Hypoxia-induced long non-coding RNA DARS-AS1 regulates RBM39 stability to promote myeloma malignancy

Jia Tong,[1,*] Xiaoguang Xu,[1,*] Zilu Zhang,[1] Chengning Ma,[2] Rufang Xiang,[1] Jia Liu,[1] Wenbin Xu,[1] Chao Wu,[1] Junmin Li,[1] Fenghuang Zhan,[3] Yingli Wu[2] and Hua Yan[1,4]

[1]Department of Hematology, Affiliated Ruijin Hospital of Shanghai Jiao Tong University School of Medicine, Shanghai, China; [2]Hongqiao International Institute of Medicine, Shanghai Tongren Hospital, Key Laboratory of Cell Differentiation and Apoptosis of the Chinese Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai, China; [3]Division of Hematology, Oncology, and Blood and Marrow Transplantation, Department of Internal Medicine, University of Iowa, Iowa City, IA, USA and [4]Department of General Practice, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

**JA and XX contributed equally as co-first authors.*

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Correspondence: *HUA YAN* - yanhua_candy@163.com

YINGLI WU - wuyingli@shsmu.edu.cn

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Method and materials

***Cellular growth assay*.** Myeloma cells were transfected with the indicated plasmids for 48h. The same initial number cells were plated in 6-well culture plates. The cell number was counted regularly using automated cell counter (Countstar).

Western blotting. Antibodies for research were as follows: RBM39 (Abnova or Proteintech); RNF147 (Proteintech); HIF-1α (Abcam); p-mTOR (Ser2448), p-P70S6K (Thr389), p-4EBP1 (Ser65), p-P65 (Ser536), mTOR, P70S6K, 4EBP1, P65 (Cell Signaling Technology, CST); Rabbit and mouse secondary antibodies were purchased from CST.

Fluorescence-activated cell sorting (FACS) analysis. Cell cycle distribution was profiled using Propidium Iodide (PI) staining followed by FACS analysis using a BD cytometer (BD). Cell apoptosis was detected by AnnexinV/PI assay apoptosis detection kit (BD). The cell populations were analyzed by flow cytometry (BD).

$CD38^+$primary MM cells and PBMCs. Patients and healthy volunteers were informed to sign the informed consent forms before sample collection. The study was conducted in accordance with the Declaration of Helsinki protocol, and the study has been approved by the Ethics Committee of Affiliated Ruijin Hospital of Shanghai Jiao-Tong University School of Medicine. Mononuclear cells (MNCs) were isolated from the bone

marrow specimens using Ficoll-Hypaque density gradient sedimentation (Pharmacia). $CD38^+$ cells of the active MM patients were obtained from the bone marrow samples using $CD38^+$ microbeads (Miltenyi). Peripheral Blood Mononuclear Cells (PBMC) obtained from healthy volunteers were separated according to the above mentioned methods.

Animal breeding and treatments. Five-week-old female NOD-SCID mice were obtained from Shanghai SLAC Laboratory Animal Co and housed under standard conditions at the animal care facility at Center of Experimental Animal of Shanghai Jiaotong University (SPF grade). All animal experiments were approved by the Experimental Animal Ethical Committee at Shanghai Jiao Tong University School of Medicine and conformed to the legal mandates and national guidelines for the care and maintenance of laboratory animals.Animals were implanted with respective tumor cells. $1x10^7$ tumor cells in 200 µl growth medium (mixed with matrigel at a 1:1 ratio) were injected subcutaneously into the dorsal flanks of five-week-old female NOD-SCID mice (n =5). At 28 days post-injection, tumors were dissected, photographed, and weighed.

Cloning Procedures. The expression plasmids encoding Flag-tagged human HIF-1α and HIF-2α, the expression plasmids encoding shRNAs targeting HIF-1α and HIF-2α were generously provided by Dr Guoqiang Cheng at Shanghai Jiao Tong University School of Medicine. pGL3-HRE-luciferase plasmids were kindly provided by Dr Y. Fujii-Kuriyama in University of Tsukuba, Japan. Full length RBM39 with Flag-tag on the N-terminal and full length DARS-AS1 were subcloned into pLVX-puro expression vector. Truncations of RBM39 (Flag-RBM39 1-240, Flag-RBM39 240-400, Flag-RBM39 400-530) were constructed by subcloning the gene sequences into pLVX-puro vector. The shRNAs targeting DARS-AS1 (GGCTTATAAGGGACTATATCT for shDARS-AS1 #1 and GGACAAGCAACTTCAAATTTC for shDARS-AS1 #2) were cloned into pSIREN plasmid according to Knockout™ RNAi Systems User Manual (Clontech Laboratories). The lentiviral vector pGIPZ containing RBM39 specific shRNA (GATCTACTGTC ATTTGTAT for shRBM39 #1 and CGATCCAAGGGATATGGAT for shRBM39 #2) and the RNF147 specific shRNAs (CAGAGCACCATAGACCTCA for shRNF147 #2 and CCGAAC TCAACATCTCTCA for shRNF147#3) were purchased from plasmid library of Shanghai Jiaotong University of medicine. The primers for constructing plasmids were showed in table S1.

Table S1 Primers for constructs (5’-3’)

shDARS-AS1 1#	F	GATCCGGCTTATAAGGGACTATATCTTTCAAGAG AAGATATAGTCCCTTATAAGCCTTTTTTG
	R	AATTCAAAAAAGGCTTATAAGGGACTATATCTTC TCTTGAAAGATATAGTCCCTTATAAGCCG
shDARS-AS1 2#	F	GATCCGGACAAGCAACTTCAAATTTCTTCAAGA GAGAAATTTGAAGTTGCTTGTCCTTTTTTG
	R	AATTCAAAAAAGGACAAGCAACTTCAAATTTCT CTCTTGAAGAAATTTGAAGTTGCTTGTCCG
Sense DARS-AS1	F	GCGGAATTCCGCTTCTAGTAGGCCAAACT
	R	GCGCTCGAGCTGTGTCCATGTGTTCTCAT
Anti-sense DARS-AS1	F	GCG GAATTCCTGTGTCCATGTGTTCTCAT
	R	GCGCTCGAG CGCTTCTAGTAGGCCAAACT
RBM39 1-240aa	F	CGCTCGAGATGGATTACAAGGATGACGACGATA AGATGCTTGAGGCTCCTTAC
	R	CGGGATCCTCAGTTTGCCATTGCTGC
RBM39 400-530aa	F	GCCTCGAGATGGATTACAAGGATGACGACGATA AGATGACAAGACTTTCCCAGC
	R	GCGGATCCTCATCGTCTACTTGGAAC
RBM39 241-530aa	F	CGCTCGAGATGGATTACAAGGATGACGACGATA AGATGCCTATGAGGCTTTATGGG
	R	GCGGATCCTCATCGTCTACTTGGAAC
RBM39 1-400aa	F	CGCTCGAGATGGATTACAAGGATGACGACGATA AGATGCTTGAGGCTCCTTAC
	R	CGGGATCCTCAGAATTCTGCCACAGC
Promotor of DARS-AS1	F	CGCGGTACCATACCATTTAACCTAC
	R	CCCTCGAGTCACCTGGGAGTTTG

Quantitative Real-time PCR (Q-PCR). Total RNA from myeloma cell lines or myeloma clinical tissues were extracted using trizol reagent (Invitrogen). Q-PCR was performed on the7900 HT Real-Time PCR System (Applied Biosystems) using SYBR green PCR mix (Applied Biosystems).The primers were showed in table S2.

Table S2 Primers for Q-PCR to detect the indicated mRNA levels(5'-3')

DARS-AS1 1#	F	CCTAACAGAGTGGTGAGGCT
	R	TGAAGTTGCTTGTCCTTCAACG
DARS-AS1 2#	F	GTGTCCCTAACAGAGTGGTG
	R	TCACCACATGTCTGATGCCTG
DARS	F	CTTTGGCCTGCCTTACGGA
	R	TTAGCATAATCTTCCGCCGC
RBM-39	F	GCAAGGACAGTCTTCTGTATGC
	R	CGACGAACTCCACATAAGCAA
HIF-1α	F	CACCACAGGACAGTACAGGAT
	R	CGTGCTGAATAATACCACTCACA
β -actin	F	CATCCTCACCCTGAAGTACCC
	R	AGCCTGGATAGCAACGTACATG

Table S3 Primers for CHIP assay (5’-3’)

Motif 1	F	GCACACATAACTTGTAGGATGGTT
	R	CTCTCAGTGACCCGGTAAGG
Motif 2	F	TAGGAACACGAGGGCTGAGT
	R	CCTGGGAGTTTGGCCTACTA
18S	F	CGGCGACGACCCATTCGAAC
	R	GAATCGAACCCTGATTCCCCGTC
LDHA	F	TTGGAGGGCAGCACCTTACTTAGA
	R	GCCTTAAGTGGAACAGCTATGCTGAC

Table S4 Differentially expressed lncRNAs under hypoxic conditions

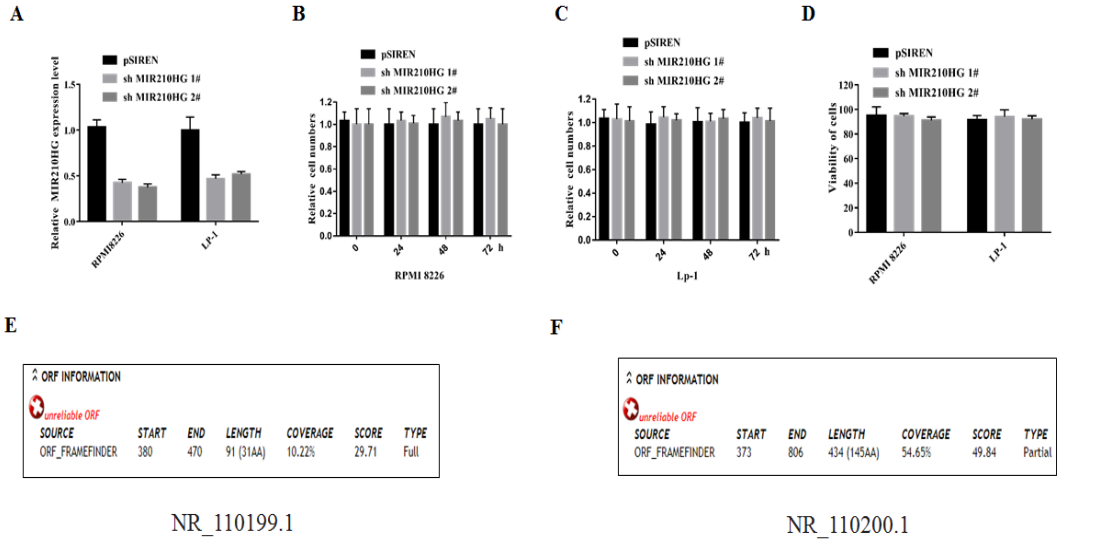
Gene	logFC	PValue	FDR
ENST00000476051.5	7.168668	4.26E-06	0.002904
NONHSAT009601.2	6.92262	2.06E-05	0.008034
NONHSAT055357.2	6.92262	2.06E-05	0.008034
ENST00000487303.1	6.830303	3.54E-05	0.011815
NONHSAT216006.1	6.830303	3.54E-05	0.011815
ENST00000474610.1	6.830303	3.54E-05	0.011815
NONHSAT145982.2	6.731672	6.13E-05	0.016373
ENST00000444744.1	6.731672	6.13E-05	0.016373
NONHSAT165543.1	6.731672	6.13E-05	0.016373
NONHSAT126413.1	6.731672	6.13E-05	0.016373
ENST00000515608.5	6.625801	0.000107	0.02305
ENST00000490757.2	6.625801	0.000107	0.02305
ENST00000492718.1	6.511541	0.000188	0.029041
NONHSAT167188.1_6	6.511541	0.000188	0.029041
ENST00000607362.1	6.511541	0.000188	0.029041

ENST00000414386.5	6.387447	0.000335	0.043599
ENST00000442712.1	6.387447	0.000335	0.043599
NONHSAT150248.1_2	6.387447	0.000335	0.043599
NONHSAT138608.2	6.387447	0.000335	0.043599
ENST00000563751.1	6.387447	0.000335	0.043599
ENST00000500447.1	3.926723	6.24E-17	3.19E-13
ENST00000424792.5	3.913799	0.000103	0.02305
ENST00000427630.1	3.906829	8.06E-09	1.57E-05
NONHSAT083665.1	3.826521	1.20E-06	0.001155
ENST00000490115.5	3.351244	6.24E-23	5.48E-19
ENST00000533920.1	3.336381	1.38E-11	3.70E-08
NONHSAT192783.1	3.326361	7.30E-05	0.018779
ENST00000474968.5	3.278976	3.58E-16	1.53E-12
ENST00000544511.1	3.265306	0.000112	0.023727
ENST00000557989.1	3.064916	0.000413	0.048613
ENST00000479855.1	2.961067	5.03E-07	0.000603

ENST00000535078.1	2.883093	0.000148	0.029041
NONHSAT008616.2	2.529347	3.83E-05	0.012607
ENST00000534540.1	2.303079	2.66E-05	0.009837
ENST00000495820.5	2.261591	2.14E-05	0.008274
ENST00000474212.5	2.207927	2.24E-08	4.03E-05
ENST00000460369.2	2.199291	3.87E-09	8.16E-06
ENST00000615516.1	1.808381	0.000126	0.026163
ENST00000565737.1	1.806216	0.000398	0.048104
ENST00000485017.1	1.780939	0.000366	0.044856
ENST00000438432.5	1.755705	5.21E-06	0.003294
ENST00000537733.1	1.717463	7.51E-05	0.019301
ENST00000507479.5	1.632792	1.53E-06	0.001339
ENST00000506424.2	1.627077	1.49E-06	0.001339
NONHSAT219817.1	1.626792	0.000223	0.033552
ENST00000554032.1	1.618442	0.000156	0.029041
ENST00000594735.5	1.561943	0.000275	0.039329

ENST00000469824.1	1.549061	0.000142	0.0288
ENST00000562842.1	1.515943	0.000381	0.046409
ENST00000481651.1	1.41226	5.51E-05	0.016284
ENST00000517565.1	1.360717	4.66E-05	0.014053
ENST00000494978.5	1.354176	5.05E-05	0.015063
NONHSAT152813.1	1.351228	9.59E-06	0.005021
ENST00000452643.5	1.088001	0.000411	0.048613
ENST00000604728.5	-1.82309	0.000267	0.038374
ENST00000497996.1	-2.05095	1.08E-05	0.005413
ENST00000500537.2	-2.72934	0.000134	0.027652
ENST00000418824.3	-3.6055	4.33E-27	7.60E-23
ENST00000483055.1	-4.01493	7.87E-05	0.020025
ENST00000393515.7_1	-4.8682	6.57E-08	0.000101
NONHSAT129023.1_7	-6.28378	0.000353	0.043736
NONHSAT216414.1_1	-6.28378	0.000353	0.043736
NONHSAT012779.2_3	-6.28378	0.000353	0.043736

ENST00000550753.5	-6.28378	0.000353	0.043736
ENST00000609088.1	-6.28378	0.000353	0.043736
NONHSAT081685.2	-6.28378	0.000353	0.043736
NONHSAT104294.2	-6.43393	0.000175	0.029041
NONHSAT220698.1	-6.43393	0.000175	0.029041
ENST00000601033.1	-6.43393	0.000175	0.029041
NONHSAT193839.1	-6.43393	0.000175	0.029041
NONHSAT198718.1	-6.56992	8.78E-05	0.02093
NONHSAT105099.2	-6.56992	8.78E-05	0.02093
NONHSAT172617.1	-6.56992	8.78E-05	0.02093
ENST00000411448.5	-6.56992	8.78E-05	0.02093
NONHSAT184209.1	-6.91458	1.19E-05	0.005532
NONHSAT195305.1	-7.10573	3.32E-06	0.002463
NONHSAT196610.1_3	-7.42556	2.87E-07	0.000375



E

ORF INFORMATION

unreliable ORF

SOURCE	START	END	LENGTH	COVERAGE	SCORE	TYPE
ORF_FRAMEFINDER	380	470	91 (31AA)	10.22%	29.71	Full

NR_110199.1

F

ORF INFORMATION

unreliable ORF

SOURCE	START	END	LENGTH	COVERAGE	SCORE	TYPE
ORF_FRAMEFINDER	373	806	434 (145AA)	54.65%	49.84	Partial

NR_110200.1

Figure S1 MIR210HG has no significant effects on the myeloma cell proliferation or apoptosis.

(A-D) Myeloma cells proliferation or apoptosis after silencing MIR210HG were assessed. MIR210HG has no significant effects on the myeloma cell proliferation or apoptosis. (E-F) DARS-AS1 has limited protein-coding potential.

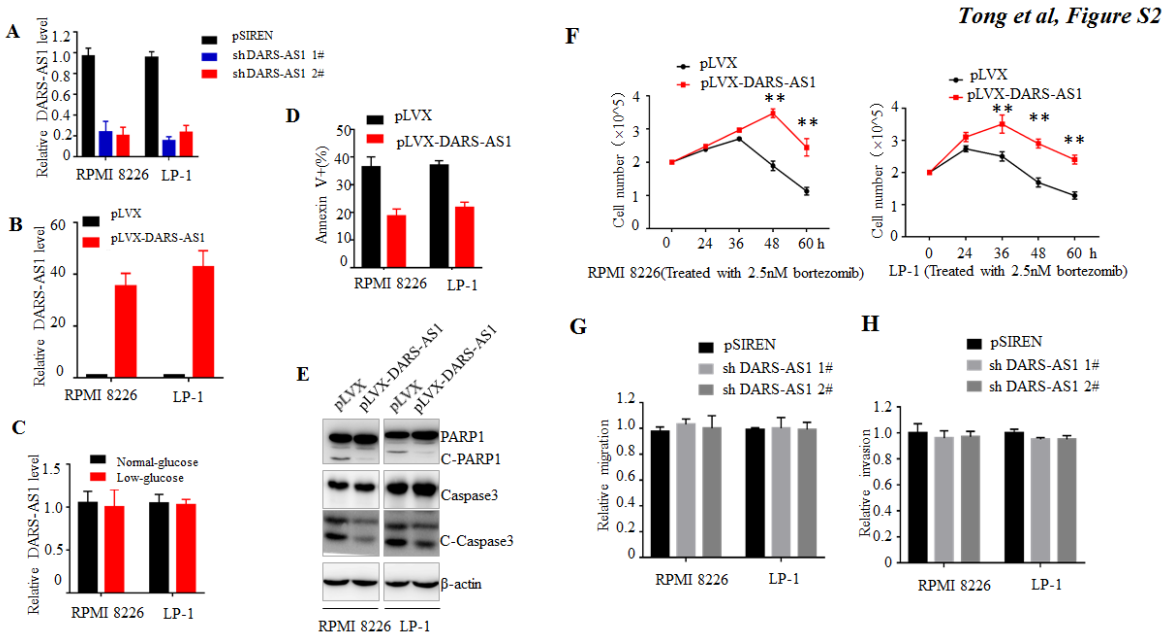


Figure S2 Functional verification of DARS-AS1 (1).

(A and B) Expression of DARS-AS1 was determined in DARS-AS1 knockdown or overexpressed myeloma cells. (C) The expression of DARS-AS1 was not changed in low-glucose culture conditions (glucose level 1 mM). (D and E) Myeloma cells with DARS-AS1 overexpression were cultured under low-glucose condition. The Annexin V^+cells decreased in DARS-AS1-overexpressed myeloma cells. Cleaved caspase-3/PARP decreased in DARS-AS1-overexpressed myeloma cells. (F) Myeloma cells with DARS-AS1-overexpression were less sensitive to bortezomib compared with the control. (G and H) DARS-AS1-silencing in myeloma cells had no influence on the migration and invasion phenotype under the hypoxic culture environment. Data represent means ± SEM (bars) from 3 independent experiments. **P<0.001.

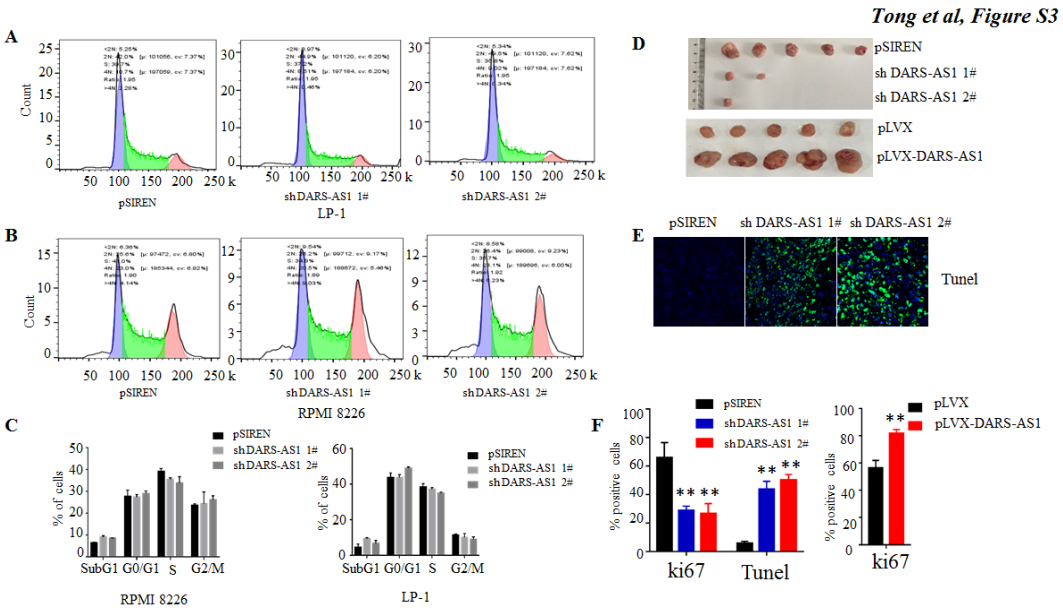


Figure S3 Functional verification of DARS-AS1 (2).

(A-C) DARS-AS1-silencing in myeloma cells had no influence on the cell cycle distribution under hypoxic culture environment. (D) The DARS-AS1-knockdown in RPMI 8226 cells reduced the myeloma tumor formation rate and tumor volume. The DARS-AS1-overexpression in RPMI 8226 cells significantly increased the tumor volume. Cells with DARS-AS1 overexpression or DARS-AS1 knockdown in NOD-SCID mice. (E) TUNEL staining of xenografts with DARS-AS1 knockdown cells. (F) Statistical analysis of Ki67 and Tunel staining for Figure 2 H and 2 J. Data represent means ± SEM (bars) from 3 independent experiments. **P<0.001.

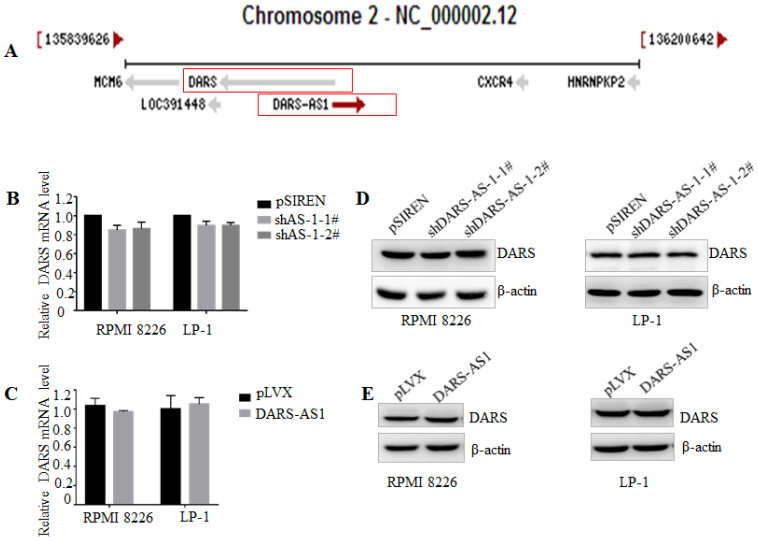


Figure S4 DARS-AS1 does not affect the expression of DARS.

(A) Location of DARS-AS1 and DARS in Genome.

(B-E) The expressions of DARS was not changed at transcriptional level (Q-PCR) and protein level (Western blotting) after downregulating DARS-AS1 or overexpressing DARS-AS1 in LP-1 and RPMI 8226 cells.

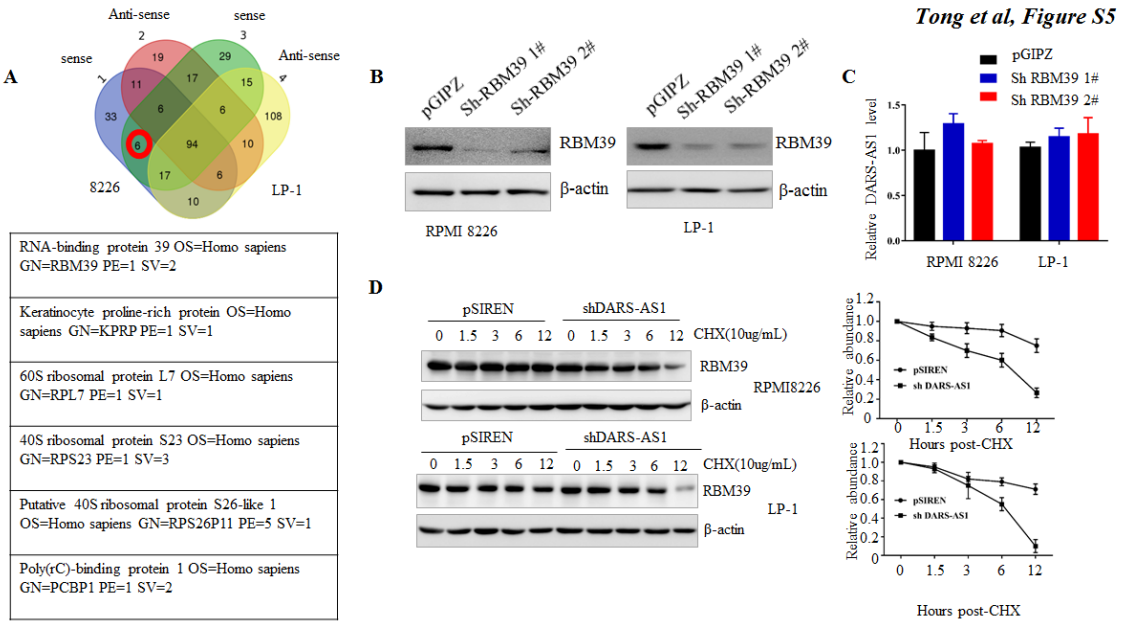


Figure S5 The interaction between with DARS-AS1 and RBM39.

(A) Mass spectrometry results show that six lncRNA-sense chain interacting proteins were identified in both LP-1 and RPMI 8226 cell lysates, after excluding the non-specific and high abundance proteins. (B-C) The expressions of DARS-AS1 were assessed by Q-PCR in RBM39-knockdown multiple myeloma cells in hypoxic culture environment. (D) DARS-AS1 knockdown or control myeloma cells were treated with the protein synthesis inhibitor CHX (10μg/mL) for 12h under the hypoxic culture environment. The degradation rate of RBM39 was significantly accelerated in DARS-AS1 knockdown myeloma cells under hypoxic environment.

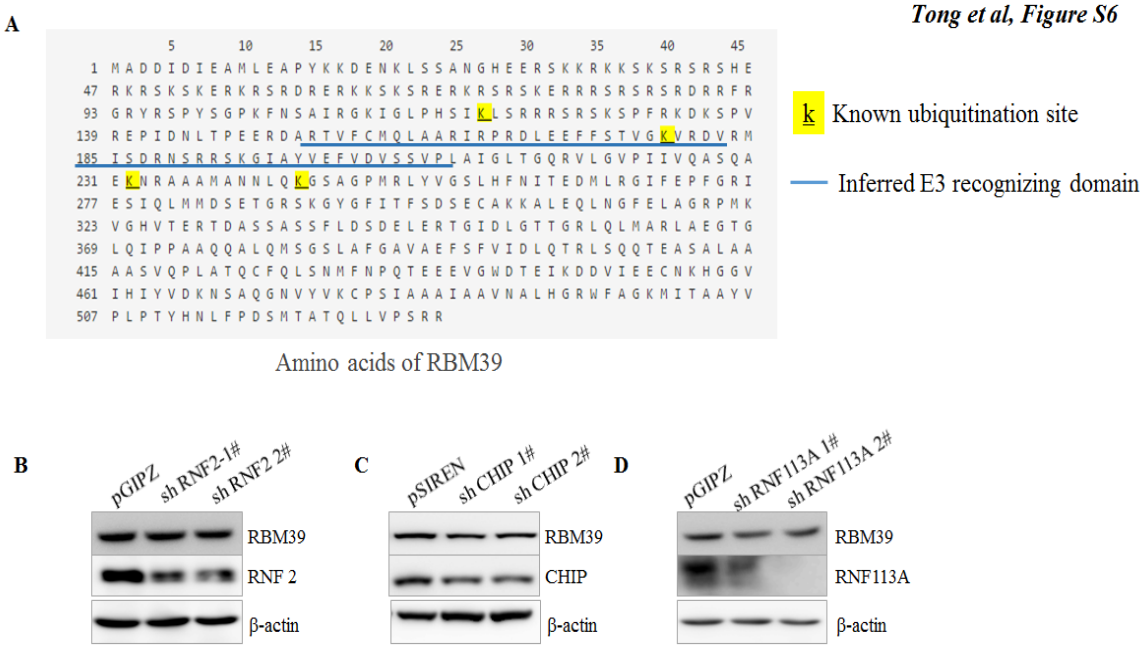


Figure S6 Screen E3 ubiquitin ligase of RBM39.

(A) The possible ubiquitin modified lysines in RBM39 was marked in yellow words. The possible E3 ubiquitin ligase interacting amino acid sequence was underlined. (B) Knockdown of RNF2, RNF113A, or CHIP did not affect the protein levels of RBM39 in 293T cells under hypoxic culture environment.

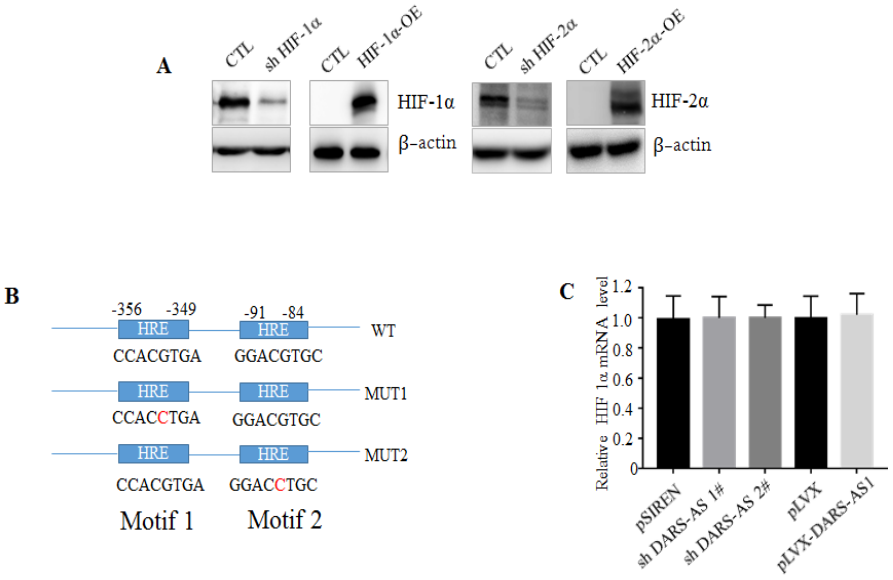


Figure S7 The relationship between HIF-1 and DARS-AS1

(A) HIF-1α or HIF-2α was knockdown or overexpressed in 293T cells. (B) Two HRE regions and the mutation sites in the promoter of DARS-AS1. (C) Q-PCR analyzed the mRNA levels of HIF-1α in DARS-AS1 overexpression or knockdown RPMI 8226 cells. Knockdown of DARS-AS1 does not affect HIF-1 mRNA levels of RPMI 8226 cells in hypoxic culture environment. Overexpression of DARS-AS1 does not affect HIF-1 mRNA levels of RPMI 8226 cells in normoxic culture environment.