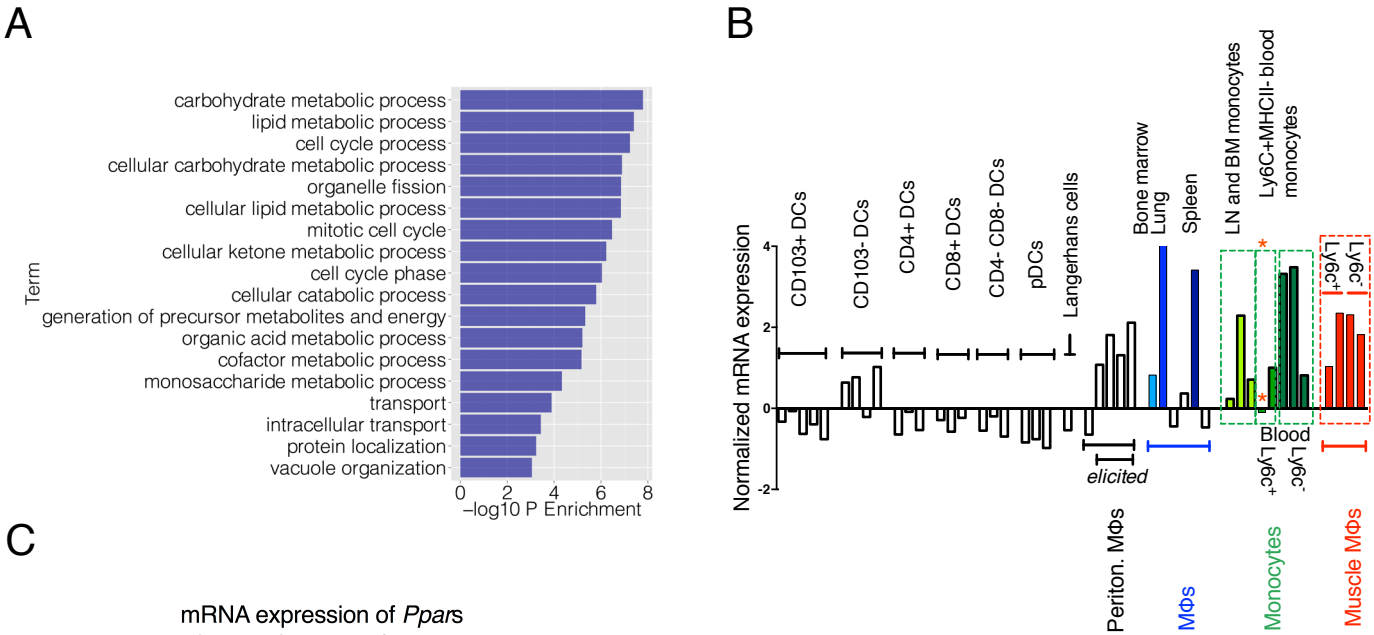
Varga et al. Macrophage PPARγ, a lipid activated transcription factor, controls the growth factor GDF3 and skeletal muscle regeneration

SUPPLEMENTAL MATERIAL

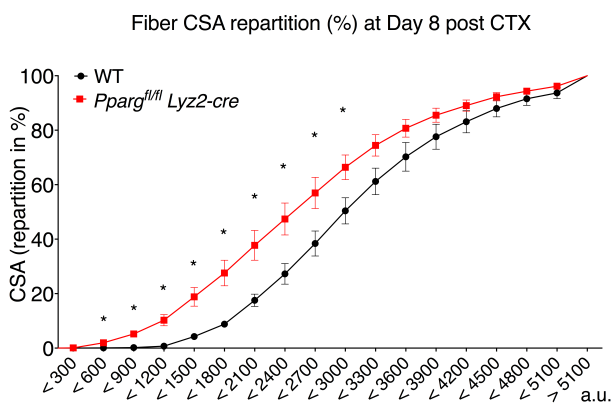


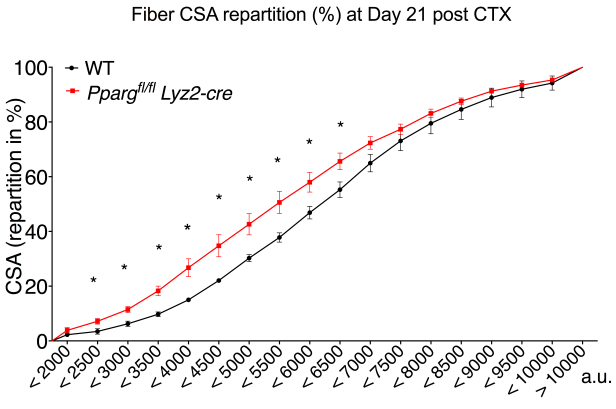
VARGA et al. Suppl Fig 1.

SUPPLEMENTAL FIGURES

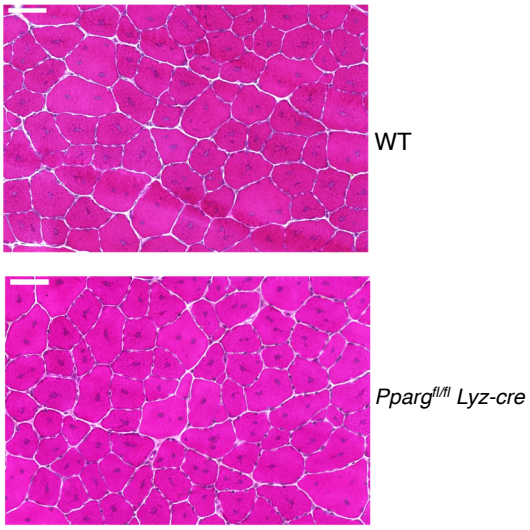
Fig S1. related to Figures 1 and 2. PPARG in muscle infiltrative macrophages during skeletal muscle regeneration (A) GO analysis of the genes that are upregulated as inflammatory Ly6c^{+} macrophages differentiate into regenerative Ly6c^{-} macrophages during muscle regeneration at day 2 past CTX injury. (B) Expression of *Pparg* in various macrophages and dendritic cells. Microarray data derived from muscle derived macrophages isolated for this study and various myeloid cell populations isolated within the Immunological Genome Project were pooled and normalized together (per gene normalization to the median expression level of *Pparg*). A selected set of samples and their normalized expression value are shown. The commonly used macrophage model, bone marrow derived macrophages, are highlighted in light blue, while the high *Pparg* expressing lung macrophage and splenic red pulp macrophage are highlighted in medium and dark blue. The most likely precursor for muscle derived macrophages, Ly6c^{+} monocytes, is labeled with a red asterisk. The detailed description of all cell types is available upon request. (C) Expression of the other *Ppar* isoforms do not suggest the involvement of PPARδ or PPARα (D) Expression of *Pparg* mRNA in day 1 WT and day 2 WT or *Pparg*$^{fl/fl}$ *Lyz2-cre* CD45^{+} cells and in (E) day 2 sorted Ly6c^{+} and Ly6c^{-} MΦs isolated from CTX injured muscle. n=3 for day 1 WT CD45^{+} and n=5 or 4 for day 2 WT or *Pparg*$^{fl/fl}$ *Lyz2-cre* CD45^{+} cells. n=5 and 5 for day 2 Ly6c^{+} or Ly6c^{-} macrophages.

A


Fiber CSA repartition (%) at Day 8 post CTX
WT
Pparg fl/fl Lyz2-cre
CSA (repartition in %)
100
80
60
40
20
0
<300
<600
<900
<1200
<1500
<1800
<2100
<2400
<2700
<3000
<3300
<3600
<3900
<4200
<4500
<4800
<5100
>5100
a.u.


Fiber CSA repartition (%) at Day 21 post CTX
WT
Pparg fl/fl Lyz2-cre
CSA (repartition in %)
100
80
60
40
20
0
<2000
<2500
<3000
<3500
<4000
<4500
<5000
<5500
<6000
<6500
<7000
<7500
<8000
<8500
<9000
<9500
<10000
>10000
a.u.

B


Day 21
WT
Pparg fl/fl Lyz-cre

C

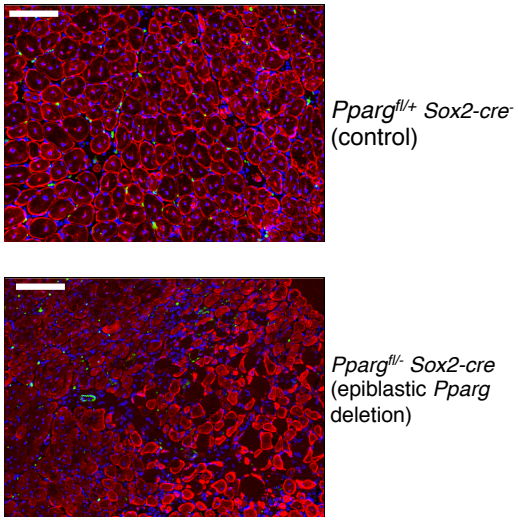
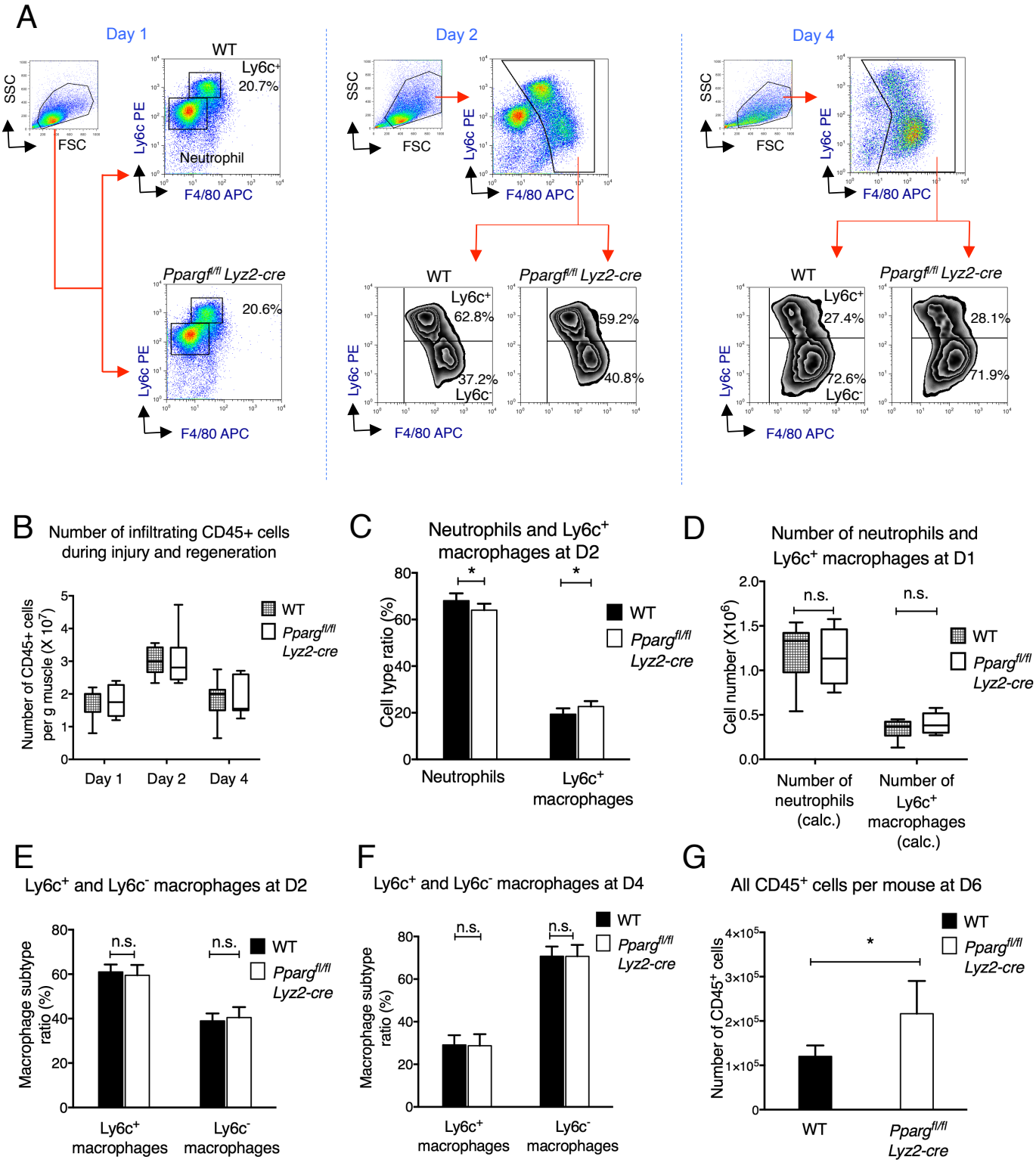
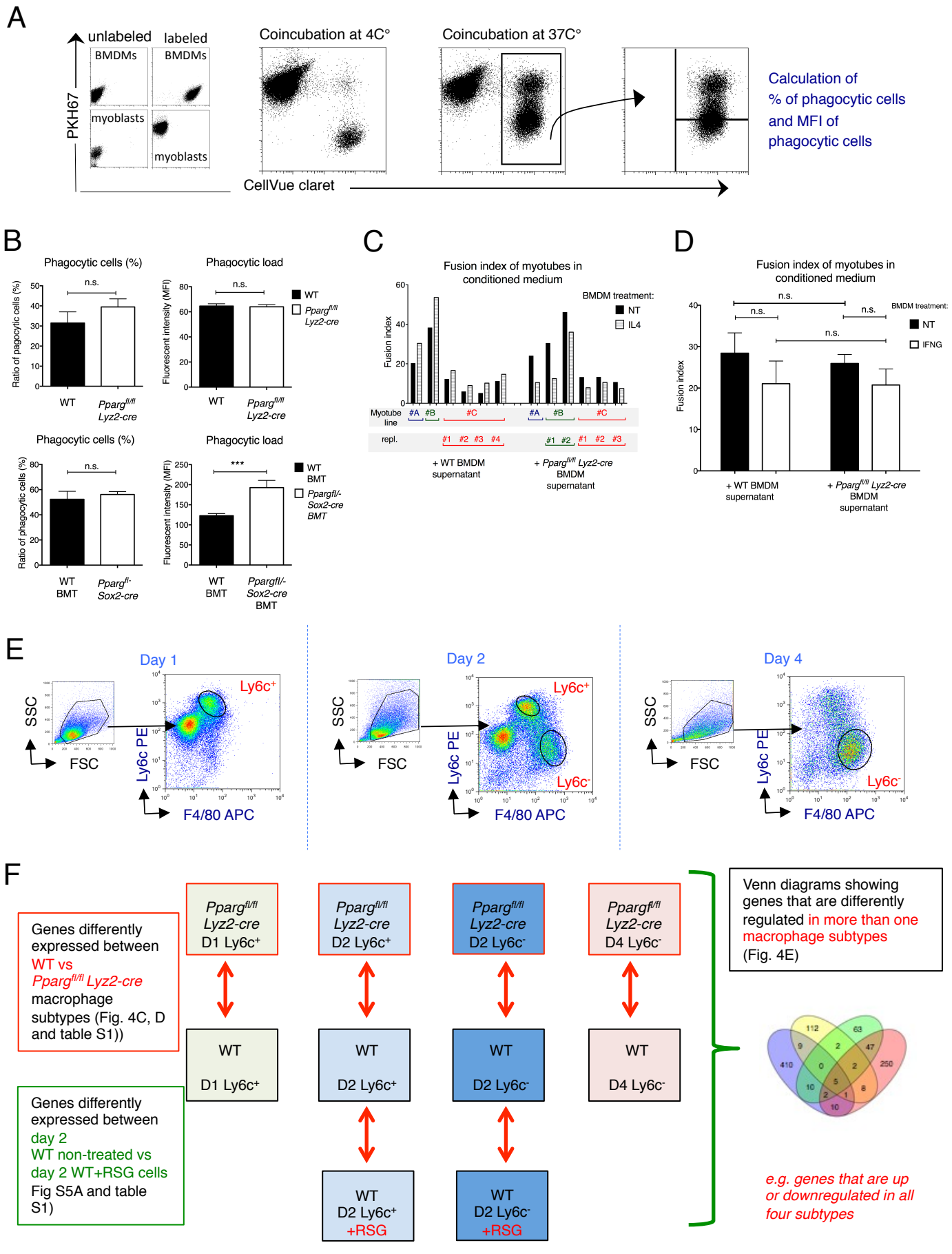

Day 8
Pparg fl/+ Sox2-cre-
(control)
Pparg fl/- Sox2-cre
(epiblastic Pparg
deletion)

Fig S2. related to Figure 1. Additional histological analysis of regeneration. Additional analysis to main Figure 1. (A) Cumulative CSA analysis of muscle section derived from WT or *Pparg*$^{fl/fl}$ *Lyz2-cre* animals at day 8 or day 21 post CTX injury. **(B)** Representative images of HE stained skeletal muscle from WT and *Pparg*$^{fl/fl}$ *Lyz2-cre* animals or post CTX induced injury (day 21) are shown. Scale bars represent 50 μm. n=5 and 6 for the day 8 *Pparg*$^{fl/fl}$ *Lyz2-cre vs.* WT comparison and 5 and 5 for the day 21 comparison. **(C)** IHC of desmin (red), F4/80 (green) and DAPI (blue) on muscle sections from full body *Pparg*$^{fl/+}$ *Sox2-cre*$^{-}$ (controls) and *Pparg*$^{fl/-}$ *Sox2-cre*$^{+}$ animals isolated at day 8 post CTX. Scale bars represent 100 μm.



VARGA et al. Suppl Fig 3.

Fig S3. related to Figure 1. Analysis of macrophage infiltration and dynamics during regeneration (A) FACS gating strategy to enumerate muscle infiltrative neutrophils, and Ly6c^{+} and Ly6c^{-} macrophages at day 1, 2 and 4 post CTX. Representative samples and gate frequencies are shown. (B) Total number of infiltrating CD45^{+} hematopoietic cells isolated from CTX injured muscles of WT and *Pparg*$^{fl/fl}$ *Lyz2-cre* animals at day 1, day 2 and day 4. (D=day) n=8, 12 and 11 for WT animals and 8, 8 and 7 for *Pparg*$^{fl/fl}$ *Lyz2-cre* mice from days 1, 2 or 4. (C) Percentage of neutrophils and Ly6c^{+} macrophages and the (D) calculated neutrophil and macrophage numbers at day 1 in injured muscles. n= 8 for both genotypes. (E and F) Percentage of Ly6c^{+} and Ly6c^{-} macrophages in injured muscles at day 2 and day 4. n=8 for WT and 11 for *Pparg*$^{fl/fl}$ *Lyz2-cre* samples at day 2 and n=12 and 7 for WT and *Pparg*$^{fl/fl}$ *Lyz2-cre* mice at day 4. (G) number of CD45^{+} cells isolated from regenerating muscles at day 6. Bar graphs show mean values +/- SD. n=4 for both genotypes.



VARGA et al. Suppl Fig 4.

Fig S4. related to Figure 2. Analysis of macrophage derived effects on muscle regeneration (A) Experimental strategy to measure *in vitro* phagocytosis in BMDMs. (B) Percentage of phagocytic BMDMs and the Median Fluorescent Intensity (MFI) in the phagocytic BMDM compartment in BMDMs derived from WT *vs.* *Pparg*$^{fl/fl}$ *Lyz2-cre* (upper panel) or WT BMT vs *Pparg*$^{fl/+}$ *Sox-cre* BMT animals (Bottom panel). n=4 for each condition or genotype. FigS4 C, D, E and F provide additional information to main Fig. 2. (C) The pro-differentiation effect of IL4-treated BMDM supernatants on myoblast fusion is independent of the myoblast clone. The experiment was carried out on 3 independently isolated myoblast lines. (D) BMDM PPARγ does not modulate the effect of IFN-γ -treated BMDM supernatants on myoblast fusion. n=3 (E) FACS gating strategy for the sorting of macrophage subsets from CTX injured muscles (F) Schematics of the transcriptomic analyses of sorted muscle derived macrophage populations.

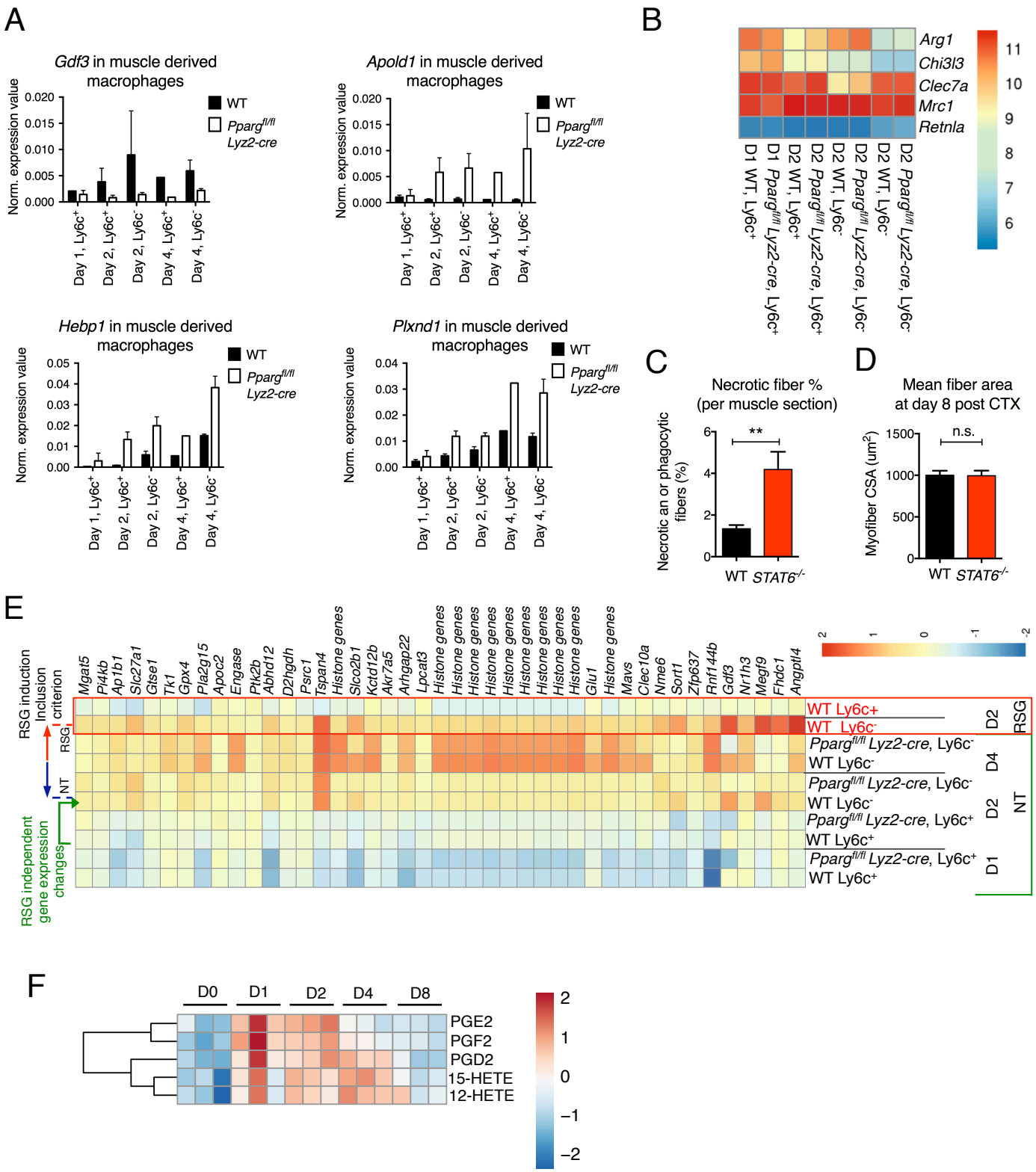
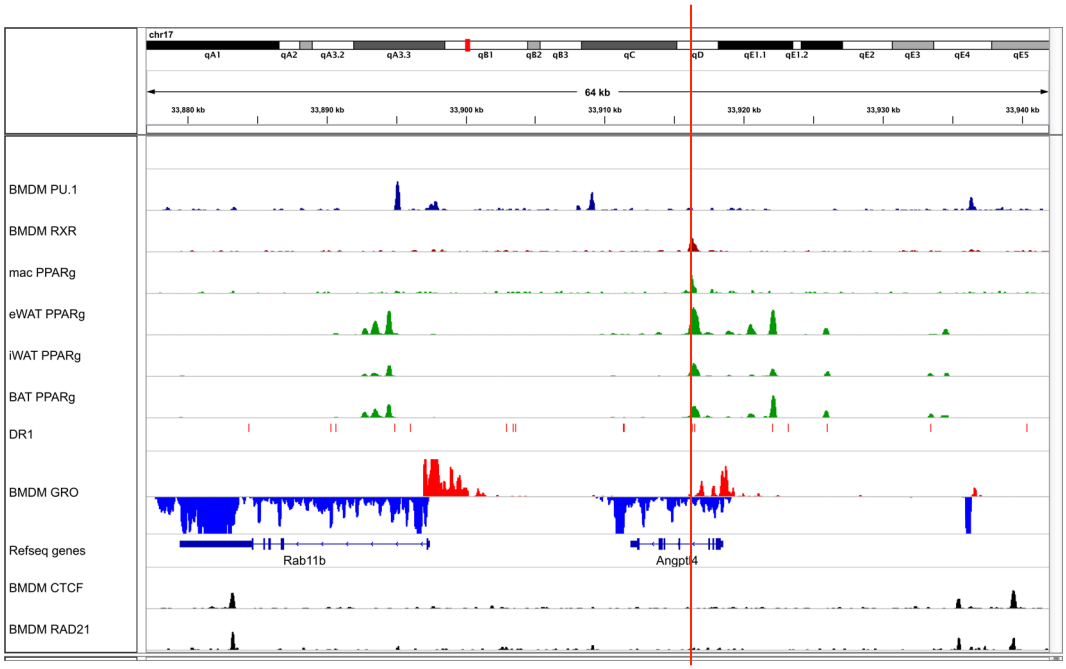

A
Gdf3 in muscle derived macrophages
Apold1 in muscle derived macrophages
Hebp1 in muscle derived macrophages
Plxnd1 in muscle derived macrophages
Norm. expression value
WT
Pparg fl/fl Lyz2-cre
Day 1, Ly6c+
Day 2, Ly6c+
Day 2, Ly6c-
Day 4, Ly6c+
Day 4, Ly6c-
B
Arg1
Chi3l3
Clec7a
Mrc1
Retnla
D1 WT, Ly6c+
D1 Pparg fl/fl Lyz2-cre, Ly6c+
D2 WT, Ly6c+
D2 Pparg fl/fl Lyz2-cre, Ly6c+
D2 WT, Ly6c-
D2 Pparg fl/fl Lyz2-cre, Ly6c-
C
Necrotic fiber %
(per muscle section)
Necrotic an or phagocytic fibers (%)
**
WT
STAT6-/-
D
Mean fiber area
at day 8 post CTX
Myofiber CSA (um2)
n.s.
E
RSG induction
Inclusion criterion
RSG
NT
RSG independent gene expression changes
Mgat5
Pi4kb
Ap1b1
Slc27a1
Gtse1
Tk1
Gpx4
Pla2g15
Apoc2
Engase
Ptk2b
Abhd12
D2hgdh
Psrc1
Tspan4
Histone genes
Slco2b1
Kctd12b
Akr7a5
Arhgap22
Lpcat3
Glu1
Mavs
Clec10a
Nme6
Sort1
Zfp637
Rnf144b
Gdf3
Nr1h3
Megf9
Fhdc1
Angptl4
WT Ly6c+
WT Ly6c-
Pparg fl/fl Lyz2-cre, Ly6c-
WT Ly6c-
Pparg fl/fl Lyz2-cre, Ly6c-
WT Ly6c-
Pparg fl/fl Lyz2-cre, Ly6c+
WT Ly6c+
Pparg fl/fl Lyz2-cre, Ly6c+
WT Ly6c+
D2
D4
D2
D1
RSG
NT
F
D0
D1
D2
D4
D8
PGE2
PGF2
PGD2
15-HETE
12-HETE

Fig S5. related to Figure 2. Additional analysis of the gene expression changes in sorted macrophage populations (A) RT-qPCR validation of the expression pattern of PPARγ-dependent genes in muscle derived sterile inflammatory macrophages. n=2, 8, 10, 1 and 4 (for day 1 Ly6c^{+}, day 2 Ly6c^{+}, day 2 Ly6c^{-}, day 4 Ly6c^{+} (pooled) and day 4 Ly6c^{-} cells) from WT and n= 5, 6, 7, 1 and 4 from *Pparg*$^{fl/fl}$ *Lyz2-cre* mice. (B) Expression of alternative macrophage markers in isolated macrophage subsets (C) STAT6 deficiency increases the number of necrotic/phagocytic fibers after CTX injury. (D) STAT6 deficiency does not impair CSA restoration after CTX injury. n= 6 or 7 for WT or *Stat6*$^{-/-}$ muscles. (E) Heatmap representation of the expression pattern of the genes that are upregulated by RSG treatment in WT Ly6c^{-} cells at day 2. Expression pattern of these genes are shown in all isolated macrophage subtypes. The RSG dependent induction is labeled with red/blue arrows on the left side. The induction in WT untreated day 2 Ly6c^{+} vs. Ly6c^{-} macrophages, which is reminiscent to the induction caused by RSG treatment, is labeled with a green arrow on the left side. Different *Hist2h3* isoforms are labeled as *Histone genes*. (F) Heatmap representation of lipid contents isolated from injured and regenerating muscles.

A



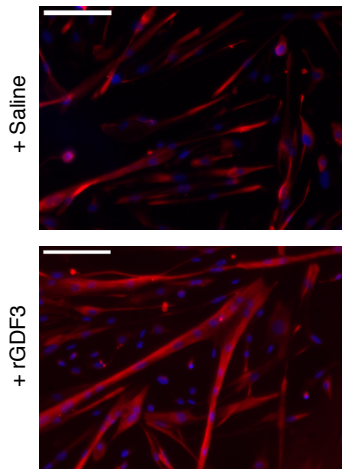
B

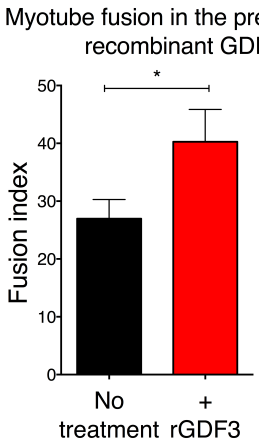
Distance from T.S.S. of *Gdf3*	BMDM PU.1 ChIPseq	BMDM RXR ChIPseq	Macrophage PPARG ChIPseq	Adipocyte PPARG ChIPseq (eWAT/iWAT / BAT)	DR1	BMDM H3K4me3	BMDM CTCF/RAD21
+38 Kb	+	+	-	+	-	+/-	-
+34 Kb	+/-	-	-	-	-	-	-
+16 Kb	+	-	-	-	-	-	-
+11 Kb	+	+	-	-	+	+	-
+10 Kb	+	+/-	-	+/-	-	+	-
+7.3 Kb	-	+/-	-	+/-	+	+	-
+5.4 Kb	+	+/-	-	-	-	+	
Gdf3							
-4.1 Kb	-	-	-	-	-	-	+
-21 Kb	+/-	+/-	+/-	+/-	+		
-25 Kb	+/-	+	+	+	-	-	-
-28 Kb	+/-	+	-	-	-	-	+/-
-40 Kb	+/-	+/-	-	-	-	-	-
-44 Kb	+	+	+/-	-	-	-	-
-47 Kb	+	+	+/-	-	-	-	-

VARGA et al. Suppl Fig 6.

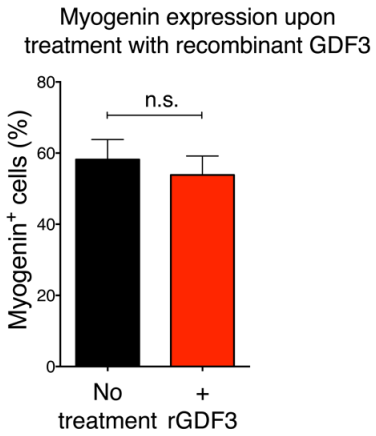
Fig S6. related to Figure 3. Epigenomic analysis of enhancers regulated by PPARγ. Additional supportive information to main Fig. 3. **(A)** Identification of the active, PPARγ -regulated enhancer around the *Angptl4* locus. Red vertical line labels the relevant enhancer. **(B)** Enhancer selection scheme for identifying active enhancers around the *Gdf3* locus.

A





B



C

LIGANDS		LIGANDS	
Gdf1	growth differentiation factor 1	*Inhbc*	inhibin beta-C
Gdf2	growth differentiation factor 2	*Inhbe*	inhibin beta E
Gdf5	growth differentiation factor 5	*Amh*	anti-Mullerian hormone
Gdf6	growth differentiation factor 6	*Nodal*	nodal
Gdf7	growth differentiation factor 7		
Mstn	myostatin	RECEPTORS	
Gdf9	growth differentiation factor 9		
Gdf10	growth differentiation factor 10	*Acvr1c*	activin A receptor, type IC
Gdf11	growth differentiation factor 11	*Acvr2b*	activin receptor IIB
Tgfb2	transforming growth factor, beta 2	*Bmpr1a*	bone morphogenetic protein receptor, type 1A
Tgfb3	transforming growth factor, beta 3	*Bmpr1b*	bone morphogenetic protein receptor, type 1B
Bmp2	bone morphogenetic protein 2	*Amhr2*	anti-Mullerian hormone type 2 receptor
Bmp3	bone morphogenetic protein 3	*Tgfbr3*	transforming growth factor, beta receptor III
Bmp4	bone morphogenetic protein 4	*Tdgf1*	teratocarcinoma-derived growth factor 1
Bmp5	bone morphogenetic protein 5	*Hfe2*	hemochromatosis type 2
Bmp6	bone morphogenetic protein 6		
Bmp7	bone morphogenetic protein 7	ANTAGONISTS	
Bmp8a	bone morphogenetic protein 8a		
Bmp8b	bone morphogenetic protein 8b	*Chrd*	chordin
Bmp10	bone morphogenetic protein 10	*Lefty1*	left right determination factor 1
Bmp15	bone morphogenetic protein 15	*Lefty2*	left right determination factor 2
Inha	inhibin, alpha	*Nog*	noggin
Inhbb	inhibin beta-B	*Sost*	sclerostin

Fig S7. related to Figures 6 and 7. GDF3 and muscle regeneration. Fig. S7A and B provides additional analysis to main Fig. 6 C and D. **(A)** Effect of GDF3 on the fusion of primary myoblasts in an *in vitro* myoblast assay optimized for measurement of the myoblasts fusion step. Representative images (left panel) and fusion indexes (+/- SEM) (right panel) are shown. n=3. **(B)** Lack of induction in myogenin protein expression detected by IHC in differentiating myoblasts in the presence of GDF3 (Mean values +/- SEM). n=3. Fig. S7C provides additional data to main Fig. 7F. **(C)** List of members of the TGF-β superfamily signaling system that are not expressed in muscle derived macrophages.

SUPPLEMENTAL TABLE LEGENDS

Table S1. related to Figure 2. Gene expression analysis of muscle infiltrative macrophages isolated from regenerating muscles of WT and *Pparg*$^{fl/fl}$ *Lyz2-cre* animals. Both RAW data and "per gene normalized (*i.e*. normalized to the median expression value of respective gene") data are shown, after preliminary data processing of microarray data. Worksheet "Day 1 to 4 WT *vs*. PPARγ KO DATA" contains all data derived from macrophages isolated 1, 2 or 4 days post CTX treatment. Worksheet "Day 2 WT NT vs RSG DATA" contains all data derived from day 2 post CTX mice that were treated +/- RSG. "COMP" worksheets contain the list of genes that are differently expressed when the two respective 2 macrophage populations are compared (based on a 2 way ANOVA carried out on the whole data set). Genes with $p \leq 0.05$ were considered significant and are listed in the comparison worksheets.

Table S2. Related to Figure 7. Gene expression analysis of differentiating primary myoblast by RNA-Seq. Expression data from undifferentiated primary myoblasts, myoblasts differentiated for 1 day and myoblasts differentiated for 1 day in the presence of GDF3 were compared. Worksheet "MyB_all_expressed_genes_anova" lists expression values, p values and log2 fold change values for all genes that are differently expressed between any two conditions by 2 way ANOVA ($p \leq 0.05$). Worksheets "Mblast vs D1" and "D1 *vs*. D1 GDF3" contain the list of genes that are differently expressed between the two relevant conditions.

SUPPLEMENTAL MATERIALS AND METHODS

Mice. Genetically modified mice and wild type (WT) C57BL/6 controls were bred under SPF conditions and used for experiments in accordance with Hungarian (license no.: 21/2011/DE MÁB) and European regulations. Experiments were conducted on 2-4 month old male mice. Breeding of genetically modified *Gdf3*$^{-/-}$ and their control C57BL/6 albino animals, and the experiments with them were accepted and conducted with the permission of Sanford Burnham Prebys Medical Discovery Institute at Lake Nona IACUC approval (protocol No. 2014-0107).

Pparg$^{fl/fl}$*Lyz2-cre* and wild type C57BL/6 mice were used in most experiments. They were generated in *Pparg*$^{fl/fl}$*Lyz2-cre* X *Pparg*$^{fl/fl}$*Lyz2-cre* and WT X WT crossings. In a separate experiment, a small cohort of *Pparg*$^{fl/fl}$*Lyz2-cre* and littermate control *Pparg*$^{+/+}$*Lyz2-cre* animals were generated from *Pparg*$^{fl/fl}$*Lyz2-cre* X *Pparg*$^{fl/+}$*Lyz2-cre* crossings. The animals from this latter cohort were CTX injected and HE stained slides generated 8 days post CTX injections were visually evaluated in a double blind fashion. This experiment detected a delay in *Pparg*$^{fl/fl}$*Lyz2-cre* animals (*vs.* WT) that was indistinguishable from the delay seen in the *Pparg*$^{fl/fl}$*Lyz2-cre* samples generated in the non-littermate crossings.

Pparg$^{fl/-}$*Sox2-cre*$^{+}$ and littermate control *Pparg*$^{fl/+}$*Lyz2-cre*$^{-}$ animals were generated in (male) *Pparg*$^{+/-}$*Sox2-Cre*$^{+}$ X (female) *Pparg*$^{fl/fl}$*Sox2-Cre*$^{-}$ crossings.

Gdf3$^{-/-}$ and littermate C57BL/6 albino controls were generated in *Gdf3*$^{+/-}$ X *Gdf3*$^{+/-}$ crossings.

Stat6$^{-/-}$ animals and littermate C57BL/6 controls were generated in *Stat6*$^{-/-}$ X *Stat6*$^{-/-}$ *and* C57BL/6 X C57BL/6 crossings, respectively.

Muscle injury. Mice were anaesthetized with isoflurane and 50 µl of cardiotoxin (12X10^{-6} mol/l in PBS) (from Latoxan) was injected in the *tibialis anterior* (TA) muscle. Muscles were recovered for flow cytometry analysis at day 1, 2 or 4 post-injury or for muscle histology at day 8 post-injury. Glycerol injury was performed according to (Heredia et al., 2013); freeze injury was performed as described in (Hardy et al., 2016); crush injury was performed as in (Mitchell et al., 1992). In some experiments, 300 ng recombinant (r) GDF3 in 50 ul PBS, was injected at day 4 post-CTX into each TA muscle.

Histological analysis of muscle regeneration. Muscles were removed and snap frozen in nitrogen-chilled isopentane (–160°C). 8 µm thick cryosections were cut and stained with hematoxylin-eosin (HE).

Picture capture and counting. For each histological analysis, at least 5 slides (per condition) were selected where the total regernerative region within the CTX injured TA muscle was at least 70%. For each TA, myofibers in at least 3 fields randomly chosen in the entire injured area were counted and measured. HE muscle sections for the day 0, day 8 and day 21 *Pparg*$^{fl/fl}$*Lyz2-cre* vs. WT comparisons were recorded with a Nikon E800 microscope at 20X magnification connected to a QIMAGING camera. Cross-sectional area (CSA) measurement of these samples was carried out using Metamorph software and the CSAs are reported in arbitrary units. HE muscle sections for the day 16 and 20 *Gdf3*$^{-/-}$ BMT *vs*. WT BMT and for the day 22 *Pparg*$^{fl/-}$*Sox2-cre* BMT *vs.* WT BMT samples, the day 8 *Stat6*$^{-/-}$ and the *Pparg*$^{fl/fl}$*Lyz2-cre* + recombinant GDF3 injected

samples, were scanned with Mirax digital slide scanner and the CSA was measured with Panoramic Viewer software. The CSAs for these latter samples are reported in µm. Quantitative analysis of necrotic and/or phagocytic *vs.* centrally nucleated myofibers was performed using the Image J software and was expressed as a percentage of the total number of myofibers. Necrotic myofibers were defined as pink pale patchy fibers and phagocyted myofibers were defined as pink pale fibers, which are invaded by basophil single cells (macrophages).

Immunofluorescent detection of muscle regeneration in day 8 CTX injected muscle: Tissue sections were fixed and permeabilized in ice cold acetone for 5 min and blocked for 30 minutes at 20 °C (room temperature) in PBS containing 2 % bovine serum albumin (BSA). Tissues were stained for 1 h at room temperature using a primary antibody diluted in 2 % BSA. The primary antibodies used for immunofluorescence are listed in Supplementary Table 1. In all cases, the primary antibody was detected using secondary antibodies conjugated to FITC (JIR 712-095-153) or Cy3 JIR (711-165-152). The nuclei were counter stained with 0.1-1 µg/ml Hoechst. Fluorescent microscopy was performed using Carl Zeiss Axio Imager Z2 microscope equipped with lasers at 488, 568 and 633 nm. Figures were analyzed and assembled using Fiji and Illustrator CS5 (Adobe).

List of primary antibodies used in immunofluorescence:

Antibody	**Dilution**	**Source**
Rabbit anti-Desmin	1/200	Abcam (ab32362)
Rat anti-F4/80	1/400	Abcam (ab664)

Macrophage cell culture for conditioned medium generation. Macrophages were obtained from bone marrow (BM) precursor cells. Briefly, total BM was obtained from mice by flushing femurs and tibiae bone marrow with DMEM. Cells were cultured in DMEM medium containing 20% FBS and 30% conditioned medium of L929 cell line (enriched in CSF-1) for 7 days. Macrophages were seeded (at 50000 cell/cm^2 for all experiments) and were activated with IFN-γ (50 ng/ml) and IL4 (10 ng/ml) to obtain M1 and M2 macrophages, respectively, in DMEM containing 10% FBS medium for 3 days. After washing steps, DMEM serum-free medium was added for 24 h, recovered and centrifugated to obtain macrophage-conditioned medium.

Myogenic precursor cell (MPC) culture. Murine MPCs were obtained from TA muscle and cultured using standard conditions in DMEM/ F12 (Gibco Life Technologies) containing 20% FBS and 2% Ultroser G (Pall Inc). Briefly, TA muscles of young mice were opened and cleared of nerves/blood vessels/fascia etc. Muscle preparations were lightly digested with collagenase and the resulting cells were plated then serially expanded. For proliferation studies, MPCs were seeded at 10000 cell/cm^2 on Matrigel (1/10) and incubated for 1 day with macrophage-conditioned medium + 2.5% FBS or with 2.5% FBS medium containing GDF3 mouse recombinant protein (300 ng/ml; R&D 958-G3-010). Cells were then incubated with anti-ki67 antibodies (15580 Abcam), which were subsequently visualized using cy3-conjugated secondary antibodies (Jackson Immunoresearch Inc). For differentiation studies, MPCs were seeded at 30000 cell/cm^2 on Matrigel (1/10) and incubated for 3 days with macrophage-conditioned medium containing 2% horse serum or with 2% horse serum medium containing GDF3 mouse recombinant protein (300 ng/ml; R&D). Cells were then incubated with anti-desmin

antibodies (32362 Abcam), in combination with a cy3-conjugated secondary antibody (Jackson Immunoresearch Inc).

In vitro effects of GDF3 on myogenesis: Myogenic cell differentiation (i.e. myoblast commitment into differentiated myocytes) was evaluated as described earlier (Saclier et al., 2013). Cells were seeded at 30000 cells/cm2 in the presence of the absence of GDF3 (100 ng/ml) for 24h. Myogenin immunostaining (sc-12732, 1/20) was performed and the number of myogenin-positive nuclei was assessed for myogenic differentiation.

Myogenic cell fusion was evaluated as described earlier (Saclier et al., 2013). Cells were first seeded at 5000 cells/cm2 in differentiation medium and the differentiating myocytes were lifted and re-seeded at 75000 cells/cm2 in the presence of the absence of GDF3 (100 ng/ml) for 3 days. The number of nuclei in the myotubes was evaluated after desmin (AB32362 1/200) immunostaining and assessed for myogenic cell fusion.

P-SMAD2 signaling was evaluated in myogenic cell that have been cultured in the presence of the absence of GDF3 (100 ng/ml) for 6h. pSMAD2 (AB3849 Millipore,1/500) immunostaining was performed and the number of positive cells was counted.

Phagocytosis assay: BMDM cells were generated as described earlier in this section. BMDMs were harvested with trypsin and careful scraping, washed twice in PBS and then stained with the lipophilic fluorescent dye CellVue (Sigma) according to the manufacturer's recommendation. Stained BMDMs were replated and let to recuperate for one day in DMEM medium. C2C12 cells were cultured in DMEM containing 10% FBS. Cells were harvested, washed and stained with the lipophilic fluorescent dye PKH67

(Sigma). Stained C2C12 cells were washed extensively and then heat killed at 55°C for 60 min. Heat killed C2C12 cells were added to BMDM cultures at 2:1 ratio and phagocytosis was commence at 37°C or 4°C (controls). Cells were harvested by scraping after 1 h and fluorescent intensity was detected with a FACScalibur instrument.

Isolation of macrophages from muscle: Fascia of the TA was removed. Muscles were dissociated in RPMI containing 0.2% collagenase B (Roche Diagnostics GmbH) at 37°C for 1 hour and filtered through a 100 μm and a 40 μm filter. $CD45^+$ cells were isolated using magnetic sorting (Miltenyi Biotec). For cell sorting, macrophages were treated with Fcγ receptor blocking antibodies and with 10% normal rat serum: normal mouse serum 1:1 mix, then stained with a combination of PE-conjugated anti-Ly6c antibody (HK1.4, eBioscience) and APC-conjugated F4/80 antibody (BM8, eBioscience). $Ly6c^+$ $F4/80^{low}$ macrophages, $Ly6c^-$ $F4/80^+$ macrophages and $Ly6c^{mid}$ $F4/80^-$ neutrophils were sorted. In each experiment, both genotypes were processed in parallel to minimize experimental variation. Cells were analyzed and/or sorted with a BD FACSAria III sorter.

Isolation of macrophages and FAP cells from CTX injured muscles: CTX injured TA muscles were dissected and fat/nerves/fascias/tendons were discarded. Muscles were pulped and treated with a collagenase/dispase cocktail. $CD45^+F4/80^+$ macrophages and $CD45^-CD31^-Sca1^+PDGFRA(CD140)^+$ FAPs were sorted for RNA isolation.

RNA isolation from sorted MFs. MF subsets were sorted from day 1, 2 and 4 post-injury muscles with a FACSAria III sorter and total RNA was isolated with TRIZOL reagent according to the manufacturer's recommendation. 20 ug glycogen (Ambion) was added as a carrier for RNA precipitation.

Microarray analysis of muscle macrophages: Global expression pattern was analyzed on Affymetrix GeneChip Mouse Gene 1.0 ST arrays. Ambion WT Expression Kit (Life Technologies, Hungary) and GeneChip WT Terminal Labeling and Control Kit (Affymetrix) were used for amplifying and labeling 150 ng of total RNA. Samples (n=3, 4 or 5) were hybridized at 45 ºC for 16 h and then standard washing protocol was performed using Affymetrix GeneChip Fluidics Station 450. The arrays were scanned on GeneChip Scanner 7G (Affymetrix). Microarray data (data acess: GSE44057) were analyzed with GeneSpring 12 GX software (Agilent BioTechnologies). Affymetrix CEL files were normalized with Robust Multichip Analysis (RMA) algorithm and median normalization.

The microarray data are publicly available (Data access: GSE71155).

Expression data processing and analysis: Data quality control and analysis was carried out following the recommendations put forward in the Imgen website (http://www.immgen.org/Protocols/ImmGen%20QC%20Documentation_ALL-DataGeneration_0612.pdf).

Data were loaded into the Genespring GX software and RMA summarization was carried out. Next, a set of filtering steps was applied to the dataset. Briefly, data distribution curve was generated and the lowest 5% of the entities with detectable signals were filtered out as not expressed. Duplicate entities, not/poorly annotated transcripts and transcripts reporting inconsistent expression values were also discarded. Further analysis was carried out on the filtered dataset. Data was analyzed either based on the RAW expression values or after following a “per gene” normalization (individual gene expression data normalized to the median of the gene).

Further analysis of gene expression and comparisons were made either within Genespring GX or using the R software package. 2-way anova tests were performed in R using functions aov and TukeyHSD of package MASS, Heatmaps were drawn with package pheatmap. Statistically significant difference was considered if $p < 0.05$.

Microarray validation by RT-qPCR: Transcript quantification was performed by quantitative real-time RT (reverse transcriptase) PCR (polymerase chain reaction) using SYBR Green assays (*Apold1, Hebp1 and Plxnd1*) or PrimeTime assays from IDT (*Gdf3 and Pparg*). Primer sequences and Taqman probes or PrimeTime assay IDs used in transcript quantification are available upon request. RT-qPCR results were analyzed with the standard delta Ct method and results were normalized to the expression of *Actb*.

Identification and quantification of lipid mediators by liquid chromatography-tandem mass spectrometry. Tibialis anterior muscles collected after CTX-induced injury were minced in ice-cold methanol and stored at -80°C. Internal deuterium-labeled standards, including d_8-5-HETE and d_4-PGE_2, were then added to assess extraction recovery and quantification. Solid phase extraction and LC-MS/MS analysis were carried out essentially as described in (Colas et al., 2014). Briefly, lipid mediators were extracted by C18 column chromatography and methyl formate fractions were taken to dryness under a stream of N_2 gas prior to suspension in methanol:water (50:50). Samples were profiled using a high performance liquid chromatograph (HPLC, Shimadzu) coupled to a QTrap5500 mass spectrometer (AB Sciex). The instrument was operated in negative ionization mode and lipid mediators were identified and quantified using multiple reaction monitoring (MRM) transitions (Colas et al., 2014) after normalization to extraction recovery based on internal deuterium-labeled standards and

external calibration curves for each mediator. The specific MRM transitions used were: PGE_2 (351>175), PGD_2 (351>233), $PGF_{2\alpha}$ (351>193), 15-HETE (319>219) and 12-HETE (319>179).

Macrophage cell culture for ChIP: Macrophages were obtained from bone marrow (BM) precursor cells. Briefly, total BM was obtained from mice by flushing femurs and tibiae bone marrow with DMEM. Cells were RBC lysed with ACK solution and then plated on non-tissue culture grade plates then cultured in DMEM medium containing 20% FBS and 30% conditioned medium of L929 cell line (enriched in CSF1) for 6 days. Macrophages were harvested from the culture plates and ChIP was carried out.

ChIP (Chromatin immunoprecipitation): Cells were double crosslinked with 0,002M DSG (Sigma) for 30 minutes and then with 1% formaldehyde (Sigma) for 10 minutes. Nuclei were isolated with ChIP Lysis Buffer (1% Triton x-100, 0.1% SDS, 150 mM NaCl, 1mM EDTA, and 20 mM Tris, pH 8.0) then chromatin were sonicated (also in ChIP Lysis Buffer) with Diagenode Bioruptor to generate 200-1000 bp fragments. Chromatin was diluted in ChIP Lysis buffer and immunoprecipitated with antibodies against pre-immune IgG (Millipore, 12-370), (pan) RXR (sc-774 Santa Cruz Biotechnology) and PPAR gamma (Perseus #PP-A3409A). Chromatin antibody complexes were precipitated with Protein A coated paramagnetic beads (Life Technologies). Chromatin antibody complexes were washed on the beads once in IP Wash Buffer 1 (1% Triton, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, and 0.1% NaDOC), twice in IP Wash Buffer 2 (1% Triton, 0.1% SDS, 500 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, and 0.1% NaDOC) and once in IP Wash Buffer

3 (0.25 M LiCl, 0.5% NP-40, 1mM EDTA, 20 mM Tris, pH 8.0, 0.5% NaDOC) and IP Wash Buffer 4 (10 mM EDTA and 200 mM Tris, pH8.0). DNA fragments were then eluted and column purified (Qiagen, MinElute). DNA was applied for QPCR analysis. QPCR results were analyzed with the standard delta Ct method and results were normalized to input signals.

Bioinformatic analysis of the active enhancers around the *Gdf3* and *Angptl4* locus: Primary analysis of the raw sequence reads has been carried out using our ChIP-seq analysis command line pipeline. Alignment to the mm9 assembly of the mouse genome was done by the Burrows–Wheeler Alignment (BWA) tool. Genome coverage (bedgraph) files were generated by makeTagDirectory and makeUCSCfile.pl (HOMER) and were used for visualization with IGV2. Putative DR1 elements (reaching score 9) were determined by annotatePeaks.pl (HOMER) using the RXR and PPARg motif matrices of HOMER. The following datasets were used for the identification of active enhancers:

Sample name	SRA identifier	GEO identifier	Cell/tissue type	Sample type	Antibody
BMDM_PU.1	SRX651749	-	bone marrow derived macrophage	ChIP-seq	PU.1
BMDM_RXR	SRX651739	-	bone marrow derived macrophage	ChIP-seq	RXR
mac_PPARg	SRX019134	GSM532739	peritoneal macrophage	ChIP-seq	PPARg
eWAT_PPARg	SRX193440	GSM1018066	epididymal white adipose tissue	ChIP-seq	PPARg
iWAT_PPARg	SRX193441	GSM1018067	inguinal white adipose tissue	ChIP-seq	PPARg
BAT_PPARg	SRX193442	GSM1018068	brown adipose tissue	ChIP-seq	PPARg
BMDM_CTCF	SRX651751	-	bone marrow derived macrophage	ChIP-seq	CTCF
BMDM_RAD21	-	-	bone marrow derived macrophage	ChIP-seq	RAD21
BMDM_H3K4me3	SRX651747	-	bone marrow derived macrophage	ChIP-seq	H3K4me3
BMDM_GRO-seq	SRX651735	-	bone marrow derived macrophage	GRO-seq	-

Western Blotting: GDF3 protein expression was measured using Western Blot analysis. The Tibialis anterior (TA) was removed from mice injected intramuscularly with cardiotoxin (CTX) at experimental time points and homogenized in RIPA buffer. $CD45^{+/-}$ cell populations were isolated from whole TA muscle using MACS Micro Magnetic Bead Separation system (Bergisch Gladbach, Germany). Cell populations were collected and lysed in RIPA buffer. Protein concentrations were determined by Qubit 2.0 Fluorometer Protein Assay (Life Technologies, Carlsbad, CA). Protein samples were prepared for SDS-PAGE with 2X Laemmli Sample Buffer (Bio-Rad, Hercules, CA) at a 1 mg/ml concentration. SDS-PAGE was completed using 4-20% Mini Protean TGX gels (Bio-Rad, Hercules, CA) at 110 volts for 1 hour. The SDS-PAGE gel was then transferred onto PVDF membrane (Thermo Fisher, Waltham, MA) at 0.35 amps for 1-2 hours at 4°C. Membranes were blocked in 5% BSA in TBS-T at room temperature for >1 hour. GDF3 was targeted using rabbit monoclonal Anti-GDF3 primary antibody (ab109617, Abcam, Cambridge, MA) at 1:1,000 dilution in 5% BSA/TBS-T overnight at 4°C. Anti-GAPDH mouse monoclonal primary antibody (AM4300, Ambion, Carlsbad, CA) was used as a protein loading control at 1:10,000 – 1:20,000 dilution in 5% BSA+TBST overnight at 4°C. Membranes were washed 3X with TBS-T for 5 minutes each for a total of 15 minutes. Goat Anti-Rabbit HRP secondary antibody was used for the detection of GDF3 at 1:10,000 dilution in 5%BSA+TBS-T at room temperature for 1 hour. Anti-Mouse HRP secondary (Cell Signaling, 7076S) and Donkey Anti-Mouse Alexa Fluor 680 secondary (ab175774) antibodies were used for the detection of GAPDH at 1:40,000 dilution at room temperature for 1 hour. Membranes were washed 3X with

TBS-T for 5 minutes each for a total of 15 minutes, followed by 2 washes in TBS for 5 minutes. Super Signal West Pico Kit allowed for ECL visualization of the blot on Hyblot CL Film (Denville, E3018).

RNA sequencing (RNA-Seq) library preparation for myoblast gene expression analysis: Myoblast cells were plated at 30.000/cm2. After cell adhesion, medium was replace to differentiation medium (DMEM/F12 containing 2% horse serum). After overnight differentiation, 150 ng/ml GDF3 was added to selected wells. Cells were harvested in trizol in 24h and RNA was isolated following the suppliers's recommendations. cDNA library for RNA-Seq was generated from 1μg total RNA using TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, poly-A tailed RNAs were purified by oligodT conjugated magnetic beads and fragmented on 94 C degree for 8 minutes, then 1st strand cDNA was transcribed using random primers and SuperScript II reverse transcriptase (Lifetechnologies, Carlsbad, CA, USA). Following this step, second strand cDNA were synthesized and then double stranded cDNA molecules were end repaired resulting blunt ends. The 3' ends of the dscDNA molecules were adenylated then Illumina TruSeq index adapters were ligated. After adapter ligation step, enrichment PCR was performed to amplify the adapter-ligated cDNA fragments. Fragment size distribution and molarity of the libraries were checked on Agilent BioAnalyzer DNA1000 chip (Agilent Technologies, Santa Clara, CA, USA).

10 pM of denatured libraries were used for cluster generation on cBot instrument, then single read 50bp sequencing run was performed on Illumina HiScan SQ instrument (Illumina, San Diego, CA, USA).

The RNA-Seq data are publicly accessible (data access: PRJNA290560/SRR2136645).

RNA-seq bioinformatics analysis: The TopHat-Cufflinks toolkit was used for mapping spliced reads, making transcript assemblies, getting and sorting gene expression data. Genes with RPKM>=1 (at least in one sample) were considered to be expressed. 2-way ANOVA tests were performed in R using functions aov and TukeyHSD of package MASS, Heatmaps were drawn with package pheatmap.

General statistical analyses. All experiments were performed using at least three different samples. Student's t-tests and 2 way ANOVA analyses were performed and $P<0.05$ was considered significant ($P<0.05$=*, $P<0.01$=**, $P<0.001$=***). Mean and SD values, or mean and SEM values are shown in graphs.

Colas, R.A., Shinohara, M., Dalli, J., Chiang, N., and Serhan, C.N. (2014). Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue. American journal of physiology Cell physiology *307*, C39-54.
Hardy, D., Besnard, A., Latil, M., Jouvion, G., Briand, D., Thepenier, C., Pascal, Q., Guguin, A., Gayraud-Morel, B., Cavaillon, J.M*., et al.* (2016). Comparative Study of Injury Models for Studying Muscle Regeneration in Mice. PloS one *11*, e0147198.
Heredia, J.E., Mukundan, L., Chen, F.M., Mueller, A.A., Deo, R.C., Locksley, R.M., Rando, T.A., and Chawla, A. (2013). Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. Cell *153*, 376-388.
Mitchell, C.A., McGeachie, J.K., and Grounds, M.D. (1992). Cellular differences in the regeneration of murine skeletal muscle: a quantitative histological study in SJL/J and BALB/c mice. Cell and tissue research *269*, 159-166.