets were ranked according to the assigned off- target class. In the last step all predicted off-targets matched with up to 2 mismatches were listed in a separate table and identical off-targets predicted for human and at least one other species were indicated.

The cell lines Panc-1 and HuH7 were purchased from ATCC and cultured under the conditions recommended by the provider. For transfection, cells were plated directly into the transfection solution at a density of 15000 cells /well in a 96-well cell culture dish (“reverse transfection”). Lipofectamine 2000 (Life Technologies) was used as transfection reagent according to the manufacturer’s protocol. All transfections were performed in quadruplicate. The test substance CEBPA51 (XD-03934) was transfected in 3 concentrations (2 nM, 10 nM and 50 nM), scrambled control XD-03291 and Aha-1 transfection control XD-00033 were transfected at the highest concentration only. After 24h incubation, cells were lysed with 150 µl of lysis mixture (Quantigene 2.0 assay kit, Panomics) diluted 1:3 with cell culture medium. Lysates were kept frozen until analysis.

The branched DNA assay (Panomics /Affymetrix, Fremont, CA) was used for mRNA quantification, in the version Quantigene 2.0 for target genes, and in the version Quantigene 1.0 for hsGAP-DH and mmGAPDH. This hybridization-based assay system provides a chemo-luminescence readout. Probe sets were custom designed by Panomics. The assay was performed according to the manufacturer’s instructions: Briefly, lysates were hybridized over night with the respective probe set and subsequently processed for signal development. Signals were measured on a Victor Light luminescence reader (Perkin Elmer). For analysis of transfection experiments, luminescence units obtained for target mRNAs were normalized to the housekeeper mRNA for GAPDH. Relative expression values obtained for transfection reagent only (“mock”) treated cells were set to 1.

Cynomolgus cross-reactivity

CYNOM-K1 cynomolgus skin fibroblasts (Sigma) were maintained in MEM supplemented with 10% FBS, 2mM L-glutamine, and penicillin/streptomycin in a 5% CO_2 incubator. Cells were transfected with Lipofectamine 2000 as described in the main text with the indicated oligonucleotide and were harvested for analysis after 48 hours.

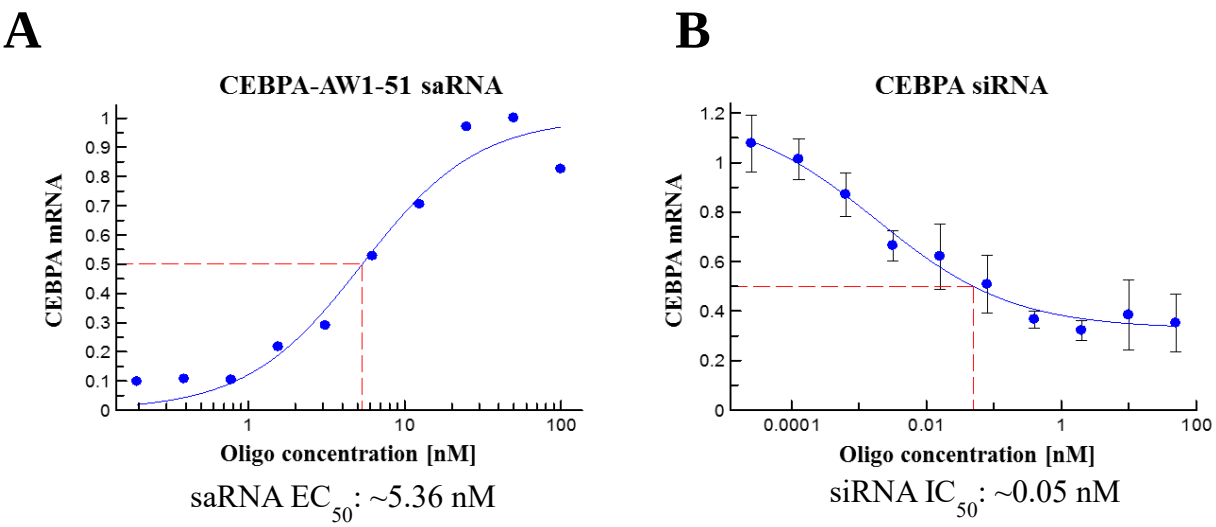
Supplemental Table 1

Name	SS Sequence (5’->3’)	AS Sequence (5’->3’)	Notes
NC	ACUACUGAGUGACAGUAGAUU	UCUACUGUCACUCAGUAGUUU	Unmodified non-targeting negative control
MM	UCGAAGUAUUCCGCGUACGUU	CGUACGCGGAAUACUUCGAUU	Unmodified non-targeting negative control

FLUC	mCmUmUAmCGmCmUGA GmUAmCmUmUmCGAdTpsdT	UCGAAGmUACUmU AGCGmUAAGdTpsdT	Modified FLUC negative control
AHSA1 siRNA	GGAmUGAAGmUGG AGAmUmUAGmUdTpsdT	ACmUAAUCUCmCA CUUmCAUCCdTpsdT	siRNA to AHSA1
AW1-42	GUCACUGGUCAGCUCCAGCUU	GCUGGAGCUGACCAGUGACUU	-8nt from AW1 hotspot
AW1-46	CAUUGUCACUGGUCAGCUCUU	GAGCUGACCAGUGACAAUGUU	-4nt from AW1 hotspot
AW1-50	CGGUCAUUGUCACUGGUCAUU	UGACCAGUGACAAUGACCGUU	AW1 hotspot
AW1-51	GCGGUCAUUGUCACUGGUCUU	GACCAGUGACAAUGACCGCUU	+1nt from AW1 hotspot
AW1-52	GGCGGUCAUUGUCACUGGUUU	ACCAGUGACAAUGACCGCCUU	+2nt from AW1 hotspot
AW1-53	AGGCGGUCAUUGUCACUGGUU	CCAGUGACAAUGACCGCCUUU	+3nt from AW1 hotspot
AW1-54	CAGGCGGUCAUUGUCACUGUU	CAGUGACAAUGACCGCCUGUU	+4nt from AW1 hotspot
AW1-55	GCAGGCGGUCAUUGUCACUUU	AGUGACAAUGACCGCCUGCUU	+5nt from AW1 hotspot
AW1-56	CGCAGGCGGUCAUUGUCACUU	GUGACAAUGACCGCCUGCGUU	+6nt from AW1 hotspot
AW1-57	GCGCAGGCGGUCAUUGUCAUU	UGACAAUGACCGCCUGCGCUU	+7nt from AW1 hotspot
AW1-58	UGCGCAGGCGGUCAUUGUCUU	GACAAUGACCGCCUGCGCAUU	+8nt from AW1 hotspot
AW1-59	UUGCGCAGGCGGUCAUUGUUU	ACAAUGACCGCCUGCGCAAUU	+9nt from AW1 hotspot
AW2-40	AUUCAUCCUCCUCGCGGGGUU	CCCCGCGAGGAGGAUGAAUUU	-10nt from AW2 hotspot
AW2-41	GAUUCAUCCUCCUCGCGGGUU	CCCGCGAGGAGGAUGAAUCUU	-9nt from AW2 hotspot
AW2-42	GGAUUCAUCCUCCUCGCGGUU	CCGCGAGGAGGAUGAAUCCUU	-8nt from AW2 hotspot
AW2-43	AGGAUUCAUCCUCCUCGCGUU	CGCGAGGAGGAUGAAUCCUUU	-7nt from AW2 hotspot
AW2-44	AAGGAUUCAUCCUCCUCGCUU	GCGAGGAGGAUGAAUCCUUUU	-6nt from AW2 hotspot
AW2-45	AAAGGAUUCAUCCUCCUCGUU	CGAGGAGGAUGAAUCCUUUUU	-5nt from AW2 hotspot
AW2-46	UGAAAGGAUUCAUCCUCCUUU	AGGAGGAUGAAUCCUUUCAUU	-4nt from AW2 hotspot
AW2-48	CUGAAAGGAUUCAUCCUCCUU	GGAGGAUGAAUCCUUUCAGUU	-2nt from AW2 hotspot
AW2-49	GCUGAAAGGAUUCAUCCUCUU	GAGGAUGAAUCCUUUCAGCUU	-1nt from AW2 hotspot
AW2-50	AGCUGAAAGGAUUCAUCCUUU	AGGAUGAAUCCUUUCAGCUUU	AW2 hotspot
AW2-51	CAGCUGAAAGGAUUCAUCCUU	GGAUGAAUCCUUUCAGCUGUU	+1nt from AW2 hotspot
AW2-52	CCAGCUGAAAGGAUUCAUCUU	GAUGAAUCCUUUCAGCUGGUU	+2nt from AW2 hotspot
AW2-53	GCCAGCUGAAAGGAUUCAUUU	AUGAAUCCUUUCAGCUGGCUU	+3nt from AW2 hotspot
AW2-54	CGCCAGCUGAAAGGAUUCAUU	UGAAUCCUUUCAGCUGGCGUU	+4nt from AW2 hotspot

AW2-55	GCGCCAGCUGAAAGGAUUCUU	GAAUCCUUUCAGCUGGCGCUU	+5nt from AW2 hotspot
AW2-56	AGCGCCAGCUGAAAGGAUUUU	AAUCCUUUCAGCUGGCGCUUU	+6nt from AW2 hotspot
AW2-57	CAGCGCCAGCUGAAAGGAUUU	AUCCUUUCAGCUGGCGCUGUU	+7nt from AW2 hotspot
AW2-58	CCAGCGCCAGCUGAAAGGAUU	UCCUUUCAGCUGGCGCUGGUU	+8nt from AW2 hotspot
AW2-59	GCCAGCGCCAGCUGAAAGGUU	CCUUUCAGCUGGCGCUGGCUU	+9nt from AW2 hotspot
AW2-60	GGCCAGCGCCAGCUGAAAGUU	CUUUCAGCUGGCGCUGGCCUU	+10nt from AW2 hotspot
AW1-51 seed1	GCGGUCAUUGUCACUGCUCUU	GAGCAGUGACAAUGACCGCUU	Mutation at second seed position
AW1-51 seed4	GCGGUCAUUGUCACUCGUCUU	GACGAGUGACAAUGACCGCUU	Mutation at third seed position
AW1-51 seed2	GCGGUCAUUGUCUCAGCUCUU	GAGCUGAGACAAUGACCGCUU	Three seed mutations
AW1-51 seed3	GCGGUCAUUGUGAGUCGACUU	GUCGACUCACAAUGACCGCUU	Four seed mutations
AW1-51 scr3	GGAUUGCGUCUCGGUCUCAUU	UGAGACCGAGACGCAAUCCUU	AW1-51 sequence scrambled

Supplemental Figure 1



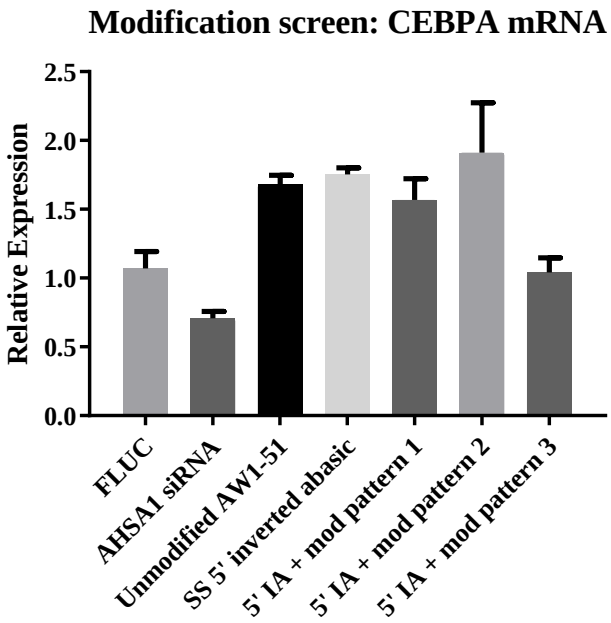
Dose response curves for CEBPA saRNA and siRNA in HepG2 cells. (A) Dose response curve for CEBPA mRNA after transfection with increasing concentrations of AW1-51 saRNA. (B) Dose response curve for CEBPA mRNA after transfection with increasing concentrations of CEBPA siRNA.

Supplemental Figure 2

A

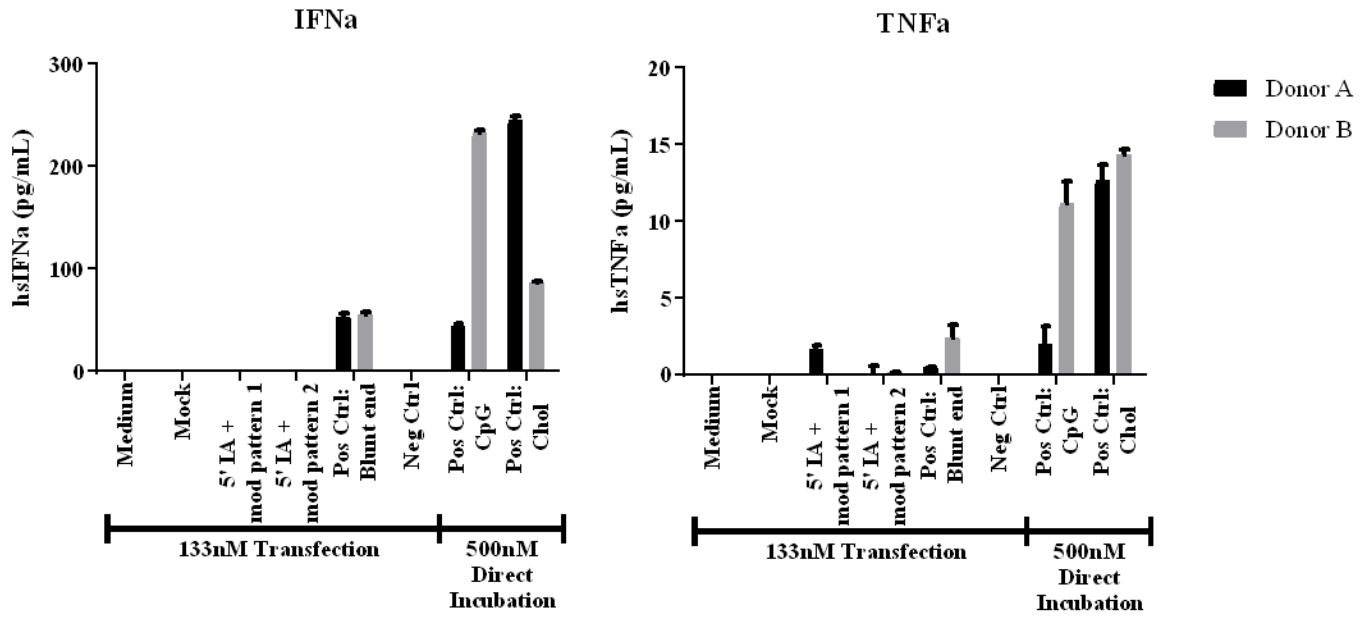
Name	SS Sequence (5'->3')	AS Sequence (5'->3')
SS 5' inverted abasic	(invabasic)GCGGUCAUUGUCACUGGUCUU	GACCAGUGACAAUGACCGCUU
5' IA + mod pattern 1	(invabasic)GCmGGmUCmAUmUGmUCmACmUGmGUCmUmU	GACCAGUGACAAUGACCGCmUmU
5' IA + mod pattern 2	(invabasic)mGmCGmGUCAUUmGUCAmCUGGUCmUmU	GACCAGUGACAAUGACCGCmUmU
5' IA + mod pattern 3	(invabasic)mGmCGGmUmCAmUmUGmUmCAmCmUGGmUmCmUmU	GACmCAGUGAmCAAUGACCGCmUmU

B



Supplemental Figure 2 (cont.)

C



Modifications of AW1-51 to avoid immune stimulation. (A) Table showing modification patterns of AW1-51 tested. (B) qPCR for CEBPA mRNA after transfection with 10nM modified AW1-51 saRNA in HepG2 cells. (C) ELISA for TNFa and IFNa from PBMCs transfected with modified AW1-51 saRNAs.

Supplemental Figure 3

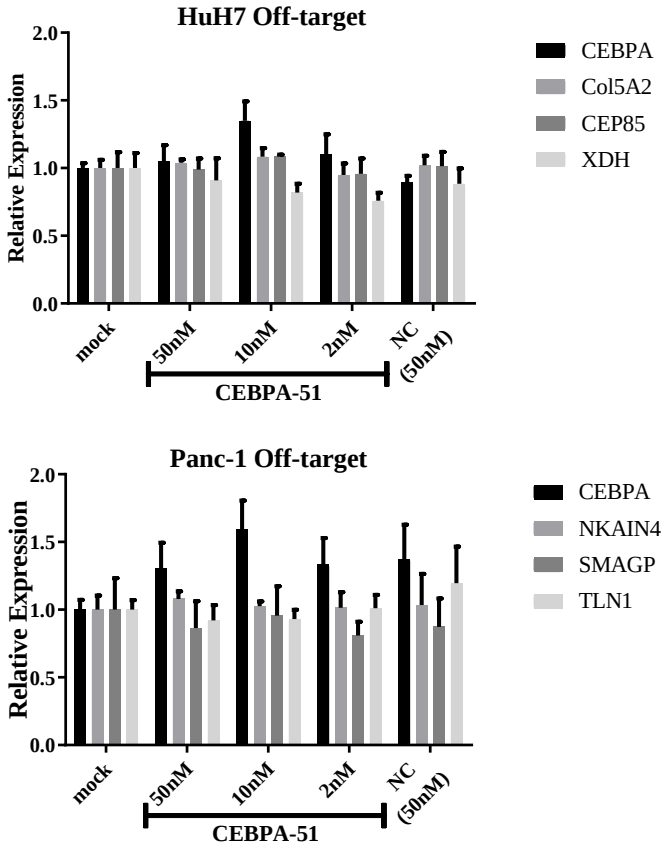
A

	antisense strand						
	miRNAs with seed regions identical to seed region of siRNA strand	Off-target frequency classified by number of mismatches					
species		score	0	1	2	3	4
human	-	3	0	0	4	79	809
rhesus monkey	-	3	0	0	2	42	534
cynomolgus monkey	n.d.	3	0	0	4	69	689
mouse	mmu-miR-470-3p, mmu-miR-1905	2	0	0	4	112	1101
rat	-	3	0	0	3	83	979

	sense strand						
	miRNAs with seed regions identical to seed region of siRNA strand	Off-target frequency classified by number of mismatches					
species		score	0	1	2	3	4
human	-	1,9	0	1	8	123	1144
rhesus monkey	-	3	0	0	5	106	876
cynomolgus monkey	n.d.	3	0	0	6	112	1074
mouse	-	2	0	0	18	170	1319
rat	-	2	0	0	18	148	1326

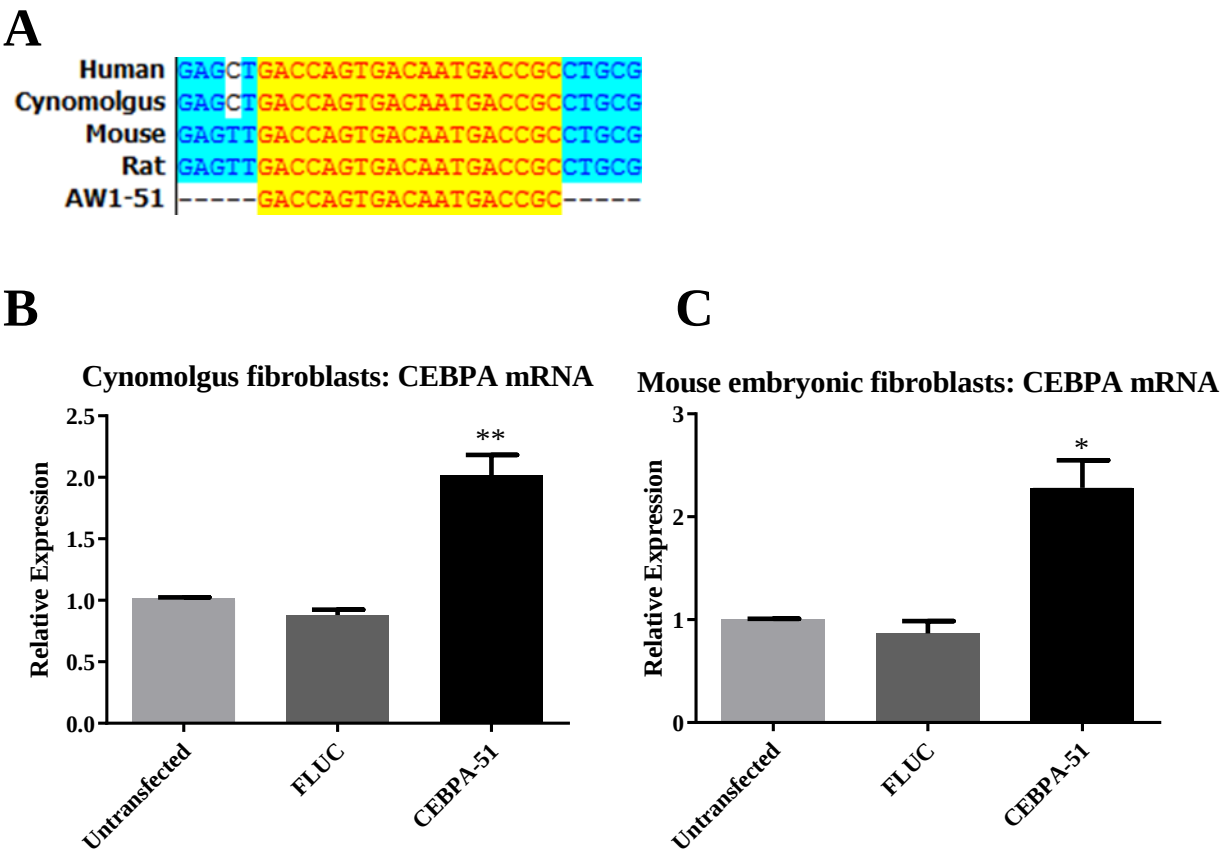
Supplemental Figure 3 (cont.)

B



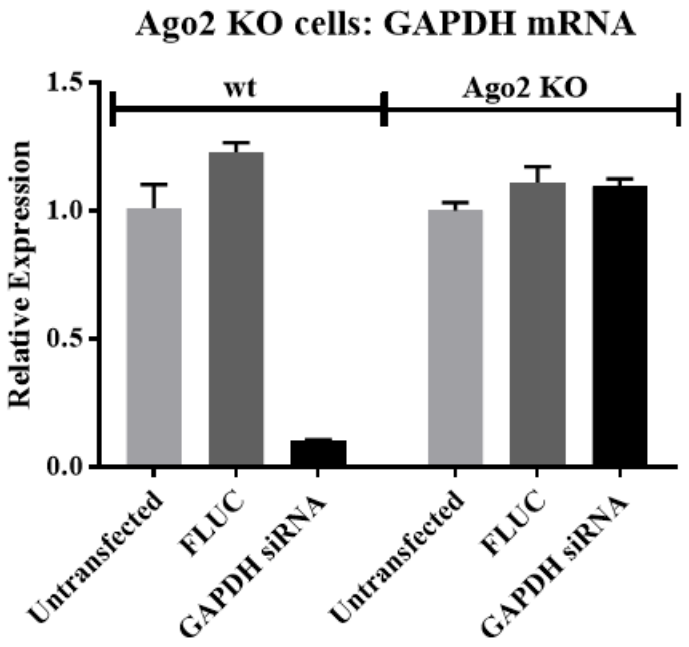
Off-target analysis of CEBPA-51 activity. (A) Table showing off-target frequency for each species analyzed. (B) Relative expression of putative off-target gene expression 24 hours after transfection with the indicated concentration of CEBPA-51.

Supplemental Figure 4



Cross-reactivity of CEBPA-51. (A) Alignment of human, cynomolgus monkey, and rodent genomic sequences at the AW1-51 target site. (B) qPCR for CEBPA mRNA after transfection of 10nM CEBPA-51 in CYNOM-K1 cells. (C) qPCR for CEBPA mRNA after transfection of 10nM CEBPA-51 in mouse embryonic fibroblasts. Statistical significance shown for CEBPA-51 compared to FLUC transfection: *, $p < 0.05$; **, $p < 0.01$.

Supplemental Figure 5



siRNA activity of Ago2 KO MEFs. Relative expression of gene expression 24 hours after transfection with control or GAPDH siRNA at 10nM.