Cell

EXTENDED EXPERIMENTAL PROCEDURES

Reagents

Super 8xTOPFLASH was from R.T. Moon (Veeman et al., 2003), full-length Fz8 was from X. He (He et al., 1997), full-length LRP6 was from C. Niehrs (Mao et al., 2001), and Shisa was from S. Aizawa (Yamamoto et al., 2005). Wnt3A, Fz8/Fc, LRP6/Fc, and IgG/Fc were from R&D. C1q, C4, active-C1s, C1-INH, C1q-depleted serum, C3-depleted serum, and C5-depleted serum were from Calbiochem. Chloroquine was from Sigma. The antibodies used were from Abcam (mouse C1q, LRP6 (extracellular domain), embryonic myosin heavy chain), Quidel (human C1q), Calbiochem (C4), Cell Signaling (LRP6 (intracellular domain), Myc, phospho-LRP6), Abgent (LRP6 (extracellular domain)), Upstate (phosphorylated histone H3, Myc, Collagen), BD PharMingen (β-catenin), SantaCruz (β-catenin, M-cadherin), Progen (Vimentin), R&D (C1r) and Sigma (human Fc, actin). BB5.1 was from Hycult. Puramatrix was from 3-D Matrix, Ltd.

Isolation and Culture of Satellite Cells and Fibroblasts from Skeletal Muscle

Hindlimb muscles were dissected and digested in DMEM containing 0.2% (w/v) Collagenase II (Worthington) for 90 min at 37°C with agitation. Digested muscles were then rinsed repeatedly in washing buffer containing 10% horse serum and dissociated into single myofibers by repeated triturating with a 18G needle attached with 10 ml syringe. The fibers were further digested with digestion buffer (Ham's F-10 containing 0.5 U/ml Dispase (Invitrogen) and 38 U/ml Collagenase II (Worthington)) for 20 min at 37°C with agitation. Digests were then dissociated with 21G needle attached with 10 ml syringe for 10 times and further digested in digestion buffer. The digests were then centrifuged at 500 g for 1 min to sediment debris and the supernatant was filtered through 70 μm cell strainer. Cells were collected by centrifugation at 1,000 g for 5 min, washed twice with basal medium (Ham's F-10 medium containing 20% fetal bovine serum, 5 ng/ml bFGF), and then plated to laminin coated 96 well plate at 5,000 cells/well (for cell proliferation assay) or to laminin coated dish at $1X10^5$ cells/35-mm dish. Cells were recovered for 2 days in basal medium and used for experiments.

Fibroblasts from skeletal muscle were isolated by repeated digestion of minced skeletal muscles in 0.25% trypsin-EDTA (GIBCO). After addition of fibroblast medium (DMEM containing 10%FBS), the digests were filtered through 70 μm, then 40 μm cell strainer. Cells were collected by centrifugation at 1000 g for 5 min, washed twice with fibroblast medium and plated on gelatin coated dish. Medium was changed 3 hr after initial isolation, and cells were further incubated for 24 hr. Cells were then collected and plated to new gelatin coated 96 well plate at 1,000 cells/well (for cell proliferation assay) or to gelatin coated dish at $5X10^3$ cells/35-mm dish.

Pull-Down Assay

To detect Fz8/Fc-bound C1q, C1q was mixed with IgG/Fc or Fz8/Fc and precipitated with protein G. Proteins bound to IgG/Fc or Fz8/Fc were eluted, fractionated by SDS-PAGE, and immunoblotted with anti-C1q antibody. To detect Fz8 CRD-AP-bound C1q, C1q was mixed with His-myc-tagged alkaline phosphatase (AP) or His-myc-tagged Fz8 CRD-AP fusion protein and precipitated with nickel-agarose. Proteins bound to AP or Fz8 CRD-AP were eluted, fractionated by SDS-PAGE, and immunoblotted with anti-C1q antibody.

RNA Analysis

Total RNA was extracted using TRIZOL. RNA was treated with DNase and reverse transcribed using QuantiTect Reverse Transcription Kit (QIAGEN). Real time quantitative PCR was performed using Universal Probe Library (UPL) (Roche) and Light Cycler TaqMan Master kit (Roche). Relative levels of gene expression were normalized to the *Gapdh* gene expression using the comparative Ct method. Primer sequences and the corresponding UPL number were retrieved from the online design program provided by Roche.

siRNA Experiments

Stealth siRNA against human LRP5, LRP6, C1r, and C1s were designed and purchased from Invitrogen. Negative control for siRNA was purchased from Invitrogen (Medium GC). Transfection of siRNA was performed using Lipofectamine RNAiMax (Invitrogen). We used two different siRNAs for each gene and obtained essentially the same results. Sequences for each siRNAs are; LRP5-1: uauaaucgcuguacugcgucagacc; LRP5-2: uuucuggcugaacagcaagaaggug; LRP6-1: aauacauguacccaaccaugggauc; LRP6-2: auaa caauccaguucaucugacuug; C1r-1: acgagcuucacccuguaucccgugg; C1r-2: aagaacagcagauccacagcauugc; C1s-1: acaacuucaaaccc auccagacagg; C1s-2: auucauuguuggcucccuguuuccc;. Knockdown efficiency were ~85% for LRP5, ~80% for LRP6, ~90% for C1r, and ~90% for C1s, as assessed by real time PCR (Figure S3D).

Protein Analysis

For serum isolation, whole blood was clotted for 30 min at 37°C, and centrifuged for 10 min at 9000 rpm. Albumin and immunoglobulins were removed (ProteoExtract Albumin/IgG Removal Kit; Calbiochem) before precipitation with Fz8/Fc or SDS-PAGE.

LC-MS/MS analysis was performed using MAGIC 2002 (Michrom BioResources Inc.) and Q-Tof2 (Waters Micromass). N-terminal amino acid sequencing was performed using Procise 494 cLC Protein Sequencing System.

β-Galactosidase Assay

X-gal staining was performed as described (Akazawa et al., 2000). Colorimetric assay for β-galactosidase activity from tissue sample was performed using β-Galactosidase Enzyme Assay System (Promega).

ELISA for C1q and Cleaved LRP6 Fragment

To quantify C1q level in the serum, 96-well plate was coated with Fz8/Fc in carbonate buffer. After blocking with skim milk, serum samples diluted 1:200 in PBS containing 10 mM EDTA were applied. Detection was performed using a mouse monoclonal antibody (Abcam) and HRP-tagged secondary antibody (Jackson). Standard was made by adding purified mouse C1q to the pooled serum containing known concentration (assessed by nephelometry) of C1q. C1q purification was performed as described previously (Wing et al., 1993).

To quantify LRP6 fragment in the serum, 96-well plate was coated with a mouse monoclonal antibody against extracellular domain of LRP5/6 in carbonate buffer. After blocking with bovine serum albumin, serum samples diluted 1:200 in PBS was applied. Detection was performed using a rabbit polyclonal antibody (Abgent) and HRP-tagged secondary antibody (Jackson). Recombinant mouse LRP6 (R&D) was used as a standard.

Animals

Cryoinjury to the gastrocnemius muscle was made by applying a chilled metal probe directly to muscle surface for 6 s. Fifty μL of 1% Puramatrix containing various reagents was layered over the injured muscle and the skin was closed 5 min later when hydrogel became solid. Tissues were embedded in OCT, and 8 μm sections were processed for immunostaining.

Mice with thoracic aorta constriction (Sano et al., 2007) and mutated cardiac α-actin transgenic mice that exhibit dilated cardiomyopathy-like phenotypes (Toko et al., 2010) were used as mouse models of heart failure.

SUPPLEMENTAL REFERENCES

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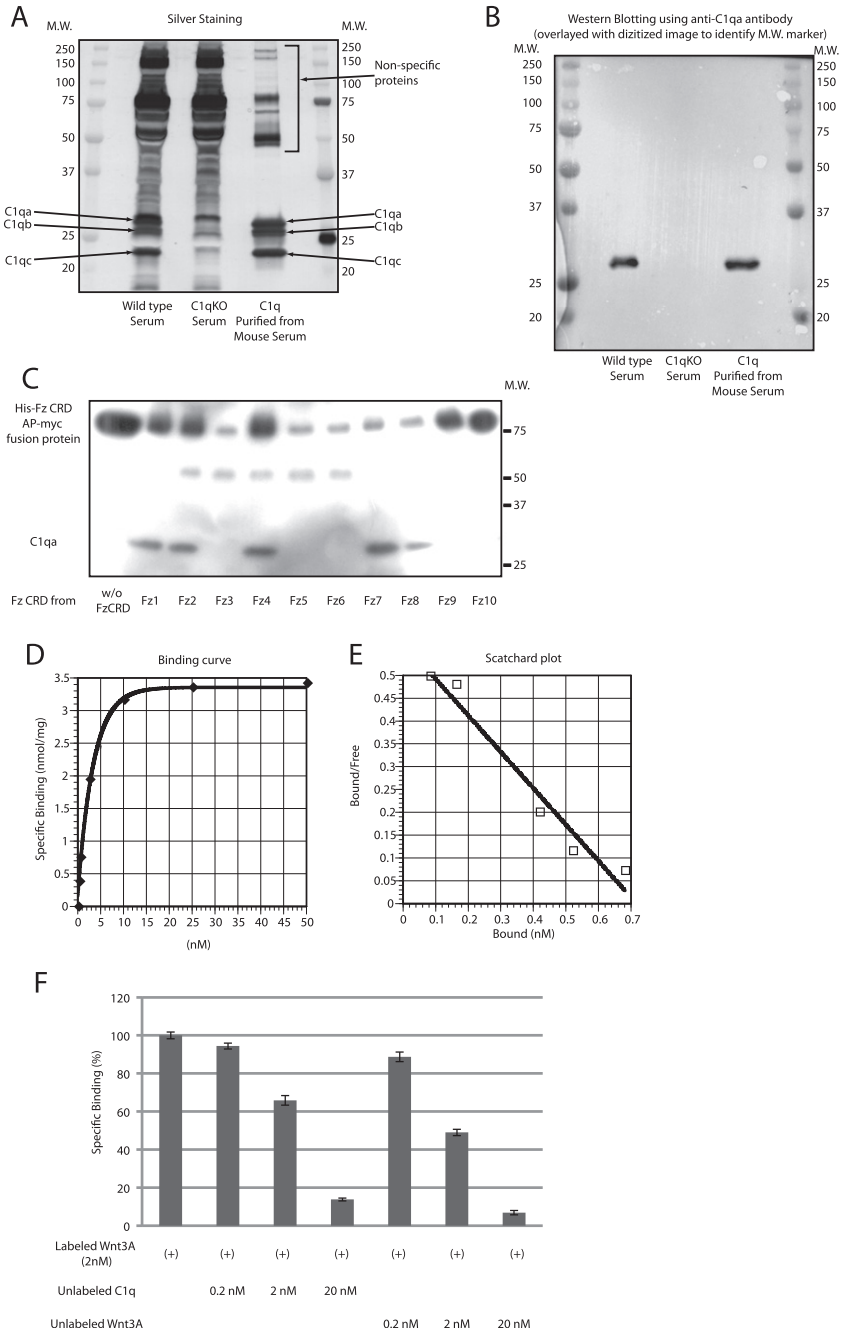

Figure S1. C1q Binds to Fz Receptors, Related to Figure 1

(A and B) Western blot analysis showing the specificity of anti-C1qa antibody. Serum was obtained from wild-type and C1qa-deficient mice (C1qKO). Serum was depleted of immunoglobulins and albumins (ProteoExtract Albumin/IgG Removal Kit; Calbiochem) and concentrated using Amicon Ultra (30K). Serum samples and purified C1q protein were fractionated with SDS-PAGE and visualized by silver staining (A). Samples obtained as in (A) were fractionated with SDS-PAGE, electrotransfered, and immunoblotted against anti-C1qa antibody. Anti-C1qa antibody selectively recognized C1qa protein as judged by the relative positions in a reduced gel. No signal was detected in the serum obtained from C1qa-deficient mice (B).

(C) Pull-down assay. CRD of Fz1 through 10 was fused with AP-His-myc and mixed with C1q. The mixture was then subjected to nickel-agarose column and the bound protein was eluted, fractionated with SDS-PAGE, and immunoblotted against anti-myc and anti-C1q antibody. C1q interacted with Fz1, 2, 4, 7, and 8 but not with Fz3, 5, 6, 9, and 10.

(D and E) Binding kinetics of Wnt3A to Fz8 CRD. A binding curve (D) and a Scatchard plot (E) are shown.

(F) Heterologous competition assay. Alkyne labeled Wnt3A was incubated with Fz8/Fc and various concentration of unlabeled C1q or Wnt3A. Wnt3A that bound to Fz8/Fc was precipitated with protein G, eluted, and quantified by ELISA using biotin-azide and HRP-streptavidin. Data are shown as the percentage against the maximum binding without competitor. C1q competes with Wnt3A for the binding with Fz8 CRD. Data are presented as mean ±SD.

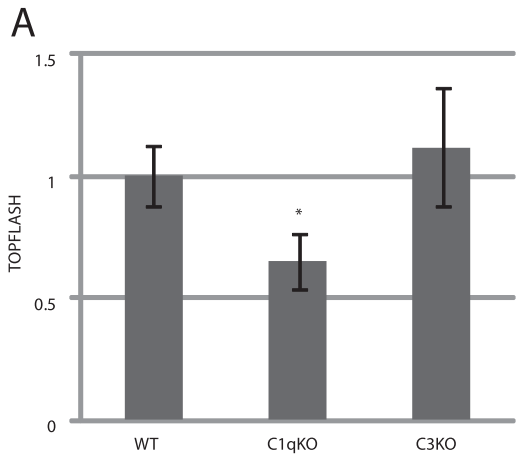

Figure S2. C1q Mediates Serum-Induced Wnt Signal Activation, Related to Figure 2

(A) TOPFLASH assay. HEK293 cells were stimulated with serum obtained from wild-type (WT), C1qa-deficient (C1qKO), or C3-deficient (C3KO) mice at the age of 3 months. Serum-induced Wnt activity was significantly lower in C1qKO mice compared with WT or C3KO mice. Data are presented as mean ±SD. *$p < 0.05$ versus wild-type ($n = 8$).

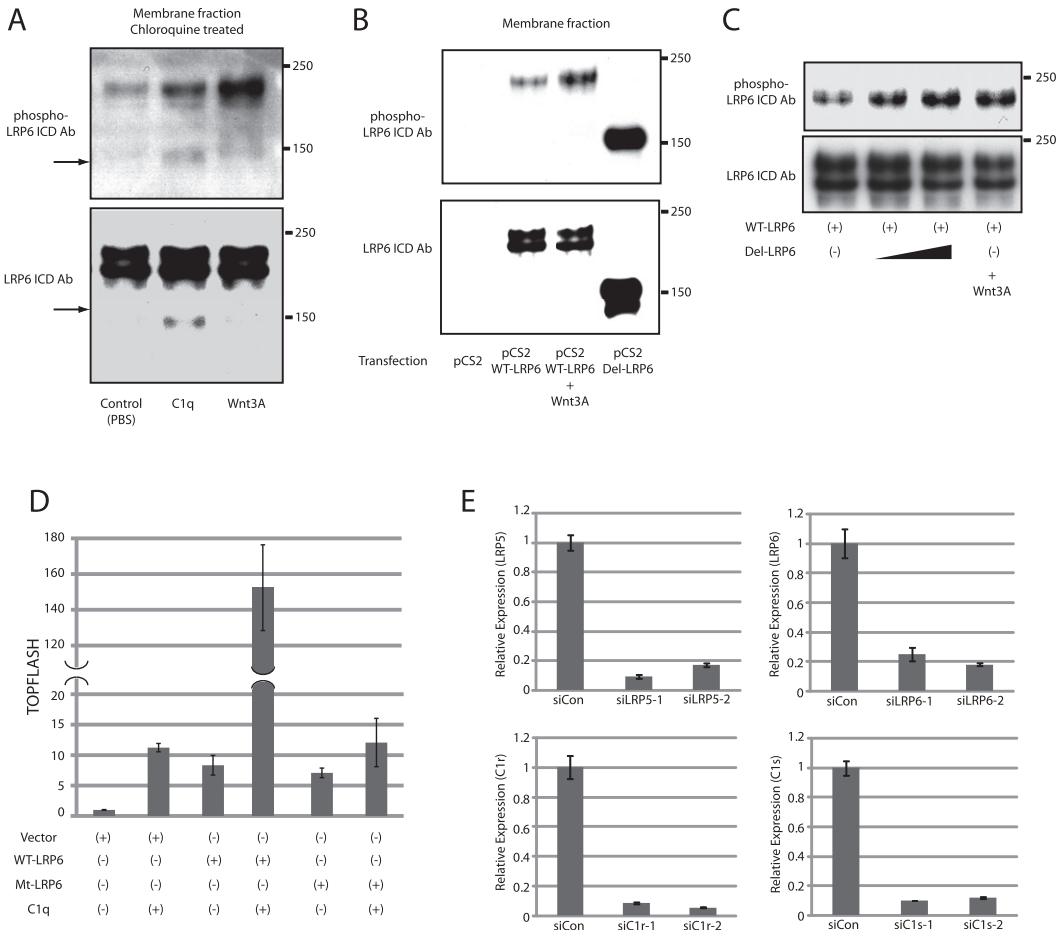

Figure S3. C1q Activates Wnt Signaling by Inducing LRP6 Cleavage, Related to Figure 5

(A) Western blot analysis for the phosphorylation of LRP6. HepG2 cells were treated with C1q (100 μg/ml) or Wnt3A (100 ng/ml) for 3 hr and 30 min, respectively. Chloroquine (50 μM) was added for last 3 hr in every sample. Membrane/organelle fraction was subjected to SDS-PAGE and subsequently blotted with anti-phosphorylated LRP6 (S1490) antibody and anti-LRP6 ICD antibody. An arrow indicates C-terminal LRP6 fragment. Phosphorylation of the full length LRP6 was observed following C1q and Wnt3A treatment, and phosphorylation of the C-terminal LRP6 fragment was observed by C1q treatment but not by Wnt3A treatment.

(B) Western blot analysis for the phosphorylation of LRP6. HepG2 cells were transfected with pCS2 (vector control), WT-LRP6 (full-length LRP6), and Del-LRP6 (LRP6 truncated at R792), and treated with or without Wnt3A protein (100 ng/ml). Strong phosphorylation of Del-LRP6 was observed.

(C) Western blot analysis for the phosphorylation of full length LRP6 upon co-transfection of LRP6 truncated at R792. HepG2 cells were transfected with WT-LRP6 with or without Del-LRP6. Phosphorylation of full length LRP6 was increased upon co-transfection of Del-LRP6. Wnt3A (100 ng/ml) was used as a positive control.

(D) TOPFLASH assay. HEK293 cells were transfected with WT-LRP6 or Mt-LRP6 and treated with C1q (100 μg/ml). Transfection of Mt-LRP6 by itself activated TOPFLASH activity by ~7-fold (lane 5), and C1q treatment of Mt-LRP6-transfected cells further increased TOPFLASH activity up to 12-fold of the control (compare lanes 5 and 6). This further increase by C1q treatment of Mt-LRP6-transfected cells presumably reflects the activation of Wnt signaling mediated by C1q-induced cleavage of endogenous LRP5/6. Lanes 3 and 4 correspond to lanes 1 and 2 of Figure 5G, and lanes 5 and 6 correspond to lanes 1 and 2 of Figure 5H. Data are presented as mean ±SD.

(E) Real-time PCR analysis. Knockdown efficiency of the siRNAs used in this study is shown by the relative mRNA expression levels of *lrp5*, *lrp6*, *C1r*, and *C1s*. Data are presented as mean ±SD.

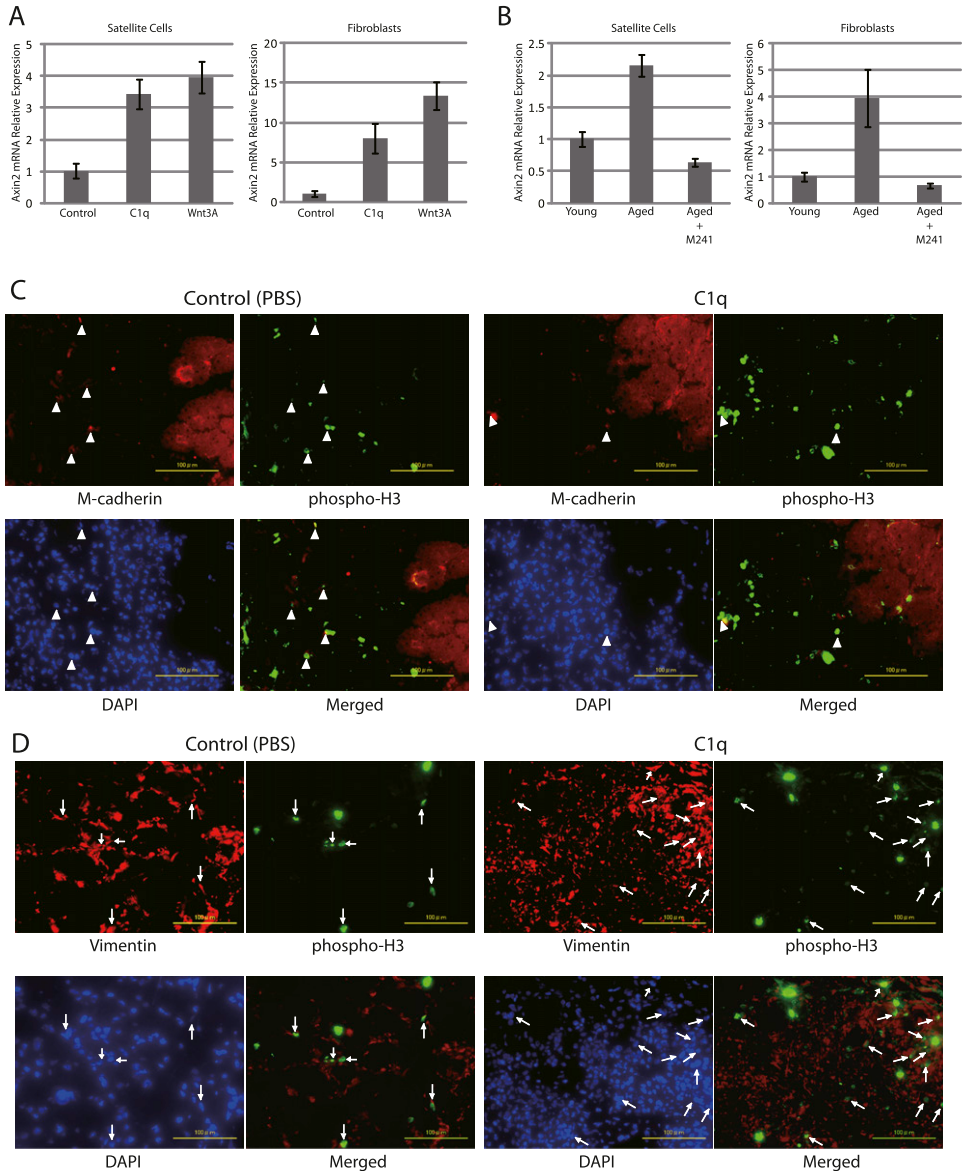

Figure S4. C1q Activates Wnt Signaling in Skeletal Muscle and Exhibits Differential Effects on Satellite Cells and Fibroblasts, Related to Figure 6

(A) *Axin2* mRNA expression. Wnt signaling activity in satellite cells and fibroblasts isolated from skeletal muscle was assessed by *Axin2* gene expression. Satellite cells and fibroblasts were harvested, treated with C1q (100 μg/ml) or Wnt3A (10 ng/ml), and RNA was extracted after 24 hr. Data are presented as mean ±SD. (B) *Axin2* mRNA expression. Wnt signaling activity in satellite cells and fibroblasts were assessed by *Axin2* gene expression. Satellite cells and fibroblasts were stimulated with 5% mouse serum derived from young (2-months old) or aged mice (2-years old) for 24 hr with or without M241. Data are presented as mean ±SD. (C and D) Immunohistochemistry. Skeletal muscle of young mice (2-months old) was cryoinjured and treated with PBS- or C1q- (50 μg/ml) containing hydrogel. Tissue samples were immunostained with M-cadherin (red staining, as a satellite cell marker) and phospho-histone H3 (phospho-H3) (green staining, as a mitotic cell marker), counterstained with DAPI (blue staining, nucleus), and proliferating satellite cells were identified as M-cadherin/phospho-H3 double positive cells (arrowheads). The number of M-cadherin/phospho-H3 double positive cells in cryoinjured skeletal muscle in vivo was reduced by C1q treatment (C). Mice were treated as in (C), and tissue samples were immunostained with Vimentin (red staining, as a fibroblast marker) and phospho-H3, counterstained with DAPI. Proliferating fibroblasts were identified as Vimentin/phospho-H3 double positive cells (arrows). The number of Vimentin/phospho-H3 double positive cells in cryoinjured skeletal muscle in vivo was increased by C1q treatment (D).