

Supplementary material

Fibronectin leucine-rich transmembrane protein 2 (FLRT2) drives monocyte differentiation into macrophages via the UNC5B-Akt/mTOR axis

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Supplementary Figures 1 to 11

Supplementary Table 1. Key resources.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat monoclonal anti-CD16/32 (FCM)	Biolegend	Cat# 101320; RRID: AB_1574973
Rat monoclonal anti-CD45 (FCM)	Biolegend	Cat# 101326; RRID: AB_4935535
Rat monoclonal anti-F4/80 (FCM)	Invitrogen	Cat# 17-4801-80; RRID: AB_2784647
Rat monoclonal anti-CD11b (FCM)	Biolegend	Cat# 101206; RRID: AB_312789
Rat monoclonal anti-CD19 (FCM)	Biolegend	Cat# 115529; RRID: AB_830706
Goat polyclonal anti-FLRT2 (WB and IF)	R&D	Cat# AF2877; RRID: AB_2106600
Rabbit polyclonal anti-FLRT2 (FACS)	Abcam	Cat# ab154023
Rabbit polyclonal anti-UNC5B (WB, IP, and IF)	Biorbyt	Cat# orb542246
Mouse monoclonal anti- β -Actin (WB)	Santa Cruz	Cat# sc-47778; RRID: AB_2714189
Mouse monoclonal anti-GAPDH (WB)	Santa Cruz	Cat# sc-137179; RRID: AB_2232048
Mouse monoclonal anti-CD36 (WB)	Santa Cruz	Cat# sc-7309; RRID: AB_627044
Goat polyclonal anti-SR-A (WB)	R&D	Cat# AF2708; RRID: AB_663950
Chicken polyclonal anti-GFP (IF)	Abcam	Cat# ab13970; RRID: AB_300798

Rabbit polyclonal anti-Myc (WB & IP)	Proteintech	Cat# 16286-1-AP; RRID: AB_11182162
Rabbit polyclonal anti-FLAG (WB)	Invitrogen	Cat# PA1-984B; RRID: AB_347227
Mouse monoclonal anti-FLAG (WB)	Proteintech	Cat# 66008-3-Ig; RRID: AB_2749837
Rabbit polyclonal anti-Akt (WB)	Cell Signaling Technology	Cat# 9272; RRID: AB_329827
Rabbit polyclonal anti-Phospho-Akt (Ser473) (WB)	Cell Signaling Technology	Cat# 9271; RRID: AB_329825
Rabbit monoclonal anti-Phospho-Akt (Thr308) (WB)	Cell Signaling Technology	Cat# 4056; RRID: AB_331163
Rabbit monoclonal anti-mTOR (WB)	Cell Signaling Technology	Cat# 2983; RRID: AB_2105622
Rabbit monoclonal anti-4E-BP1 (WB)	Cell Signaling Technology	Cat# 9644; RRID: AB_2097841
Rabbit monoclonal anti-Phospho-4E-BP1 (Thr37/46) (WB)	Cell Signaling Technology	Cat# 2855; RRID: AB_560835
Rabbit polyclonal anti-S6K (WB)	Cell Signaling Technology	Cat# 9202; RRID: AB_331676
Rabbit monoclonal anti-Phospho-S6K (Thr389) (WB)	Cell Signaling Technology	Cat# 9234; RRID: AB_2269803
Rabbit monoclonal anti-S6 (WB)	Cell Signaling Technology	Cat# 2217; RRID: AB_331355
Mouse monoclonal anti-Rac1(WB)	Santa Cruz	Cat# sc-514583; RRID: AB_2818941
Normal Rabbit Control IgG	Sino Biological	Cat# CR1
Rabbit polyclonal anti-	Cell Signaling	Cat# 2974; RRID: AB_2262884

Phospho- mTOR (Ser2481) (WB)	Technology	
Rabbit polyclonal anti-CD11B (WB)	Beyotime	Cat# AF6396
Bacterial and Virus Strains		
Stbl3 Chemically Competent Cell	Kangti Life Technology Co., Ltd.	Cat# KTSM110L
RFP overexpression lentivirus	This paper	N/A
Chemicals		
Zombie Aqua™ Fixable Viability Kit	BioLegend	Cat# 423101
Anti-FLAG® M2 Magnetic Beads	Sigma-Aldrich	Cat# M8823
Protein A/G Magnetic Beads	MedChemExpre ss	Cat# HY-K0202
Phorbol 12-myristate 13- acetate	Sigma-Aldrich	Cat# P8139; CAS: 16561-29-8
Dimethyl sulfoxide	Sigma-Aldrich	Cat# D2650; CAS: 67-68-5
Crystal Violet	Sigma-Aldrich	Cat# C0775; CAS: 548-62-9
Zymosan A (<i>S. cerevisiae</i>) BioParticles™	Invitrogen	Cat# Z23374
LY-294,002 hydrochloride	Sigma-Aldrich	Cat# L9908; CAS: 934389-88-5
Rapamycin	Sigma-Aldrich	Cat# V900930; CAS: 53123-88-9
Calcein AM	Beyotime	Cat# C2012; CAS: 148504-34-1
MHY1485	Sigma-Aldrich	Cat# SML0810; CAS: 326914-06-1
DAPI	Invitrogen	Cat# D1306; CAS: 28718-90-3
Recombinant human M-CSF protein	R&D	Cat# 216-MCC

Critical Commercial Assays		
qPCR	Accurate Biology	Cat# AG11701
Experimental Models: Cell Lines		
Human: HEK239T	ATCC	Cat# CRL-3216
Human: HUVEC	ATCC	Cat# PCS-100-013
Human: Peripheral blood mononuclear cell	LDEBIO	Cat# 1501-50M
Experimental Models: Organisms		
Mouse: C57BL/6J	Guangdong Medical Laboratory Animal Center	N/A
Mouse: B6.129P2- <i>Lyz2^{tm1(cre)Ifo/J}</i>	The Jackson Laboratory	Cat# JAX: 004781
Mouse: C57BL/6- <i>Flrt2^{em1(flox)Smoc}</i>	This paper	N/A
Oligonucleotides		
shRNA targeting sequence: hFLRT2 #1: 5'- CATCTGATCAGGCTCTATT TGCTC-3'	This paper	N/A
shRNA targeting sequence: hFLRT2 #2: 5'- GTCTCCTTAAATAACGATC	This paper	N/A

AACTC-3'		
shRNA targeting sequence: hFLRT2 #3: 5'- CGTCAGGGAATTAAATATG AACTC-3'	This paper	N/A
shRNA targeting sequence: hUNC5B #1: 5'- AGGTGGAATGGCTCAAGA ATGAG-3'	This paper	N/A
shRNA targeting sequence: hUNC5B #2: 5'- AGGAGCCGAAACCGCTAA TGTTCA-3'	This paper	N/A
shRNA targeting sequence: hUNC5B #3: 5'- TGTCGGACACTGCCAACT ATAC-3'	This paper	N/A
shRNA targeting sequence: negative control 5'- CGTACGCGGAATACTTCG A-3'	This paper	N/A
Primers for plasmid construction, see Supplementary Table 2	This paper	N/A

Primers for qPCR, see Supplementary Table 3	This paper	N/A
Primers used to genotype <i>FLRT2^{fl/fl}</i> mice: F: 5'- TAGAGGTCCAGCGTTAGA AAG- 3'; R: 5'- TGAGCCCACCTGACATTAT C- 3'	This paper	N/A
Primers used to genotype <i>Lyz2^{Cre}</i> mice: F: 5'- AGCGATGGATTTCGTCTC TGG- 3'; R: 5'- AGCTTGCATGATCTCCGGT ATTGAA- 3'	This paper	N/A
Recombinant DNA		
pLvTHM-Venus (expression vector for GFP)	This study	N/A
pLvTHM-Venus-hFLRT2 (expression vector for recombinant human FLRT2)	This study	N/A
pLvTHM-mApple (expression vector for RFP)	This study	N/A
pLvTHM-Venus-hUNC5B (expression vector for recombinant human UNC5B)	This study	N/A

pWPI-3XFlag/Strep (expression vector for Flag)	This study	N/A
pWPI-3XFlag/Strep-hFLRT2 (expression vector for recombinant human FLRT2)	This study	N/A
pWPI-3XFlag/Strep-hFLRT2- ED (expression vector for recombinant human FLRT2- ED)	This study	N/A
pWPI-3XFlag/Strep-hFLRT2- ID (expression vector for recombinant human FLRT2- ID)	This study	N/A
pKMyC (expression vector for Myc)	This study	N/A
pKMyC-hUNC5B (expression vector for recombinant human UNC5B)	This study	N/A
Software		
ImageJ 1.51 s	N/A	https://imagej.nih.gov/ij/
GraphPad Prism 8	N/A	https://www.graphpad.com

Supplementary Table 2. Sequences of primers used for plasmid construction.

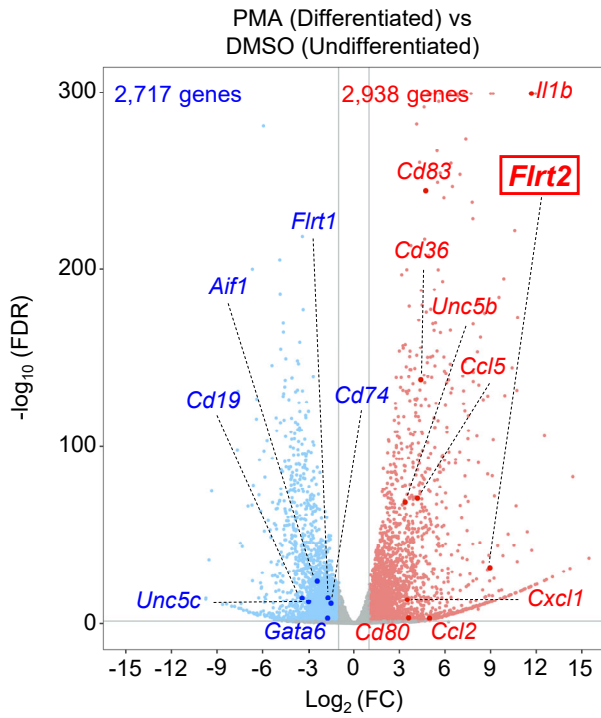
Name		Sequence 5'-3'
hFLRT2	Forward	ATGGGCCTACAGACCACAAAG
	Reverse	TCACGTATGGCAGTGCTCCAG
hUNC5B	Forward	ATGGGGGCCCCGAGCGGAGCT
	Reverse	TCAGCAGTCCCCGTCGGTGGC
hFLRT2- ECD	Forward	ATGGGCCTACAGACCACAAAG
	Reverse	TCAAAAGGGGGAGCCCATGCT
hFLRT2- ICD	Forward	ATGTGCTGGCATATGCACAAA
	Reverse	TCACGTATGGCAGTGCTCCAG

Supplementary Table 3. Sequences of the primers used for qPCR.

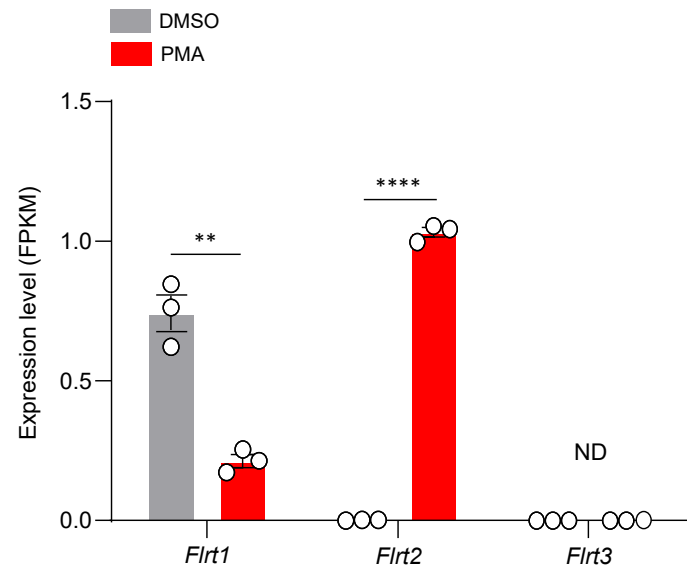
Gene	Species		Sequence
<i>Flrt2</i>	Homo	Forward	ACCACCCATGCCTCCTATCT
		Reverse	AAAGACGCTGAGCAAGACCA
<i>Unc5b</i>	Homo	Forward	ACTCACGGGGCAATGACTGG
		Reverse	AGGTCCAGGATCACACCCGT
<i>Itgam</i>	Homo	Forward	GCCGGTGAAATATGCTGTCT
		Reverse	GCGGTCCCATATGACAGTCT
<i>Cd14</i>	Homo	Forward	TCCCGGCCATCCAGAATCTA
		Reverse	AGCGAACGACAGATTGAGGG
<i>Csflr</i>	Homo	Forward	TGTGGAGATGAGGCCTGTCT
		Reverse	TTCTTGGAAGCGAGGAAGGC
<i>Msr1</i>	Homo	Forward	TTGTGACGATCGCTGGGAAG
		Reverse	ATGTGAACAGGCTCTTGTCCC
<i>Cd36</i>	Homo	Forward	CAAAACGGCTGCAGGTCAAC
		Reverse	AAGCAACAAACATCACCACACC
<i>Olr1</i>	Homo	Forward	GCAGAAGAAGCTTCACAGGAGT
		Reverse	TTTCTCCATGCCAGATCCAGTC
<i>Gapdh</i>	Homo/Mus	Forward	CAAGGTCATCCATGACAACTTTG
		Reverse	GTCCACCACCCTGTTGCTGTAG
<i>Flrt2</i>	Mus	Forward	CTCTCTGTTCCAAGCCCCAG
		Reverse	ACGAGACTGTGGCCCATTTT
<i>Unc5b</i>	Mus	Forward	GGAACTACCAAGAGTCGCCG
		Reverse	TCAATGGTGAGCAGGAAGTTAGTG
<i>Itgam</i>	Mus	Forward	CATCTGCCAAGACGATCTCA
		Reverse	TTCTGGCTTGCTGAATCCTT
<i>Cd14</i>	Mus	Forward	GCTCAACTTTTCCTGCGAAC
		Reverse	CCCGCAGTGAATTGTGACTA
<i>Csflr</i>	Mus	Forward	GACCTGCTCCACTTCTCCAG

		Reverse	GATGTCCCTAGCCAGTCCAA
<i>Msr1</i>	Mus	Forward	GGGCTTACTGGACAAACTGGT
		Reverse	CGCCTACACTCCCCTTCTCT
<i>Cd36</i>	Mus	Forward	TCGGAACTGTGGGCTCATTG
		Reverse	TCTTTGCCACGTCATCTGGG
<i>Olr1</i>	Mus	Forward	TTCCATGGGCCCTTTAGCTG
		Reverse	GTAAAGAAACGCCCCTGGTCT

a

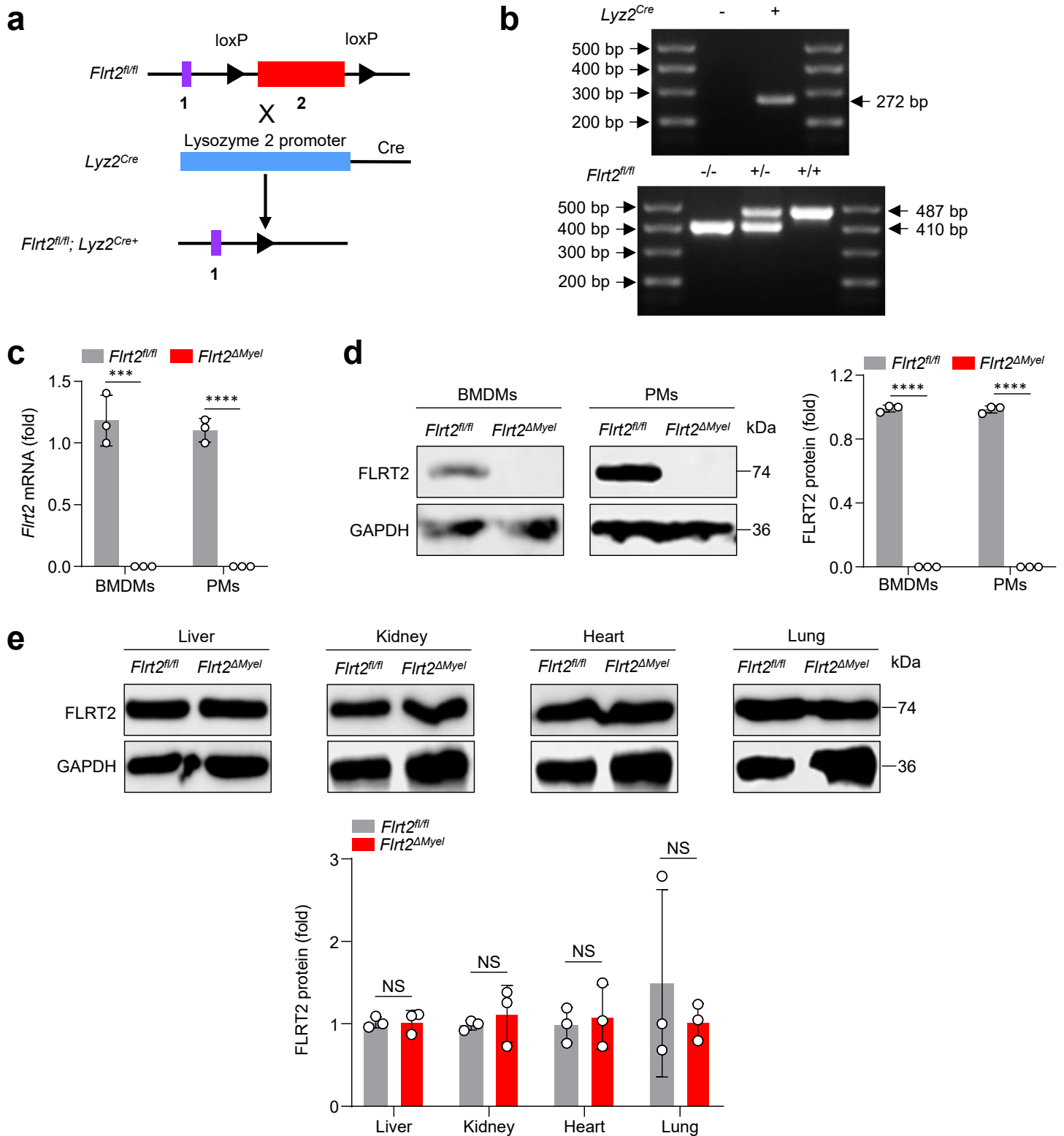


b



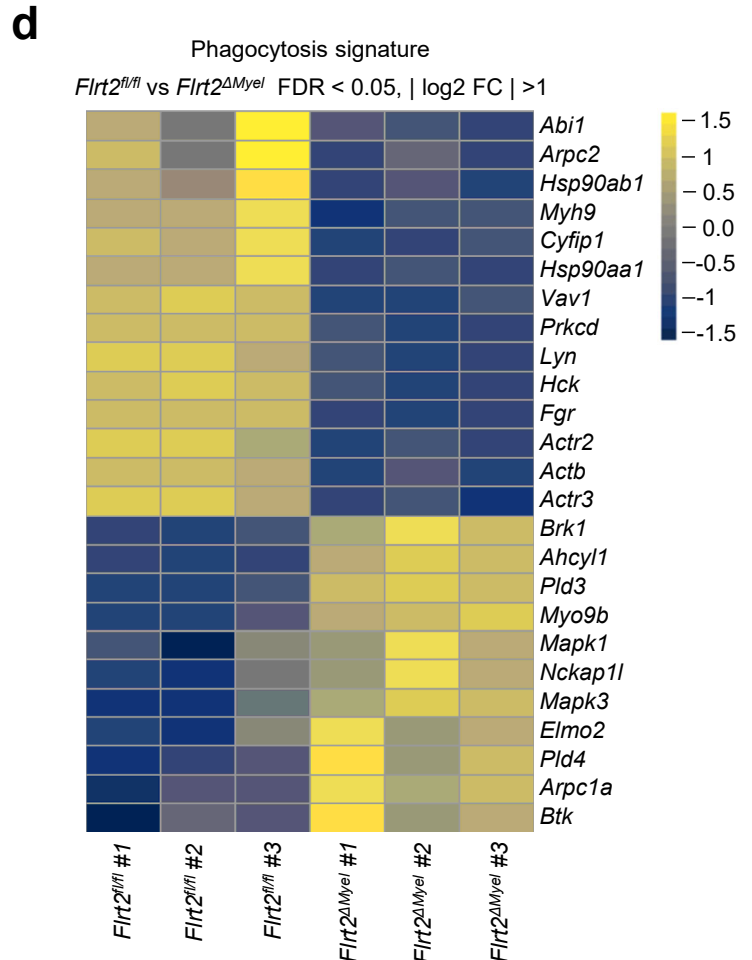
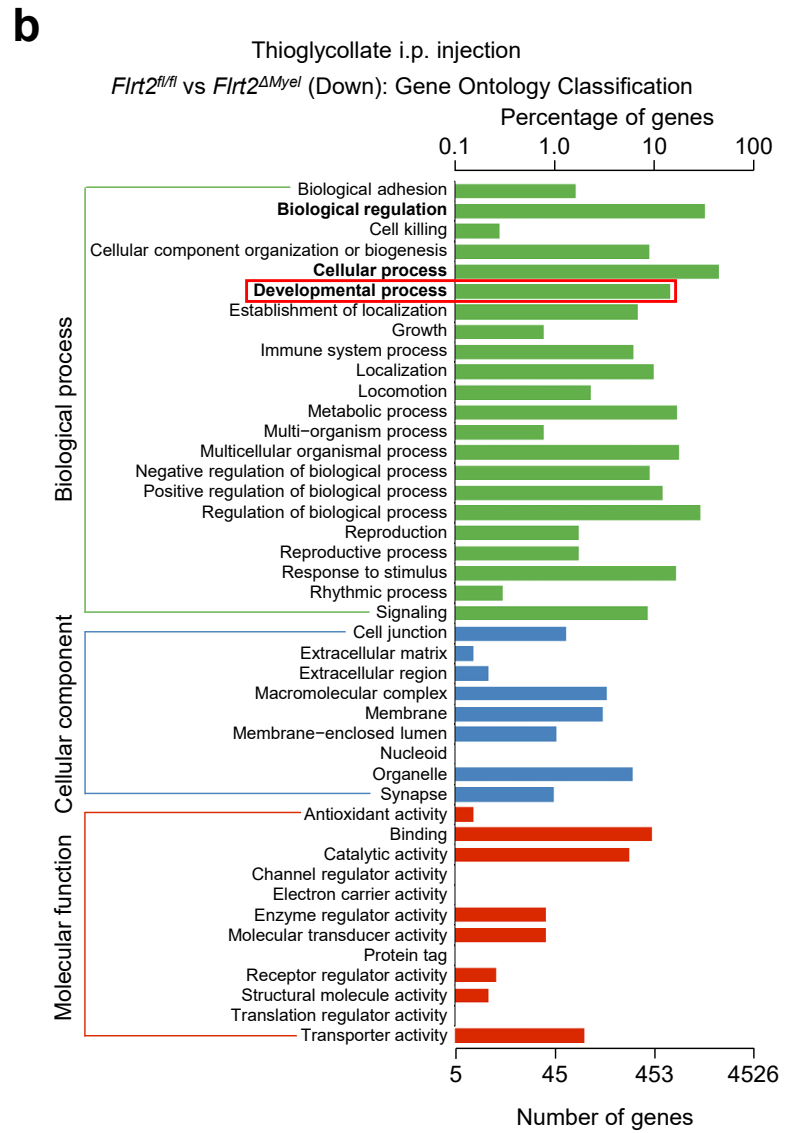
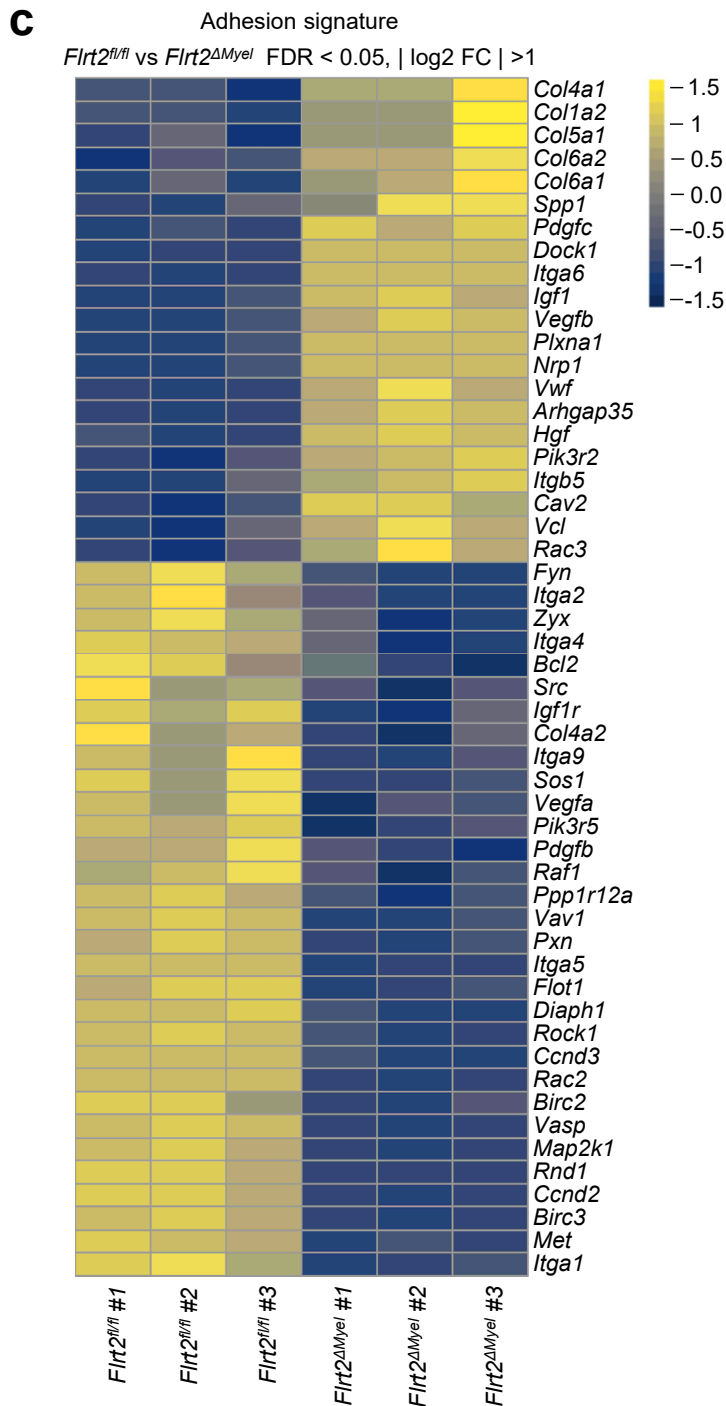
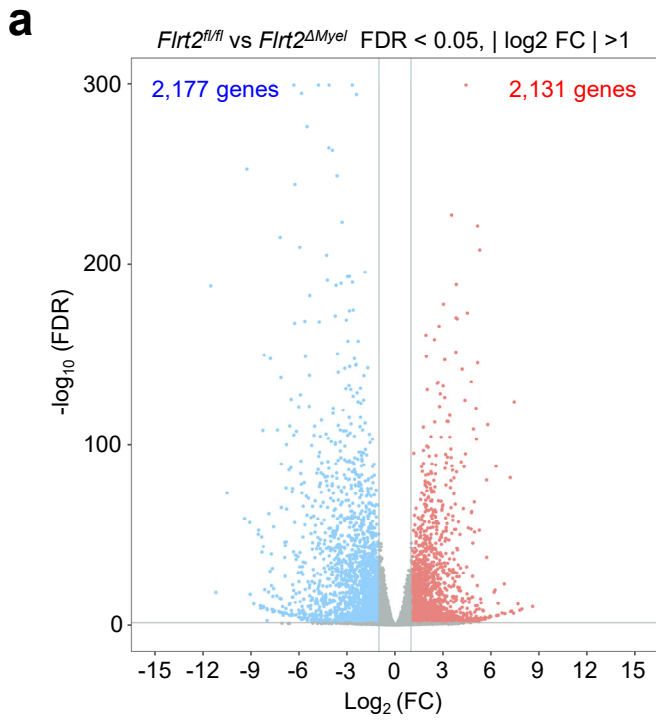
Supplementary Figure 1. FLRT2 is markedly upregulated in PMA-treated THP-1 cells. **a** Volcano plot of RNA-seq data showing differentially expressed genes (DEGs) in THP-1 cells treated with DMSO or PMA (100 ng/ml) for 24 h. Genes with substantially increased and decreased peak expressions are highlighted in red and blue, respectively. Biological replicates, $n = 3$. **b** RNA-seq analysis of *Flrt1*, *Flrt2*, and *Flrt3* expression in the cells. Data are means \pm SD. P values were determined using unpaired, two-tailed Student's t -tests. ND, not detected. ** $P < 0.01$, **** $P < 0.0001$.

Supplementary Figure 2



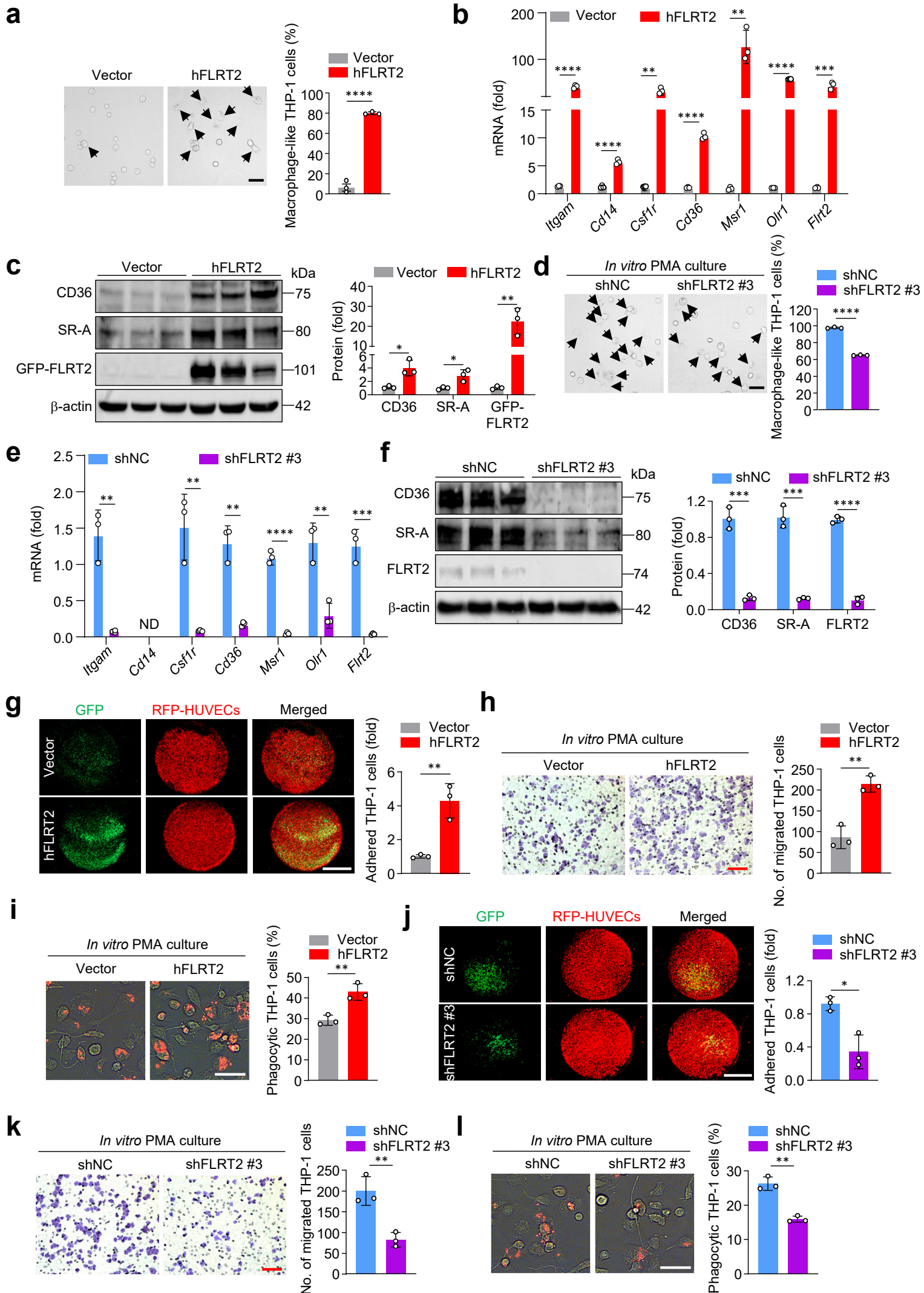
Supplementary Figure 2. Generation and identification of myeloid cell-specific FLRT2 knockout (*Flrt2^{ΔMyel}*) mice. **a** Diagram illustrating how to generate *Flrt2^{ΔMyel}* mice. **b** Representative genotyping analysis of 3-week-old mice. *Flrt2^{fl/fl}; Lyz2^{Cre+}* and *Flrt2^{fl/fl}; Lyz2^{Cre-}* mice serve as *Flrt2^{ΔMyel}* and *Flrt2^{fl/fl}* mice, respectively. **c** qPCR analysis of *Flrt2* mRNA in BMDMs and PMs isolated from *Flrt2^{fl/fl}* and *Flrt2^{ΔMyel}* mice (n = 3 mice per group). **d, e** Immunoblot analysis of FLRT2 protein in bone marrow-derived macrophages (BMDMs), peritoneal macrophages (PMs), liver, kidney, heart, and lung isolated from *Flrt2^{fl/fl}* and *Flrt2^{ΔMyel}* mice (n = 3 mice per group). Data are means ± SD. *P* values were determined using unpaired, two-tailed Student's *t*-tests. NS, not significant. ****P* < 0.001, *****P* < 0.0001.

Supplementary Figure 3

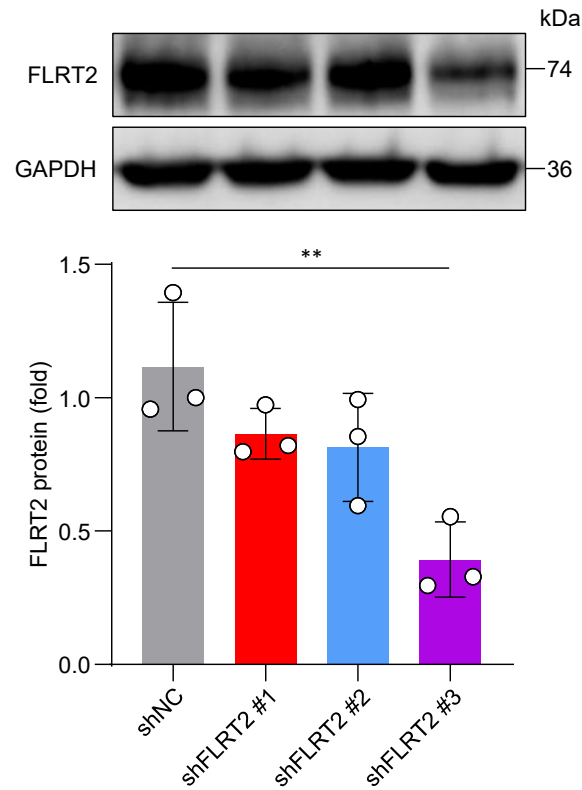


Supplementary Figure 3. RNA-seq analysis of peritoneal macrophages (PMs) of *Flrt2^{fl/fl}* and *Flrt2^{ΔMyel}* mice. **a** PMs were isolated from *Flrt2^{fl/fl}* and *Flrt2^{ΔMyel}* mice after 3 days of i.p. thioglycolate injection, and subjected to RNA-seq analysis. Volcano plots showing total differentially expressed genes (DEGs) in PMs (n = 3 mice per group). **b** Gene ontology classification of downregulated DEGs in PMs of *Flrt2^{ΔMyel}* mice in relative to *Flrt2^{fl/fl}* mice. **c, d** Heat map showing dysregulated genes relevant to macrophage adhesion (**c**) and phagocytosis (**d**) in PMs of *Flrt2^{fl/fl}* and *Flrt2^{ΔMyel}* mice (n = 3 mice per group).

Supplementary Figure 4

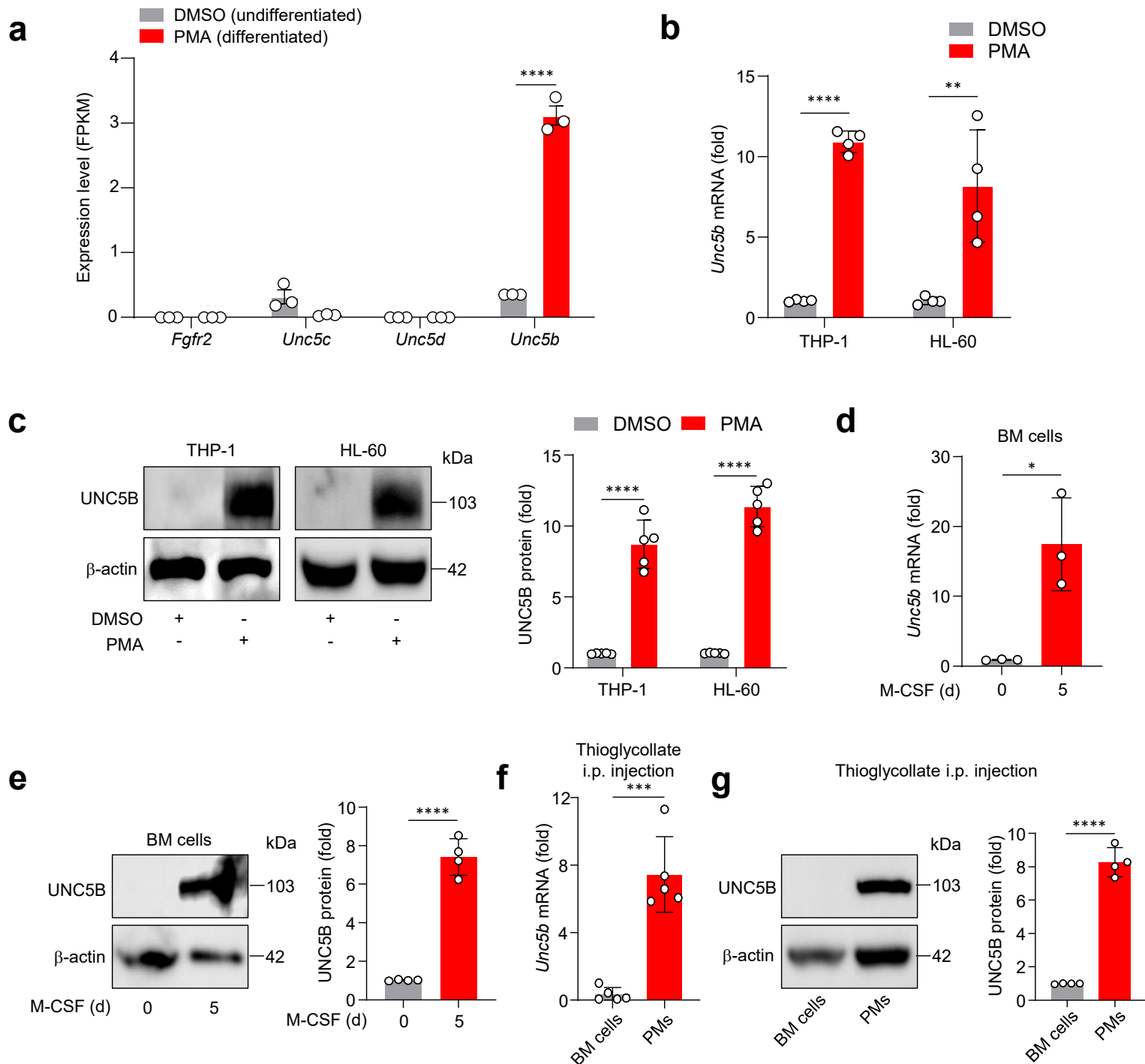


Supplementary Figure 4. FLRT2 promotes THP-1 monocyte differentiation into macrophages and enhances the adhesion, migration, and phagocytosis of differentiated THP-1-derived macrophages. **a, d** Representative phase-contrast light microscopy images showing morphological alterations of THP-1 cells transfected and treated as indicated ($n = 3$). Scale bar, 50 μm . **b, e** qPCR analysis of *Itgam*, *Cd14*, *Csf1r*, *Cd36*, *Msr1*, *Olr1*, and *Flrt2* mRNA in THP-1 cells transfected as indicated ($n = 3$). **c, f** Immunoblot analysis of CD36, SR-A, GFP-FLRT2, and FLRT2 proteins in THP-1 cells transfected as indicated ($n = 3$). **g** HUVECs expressing RFP were co-cultured with THP-1 cells expressing GFP or hFLRT2-GFP. After 6 h co-culture, pictures were taken using a fluorescence microscope ($n = 3$). Scale bar, 5 mm. **h, i** THP-1 cells were transfected with control or hFLRT2 vector for 24 h and treated with 100 ng/ml PMA for an additional 24 h. **h** Transwell cell migration assays were performed, and numbers of migrated cells were counted ($n = 3$). Scale bar, 100 μm . **i** Phagocytosis assay was performed by culturing the cells in Texas red-conjugated zymosan particles for 2 h at 37°C ($n = 3$). Cells were viewed for internalization of the particles by fluorescence microscopy. Scale bar, 50 μm . **j** HUVECs expressing RFP were co-cultured with THP-1 cells expressing GFP-shControl or GFP-shFLRT2 #3. After 6 h co-culture, pictures were taken with a fluorescence microscope ($n = 3$). Scale bar, 5 mm. **k, l** THP-1 cells were transfected with negative control shRNA (shNC) or FLRT2 targeting shRNA #3 (shFLRT2 #3) for 24 h and treated with 100 ng/ml PMA for an additional 24 h. **k** Migration assay was performed as described in (**h**). **l** Phagocytosis assay was performed as described in (**i**). Data are means \pm SD. P values were determined using unpaired, two-tailed Student's t -tests. ND, not detected. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

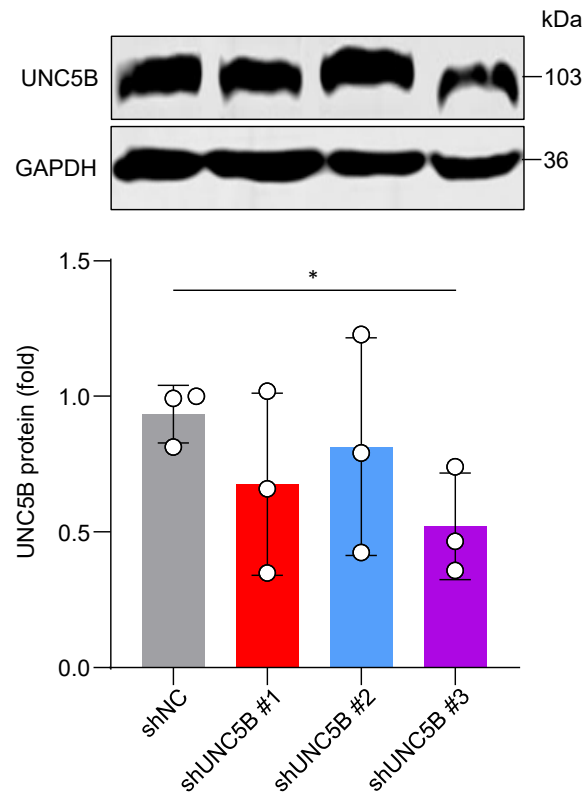


Supplementary Figure 5. FLRT2 is effectively silenced by shFLRT2 #3. Immunoblot analysis of FLRT2 protein of negative control shRNA (shNC) or FLRT2 targeting shRNA (shFLRT2)-transfected HEK293T cells (n = 3). Data are means \pm SD. *P* values were determined using unpaired, two-tailed Student's *t*-tests. ***P* < 0.01.

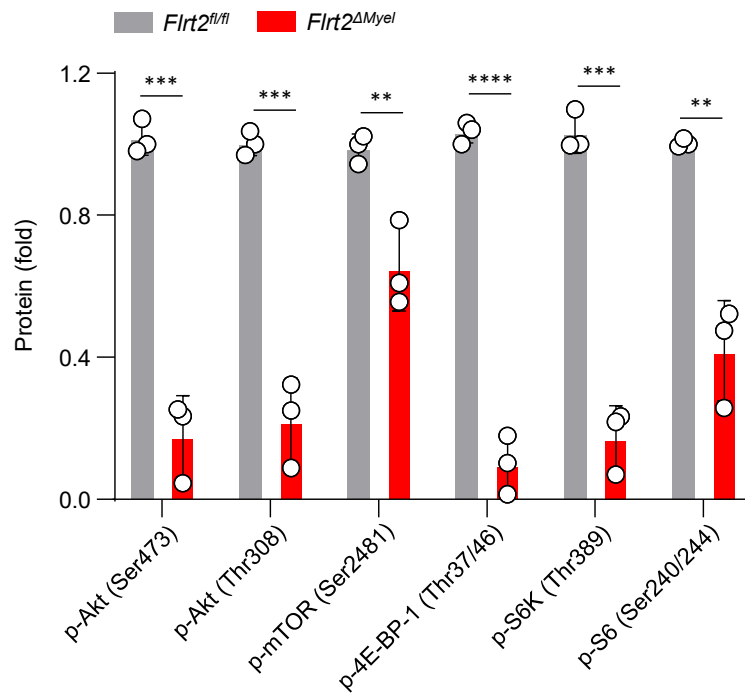
Supplementary Figure 6



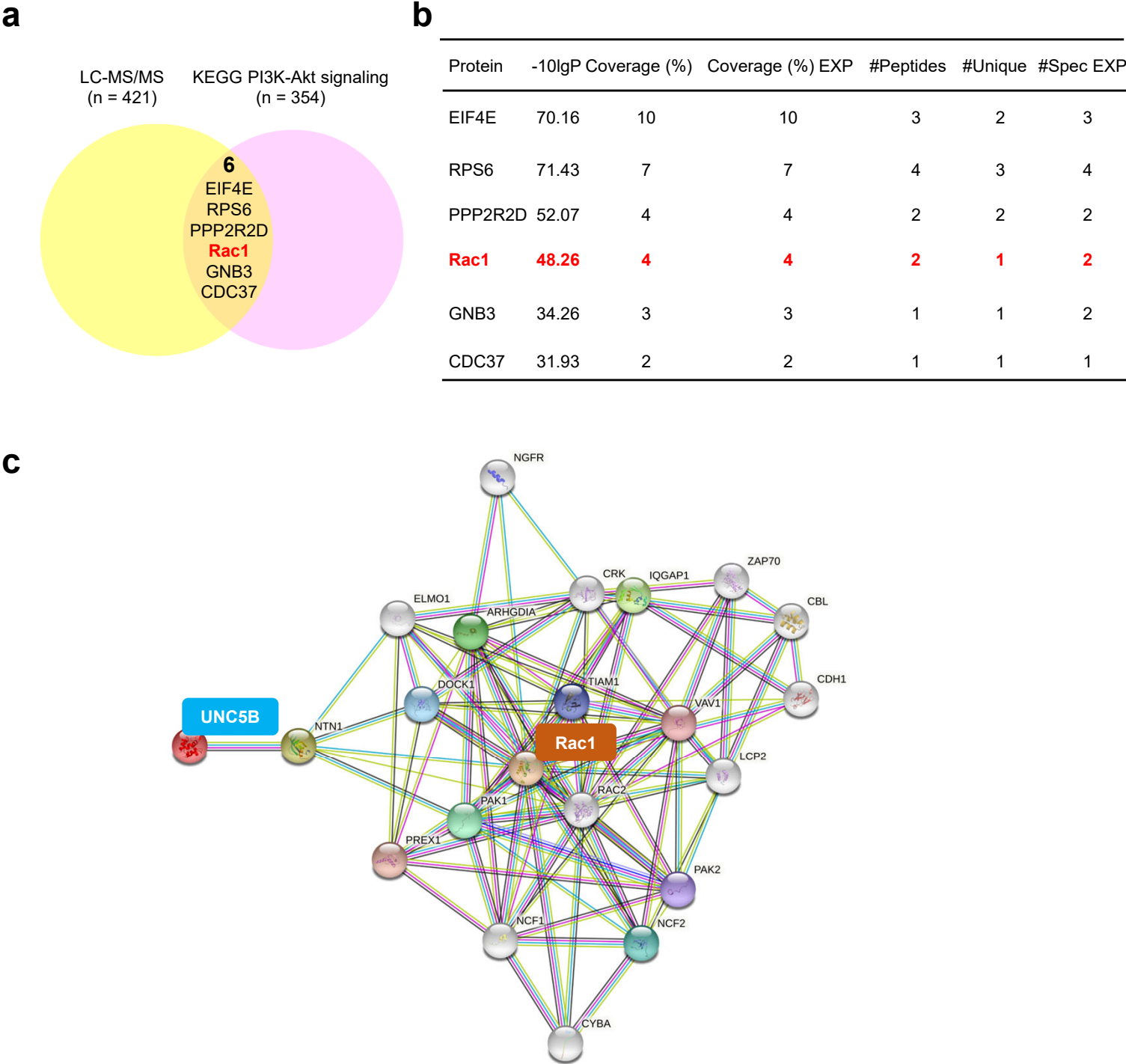
Supplementary Figure 6. UNC5B expression is significantly promoted during monocyte-to-macrophage differentiation. **a** RNA-sequencing analysis of *Fgfr2*, *Unc5c*, *Unc5d*, and *Unc5b* expression in DMSO- and PMA-treated THP-1 cells (n = 3). **b**, **c** qPCR analysis of *Unc5b* mRNA (**b**, n = 4) and immunoblot analysis of UNC5B protein (**c**, n = 5) in THP-1 or HL-60 treated with DMSO or PMA (100 ng/ml) for 24 h. **d**, **e** qPCR analysis of *Unc5b* mRNA (**d**, n = 3) and immunoblot analysis of UNC5B protein in BM cells induced by 25 ng/ml M-CSF for the indicated time (**e**, n = 4). **f**, **g** qPCR analysis of *Unc5b* mRNA (**f**, n = 5 mice per group) and immunoblot analysis of UNC5B protein (**g**, n = 4 mice per group) in BM cells and PMs isolated from C57BL/6J mice after 3 days of i.p. thioglycollate injection. Data are means \pm SD. *P* values were determined using unpaired, two-tailed Student's *t*-tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.



Supplementary Figure 7. UNC5B is effectively silenced by shUNC5B #3. Immunoblot analysis of FLRT2 protein of shNC or UNC5B targeting shRNA (shUNC5B)-transfected HEK293T cells (n = 3). Data are means \pm SD. *P* values were determined using unpaired, two-tailed Student's *t*-tests. **P* < 0.05.

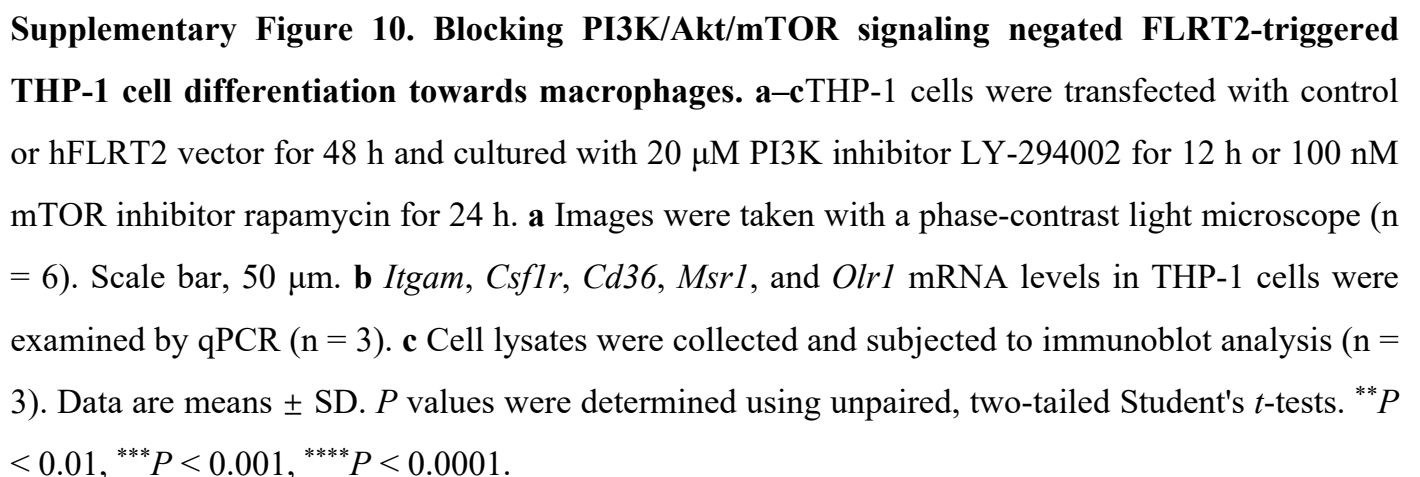


Supplementary Figure 8. Akt/mTOR pathway is inhibited in FLRT2-null PMs (related to main Figure 6b). Quantification of immunoblots of p-Akt (Ser473), p-Akt (Thr308), Akt, p-mTOR (Ser2481), mTOR, p-4E-BP-1 (Thr37/46), 4E-BP-1, p-S6K (Thr389), S6K, p-S6 (Ser240/244), and S6 proteins in PMs isolated from *Flrt2^{fl/fl}* and *Flrt2^{ΔMyel}* mice i.p. injected with thioglycollate for 3 days (n = 3 mice per group). Data are means \pm SD. *P* values were determined using unpaired, two-tailed Student's *t*-tests. ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

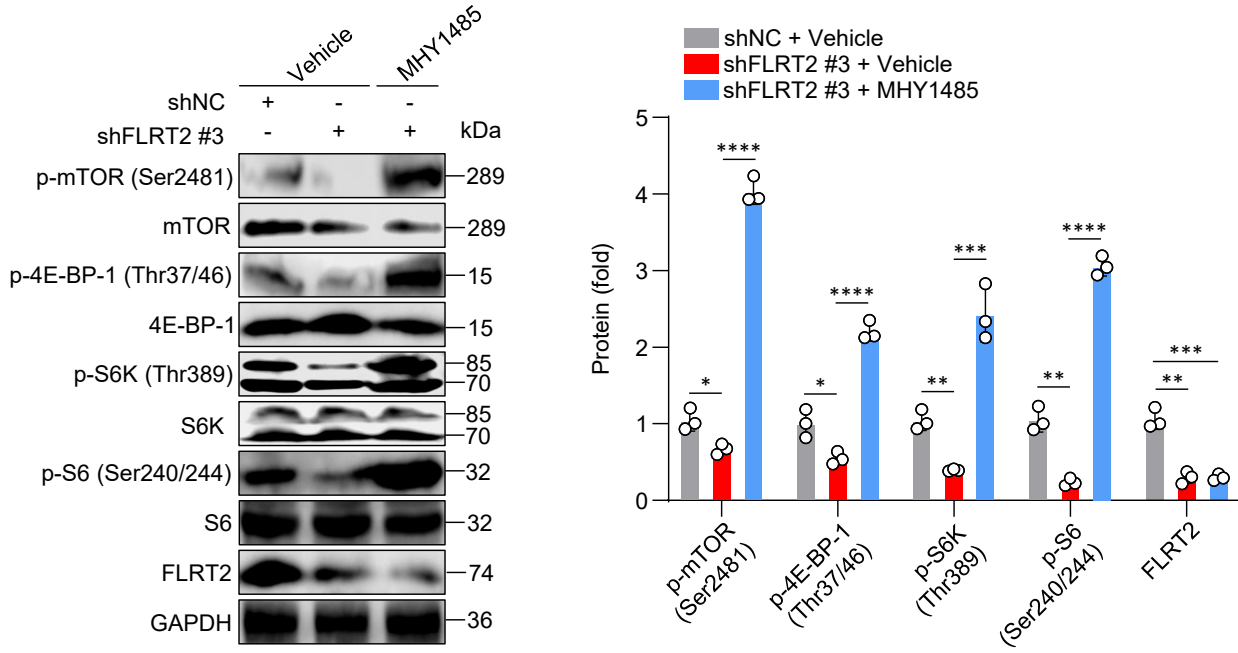


Supplementary Figure 9. UNC5B interacts with Rac1. **a** Venn diagram depicting the overlap of proteins identified by mass spectrometry associated with PI3K-Akt signaling pathway. **b** Mass spectrometry data of 6 overlapping proteins. **c** Protein-protein interaction (PPI) analysis was performed using the STRING database (STRING v11.0) (<https://string-db.org/>).

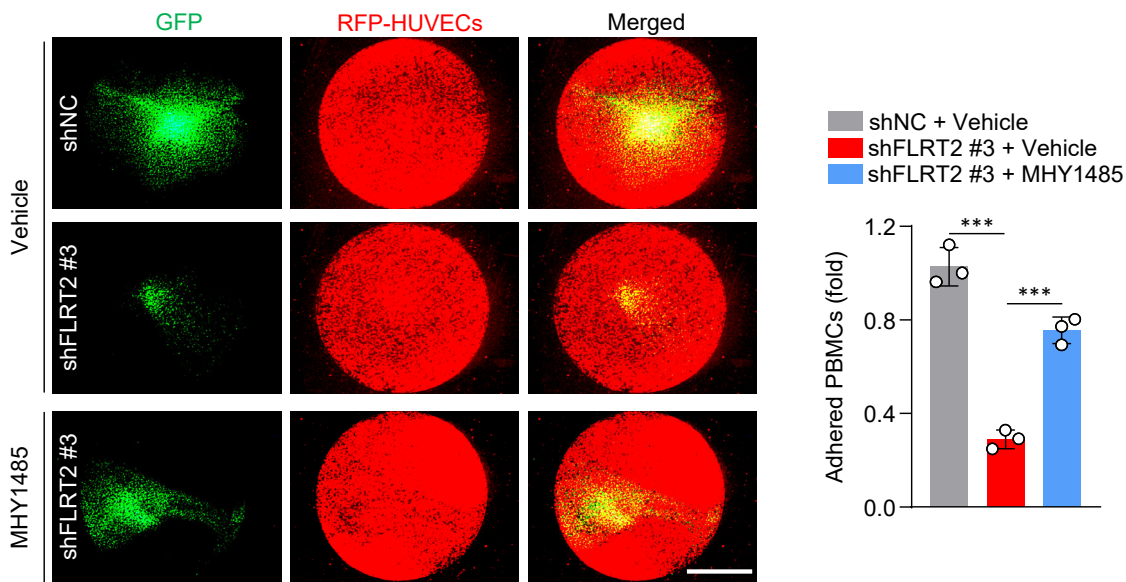
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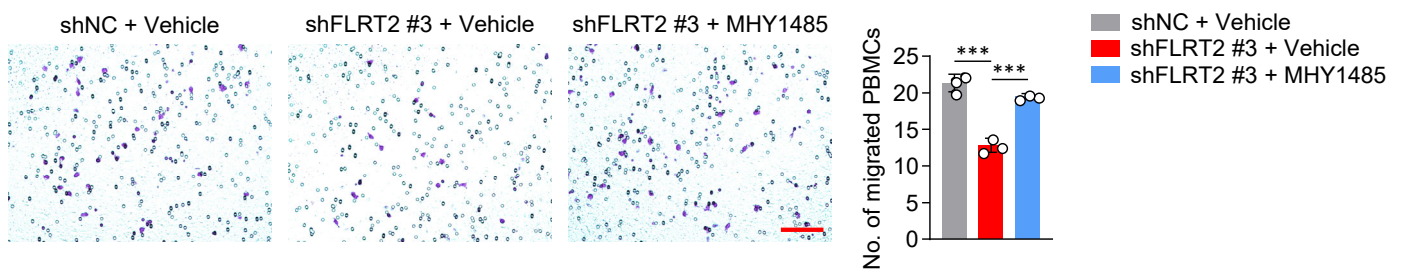
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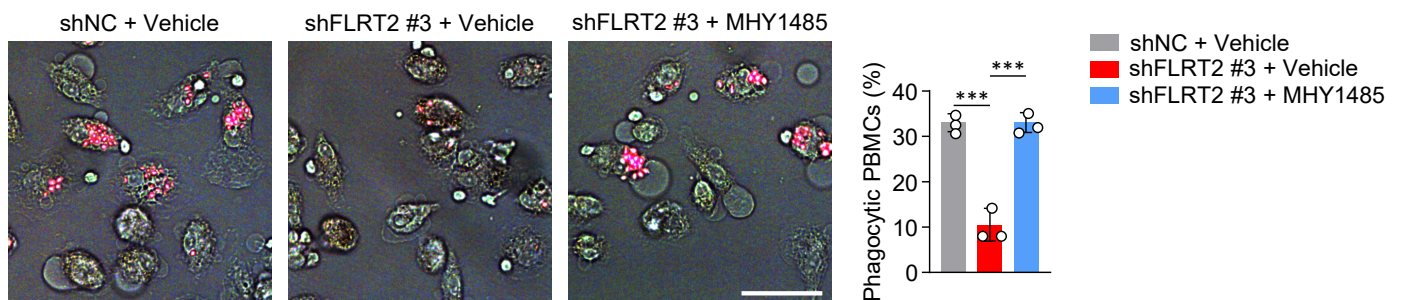
b



c



d



Supplementary Figure 11. MHY1485, an mTOR agonist, improved impaired functions of M-CSF-primed PBMC-derived macrophages with FLRT2 loss. **a–d** PBMCs were transfected with shNC or shFLRT2 for 48 h, and then incubated with 2 μ M MHY1485 for additional 6 h, followed by further analyses. **a** Immunoblot analysis showing p-mTOR (Ser2481), mTOR, p-4E-BP-1 (Thr37/46), 4E-BP-1, p-S6K (Thr389), S6K, p-S6 (Ser240/244), S6, and FLRT2 protein levels in PBMCs indicated above ($n = 3$). **b** PBMCs were co-cultured with HUVECs expressing RFP for 6 h, followed by fluorescent microscopy ($n = 3$ mice per group). Scale bar, 5 mm. **c** Transwell cell migration assays were performed, and numbers of migrated cells were counted ($n = 3$). Scale bar, 100 μ m. **d** Phagocytosis assays were performed by culturing the PBMCs indicated above in Texas red-conjugated zymosan particles for 2 h at 37°C ($n = 3$). Cells were viewed for internalization of the particles by fluorescence microscopy. Scale bar, 50 μ m. Data are means \pm SD. P values were determined using unpaired, two-tailed Student's t -tests. * $P < 0.05$, ** $p < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.