Perhexiline, a KLF14 activator, reduces atherosclerosis by modulating apoA-I production

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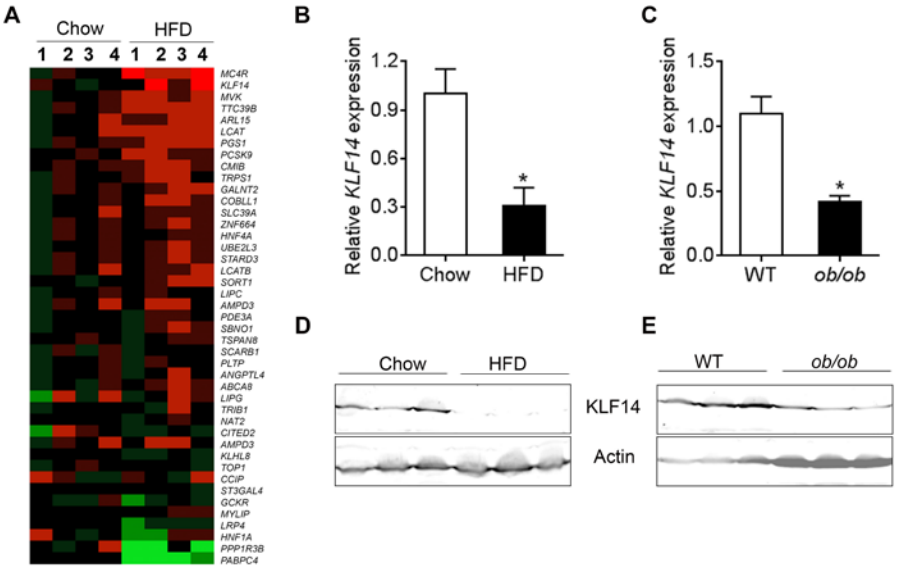
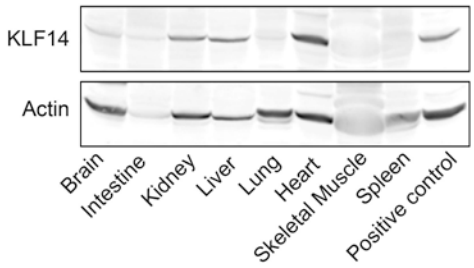
Supplemental Figure 1



Supplemental Figure 1

Hepatic KLF14 expression is reduced in dyslipidemia mouse models. (**A**) Heat map of replicate experiments displays the HDL-C trait related gene expression in livers from C57BL/6 mice fed chow diet or HFD for 12 weeks. Expression of genes was determined by qRT-PCR and normalized with 18S RNA. Primer pairs for screen are shown in Supplemental Table. (**B** and **C**) *Klf14* expression in liver from C57BL/6 mice fed chow diet or HFD for 12 weeks or wild-type or *ob/ob* mice, respectively, was determined by real-time qRT-PCR and normalized to 18S RNA (n = 4). (**D** and **E**) Hepatic KLF14 and GAPDH levels were determined in livers from the indicated animals by Western-blot (n = 3). *, $p < 0.05$, Student's t test. Chow, chow diet; HFD, high fat diet; WT, wild type C57BL/6 mice; *ob/ob* mice, leptin-deficient mice.

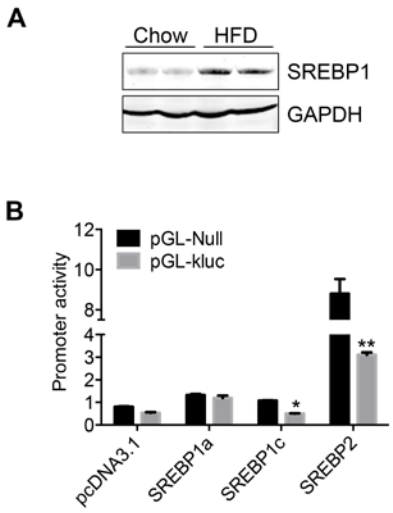
Supplemental Figure 2



Supplemental Figure 2

Expression of KLF14 in mouse tissues. Expression of KLF14 was detected by Western-blot using whole-tissue lysates from wild-type C57BL/6 adult mice. Total cell lysates from HepG2 cells transfected with Ad*KLF14* were used as a positive control.

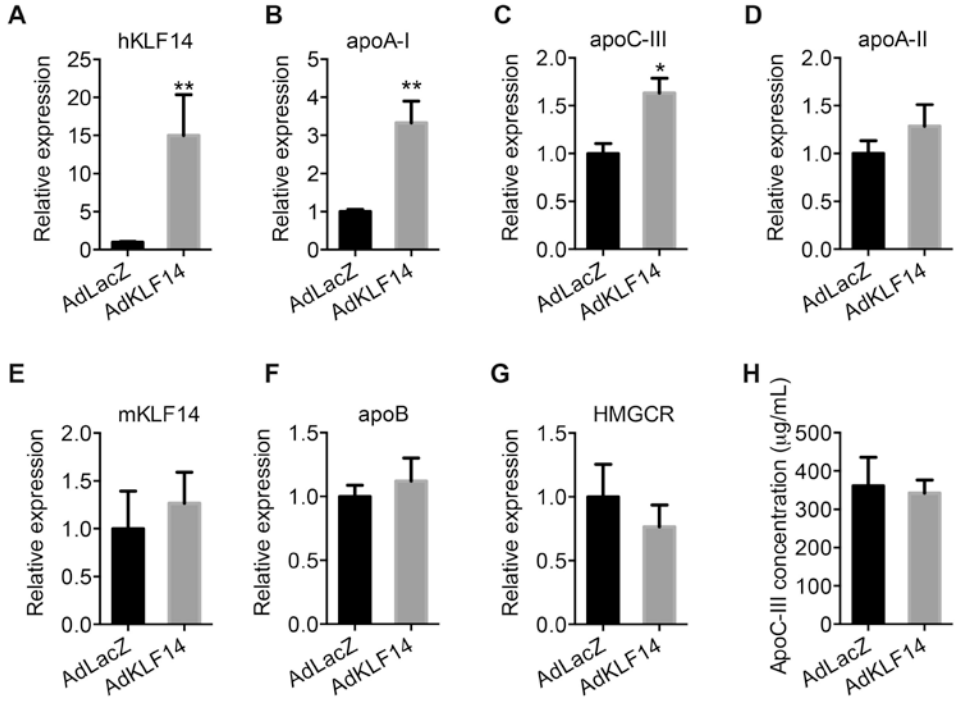
Supplemental Figure 3



Supplemental Figure 3

SREBPs inhibit the activation of *Klf14*. (**A**) The expression of SREBP1 was detected in the livers from C57BL/6 mice fed chow diet or HFD for 12 weeks by Western Blot. Chow, chow diet; HFD, high fat diet. (**B**) Luciferase activity of reporters was analyzed in HepG2 cells cotransfected with *KLF*-luc and pcDNA3.1-SREBP1a, pcDNA3.1-SREBP1c or pcDNA3.1-SREBP2 constructs after 24 hours. **, $p < 0.01$. Two-way ANOVA and Multiple comparisons. Values represent mean $\pm$ SEM; n = 3.

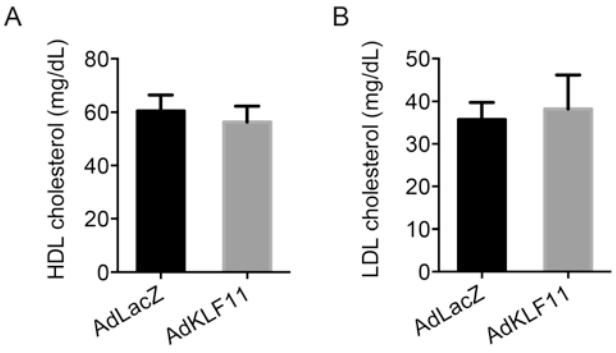
Supplemental Figure 4



Supplemental Figure 4

Effects of KLF14 overexpression on the expression levels of genes involved in lipoprotein metabolism *in vivo*. Adenovirus containing LacZ (AdLacZ) or human *KLF14* (Ad*KLF14*) (5×10^8 pfu per mouse) were administered via tail vein injection in C57BL/6 mice previously fed HFD for 12 weeks (n=10). Six days post-injection, liver samples of those animals were used in qRT-PCR to determine mRNA expression of lipoprotein metabolism genes including human *KLF14* (**A**), *ApoA-I* (**B**), *ApoC-III* (**C**), *ApoA-II* (**D**), mouse *Klf14* (**E**), *ApoB* (**F**), *HMGCR* (**G**). Data are expressed relative to 18S RNA as mean ± SEM. *, $p< 0.05$; **, $p< 0.01$, Student's *t* test. Results were replicated in one or more independent experiments. apoA-I, Apolipoprotein A-I; apoA-II, Apolipoprotein A-II; apoC-III, Apolipoprotein C-III; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase. (**H**) The serum apoC-III levels were determined by ELISA (n=10).

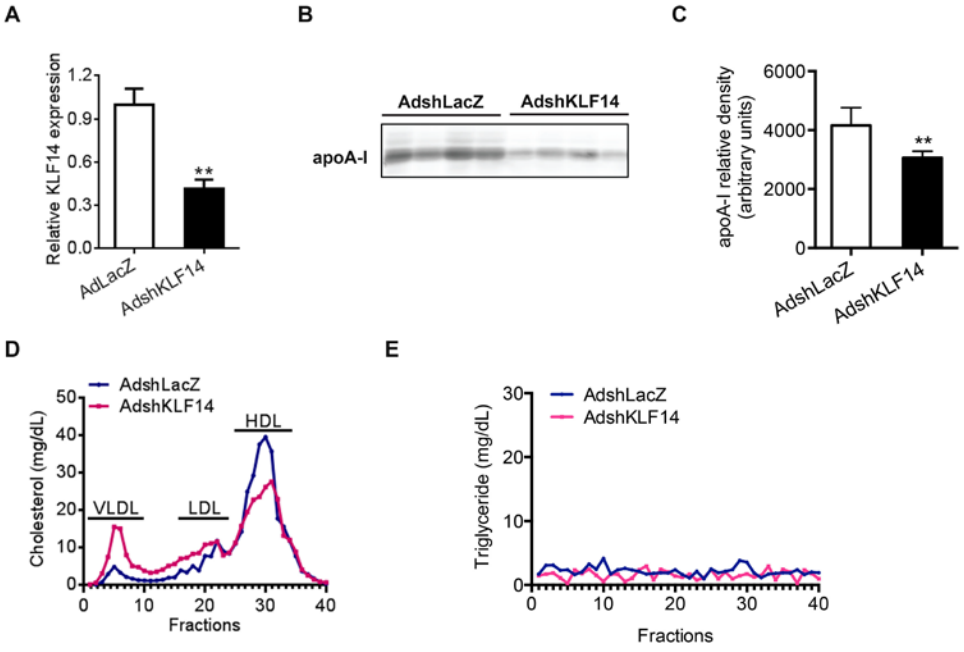
Supplemental Figure 5



Supplemental Figure 5

Overexpression of *KLF11* does not regulate HDL-C and LDL-C levels in vivo. Adenoviral vectors containing LacZ (AdLacZ) or human *KLF11* (Ad*KLF11*) (5×10^8 pfu per mouse) were administered via tail vein injection to C57BL/6 mice fed HFD for 12 weeks. Plasma samples were collected at day 6 and subjected individually to analytical chemistry to measure HDL-C (**A**) and LDL-C (**B**) levels. Values represent mean ± SEM. n = 6 per group.

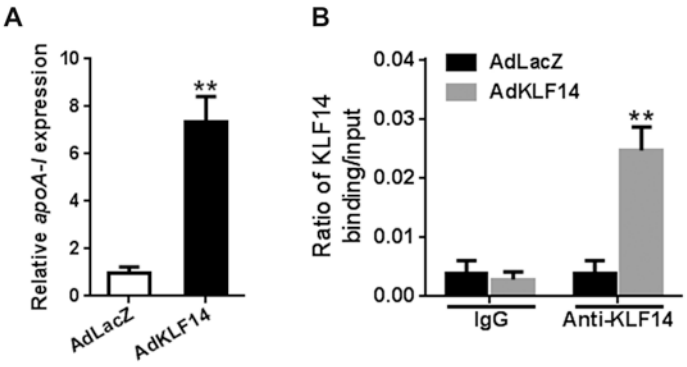
Supplemental Figure 6



Supplemental Figure 6

Adenoviral vectors containing shRNA-LacZ (AdshLacZ) or shRNA-KLF14 (AdshKLF14) (1×10^9 pfu per mouse) were administered via tail vein injection to C57BL/6 mice fed HFD for 12 weeks. Serum samples were collected at day 6. (**A**) *KLF14* mRNA levels were determined by quantitative real-time PCR. Values represent mean ± SEM. **, $p < 0.01$, Student's *t* test. (**B** and **C**) Western blot analysis of apoA-I in 3 µL of serum samples from the mice injected with AdshLacZ or AdshKLF14. **, $p < 0.01$, Student's *t* test. (**D** and **E**) Serum samples collected at day 6 after AdshLacZ or AdshKLF14 injection were pooled and the lipid profile was analyzed by FPLC followed by measurement of cholesterol levels in the fractions.

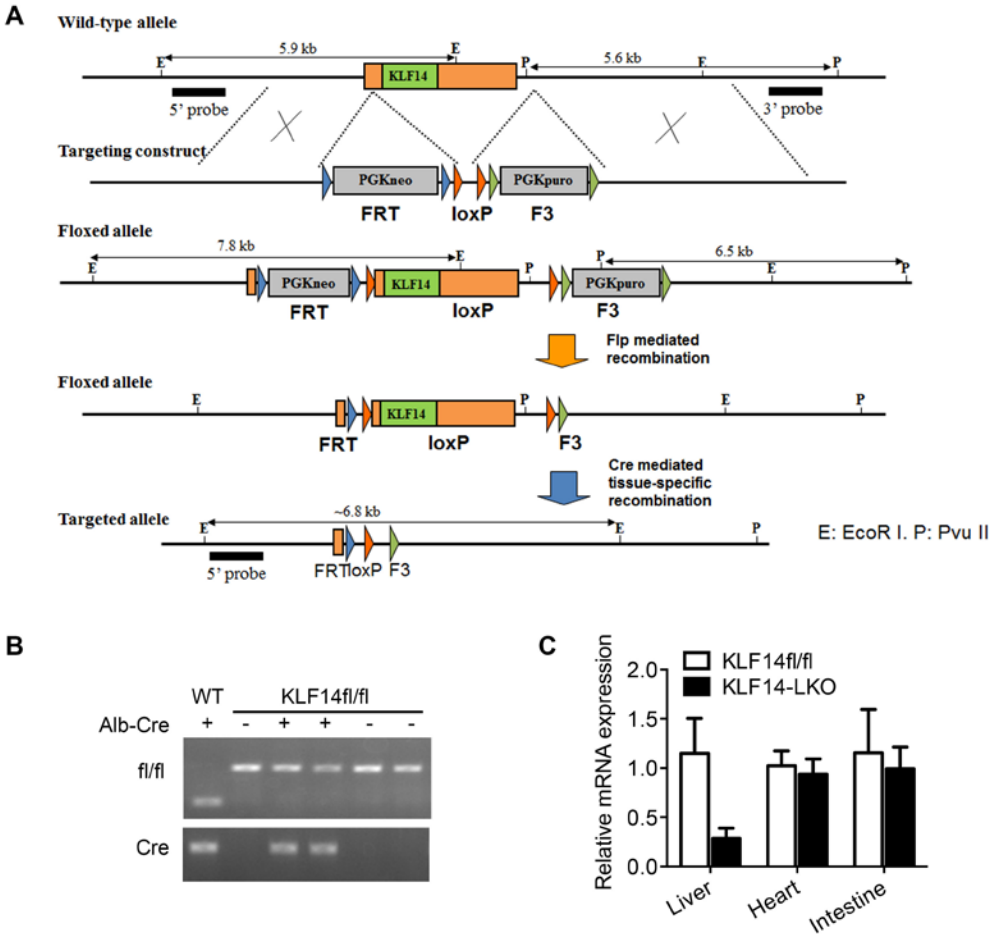
Supplemental Figure 7



Supplemental Figure 7

KLF14 regulates the transcription of mouse *ApoA-I*. (**A**) Primary hepatocytes from C57BL/6 mice were infected with AdLacZ or AdKLF14 for 24h and *APOA-I* mRNA levels were determined by quantitative real-time PCR. Values represent mean ± SEM. **, $p < 0.01$, Student's *t* test. (**B**) ChIP assay revealed significant enrichment of KLF14 protein on the mouse *ApoA-I* promoter in primary hepatocytes isolated from C57BL/6 mice. Values represent mean ± SEM. n = 3. **, $p < 0.01$, Two-way ANOVA and Multiple comparisons.

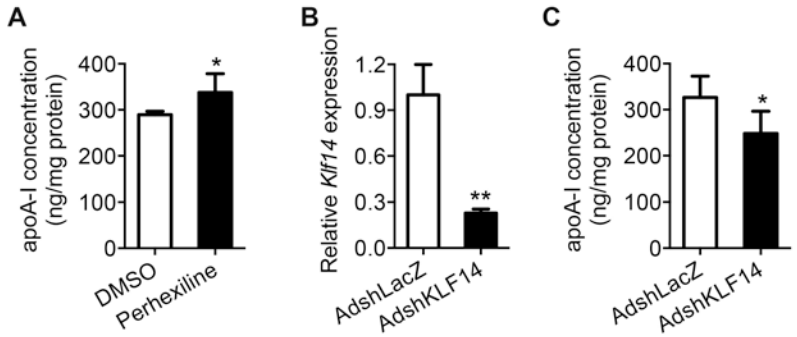
Supplemental Figure 8



Supplemental Figure 8

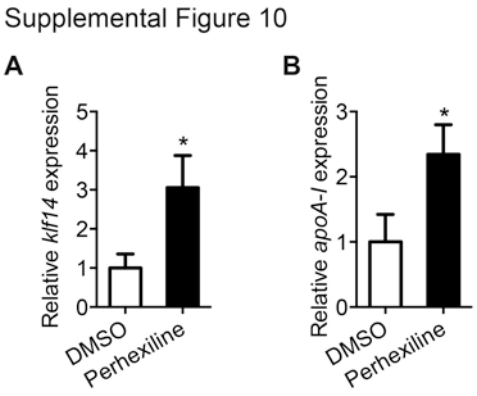
Generation of liver specific knockout of *Klf14* in mice. (**A**) Strategy for conditional disruption of the *Klf14* gene. The wild-type *Klf14* gene is shown in the upper line. For conditional gene targeting, the only exon was flanked by loxP sites (triangles). Homologous recombination, subsequent Flp-mediated removal of the frt-flanked neo, and Cre-mediated deletion of the *Klf14* gene is outlined below. (**B**) Genotyping of mice harboring wild-type (WT), loxP flanked (floxed, KLF14fl/fl) and *Alb*-Cre alleles. (**C**) qRT-PCR analysis revealed strong reduction of *Klf14* mRNA levels in the liver from KLF-LKO mice, but not the heart and intestine tissues. Values represent mean ± SEM. n = 3.

Supplemental Figure 9



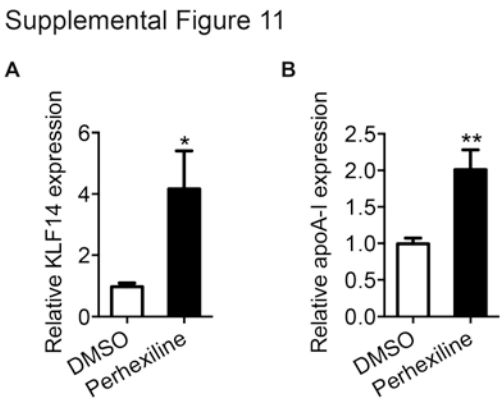
Supplemental Figure 9

(**A**) HepG2 cells were incubated with 10 μM perhexiline for 24 hours in DMEM containing 0.2% BSA. The apoA-I concentrations in the medium were detected by ELISA. Values represent mean ± SEM; n = 6. *, $p < 0.05$. (**B**) HepG2 cells were infected with AdshLacZ or AdshKLF14 for 48 hours and the knockdown efficiency of *Klf14* was detected by qRC-PCR. Values represent mean ± SEM; n = 3. **, $p < 0.01$, Student's *t* test. (**C**) HepG2 cells were infected with AdshLacZ or AdshKLF14 for 72 hours and then changed to DMEM containing 0.2% BSA. The apoA-I concentrations in the medium were detected by ELISA. Values represent mean ± SEM; n = 6. *, $p < 0.05$, Student's *t* test.



Supplemental Figure 10

Perhexiline upregulates KLF14 and apoA-I expression in Caco2 cells. qRT-PCR analysis showing the expression levels of *Klf14* (**A**) and *ApoA-I* (**B**) in Caco2 cells in the presence of perhexiline (10 μM) or DMSO for 30 hours. Data are expressed relative to 18S RNA. Values represent mean ± SEM; n = 3. *, $p < 0.05$, Student's *t* test.



Supplemental Figure 11

C57BL/6J mice placed on HFD for 12 weeks were treated with DMSO or perhexiline maleate salt (10mg/Kg/day) for five consecutive days by gavage administration and samples collected at day 7 (n = 10 per group). Total RNA was isolated from liver and the expression of *Klf14* (**A**) and *ApoA-I* (**B**) were determined qRT-PCR. Values represent mean ± SEM. *, $p < 0.05$; **, $p < 0.01$, Student's *t* test.

Supplemental Table 1

Compound Name	Fold change
DMSO	1.0
Amphotericin B	9.4
Perhexiline Maleate	5.1
Flufenazine Hydrochloride	5.1
Estradiol Cypionate	3.3
Gentian Violet	3.1
Acriflavinium Hydrochloride	3.1
Pyrithione Zinc	2.8
Nystatin	2.2

KLF14-luc-transfected 293 cells were used for high-throughput screening of a chemical library of NIH/JDRF Custom Collection including 1040 compounds. The luciferase activities were measured 24h after compound treatment. From the primary screening, 8 compounds were identified that activate KLF14-luc activity 2-fold or more.

Supplemental Table 2

Table S2. Primers used for real-time qRT-PCR

Gene*	Name	FP	RP
18S	18S RNA	ggaagggcaccaccaggagt	tgcagccccggacatctaag
mKLF14	kruppel-like factor 14	cctcaagtcacaccagcgta	cgacctcggtactcgatcat
mapoA-I	apolipoprotein A-I	gtggctctggtcttcctgac	acggttgaacccagagtgtc
mapoA-II	apolipoprotein A-II	ttgatggagaaggccaagac	cggtttctcctcaaggttca
mApoC-III	apolipoprotein C-III	acatggaacaagcctccaag	tggttggtcctcagggttag
mABCA1	ATP-binding cassette transporter 1	gggagtcccagaaaaggaag	tgtggttggttcatccagaa
mABCG1	ATP-binding cassette sub-family G member 1	gtaccatgacatcgctggtg	agccgtagatggacaggatg
mLDLR	low density lipoprotein receptor	gaggagcagccacatggtat	gctcgtcctctgtggtcttc
mSR-BI	scavenger receptor class B member 1	attcccacgtatcgcttcac	gctcctttgggttagggttc
hapoA-I	apolipoprotein A-I	tggatgtgctcaaagacagc	aggccctctgtctccttttc
hapoA-II	apolipoprotein A-II	gagctttggttcggagacag	tgtgttccaagttccacgaa
hKLF14	kruppel-like factor 14	tacaagtcgtcgcacctcaa	gtccccggtactcgatcata
mMC4R	melanocortin receptor 4	tcatctgtagcctggctgtg	ggtactggagcgcgtaaaag
mMVK	mevalonate kinase	gaagcaggctgaccaagttc	cagatggtgctggttcatgt
mANGPTL 4	angiopoietin-like 4	tccaatttcccatccatttg	ggctcttggcacagttaagg

mNAT2	arylamine N-acetyltransferase	ctgggctttgaaaccacaat	ctgaggctgatcctttccag
mSORT1	sortilin 1	caggagacaaatgccaaggt	ccttccgccacagacatatt
mTRIB1	tribbles homolog 1	gaggtgctccttggtgagag	tcggtggagaagacgaactt
mHNF1a	hepatocyte nuclear factor 1-alpha	tcacagacaccaacctcagc	gaggacactgtgggactggt
mPCSK9	proprotein convertase subtilisin/kexin type 9	tccattgggaagtggaagac	acctgctctgaaggacctga
mGCKR	glucokinase (hexokinase 4) regulator	cagcgtgagttaagcaccaa	tcagtgatggagcacctgag
mLIPC	lipase, hepatic	tctcggagcaaagttcacct	tatgaatggcgtccacaaaa
mLIPG	lipase, endothelial	cttccagtgcacagactcca	gggtgtccccactgttattg
mTOP1	topoisomerase (DNA) I	gccaaggtgttccgtaccta	cccttcgagcatctgctaac
mMYLIP	myosin regulatory light chain interacting protein	tagagtggcatgctgtgagg	ctccttggtgacggtcaagt
mST3GAL 4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4	cgatggacttccactggatt	gcagaggtgtagagccaagg
mCOBLL1	cordon-bleu WH2 repeat protein-like 1	ctgtgccacaagcacagatt	ctggcgatgctgttagatga
mKLHL8	kelch-like family member 8	tgggtgtgatctctgtggaa	tctccacgtcactgaagcac
mTSPAN8	tetraspanin 8	ctggccatatgggtgagagt	tttcacagctccacagcatc
mPABPC4	poly(A) binding protein, cytoplasmic 4	ccaggggtgaatctctaca	tcatctcggtgacagctttg
mLCAT	lecithin-cholesterol	aaagaggagcagcgcataac	gcccacaccgtagagacaat

	acyltransferase		
mSLC39A	Zinc transporter ZIP11	agcctaacggacacatccac	agtacaagatgccccaatcg
mPPP1R3B	protein phosphatase 1, regulatory subunit 3B	tgctgaaggataaggccatc	gccgttacactcgtagcaca
mTTC39B	tetratricopeptide repeat domain 39B	acaggtggatggtctgaagc	cctcagccttctccacagtc
mSTARD3	StAR-related lipid transfer (START) domain containing 3	ggcagggaaaggaagctact	cctgatacaccagctcagca
mARL15	ADP-ribosylation factor-like 15	gttgctggctttttcaggag	aagcgctcgaaaacacagat
mPLTP	phospholipid transfer protein	aaatcagtctgcgctggagt	gcaggacggttcttgtcaat
mGALNT2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2)	ctggacaccttgggacactt	gagttgccttcgatctgctc
mPGS1	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	acgctgattggctctcctaa	ttcttgattagcggggtcac
mHNF4a	hepatic nuclear factor 4 alpha	gattgccaacatcacagacg	aggagcagcacgtccttaaa
mUBE2L3	ubiquitin-conjugating enzyme E2L 3	agcttgaagagatccgcaaa	tgtgatcttgggtggtttga
mCITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	tgggcgagcacatacactac	ggtaggggtgatggttgaaa

mTRPS1	trichorhinophalangeal syndrome I	gcccagggttcattgactaa	gggtgttttgcaggtctcat
mAMPD3	adenosine monophosphate deaminase 3	ctgcccctgttcaaagctac	agcaccatgatgttggcata
mLRP4	low density lipoprotein receptor-related protein 4	ccaagccagccgtgtataat	tgctctgtctccgtgtcatc
mPDE3A	phosphodiesterase 3A, cGMP-inhibited	gaggacgaagcctgtgaaag	ctcttggcttccccttctct
mSBNO1	strawberry notch homolog 1	accaaacactgggaagcaac	cacttttgtccagacgctca
mZNF664	zinc finger protein 664	catattcattggcgagacca	agctccagttgaaggctttg
mSCARB1	scavenger receptor class B, member 1	tcgaattctggggtcttcac	aatgccttcaaacacccttg
mLCATB	lactamase, beta	tgctgacaactgtccaggag	tcacccactgtggacagaaa
mCMIP	c-Maf inducing protein	ctgctgtccgactacgatga	cagggctgtagagctggaac
mABCA8	ATP-binding cassette, sub-family A (ABC1), member 8	caggaccagctgaagtctcc	ccctgattgcttgccatatt
mAMPD3	adenosine monophosphate deaminase 3	ctgcccctgttcaaagctac	agcaccatgatgttggcata

***** m, in front of the gene name indicates mouse; h, indicates human.

FP: Forward primer; RP: Reverse primer.

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Conflict of interest: The authors have declared that no conflict of interest exists.

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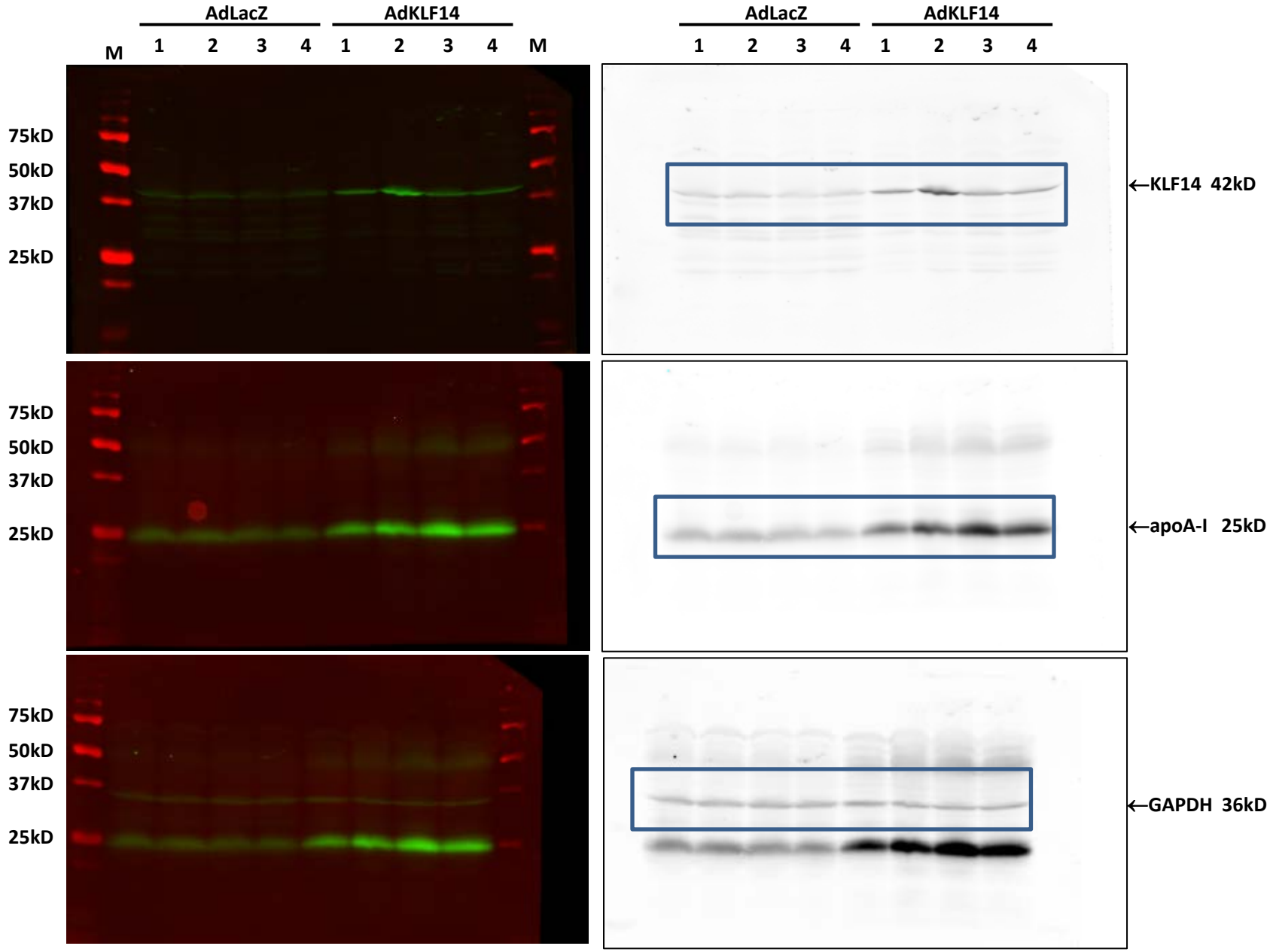

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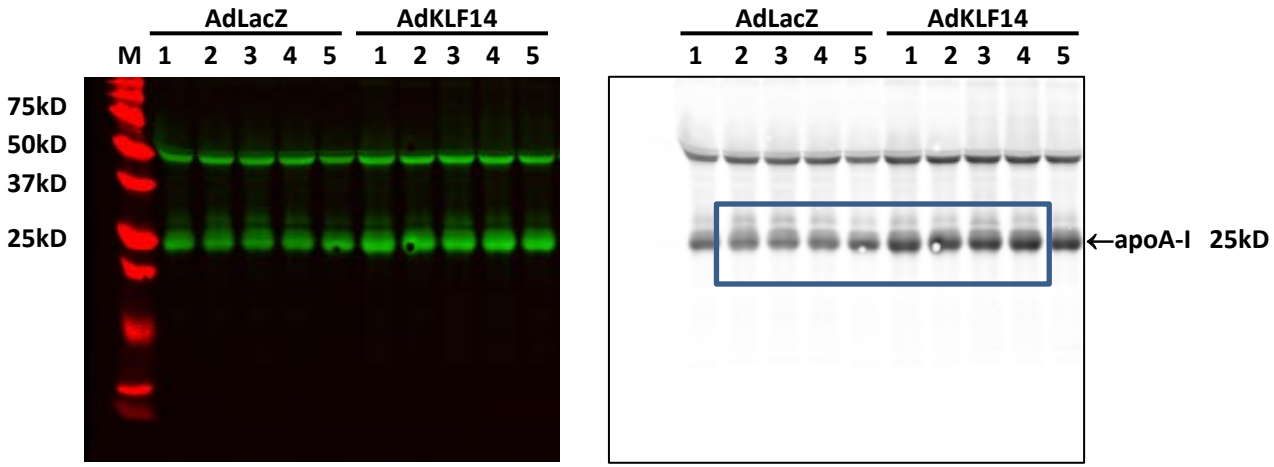

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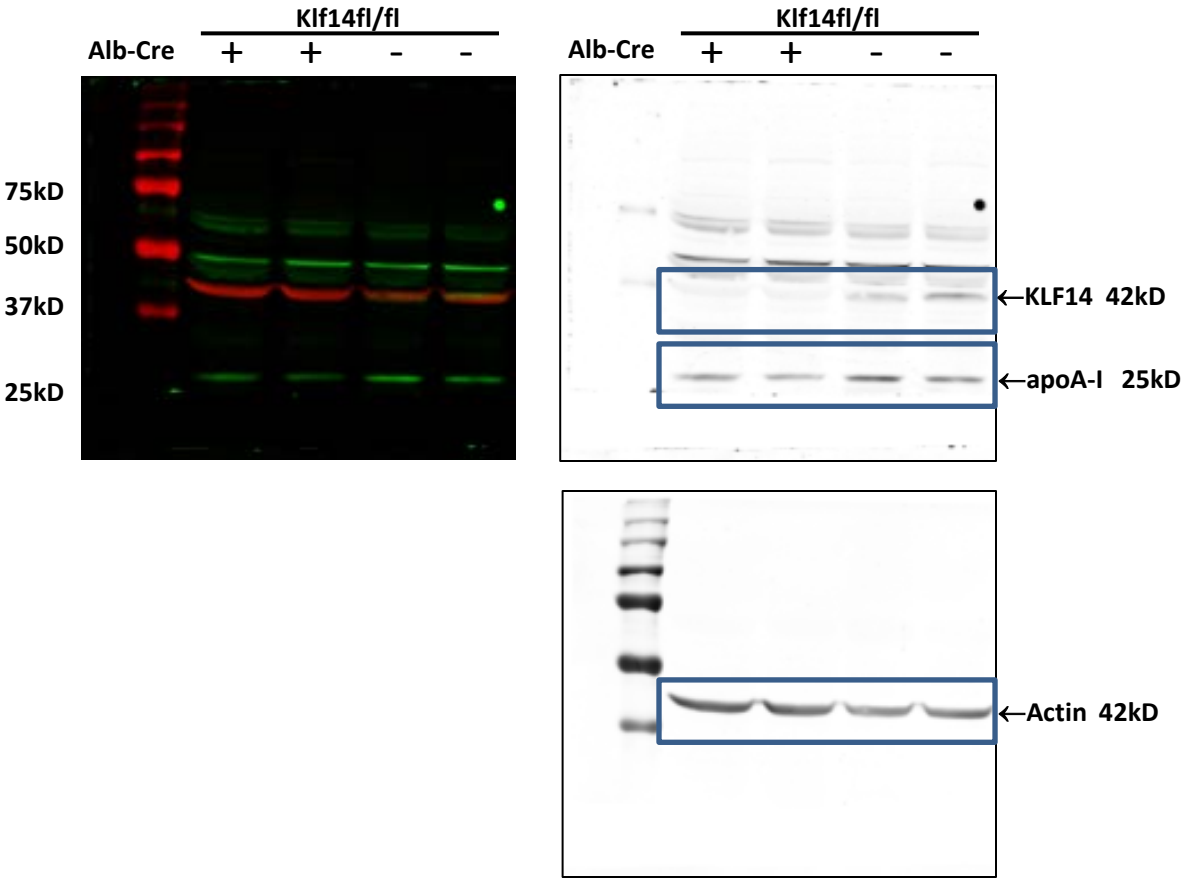

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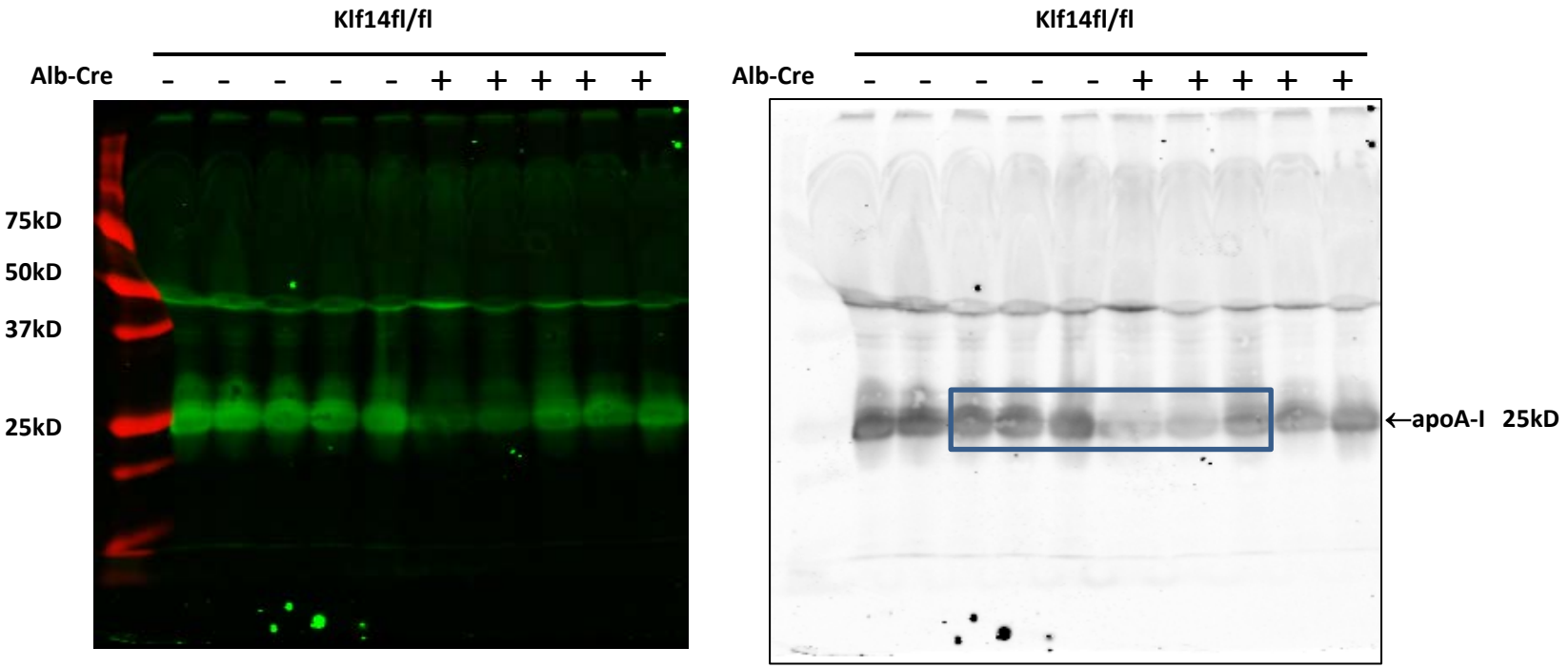

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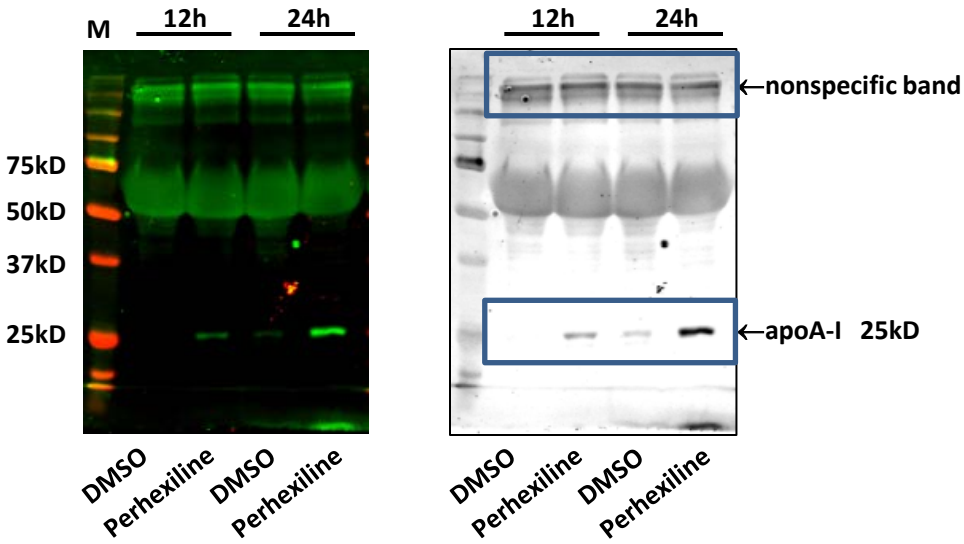

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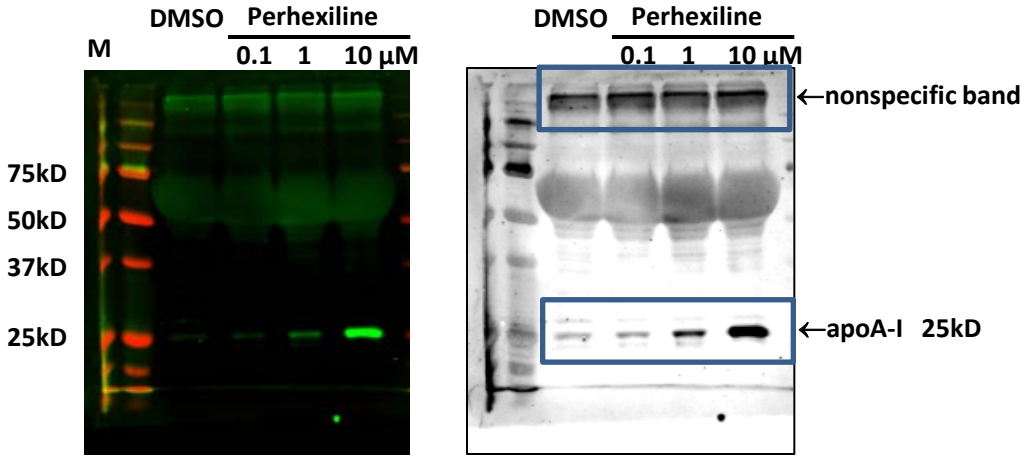

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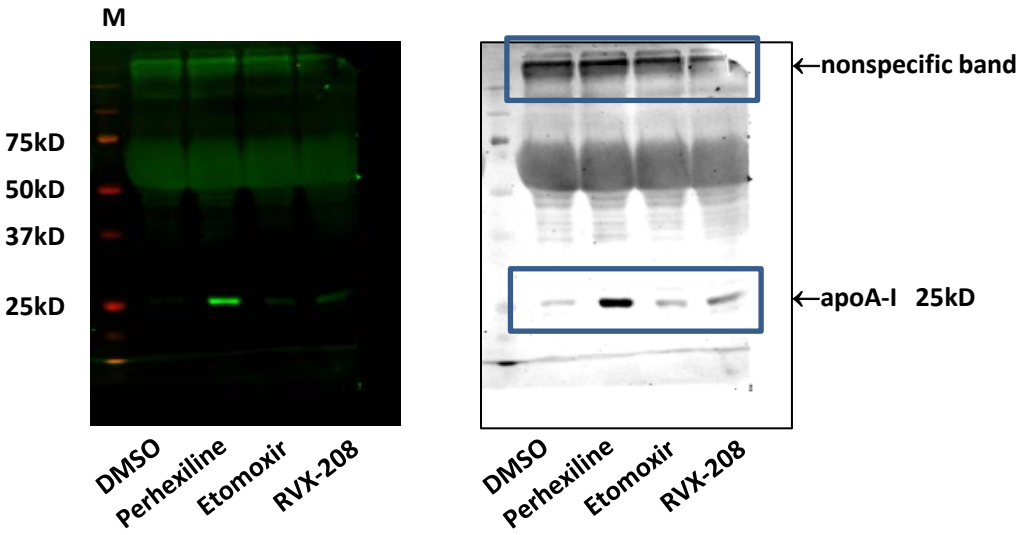

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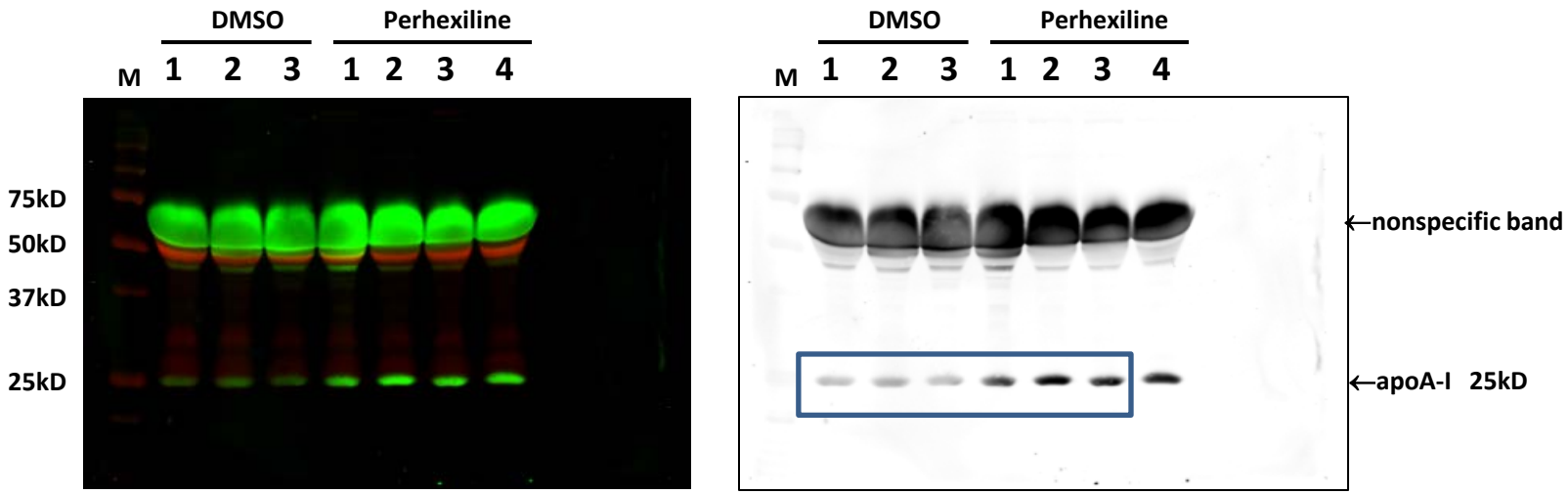

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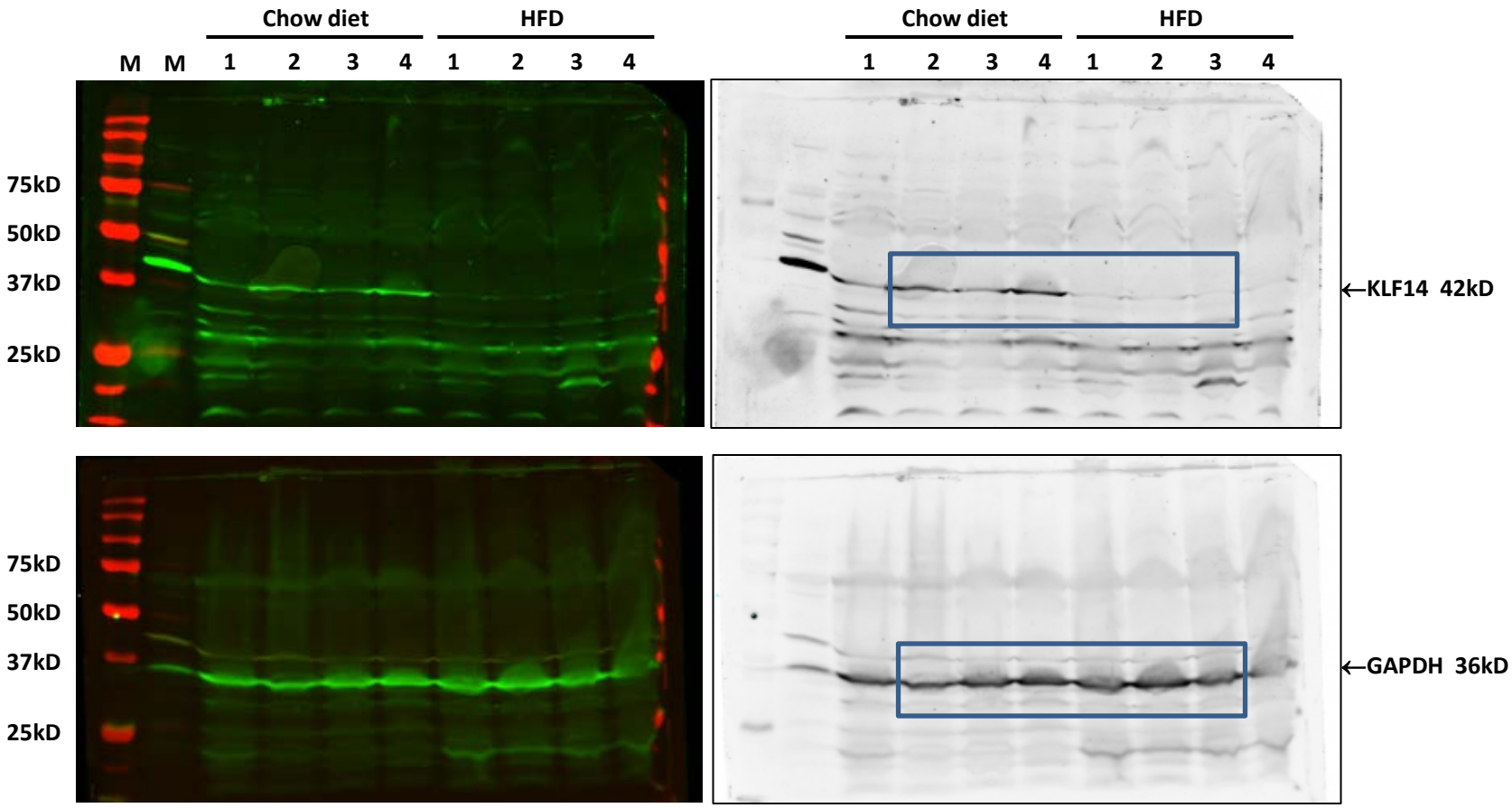
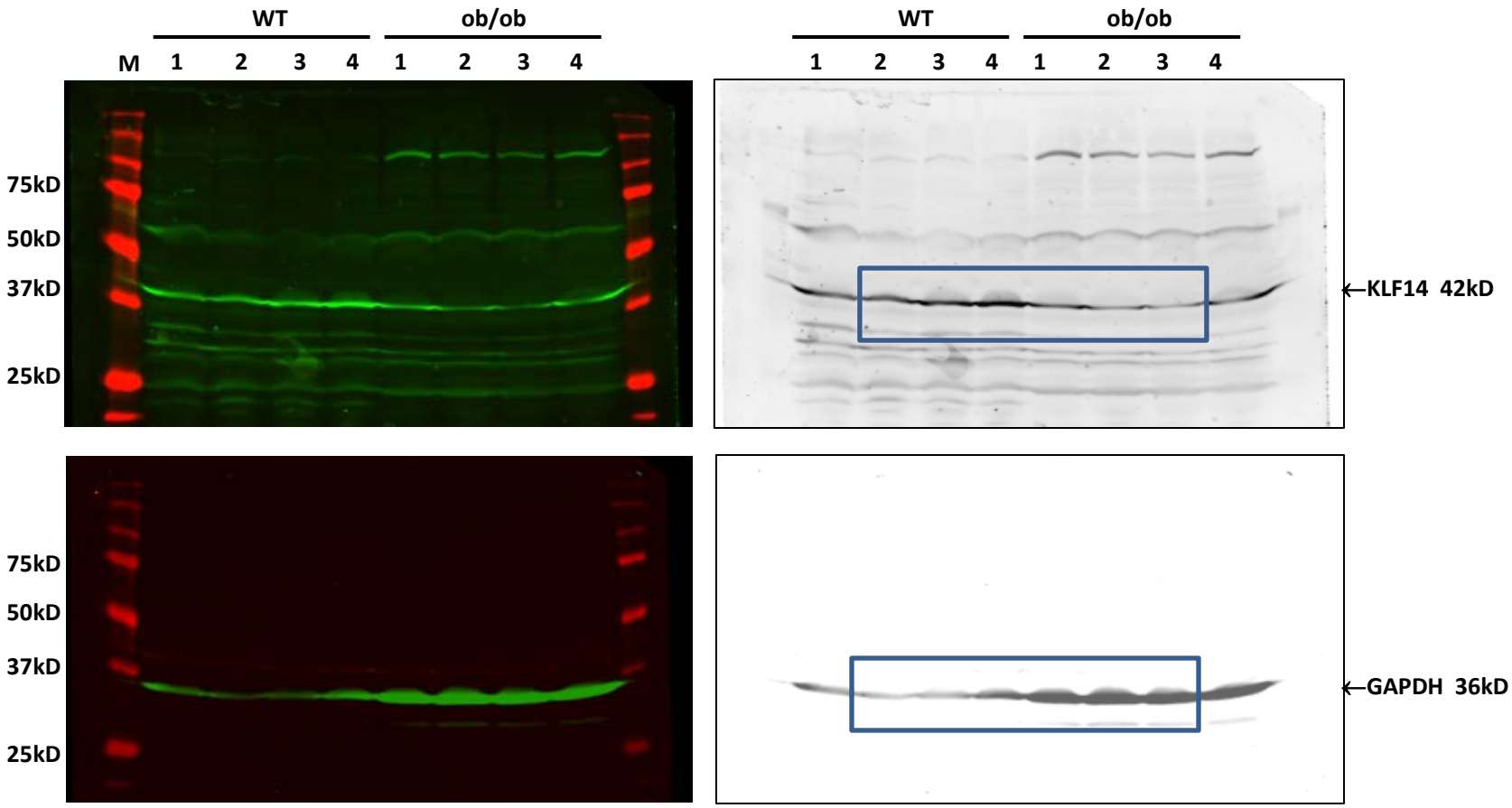
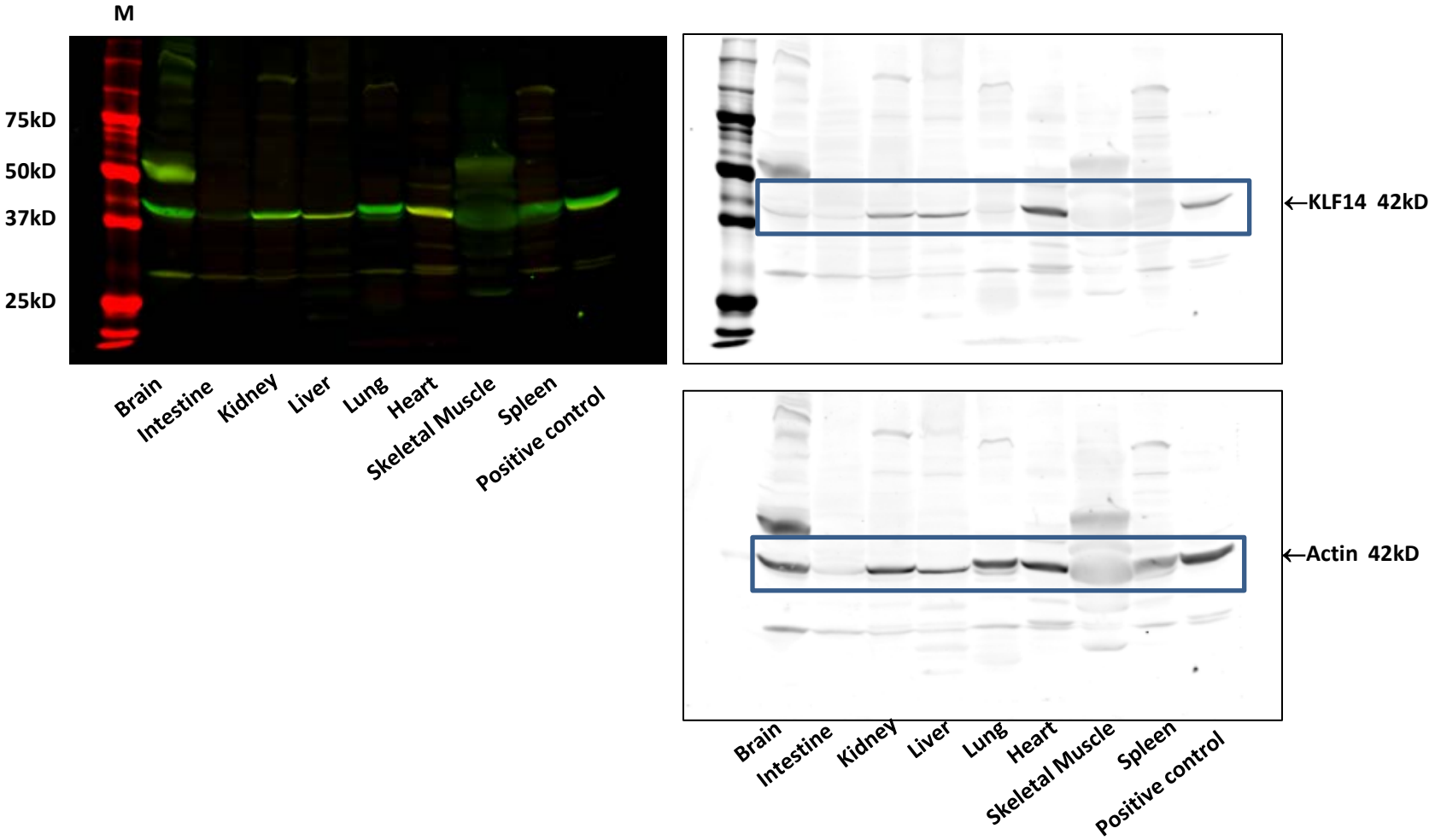


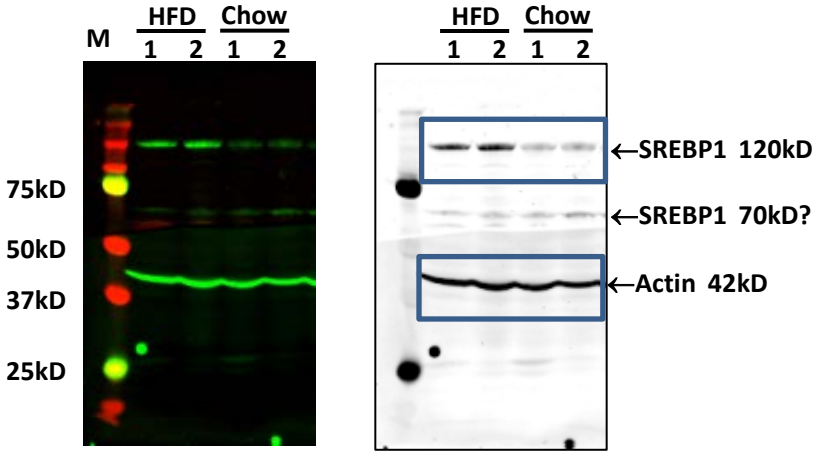
Full unedited gel for Supplemental Figure 1E



Full unedited gel for Supplemental Figure 2



Full unedited gel for Supplemental Figure 3A



Full unedited gel for Supplemental Figure 6B