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Supplemental information

DDX3 modulates the tumor microenvironment via its role in endoplasmic reticulum-associated translation

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SUPPLEMENTARY TABLES

Table S1. List of the Top Growth Factors or Cytokines That Were Down-Regulated by DDX3 Knockdown (Related to Figure 1)

Regulator	RIBOseq Log2 (siD#1/siC)	Molecular Type	Activation State	z-score	p-value of overlap	TE Log2 (siD#1/siC)
AREG	-3.1518	Growth factor	Inhibited	-2.359	1.75E-04	-0.80479
NRG1	-2.21476	Growth factor	Inhibited	-3.087	7.34E-10	-0.53487
EDN1	-2.08081	Cytokine	Inhibited	-2.945	1.64E-05	-0.82668
IL1A	-2.05664	Cytokine	Inhibited	-2.389	4.93E-03	-0.71868
IL1B	-1.84014	Cytokine	Inhibited	-3.249	9.45E-06	-0.70392
TGFA	-1.31945	Growth factor	Inhibited	-2.574	5.49E-04	-1.37732

Table S2. Identification of DDX3-Interacting Partners by Using Immunoprecipitation-Mass Spectrometry (Related to Figure 5)

Top 10 candidates in each protein bands (Figure S5) are shown.

Band	TOP10 non-redundant Gene symbol
1	ZC3H18, ATXN2, EIF3A, THRAP3,PELP1, DCD, MAP7D1, BCLAF1, TJP2, TJP1
2	LARP4, SRPK2, EIF3C, DDX54, SRPK1, YTHDC1, ILF3, NOM1, EIF3B, SND1
3	NUFIP2, EIF4B, ZC3HAV1, ZFP91, FXR2, LCA5, NUMB, SPTY2D1, TDRD3, MAP3K20
4	DDX3X, SRP68, SRP72, HSPA8, DDX17, PABPC1, DDX5, HSPA5, FXR1, DDX1
5	RTCB, TUBB, TUBB4B, IMPDH2, TUBB4A, TUBB3, TUBA1B, SERBP1, TUBA1A, TUBB6
6	RPL4, RPL3, YBX1, BYSL, RBM34, EIF3E, LUC7L2, YBX3, FAM98B, TUBB2A
7	HNRNPA2B1, NPM1, EIF3I, GAPDH, RPSA, HNRNPA3, APOBEC3A, FYTTD1, RPL15, RPL34
8	RPL5, HNRNPA1, RPLP0, PWP1, CCDC59, CCDC137, RACK1, TRA2B, ABT1, FAM60A
9	PYCR1, MRPS18B, UTP23, RBM7, TAF15, RPL22, HIST1H1C, FGFBP1, PCDHGC4, C6orf136
10	RPL8, RPL7, RPS6, RPL7A, RPS3A, RPS4X, RPS2, CHTOP, ALYREF, RPS3
11	RPL18, RPS8, RPL10, RPL13, RPL19, RPL9, RPL14, RPL17, PYCR2, RPL13A
12	RPL24, RPS5, RPL18A, RPL11, RPL21, RPL29, RPL23A, RPS9, RPS7, RPL6
13	RPL26, RPL28, RPL26L1, RPL32, RPS10, RPS13, RPL12, RPS11, RPL27A, RPS14

Table S3. GO Analysis of Potential DDX3-Interacting Partners Identified by Immunoprecipitation-Mass Spectrometry (Related to Figure 5)

Biological process (GO)	Observed count	Background count	*P* value
Translational initiation	78	142	8.37E-70
Translation	99	362	1.34E-66
SRP-dependent cotranslational protein targeting to membrane	64	92	5.69E-62
mRNA metabolic process	114	667	3.64E-59
Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	65	118	1.94E-58
Peptide metabolic process	101	497	4.69E-58
Protein localization to endoplasmic reticulum	65	123	1.24E-57
Amide biosynthetic process	100	495	2.71E-57
Nuclear-transcribed mRNA catabolic process	71	191	7.43E-55
Ribonucleoprotein complex biogenesis	90	409	6.51E-54

Table S4. GO Analysis of Potential DDX3-Interacting Partners Identified by SILAC (Related to Figure 5)

Biological process (GO)	Observed count	Background count	*P* value
Translational initiation	45	142	9.23E-53
Translation	49	362	2.51E-42
SRP-dependent cotranslational protein targeting to membrane	34	92	2.84E-41
Amide biosynthetic process	52	495	1.37E-40
Peptide metabolic process	52	497	1.42E-40
Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	33	118	4.44E-37
Cellular amide metabolic process	55	732	2.21E-36
Establishment of protein localization to organelle	43	396	1.13E-33
mRNA catabolic process	35	207	6.86E-33
Nuclear-transcribed mRNA catabolic process	34	191	1.40E-32

Table S5. List of shRNA Target Sequences (Related to STAR Methods)

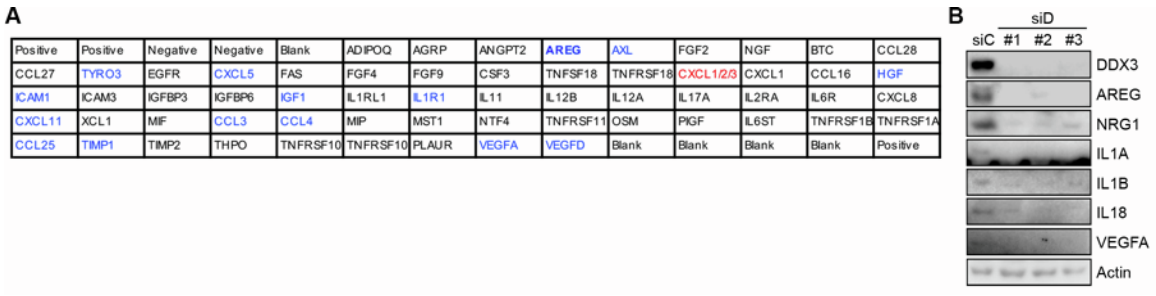
shRNA	Sense target sequence
shC	AACUUUCGCUUAUUGGACUAA
shDDX3	ACAUUGAGCUUACUCGUUAUA
shAREG	GACCUCAAUGACACCUACUCU
shSRP9	GGCAUUCUGAUGGGAACUUGU
shSRP14	GCACAGUAAAGGGCAUACAUU
shSRP19	GAAGGCGAAUCCCCAUAAGUA
shSRP54	GGUAUUGAAUGCUAUGCUAAA
shSRP68	GAGCUUCUGACCGAUAAUAGA
shSRP72	GCGCUCUCAAGACCGUCAAUA
shALYREF	GCUUGUCACGUCACAGAUUGA
shATXN2	GGUUCAUAUACUUACAUCAGU
shCASC3	GAACGGUGAGCGGCUAAACAA
shDDX6	GGUAGGGAUAUCUUAGCUAGA
shDDX42	GUUCGAUCCAUAGCAAGUCAU
shDDX55	GGAAGAGUCAUACAUCAAUUU
shEIF2B3	GCAGUAGUGAUGGCAGUAGGU
shEIF2S3	GCCCUUAGCCGAAGAGUUGAA
shEIF4E	GGAAACCACCCCUACUCCUAA
shEIF4H	GAUCUCAGCAUAAGGAGUGUA
shEIF5	GACGUUGCAAAGGCGCUUAAU
shEIF5A	GUAAGAUCGUCGAGAUGUCUA
shEIF5B	GAGAAGAGGAAGAACGUAUAA
shEIF6	GUGCUUAUCGCCUGGAUCUAU
shELAVL1	GAUCAAAGACGCCAACUUGUA
shIGF2BP1	GCUGGCUCAGUAUGGUACAGU
shLARP4	GCCAGAAGCAAGGGCUAGUAA
shMETAP2	GAAGACUGUUCACGCAAGUUA
shSYNCRIP	GACGGUGCAUUGGCAGUUCUU
shYBX3	GAAUAACCCACGGAAAUAUCU

Table S6. List of Primers Used in the Study (Related to STAR Methods)

Primer	Sequence
SRP9 qPCR F	CTGCCGAGAAGCTTTACCTC
SRP9 qPCR R	GGGCTTCCTTGGCTACCATA
SRP14 qPCR F	AGCTAACATGGATGGGCTGA
SRP14 qPCR R	GTTGTTGCTGCTGTTGTTGG
SRP19 qPCR F	GAAGACCATCGCAGAGGGAA
SRP19 qPCR R	GGCTCCCATCTTCCTGTTTG
SRP54 qPCR F	CAGTACGGATGGTGCCAAAG
SRP54 qPCR R	GACATGTCGCCACCTTTGAA
SRP68 qPCR F	CAAGTGCGGTCAGAGAAGTG
SRP68 qPCR R	ACCGTTCAACCAGAGGCTTA
SRP72 qPCR F	ATCTCGTCCGAAACTCCCAA
SRP72 qPCR R	CCAGGTTCTCTGGAACCACT
hRL qPCR F1	CCAAGCAAGATCATGCGGAA
hRL qPCR R1	TAACCTCGCCCTTCTCCTTG
hFL qPCR F1	CTTTCATCTGCCAGGCATCC
hFL qPCR R1	CACCTTGGCCTCGAAGAATG
AREG qPCR F	GGTGCTGTCGCTCTTGATAC
AREG qPCR R	TTCACGCTTCCCAGAGTAGG
ACTB qPCR F	GAGGCACTCTTCCAGCCTT
ACTB qPCR R	AAGGTAGTTTCGTGGATGCC
GAPDH qPCR F	ATGGGTGTGAACCATGAGAA
GAPDH qPCR R	GTGCTAAGCAGTTGGTGGTG
IL1B qPCR F	AGCTGAGGAAGATGCTGGTT
IL1B qPCR R	GTGATCGTACAGGTGCATCG
IL23A qPCR F	AGTCAGTTCTGCTTGCAAAGG
IL23A qPCR R	AGTAGGGAGGCATGAAGCTG
MAF qPCR F	CTTGCACTTTGCACAGAGGT
MAF qPCR R	CCTCTTCTGCTTGGCTCTCT
VEGFA qPCR F	ACAAGATCCGCAGACGTGTA
VEGFA qPCR R	TCACATCTGCAAGTACGTTCG
EDN1 qPCR F	AGACAAACCAGGTCGGAGAC
EDN1 qPCR R	TGTGGGTCACATAACGCTCT
NRG1 qPCR F	AACGTCATCTCCAGTGAGCA
NRG1 qPCR R	GATGCTTTCAGTGTGTCCGT
TGFA qPCR F	CTGCCATTCTGGGTACGTTG
TGFA qPCR R	GTGATGGCCTGCTTCTTCTG
XBP1 qPCR F	CAGACTACGTGCACCTCTGC
XBP1 qPCR R	CTGGGTCCAAGTTGTCCAGAAT
CD163 qPCR F	AATTCCTCAGGAGGCCATTC
CD163 qPCR R	TGCTCCATTCAATAGTCCAGG
IL10 qPCR F	GTGGAGCAGGTGAAGAATGC
IL10 qPCR R	GCCACCCTGATGTCTCAGTT

MRC1 qPCR F	CAGCGCTTGTGATCTTCATT
MRC1 qPCR R	TACCCCTGCTCCTGGTTTTT
IL1A qPCR F	TTCAAGGAGAGCATGGTGGT
IL1A qPCR R	AAAGGTGCTGACCTAGGCTT
IL18 qPCR F	CAGTCTACACAGCTTCGGGA
IL18 qPCR R	TGCCACAAAGTTGATGCAAT
IL12B qPCR F	GTTTCAGGGCCATTGGACT
IL12B qPCR R	GAGATGCCAGAAAAACCAGG
HLADR qPCR F	TGGAGTCCCTGTGCTAGGAT
HLADR qPCR R	ATAGAACTCGGCCTGGATGA
NOS2 qPCR F	GCCAAGAACGTGTTCACCAT
NOS2 qPCR R	GCCATCCTCACAGGAGAGTT
CDH1 qPCR F	GCGTGTGTGACTGTGAAGGG
CDH1 qPCR R	GTCCCGGGTGTCATCCTCTG
NheI+AREG 5' UTR F	TAT GCTAGC AGACGTTCGCACACCTGGGT
NheI+AREG 5' UTR R	TAT GCTAGC ATTGGTCCTTCGCAGCGGCG
XhoI+AREG 3' UTR F	TAT CTCGAG CTGAAGATAAAATTACAGGATATC
NotI+AREG 3' UTR R	TAT GCGGCCGC TTTGGTTAAAAAAGTTTAATGAGC
HindIII+AREG CDS F	TAT AAGCTT ACG ATGAGAGCCCCGCTGCTACC
BamHI+AREG CDS R	TAT GGATCC TGCTATAGCATGTACATTTCCA

SUPPLEMENTARY FIGURE LEGENDS



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Positive	Positive	Negative	Negative	Blank	ADIPOQ	AGRP	ANGPT2	AREG	AXL	FGF2	NGF	BTC	CCL28
CCL27	TYRO3	EGFR	CXCL5	FAS	FGF4	FGF9	CSF3	TNFSF18	TNFRSF18	CXCL1/2/3	CXCL1	CCL16	HGF
ICAM1	ICAM3	IGFBP3	IGFBP6	IGF1	IL1RL1	IL1R1	IL11	IL12B	IL12A	IL17A	IL2RA	IL6R	CXCL8
CXCL11	XCL1	MIF	CCL3	CCL4	MIP	MST1	NTF4	TNFRSF11	OSM	PlGF	IL6ST	TNFRSF1B	TNFRSF1A
CCL25	TIMP1	TIMP2	THPO	TNFRSF10	TNFRSF10	PLAUR	VEGFA	VEGFD	Blank	Blank	Blank	Blank	Positive

Figure S1. DDX3 is Required for the Expression of Multiple Growth Factors and Cytokines (Related to Figure 1)

(A) The list of antibodies on the array used in Figure 1B. The proteins of which the signals were downregulated or upregulated by ≥2-fold (Figure 1B) are marked as blue and red, respectively.

(B) Immunoblotting of growth factors and cytokines as indicated in the lysates of siC-transfected or siD (#1-3)-transfected SAS cells.

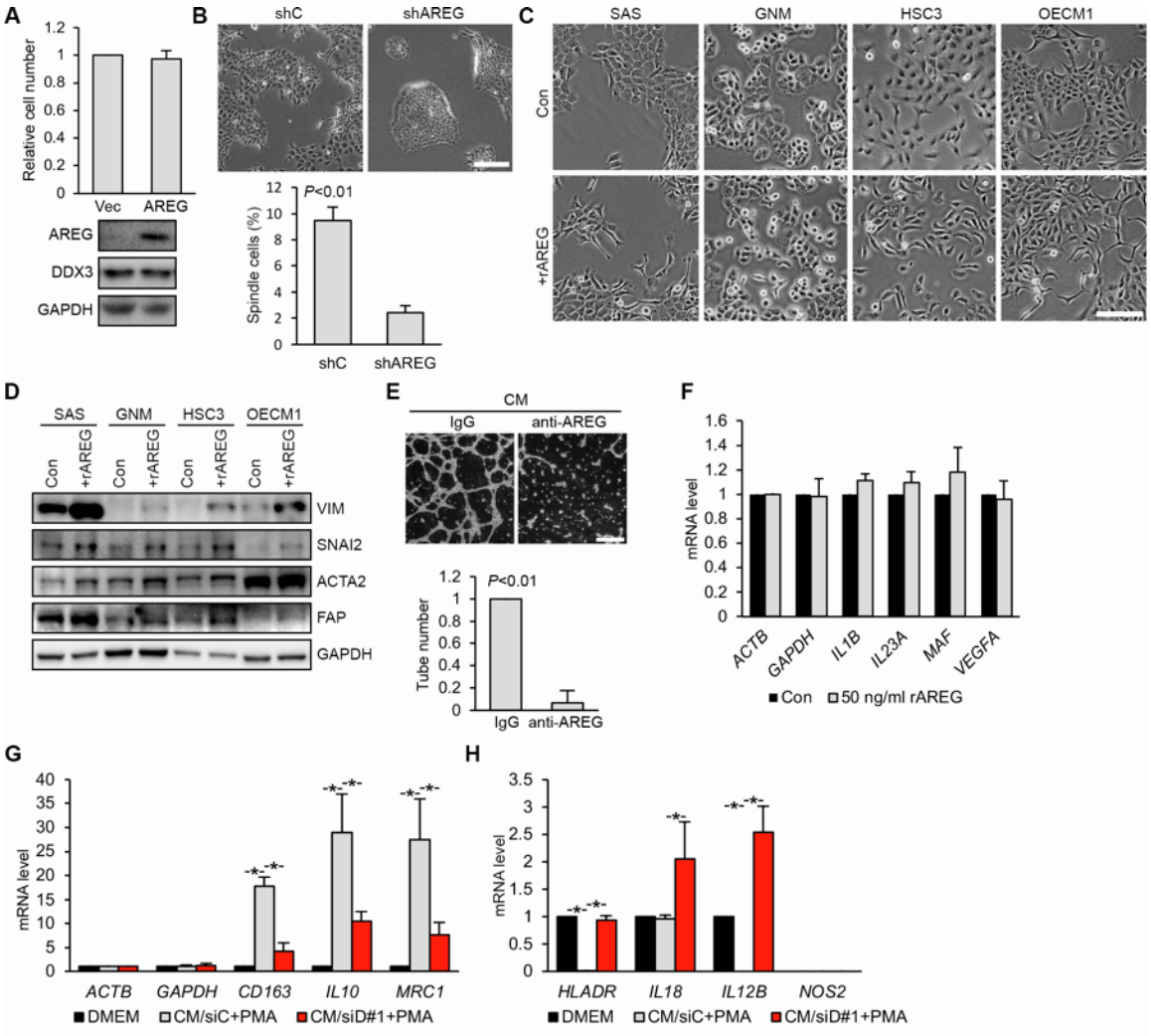

Figure S2. The Potential of AREG in Promoting Cell Migration, Angiogenesis and Macrophage Differentiation (Related to Figure 2)

(A) SAS cells were transfected with pP2A (vec) or pP2A-AREG for 72 hours. Bar graph shows relative cell number; average and standard deviation were from three independent experiments. Immunoblotting was performed using antibodies as indicated.

(B) Morphology analysis of cells transfected with shC or shAREG. The scale bar represents 50 μm.

(C) Representative images of morphology of indicated cells cultured in control or rAREG-containing medium. The scale bar represents 50 μm.

(D) Immunoblotting of the lysates of control or rAREG-treated OSCC cell lines as in panel C with antibodies against indicated proteins.

(E) Angiogenesis assay of EA.hy926 cells cultured in CM of SAS cells treated with 1 μg/ml of control IgG or anti-AREG.

(F) RT-qPCR analysis of the indicated mRNAs from THP-1 cells cultured in DMEM with or without 50 ng/ml of rAREG.

(G and H) RT-qPCR analysis of indicated mRNAs from THP-1 cells that were cultured in DMEM or CM of siC or siD#1 SAS cells supplemented with PMA; **P* < 0.01.

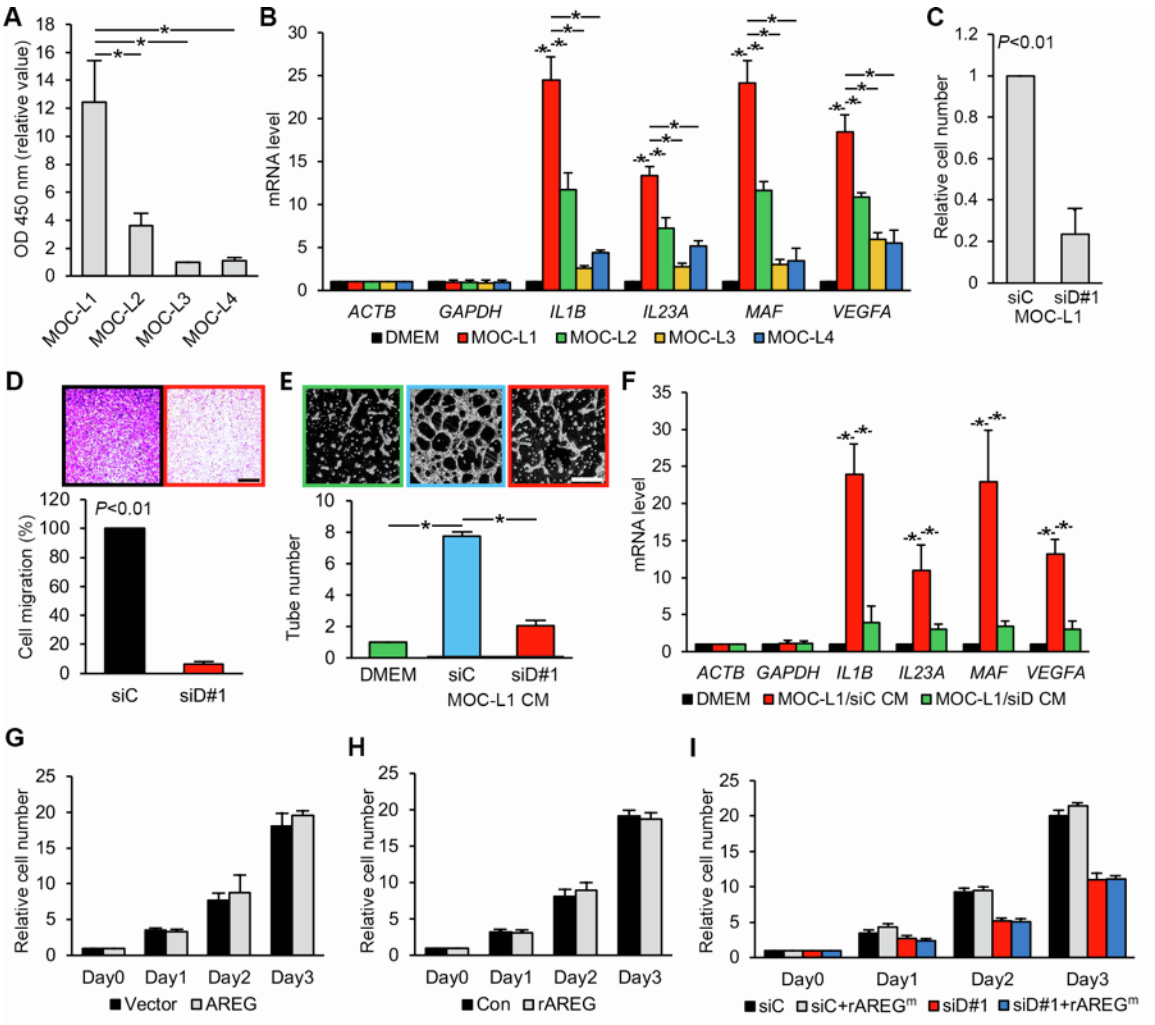

Figure S3. DDX3 Promotes Cell Growth and Migration of Mouse MOC-L1 Cells and Enhances Their Activity in Remodeling Stromal Cells (Related to Figure 3)

(A) ELISA assays of mouse AREG protein in CM of indicated lines of mouse OSCC cells.

(B) RT-qPCR analysis of indicated transcripts in THP-1 cells cultured in CM derived from indicated mouse OSCC cells.

(C) Cell growth assays of MOC-L1 cells transfected with siC or siD#1.

(D) Boyden chamber assays of MOC-L1 cells transfected with siC or siD#1. Image and bar graph are analogous to Figure 2B.

(E) Angiogenesis assays of EA.hy296 cells cultured in DMEM or CM of MOC-L1 transfected with siC or siD#1. Image and bar graph are analogous to Figure 2C.

(F) RT-qPCR analysis of indicated transcripts in THP-1 cells that were cultured in DMEM or CM from MOC-L1 transfected with siC or siD#1. Bar graph is analogous to Figure 2D; $*P < 0.01$.

(G) MOC-L1 cells were transfected with the control or AREG overexpression vector. The number of cells was counted at different time points as indicated. Bar graph shows the relative cell number of mock or AREG-overexpressed cells at different days, representing cell growth.

(H) Cell growth assay was performed as in panel G, except that MOC-L1 cells were mock treated or treated with 50 ng/ml rAREG^{m}.
(I) Cell growth assay was performed in mock or DDX3 knockown-MOC-L1 cells that were treated rAREG^{m} as in panel H.

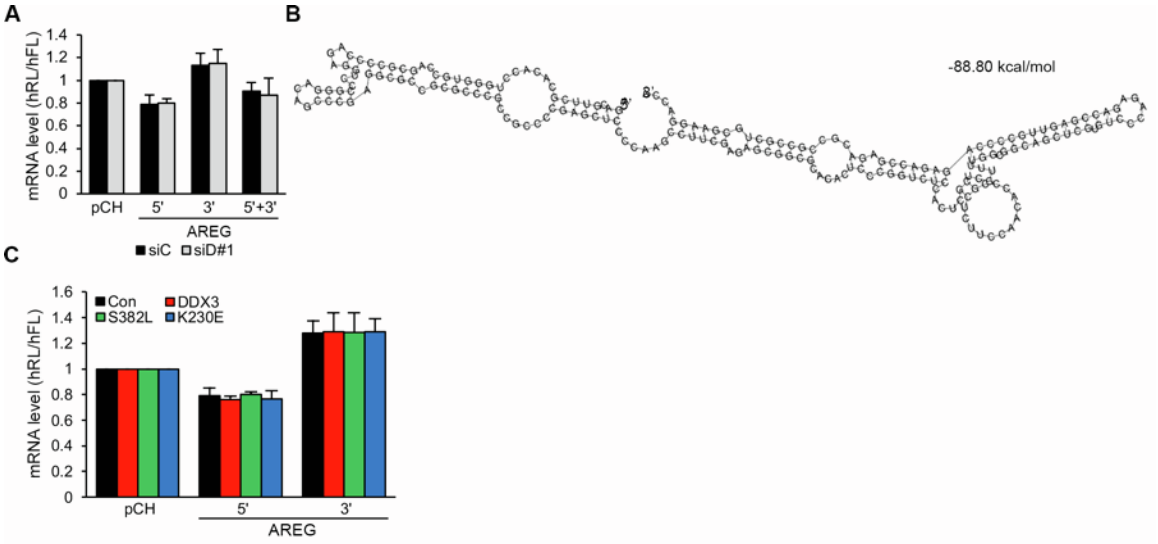

Figure S4. DDX3 Knockdown Has no Effect on AREG Reporter mRNA Levels (Related to Figure 4)
(A) RT-qPCR analysis of reporter mRNAs in the *in vivo* translation reactions (Figure 4B).
(B) RNAfold prediction for the secondary structure of *AREG* 5' UTR.
(C) RT-qPCR analysis of reporter mRNAs in the *in vivo* translation reactions (Figure 4C).

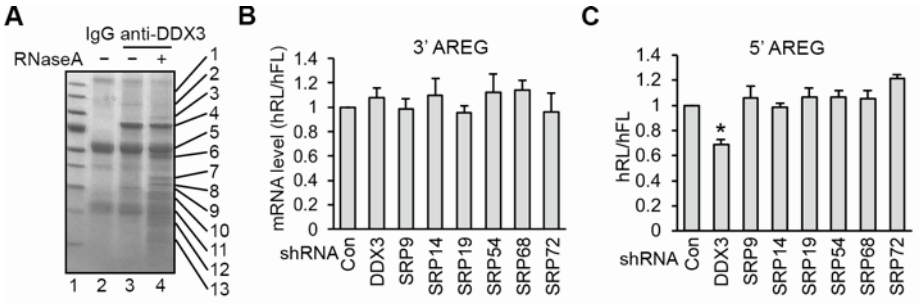

Figure S5. The SRP is a DDX3-Interacting Partner and Has no Effect on AREG 5' UTR-Mediated Translation (Related to Figure 5)

(A) Immunoprecipitation was performed using anti-DDX3 in SAS cell lysates. Coomassie blue staining shows IgG or DDX3 co-immunoprecipitates that were not treated or treated with RNase. Thirteen bands of RNase-treated immunoprecipitates (lane 4) were subjected to mass spectrometry analysis. The top 10 non-redundant proteins in each band are listed in Table S2.

(B) RT-qPCR analysis of reporter mRNAs in the *in vivo* translation reactions (Figure 5B).

(C) *In vivo* translation assay was performed as in Figure 5B except that the AREG 5' UTR reporter was used.

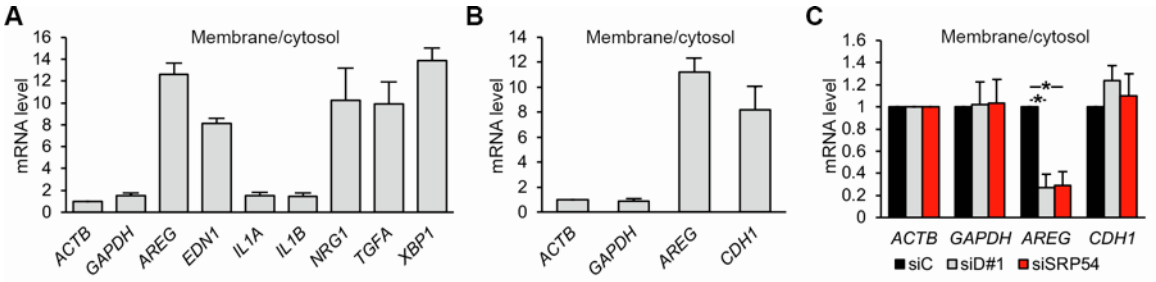

Figure S6. Identification of Membrane-Associated mRNAs (Related to Figure 6)

(A and B) Cell fractionation was performed as in Figure 6. RT-qPCR of the indicated transcripts in the membrane and cytosol fractions. Bar graphs shows relative membrane/cytosol ratios.

(C) RT-qPCR analysis of the indicated mRNAs in both the membrane and cytosol fractions of siRNA-transfected SAS cells. Bar graph shows the membrane-to-cytosol ratio of each mRNA in indicated knockdown (siD#1, siSRP54) cells relative to control (siC).