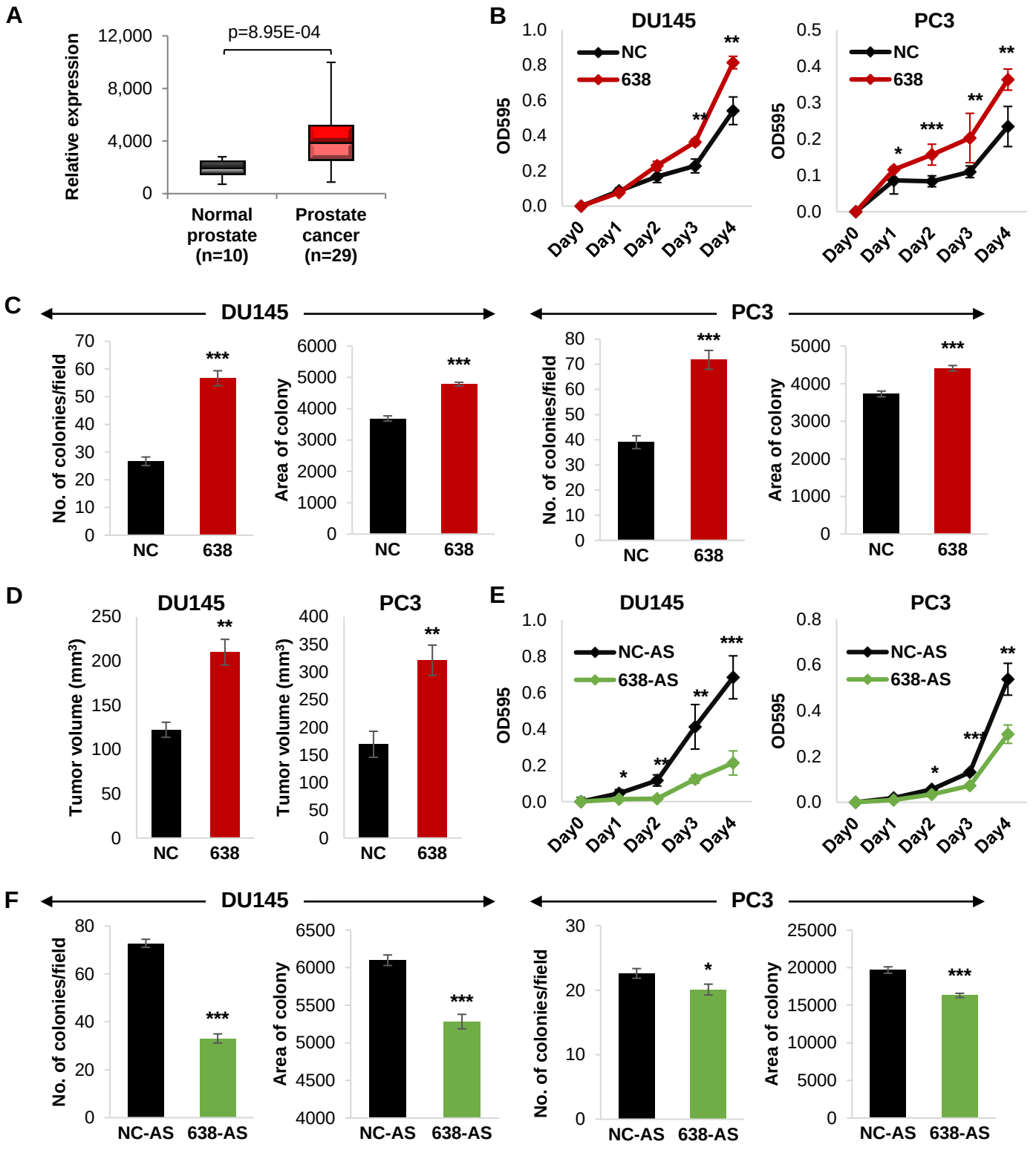
Supplementary Data

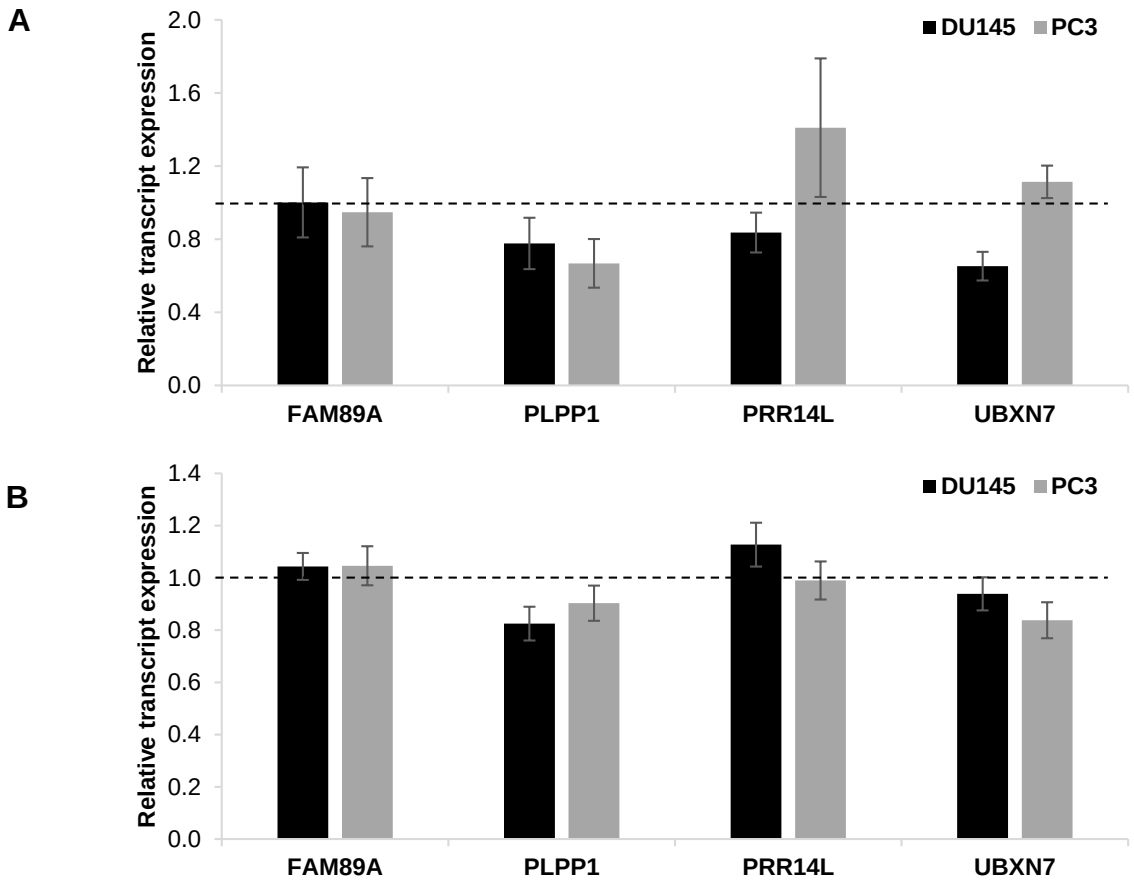
A FTH1 gene:pseudogene:microRNA network regulates tumorigenesis in prostate cancer

Jia Jia Chan[1], Zhi Hao Kwok[1], Xiao Hong Chew[1], Bin Zhang[1], Chao Liu[2], Tuck Wah Soong[2,3], Henry Yang[1], Yvonne Tay[1,4,*]

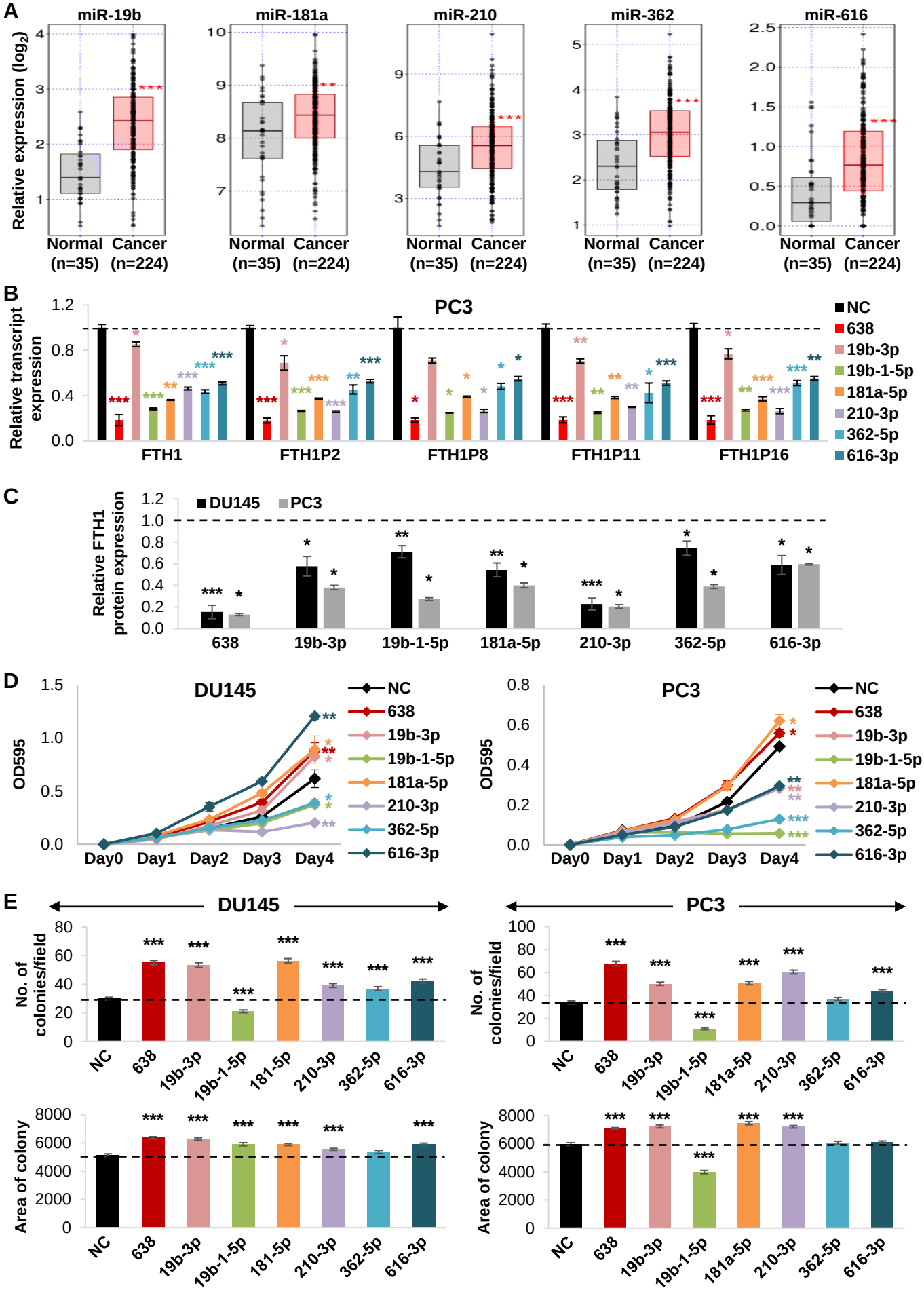
[1] Cancer Science Institute of Singapore, National University of Singapore, Singapore 117599, Singapore
[2] Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore
[3] National Neuroscience Institute, Singapore 308443, Singapore
[4] Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore



Supplementary Figure S1. Effect of miR-638 overexpression in prostate cancer.
(**A**) GEO data (GSE45604) analysis of miR-638 expression in prostate cancer compared to normal prostate. (**B-D**) Effect of miR-638 overexpression on proliferation (**B**), anchorage-independent growth (**C**) and tumor xenograft growth of DU145 and PC3 cells subcutaneously injected into nude mice (**D**).
(**E, F**) Effect of miR-638 inhibition on proliferation (**E**) and anchorage-independent growth (**F**) *in vitro*.
NC and NC-AS denote non-targeting control mimic and non-targeting control antisense mimic respectively. (**B, E**) Mean ± SD; n ≥ 3. (**C, D, F**) Mean ± SEM; n ≥ 3. *p < 0.05; **p < 0.01; ***p < 0.001.



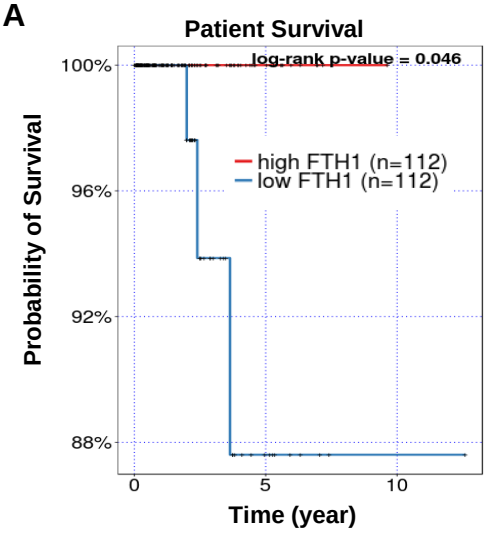
Supplementary Figure S2. Validation of the negative controls for miR-638.
(**A, B**) qRT-PCR validation of the expression of genes that are not targeted by miR-638 in biotinylated miR-638 pulldown (**A**) and miR-638 overexpression (**B**) samples.
Mean ± SEM; n ≥ 3. *p < 0.05; **p < 0.01; ***p < 0.001.



Supplementary Figure S3

Supplementary Figure S3. Properties of other FTH-targeting miRNAs in prostate cancer.
(**A**) TCGA data analysis of the expression of selected oncogenic miRNAs in prostate cancer compared to normal prostate.
(**B**) Effect of selected miRNA overexpression on the transcript expression of FTH1 and its pseudogenes in PC3 cells.
(**C**) Quantification of the effect of selected miRNA overexpression on FTH1 protein expression.
(**D**) Effect of selected miRNA overexpression on proliferation *in vitro*.
(**E**) Quantification of selected miRNA overexpression on anchorage-independent growth *in vitro*.
(**B, C, E**) Mean ± SEM; n ≥ 3. (**D**) Mean ± SD; n ≥ 3. *p < 0.05; **p < 0.01; ***p < 0.001.

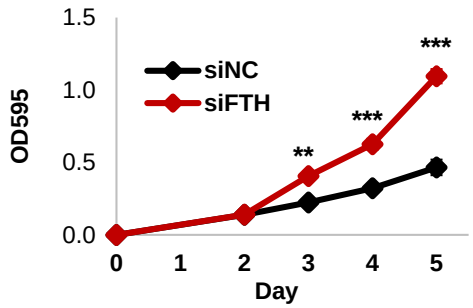
A



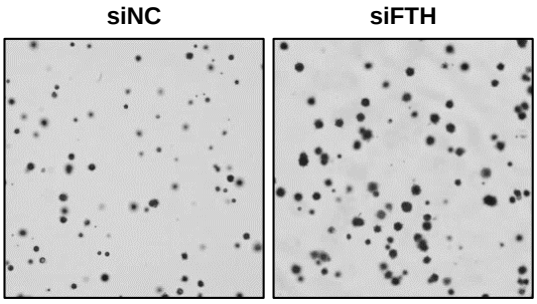
B

siRNA sequence	Targets	leftmost position of target site
AAUCUGGCUUGGCGGAAUA	FTH1	723
	FTH1P8	786
GCGAUGAUGUGGCUUUGAA	FTH1	366
	FTH1P11	433
GGGAGACAGUGAUAAUGAA	FTH1	763
	FTH1P11	830
	FTH1P16	834
CAAACUGGCCACUGACAAA	FTH1	592
	FTH1P2	316
	FTH1P8	655
	FTH1P11	659

C



D



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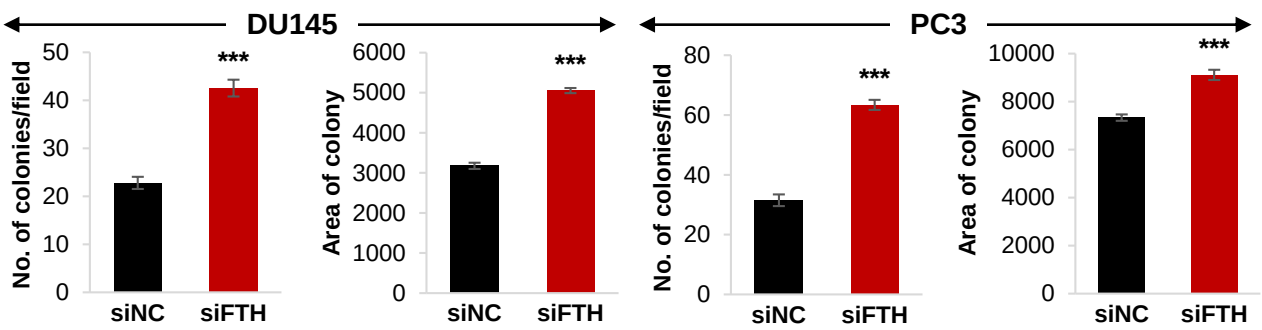

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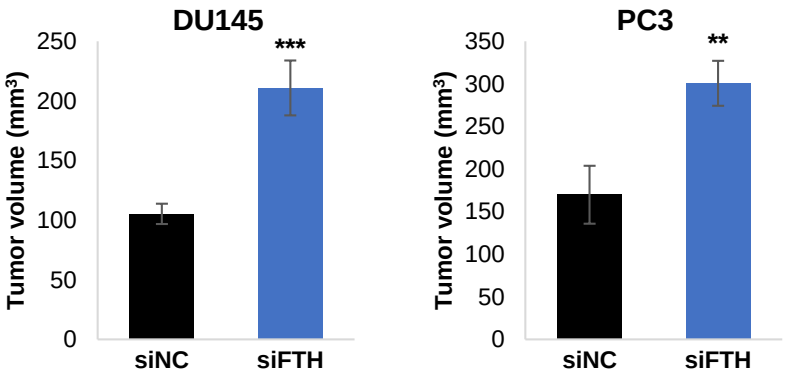
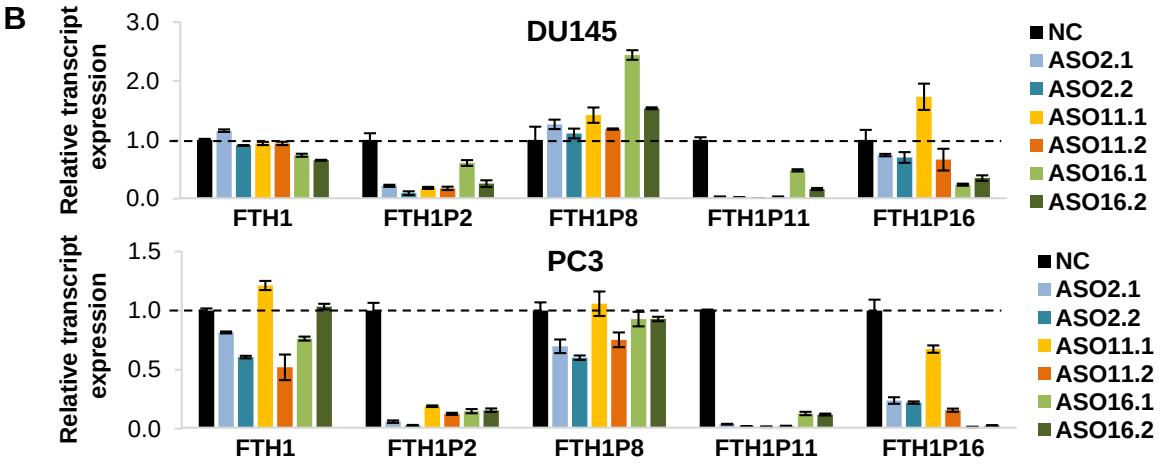
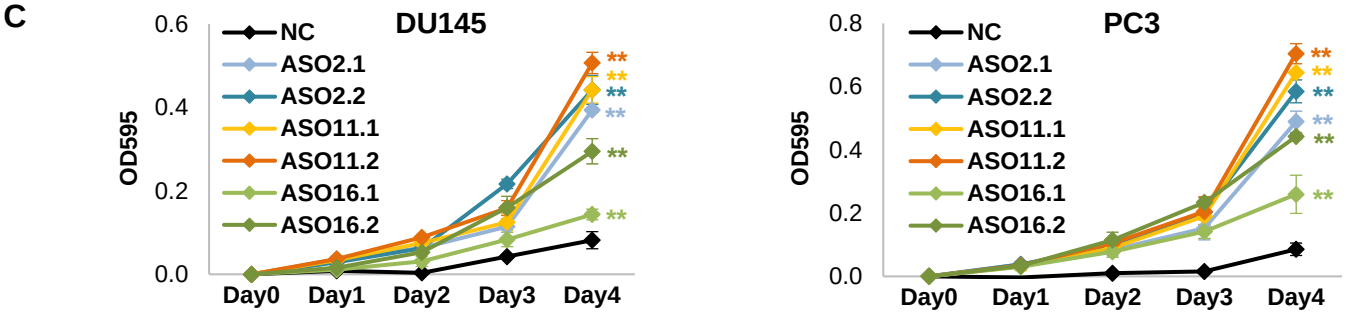


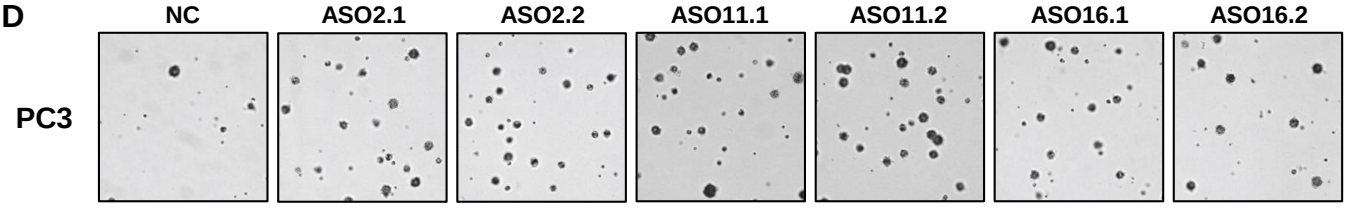
Supplementary Figure S4. Effects of silencing FTH1 and its pseudogenes in prostate cancer.
(**A**) TCGA-derived Kaplan-Meier survival curve of prostate cancer patients with differential FTH1 expression.
(**B**) siRNA sequences in the FTH1 siRNA pool and the position of the sites targeted on the respective transcripts.
(**C, D**) Effect of siRNA-mediated knockdown of FTH1 and its pseudogenes on proliferation (**C**) and anchorage-independent growth (**D**) in PC3 cells.
(**E**) Quantification of the effect of siRNA-mediated knockdown of FTH1 and its pseudogenes on anchorage-independent growth *in vitro*.
(**F**) Effect of siRNA-mediated knockdown of FTH1 and its pseudogenes on tumor xenograft growth of DU145 and PC3 cells subcutaneously injected into nude mice.
(**C**) Mean ± SD; n ≥ 3. (**E, F**) Mean ± SEM; n ≥ 3. *p < 0.05; **p < 0.01; ***p < 0.001.

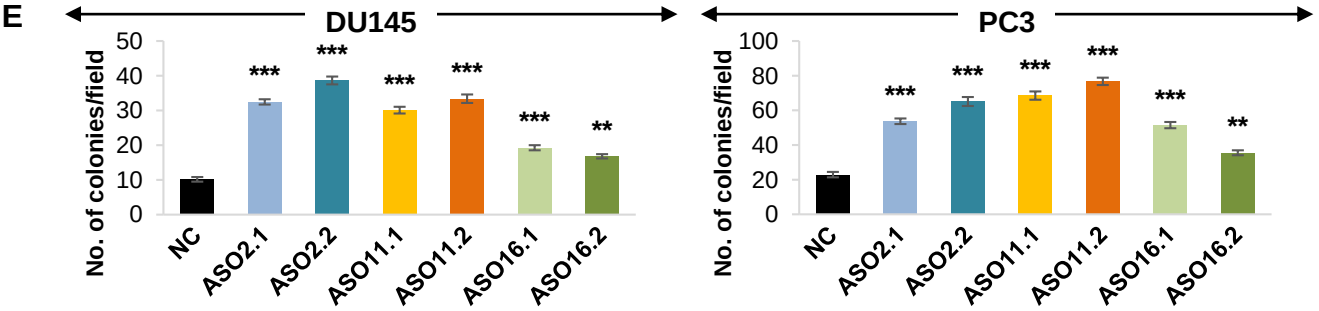
A

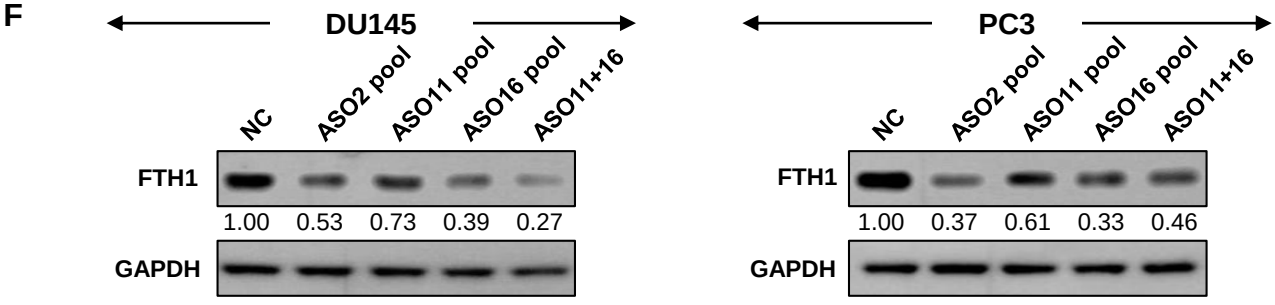
Target	ASO name	ASO sequence	Number of nucleotide mismatches between ASO and gene				
			FTH1	FTH1P2	FTH1P8	FTH1P11	FTH1P16
FTH1P2	ASO2.1	CATCCACTTAAGTTCGTTGA	>7		>6	9	>6
	ASO2.2	TTGTACCAAAACATCCACTT	8		8	>7	8
FTH1P11	ASO11.1	AGACCGTCTCCGCCTTCTCT	8	>7	8		>7
	ASO11.2	TTTAAAACCCCAGACTTTTC	>7	>4	9		>7
FTH1P16	ASO16.1	CTCGGCAAAGAACGTCTCTA	4	8	2	4	
	ASO16.2	AGTTGCTACGTAACAGTACA	>7	7	>5	9	





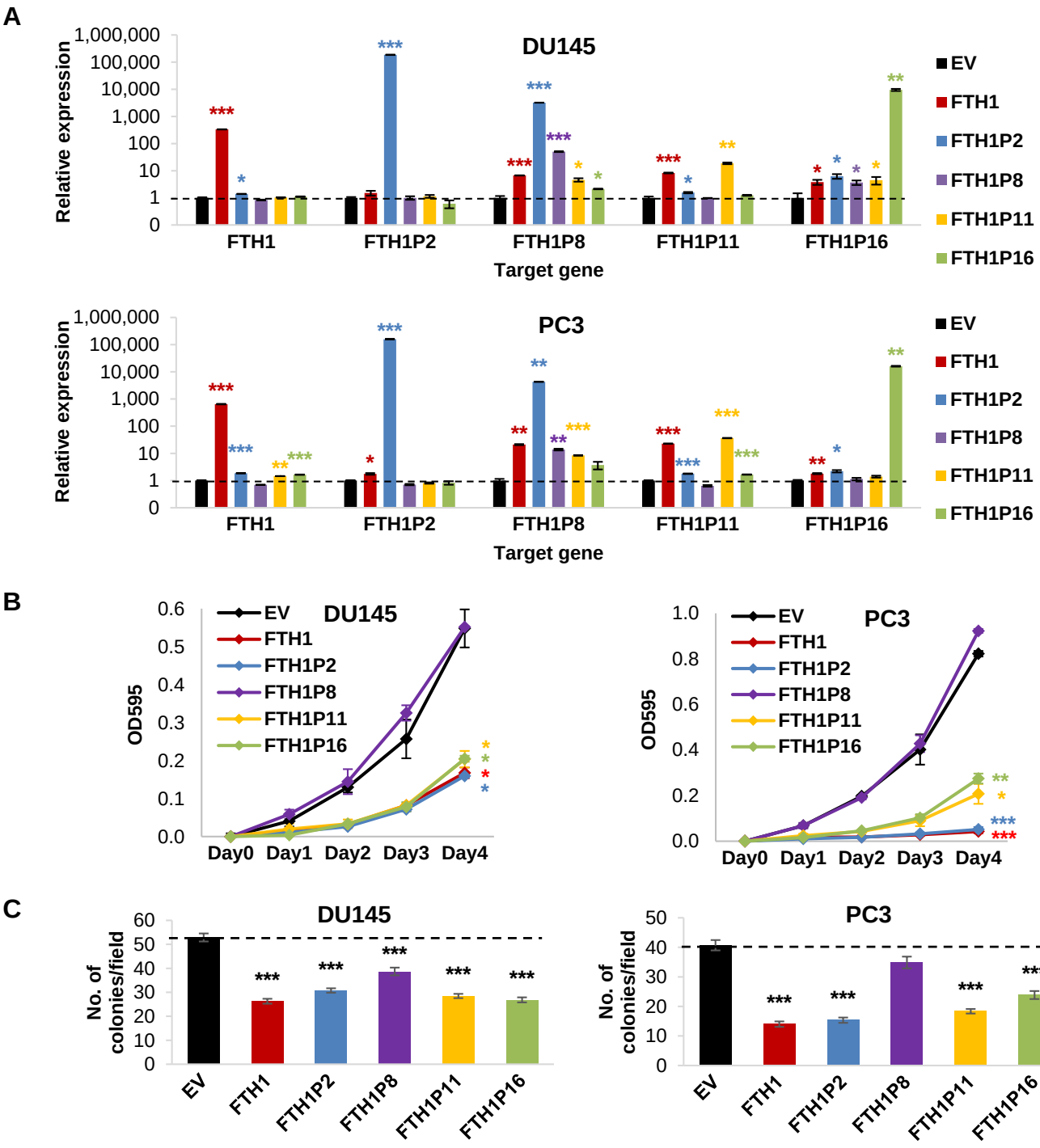




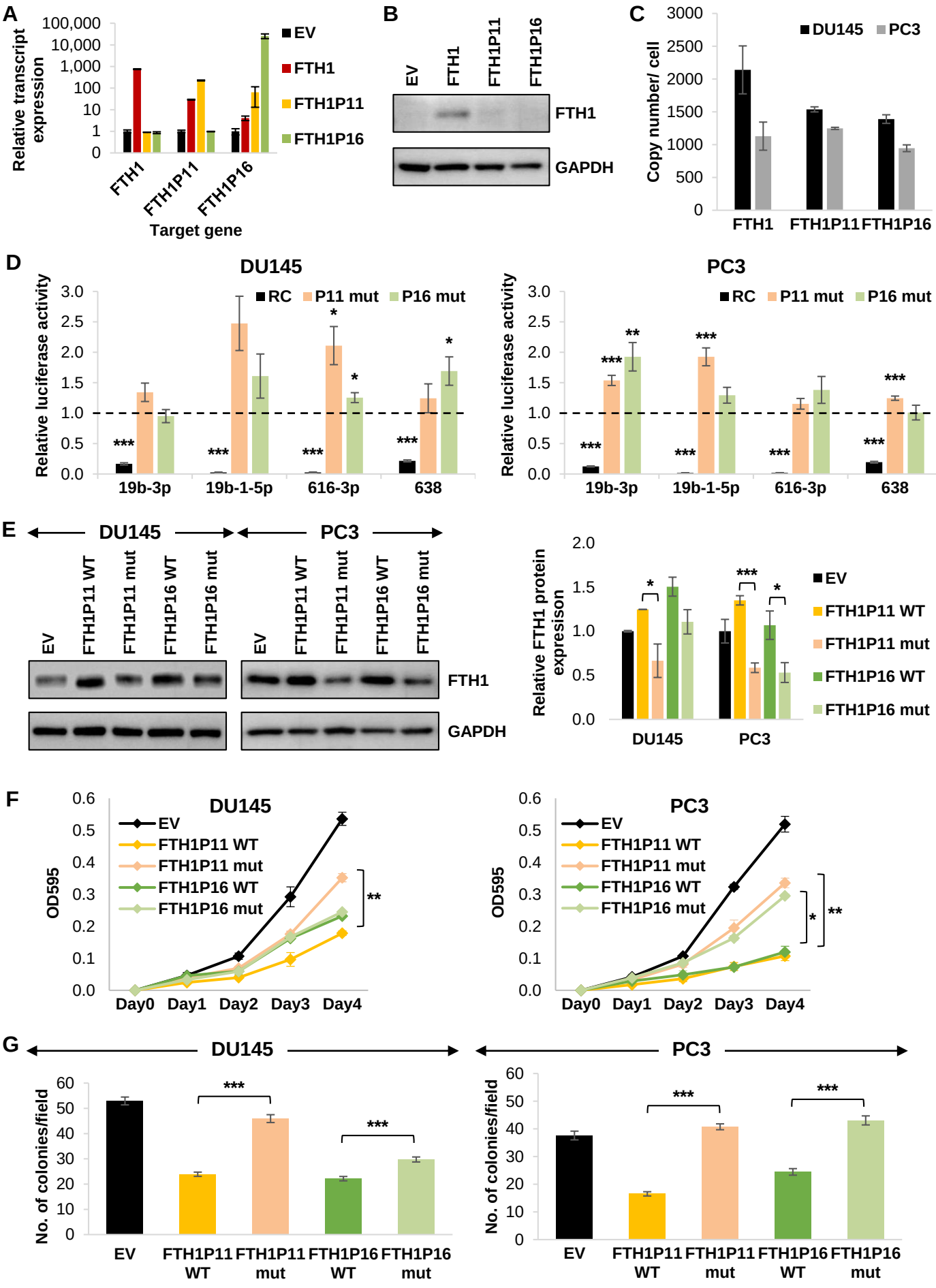


Supplementary Figure S5

Supplementary Figure S5. Effects of ASO-mediated knockdown of FTH1 pseudogenes in prostate cancer.
(**A**) Antisense oligonucleotide (ASO) sequences targeting the FTH1 pseudogenes. The number of nucleotide mismatches is derived from sequence alignments of each ASO and transcript. Alignments with breaks in the middle of individual ASO sequences are indicated with “>”.
(**B**) qRT-PCR validation of ASO-mediated FTH1 pseudogene knockdown.
(**C**) Effect of ASO-mediated knockdown of the FTH1 pseudogenes on cell proliferation.
(**D, E**) Effect of (**D**) and quantification of the effect of (**E**) ASO-mediated knockdown of the FTH1 pseudogenes on anchorage-independent growth *in vitro*.
(**F**) Effect of ASO-mediated knockdown of the FTH1 pseudogenes on FTH1 protein expression. Pool denotes the combination of two ASOs designed to target the same pseudogene.
(**B, E**) Mean ± SEM; n ≥ 3. (**C**) Mean ± SD; n ≥ 3. *p < 0.05; **p < 0.01; ***p < 0.001.

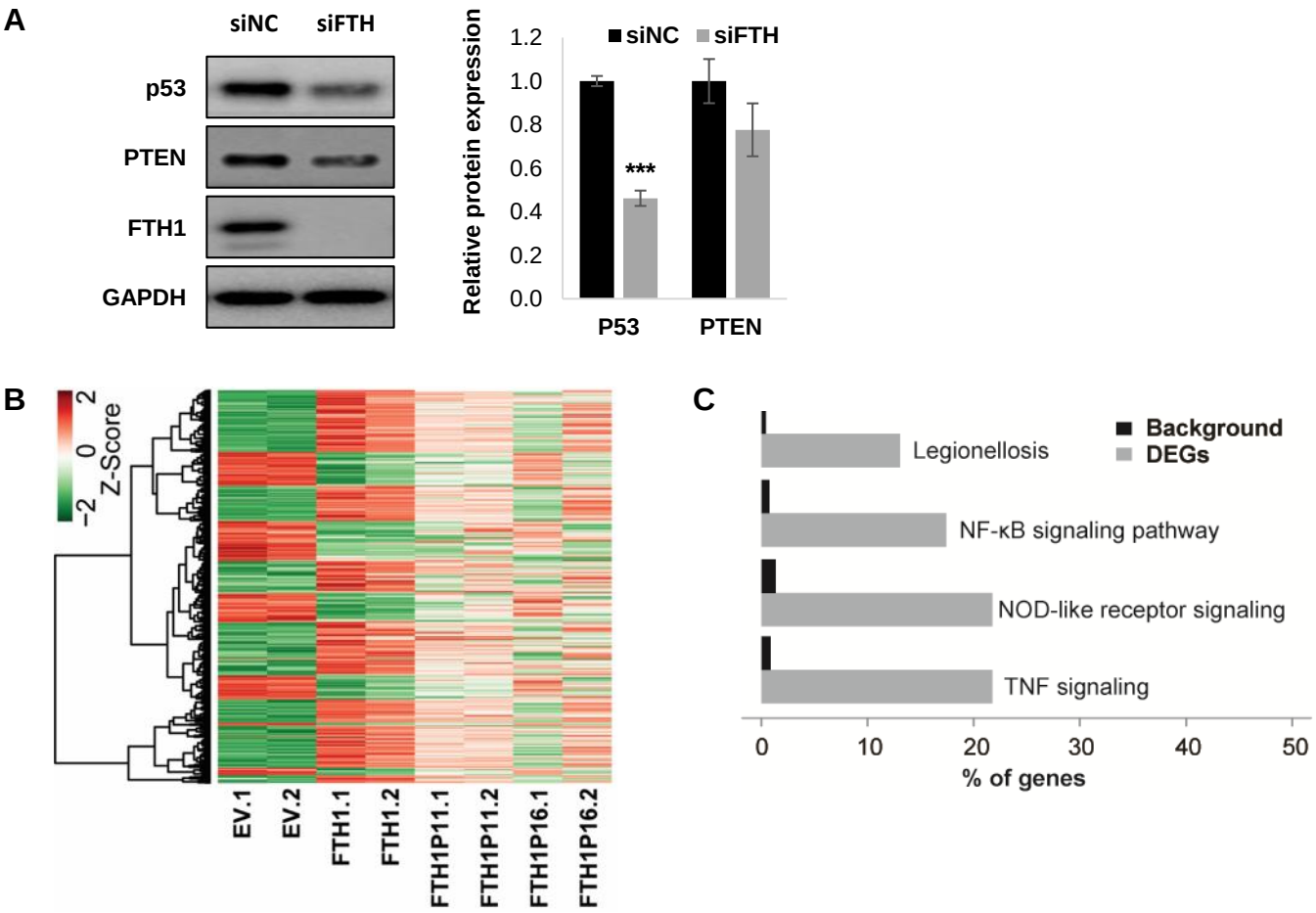


Supplementary Figure S6. Quantification of the effects of overexpressing FTH1 and its pseudogenes.
(**A**) qRT-PCR validation of the overexpression of FTH1 and its pseudogenes.
(**B**) Effect of overexpressing FTH1 and its pseudogenes on proliferation *in vitro*.
(**C**) Quantification of the effect of overexpressing FTH1 and its pseudogenes on anchorage-independent growth *in vitro*.
(**A, C**) Mean ± SEM; n ≥ 3. (**B**) Mean ± SD; n ≥ 3. *p < 0.05; **p < 0.01; ***p < 0.001.



Supplementary Figure S7

Supplementary Figure S7. Effect of MRE mutations on FTH1 pseudogenes.
(**A**) qRT-PCR validation of the overexpression of FTH1, FTH1P11 and FTH1P16 in Hap1 FTH1 knockout cell line.
(**B**) Effect of the overexpression of FTH1, FTH1P11 and FTH1P16 on FTH1 protein expression in Hap1 FTH1 knockout cell line.
(**C**) Estimated copy number per cell of FTH1 and its pseudogenes in DU145 and PC3 cells.
(**D**) Effect of selected miRNA overexpression on the luciferase reporter activity of the reverse complement (RC) control, and FTH1P11 and FTH1P16 MRE mutants (denoted by P11 and P16 mut).
(**E**-**G**) Effect of overexpressing wild-type (WT) and mutant FTH1P11 and FTH1P16 (mut) on FTH1 protein expression (**E**), cell proliferation (**F**) and anchorage-independent growth (**G**) *in vitro*.
(**A, C, D, E, G**) Mean ± SEM; n ≥ 3. (**F**) Mean ± SD; n ≥ 3. *p < 0.05; **p < 0.01; ***p < 0.001.



Supplementary Figure S8. Other regulatory pathways involving the FTH1 gene:pseudogene network.
(**A**) Effect of siRNA-mediated knockdown of FTH1 and its pseudogenes on protein expression of p53 and PTEN. Mean ± SEM; n ≥ 3. *p < 0.05; **p < 0.01; ***p < 0.001.
(**B, C**) Microarray analysis of differentially expressed genes (DEGs) (**B**) and KEGG pathway analysis of DEGs (**C**) upon FTH1, FTH1P11 and FTH1P16 overexpression.

Supplementary Table S1. Predicted miR-638 MREs on FTH1 and its pseudogenes.

Transcript	MRE no.	leftmost position of predicted target site	folding energy (-Kcal/mol)	MRE/ miRNA heteroduplex	p-value
FTH1	1	390	-11.1	TTGCCAAATACTTTCTTCACCAATCTCA ||| :||: |:: | |||:| TCCGG-CGGTGGGCGGG--CGCTAGGGA	9.15E-02
	2	491	-14.9	ATCAAGAAACCAGACTGTGATGACT | ||| |:|:||| || TCCGGCGGTGGGCGGGCGCTAGGGA	1.96E-02
	3	594	-14.7	AACTGGCCACTGACAAAAATGACCCCC : |||||: | :|| ||| TCCGGCGGTGGGCG--GGCGCTAGGGA	3.12E-02
	4	937	-14.1	AATTTGGTACCCAGGTGTTGTCTTT ::| :|||| :|: :||::| TCCGGCGGTGGGCGGGCGCTAGGGA	1.21E-01
	5	989	-16.9	TCCAGGCTATCTTCC--AGATTCCT |:|:|: || |||:||| TCCGGCGGTGGGCGGGCGCTAGGGA	1.15E-02
FTH1P2	6	16	-10.3	AGGAATGGTACAAATCAACGAACTTA ||| :| :|| :| ||| |:: TCC-GGCGGTGGGCGGGCGCTAGGGA	3.79E-01
	1	114	-11.1	TTGCCAAATACTTTCTTCACCAATCTCA ||| :||: |:: | |||:| TCCGG-CGGTGGGCGGG--CGCTAGGGA	9.06E-02
	2	215	-14.9	ATCAAGAAACCAGACTGTGATGACT | ||| |:|:||| || TCCGGCGGTGGGCGGGCGCTAGGGA	1.90E-02
	3	318	-14.7	AACTGGCCACTGACAAAAATGACCCCC : |||||: | :|| ||| TCCGGCGGTGGGCG--GGCGCTAGGGA	3.02E-02
	7	550	-19.8	CCCTGGTCACC-AAGAGTTATCCCT : |:|||| |: ||||| TCCGGCGGTGGGCGGGCGCTAGGGA	3.60E-01
FTH1P8	1	455	-11.1	TTGCCAAATACTTTCTTCACCAATCTCA ||| :||: |:: | |||:| TCCGG-CGGTGGGCGGG--CGCTAGGGA	9.15E-02
	2	556	-14.9	ATCAAGAAACCAGACTGTGATGACT | ||| |:|:||| || TCCGGCGGTGGGCGGGCGCTAGGGA	2.03E-02
	3	657	-14.7	AACTGGCCACTGACAAAAATGACCCCC : |||||: | :|| ||| TCCGGCGGTGGGCG--GGCGCTAGGGA	2.03E-02
	8	901	-19.2	AGGCAGTGCATGCATGTTGGGGTTTCCT |||| || ::| :|:: | | |:||| TCCG--GCG-GTGGGCGGGCGCTAGGGA	2.73E-01

FTH1P11	2	558	-14.9	ATCAAGAAACCAGACTGTGATGACT | ||| |:|:||| || TCCGGCGGTGGGCGGGCGCTAGGGA	1.96E-02
	3	661	-14.7	AACTGGCCACTGACAAAAATGACCCCC : |||||: | :|| ||| TCCGGCGGTGGGCG--GGCGCTAGGGA	1.34E-02
FTH1P16	9	11	-10.3	ATACCATGACATC--ACCACAGTCTTA || ||:| || | :||:: TCCGG--CGGTGGGCGGGCGCTAGGGA	1.82E-02
	10	183	-22.8	CGGCCGCC-CATAGCCAGCCCTCCCT |||| | ||| || ||||| TCCGGCGGTG-GGCGGGCGCTAGGGA	1.83E-01
	11	270	-26.7	CCACTGCCACCGCCGCCACCTCTCCTT |:||||| |||| | |||:| TCCGGCGGTGG--GCGGGCGCTAGGGA	6.34E-02
	1	461	-11.1	TTGCCAAATACTTTCTTCACCAATCTCA ||| :||: |:: | |||:| TCCGG-CGGTGGGCGGG--CGCTAGGGA	9.15E-02
	2	562	-14.1	ATCAAAAAACCAGACTGTGATGACT ||| |:|:||| || TCCGGCGGTGGGCGGGCGCTAGGGA	2.80E-02
	12	665	-14.7	AATTGGCCACTGACAAAAATGACCCCC : |||||: | :|| ||| TCCGGCGGTGGGCG--GGCGCTAGGGA	4.16E-02

Supplementary Table S1

Supplementary Table S2. Predicted MREs of miRNAs that target FTH1 and its pseudogenes.

miRNA Name	Transcript	leftmost position of predicted target site	folding energy (-Kcal/mol)	MRE/ miRNA heteroduplex	p-value
hsa-miR-19b-3p	FTH1P2	295	-16.7	TCAGTCACTACTGGAACTGCACA ||||| |||| |||||| AGTCAAAACGTACCTAAACGTGT	3.02E-02
	FTH1P8	634	-16.8	TCAGT-CACCACTGGAACTGCACA ||||| || |||| |||||| AGTCAAAACGT-ACCTAAACGTGT	2.03E-02
	FTH1P16	642	-16.7	TCAGTCACTACTGGAACTGCACA ||||| |||| |||||| AGTCAAAACGTACCTAAACGTGT	4.16E-02
hsa-miR-19b-1-5p	FTH1P2	165	-15	AACTGATG-AAGC-TGCAGAACC |||| ||:| ||||:||| CGACCTACGTTTGGACGTTTTGA	2.94E-02
	FTH1P11	508	-15	AACTGATG-AAGC-TGCAGAACC |||| ||:| ||||:||| CGACCTACGTTTGGACGTTTTGA	3.03E-02
	FTH1P16	513	-15.4	ACTG-ATG-AAGC-TGCAGAACC ||| ||| ||:| ||||:||| CGACCTACGTTTGGACGTTTTGA	3.03E-02
hsa-miR-181a-5p	FTH1P8	893	-19	GGTCACCAAGGCAGTGCATGCATGTT : ||||| |:|||:| || ||||| TGAGTGG--CTGTCGC-AACTTACAA	2.73E-01
	FTH1P16	899	-19	GGTCACCAAGGCAGTGCATGCATGTT : ||||| |:|||:| || ||||| TGAGTGG--CTGTCGC-AACTTACAA	1.74E-01
hsa-miR-210-3p	FTH1P2	299	-16.2	TCA-CTACTGGAAC-TGCACAA ||| | ||| || :||||| AGTCGGCGACAGTGTGCGTGTC	3.02E-02
	FTH1P8	638	-18.3	TCA-CCACTGGAAC-TGCACAA ||| || ||| || :||||| AGTCGGCGACAGTGTGCGTGTC	2.03E-02

hsa-miR-362-5p	FTH1P2	478	-17.9	GCACAC-CCTGGGGGACAGTGATA :| ||| |||:|| ||: ||| TGAGTGTGGATCCAAGGTTCCTAA	1.76E-01
	FTH1P11	821	-16.8	GCACAC-CCTGGGAGACAGTGATA :| ||| |||:|| ||: ||| TGAGTGTGGATCCAAGGTTCCTAA	1.46E-01
	FTH1P16	825	-16.8	GCACAC-CCTGGGAGACAGTGATA :| ||| |||:|| ||: ||| TGAGTGTGGATCCAAGGTTCCTAA	1.74E-01
hsa-miR-616-3p	FTH1P2	176	-16	CTGC-AGAACCAACGAGGTGGCC |||| :|||| ::||:| GACGAGTTTGG-GAGGTTACTGA	2.94E-02
	FTH1P2	311	-21.6	CTGCACAAACTGGCCACTGACA |||| |||||: ||| |||| GACGAGTTTGGGAGGTTACTGA	3.02E-02
	FTH1P8	517	-16	CTGC-AGAACCAATGAGGTGGCC |||| :|||| ::||:| GACGAGTTTGG-GAGGTTACTGA	1.29E-02
	FTH1P8	650	-21.6	CTGCACAAACTGGCCACTGACA |||| |||||: ||| |||| GACGAGTTTGGGAGGTTACTGA	2.03E-02
	FTH1P11	519	-16	CTGC-AGAACCAACGAGGTGGCC |||| :|||| ::||:| GACGAGTTTGG-GAGGTTACTGA	3.03E-02
	FTH1P11	654	-20	CTGTACAAACTGGCCACTGACA |||: |||||: ||| |||| GACGAGTTTGGGAGGTTACTGA	1.34E-02
	FTH1P16	523	-16	CTGC-AGAACCAACGAGGTGGCC |||| :|||| ::||:| GACGAGTTTGG-GAGGTTACTGA	3.03E-02
	FTH1P16	658	-19.2	CTGCACAAATTGGCCACTGACA |||| ||||:: ||| |||| GACGAGTTTGGGAGGTTACTGA	4.16E-02

Supplementary Table S2

Supplementary Table S3. Mutated MREs and primer sequences used for mutagenesis.

Gene	miRNA Name	Mutated MRE sequence	Mutagenesis primer sequence
FTH1P11	hsa-miR-19b-1-5p	AACTGATGAAGCTGGTCTTGC	TCGGCCACCTCGTTGCAAGACCAGCTT CATCAGTTTC
	hsa-miR-616-3p	CTGGTCTTGCAACGACCACCGC*	CTGAAGGAAGATTCGCGGTGGTCGTTG CAAGACCAGCT
		CTGTACAAACTGGCCTCACTGA	TGGGGGTCATTTTTCAGTGAGGCCAGT TTGTACAG
	hsa-miR-638	AACTGGCCTCACTGAAAAATCTCGGGC*	GAAGTCACACAAATGCCCGAGATTTTT CAGTGAGGCCA**
FTH1P16	hsa-miR-19b-3p	TCAGTCACTACTGGAACACGTGA	GAAGTCACACAAATGCCCGAGATTTTT GTCAGTGGCCAATTTCACGTGTTCCAG TAGTG**
	hsa-miR-19b-1-5p	ACTGATGAAGCTGGTCTTGC	GATTCGGCCACCTCGTTGCAAGACCAG CTTCATCAGTTTCTCAGC
	hsa-miR-616-3p	CTGGTCTTGCAACGACCACCGC*	CTGAAGGAAGATTCGCGGTGGTCGTTG CAAGACCAGC**
	hsa-miR-638	ATACCATGACATCACCACACAGAAA	GCTACGTAACAGTACAAGTTTTTTCTG TGTGGTGATGTCATGG
		CCACTGCCACCGCCGCCACCTCAGGAT	CATGGCGGCGACTATCCTGAGGTGGCG GCG
		TTGCCAAATACTTTCTTCACCATAGAGA	GCTCCCTCTCCTCATCTCTATGGTGAA GAAAGTATTTGGC
		AATTGGCCACTGACAAAAATCTCGGGC	GAAGTCACACAAATGCCCGAGATTTTT GTCAGTGGC

Mutated nucleotides are in red

* MRE contains overlapping mutated sequence from another closely spaced MRE

** Primers used in sequential mutagenesis to mutate closely spaced MREs