Cell Metabolism, Volume *31*

Supplemental Information

Endothelial Lactate Controls Muscle Regeneration from Ischemia by Inducing M2-like Macrophage Polarization

Jing Zhang, Jonathan Muri, Gillian Fitzgerald, Tatiane Gorski, Roberto Gianni-Barrera, Evi Masschelein, Gommaar D'Hulst, Paola Gilardoni, Guillermo Turiel, Zheng Fan, TongTong Wang, Mélanie Planque, Peter Carmeliet, Luc Pellerin, Christian Wolfrum, Sarah-Maria Fendt, Andrea Banfi, Christian Stockmann, Inés Soro-Arnáiz, Manfred Kopf, and Katrien De Bock

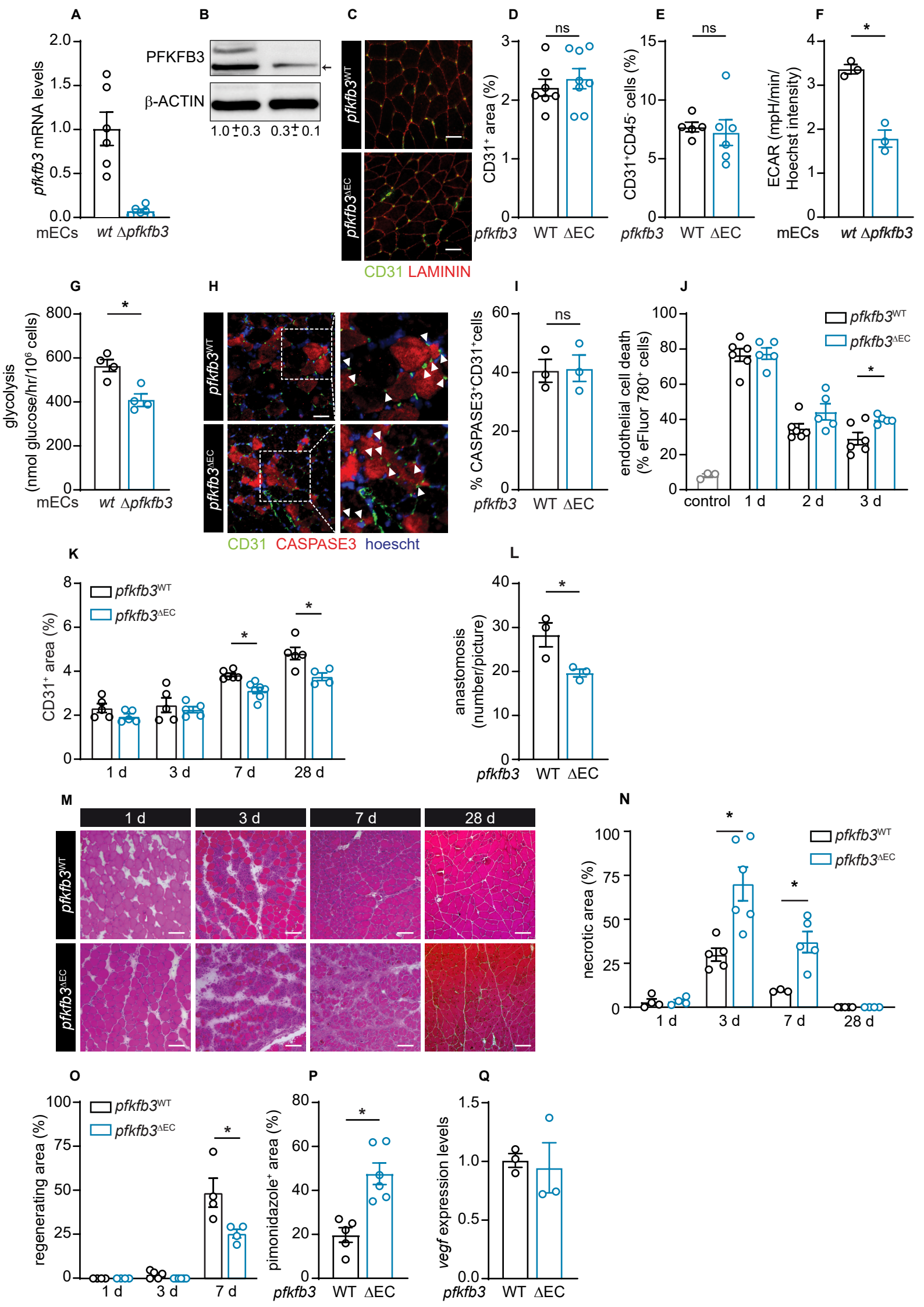
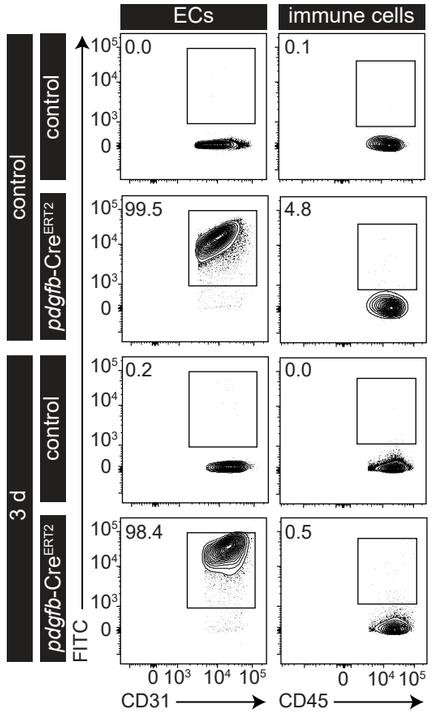

A
pfkfb3 mRNA levels
mECs
wt
Δpfkfb3
B
PFKFB3
β-ACTIN
1.0±0.3
0.3±0.1
C
pfkfb3WT
pfkfb3ΔEC
CD31 LAMININ
D
ns
CD31+ area (%)
pfkfb3
WT
ΔEC
E
ns
CD31+CD45- cells (%)
F
*
ECAR (mpH/min/ Hoechst intensity)
G
*
glycolysis (nmol glucose/hr/10^6 cells)
H
CD31 CASPASE3 hoescht
I
ns
% CASPASE3+CD31+cells
J
endothelial cell death (% eFluor 780+ cells)
control
1 d
2 d
3 d
K
CD31+ area (%)
1 d
3 d
7 d
28 d
L
anastomosis (number/picture)
M
N
necrotic area (%)
O
regenerating area (%)
P
pimonidazole+ area (%)
Q
vegf expression levels

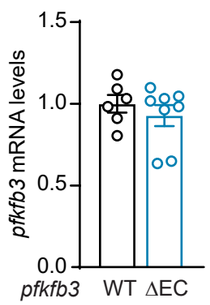
Figure S1. Endothelial PFKFB3 controls EC glycolysis and ischemia induced revascularization. Related to Figure 1.

(**A**-**B**) *Pfkfb3* mRNA (**A**) and PFKFB3 protein (**B**) levels (n=3) in isolated muscle endothelial cells from *pfkfb3*^{WT} and *pfkfb3*$^{\Delta EC}$ mice. PFKFB3 protein levels are corrected for β-ACTIN. The arrow indicates 50 kDa. (**C**-**D**) Representative images of immunostainings for CD31 (green) and laminin (red) (**C**) and quantification of CD31$^+$ area (**D**) in *pfkfb3*^{WT} and *pfkfb3*$^{\Delta EC}$ muscle. (**E**) Flow cytometry based quantification of the CD31$^+$CD45$^-$ endothelial cell population (% of total mononuclear population) in muscle of *pfkfb3*^{WT} and *pfkfb3*$^{\Delta EC}$ mice. (**F**-**G**) Extracellular acidification rate (ECAR) (**F**) and glycolytic flux (**G**) in mECs isolated from *pfkfb3*^{WT} and *pfkfb3*$^{\Delta EC}$ mice. (**H**-**I**) Representative images of CASPASE3 (red), CD31 (green) and hoechst (blue) immunostainings (**H**) and quantification (**I**) of CASPASE3$^+$CD31$^+$ cells in muscles 1 day after HLI. (**J**) Quantification of efluor780$^+$CD31$^+$CD45$^-$ dead endothelial cells in the control leg and in ischemic muscle at the indicated times. (**K**) Quantification of CD31$^+$ area on transverse cross sections of muscles harvested at the indicated times. (**L**) Quantification of anastomosis on longitudinal sections of muscles harvested 28 d after HLI. (**M**-**O**) Representative images of low magnification hematoxylin-eosin (H&E) stainings (**M**) and quantification of necrotic (**N**) and regenerating (**O**) areas at the indicated times. (**P**) Quantification of pimonidazole positive area 3 d after HLI. (**Q**) Quantification of *Vegf* gene expression levels in MPCs, isolated 3 d after HLI. Scale bar, 50 μm. Student's *t* test (two-tailed, unpaired) in **A**, **D**, **E**, **F**, **G**, **I**, **J**, **L**, **P**, **Q** (*$p \leq 0.05$; ns, not significant). Two-way ANOVA with Tukey's multiple comparisons test in **K**, **N**, **O** (*$p < 0.05$). Each dot represents a single mouse. Bar graphs represents mean ± SEM.

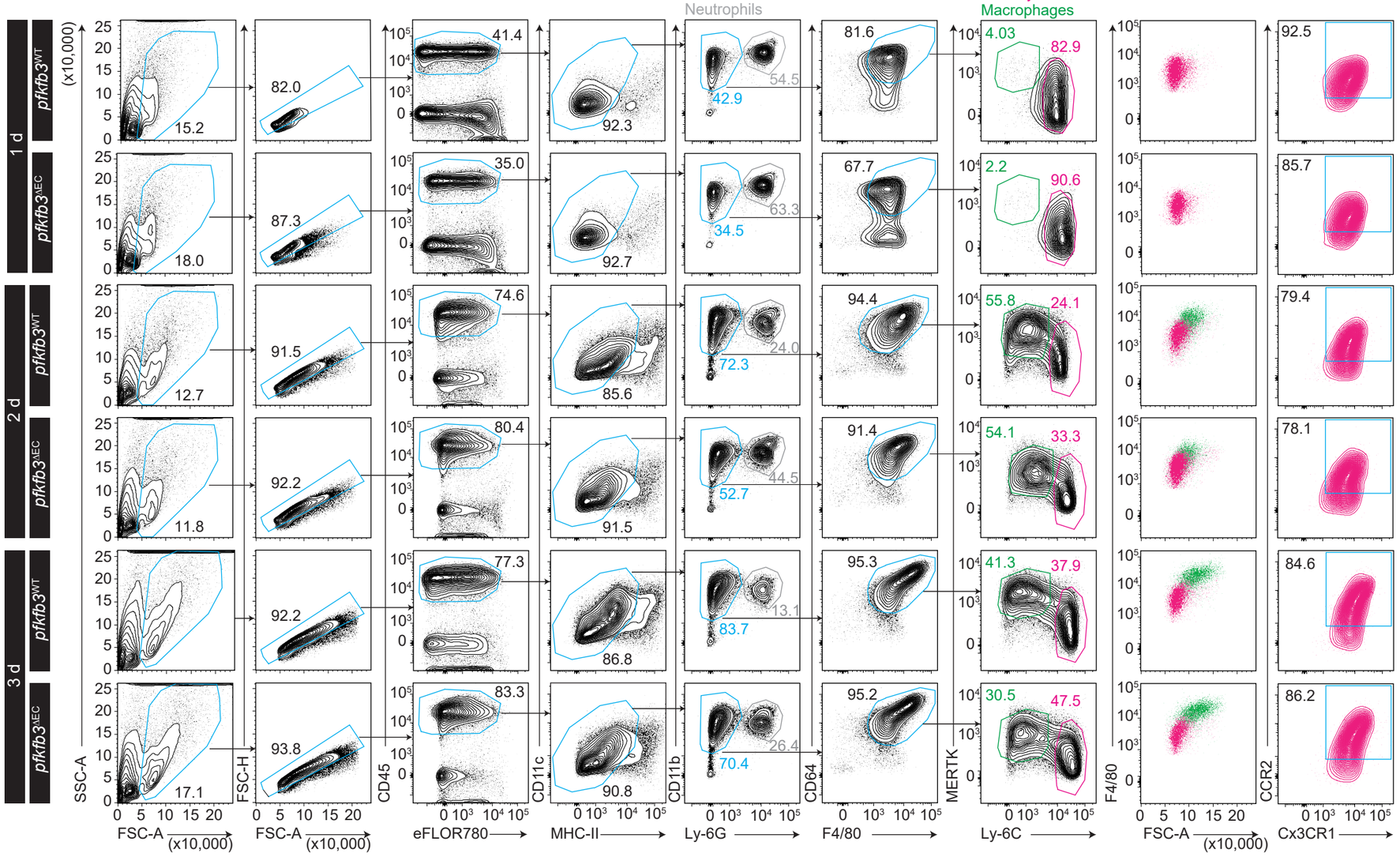
A


ECs
immune cells
control
pdgfb-CreERT2
3 d
0.0
0.1
99.5
4.8
0.2
0.0
98.4
0.5
FITC
CD31
CD45

B


pfkfb3 mRNA levels
1.5
1.0
0.5
0.0
pfkfb3
WT
ΔEC

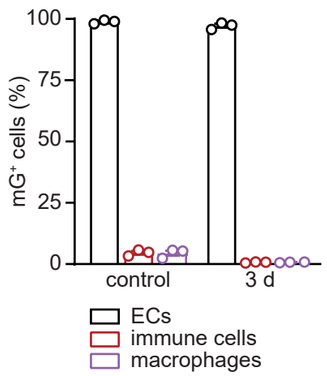
C


1 d
2 d
3 d
pfkfb3WT
pfkfb3ΔEC
(x10,000)
Neutrophils
Monocytes
Macrophages
15.2
82.0
41.4
92.3
54.5
42.9
81.6
4.03
82.9
18.0
87.3
35.0
92.7
63.3
34.5
67.7
2.2
90.6
12.7
91.5
74.6
85.6
24.0
72.3
94.4
55.8
24.1
11.8
92.2
80.4
91.5
44.5
52.7
91.4
54.1
33.3
17.1
93.8
83.3
86.8
13.1
83.7
95.3
41.3
37.9
90.8
26.4
70.4
95.2
30.5
47.5
SSC-A
FSC-A
FSC-H
CD45
CD11c
CD11b
Ly-6G
CD64
MERTK
Ly-6C
F4/80
CCR2
eFLOR780
MHC-II
Cx3CR1

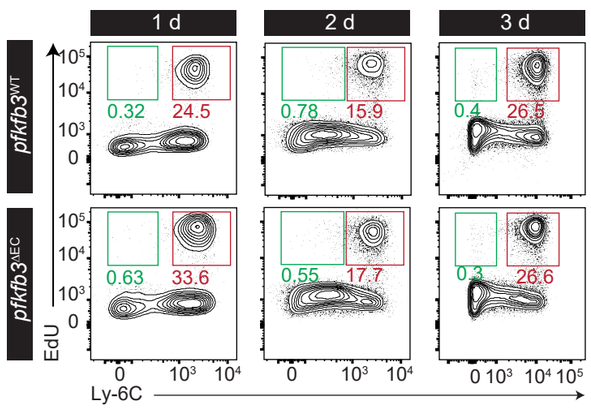
D

E

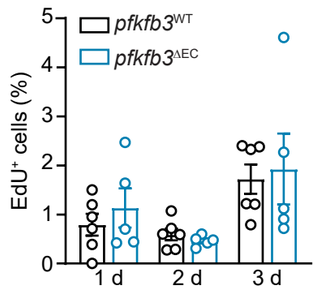
92.5
85.7
79.4
78.1
84.6
86.2


mG+ cells (%)
100
75
50
25
0
control
3 d
ECs
immune cells
macrophages

F


1 d
2 d
3 d
pfkfb3WT
pfkfb3ΔEC
0.32
24.5
0.78
15.9
0.4
26.5
0.63
33.6
0.55
17.7
0.3
26.6
EdU
Ly-6C

G


pfkfb3WT
pfkfb3ΔEC
EdU+ cells (%)
5
4
3
2
1
0
1 d
2 d
3 d

H

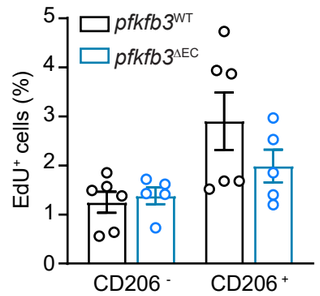

pfkfb3WT
pfkfb3ΔEC
EdU+ cells (%)
5
4
3
2
1
0
CD206 -
CD206 +

Figure S2. Endothelial PFKFB3 is crucial for M2-like polarization of macrophages in the muscle. Related to Figure 2.

(**A**) Flow cytometry analysis (**top**) and quantification (**bottom**) of $mGFP^+CD31^+$ cell and $mGFP^+CD45^+$ cell distribution in the muscle of *pdgfb-Cre*^{ERT2} x *rosa*^{mTmG} mice and their WT littermates before and 3 d after HLI. ECs, $CD31^+$; immune cells, $CD45^+$; macrophages, $CD45^+CD11b^+F4/80^+CD64^+Ly\text{-}6G^-Ly\text{-}6C^-$. (**B**) *Pfkfb3* gene expression in $CD45^+$ cells sorted from *pfkfb3*^{WT} and *pfkfb3*$^{\Delta EC}$ muscle 12 h after HLI. (**C**) Gating strategy for neutrophils, monocytes and macrophages isolated from ischemic muscle at the indicated time. (**D**) F4/80 expression and cellular size (FSC-A) of macrophages (green) and monocytes (pink) gated as described in (C). (**E**) CCR2 and CX3CR1 expression on monocytes (pink) gated as described in (C). (**F**) Flow cytometry analysis of EdU^+ macrophage ($Ly\text{-}6C^-$) and monocyte ($Ly\text{-}6C^+$) isolated from ischemic muscle at the indicated time. Shown are representative FACS plots pre-gated on $CD45^+CD11c^-MHC\text{-}II^-Ly\text{-}6G^-CD11b^+CD64^+F4/80^+$. (**G**) Quantification of EdU^+ macrophages (% of total macrophages) in muscle at the indicated times after HLI determined by flow cytometry. (**H**) Quantification of EdU^+ $CD206^+$ macrophages and EdU^+ $CD206^-$ macrophages in muscle 72 h after HLI. Student's *t* test (two-tailed, unpaired) in **B** ($^*p < 0.05$). Two-way ANOVA with Tukey's multiple comparisons test in **G**, **H** ($^*p < 0.05$). Each dot represents a single mouse (**B**, **G**, **H**). Bar graphs represents mean ± SEM.

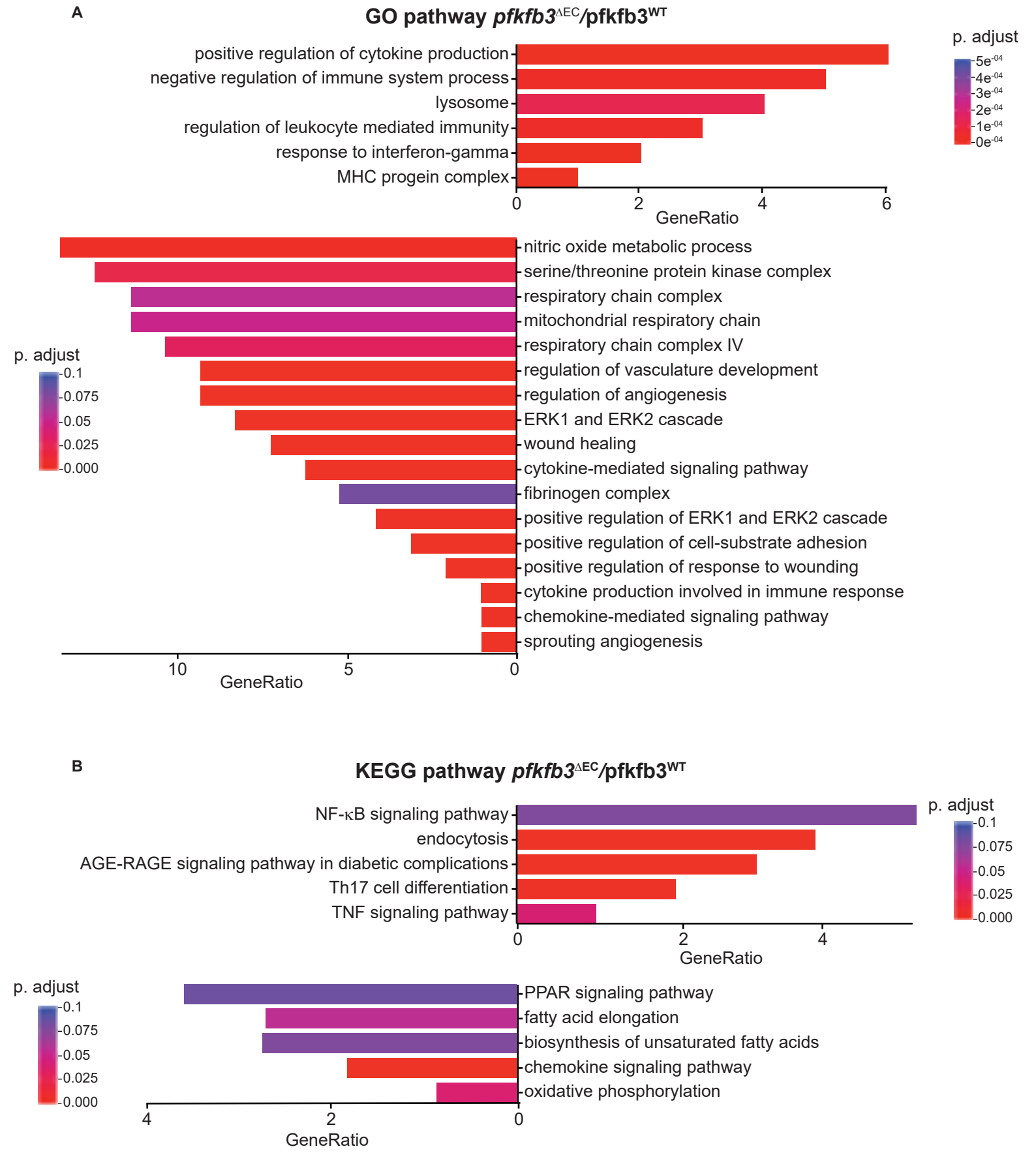

A
GO pathway pfkfb3ΔEC/pfkfb3WT
positive regulation of cytokine production
negative regulation of immune system process
lysosome
regulation of leukocyte mediated immunity
response to interferon-gamma
MHC progein complex
0
2
4
6
GeneRatio
p. adjust
5e-04
4e-04
3e-04
2e-04
1e-04
0e-04
nitric oxide metabolic process
serine/threonine protein kinase complex
respiratory chain complex
mitochondrial respiratory chain
respiratory chain complex IV
regulation of vasculature development
regulation of angiogenesis
ERK1 and ERK2 cascade
wound healing
cytokine-mediated signaling pathway
fibrinogen complex
positive regulation of ERK1 and ERK2 cascade
positive regulation of cell-substrate adhesion
positive regulation of response to wounding
cytokine production involved in immune response
chemokine-mediated signaling pathway
sprouting angiogenesis
10
5
0
GeneRatio
p. adjust
0.1
0.075
0.05
0.025
0.000
B
KEGG pathway pfkfb3ΔEC/pfkfb3WT
NF-κB signaling pathway
endocytosis
AGE-RAGE signaling pathway in diabetic complications
Th17 cell differentiation
TNF signaling pathway
0
2
4
GeneRatio
p. adjust
0.1
0.075
0.05
0.025
0.000
PPAR signaling pathway
fatty acid elongation
biosynthesis of unsaturated fatty acids
chemokine signaling pathway
oxidative phosphorylation
4
2
0
GeneRatio
p. adjust
0.1
0.075
0.05
0.025
0.000

Figure S3. Endothelial PFKFB3 is crucial for M2-like polarization of macrophages in the muscle. Related to Figure 2.

(**A-B**) Gene ontology (GO) (**A**) and KEGG (**B**) pathway analysis for cellular components on macrophages isolated from ischemic muscles 3 d after HLI.

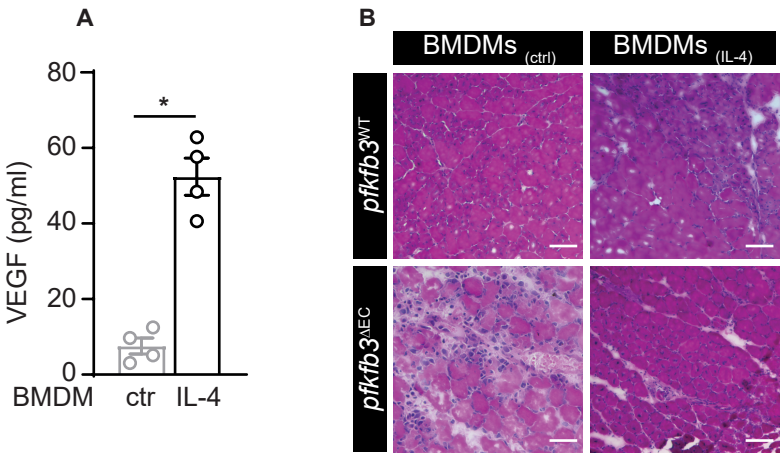

A
80
60
40
20
0
VEGF (pg/ml)
*
BMDM
ctr
IL-4
B
BMDMs (ctrl)
BMDMs (IL-4)
pfkfb3WT
pfkfb3ΔEC

Figure S4. Restoring M2 macrophage content in muscle of *pfkfb3*$^{\Delta EC}$ mice improves muscle perfusion and regeneration. Related to Figure 3.

(**A**) VEGF secretion by BMDMs after stimulation with IL-4. (**B**) Representative low magnification images of hematoxylin-eosin (H&E) staining (scale bar, 50 μm). Student's *t* test (two-tailed, unpaired) in **A** (*$p < 0.05$). Each dot represents an independent experiment (**A**). Bar graph represents mean ± SEM.

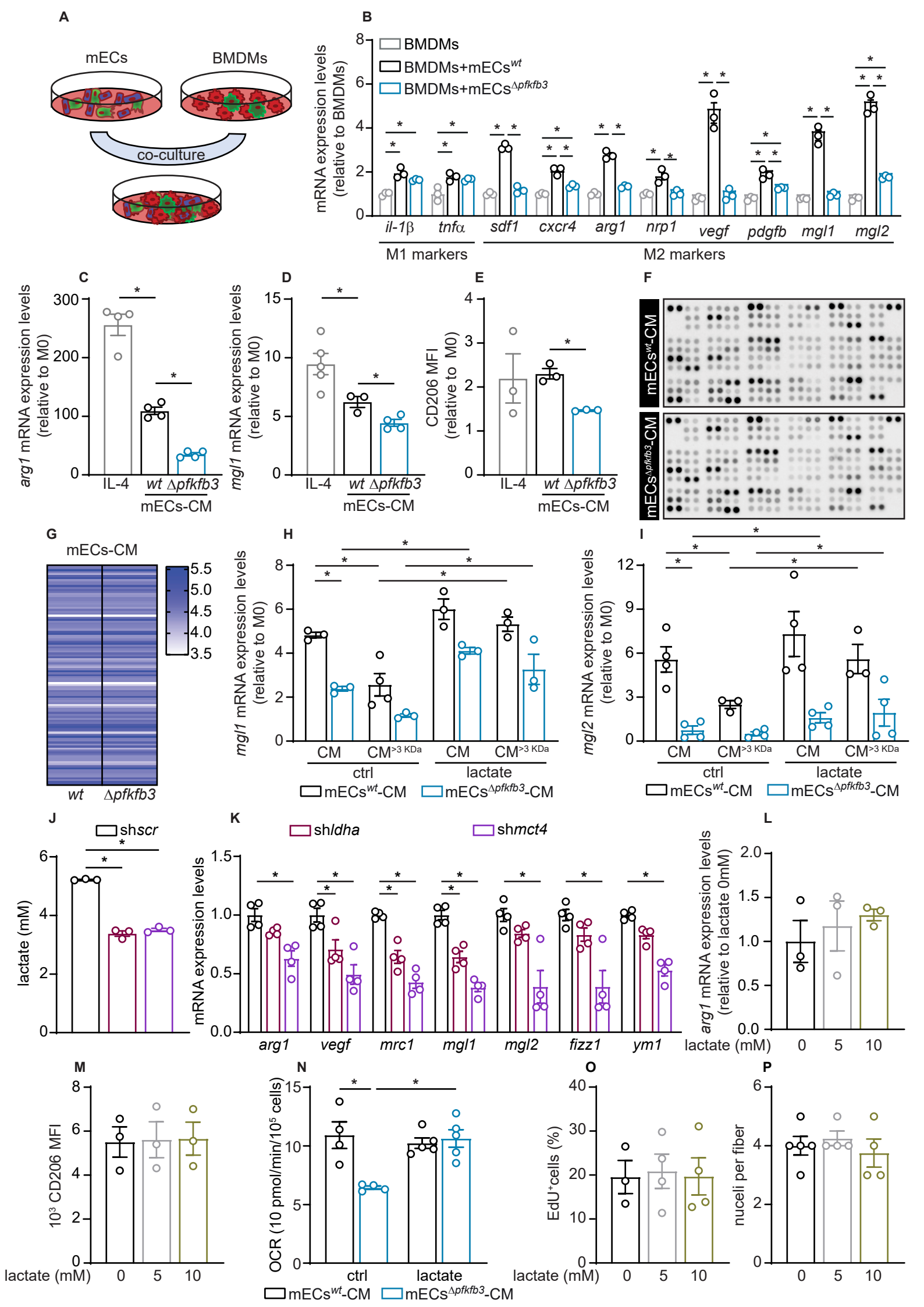

A
mECs
BMDMs
co-culture
B
BMDMs
BMDMs+mECs^wt
BMDMs+mECs^Δpfkfb3
mRNA expression levels (relative to BMDMs)
il-1β
tnfα
sdf1
cxcr4
arg1
nrp1
vegf
pdgfb
mgl1
mgl2
M1 markers
M2 markers
C
arg1 mRNA expression levels (relative to M0)
IL-4
wt Δpfkfb3
mECs-CM
D
mgl1 mRNA expression levels (relative to M0)
E
CD206 MFI (relative to M0)
F
mECs^wt-CM
mECs^Δpfkfb3-CM
G
mECs-CM
wt
Δpfkfb3
H
mgl1 mRNA expression levels (relative to M0)
CM
CM>3 KDa
ctrl
lactate
mECs^wt-CM
mECs^Δpfkfb3-CM
I
mgl2 mRNA expression levels (relative to M0)
J
shscr
lactate (mM)
K
shldha
shmct4
mRNA expression levels
arg1
vegf
mrc1
mgl1
mgl2
fizz1
ym1
L
arg1 mRNA expression levels (relative to lactate 0mM)
lactate (mM)
M
10³ CD206 MFI
N
OCR (10 pmol/min/10⁵ cells)
O
EdU⁺cells (%)
P
nuceli per fiber

Fig. S5. Endothelial lactate controls macrophage polarization and function upon muscle ischemia. Related to Figure 4.

(**A**) Schematic illustration of co-culture of mECs with BMDMs. (**B**) Gene profiling of $CD11b^{+}F4/80^{+}$ BMDMs 24 h after co-culture of BMDMs with mECs isolated from *pfkfb3*^{WT} and *pfkfb3*$^{\Delta EC}$ mice. (**C**-**D**) *Arg1* (**C**) and *mgl1* (**D**) gene expression in BMDMs after stimulation with IL-4 or mECs-CM. (**E**) Quantification of CD206 mean fluorescence intensity (MFI) in BMDMs by flow cytometry after stimulation with IL-4 or mECs-CM. (**F**-**G**) Representative images (**F**) and heat map analysis (**G**) of cytokine profile array of mECs^{wt} –CM and mECs$^{\Delta pfkfb3}$-CM. (**H**-**I**) Gene expression analysis of *mgl1* (**H**) and *mgl2* (**I**) in BMDMs upon incubation with CM derived from mEC^{wt} or mEC$^{\Delta pfkfb3}$. (**J**) Lactate concentration in CM derived from *scr*, *ldha* and *mct4* knockdown HUVECs. (**K**) Gene expression analysis of M2 markers in BMDMs stimulated with *scr*, *ldha* and *mct4* knockdown HUVEC-derived CM. (**L**) *Arg1* gene expression BMDMs cultured in the presence of increasing concentrations of lactate. (**M**) Flow cytometry based quantification of CD206 mean fluorescence intensity (MFI) in BMDMs cultured in the presence of increasing concentrations of lactate. (**N**) Maximal oxygen consumption rate (OCR) of BMDMs after mECs^{wt}-CM, mECs$^{\Delta pfkfb3}$-CM, mECs^{wt+lac}-CM, mECs$^{\Delta pfkfb3+lac}$-CM, mECs^{wt+AZD}-CM, mECs$^{\Delta pfkfb3+AZD}$-CM stimulation. (**O**-**P**) MPC proliferation measured as percentage of EdU^{+} cells (**O**) and fusion analysis after DESMIN labeling (**P**) in the presence of increasing concentrations of lactate. One-way ANOVA with Tukey's multiple comparisons test in **B**, **C**, **D**, **E**, **J**, **K**, **L**, **M**, **O**, **P** (*$p < 0.05$). Two-way ANOVA with Tukey's multiple comparisons test in **G**, **H**, **I, N** (*$p < 0.05$). Each dot represents an independent experiment. Bar graphs represents mean ± SEM.

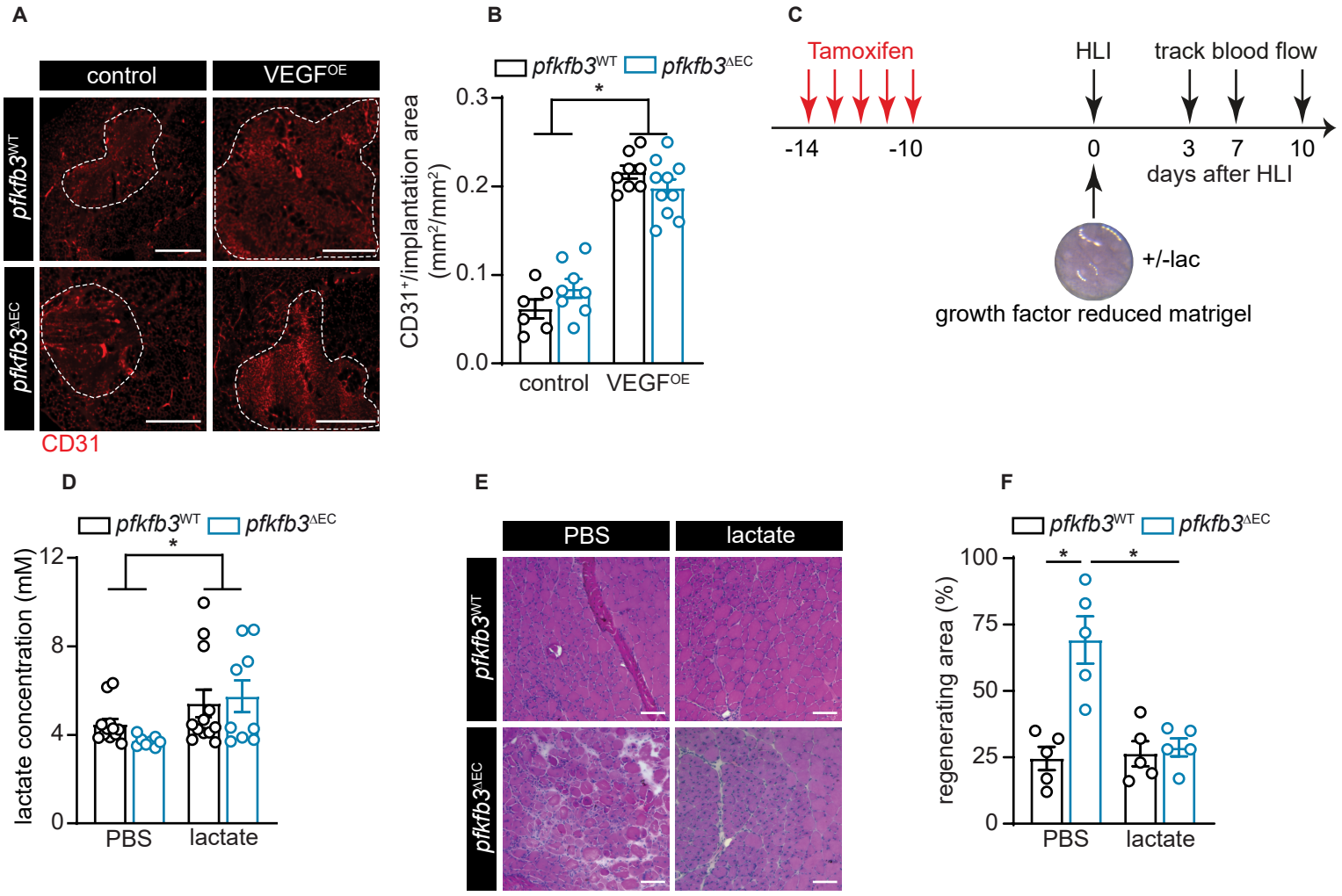

A
control
VEGF^OE
pfkfb3^WT
pfkfb3^ΔEC
CD31
B
pfkfb3^WT
pfkfb3^ΔEC
*
CD31+/implantation area (mm2/mm2)
0.3
0.2
0.1
0.0
control
VEGF^OE
C
Tamoxifen
HLI
track blood flow
-14
-10
0
3
7
10
days after HLI
+/-lac
growth factor reduced matrigel
D
pfkfb3^WT
pfkfb3^ΔEC
*
lactate concentration (mM)
12
8
4
0
PBS
lactate
E
PBS
lactate
pfkfb3^WT
pfkfb3^ΔEC
F
pfkfb3^WT
pfkfb3^ΔEC
*
*
regenerating area (%)
100
75
50
25
0
PBS
lactate

Fig. S6. Increasing muscle lactate levels in *pfkfb3*$^{\Delta EC}$ mice restores M2 macrophage content and improves muscle reperfusion and regeneration. Related to Figure 5.

(**A**-**B**) Representative images of CD31 immunofluorescent staining (**A**) and quantification of CD31^{+} area (**B**) in muscle 12 d after vegf overexpression myoblast injection. (**C**) Schematic illustration of lactate (lac) explant experiments. (**D**) Lactate concentration in serum of *pfkfb3*^{WT} and *pfkfb3*$^{\Delta EC}$ mice obtained 3 d after HLI treated with PBS (control) or lactate. (**E-F**) Representative low magnification images of hematoxylin-eosin (H&E) staining (**E**) and quantification of regenerating area (**F**) 12 days after HLI. Scale bar, 50 μm. Two-way ANOVA with Tukey's multiple comparisons test in **B**, **D**, **F** (*$p < 0.05$). Each dot represents a single mouse (**B**, **D**, **F**). Bar graphs represents mean ± SEM.

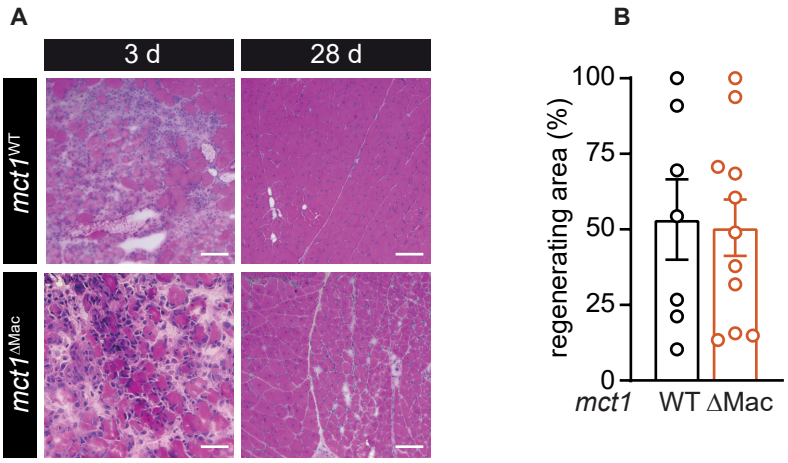

A
3 d
28 d
mct1WT
mct1ΔMac
B
regenerating area (%)
100
75
50
25
0
mct1
WT
ΔMac

Fig. S7. Loss of MCT1 in macrophages impairs M2-like macrophage polarization and muscle recovery from ischemia. Related to Figure 7.

(**A-B**) Representative low magnification images of hematoxylin-eosin (H&E) staining at the indicated time points (**A**) and quantification of regenerating area (**B**) 28 d after HLI. Student's *t* test (two-tailed, unpaired) in **B** (*p < 0.05). Each dot represents a single mouse (**B**). Bar graphs represents mean ± SEM.

Table S1. LC-MS/MS mediated determination of metabolites in conditioned media collected from *wt* and Δ*pfkfb3* mECs after culturing at confluence for 2 days (n=3). Related to Figure 5.

metabolites	**pfkfb3^{WT} (mean ± SEM)**	**pfkfb3$^{\Delta EC}$ (mean ± SEM)**
succinate	16.80 ± 7.60	30.20 ± 6.88
fumarate	5.83 ± 1.58	7.37 ± 0.29
citrate	13.33 ± 2.24	10.07 ± 1.86
glutamate	316.73 ± 66.48	283.23 ± 51.12
malate	18.90 ± 1.00	21.73 ± 1.03
a-KG	1.07 ± 0.61	1.233 ± 0.28
arginine	12.93 ± 0.48	12.27 ± 0.32
aspartate	287.27 ± 27.2	260.3 ± 41.16

Table S2. Sequences of primers used for RT-PCR. Related to Figure 4 and Figure S5 .

gene	Forward	reverse
18s	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA
pfkfb3	TATGAAGCCAGCTACCAGCC	TCTGGATGTGGTCCTGCAC
il-1β	AGTTGACGGACCCCAAAAG	AGCTGGATGCTCTCATCAGG
tnfα	CTCTTCTGTCTACTGAACTTCGG	AAGATGATCTGAGTGTGAGGGT
cxcl10	GCTGCCGTCATTTTCTGC	TCTCACTGGCCCGTCATC
sdf1	CCAAACTGTGCCCTTCAGAT	ATTTCGGGTCAATGCACACT
cxcr4	TGGAACCGATCAGTGTGAGT	GGGCAGGAAGATCCTATTGA
arg1	CCACAGTCTGGCAGTTGGAAG	GGTTGTCAGGGGAGTGTTGATG
nrp1	TCCTGGGAAACTGGTATATCTATGA	CATTCCAGAGCAAGGATAATCTG
vegf	CTTGTTCAGAGCGGAGAAAGC	ACATCTGCAAGTACGTTCGTT
pdgfb	AGCAGAGCCTGCTGTAATCG	GGCTTCTTTCGCACAATCTC
mgl1	CAGAATCGCTT AGCCAATGTGG	TCCCAGTCCGTGTCCGAAC
mgl2	TTCAAGAATTGGAGGCCACT	CAGACATCGTCATTCCAACG
mrc1	CTCTGTTCAGCTATTGGACGC	CGGAATTTCTGGGATTCAGCTTC
ldha	TCTCTGTAGCAGATTTGGCAGA	AAGACATCATCCTTTATTCCGTAAA
mct4	AGCAGGTATCCTTGAGACGG	GATGGCAAAGCAGATGGTGT
mct1	GGGCTAAAGCCACAGTCCAT	TCTGCTAAGTGCCACACAGG