

Supplementary Figures

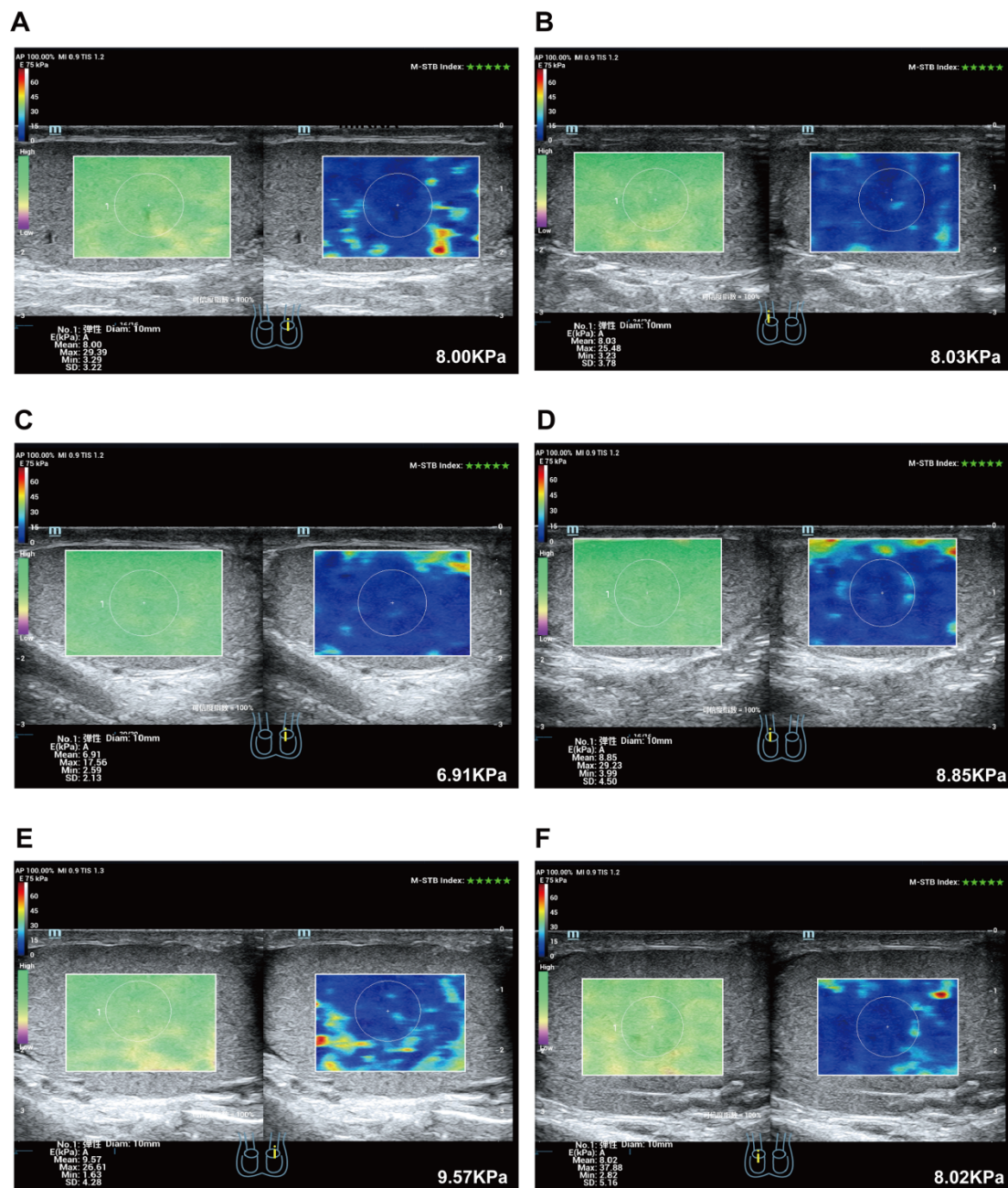


Figure S1 Correlation between varicocele (VC) severity and testicular elasticity. The association between VC severity and testicular elasticity in NC controls (A, B), left-sided VC with normal spermogram parameters (C, D), and left-sided VC with abnormal spermogram parameters (E, F).

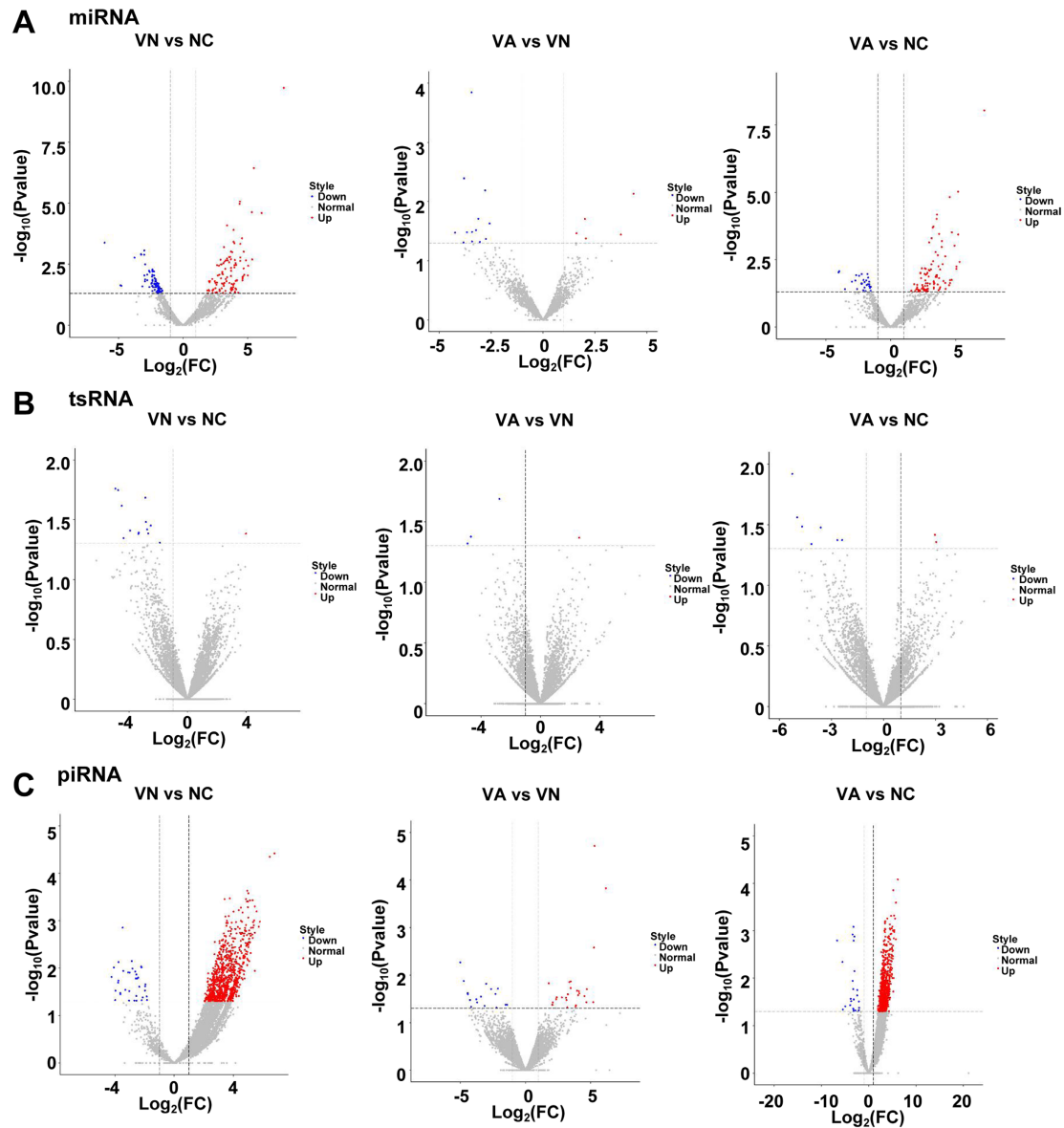


Figure S2 Identification of differentially expressed small RNAs in the testicular tissues of patients with varicocele (VC). Volcano plot of differentially expressed miRNAs (A), tsRNAs (B), and piRNAs (C). NC represents testicular tumor patients undergoing radical orchiectomy with WHO-normal semen parameters, from whom histologically normal adjacent testicular tissues were obtained. VN represents VC patients with WHO-normal semen parameters, and VA represents VC patients with asthenospermia, with testicular tissues collected accordingly.

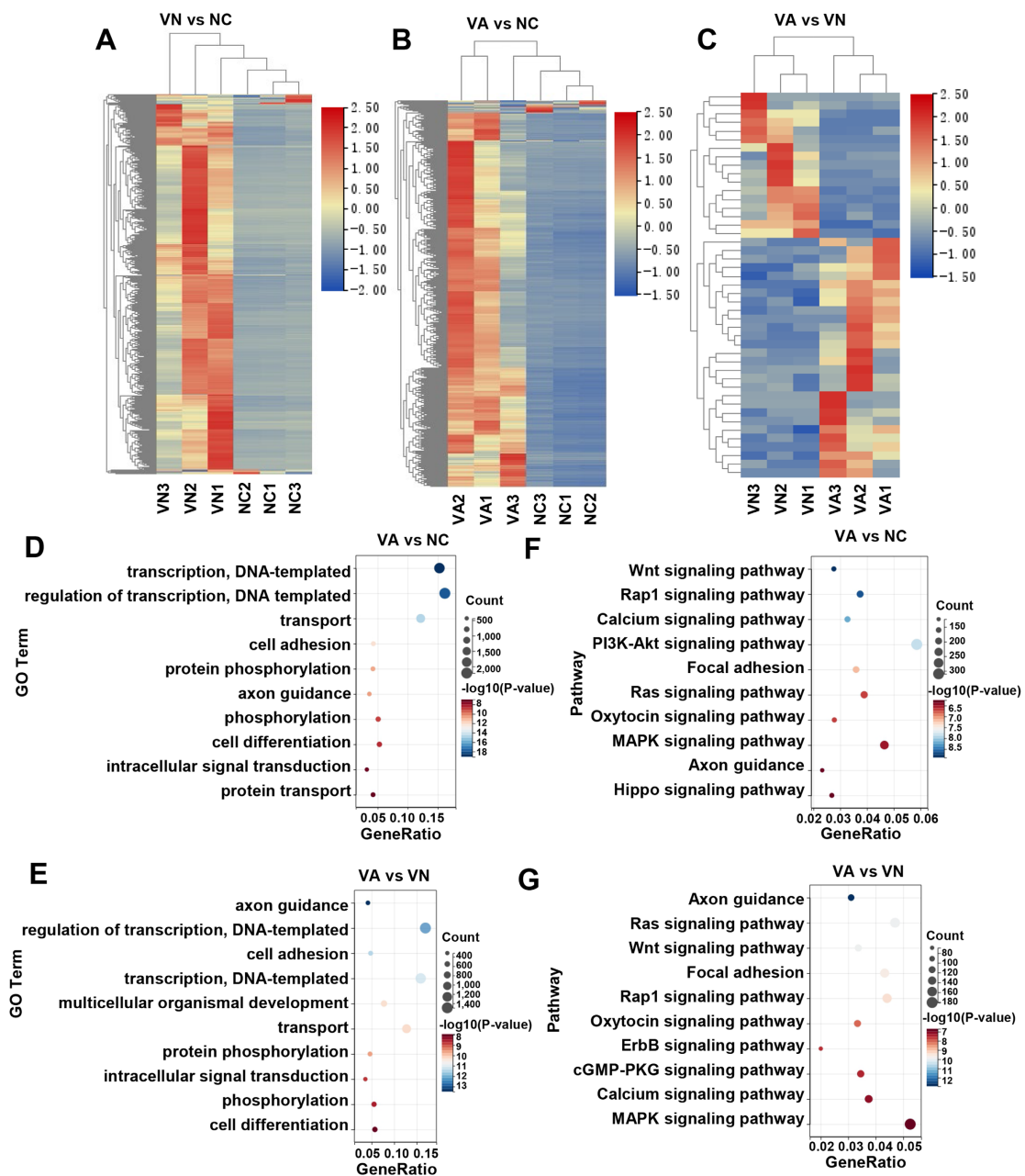


Figure S3 Functional analysis of differentially expressed piRNAs. (A-B) Heatmap of differentially expressed piRNAs in the VN vs NC group (A), VA vs NC group (B), and VA vs VN group (C). (D, E) GO analysis of differentially expressed piRNAs between the VA and NC (D) or VN (E) groups. (F, G) KEGG pathway enrichment analysis of differentially expressed piRNAs between the VA and NC (F) or VN (G) groups.

A**Sequence annotation of piRNA-46403**

Organism	<i>Homo sapiens</i>
Length	31
Sequence	TCTTCTCGGAGCTGCTGTCCAACTGTACTC
piRNA loci	chr15 : 101762373 - 101762404 (+)
Genome Assembly	GRCh38/hg38
%GC content	51.61
Nucleotide Bias	1T 10A
Alias ID	gi 108062927, DQ578291.1, piR-46403

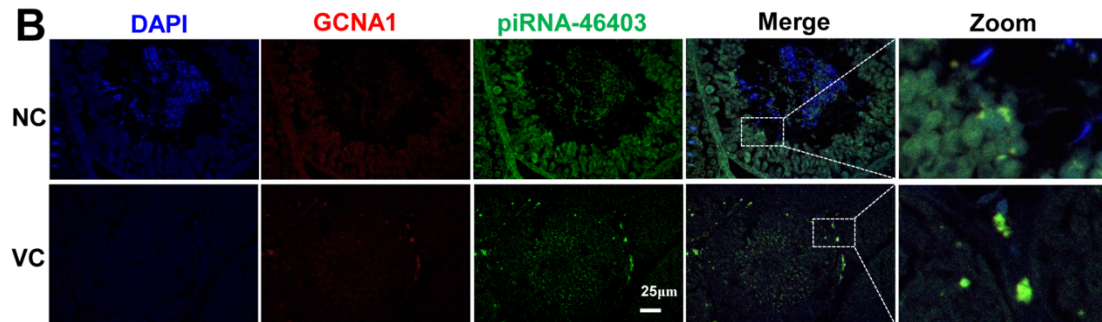
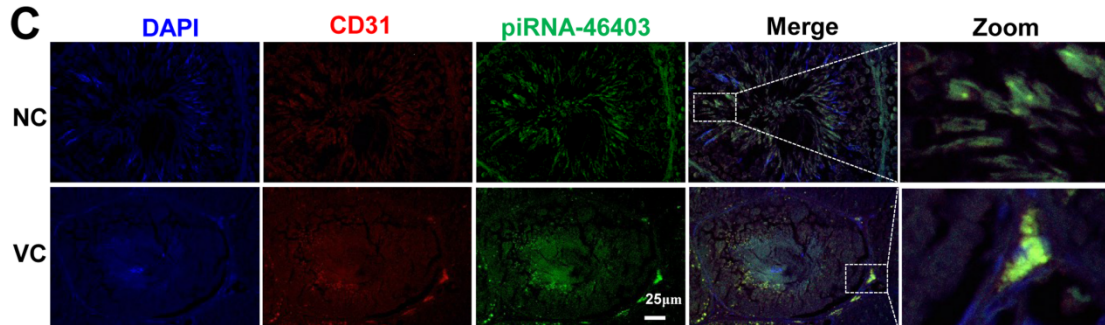
B**C**

Figure S4 The expression of piRNA-46403 in spermatogonia (marker GCNA1) and endothelial cells (marker CD31) of testicular tissues. (A) Sequence characteristics of piRNA-46403. (B) Immunofluorescence and FISH were utilized to assess co-localization and expression of piRNA-46403 and GCNA1 in testicular tissues. (C) Immunofluorescence and FISH were used to detect co-localization and expression of piRNA-46403 and CD31 in testicular tissues. Scale bar=25μm.

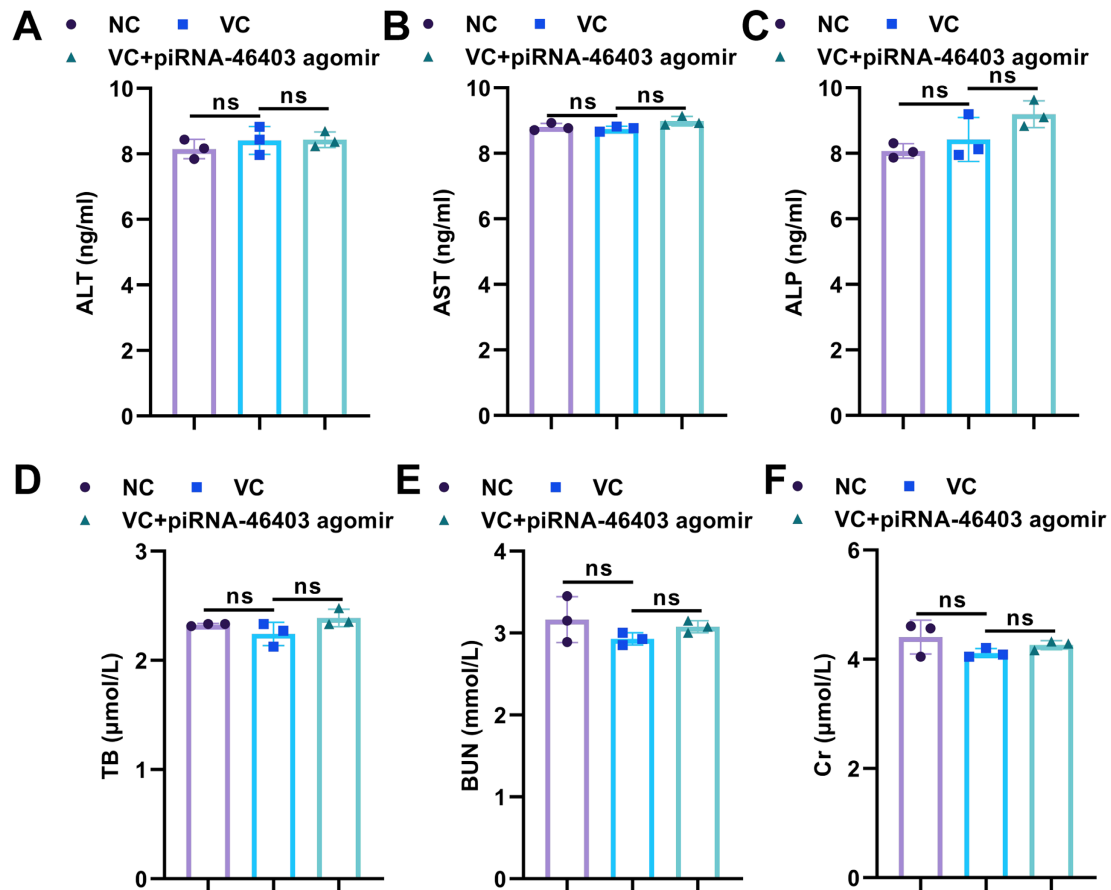


Figure S5 Effects of piRNA-46403 overexpression on liver and renal function in a varicocele (VC) rat model. (A-F) Serum levels of liver function markers (A) ALT, (B) AST, (C) ALP, (D) TB, and renal function markers (E) BUN, and (F) Cr, were measured. ns, not significant.

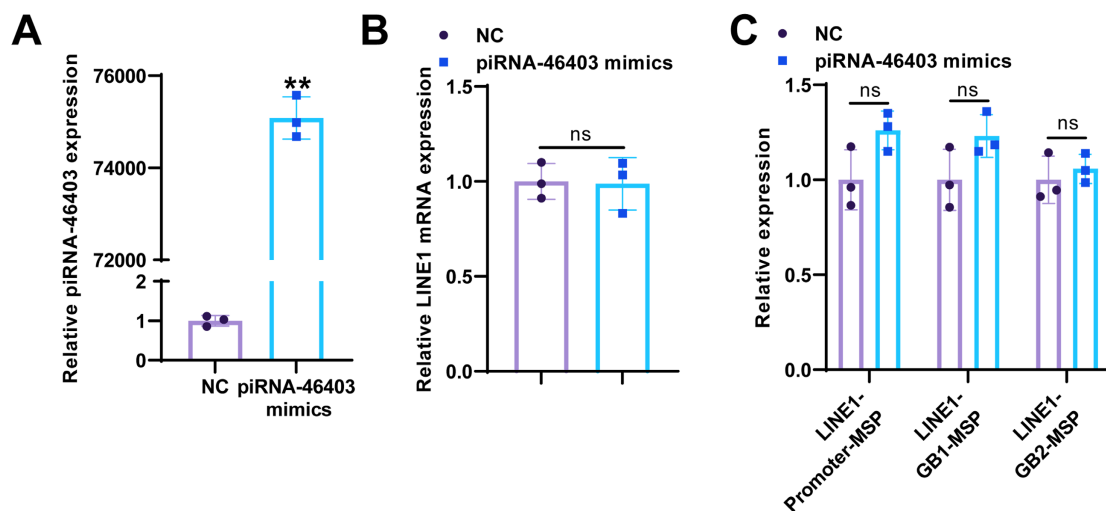


Figure S6 The LINE1 expression and methylation levels in piRNA-46403-overexpressed Sertoli cells. (A) qRT-PCR analysis of the overexpression efficiency of piRNA-46403. (B) The effect of piRNA-46403 overexpression on LINE1 expression in Sertoli cells was assessed by qRT-PCR. (C)

LINE1 methylation levels in Sertoli cells following piRNA-46403 overexpression were evaluated by qRT-PCR. ns, not significant, **P<0.01.

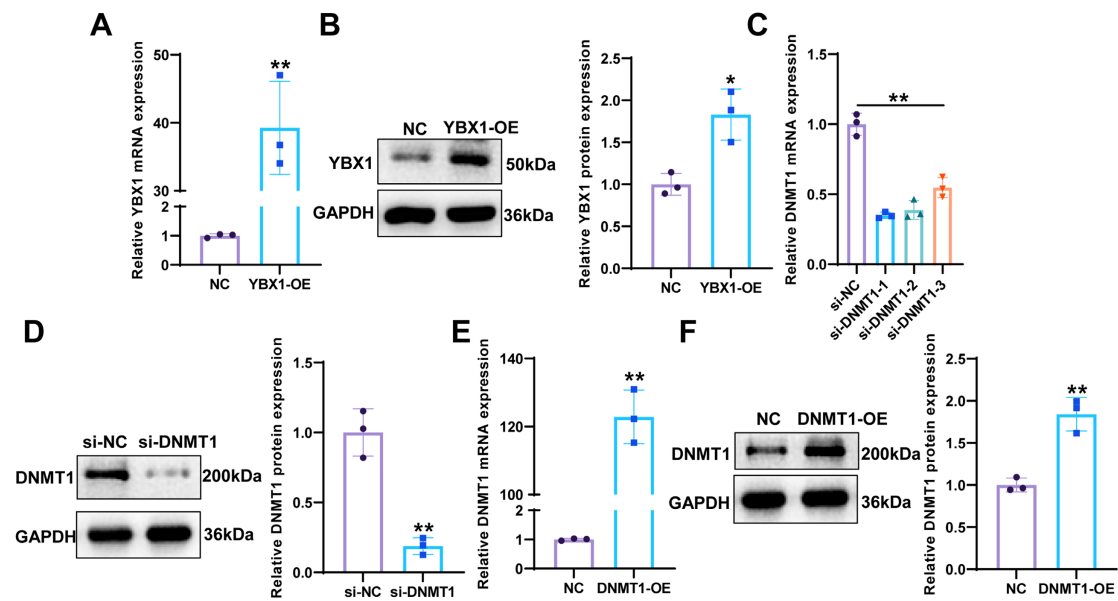


Figure S7 Verification of piRNA-46403 and YBX1 overexpression, as well as DNMT1 knockdown and overexpression, in Sertoli cells. (A, B) The overexpression efficiency of YBX1 was verified by qRT-PCR (A) and western blot (B). (C, D) qRT-PCR and western blot confirmed the knockdown efficiency of DNMT1. (E, F) qRT-PCR and western blot were used to validate the overexpression efficiency of DNMT1. *P<0.05; **P<0.01.

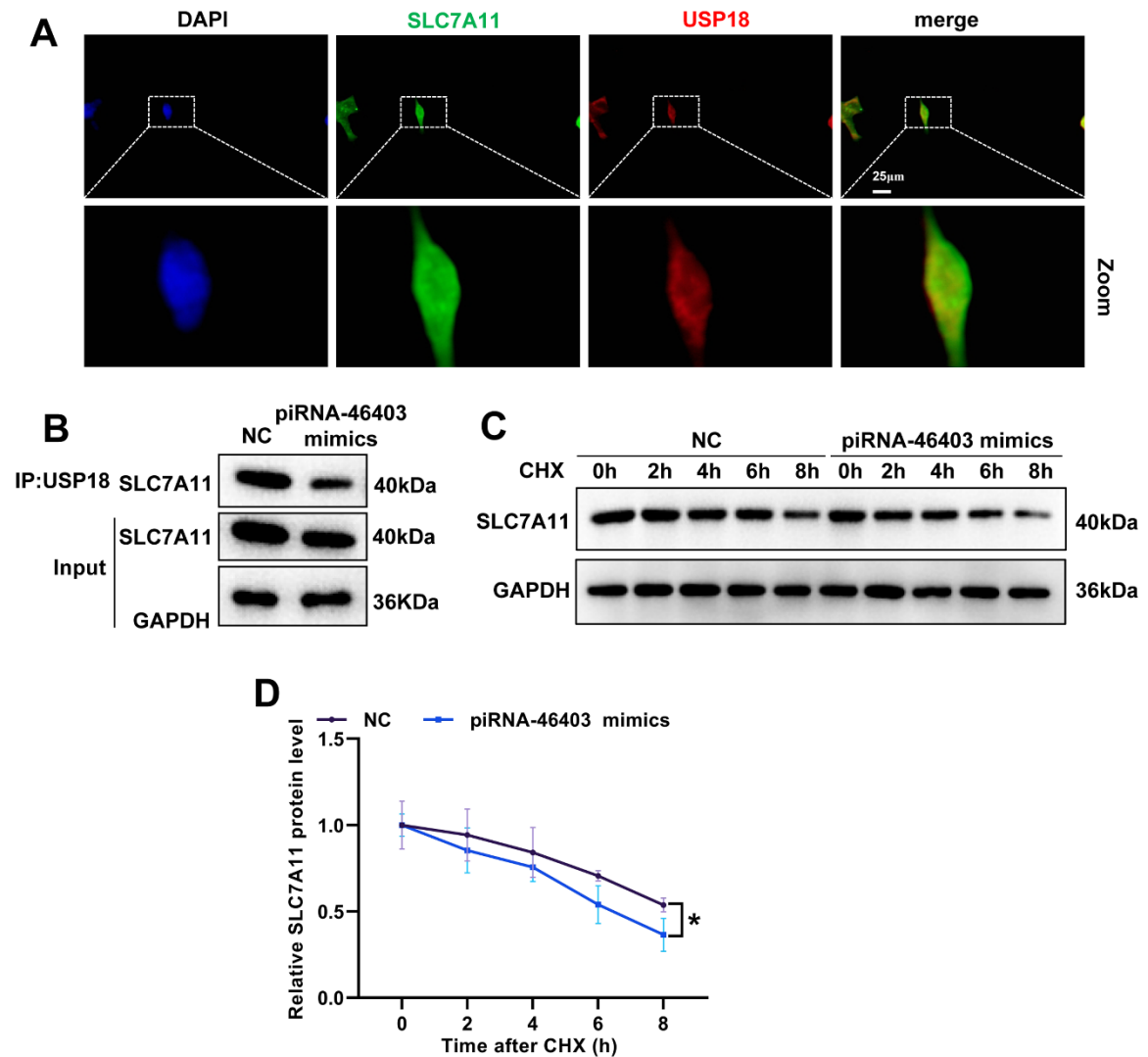


Figure S8 piRNA-46403 regulates SLC7A11 protein stability via USP18. (A) Immunofluorescence was performed to detect the subcellular localization of USP18 and SLC7A11. (B) Co-immunoprecipitation was used to examine the interaction between USP18 and SLC7A11. (C, D) Protein stability of SLC7A11 was assessed by CHX chase assay. * $P < 0.05$.

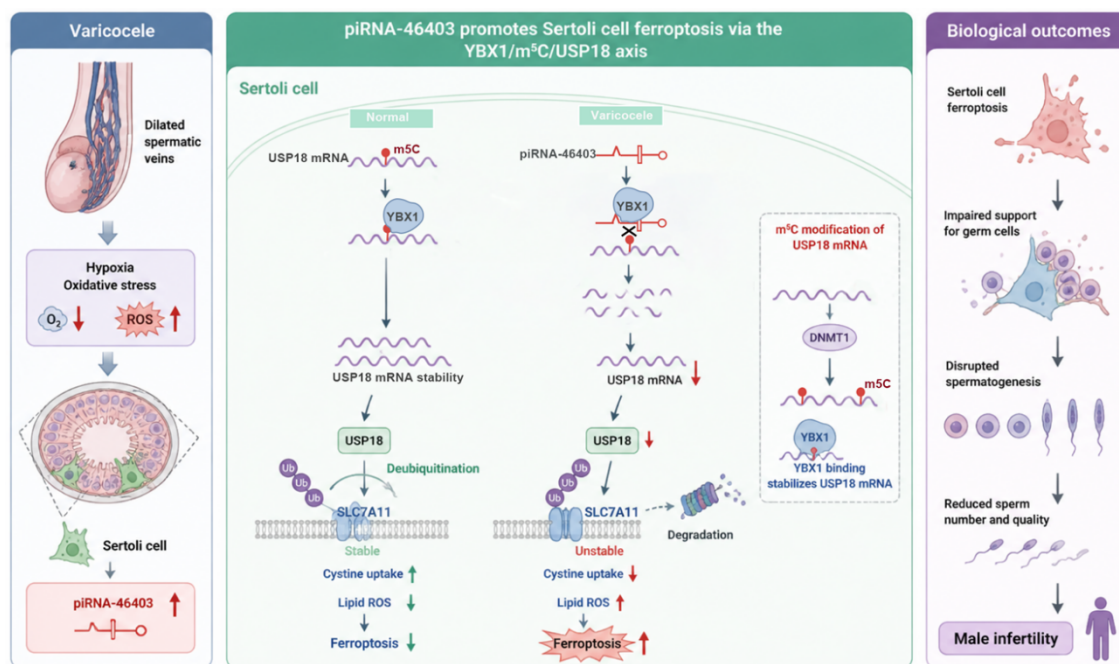


Figure S9 Schematic diagram of piRNA-46403/YBX1/USP18 axis regulating Sertoli cell ferroptosis in varicocele-mediated male infertility.

Table S1 Clinical characteristics of the participants

Characteristics	NC (n=5)	VN (n=7)	VA (n=10)	P value (NC vs VN)	P value (NC vs VA)	P value (VN vs VA)
Age (years)	23.60±2.97	22.43±4.28	25.10±3.90	0.8628	0.7600	0.3570
sperm concentration (M/ml)	87.86±50.50	41.66±13.43	63.51±54.75	0.2103	0.5915	0.5930
Total sperm motility (%)	53.00±5.20	56.86±6.59	36.20±13.40	0.7976	0.0192	0.0017
Forward motility of sperm (%)	43.60±6.35	42.86±5.67	22.20±8.15	0.9825	<0.0001	<0.0001
abnormal morphology (%)	89.40±7.37	89.86±1.86	95.90±1.52	0.9757	0.0121	0.0096

Note: NC represents testicular tumor patients undergoing radical inguinal orchiectomy, with preoperative semen parameters within WHO-normal reference ranges, VN represents VC patients with normal semen, VA represents VC patients with asthenospermia.

Table S2 Primer sequences

Primers	Sequence (5' to 3')
U6-F	CGATACAGAGAAGATTAGCATGGC
U6-R	AACGCTTCACGAATTTGCGT
piRNA-58317-F	ggacctgcctcttggt
piRNA-58317-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGA TACGACACATGAT
piRNA-36388-F	gatcaagatgcctgaggattc
piRNA-36388-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGA TACGACAGGCACT
piRNA-34615-F	gagatcaagatgcctgagg
piRNA-34615-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGA TACGACGGCACT
piRNA-42954-F	gcagtccatctgtgcaag
piRNA-42954-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGA TACGACGGTGTGG
piRNA-46403-F	gtcttctcggagctgct
piRNA-46403-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGA TACGACGAGTACA
General primer-R	AGTGCGTGTCGTGGAGTCG
GAPDH-F	ACAAC TTTGGTATCGTGGAAGG
GAPDH-R	GCCATCACGCCACAGTTTC
USP18-F	AACGTGCCCTTGTTTGTCCAA
USP18-R	GAGTCCTTCACCCGGATCGTA
YBX1-F	CCCCAGGAAGTACCTTCGC
YBX1-R	AGCGTCTATAATGGTTACGGTCT
DNMT1-F	CCTAGCCCCAGGATTACAAGG
DNMT1-R	ACTCATCCGATTTGGCTCTTTC
L1-ORF1-F	GAATGATTTTGACGAGCTGAGAGAA
L1-ORF1-R	GTCCTCCCGTAGCTCAGAGTAATT
LINE1-Promoter-MSP-F	GTCGAATAGGAATAGTTTCGG
LINE1-Promoter-MSP-R	ACTCCCTAACCCCTTACGCT
LINE1-GB1-MSP-F	GTTTAGATTTAGGAAATATAGAGAAC
LINE1-GB1-MSP-R	CTAACTTATAAAATTTCTACCGAA
LINE1-GB2-MSP-F	GTTGGATGGAGAATGATTTTGAC
LINE1-GB2-	TTAATCACATCGACTCCTAAAAC

MSP-R	
Actin-MSP-F	TGGTGATGGAGGAGGTTTAGTAAG
Actin-MSP-R	AACCAATAAAACCTACTCCTCCCTTAA

Table S3 Pull-down combined with mass spectrometry to identify proteins bound to piRNA-46403

Accession	Coverage [%]	Peptides	PS Ms	Unique Peptides	A As	MW [kDa]	calc. pI	Abundances (Grouped): F1
H6VRF8	52	33	56	28	644	66	8.12	759192329.9
P35527	51	28	38	27	623	62	5.24	271530654.2
A0A1B0GVI3	51	26	39	23	624	63.3	5.26	232855905.2
A0A024R0Y2	16	31	34	31	2268	257.1	6.61	27643788.25
P35908	38	22	30	14	639	65.4	8	129316770.4
Q15149	8	34	34	34	4684	531.5	5.96	13213479.12
A0A804HL40	21	26	27	26	1393	157.7	6.18	27928447.44
Q92945	35	23	25	19	711	73.1	7.3	78753119.78
A6NLN1	31	10	18	8	527	56.5	9.38	124854796.6
Q969I0	59	21	28	15	365	41.1	5	66954764.25
A0A7I2V4F0	31	18	20	18	734	81.7	7.33	23865309.06
P23246	25	14	19	13	707	76.1	9.44	88229323.83
P19338	28	18	20	18	710	76.6	4.7	66896886
P13647	29	19	22	10	590	62.3	7.74	22766705.91
B2R853	29	17	19	5	564	60	7.72	6123293.625
P02768	14	10	21	10	609	69.3	6.28	3319934306
P02533	36	14	16	5	472	51.5	5.16	21424679.56
Q96AE4	26	16	17	11	64	67.5	7.61	19547498.47

					4			
Q2TSD0	36	9	11	9	33 5	36	8.46	19193172.47
A0A7I2V 535	21	11	17	10	50 1	57.6	9.33	15979546.06
A0A7I2Y QN2	22	12	14	12	56 1	62.5	7.56	21214001.88
P78386	28	16	18	8	50 7	55.8	6.55	69008349.78
Q86YZ3	9	9	10	9	28 50	282.2	10.0 4	9559612.938
P08727	28	13	15	6	40 0	44.1	5.14	15771512.22
Q6FI03	23	8	11	7	46 6	52.1	5.59	16913512.38
O95678	13	9	11	2	55 1	59.5	7.74	13207487.13
A0A087X 0X3	19	13	13	13	73 0	77.5	8.78	10805741.88
Q96I24	20	11	12	8	57 2	61.6	8.38	7448383.031
E9PKE3	21	10	10	8	62 7	68.8	5.52	7530984.656
A0A140T A69	19	10	11	4	43 6	49.4	5.06	50078611.75
Q53HQ7	21	8	10	8	46 2	50.2	8.94	24085858.75
B4DZC3	11	9	9	9	89 6	102.4	7.64	4834669.141
A0A0G2J LI4	17	9	9	6	50 9	57.1	8.24	6063420.156
Q14525	15	8	10	3	40 4	46.2	4.84	13125512.41
Q14533	15	8	10	2	50 5	54.9	5.47	3907576.125
P05783	19	9	9	8	43 0	48	5.45	18184241.25
A0A087 WZ13	19	9	10	9	73 9	77.8	8.84	3733954.656
B4DUQ1	23	8	8	8	43 9	48.5	5.92	7875947.281
Q53G99	24	8	8	8	37 5	41.7	5.59	7452057.281
A0A024R	12	4	6	3	44	50.8	5.38	4886935.25

DB2					9			
P08779	17	8	9	1	47 3	51.2	5.05	527927.3125
Q04695	15	8	9	2	43 2	48.1	5.02	496210.0938
Q92764	11	6	8	2	45 5	50.3	4.91	2851267.844
P04075	23	6	6	6	36 4	39.4	8.09	4927416.906
Q9Y6M1	13	7	7	7	59 9	66.1	8.46	3585028.406
A0A7G6J 4N4	12	8	8	8	80 2	87.5	5.27	4247734.719
A0A024R DF4	22	7	8	5	30 6	32.8	8.16	13384592.19
A0A5H1Z RQ2	8	6	6	3	73 1	80.4	8.37	1663879.406
Q7Z794	5	3	6	1	57 8	61.9	5.99	347366.1563
Q14444	10	6	7	6	70 9	78.3	5.25	9552723.094
Q9Y3I0	14	5	5	5	50 5	55.2	7.23	2367852.266
B2R6U8	25	5	5	5	22 7	26.2	8.82	4009029.563
Q14532	11	5	6	2	44 8	50.3	4.84	975876.2188
P84103	20	4	5	3	16 4	19.3	11.6 5	8565725.313
Q00341	6	6	6	6	12 68	141.4	6.87	1996568.063
P11498	6	6	6	6	11 78	129.6	6.84	1869713.688
Q6FHZ0	13	5	5	5	33 8	35.5	8.68	2120885.969
A0A1X7S BS1	8	5	5	5	74 4	81.7	5.83	4873667.188
Q9UHD8	11	6	6	6	58 6	65.4	8.97	2656234.406
O95758	9	5	5	3	55 2	59.7	9.04	1776988.688
P05141	14	4	4	4	29 8	32.8	9.69	1593723.688
A8MXP9	6	5	5	5	89	99.9	6.04	522080.4063

					5			
Q701L7	7	4	4	1	51 3	56.6	6.74	1110232.125
Q9GZT3	39	4	4	4	10 9	12.3	10.2 4	2358221.188
J3QTR3	25	3	13	3	10 6	12.2	10.1 4	92919872.25
Q16629	11	2	3	1	23 8	27.4	11.8 2	533313.0625
Q96IR1	19	5	5	5	24 3	27.2	9.94	1846677.781
D6W5Y5	11	3	3	3	29 7	31.9	9.61	1455926.75
A0A7I2V 649	6	4	4	4	59 0	65.1	9.36	1429164.406
F8VZX2	14	4	4	4	32 1	33.8	8.24	2550056.781
A0A024Q Z91	5	4	4	4	97 5	103.5	8.12	1268691.344
A0A7P0T AI0	9	4	4	2	61 6	68.1	5.14	677325.4688
P81605	26	3	3	3	11 0	11.3	6.54	16365822.19
A1A508	9	2	3	1	24 7	26.5	7.21	989542536.9
F8W1S1	4	3	3	1	54 3	59.4	7.44	904594.625
Q99729	8	3	3	1	33 2	36.2	8.21	
A8K6U7	6	4	5	4	85 6	95.6	6.92	706163.875
P15924	1	4	4	4	28 71	331.6	6.81	1292559.594
D3DQ69	6	2	2	2	45 0	49.1	9.33	349826.625
Q4VB24	13	3	3	3	21 9	21.9	11.0 3	2596147.438
Q02413	3	2	2	2	10 49	113.7	5.03	916593.75
Q8N5L9	13	4	4	4	29 3	31.3	10.2 4	1262010.719
Q5D862	1	2	2	2	23 91	247.9	8.31	703872.125
E7EQ64	11	2	4	1	26	28.1	7.25	366418.3438

					1			
X6RDA4	18	3	3	3	24 8	27.3	5.45	1495273.844
Q96PK6	7	3	3	3	66 9	69.4	9.67	1395477.813
P08670	6	3	3	2	46 6	53.6	5.12	837176.2188
Q6NUR7	4	3	3	3	58 6	69.2	6.27	2152879.563
A0A0B4J 259	15	2	2	2	13 7	15.3	8.69	2489623.625
Q01085	11	3	3	3	37 5	41.6	7.74	494706.75
P05109	24	2	3	2	93	10.8	7.03	2475366.313
P62805	31	3	3	3	10 3	11.4	11.3 6	1911921.969
P31151	23	2	3	2	10 1	11.5	6.77	1419167.5
Q9Y224	11	3	3	3	24 4	28.1	6.65	2851891.625
V9HWC6	14	3	3	3	20 8	22.7	9.32	1603954
P0DPH7	7	3	4	3	45 0	49.9	5.1	997579.75
P23396	9	2	2	2	24 3	26.7	9.66	485371.4219
Q8N684	4	2	2	2	47 1	52	8	816108.6875
A0A125Q YY8	12	1	1	1	11 2	12.2	8.5	513955.2813
P62753	6	2	2	2	24 9	28.7	10.8 4	953792.875
E9PKN0	3	2	2	2	78 4	88.9	9.85	500066.3281
F8WJN3	5	2	2	2	47 8	52.2	6.43	2710251.125
Q16778	19	2	2	2	12 6	13.9	10.3 2	937561.5
B5BU25	4	2	2	2	47 1	53.1	9.09	450760.0625
P60866	19	2	2	2	11 9	13.4	9.94	1084546.5
P50991	5	3	3	3	53 9	57.9	7.83	439562.8281

Q53FN0	9	2	2	2	22 1	25.5	11.8 5	1844762.375
I6L9E8	6	2	2	2	51 8	55.1	8.88	989278.0313
E7EX73	2	2	2	2	14 36	158.5	5.21	253488.7188
P08729	4	2	2	1	46 9	51.4	5.48	629484.1875
Q8TBR3	3	2	2	2	52 6	53.4	9.38	2413567.063
E9PB61	4	1	1	1	26 4	27.5	11.0 5	682230.5625
Q6PKI6	10	2	2	1	26 6	29.4	10.2 3	886473.4375
B4DYY5	2	2	2	2	10 78	113.5	6.68	1121159.469
Q8N1N4	3	2	2	1	52 0	56.8	6.02	518363.4063
P16989	7	2	2	1	37 2	40.1	9.77	762863.2813
Q13151	7	1	1	1	30 5	30.8	9.29	1615472.25
Q96EP5	4	1	1	1	40 7	43.4	8.56	
C9JFR7	19	2	2	2	10 1	11.3	9.66	750118.7813
H0Y8K0	5	2	2	2	45 8	50	9.38	183438.5469
Q0QEL2	5	1	1	1	22 4	24.9	6.86	251276.625
P39748	5	2	2	2	38 0	42.6	8.62	791054.7813
B4DM21	4	2	2	2	49 0	55.1	7.72	1529679.094
Q75MU2	8	1	1	1	15 6	17.1	5.31	463932
A0A024R CT9	5	1	1	1	32 8	34.3	10.3 7	472889.1875
A0A3S8N FS6	2	1	1	1	41 4	47.1	6.04	379294.0938
M0QYW4	5	1	1	1	14 1	15.5	8.31	183389664
P26373	5	1	1	1	21 1	24.2	11.6 5	246923.5781

B0QYK0	1	1	1	1	61 8	64.9	9.38	2002922.875
P46783	5	1	1	1	16 5	18.9	10.1 5	
A0A024R A87	2	1	1	1	38 4	44.8	8.22	437596.1875
A2A3S3	3	1	1	1	36 1	39.3	8.43	516826.5313
Q08188	1	1	1	1	69 3	76.6	5.86	438664.5313
B2R5U1	1	1	1	1	88 5	99.6	6.96	244564.2969
Q16777	15	1	1	1	12 9	14	10.9	
Q9BTI9	4	1	1	1	22 8	25	4.86	592357.5
M0QX76	20	1	1	1	50	5.6	9.63	
Q92879	2	1	1	1	48 6	52	8.46	520293.625
C9J0E4	19	1	1	1	63	7.1	7.24	801828.875
Q8TA90	2	1	1	1	51 7	57.5	6.93	126749.2813
P26196	2	1	1	1	48 3	54.4	8.66	
E9PLX7	10	1	1	1	11 3	12.5	11.4 4	283013.4375
H0YNP5	6	1	1	1	17 5	19.7	5.21	496822.3125
B2RDE0	2	1	1	1	70 3	77.4	7.06	248896.7344
K7EMH1	15	1	1	1	89	10.4	9.66	356944.4688
A0A6Q8P F80	3	1	1	1	34 2	38.6	6.49	186693.4844
Q9UG93	3	1	1	1	36 9	40.6	5.86	353971.25
F5GXS0	1	1	1	1	16 98	187.6	7.33	529410.6875
H7C2W9	8	1	1	1	10 8	12.8	11	601847.5
E9PFZ2	1	1	1	1	94 6	108.8	5.77	270034.8438
C9JH19	2	1	1	1	45 1	48.6	7.84	296249.5
Q6ZQN2	2	1	2	1	17	180.6	6.15	531994.6875

					14			
Q5T750	3	1	1	1	25 0	26.2	7.97	1145045.125
A0A2R8Y 811	9	1	1	1	15 0	16.1	10.0 5	418193.9375
H0YHA7	8	1	1	1	16 7	19	11.7 8	172231.0156
Q13061	1	1	1	1	72 9	81.5	9.42	796374.3125
K7ENJ4	4	1	1	1	19 9	21.3	10.4 5	391937.6563
B5MCT8	5	1	1	1	13 9	16.6	11.0 6	494366.75
A8K4W0	3	1	1	1	26 4	29.9	9.73	
A0A8V8T MM5	3	1	1	1	34 4	37.4	6.39	591161.3125
A0A0J9Y YL3	2	1	1	1	50 5	54.6	5.34	292391.6563
A0A024R 7L5	1	1	1	1	11 18	123	6.68	124068.5469
B3KP18	1	1	1	1	79 2	90.5	5.45	557374.8125
E5RI99	11	1	1	1	11 4	12.6	9.55	195426.8906
Q9UKV8	1	1	1	1	85 9	97.1	9.19	142599.5
P05089	3	1	1	1	32 2	34.7	7.21	
K7EJV9	8	1	1	1	17 0	19.4	10.4 8	431069
H0YEX5	3	1	1	1	31 5	35	4.81	195986.0156
B2R9K8	2	1	1	1	53 1	57.9	6.8	95819.09375
H7C2E8	8	1	1	1	10 2	11.6	7.88	196167.0469
Q8TB74	2	1	1	1	29 8	34.3	7.3	853710.6875
P27694	2	1	1	1	61 6	68.1	7.21	359885.25
H0Y7V4	0	1	1	1	41 88	478.6	6.11	228409.9531
Q9Y663	3	1	1	1	40	44.9	9.5	3228489.75

					6			
Q68D27	3	1	1	1	57 5	63.9	6.87	214181.7188
Q96P63	2	1	1	1	40 5	46.2	5.53	
B4DF70	6	1	1	1	18 3	20.1	8.78	227575.7656
F4MH93	2	1	1	1	48 2	54.9	6.99	2479155.5
C9JD32	9	1	1	1	91	9.7	11.4 9	949472
A0A494C 0Y9	3	1	1	1	28 0	29.5	9.74	153896.1094
L0R6G7	5	1	1	1	13 9	16.7	12.8 6	644164.3125
P25311	4	1	1	1	29 8	34.2	6.05	
P06702	7	1	1	1	11 4	13.2	6.13	323191.7188
M0R1H5	10	1	1	1	79	9.5	10.6 5	

Table S4 The binding ability of proteins

Protein	RNA_ID	Top_RNA_fragment	Interaction_Propensity	Z_score
DNMT1	NM.017414.4	1377-1452	49.93	1.62
DNMT3B	NM.017414.4	1377-1452	46.11	1.4
NSUN2	NM.017414.4	1377-1452	40.52	1.08
TRDMT1	NM.017414.4	1562-1637	26.38	0.26
NSUN7	NM.017414.4	1377-1452	25.84	0.23
NSUN3	NM.017414.4	1562-1637	17	-0.28
NSUN6	NM.017414.4	1377-1452	16.19	-0.32
NSUN4	NM.017414.4	1562-1637	14.62	-0.41
NSUN5	NM.017414.4	1562-1637	10.38	-0.66