SUPPLEMENTAL MATERIALS

Lipin-1 Regulates Autophagy Clearance and Intersects with Statin Drug Effects in Skeletal Muscle

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Gene Expression Analyses

Total RNA was isolated with TRIzol reagent and reverse-transcribed using Omniscript RT kit and oligo(dT) primers (Invitrogen). Conventional PCR and qPCR were performed as described previously (Zhang et al., 2012). Expression levels were normalized to the endogenous control TATA box-binding protein (TBP) and 18S rRNA. Results were expressed as fold-induction or -repression by normalizing to the control conditions. PCR primer sequences are provided below.

Lipin-1: CCTTCTATGCTGCTTTTGGGAACC' and 'GTGATCGACCACTTCGCAGAGC';

Acly: 'CTCACACGGAAGCTCATCAA' and 'ACGCCCTCATAGACACCATC';

Fasn: 'CAGCAGAGTCTACAGCTACCT' and 'ACCACCAGAGACCGTTATGC';

Cpt1b: 'CCCCCTCATGGTGAACAGCAAC' and 'TCCAGTTTGCGGCGATACATGA';

Acadm: 'AGGTTTCAAGATCGCAATGG' and 'CTCCTTGGTGCTCCACTAGC';

Acadl: 'CCTCACCACACAGAATGGGAGA' and 'TGAGAGCAAGTCCCCACCAATG';

Acox1: 'CAGGAAGAGCAAGGAAGTGG' and 'CCTTTCTGGCTGATCCCATA';

p62(Sqstm1): 'GAAGAATGTGGGGGAGAGTGTGG' and 'TGCCTGTGCTGGAACTTTCTGG';

Tbp: 'ACCCTTCACCAATGACTCCTATG' and 'ATGATGACTGCAGCAAATCGC';

18s rRNA: 'ACCGCAGCTAGGAATAATGGA' and 'GCCTCAGTTCCGAAAACCA'.

Electron Microscopy

Sections (1 μm) were prepared from segments of soleus muscle following fixation in 2.5% glutaraldehyde/0.1M sodium phosphate buffer, pH 7.4. The blocks were secondarily fixed in 1% osmium tetroxide, dehydrated in graded methanol and propylene oxide, and embedded in Epon 812 resin. Ultra-thin sections were cut and stained in uranylacetate/lead citrate and examined at various magnifications in a *Philips EM 208 S* transmission electron microscope operating at 80kV.

Mitochondrial DNA Content

Total genomic DNA from lower hindlimb muscle was isolated by phenol/chloroform/isoamyl alcohol extraction. Mitochondrial and nuclear DNA were amplified by real time PCR with 25 ng of DNA and primers in the mitochondrial D-loop region and nuclear genome *Tert* gene, respectively. The primer used for D-loop was: 'AATCTACCATCCTCCGTGAAACC-3' and 'TCAGTTTAGCTACCCCCAAGTTTAA'. The primer used for *Tert*: 'TAGCTCATGTGTCAAGACCCTCTT' and 'GCCAGCACGTTTCTCTCGTT'.

Cell Culture

Mouse C2C12 myoblasts and human HEK293 cells (American Type Culture Collection) were maintained at subconfluence in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FBS) plus penicillin/streptomycin. When C2C12 cells reached 90% confluence, medium was replaced with DMEM supplemented with 2% horse serum to induce myotube

formation. Primary mouse embryonic fibroblasts (MEFs) were prepared from 14-day wild-type or *fld* mouse embryos as described (Zhang et al., 2012). Primary MEFs were transfected with GFP-LC3 plasmids with BioT (Bioland Scientific LLC., Paramount, CA)

Western Blot Analysis

Proteins were extracted from the lower hindlimb muscle of mice or cultured cells, electrophoresed on NuPAGE Tris acetate or Bis-Tris gels (Invitrogen), transferred to membranes and detected with antibodies against the corresponding antibodies. The images were quantified by densitometry using Quantity One-4.6.9 software (Bio-Rad). The anti-Akt, phosphor-Akt (S473), mTOR, phosphor-mTOR (S2481), Raptor, S6K1 (p70S6K1), phosphor-S6K1 antibodies, phospho-PKD /PKCμ(S744/748), and anti-phospho-PKC antibody sampler kit were purchased from Cell Signaling Technology (Beverly, MA). The anti-LC3 antibody was from Novus Biologicals (Littleton, CO), the anti-p62 antibody was from Sigma-Aldrich (St. Louis, MO), the anti-FoxO3 and phosphor-FoxO3 (S253) antibodies were from Millipore (Temecula, CA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody from GeneTex, Inc. (Irvine, CA).

Reference

Zhang, P., Takeuchi, K., Csaki, L.S., and Reue, K. (2012). Lipin-1 phosphatidic acid phosphatase activity modulates phosphatidate levels to promote peroxisome proliferator-activated receptor γ (PPARγ) gene expression during adipogenesis. J. Biol. Chem. 287, 3485-3494.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (related to Fig. 1). **Lipid and gene expression profiles in muscle of wild-type and *fld/fld* mice.**

(A) Neutral lipid droplet accumulation in skeletal muscle of statin-treated *fld/fld* mice. *Upper*, Oil Red O (ORO) stained sections from tibialis anterior muscle. *Lower*, NADH-tetrazolium reductase (NADH-TR) staining of adjacent sections used for ORO staining showing that lipid accumulation occurs primarily in type I fibers (dark blue staining). Scale bars = 50μm.

(B) Reduced mRNA levels of ATP citrate lyase (*Acly*) and fatty acid synthase (*Fasn*) in lower hindlimb muscle from *fld/fld* mice (n = 3-6). *, $p < 0.05$.

(C) mRNA levels of gene targets of the lipin-1 coactivator function. No significant differences were observed among genotypes or treatments (n = 3-6; $p > 0.05$). *Cpt1b*, carnitine palmitoyl transferase 1b; *Acadm*, acyl-CoA dehydrogenase, medium chain; *Acadl,* acyl-CoA dehydrogenase, long chain*;* *Acox1*, acyl-CoA oxidase I

(D) Electrospray ionization mass spectrometric quantification of phospholipids and sphingolipids in hindlimb muscle from wild type and *fld/fld* mice under chow diet. PC, phosphatidylcholines; LysoPC, lysophosphatidylcholines; SM, sphingomyelins; HexCer, hexosylceramides; DiHexCer, dihexosylceramides; phosphatidylcholines (ePC), and ceramides (n = 3). *, $p < 0.05$; **, $p < 0.01$.

Figure S2 (related to Fig. 2) Muscle lipin-1 transgene reduces muscle turnover and restore lipid homeostasis in *fld/fld* mice.

(A) Frequency distribution of myofiber area in tibialis anterior and soleus muscle from mice under basal conditions and statin treatment. Average area of corresponding muscle fibers shown in the right.

(B) Muscle lipin-1 transgene prevents neutral lipid droplet accumulation in tibialis anterior muscle of *fld/fld* mice under statin treatment. *Upper*, Oil Red O (ORO) stained sections. *Lower*, NADH-tetrazolium reductase (NADH-TR) stained of adjacent sections.

(C) Levels of ePC and ceramide in lower hindlimb muscle from mice of the *fld/fld*–MCK Tg and littermates of all genotypes, as indicated (n = 3-6). *, $p < 0.05$ *vs.* indicated groups.

(D) Muscle lipin-1 transgene reduces the frequency of centrally nucleated myofibers in *fld/fld* mice under statin treatment. *Left*, representative centrally nucleated myofibers (arrowheads) in transverse section of hindlimb muscle (Methyl Green stain). Scale bars in panels (B) and (D) correspond to 50μm.

Figure S3 (related to Fig. 3) Accumulation of aberant mitochondrial and autophagy substrate in in *fld/fld* muscle

(A) Large magnification of the electron micrographs in Figure 3A. Soleus muscle from *fld/fld* mice accumulate abnormally shaped mitochondria with disorganized cristae. Scale bar = 0.5μm.

(B) Quantitation of p62 protein and mRNA levels from the experiment shown in lower panel of Figure 3D (n = 3-6). *, $p < 0.05$; **, $p < 0.01$ *vs.* indicated groups; §, $p < 0.05$ *vs.* all other groups.

Figure S4 (related to Fig. 4) Accumulation of aberrant mitochondrial and autophagy substrate in in *fld/fld* muscle

(A) Immunoblot analysis of LC3-II intensity (normalized to LC3-I) in primary MEFs infected with adenoviruses expressing a control gene (LacZ), wild-type lipin-1 (WT-lipin-1), or PAP–deficient lipin-1 (PAPdef-lipin-1), under control conditions (C), after 2μM rapamycin (R), or starvation conditions in HBSS (S) for 8 hours. *, $p < 0.05$.

(B) Confocal microscopy showing co-localization of endogenous LC3 (green) and LAMP-1 (red) in primary MEFs in the experiment shown in Figure 4B. Scale bars = 10μm.

Figure S5 (related to Fig. 5) Lipin-1 PAP activity is required for the PKD-Vps34 Pathway in Autophagy Clearance

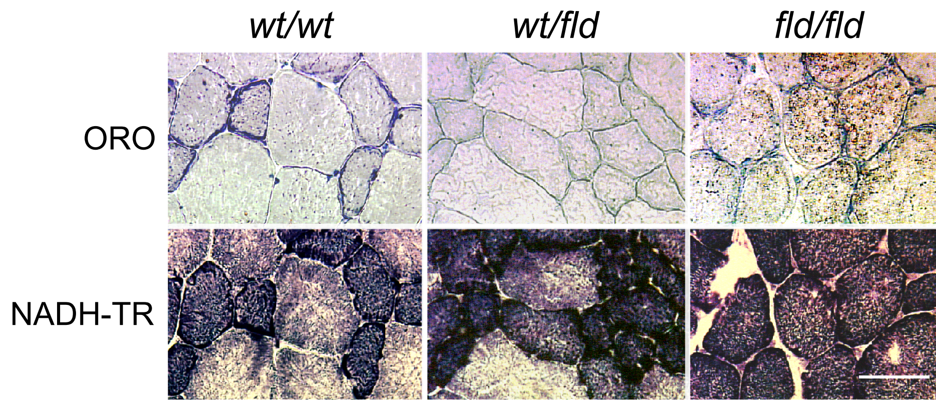
(A) Autophagosome (LC3 puncta) formation in *fld*-MEFs transfected with GFP-LC3 under control (10% FBS) or 60-min starvation. Cells were incubated without or with 1,2-dipalmitoyl-sn-glycerol (DPG, 100 μM), as indicated. n = 80 cells per group. **, $p < 0.01$.

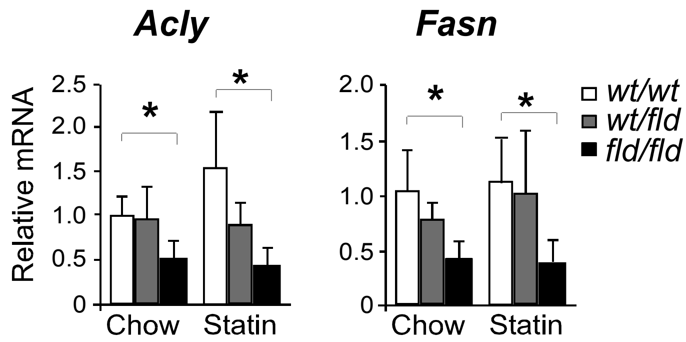
(B) Quantitation of band intensity of phospho-PKD (normalized to total PKD) in Figure 5D. *, $p < 0.05$.

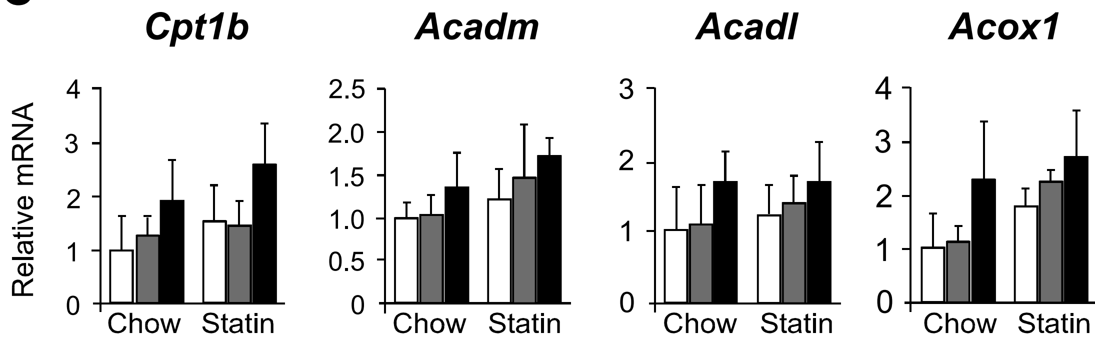
Figure S6 (related to Fig. 6) Statins increase levels of PA in C2C12 myocytes

Biochemical quantitation of PA in C2C12 myocytes treated with 10μM atorvastatin, lovastatin, simvastatin or pravastatin for 24 h (n = 3). *, $p < 0.05$.

Figure S1.







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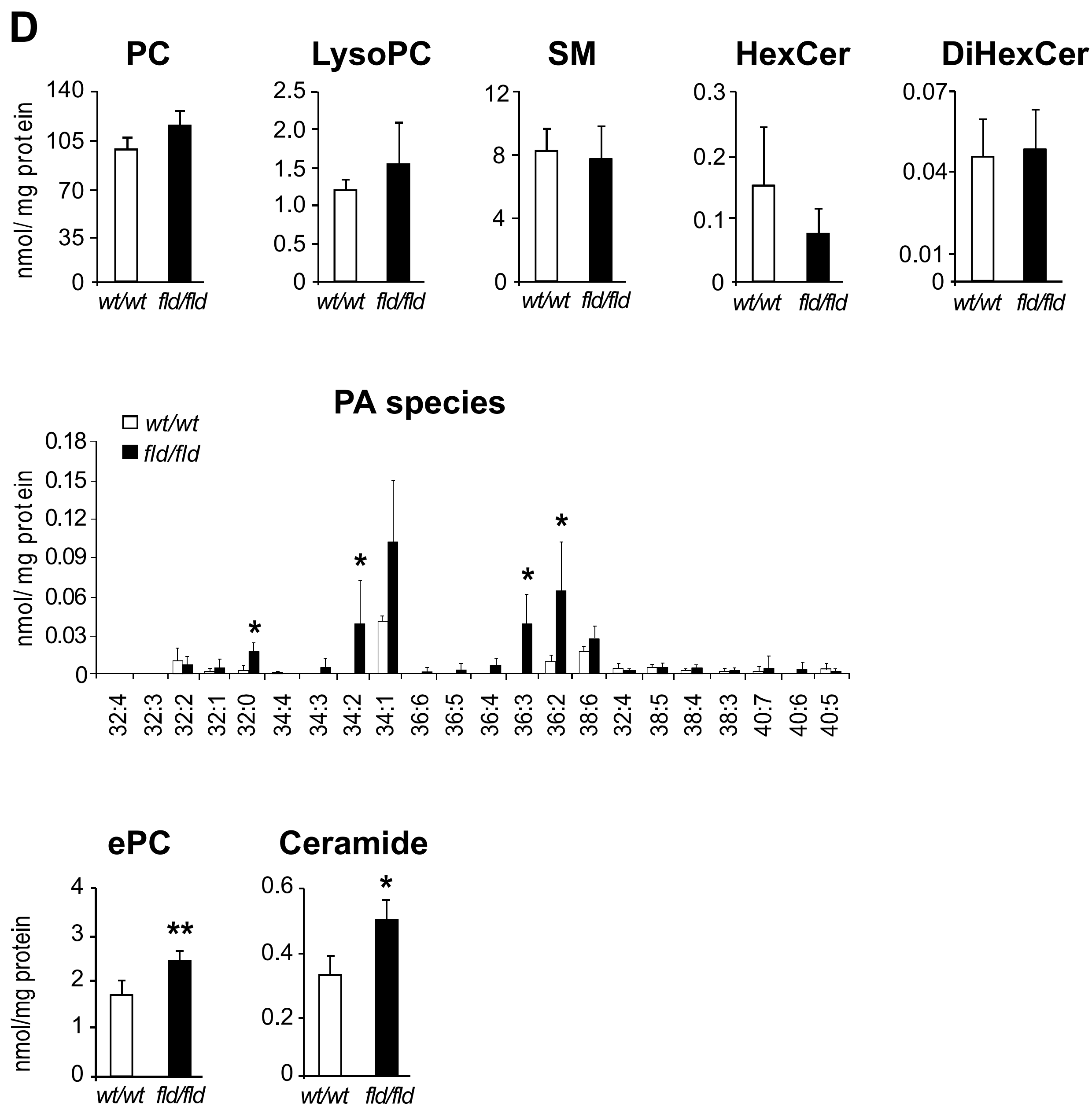

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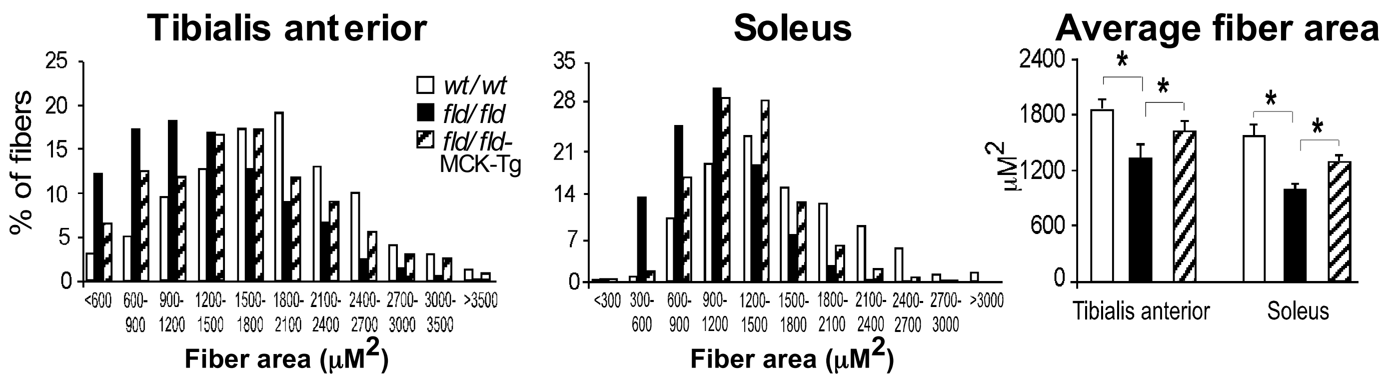
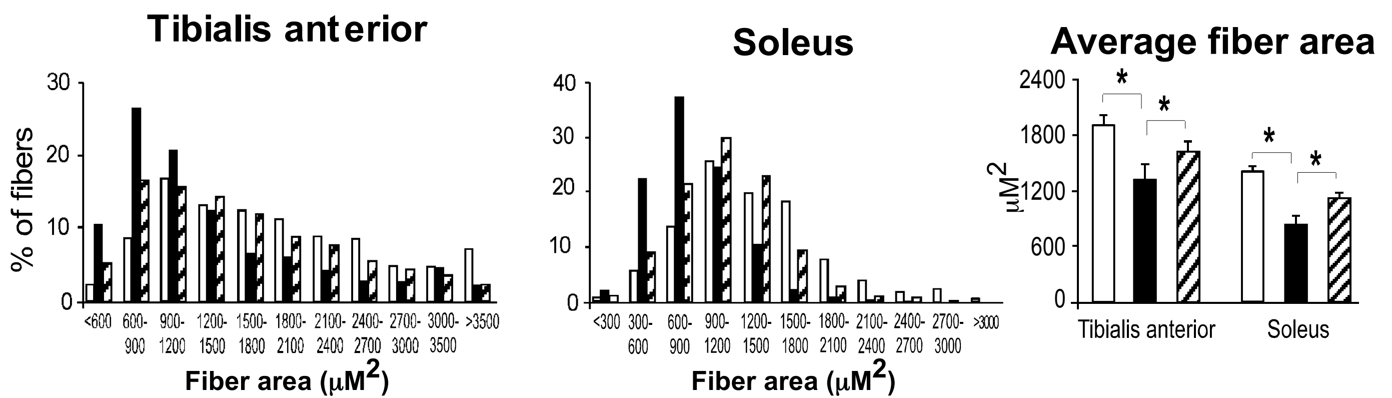
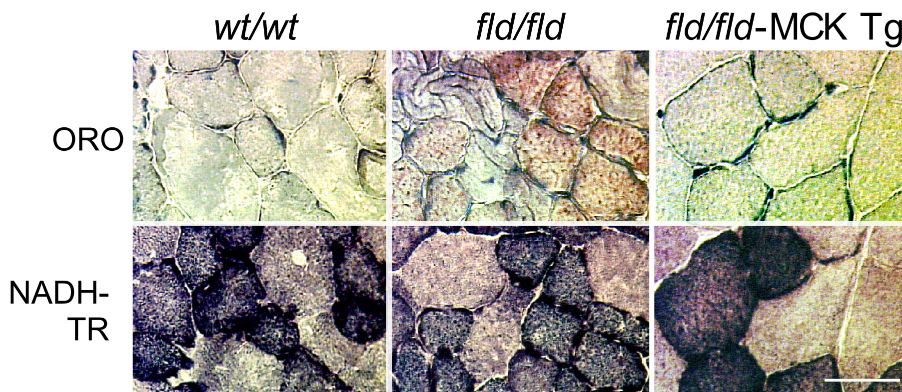
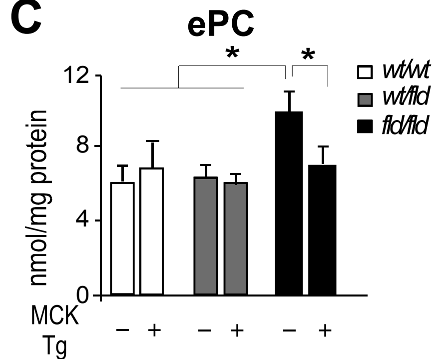
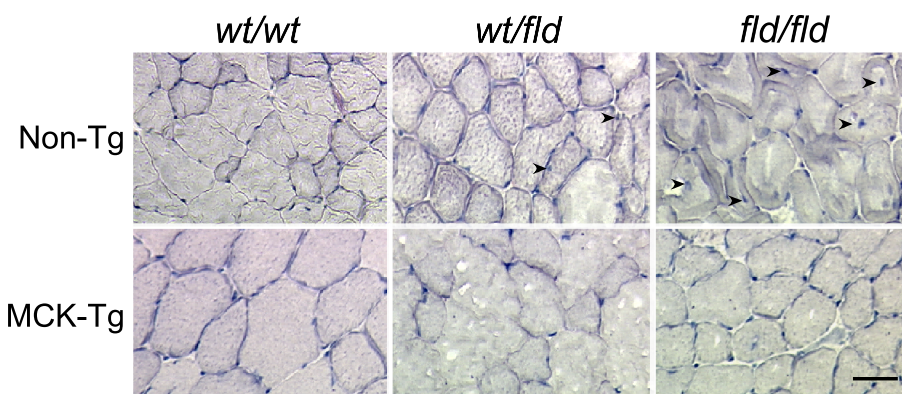










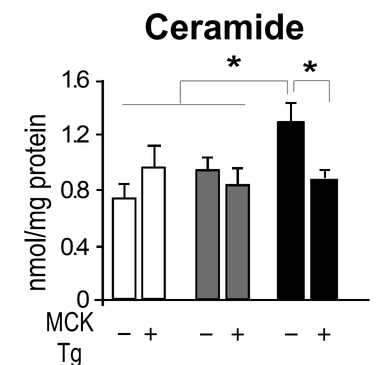
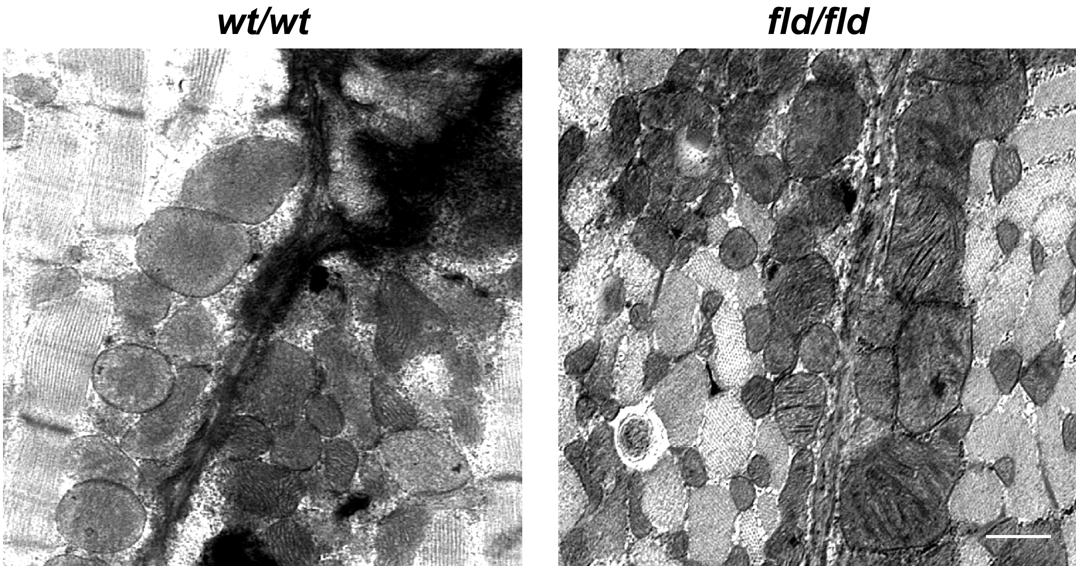


Figure S3.



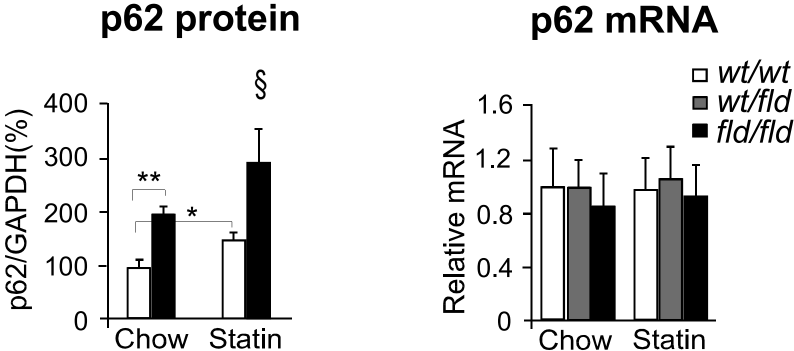
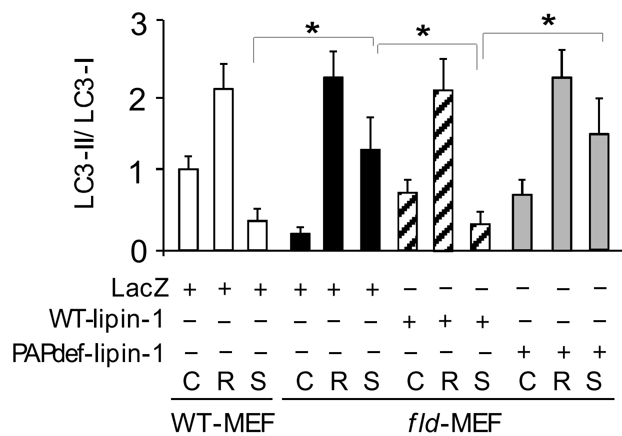


Figure S4.

A



B

LC3/LAMP-1/DAPI

WT-MEF
+LacZ

fld-MEF
+LacZ

fld-MEF
+WT-lipin-1

fld-MEF
+PAPdef-
lipin-1

Figure S5

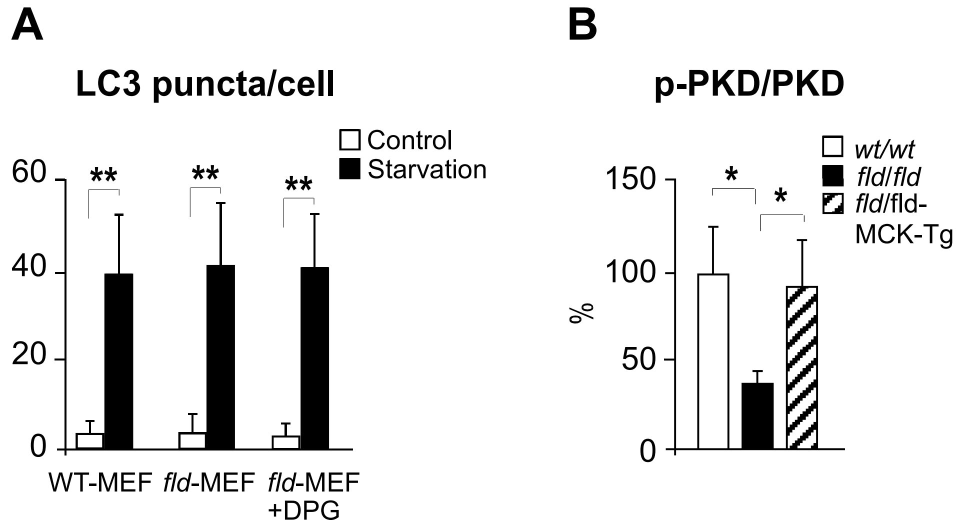


Figure S6