Supplementary Materials for

Title: Intercepting IRE1 Kinase-FMRP Signaling Prevents Atherosclerosis Progression

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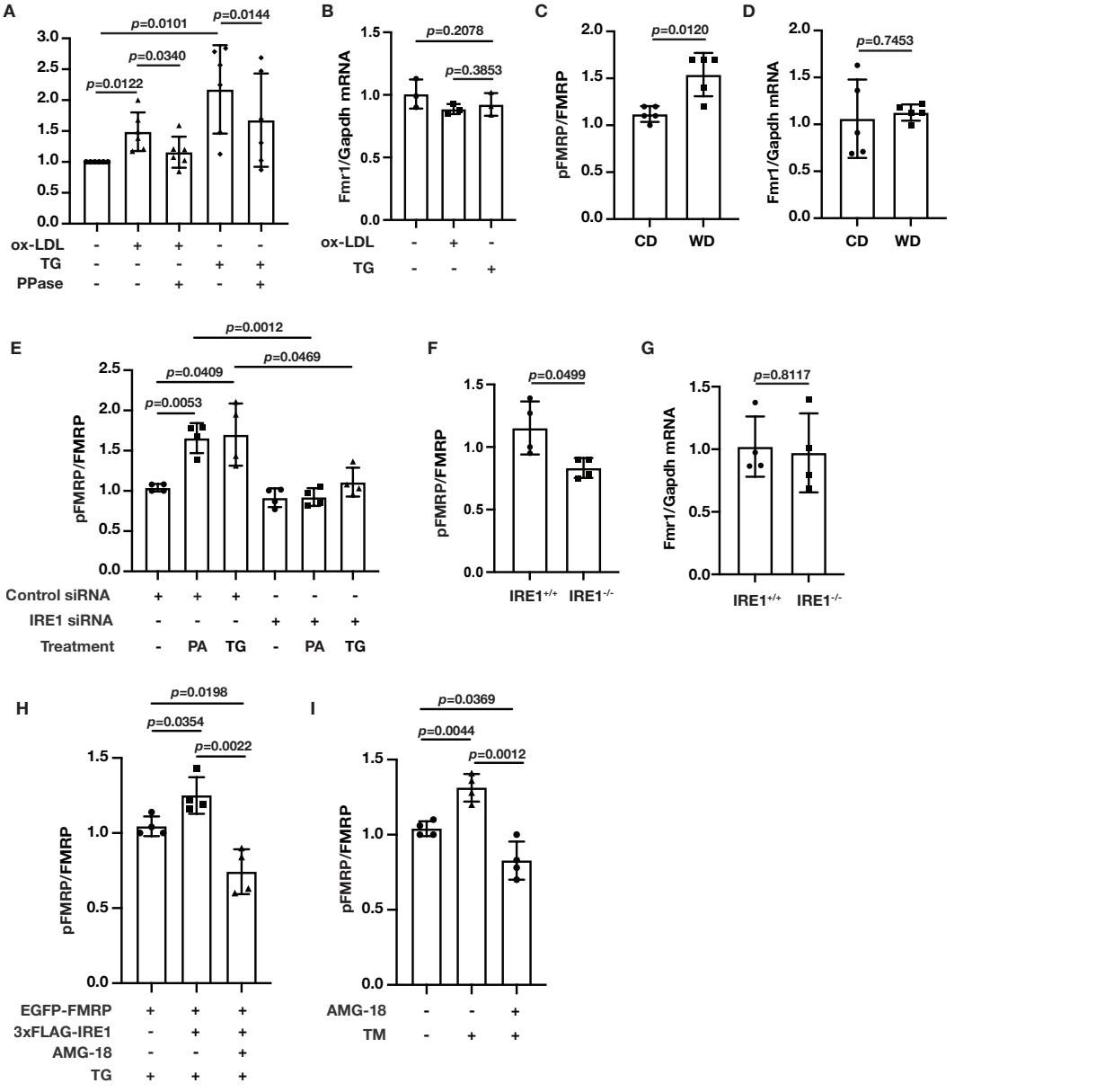
This PDF file includes:

Figures and Figure Legends for Appendix Fig.S1-S6


A
p=0.0101
p=0.0144
p=0.0340
p=0.0122
pFMRP/FMRP
ox-LDL - + + - -
TG - - - + +
PPase - - + - +
B
p=0.2078
p=0.3853
Fmr1/Gapdh mRNA
ox-LDL - + -
TG - - +
C
p=0.0120
pFMRP/FMRP
CD WD
D
p=0.7453
Fmr1/Gapdh mRNA
CD WD
E
p=0.0012
p=0.0469
p=0.0409
p=0.0053
pFMRP/FMRP
Control siRNA + + + - - -
IRE1 siRNA - - - + + +
Treatment - PA TG - PA TG
F
p=0.0499
pFMRP/FMRP
IRE1+/+ IRE1-/-
G
p=0.8117
Fmr1/Gapdh mRNA
IRE1+/+ IRE1-/-
H
p=0.0198
p=0.0354
p=0.0022
pFMRP/FMRP
EGFP-FMRP + + +
3xFLAG-IRE1 - + +
AMG-18 - - +
TG + + +
I
p=0.0369
p=0.0044
p=0.0012
pFMRP/FMRP
AMG-18 - - +
TM - + +

J

K

AA	Sequence	z	XCorr	DeltaCN	[M+H]+	A-Score
*pS*362	HLDIKEN*pS*THFSQPNSTK	3	2.8194	0.3306	2162.9890	14.23
	KHLDIKEN pSTHFSQPNSTK	3	3.2526	0.3569	2291.0808	33.7
	KHLDIKEN*pS*THFSQPNSTK	4	3.4197	0.2304	2291.0813	34.46
*pS*494	RGPGYTSGTN*pS*EASNA	2	3.0995	0.4448	1648.6614	13.27
*pS*497	RGPGYTSGTNSEA*pS*NASETESDHRDELSDWSLAPTEEERE	4	3.457	0.3924	4476.8633	7.81
*pS*500	*pS*ETESDHRDELSDW	2	3.1722	0.3710	1785.6604	22.85
	NA*pS*ETESDHRDELSDW	2	3.1225	0.3763	1970.7406	30.93
>	**NA*pS*ETESDHRDELSDW**	**2**	**4.0257**	**0.4280**	**1970.7393**	**39.56**
	NA*pS*ETESDHRDELSDW	2	3.3907	0.3348	1970.7386	33.68
*pT*502	RGPGYTSGTNSEASNASE*pT*ESDHRDELSDWSLAPTEEERE	4	6.2067	0.5107	4476.8564	4.6
*pS*504	SETE*pS*DHRDELSDW	2	3.431	0.3944	1785.6588	15.5
*pT*592	SVH*pT*KTLQNTSSEGSR	3	3.996	0.2936	1811.8281	6.17
	SVH pTKTLQNTSSEGSR	3	4.4606	0.3795	1811.8282	7.53
	SVH*pT*KTLQNTSSEGSR	3	3.5417	0.3859	1811.8276	11.79
*pT*594	SVHTK*pT*LQNTSSEGSR	3	3.1378	0.3386	1811.8282	10.36

Appendix Figure S1. Supplemental figure related to Figure 1.

A Western blot quantifications for pFMRP/FMRP ratio in Fig 1C.

B qRT-PCR analysis of Fmr1 mRNA from the samples in Fig 1C (n=4 biological replicates).

C Western blot quantifications for pFMRP/FMRP ratio in Fig 1D.

D qRT-PCR analysis for Fmr1 mRNA from the samples in Fig 1D (n=3 biological replicates).

E Western blot quantifications for pFMRP/FMRP ratio in Fig 1E.

F Western blot quantifications for pFMRP/FMRP ratio in Fig 1F.

G qRT-PCR analysis for Fmr1 mRNA from the samples in Fig 1F (n=6 biological replicates).

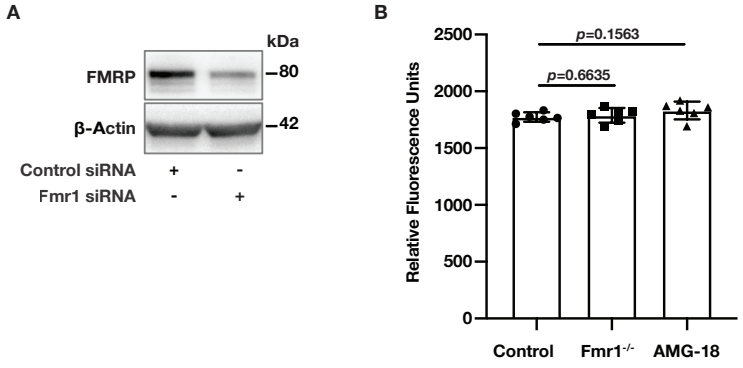
H Western blot quantifications for pFMRP/FMRP ratio in Fig 1G.

I Western blot quantifications for pFMRP/FMRP ratio in Fig 1H.

J pFMRP sequence covered (83%) with two different digestion strategies – Proteinase K (red text) and Trypsin-GluC (highlighted yellow) using LC-MS/MS in samples from Fig 1H.

K LC-MS/MS identified phosphorylated peptides from the samples in Fig 1H; with sites of phosphorylation (AA), charge state (z), identification parameters (XCorr and DeltaCN – greater values indicate higher confidence by peptide-spectrum match/PSM), measured precursor mass ([M+H]+), and a confidence score for localization of phosphorylation on an amino acid (A-Score, value > 19 and > 13, represent > 99% and > 95% confidence, respectively); the peptide marked with (>) was evaluated further.

Data information: Data are mean ± SEM. Unpaired *t*-test with Welch's correction.



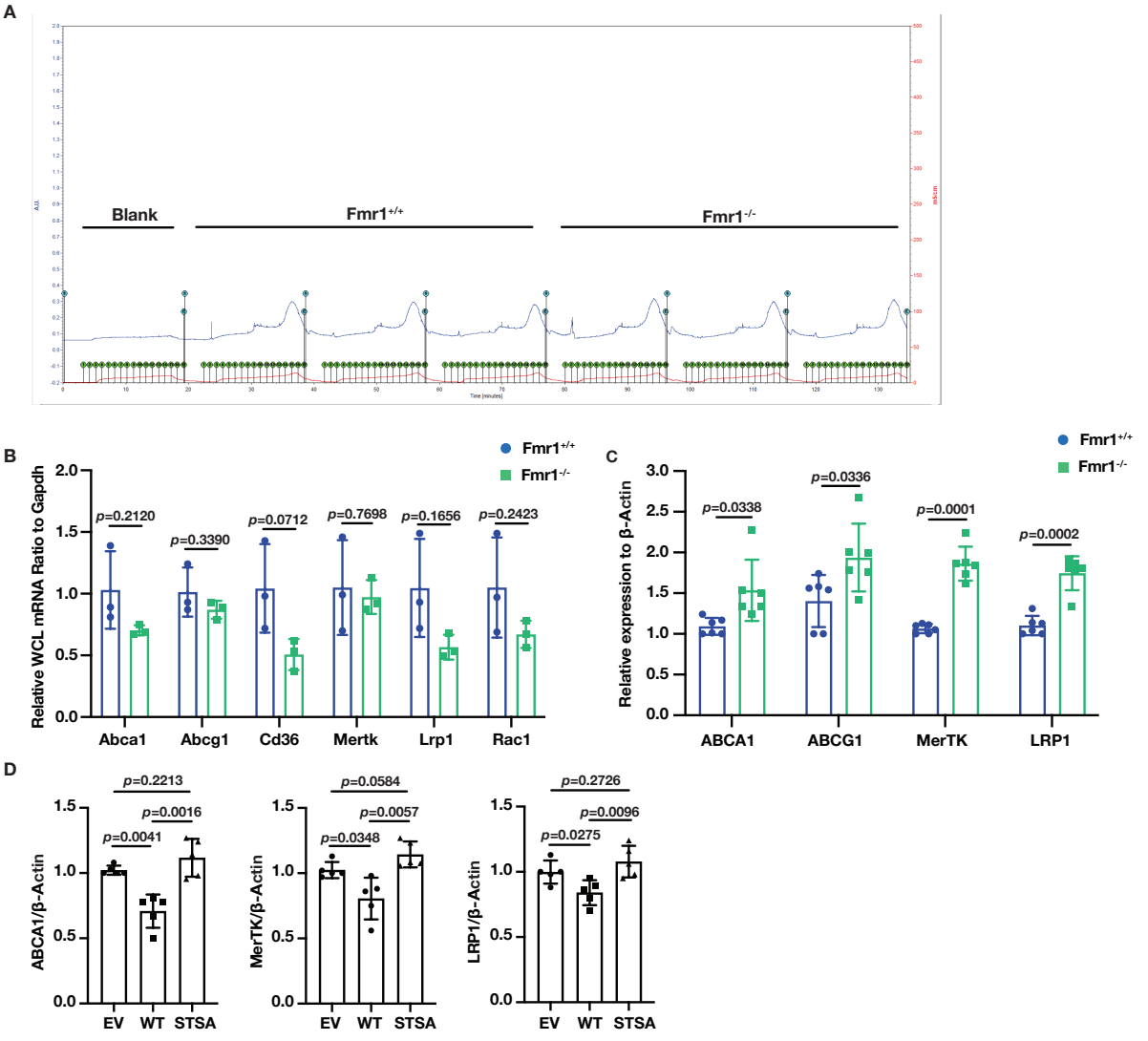
Appendix Figure S2. Supplemental figure related to Figure 2.

A Protein lysates from macrophages used in Fig 2C were analyzed by western blotting using specific antibodies for FMRP and β-Actin.

B $Fmr1^{-/-}$ or AMG-18 pre-treated cells (10 μM, 1 hour) were treated with fluorescently labeled cholesterol for 4 hours (n=6 biological replicates).

Data information: Data are mean ± SEM. Unpaired *t*-test with Welch's correction.

Appendix Figure S3



Appendix Figure S3. Supplemental figure related to Figure 4.

A mRNA distribution of fractions from $Fmr1^{+/+}$ and $Fmr1^{-/-}$ RNA samples after sucrose gradient.

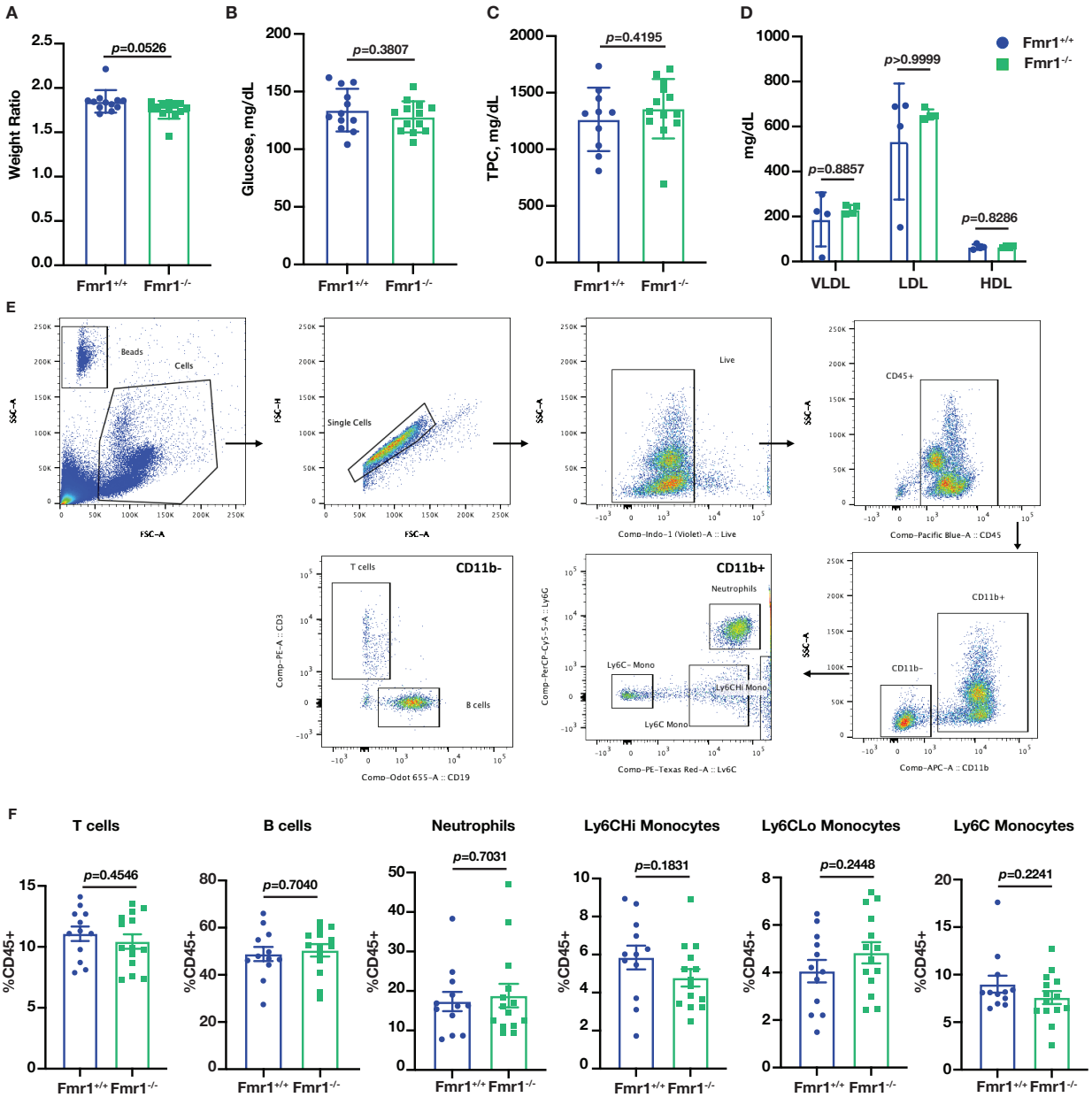
B qRT-PCR analysis of Abca1, Abcg1, Mertk, Lrp1, Cd36 and Rac1 in total mRNA levels from same samples used in polysome fractions (n=3 biological replicates).

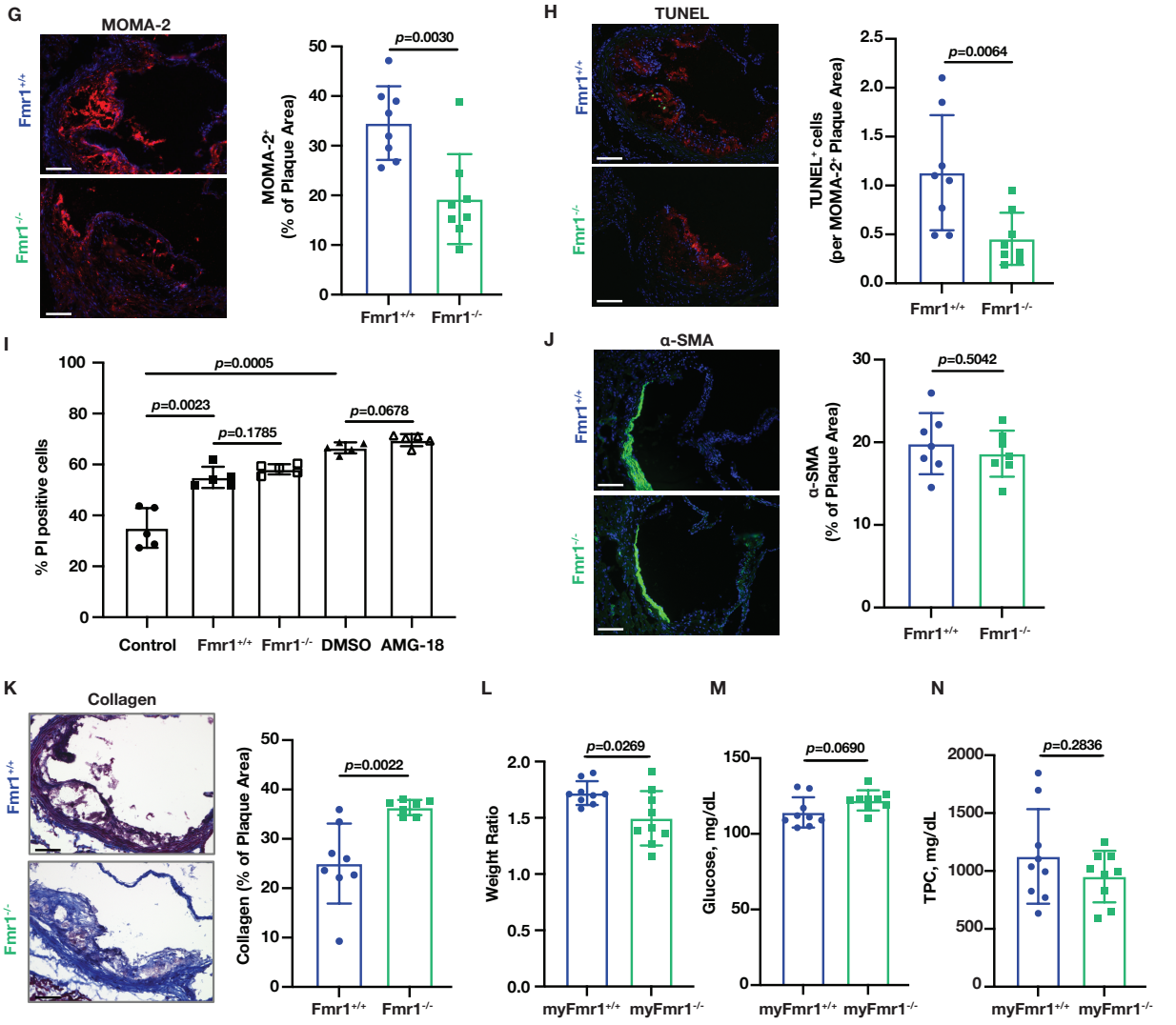
C Western blot quantifications for ABCA1, ABCG1, MerTK and LRP1 in Fig 4C. The fold change of protein expression level was calculated relative to β-Actin.

D Western blot quantifications for ABCA1, MerTK and LRP1 in Fig 4D. The fold change of protein expression level was calculated relative to β-Actin.

Data information: Data are mean ± SEM. Unpaired t-test with Welch's correction.

Appendix Figure S4




G
MOMA-2
Fmr1+/+
Fmr1-/-
MOMA-2+ (% of Plaque Area)
p=0.0030
Fmr1+/+ Fmr1-/-
H
TUNEL
TUNEL+ cells (per MOMA-2+ Plaque Area)
p=0.0064
I
% PI positive cells
p=0.0005
p=0.0023
p=0.1785
p=0.0678
Control Fmr1+/+ Fmr1-/- DMSO AMG-18
J
α-SMA
α-SMA (% of Plaque Area)
p=0.5042
K
Collagen
Collagen (% of Plaque Area)
p=0.0022
L
Weight Ratio
p=0.0269
myFmr1+/+ myFmr1-/-
M
Glucose, mg/dL
p=0.0690
N
TPC, mg/dL
p=0.2836

Appendix Figure S4. Supplemental figure related to Fig 5.

A The ratio of mouse weight at the beginning of the experiment over the weight at the end of the experiment in Fig 5A (n=12-13 mice per group).

B-D Plasma measurements for (B) glucose (n=12-13 mice per group), (C) cholesterol (n=12-13 mice per group) and (D) lipoproteins (n=4 mice per group) from mice in Fig 5A.

E Gating strategy for the flow cytometry analysis of mouse peripheral blood.

F Abundance of B cells, T cells and monocytes in the peripheral blood as % of total $CD45^+$ cells from the $Fmr1^{+/+}$ and $Fmr1^{-/-}$ mice in Fig.5A (n=12-13 mice per group).

G Macrophage area was calculated from MOMA-2 (red)-stained aortic root sections as % of $MOMA2^+$ stained area to total plaque area (n=8 mice per group; Scale bar = 100 μm).

H Apoptosis was calculated from the number of $TUNEL^+$ cells (green) in the MOMA-2-stained (red) plaque area (n=8 mice per group; Scale bar = 100 μm).

I $Fmr1^{+/+}$ and $Fmr1^{-/-}$ BMDMs or AMG-18 pre-treated cells (10 μM, 1 hour) were treated with PA (500 μM) treatment for 12 hours and then stained with Propidium iodide (PI) (n=5 biological replicates).

J % Smooth muscle actin (α-SMA) was calculated from aortic root sections stained with α-SMA (green) as % α-SMA^+ area to the total plaque area (n=8 mice per group, Scale bar 100 μm).

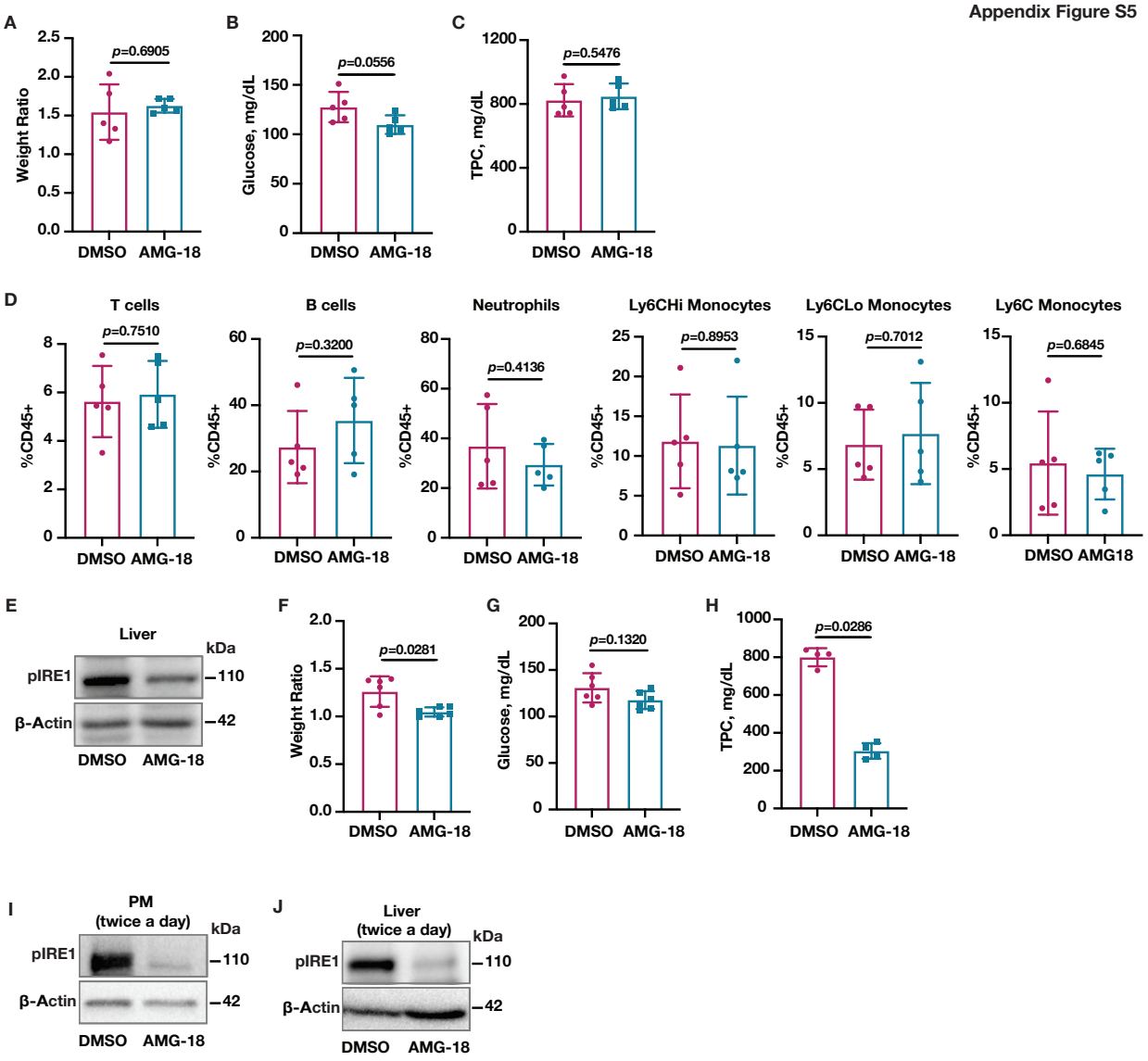
K % collagen area was calculated from Masson's Trichrome staining as the percentage of collagen (blue) area in total plaque area (n=8 mice per group; Scale bar = 100 μm).

L The ratio of mouse weight at the beginning of the experiment over the weight at the end of the experiment in Fig. 5F (n=9 mice per group).

M-N Plasma measurements for (M) glucose (n=9 mice per group) and (N) cholesterol (n=9 mice per group) from mice in Fig 5F.

Data information: Data are mean ± SEM; Mann Whitney U test.

Appendix Figure S5



Appendix Figure S5. Supplemental figure related to Fig 6.

A The ratio of mouse weight at the beginning of the experiment over the weight at the end of the experiment in Fig. 6A (n=5 mice per group).

B-D Plasma measurements for (B) glucose (n=5 mice per group) and (C) cholesterol (n=5 mice per group) from mice in Fig. 6A. (D) Abundance of B cells, T cells and monocytes in the peripheral blood as % of total $CD45^+$ cells from the vehicle (DMSO) or AMG-18 (30 mg/kg) once a day injected mice in Fig. 6A (n=5 mice per group).

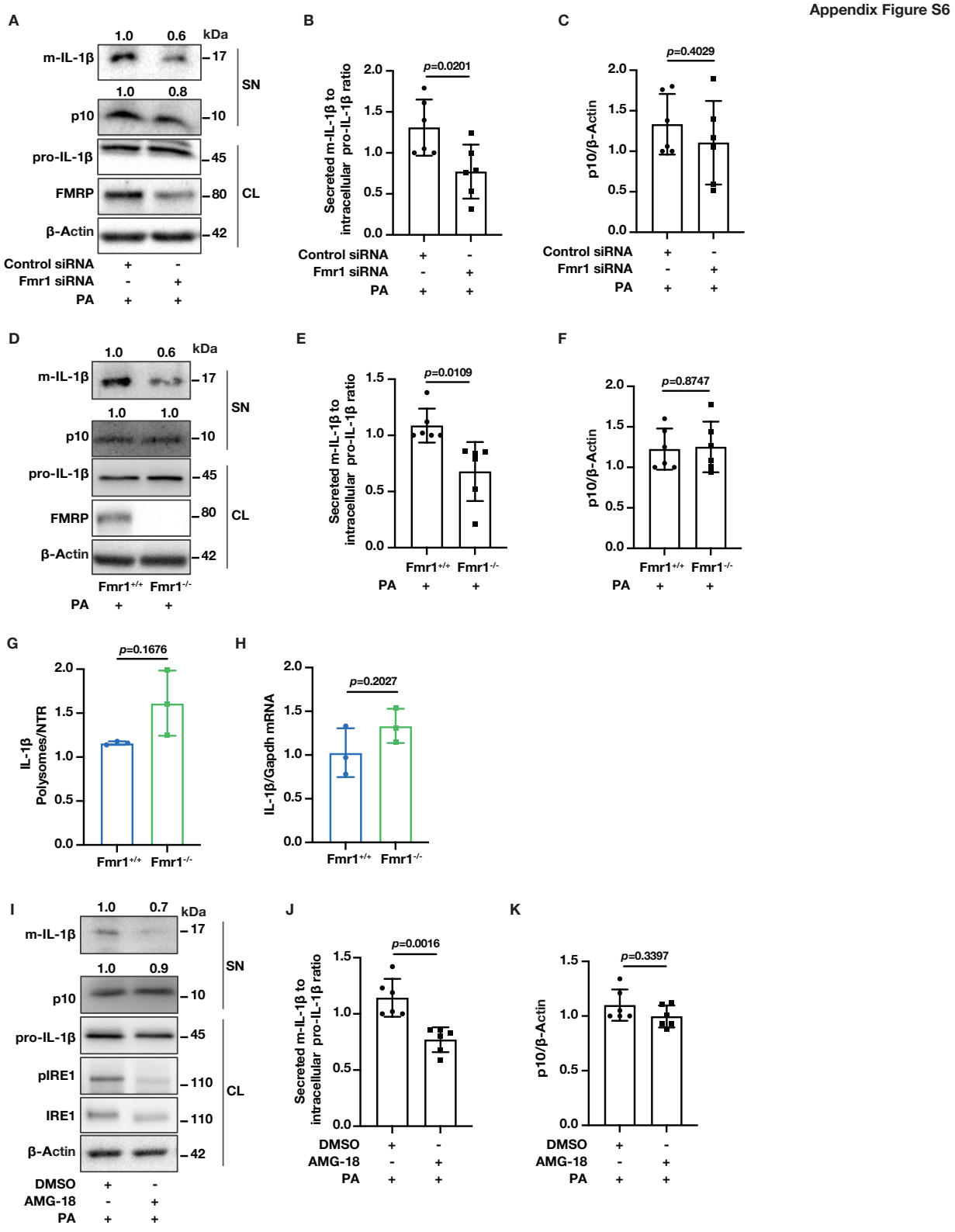
C Protein lysates of liver from mice in Fig. 6A. Proteins were analyzed by western blotting using specific antibodies for pIRE1 and β-Actin (n=5 mice per group).

F The ratio of mouse weight at the beginning of the experiment over the weight at the end of the experiment in Fig. 6F (n=6 mice per group).

G-H Plasma measurements for (G) glucose (n=6 mice per group) and (H) cholesterol (n=6 mice per group) from Fig 6F.

I-J Protein lysates of (I) thioglycolate-elicited PM and (J) liver from mice in Fig 6F. Proteins were analyzed by western blotting using specific antibodies for pIRE1 and β-Actin (n=6 mice per group).

Data information: Data are mean ± SEM; Mann Whitney U test.


A
1.0
0.6
kDa
m-IL-1β
17
SN
1.0
0.8
p10
10
pro-IL-1β
45
FMRP
80
CL
β-Actin
42
Control siRNA + -
Fmr1 siRNA - +
PA + +
B
Secreted m-IL-1β to intracellular pro-IL-1β ratio
p=0.0201
Control siRNA + -
Fmr1 siRNA - +
PA + +
C
p10/β-Actin
p=0.4029
Control siRNA + -
Fmr1 siRNA - +
PA + +
D
1.0
0.6
kDa
m-IL-1β
17
SN
1.0
1.0
p10
10
pro-IL-1β
45
FMRP
80
CL
β-Actin
42
Fmr1+/+ Fmr1-/-
PA + +
E
Secreted m-IL-1β to intracellular pro-IL-1β ratio
p=0.0109
Fmr1+/+ Fmr1-/-
PA + +
F
p10/β-Actin
p=0.8747
Fmr1+/+ Fmr1-/-
PA + +
G
IL-1β Polysomes/NTR
p=0.1676
Fmr1+/+ Fmr1-/-
H
IL-1β/Gapdh mRNA
p=0.2027
Fmr1+/+ Fmr1-/-
I
1.0
0.7
kDa
m-IL-1β
17
SN
1.0
0.9
p10
10
pro-IL-1β
45
pIRE1
110
CL
IRE1
110
β-Actin
42
DMSO + -
AMG-18 - +
PA + +
J
Secreted m-IL-1β to intracellular pro-IL-1β ratio
p=0.0016
DMSO + -
AMG-18 - +
PA + +
K
p10/β-Actin
p=0.3397
DMSO + -
AMG-18 - +
PA + +

Appendix Figure S6. IRE1 Kinase domain and FMRP regulates IL-1β secretion in macrophages.

A BMDMs were transfected with Fmr1- or control-siRNA and 24 hours after transfection cells were primed with LPS (200 μM) for 3 hours followed by PA (500 μM) treatment for 16 hours (n=6 biological replicates).

B Quantification of secreted m-IL-1β to intracellular pro-IL-1β ratio normalized to β-Actin in Appendix Figure S6A.

C Quantification of secreted p10 (caspase 1) to β-Actin in Appendix Figure S6A.

D $Fmr1^{+/+}$ and $Fmr1^{-/-}$ BMDMs were primed with LPS (200 μM) for 3 hours followed by PA (500 μM) treatment for 16 hours (n=6 biological replicates).

E Quantification of secreted m-IL-1β to intracellular pro-IL-1β ratio normalized to β-Actin in Appendix Figure S6D.

F Quantification of secreted p10 (caspase 1) to β-Actin in Appendix Figure S6D.

G The ratio of the pro-IL-1β mRNA in polysome to NTR fraction (n=3 biological replicates).

H qRT-PCR analysis of pro-IL-1β in total mRNA levels from same samples used in polysome fractions (n=3 biological replicates).

I BMDM cells were pre-treated with AMG-18 (10 μM) for 1 hour and the primed with LPS (200 μM) for 3 hours followed by PA (500 μM) treatment for 16 hours (n=6 biological replicates).

J Quantification of secreted m-IL-1β to intracellular pro-IL-1β ratio normalized to β-Actin in Appendix Figure S6I.

K Quantification of secreted p10 (caspase 1) to β-Actin in Appendix Figure S6I.

Data information: A representative blot is shown. In A and I data are cumulative results of 2 independent experiments. In D data are cumulative results of 3 independent experiments. Supernatants were analyzed by western blotting using specific antibody for IL-1β and caspase-1 and protein lysates were analyzed by western blotting using specific antibodies for FMRP, pIRE1, IRE1 and β-Actin and fold inductions are depicted above the blots. Data are mean ± SEM. Unpaired *t*-test with Welch's correction.