Supplementary Information

Title

Epigenetic regulation of NKG2D ligands is involved in exacerbated atherosclerosis development in Sirt6 heterozygous mice

Authors

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Supplementary tables

Supplementary Table 1. Blood pressure and heart rate in $ApoE^{-/-}$ mice and $Sirt6^{+/-}ApoE^{-/-}$ mice which were fed with Western diet for 16 weeks.

	$ApoE^{-/-}$ (n=11)	$Sirt6^{+/-}ApoE^{-/-}$ (n=10)	P value
HR (beats/min)	602 ± 17	620 ± 12	0.41
SBP (mmHg)	118 ± 8	117 ± 4	0.91
MBP (mmHg)	66 ± 4	69 ± 4	0.68
DBP (mmHg)	81 ± 4	84 ± 4	0.51

Student's t test was applied to calculate the P value.

Supplementary Table 2. Levels of plasma lipids in $ApoE^{-/-}$ mice and $Sirt6^{+/-}ApoE^{-/-}$ mice which were fed with Western diet for 16 weeks.

	$ApoE^{-/-}$ (n=15)	$Sirt6^{+/-}ApoE^{-/-}$ (n=11)	P value
Total cholesterol (mmol/L)	24.21 ± 1.42	23.27 ± 2.32	0.74
Triglyceride (mmol/L)	0.13 ± 0.03	0.14 ± 0.04	0.86
HDL cholesterol (mmol/L)	0.38 ± 0.05	0.40 ± 0.06	0.79
LDL cholesterol (mmol/L)	7.96 ± 0.64	7.45 ± 0.81	0.63
Glucose (mmol/L)	5.60 ± 0.65	5.00 ± 0.73	0.27

Student's t test was applied to calculate the P value.

Supplementary Table 3. Top pathways affected by Sirt6 kncokout.

Ingenuity Canonical Pathways	-log(p-value)	Ratio
Hepatic Fibrosis / Hepatic Stellate Cell Activation	8.45E00	1.32E-01
Atherosclerosis Signaling	5.36E00	1.3E-01
Axonal Guidance Signaling	5.66E00	8.1E-02
Sertoli Cell-Sertoli Cell Junction Signaling	4.92E00	1.06E-01
Agranulocyte Adhesion and Diapedesis	4.58E00	1.01E-01
Granulocyte Adhesion and Diapedesis	4.44E00	1.02E-01
Role of Tissue Factor in Cancer	4.02E00	1.18E-01
Reelin Signaling in Neurons	3.47E00	1.27E-01
Leukocyte Extravasation Signaling	3.34E00	8.59E-02
Inhibition of Angiogenesis by TSP1	3.04E00	1.76E-01
Dendritic Cell Maturation	2.91E00	8.38E-02
Role of NFAT in Cardiac Hypertrophy	2.91E00	8.38E-02

Supplementary Table 4. Genes involed in atherosclerosis pathway.

Symbol	Entrez Gene Name	Affymetrix	Fold Change (ko/wt, log2)
H60b	H60b mRNA for histocompatibility-60b	1440145_at	5.96
LPL	lipoprotein lipase	1431056_a_at	2.231
ICAM1	intercellular adhesion molecule 1	1424067_at	2.325
IL6	interleukin 6	1450297_at	2.359
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	1416298_at	2.544
APOD	apolipoprotein D	1416371_at	2.777
IL1RN	interleukin 1 receptor antagonist	1425663_at	2.796
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	1456498_at	2.989
VCAM1	vascular cell adhesion molecule 1	1436003_at	2.999
COL1A2	collagen, type I, alpha 2	1446326_at	-2.454
PDGFD	platelet derived growth factor D	1426319_at	-3.051
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	1418945_at	-3.142
SELP	selectin P (granule membrane protein 140kDa, antigen CD62)	1420558_at	-3.244
CLU	clusterin	1437689_x_at	-3.367

Supplementary Table 5. Expression of NKG2D ligands in MEF (selected from Supplementary Microarray Data).

Gene Symbol	Ratio ($Sirt6^{-/-}$ vs. $Sirt6^{+/+}$)	$Sirt6^{-/-}$_ Signal	$Sirt6^{-/-}$_ Detection	$Sirt6^{+/+}$_ Signal	$Sirt6^{+/+}$_ Detection
H60a	46.1984	905.7	P	2.3	A
H60b	5.96336	256.9	P	11.7	A
Rae1a/Rae1b/Rae1c/Rae1d /Rae1e	0.831352	395.3	P	532	P

"P": indicate presence. "A": indicates absence.

Supplementary Table 6. Primers used for realtime PCR**.**

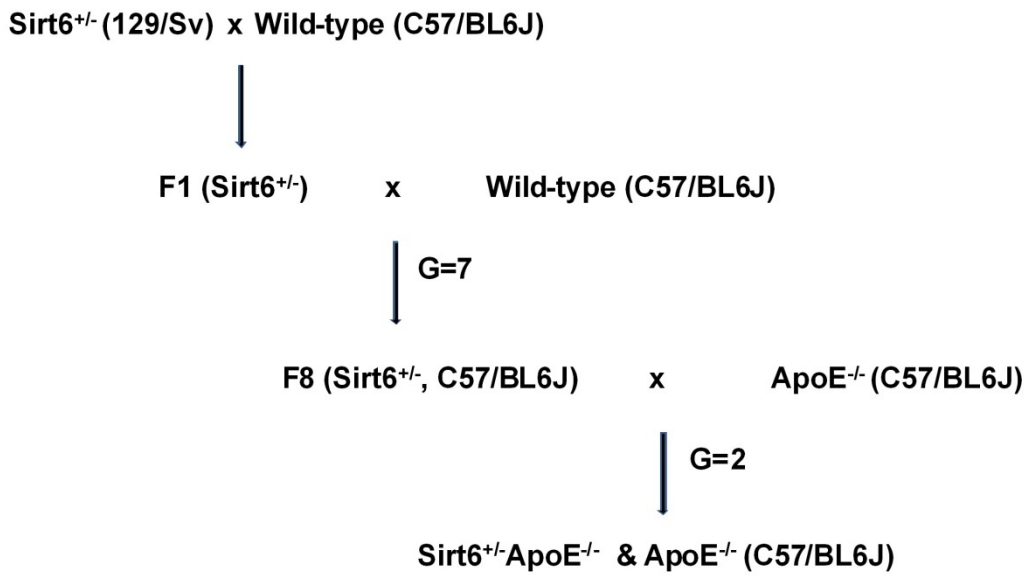
Primer name	**Primer sequence**
Mouse Sirt6 Forward	CCTGGTCAGCCAGAACGTAG
Mouse Sirt6 Reverse	TACTGCGTCTTACACTTGGGA
Human Sirt6 Forward	CCAAGTTCGACACCACCTTT
Human Sirt6 Reverse	GGCACATTCTTCCACAAACA
Mouse TNF-α Forward	CCCCAAAGGGATGAGAAGTT
Mouse TNF-α Reverse	CACTTGGTGGTTTGCTACGA
Mouse IFN-γ Forward	CACGGCACAGTCATTGAAAG
Mouse IFN-γ Reverse	GTCACCATCCTTTTGCCAGT
Mouse IL-1β Forward	TACAGGCTCCGAGATGAACA
Mouse IL-1β Reverse	AGGCCACAGGTATTTTGTCG
Mouse H60b Forward	GCTGCCTCAACAAATTGTCA
Mouse H60b Reverse	CAGACCCTGGGTGTCAGAAT
Mouse Rae-lα Forward	GGGGAATGTTTGACACAACC
Mouse Rae-lα Reverse	CCCTGGCTTTGCAGATAAAT
Mouse Rae-lδ Forward	AGCTATGGATACACCAACGGG
Mouse Rae-lδ Reverse	ACGAAGCACTTCACTTCATCTG
Mouse Rae-lε Forward	GACCAAGCGCCATCATTTTAT
Mouse Rae-lε Reverse	AGCACTTCACGTCACACCAG
Human MICA Forward	TAGAATCCGGCGTAGTCCTG
Human MICA Reverse	CTGCATGTCACGGTGATGTT

Human MICB Forward	GTGGCCATCAGGAGAACAGT
Human MICB Reverse	GACGCCAGGTCAGTGTGATA
Mouse β-actin Forward	GGCTGTATTCCCCTCCATCG
Mouse β-actin Reverse	CCAGTTGGTAACAATGCCATGT
Human β-actin Forward	CTCTTCCAGCCTTCCTTCCT
Human β-actin Reverse	AGCACTGTGTTGGCGTACAG

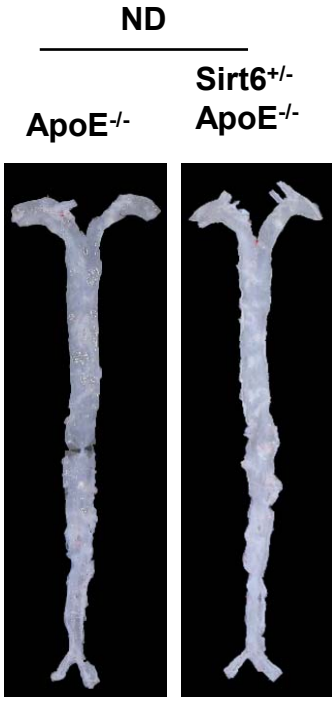
Supplementary Table 7. Primers Used in ChIP

Primer name	**Primer sequence**
mH60b promoter Forward (-158/-53bp)	GTGGTGCACGCCTTTAATTC
mH60b promoter Reverse (-158/-53bp)	CTCTGTGGGCAGGGGATG
hMICA promoter Forward (-112/+34bp)	CCCCAGTTTCATTGGATGAG
hMICA promoter Reverse (-112/+34bp)	CAGCCAGAAGCAGAAAGACC
hMICB promoter Forward (-139/-15bp)	CTAAGTTCCGGGCCTCAGTT
hMICB promoter Reverse (-139/-15bp)	CTACGTCGCCACCTTCTCAG

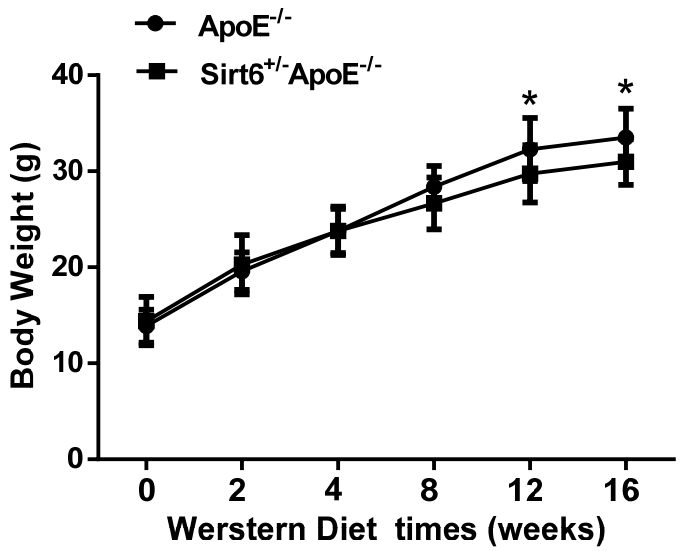
Supplementary Figures and Figure Legends



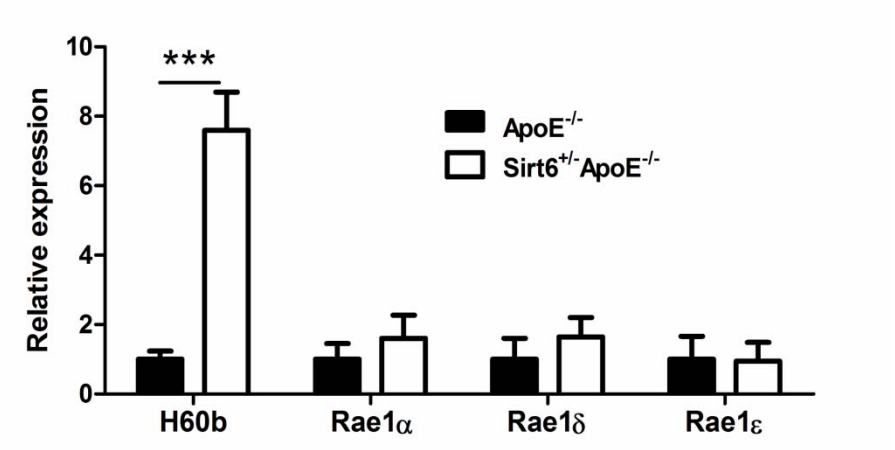
Supplementary Figure 1. Generation of $Sirt6^{+/-}ApoE^{-/-}$ mice.



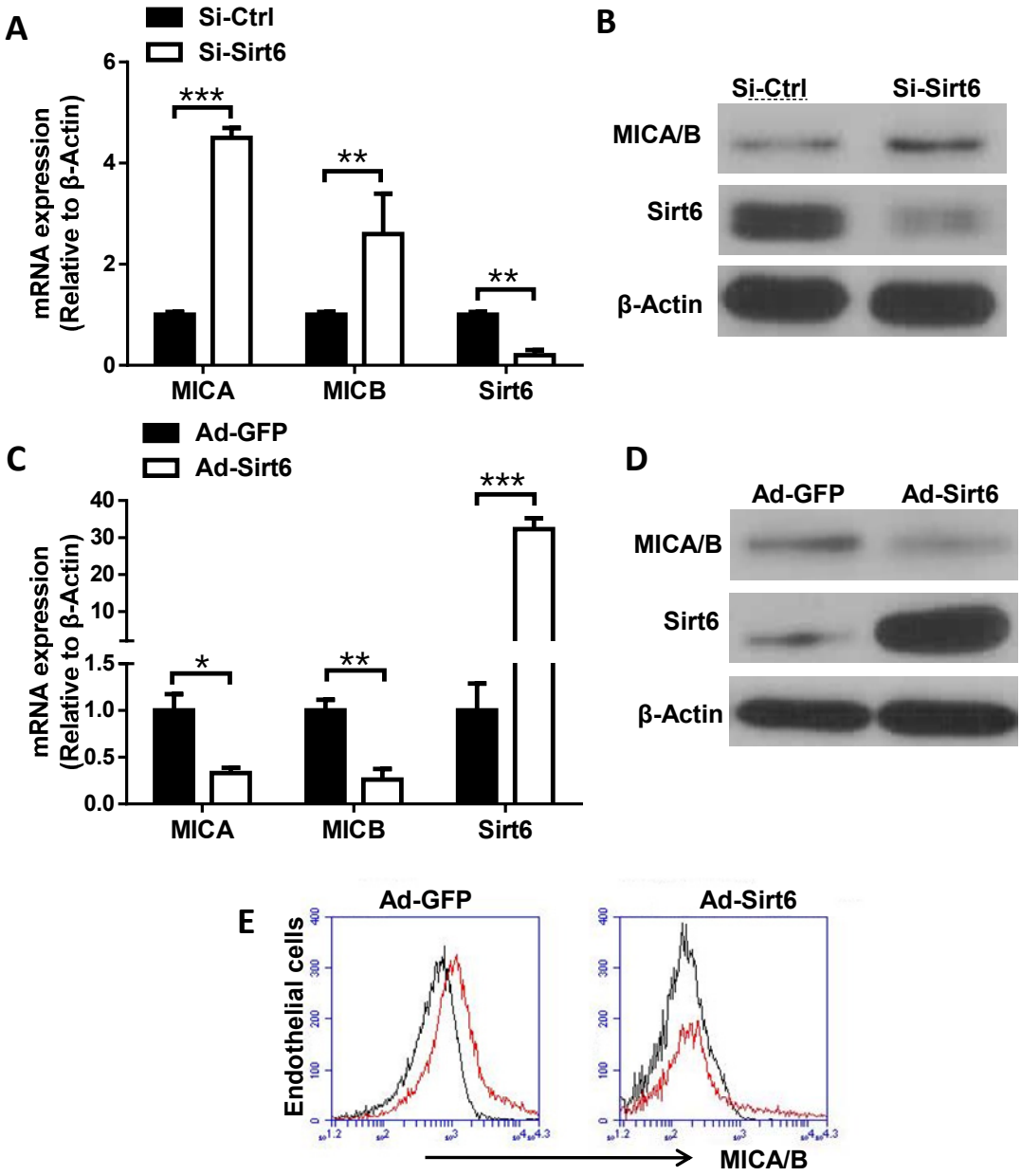
Supplementary Figure 2. Representative photographs showing Oil Red O staining of aortas of $ApoE^{-/-}$ mice and $Sirt6^{+/-}ApoE^{-/-}$ mice (n=5 each group) fed with normal chow diet (ND).



Supplementary Figure 3. Body weight of $ApoE^{-/-}$ mice and $Sirt6^{+/-}ApoE^{-/-}$ mice which were fed with Western diet for 16 weeks (n=15 each group at each time point). P value was obtained by two-way analysis of variance (ANOVA) plus a *post hoc* analysis using the Bonferroni test. (*P<0.05)

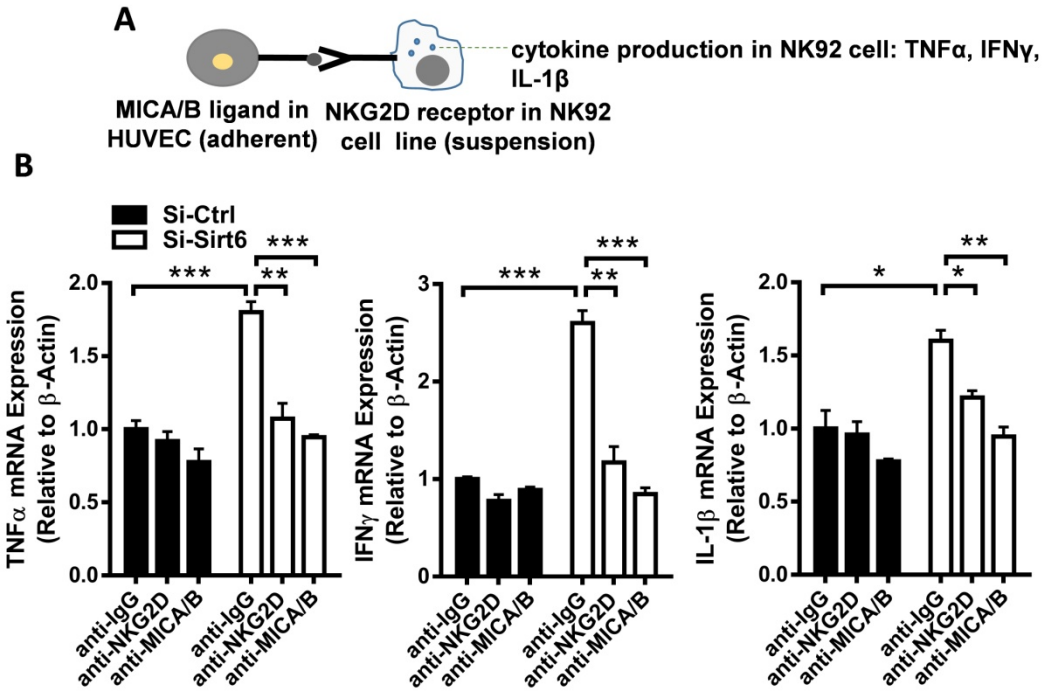


Supplementary Figure 4. ApoE$^{-/-}$ mice and Sirt6$^{+/-}$ApoE$^{-/-}$ were fed with Western diet for 16 weeks. Expression levels for NKG2D ligands in aortas were determined (n=4). Student's t test was applied to calculate the P value. (***P<0.005)

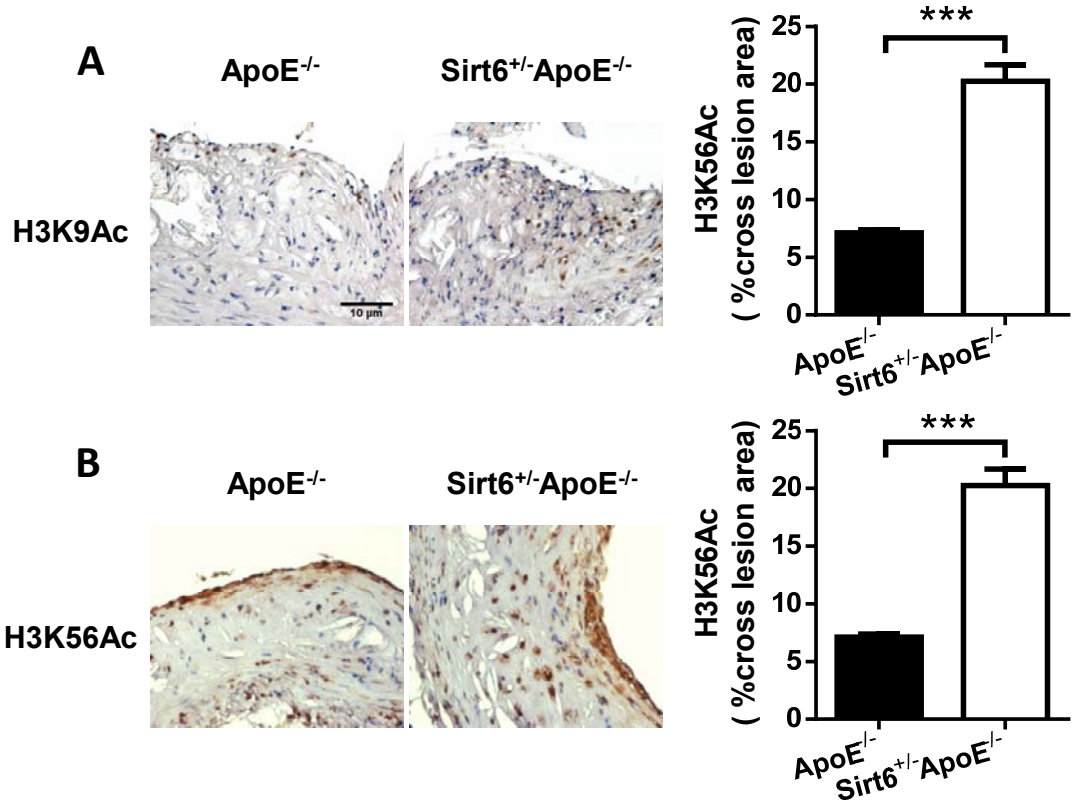


Supplementary Figure 5. Sirt6 inhibits MICA/B ligands expression in HUVECs. (A-B) HUVECs were transfected with Si-Ctrl or Si-Sirt6 and mRNA (A) and protein (B) levels of MICA/B were determined. (C-D) HUVECs were infected with Ad-GFP or Ad-Sirt6, mRNA(C) and protein (D) levels of MICA/B were determined. (E) HUVECs were infected with Ad-GFP or Ad-Sirt6, MICA/B quantity was determined by flow cytometry. Student's t test was applied to calculate the P value. (*P<0.05,

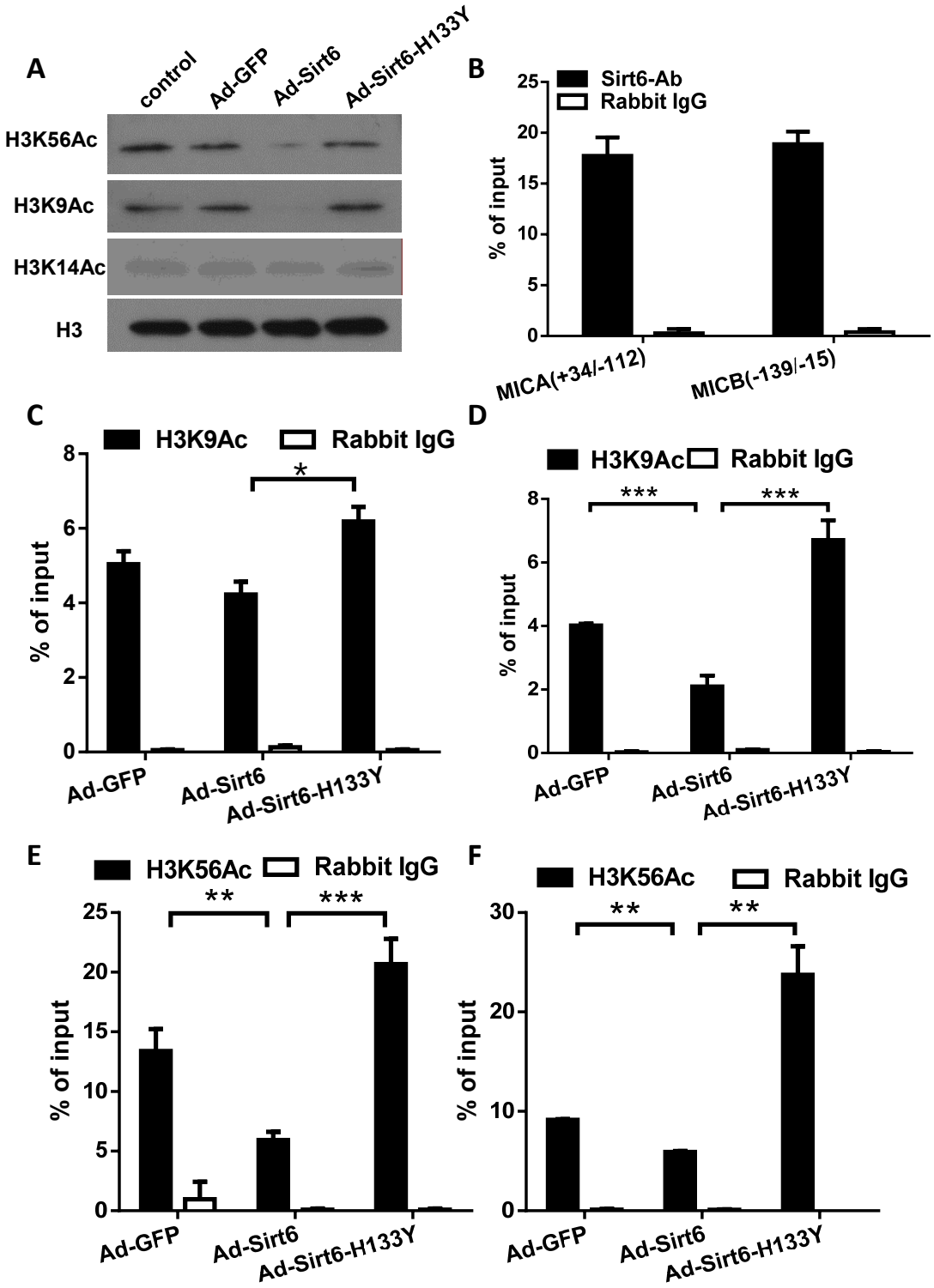
**P<0.01, ***P<0.005)



Supplementary Figure 6. MICA/B mediates increased cytokine expression in NK92 cell by Sirt6 downregulation. (A) Schematic diagram of the experimental process. Adherent HUVECs were transfected with Si-Ctrl or Si-Sirt6. 24 hours later, cells were treated with 30 μg/ml ox-LDL for 24 hours, then cocultured with NK92 cells at 1:10 ratio for 6 hours. The NK92 cells were collected and used for determination of cytokine expression by realtime PCR. (B) Experiments were performed as in (A). To block ligand-receptor interaction, isotype IgG antibody or NKG2D antibody (R&D, BAM1547, 3 μg/ml) or MICA/B antibody (R&D, MAB13001, 3 μg/ml) were added for 2 hours before co-incubation with NK92 cells. P value was obtained by two-way analysis of variance (ANOVA) plus a *post hoc* analysis using the Bonferroni test. (*P<0.05,**P<0.01, ***P<0.001)



Supplementary Figure 7. H3K9Ac and H3K56Ac levels were increased in Sirt6 heterozygous atherosclerotic plaques. (A-B) Representative immunostaining was performed to detect H3K9Ac (A) and H3K56Ac (B) levels in atherosclerotic plaques of $ApoE^{-/-}$ mice and $Sirt6^{+/-}ApoE^{-/-}$ mice fed with Western diet for 16 weeks. The indicated area was normalized to total cross lesion area and the statistical analysis is shown on the right side (n=6 each group). Student's t test was used to calculate the P value in A and B. (***P<0.005)



Supplementary Figure 8. Sirt6 binds to the promoters of MICA/B genes and deacetylates H3K9 and H3K56. (A) Total H3K9Ac and H3K56Ac in Ad-GFP, Ad-Sirt6 and Ad-Sirt6-H133Y infected HUVECs. (B) ChIP assays for Sirt6 binding to

the MICA/B promoters in HUVECs. (C-F) HUVECs were infected with Ad-GFP, Ad-Sirt6 or Ad-Sirt6-H133Y. The H3K9Ac levels in MICA (C) and MICB (D) and H3K56Ac levels in MICA (E) and MICB (F) gene promoters were determined by ChIP assays. P value was obtained by two-way analysis of variance (ANOVA) plus a *post hoc* analysis using the Bonferroni test. (*P<0.05, **P<0.01, ***P<0.001)