LETTER TO THE EDITOR

High-cholesterol diet fuels myeloma progression, dysregulates adipokine expression *in vivo*, and impairs treatment response *ex vivo*

Authors

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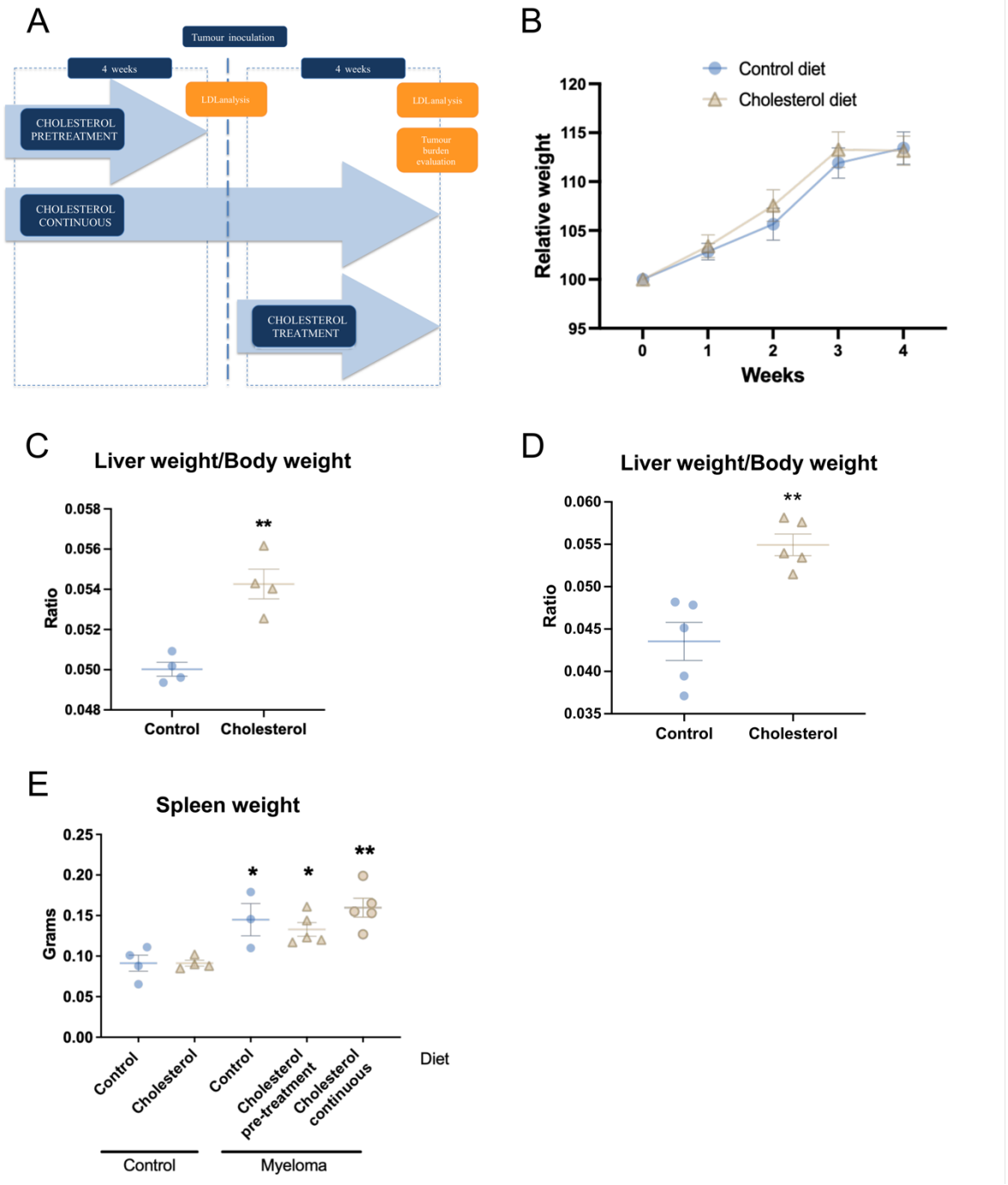
SUPPLEMENTARY FIGURES

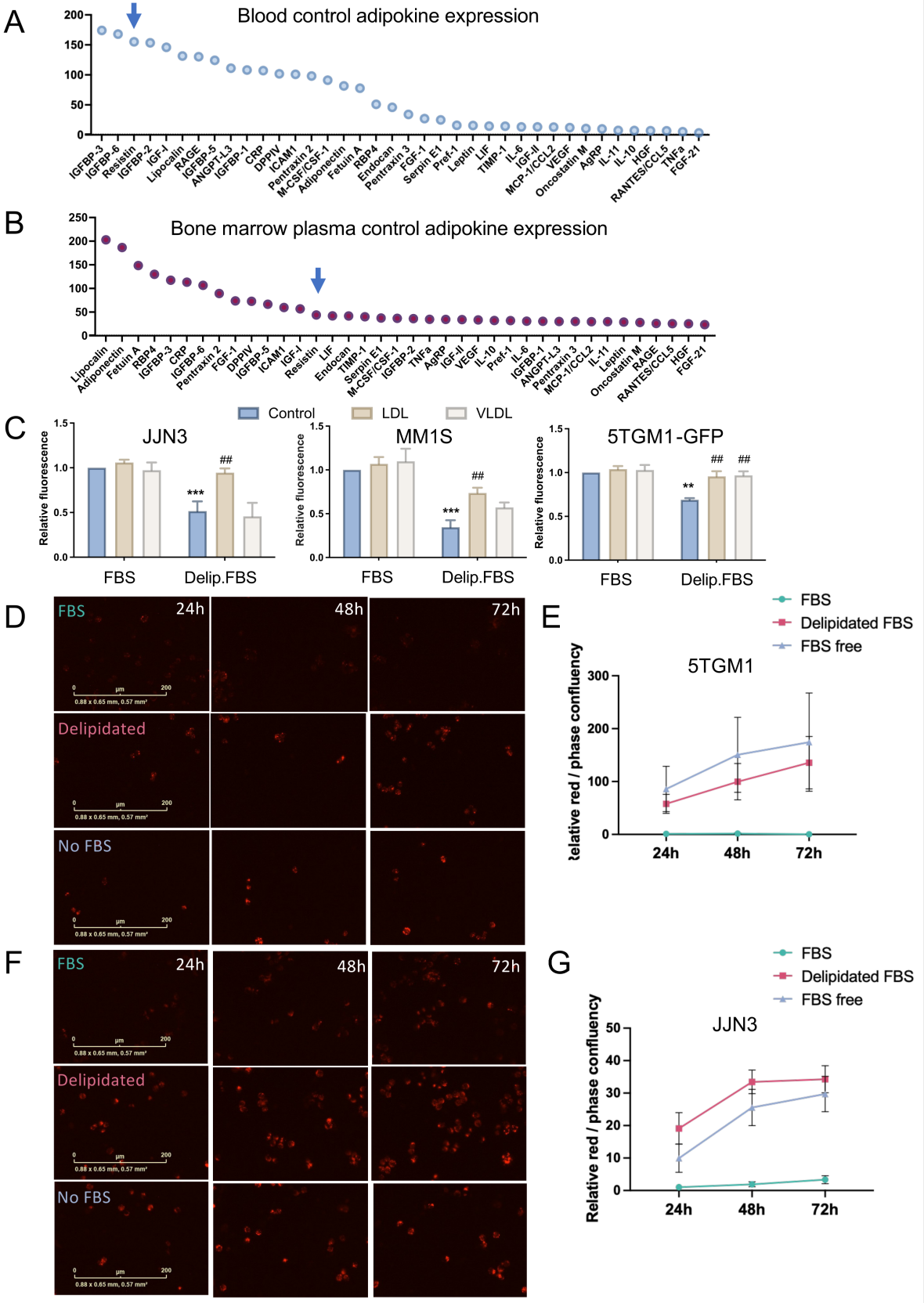
Supplementary figure 1. Cholesterol diet does not increase body weight or spleen weight but induces signs of fatty liver.



Supplementary figure 1. Cholesterol diet does not increase body weight or spleen weight but induces signs of fatty liver.

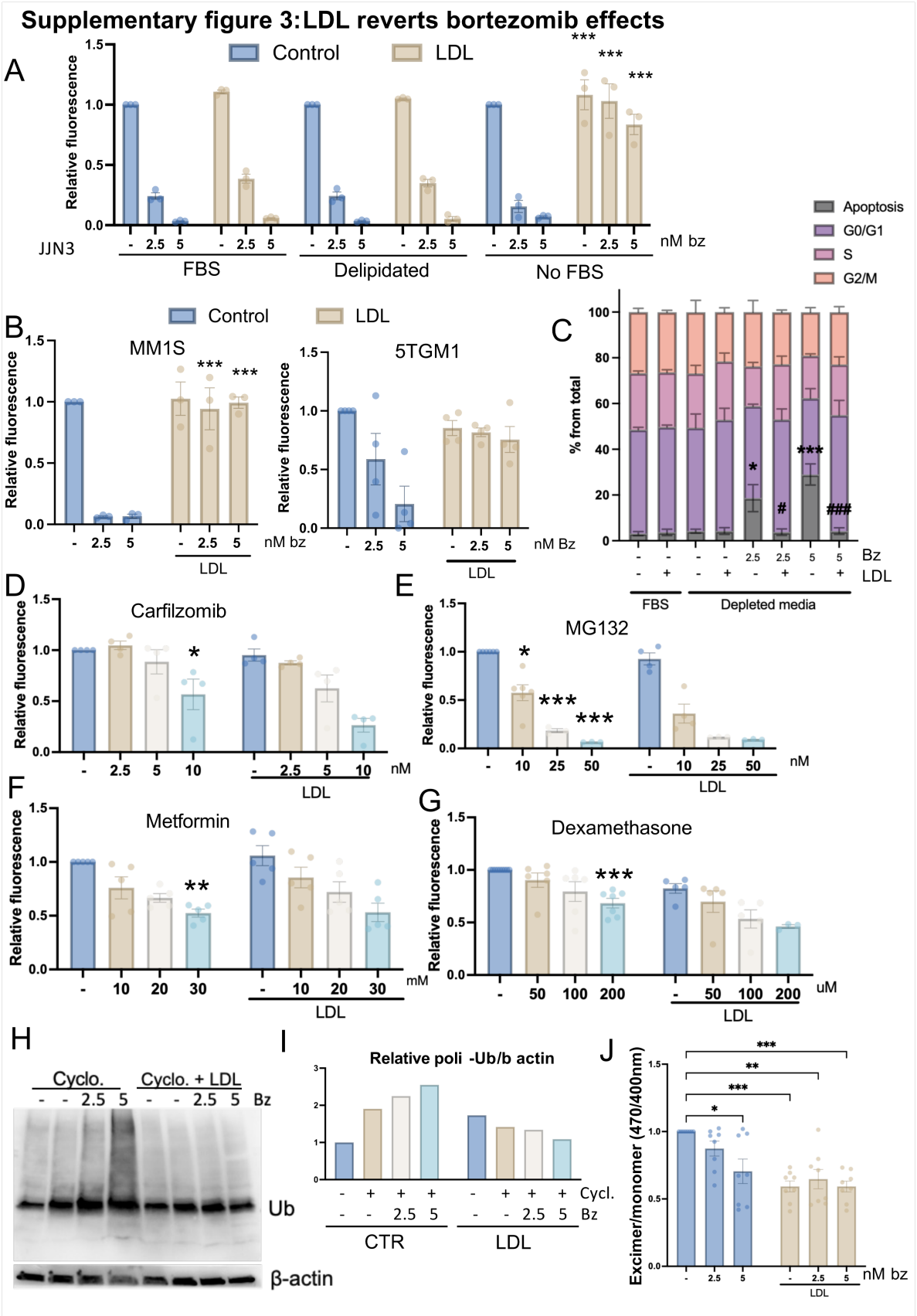
(**A**) Schematic figure showing the experimental setting for in vivo experiments. Mice were fed with either control or high cholesterol diet for 4 weeks and serum LDL was measured. Then animals were randomly distributed into either control or tumour-bearing mice (myeloma) and inoculated with 5TGM1-GFP cells. Cholesterol pre-treatment myeloma mice had cholesterol diet halted at time of inoculation whereas cholesterol continuous group were fed with cholesterol diet until sacrifice, where tumour burden was assessed. In separate experiments, cholesterol diet was introduced at time of tumour inoculation (cholesterol treatment). (**B**) C57BL/6 KaLwRij body weight after 4 weeks of high cholesterol diet. (**C**) Liver weight/body weight ratio in control and cholesterol-fed animals for 4 weeks. (**D**) Liver weight/body weight ratio in control and cholesterol-fed animals for 8 weeks. (**E**) Spleen weights where the diet was given pre-inoculation (cholesterol pre-treated) or continuously before and after tumour inoculation (cholesterol continuous). For 2 group comparison, two-tailed Student's t-test was performed. $^{**}p < 0.01$. For more than 2 groups one-way anova analysis was performed. If not otherwise indicated, $^{*}p< 0.05$, $^{**}p < 0.01$ compared to control with no-tumour. Results are presented as mean ± SEM.

Supplementary figure 2: Effect of LDL on myeloma cell viability



Supplementary Figure 2. Effect of LDL on myeloma cell viability

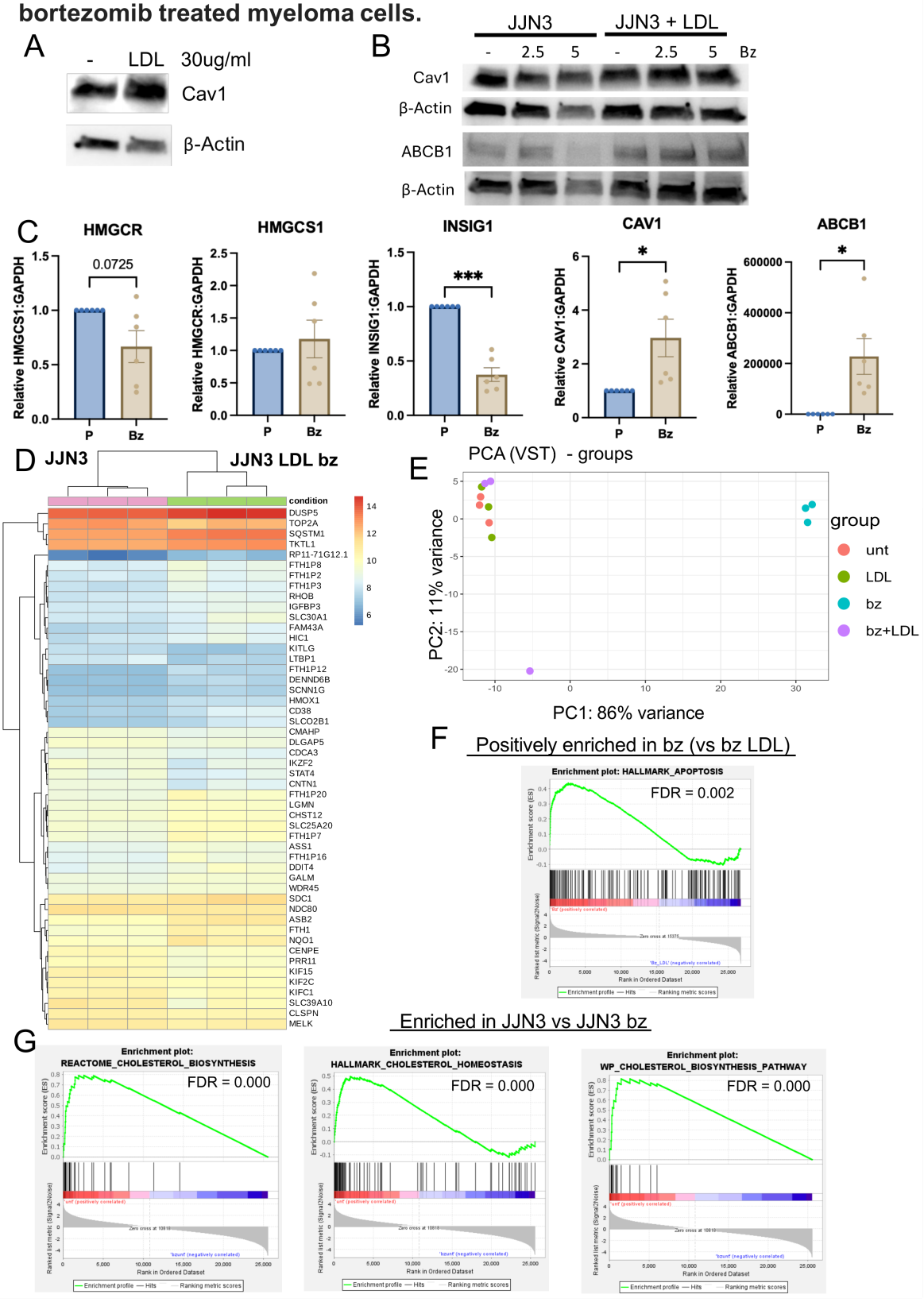
Relative expression of adipokines in blood **(A)** and bone marrow plasma **(B)** of non-tumour C57Bl/KaLWRIj mice. (**C**) JJN3, MM1s and 5TGM1-GFP cells were cultured in 10% FBS media or lipid-depleted media ± 6 µg/ml LDL or VLDL. Viability was assessed after 72 (JJN3, MM1s) or 96 hours (5TGM1) . 5TGM1-GFP (**D & E**) and JJN3 (**F & G**) were seeded using 10% FBS media, delipidated FBS media or serum free media together with Red-LDL for 24, 48 or 72 hours. Images were taken using Incucyte®Live-Cell analysis system (**D & F**). Quantification was performed using confluency of red signal over confluency of bright images (n=3) (**E & G**). For C $**p < 0.01$ and $***p < 0.001$ compared to control FBS condition. $##p < 0.01$ compared to control delipidated condition. Results are presented as mean ± SEM.


Supplementary figure 3:LDL reverts bortezomib effects
A
Control
LDL
Relative fluorescence
JJN3
FBS
Delipidated
No FBS
nM bz
Apoptosis
G0/G1
S
G2/M
B
MM1S
5TGM1
Control
LDL
nM bz
nM Bz
C
% from total
Bz
LDL
FBS
Depleted media
D
Carfilzomib
nM
E
MG132
F
Metformin
mM
G
Dexamethasone
uM
H
Cyclo.
Cyclo. + LDL
Bz
Ub
β-actin
I
Relative poli -Ub/b actin
Cycl.
CTR
J
Excimer/monomer (470/400nm)

Supplementary Figure 3. LDL reverts bortezomib effects

(**A**) JJN3 cells were cultured in 10% FBS, delipidated or no FBS media and treated with 30 µg/ml LDL for 3 hours before 24 hour bortezomib treatment (2.5 or 5nM). Viability was quantitated by alamar blue (n = 3). (**B**) MM1S and 5TGM1 cells were cultured in no FBS media and treated was (A). Viability was quantitated by alamar blue (n = 3-4). (**C**) JJN3 were cultured in serum-free conditions ± LDL for 3 hours before bortezomib treatment for 24 hours and cell cycle analysis was performed using propidium iodide. JJN3 cells were treated with carfilzomib (**D**), MG132 (**E**), Metformin (**F**) or Dexamethasone (**G**) ± LDL. Cycloheximide experiments were performed and proteasome activity assessed by expression of poli-ubiquitin. (**H**) Representative western-blot and (**I**) quantification (as relative ubiquitin signal/β-actin) shows ubiquitinated-protein accumulation (n=3). (**J**) Changes in membrane fluidity were evaluated. For A ***$p < 0.001$ compared to control non-LDL condition. For D-G & J, *$p< 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control (no bortezomib, no LDL).

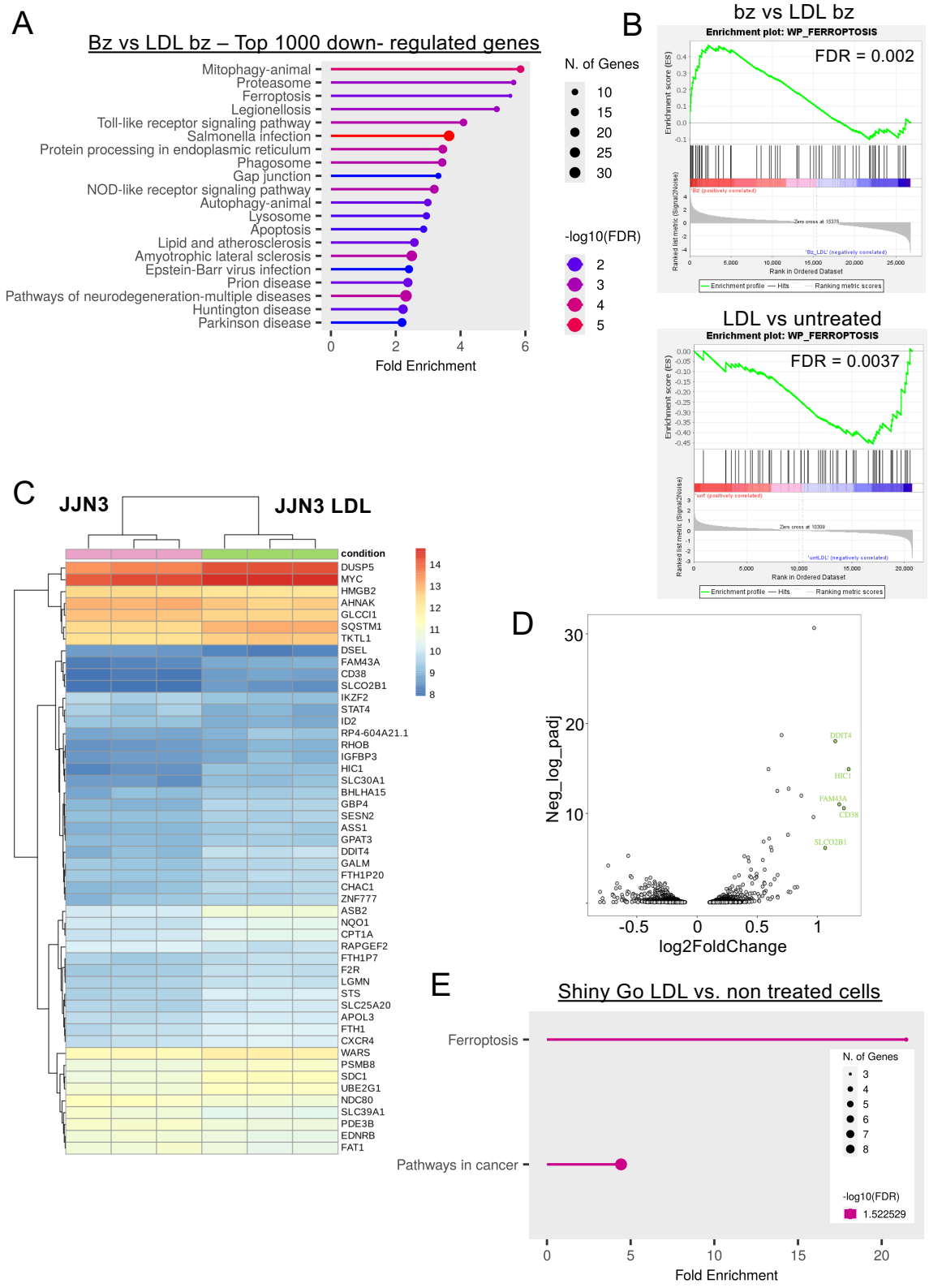
Supplementary figure 4. LDL restores the gene expression signature of bortezomib treated myeloma cells.



Supplementary Figure 4. LDL restores the gene expression signature of bortezomib treated myeloma cells.

(**A**) Expression of caveolin-1 on JJN3 myeloma cells after 30μg/ml LDL treatment for 24. (**B**) Expression of caveolin-1 and MDR/ABCB1 protein in JJN3 cells treated as per drug resistance experiments. (**C**) Expression of cholesterol-related genes in bortezomib resistant (Bz) and parental (P) L363 myeloma cells. (**D**) JJN3 myeloma cells were treated in the presence and absence of LDL and bortezomib and RNA-Seq performed. Heatmap showing DEG analysis of JJN3 vs. JJN3 LDL + bortezomib. (**E**) PCA plot from untreated (unt), bortezomib (bz), LDL treated (LDL) and LDL + bortezomib (bz + LDL) groups. (**F**) Bortezomib vs. bortezomib + LDL GSEA enrichment plot of HALLMARK_APOPTOSIS gene set. (**G**) Control vs. bortezomib GSEA enrichment plot of reactome cholesterol biosynthesis, hallmark cholesterol homeostasis and WP cholesterol biosynthesis pathway gene sets. For C, two-tailed Student's t-test was performed. *$p < 0.05$, ***$p < 0.001$.

Supplementary figure 5: LDL treatment affects the ferroptosis pathway



Supplementary Figure 5. LDL treatment affects ferroptosis pathway

(**A**) ShinyGO analysis of JJN3 treated with bortezomib vs LDL + bortezomib. (**B**) GSEA analysis using the WP_FERROPTOSIS gene set in bortezomib vs LDL + bortezomib and LDL vs. control . (**C**) JJN3 myeloma cells were treated in the presence and absence of LDL and bortezomib and RNA-Seq performed. Heatmap (**C**) and volcano plot (**D**) showing DEG analysis of LDL vs control. (**E**) ShinyGO analysis of differentially expressed genes in control vs. LDL.