

TBX1 targets the miR-200–ZEB2 axis to induce epithelial differentiation and inhibit stem cell properties

Supplementary Materials

Supplementary Table S1. Primer sequences for plasmid construction.

Primer	Sequence
miR-200 promoter_–1574_FW_NheI	5'-ACCTGCTAGCCATTCCCGACAGTCACAG-3'
miR-200 promoter_–321_FW_NheI	5'-GTGGGCTAGCAGAGGTGGAGAGGCGAGAGT-3'
miR-200 promoter_–158_FW_NheI	5'-ATCGGCTAGCCTGCGTCACCGTCACT-3'
miR-200 promoter_–110_FW_NheI	5'-GTGAGCTAGCCAGGGGACACACCTGTGCG-3'
miR-200 promoter_–64_FW_NheI	5'-GTCGGCTAGCGAGCTCGCACCTGTGTGG-3'
miR-200 promoter_–64_Mutant_FW_NheI	5'-GTCGGCTAGCGAGCTCGTCTGTGTGG-3'
miR-200 promoter_+120_RV_BglII	5'-CAGGAGATCTCCTGGCACAGGAAGTCAGTT-3'
miR-200 promoter_+19_RV_BglII	5'-TGGAAGATCTGGGGCCTCGGGAGGGAAGAG-3'
miR-203 promoter_–667_FW_XhoI	5'-CCGCTCGAGATGGCTCCAGACTTGGGGCA-3'
miR-203 promoter_+77_RV_HindIII	5'-CCCAGCTTCGACTGATCCTCCACGGC-3'
miR-203 promoter_–268_FW_XhoI	5'-CCGCTCGAGAAGTGAGAGGGGCTGGGGTGG-3'
miR-203 promoter_–248_FW_XhoI	5'-CCGCTCGAGGGGTGTGTCCAGCCCAGCCCC-3'
miR-203 promoter_–228_FW_XhoI	5'-CCGCTCGAGCCACACCCACCGGAGAGCTAG-3'
miR-203 promoter_–177_FW_XhoI	5'-CCGCTCGAGCCTCACCTGTTCCGGCCACCC-3'
miR-203 promoter_–228_Mutant_FW_XhoI	5'-CCGCTCGAGCCATGGCCACCGGAGAGCTAG-3'
Zeb2_FW_ClaI	5'-AAAATCGATATGAAGCAGCCGATCATG-3'
Zeb2_RV_SalI	5'-AAAGTCGACTTATTCCATGCCATCTTCC-3'

Underlined letters indicate the mutation in the promoter sequence.

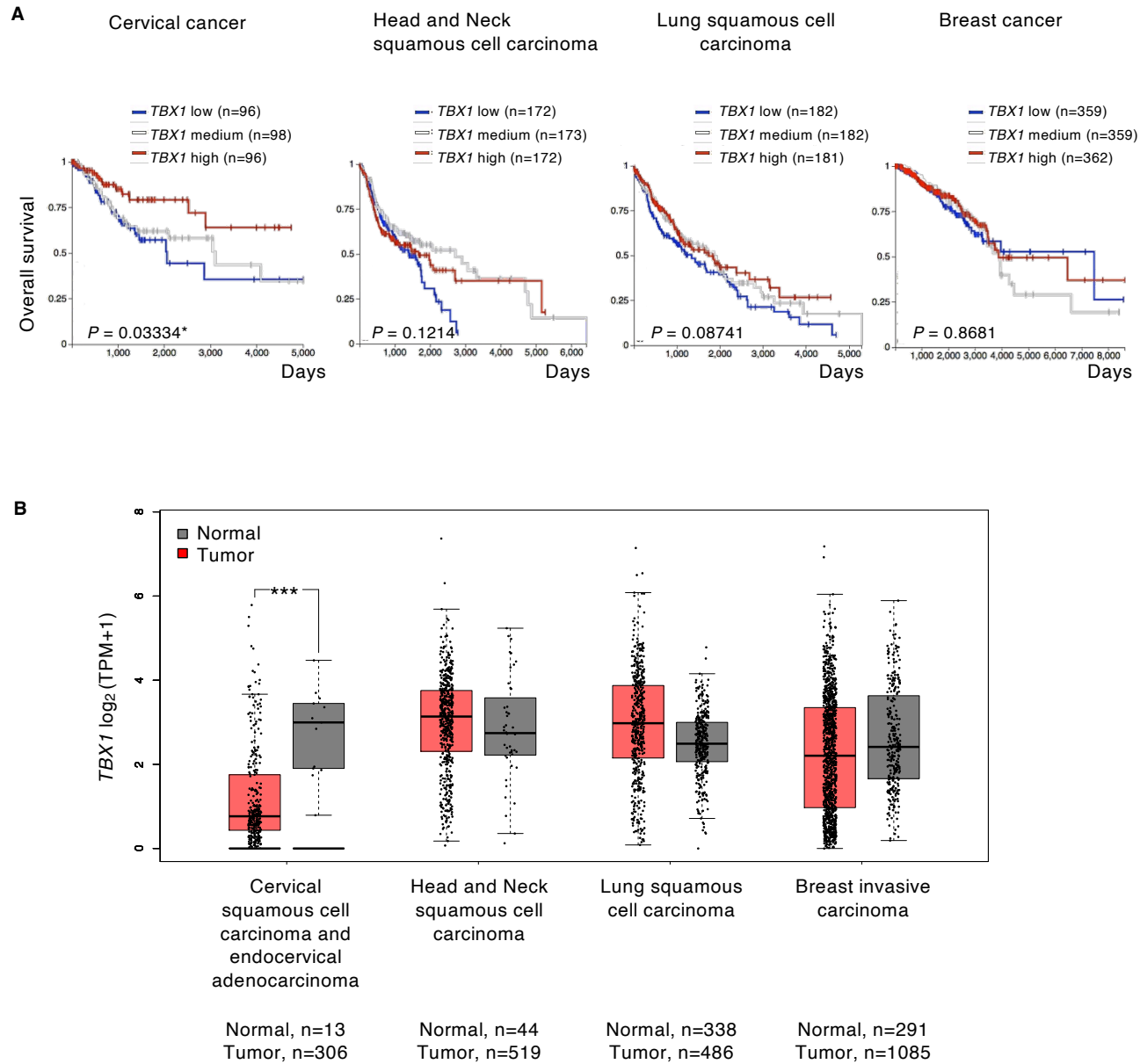
Supplementary Table S2. Primer sequences for qPCR.

Gene	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
<i>TBX1</i>	GCGCTGTGGGACGAGTTCAATCAG	GCACAAAGTCCATGAGCAGCATGTAGTC
<i>ZEB1</i>	TTACACCTTTGCATACAGAACCC	TTTACGATTACACCCAGACTGC
<i>ZEB2</i>	CAAGAGGCGCAAACAAGCC	GGTTGGCAATACCGTCATCC
<i>BMI1</i>	CGTGTATTGTTTCGTTACCTGGA	TTCAGTAGTGGTCTGGTCTTGT
<i>SOX2</i>	TACAGCATGTCCTACTCGCAG	GAGGAAGAGGTAACACAGGG
<i>ETV7</i>	CTGCTGTGGGATTACGTGTATC	GTTCTTGTGATTTCCCCAGAGTC
<i>KRT17</i>	GCCGCATCCTCAACGAGAT	CGCGGTTTCAGTTTCTCTGTC
<i>TBP</i>	CAGCAACTTCCTCAATTCCTTG	GCTGTTTAACTTCGCTTCCG
<i>pri-miR-200b-200a-429</i>	TCGAAACTCTCCAGAGACG	AGACCTGCAAGGGTGAGCTT
<i>Vegfa</i>	GCTTCCTACAGCACAGCAGA	AATGCTTTCTCCGCTCTGAA
<i>Kdr</i>	CAAACCTCAATGTGTCTCTTTGC	AGAGTAAAGCCTATCTCGCTGT
<i>Flt1</i>	CACTGACATACCCAAACTTGTGC	GTCCCATGTTATTCTTTGCCCAT
<i>Zeb2</i>	ATGAGCTTCCTACCGCATATG	GGCCTGACATGTAGTCTTGTG
<i>Zeb1</i>	AGACCAGACAGTATTACCAGGAG	ACATTGCGATCATGGTTTTGC
<i>Krt15</i>	GTCAATCTCCAGGACAACGC	CAGATCGGGACTACAGCCAT
<i>Krt5</i>	CACAAACTCATTTCTAGCCG	CAGAGCTGAGGAACATGCAG
<i>Krt18</i>	GACTCTAAAGTCATCGGCGG	CTGGGGCCACTACTTCAAGA
<i>Krt19</i>	CTGAAGTCATCTGCAGCCAG	AGACCATCGAGGACTTGCG
<i>Krt17</i>	ACCATCCGCCAGTTTACCTC	CTACCCAGGCCACTAGCTGA
<i>Sfn</i>	TTGGCTGAACAGGCCGAAC	GTAAGCTACGGAAAGCAGGTTT
<i>Tbx1</i>	GCGCTGTGGGACGAGTTCAATCAG	GCACAAAGTCCATGAGCAGCATGTAGTC
<i>Kdr</i>	CAAACCTCAATGTGTCTCTTTGC	AGAGTAAAGCCTATCTCGCTGT
<i>Flt1</i>	CACTGACATACCCAAACTTGTGC	GTCCCATGTTATTCTTTGCCCAT
<i>Actb</i>	ATGGAGGGGAATACAGCCC	TTCTTTGCAGCTCCTTCGTT

Supplementary Table S3. Primer sequences for ChIP.

Primer	Sequence
miR-200 promoter_-110_FW	5'-CAGGGGACACACCTGTCG-3'
miR-200 promoter_+19_RV	5'-GGGGCCTCGGGAGGGAAGAG-3'
miR-203 promoter_-298_FW	5'-TCTCTTTACCCCTCCCCTTC-3'
miR-203 promoter_-66_RV	5'-CCGAACCGTCTCGTCTGG-3'

Supplementary Figure S1

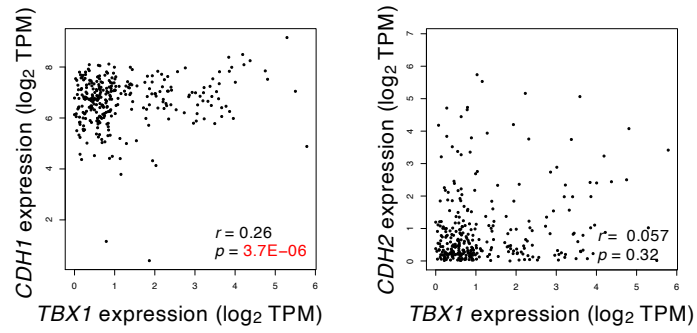


Supplementary Figure S1. Overall survival of patients with *TBX1*-expressing carcinomas.

(A) Overall survival of patients with primary head and neck cancer, lung squamous cell carcinoma, and breast cancer with high, medium, or low *TBX1* expression. Two-sided *P*-values were computed using a log-rank (Mantel-Cox) test.

(B) Box plots obtained from GEPIA using the GTEx database to compare the expression of *TBX1* in tumors and matched normal specimens. TPM, transcripts per million. ****P* ≤ 0.001; one-way ANOVA.

Supplementary Figure S2

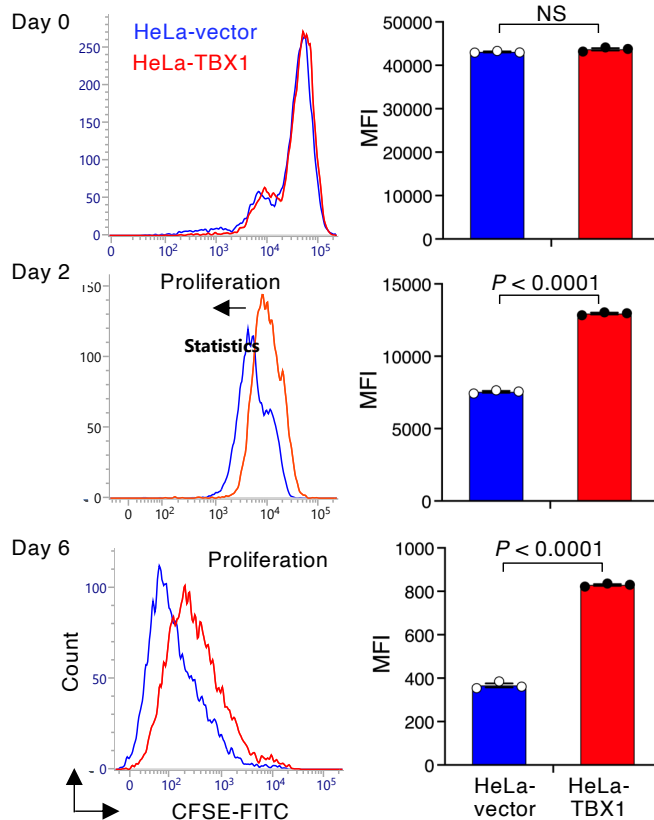


Supplementary Figure S2. Correlation of *TBX1* and *CDH1* or *CDH2* gene expression.

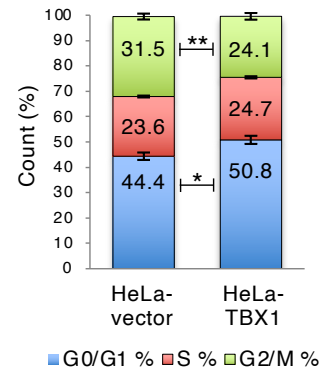
Positive correlation between *TBX1* and *CDH1*, but not *CDH2*, was observed in cervical cancer tissues analyzed by GEPIA using TCGA database. r , Pearson correlation coefficient; p , significance level.

Supplementary Figure S3

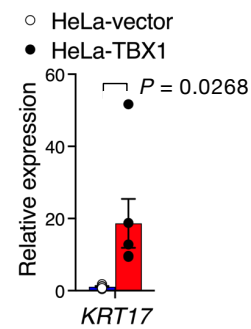
A



B



C



Statistics

Supplementary Figure S3. TBX1 suppresses proliferation and induces expression of the epithelial differentiation marker *KRT17*.

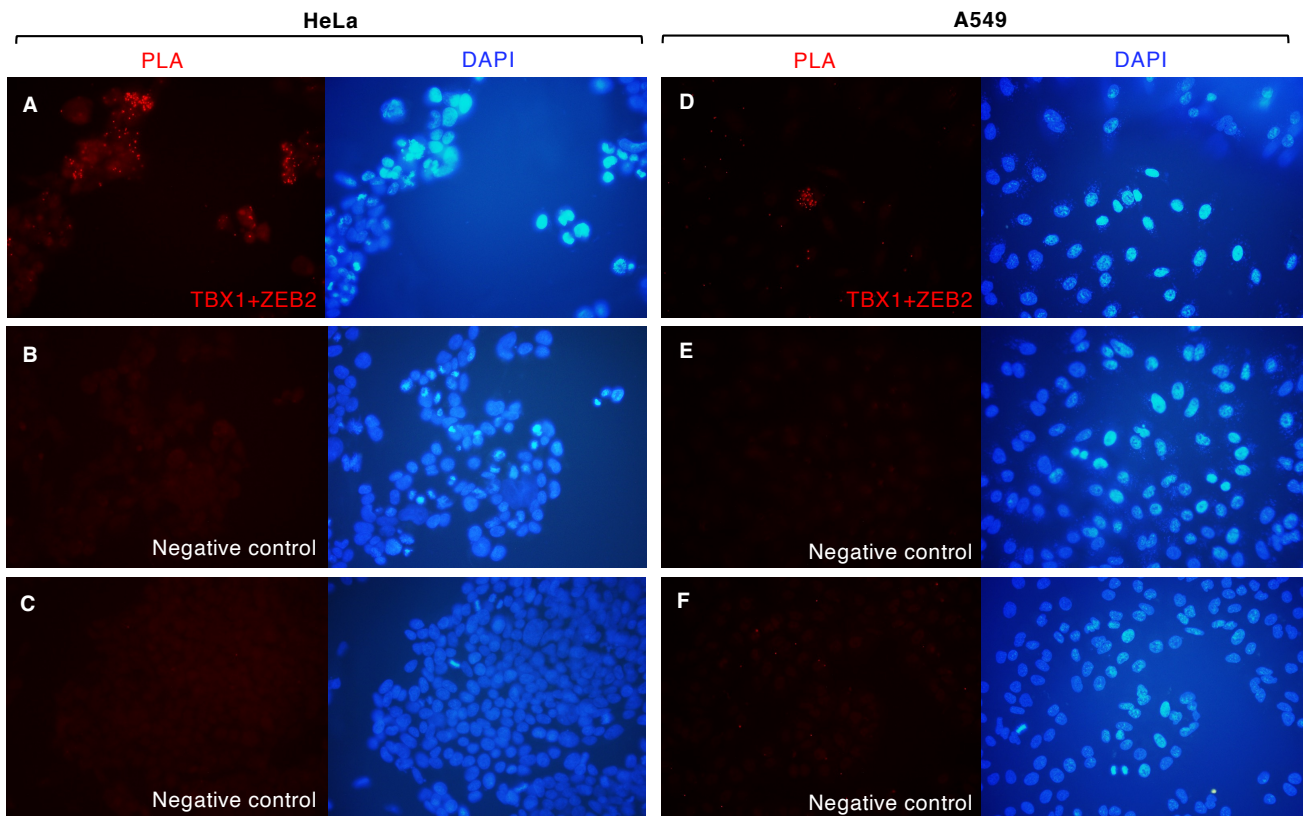
(A) Flow cytometry analysis of empty vector and TBX1 stably transfected HeLa cells labeled with the CFSE fluorescent dye: representative day 0, day 2, and day 5 histograms (left) and means $\pm$ s.e.m. of quantified results from three experiments with replicate wells (right) are shown. NS, not significant.

(B) HeLa cells overexpressing TBX1 show reduced proliferation as determined by cell cycle analysis ($n = 6$). * $P \leq 0.05$, ** $P \leq 0.01$.

(C) Expression of *KRT17* examined by qPCR in HeLa cells stably transfected with control pEBMulti plasmid (vector) or pEBMulti-TBX1 plasmid (TBX1) ($n = 6$).

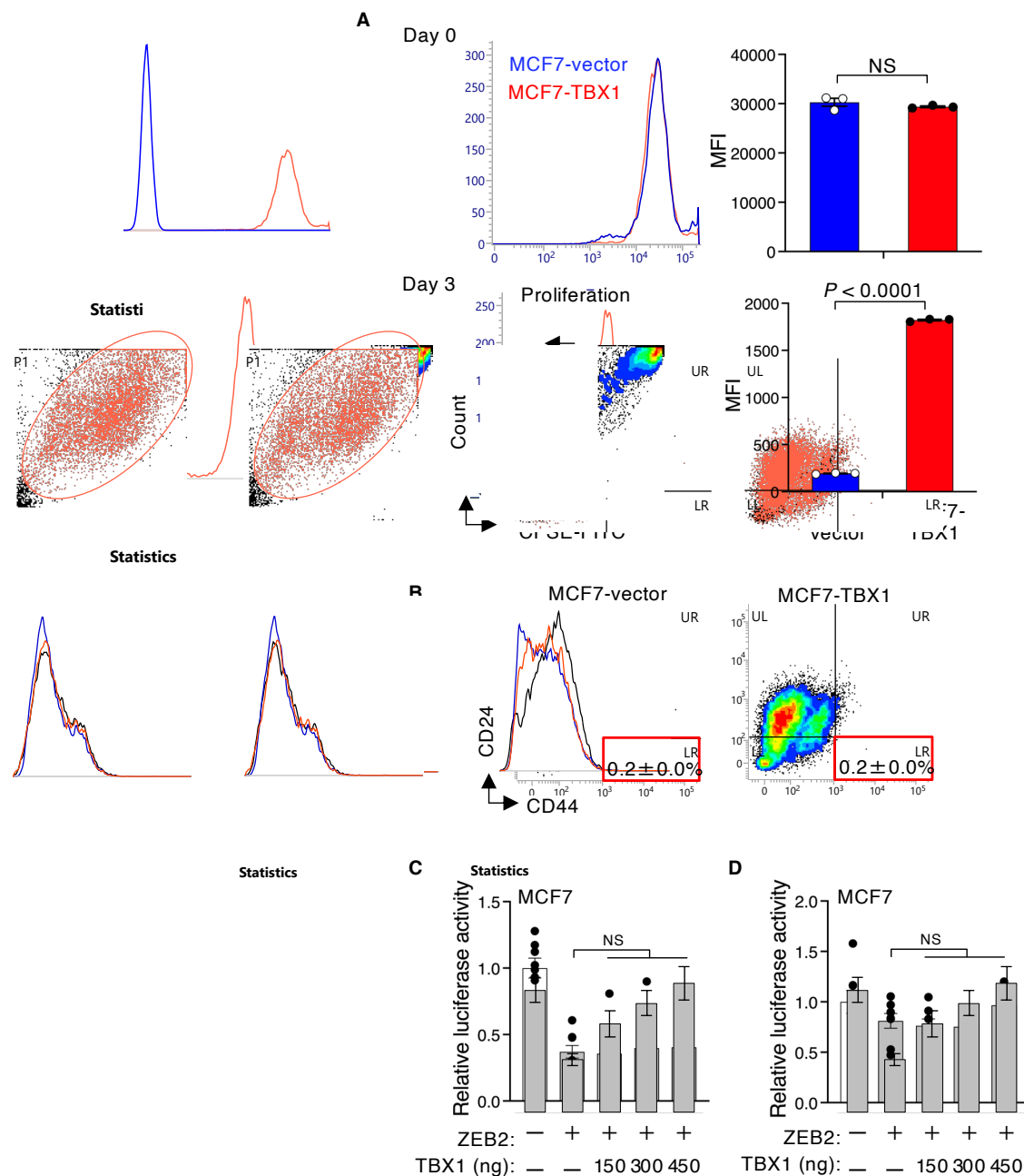
For A-C, the results are presented as mean $\pm$ s.e.m.; unpaired two-tailed Student's *t*-test.

Supplementary Figure S4



Supplementary Figure S4. *In situ* proximity ligation assay (PLA) detection of the TBX1 and ZEB2 colocalization.
 (A,D) HeLa (A) and A549 cells (D) cotransfected with Myc-tagged TBX1 and Flag-tagged ZEB2. PLA shows a very close proximity between TBX1 and ZEB2, indicated by fluorescent spots (red).
 (B,E) PLA without antibodies (assay negative control).
 (C,F) PLA without transfection of constructs encoding TBX1 and ZEB2 (assay negative control).

Supplementary Figure S5



Supplementary Figure S5. TBX1 suppresses the proliferation of MCF7 cells.

(A) Flow cytometry analysis of empty vector and TBX1 stably transfected MCF7 cells labeled with the CFSE fluorescent dye: representative day 0 and day 3 histograms (left) and means $\pm$ s.e.m. of quantified results from three experiments with replicate wells (right) are shown.

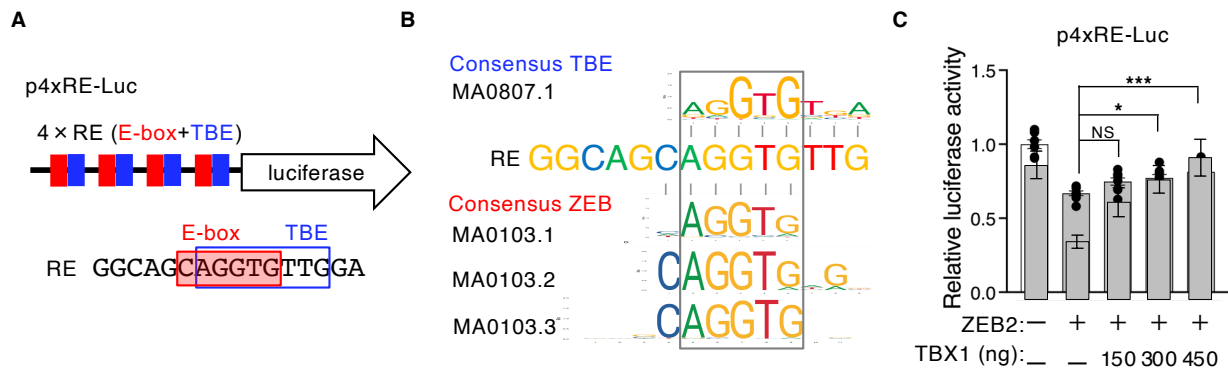
(B) Flow cytometric analysis of CD24 and CD44 expression in empty vector (MCF7-vector) and TBX1 stably transfected MCF7 cells (MCF7-TBX1). The percentage of CD44⁺CD24⁻ stem cell population (lower right box) is indicated ($n = 6$).

(C) Relative luciferase activity in MCF7 cells transiently co-transfected with the *miR-200b-200a-429* promoter construct (-321/+120), ZEB2 (150 ng), and the indicated amounts of TBX1 ($n = 8$).

(D) Relative luciferase activity in MCF7 cells transiently co-transfected with the *miR-203* promoter construct (-667/+77), ZEB2 (150 ng), and the indicated amounts of TBX1 ($n = 8$).

For A, C, and D, the results are presented as mean $\pm$ s.e.m.; NS, not significant; unpaired two-tailed Student's t-test.

Supplementary Figure S6



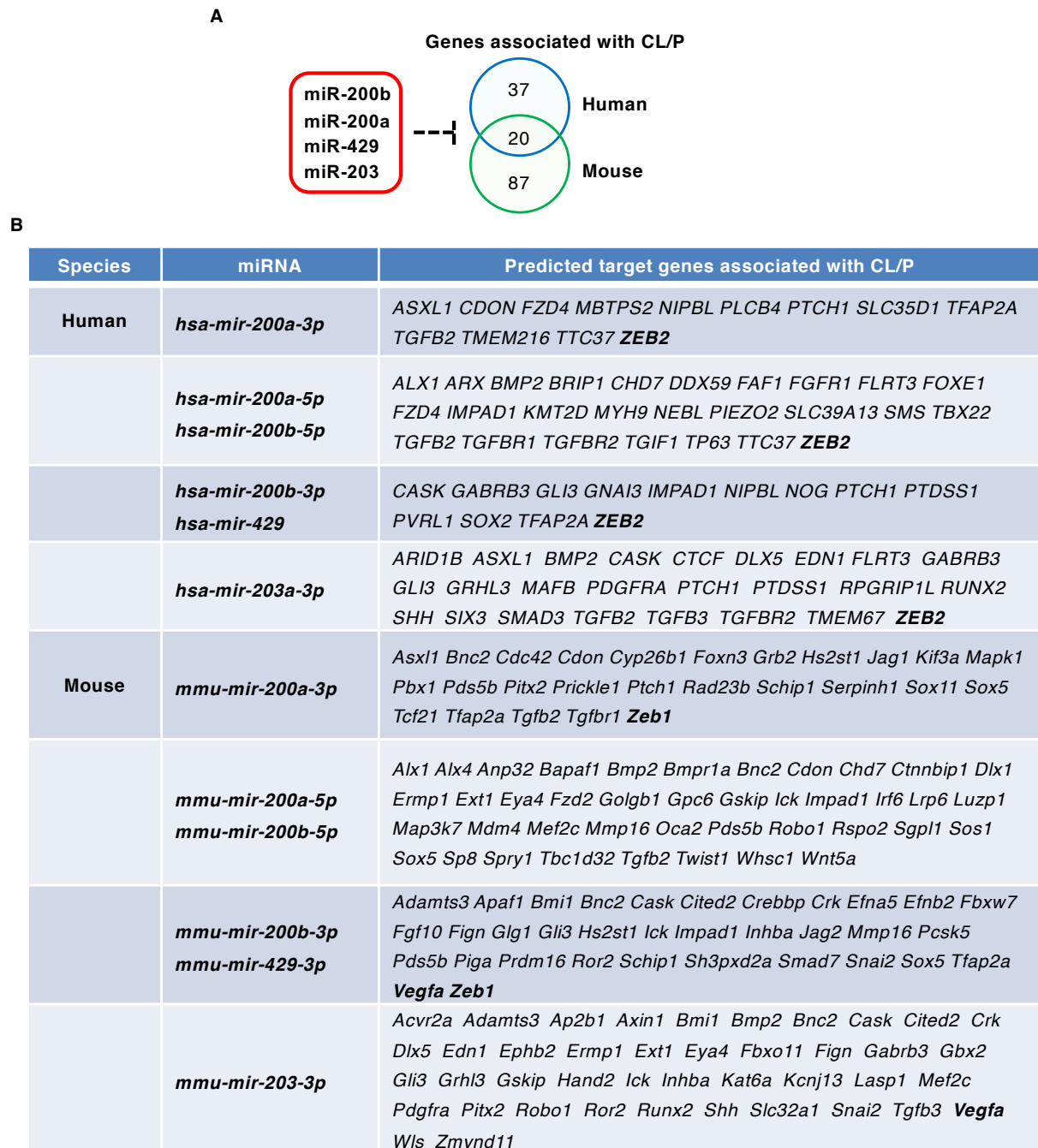
Supplementary Figure S6. TBX1 hardly affects the ZEB2-dependent inhibition of p4xRE-Luc.

(A) Schematic of the luciferase reporter plasmid (p4xRE-Luc) containing four copies of E-box and TBE.

(B) JASPAR sequence logos of E-box and TBE motifs. Box, overlapping core sequences.

(C) Relative luciferase activity in HeLa cells transiently co-transfected with p4xRE-Luc reporter (150 ng), ZEB2 (150 ng), and the indicated amounts of TBX1 ($n = 8$, $*P \leq 0.05$; $***P \leq 0.001$; NS, not significant; one-way ANOVA).

Supplementary Figure S7

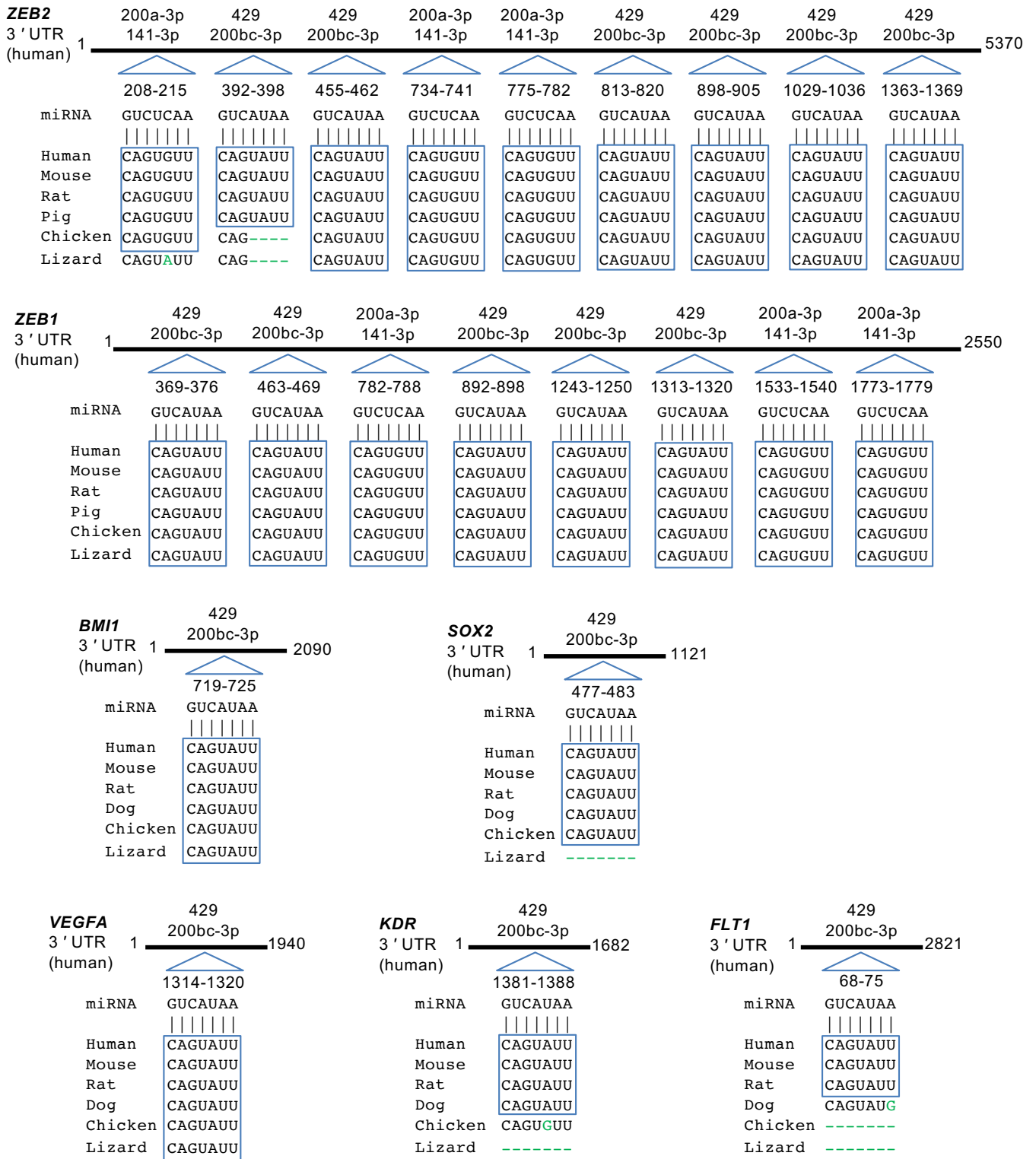


Supplementary Figure S7. miR-200 and miR-203 predicted target genes associated with CL/P.

(A) Venn diagram depicting the overlap of human and mouse genes associated with CL/P. The numbers in each area represent the number of miR-200 and miR-203 predicted target genes in B.

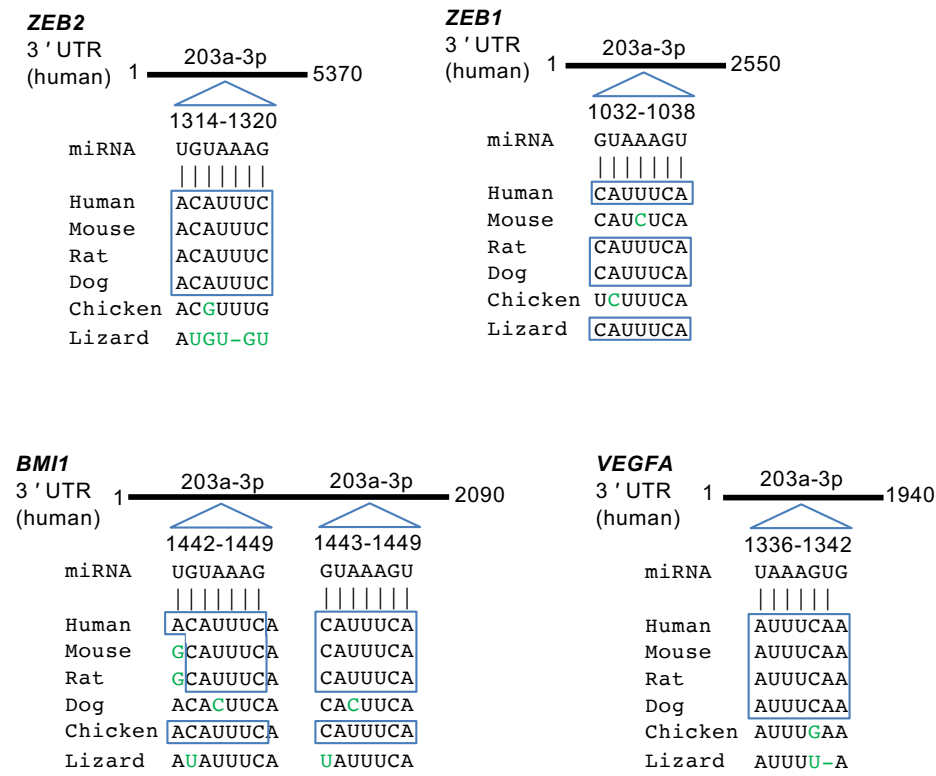
(B) miR-200 and miR-203 predicted target genes associated with CL/P in humans and mice.

Supplementary Figure S8



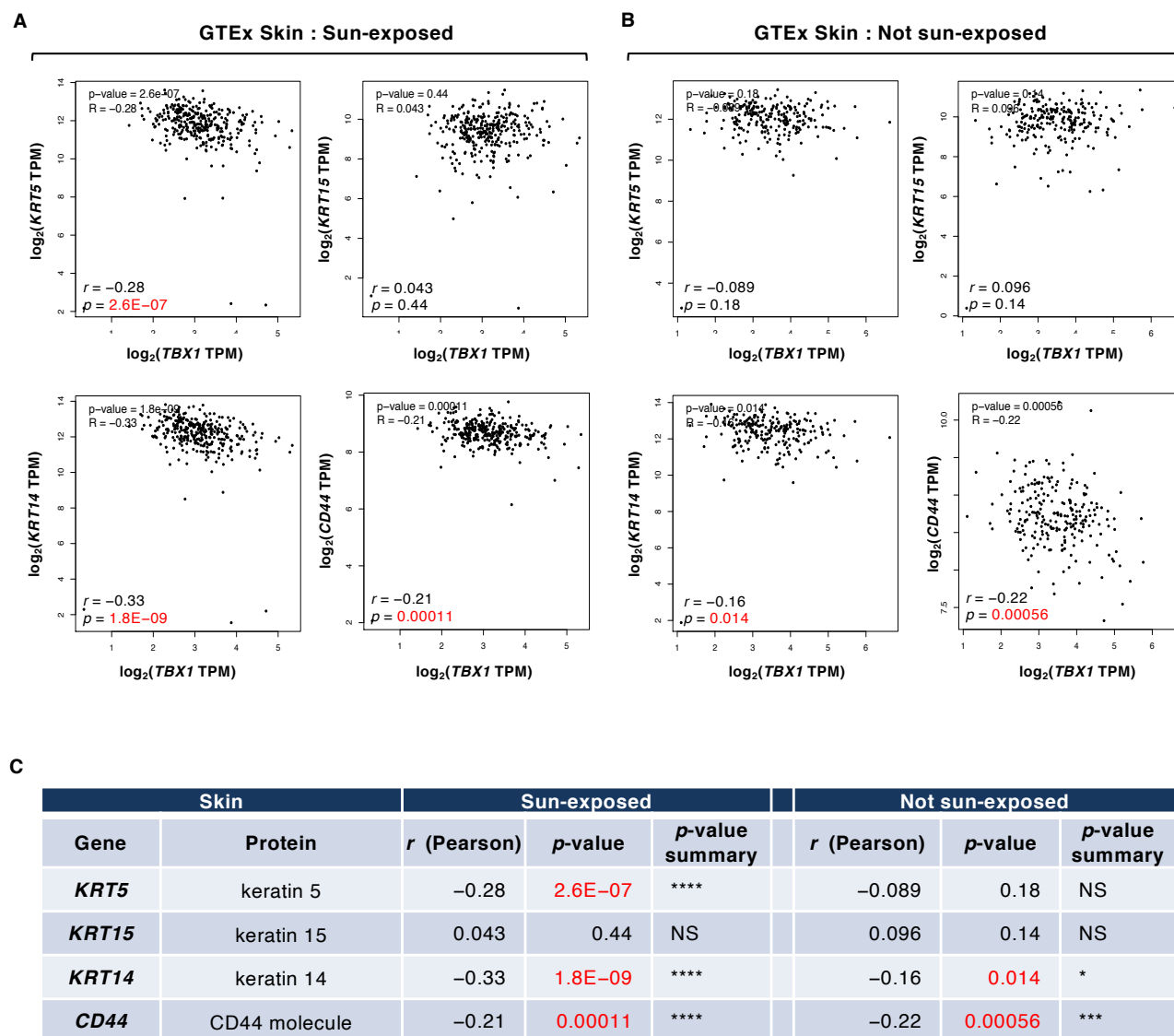
Supplementary Figure S8. A schematic diagram of binding sites for miR-200 predicted by TargetScan.
Blue boxes highlight sequence conservation among multiple species.

Supplementary Figure S9



Supplementary Figure S9. A schematic diagram of binding sites for miR-203 predicted by TargetScan. Blue boxes highlight sequence conservation among multiple species.

Supplementary Figure S10

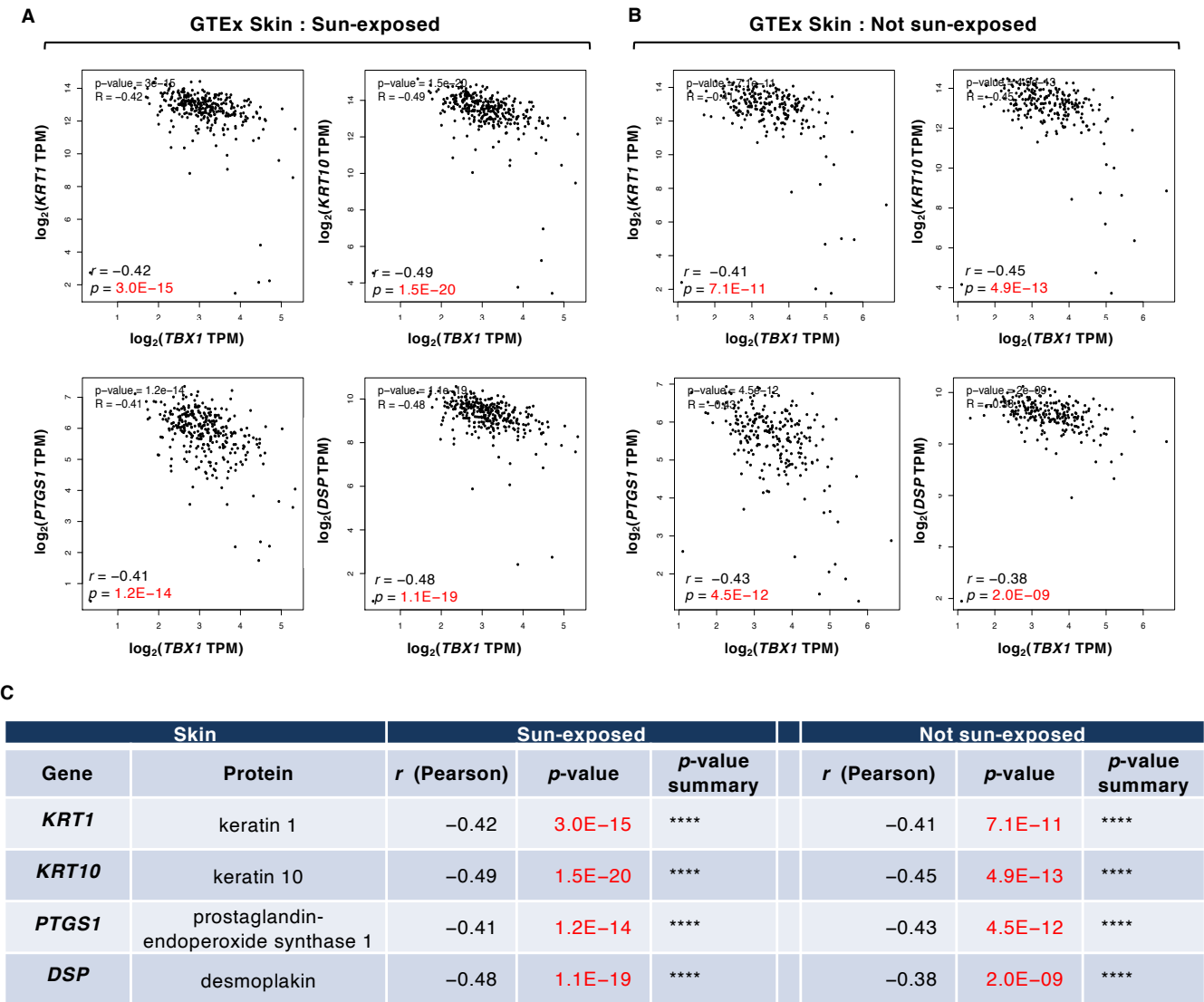


Supplementary Figure S10. Gene expression correlation between *TBX1* and basal cell markers.

(A-C) Correlation between the mRNA expression of *TBX1* and basal cell-specific genes in (A) sun-exposed (lower leg, $n = 701$) and (B) unexposed (suprapubic, $n = 604$) skin tissues.

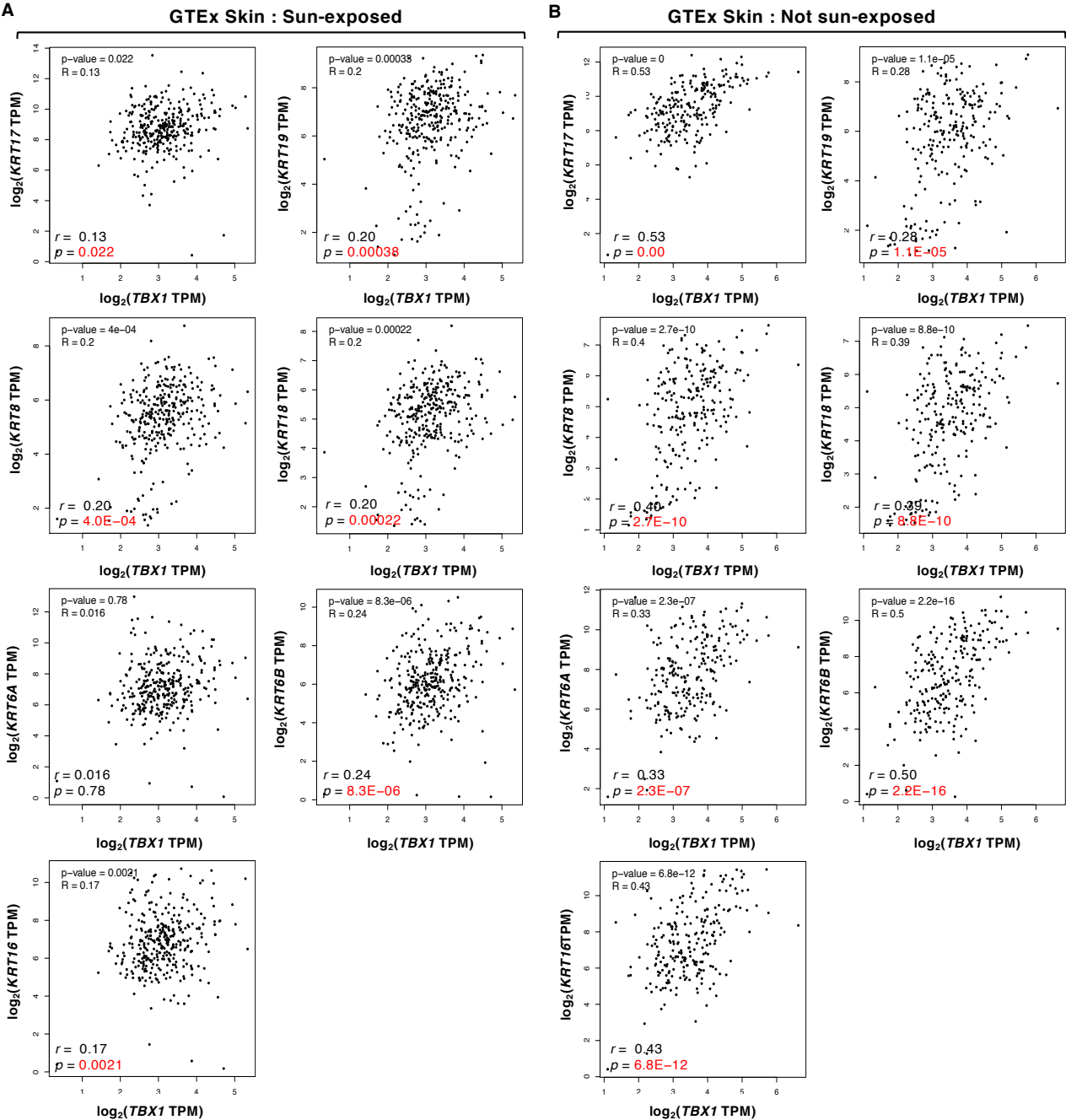
(C) Table summarizing the results in A, B. Significant negative correlations between the mRNA expression levels of *TBX1* and basal cell markers are indicated in red. * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$; NS, not significant.

Supplementary Figure S11



Supplementary Figure S11. Gene expression correlation between *TBX1* and suprabasal cell markers.
(A-C) Pearson correlation coefficient between mRNA expression of *TBX1* and suprabasal cell markers in (A) sun-exposed (lower leg) and (B) unexposed (suprapubic) skin tissue.
(C) Table summarizing the results in A, B. Significant negative correlations between mRNA expression levels of *TBX1* and suprabasal cell markers are indicated in red. **** $p \leq 0.0001$.

Supplementary Figure S12



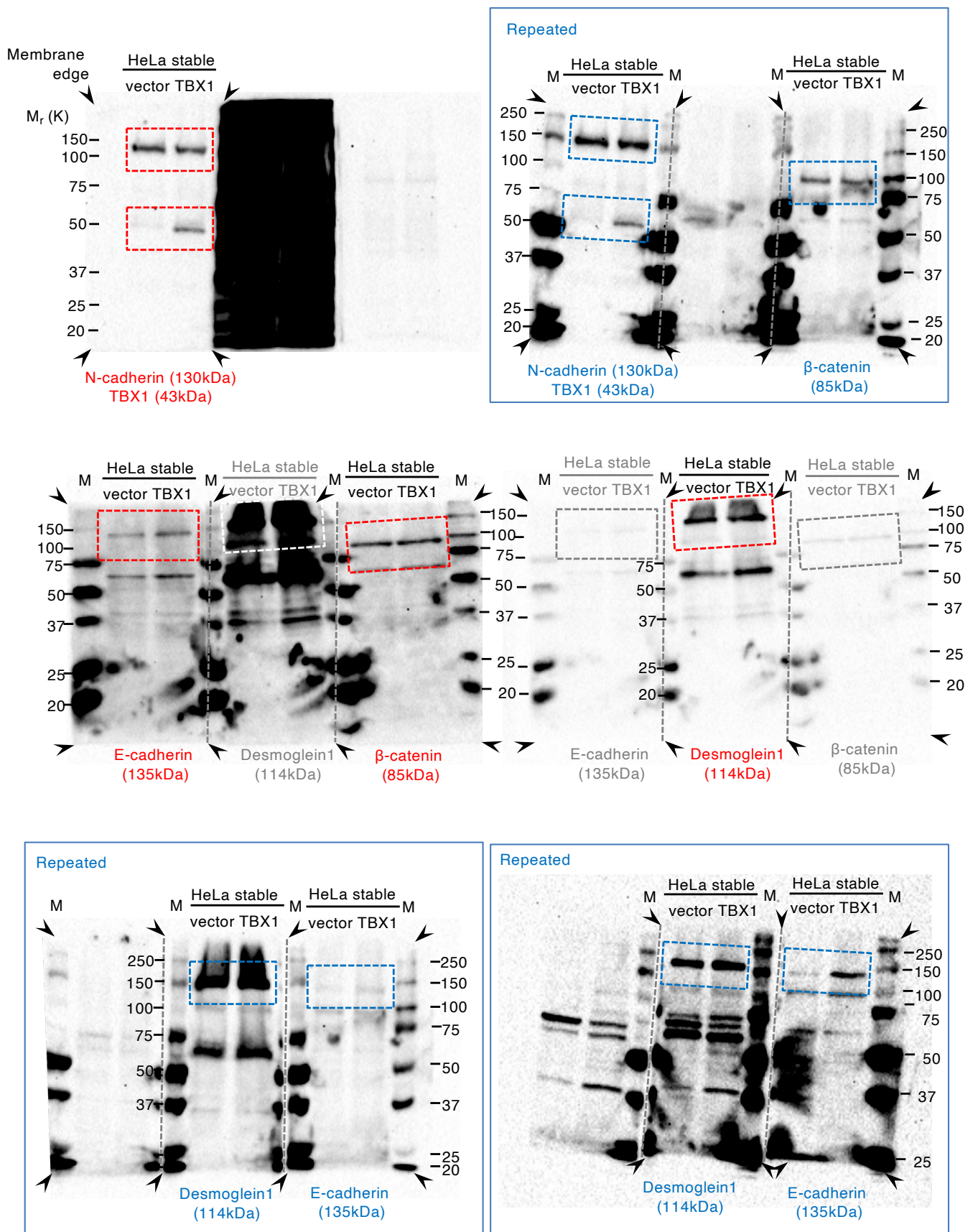
Skin		Sun-exposed			Not sun-exposed		
Gene	Protein	r (Pearson)	p -value	p -value summary	r (Pearson)	p -value	p -value summary
<i>KRT17</i>	keratin 17	0.13	0.022	*	0.53	0.00	****
<i>KRT19</i>	keratin 19	0.20	0.00038	***	0.28	1.1E-05	****
<i>KRT8</i>	keratin 8	0.20	4.0E-04	****	0.40	2.7E-10	****
<i>KRT18</i>	keratin 18	0.20	0.00022	***	0.39	8.8E-10	****
<i>KRT6A</i>	keratin 6A	0.016	0.78	NS	0.33	2.3E-07	****
<i>KRT6B</i>	keratin 6B	0.24	8.3E-06	****	0.50	2.2E-16	****
<i>KRT16</i>	keratin 16	0.17	0.0021	**	0.43	6.8E-12	****

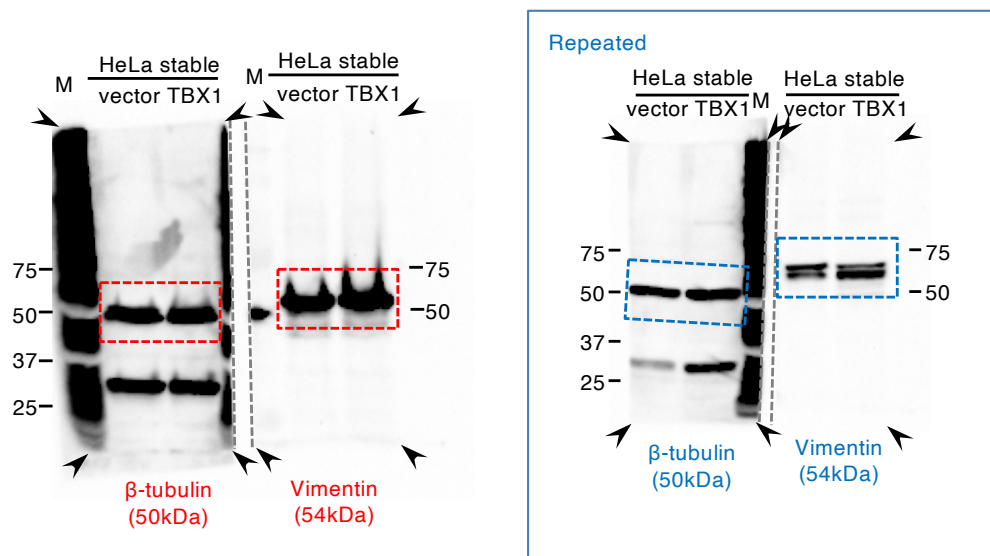
Supplementary Figure S12. Gene expression correlation between *TBX1* and keratin markers

(**A-C**) Pearson correlation coefficient between mRNA expression of *TBX1* and keratin markers in (**A**) sun-exposed (lower leg) and (**B**) unexposed (suprapubic) skin tissue.

(**C**) Table summarizing the results in **A, B**. Significant positive correlations between mRNA expression levels of *TBX1* and keratin markers are indicated in red. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$; NS, not significant.

Supplementary Figure S13





Supplementary Figure S13. Full-length images of representative Western blots related to Figure 1G. Images of selected portions are highlighted in red boxes. Arrowheads indicate membrane edges. M, Precision Plus Protein™ Dual Color Standards (Bio-Rad).

Supplementary Figure S14

Figure 3H

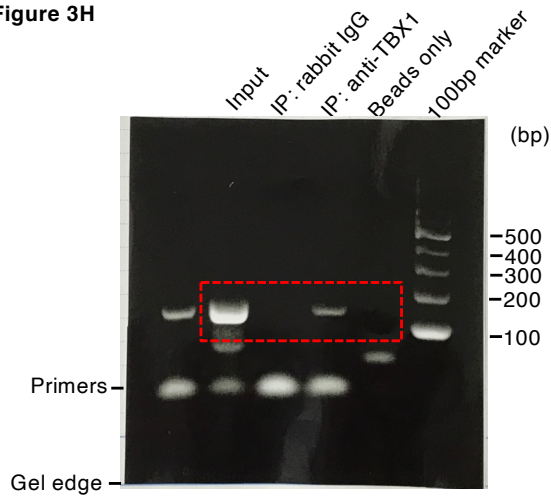


Figure 4E

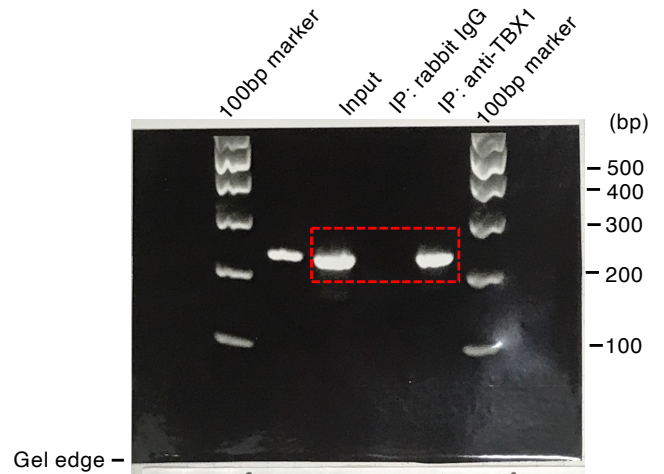


Figure 3H
Repeated

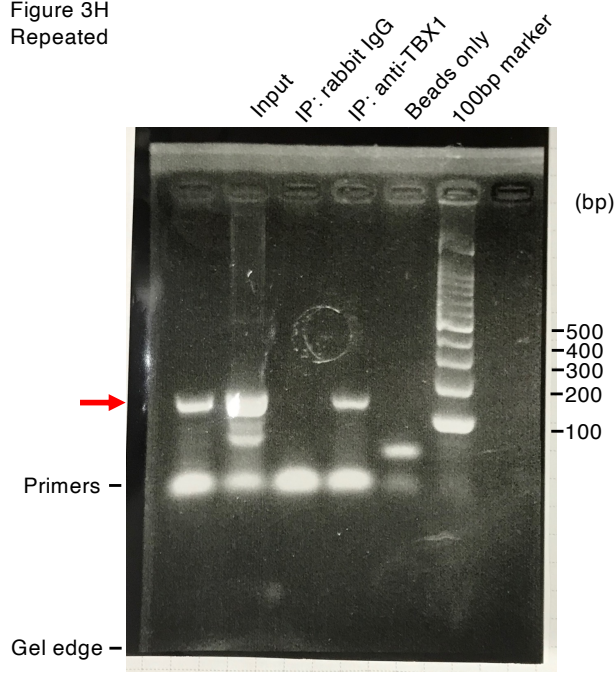
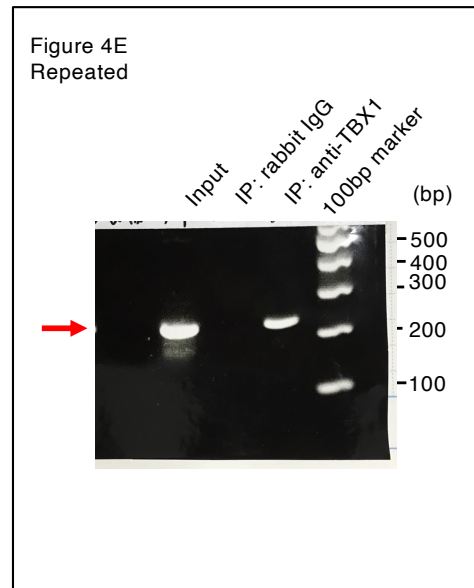


Figure 4E
Repeated



Supplementary Figure S14. Full-length images of representative ChIP assays related to Figures 3H and 4E.

Images of selected portions are highlighted in red boxes.