Critical role of Frizzled1 in age-related alterations of Wnt/β-catenin signal in myogenic cells during differentiation

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(Degree)
博士（医学）

(Date of Degree)
2014-03-25

(Date of Publication)
2015-03-01

(Resource Type)
doctoral thesis

(Report Number)
甲第6061号

(URL)
https://hdl.handle.net/20.500.14094/D1006061

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骨格筋分化過程での加齢に伴う Wnt/β-catenin シグナルの変化において Frizzled 1 が担う重要な役割

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Key words: Muscle satellite cells, Wnt/β-catenin signal, Frizzled-family of receptor 1, Aging, Myogenic differentiation, Sarcopenia

Critical role of Frizzled1 in age-related alterations of Wnt/β-catenin signal in myogenic cells during differentiation

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Abstract

Activation of Wnt/β-catenin signal in muscle satellite cells (mSCs) of aged mice during myogenic differentiation has been appreciated as an important age-related feature of the skeletal muscles, resulting in impairment of their regenerative ability following muscle injury. However, it remains elusive about molecules involved in this age-related alteration of Wnt/β-catenin signal in myogenic cells. To clarify this issue, we performed expression analyses of Wnt receptor genes using real-time RT-PCR in mSCs isolated from the skeletal muscles of young and aged mice. Here, we show that expression of *Frizzled1* (*Fzd1*) was detected at high levels in mSCs of aged mice. Higher expression levels of *Fzd1* were also detected in mSC-derived myogenic cells from aged mice and associated with activation of Wnt/β-catenin signal during their myogenic differentiation *in vitro*. We also provide evidence that suppressed expression

of Fzd1 in myogenic cells from aged mice results in a significant increase of myogenic differentiation, and its forced expression in those from young mice results in its drastic inhibition. These findings indicate the critical role of Fzd1 in altered myogenic differentiation associated with aging.

Short title

Role of Fzd1 in age-related myogenesis

Introduction

The aging of multi-cellular organisms is characterized by structural and functional alterations from the molecular to the organismal levels. While functional alterations at steady-state conditions might be subtle in several tissues/organs, it is generally appreciated that reduced abilities to respond properly to intrinsic and/or extrinsic changes are often observed in aged tissues and organs. “Sarcopenia” is one of the most serious age-related biological and medical conditions, characterized by decrease and atrophy of muscle fibers accompanying loss of muscle mass and their impaired function (Frontera *et al.* 2000). The age-related loss of muscle mass is further emphasized by its decreased regenerative potential with aging (Narici & Maffulli 2010).

The skeletal muscles are locomotive tissues with very little cellular turnover. Importantly, upon damage by physical accidents, exercise and so on, the skeletal muscles exhibit remarkable ability to initiate a rapid and extensive repair process, preventing the loss of muscle mass and function (Chargé & Rudnicki 2004). The initial phase of muscle regeneration is characterized by activation of adult muscle stem cells, designated as muscle satellite cells (mSCs) (Mauro 1961). This phase is rapidly followed by proliferation and differentiation of activated mSCs, eventually leading to proper formation of newly established myofibers (McGeachie & Grounds 1987; Chargé & Rudnicki 2004). Activation and differentiation of mSCs during regeneration resembles embryonic myogenesis in several aspects, i.e. temporal gene expression profiles of regulatory genes, such as *myogenic regulatory factor 5* (*Myf-5*), *Myogenin* and *Myosin heavy chain* (*Myhc*) (Chargé & Rudnicki 2004). The phase of myogenesis during muscle regeneration is dependent on concerted expression of these genes. The genetic hierarchy, controlling the myogenesis during regeneration, defines the initial and critical role of *Paired box protein 7* (*Pax7*), which is specifically expressed in mSCs (Seale *et al*. 2000).

The Wnt-family of proteins are secreted cysteine-rich glycoproteins with lipid modifications, that can activate β-catenin-dependent and/or –independent Wnt signal

pathways. The respective Wnt proteins exert their biological functions through the binding to their cognate receptors, including the Frizzled-family of receptors (Fzds), low-density lipoprotein receptor-related protein (Lrp5 and 6), the Ror-family of receptor tyrosine kinases and Receptor-like tyrosine kinase (Ryk) (Minami *et al*. 2010; Anastas & Moon 2013). Binding of several Wnt proteins to their Fzds and LRP5/6 receptor complex activates Wnt/β-catenin signal pathway, that induces stabilization of β-catenin, resulting in an increase of cytoplasmic β-catenin and its translocation to the nucleus where it complexes with members of the TCF/LEF family of transcription factors to mediate transcriptional activation of target genes such as *Axin2, Cyclind1*and others (Anastas & Moon 2013).

Recent studies have shown that activation of Wnt/β-catenin signal is associated with aging in various tissues (Liu *et al.* 2007; Marchand *et al*. 2011). In the skeletal muscles, relatively enhanced Wnt/β-catenin signal can be observed in mSCs and myogenic cells from aged mice compared to those from young mice, resulting in an impairment of their functions, that can be suppressed by Wnt inhibitors (Brack *et al*. 2007; Naito *et al*. 2012). However, it remains elusive which member of Wnt proteins and its receptors are involved in age-related activation of Wnt/β−catenin signal in the skeletal muscle cells. As an attempt to clarify this issue, we first performed expression analyses of Wnt receptor genes, including *Fzds* and *Lrp5/6*, in mSCs and myogenic cells from young (2-3 months old) and aged (over 22 months old) mice.

Here, we show that *Fzd1* is highly expressed in mSCs and primary myogenic cells of aged mice, in which Wnt/β-catenin signal is activated. It was also found that Fzd1 was involved in age-related suppression of myogenic differentiation. These findings indicate the critical role of Fzd1 in altered myogenic differentiation associated with aging.

Results

We first examined quantitative and qualitative differences in the skeletal muscles from young and aged mice. The weight of the tibialis anterior (TA) muscles from aged mice was decreased when compared to that from young mice, while its body weight was rather increased compared with young mice (Fig.1A). Muscle glycogen content, reflecting the muscle function, was also decreased in the muscle from aged mice compared with that from young mice (Fig.1B), suggesting that aged mice exhibit sarcopenia. Since it has been shown that activation of Wnt/β-catenin signal can be seen in mSCs and their derived myogenic cells of the skeletal muscles from aged mice compared to those from young mice, we examined expression levels of a representative target gene of Wnt/β-catenin signal (*Axin2*) following skeletal muscle injury of young

and aged mice with cardiotoxin (Ctx) (see Experimental procedures). As shown in Fig.1C, expression of *Axin2* was induced at higher levels during myogenic differentiation and/or muscle regeneration in the TA muscles from aged mice compared to those from young mice following Ctx-induced TA muscle injury, indicating that Wnt/β-catenin signal was activated at higher levels in the muscle tissues from aged mice than in those from young mice following muscle injury. We next examined a possible age-related alteration in myogenic differentiation and/or muscle regeneration in the TA muscles following Ctx-induced muscle injury. It was found that damaged muscles from aged mice were mainly composed of very thin myofibers (<500 μm^2) at day 12 (Fig.1D and E), indicating that myogenic differentiation and/or regeneration after muscle injury was delayed in aged mice.

To investigate differences in properties of mSCs between young and aged mice, we performed fluorescence-activated cell sorting (FACS) analysis using satellite cell-specific antibody, SM/C-2.6 antibody. FACS dot plots showed that the relative contents of mSCs were decreased in the TA muscles from aged mice (Fig.2A and B), while expression of *Pax7* in mSCs from aged mice was comparable to that in mSCs from young mice (Fig.2C). Next, we performed comparative expression analysis of Wnt receptors (all *Fzds*, *Lrp5* and *Lrp6*) in mSCs of the TA muscles from young and aged mice. Expression of *Fzd1* in mSCs from aged mice exhibited significantly higher levels than that from young mice, while expression of any other receptor genes in mSCs from both young and aged mice were almost comparable (Fig.2D), suggesting that high levels of *Fzd1* in mSCs might be involved in activation of Wnt/β-catenin signal in myogenic cells from aged mice during myogenic differentiation.

To further examine this possibility, we performed *in vitro* differentiation assay using isolated mSC-derived myogenic cells from young and aged mice. *Fzd1* and the typical target gene of Wnt/β-catenin signal (*Axin2*) were highly expressed in myogenic cells from aged mice up to day 7 after differentiation (Fig.3A, upper panels). Under these conditions, expression of *Myf-5* was gradually decreased, while expression of *Myogenin* was increased at day 3 and decreased thereafter during myogenic differentiation of the cells from both young and aged mice. Interestingly, *Myhc* expression during myogenic differentiation was remarkably suppressed in myogenic cells from aged mice compared with that from young mice (Fig.3A, middle panels). Furthermore, expression levels of all fibrotic marker genes were significantly higher in myogenic cells from aged mice than in those from young mice during differentiation (Fig.3A, lower panels). Consistent with the results, the number of nuclei belonging to Myhc-positive (differentiated) cells were significantly decreased in the cells from aged mice (Fig.3B), indicating that

myogenic differentiation was inhibited in myogenic cells from aged mice, where expression of *Fzd1* and *Axin2* was kept at higher levels during differentiation.

To investigate a possible involvement of Fzd1 in the activation of Wnt/β-catenin signal which might regulate differentiation of myogenic cells, we analyzed the effects of suppressed or forced expression of Fzd1 on Wnt3a-induced activation of Wnt/β-catenin signal in a myogenic cell line, C2C12 cells. We first confirmed that expression of Fzd1 was suppressed at both mRNA and protein levels following treatment with two different siRNA oligonucleotides targeting *Fzd1* (Fig.4A). Suppressed expression of *Fzd1* inhibited the induction of *Axin2* expression in C2C12 cells stimulated with high concentration (100 ng/ml) of Wnt3a (Fig.4A) during *in vitro* differentiation, while forced expression of Fzd1 enhanced accumulation of total and dephosphorylated (active) β-catenin and of phosphorylated LRP6 in the presence of low concentration (5 ng/ml) of Wnt3a which caused little response in GFP-expressing cells during *in vitro* differentiation (Fig.4B). Furthermore, it was found that forced expression of Fzd1 also increased TOPflash activity following Wnt3a stimulation at low concentrations (Fig.4C). Taken together, these results indicate that suppressed or forced expression of Fzd1 in myogenic cells can inhibit or activate Wnt3a-induced Wnt/β-catenin signal in the cells, respectively, and that Fzd1 might play an important role in activating Wnt/β-catenin signal in the cells.

To further examine the role of Fzd1 in the regulation of myogenesis, we analyzed the effects of suppressed or forced expression of Fzd1 in myogenic cells from aged and young mice on their myogenic differentiation under *in vitro* differentiation condition. Suppressed expression of Fzd1 in myogenic cells from aged mice resulted in a significant increase in the number of nuclei belonging to Myhc-positive (differentiated) cells (Fig.5A and B). On the other hand, forced expression of Fzd1 in myogenic cells from young mice resulted in a drastic decrease in the number of nuclei belonging to Myhc-positive (differentiated) cells (Fig.5C). Collectively, these results indicate that Fzd1 is involved in age-related suppression of myogenic differentiation.

Discussion

"Sarcopenia" is one of the most serious age-related biological and medical conditions of the skeletal muscles (Frontera *et al.* 2000), characterized by loss of muscle mass and their impaired functional property (Fig.1A and B). The age-related loss of skeletal muscle mass is further documented by delayed or suppressed myogenic differentiation and activation of Wnt/β-catenin signal, exemplified by induction of its target gene *Axin2*, associated with aging (Fig.1C-E). Recent studies have shown that alterations of

extracellular stimuli have been implicated in age-related alterations of myogenic differentiation (Naito *et al.* 2012; Fujimaki *et al.* 2013). It has been envisaged that age-related activation of Wnt/β-catenin signal during myogenic differentiation results in delayed or suppressed myogenic differentiation and thereby impairing the function of the skeletal muscles (Brack *et al*. 2007; Carlson *et al.* 2009). During our survey of *Fzd* genes (encoding Wnt receptors) whose expression are associated with aging by comparing their expression levels in mSCs of the TA muscles from young and aged mice, we identified *Fzd1* as a candidate gene involved in age-related activation of Wnt/β-catenin signal during myogenic differentiation (Fig.2D). In fact, Fzd1 has been shown to mediate β-catenin-dependent and/or –independent Wnt signal pathways in a cell context-dependent manner (Badiglian Filho *et al.* 2009; Trowe *et al.* 2012; Zhang *et al.* 2012). During development *Fzd1* is expressed in the dermatome and myotome and is involved in somitogenesis (Schmidt *et al.* 2000; Zilberberg *et al.* 2004; Borello *et al.* 2006; Brunelli *et al.* 2007), although little is known about the role of Fzd1 in activation of Wnt/β-catenin signal and myogenic differentiation in aged animals.

By taking an advantage of *in vitro* differentiation assays using isolated mSC-derived myogenic cells from young and aged mice, we show that expression levels of *Fzd1* and *Axin2* (a representative target gene of Wnt/β-catenin signal) in myogenic cells from aged mice are higher than those from young mice during their differentiation (Fig.3A, upper panels). In addition to their high basal expression levels in myogenic cells from aged mice, expression of both genes is induced during differentiation of the cells *in vitro*, suggesting that *Fzd1* might be a critical determinant which activates Wnt/β-catenin signal and inhibits myogenic differentiation (Fig.3). We failed to detect apparent difference in expression levels of *Axin2* in uninjured muscle tissues from young and aged mice (Fig. 1C), although *Fzd1* is expressed at higher levels in mSCs from aged mice than in those from young mice (Fig. 2D). This might be due to relatively very low contents of mSCs within the muscle tissues to evaluate altered expression of *Axin2* in mSCs between young and adult mice. Alternatively, expression levels of canonical Wnt ligands might be insufficient to activate Wnt/β-catenin signal in mSCs within uninjured tissues. Therefore, it is of importance to unravel a Wnt ligand(s) for Fzd1 in mSCs during myogenic differentiation after injury in aged mice. In our analysis using primary mSC-derived myogenic cells, it was also found that, among canonical *Wnt* genes examined, expression of *Wnt3a*, but not *Wnt8a*, *Wnt8b*, and *Wnt10b* genes, was detected in myogenic cells and increased during differentiation (Fig.S1), although *Wnt3a* is more significantly induced in myogenic cells from young mice than those from aged mice. Thus, it is conceivable that Wnt3a and Fzd1 mediate activation of Wnt/β-catenin signal

during myogenic differentiation in aged mice. In accordance with this notion, it should be noted that suppressed or forced expression of Fzd1 in the myogenic cell line, C2C12 cells, can activate or inhibit Wnt3a-induced Wnt/β-catenin signal in the cells, respectively, during *in vitro* differentiation (Fig.4). Myogenic differentiation of mSCs can be broadly classified into two phases, an early and a late phase of differentiation. At an early phase of differentiation, cells begin to express Myogenin, an early marker of myogenic commitment. Subsequently cells expressing Myhc, a marker for the multinucleated myotubes, are detected (phase of cell fusion, at a late phase). We found that expression of *Myhc* is significantly lower in myogenic cells from aged mice than in those from young mice, while *Myogenin* expression in myogenic cells during differentiation is comparable between young and aged mice, suggesting that myogenic differentiation may be suppressed at a late phase of differentiation in cells from aged mice (Fig.3A). In fact, Myhc-positive (differentiated) cells from aged mice contain a few nuclei compared to those from young mice, indicating that myogenic cells from aged mice have lower ability to fuse themselves and form myotubes during differentiation (Fig.3B). Moreover, we show that suppressed expression of Fzd1 in myogenic cells from aged mice can augment fused multinucleated Myhc-positive cells, and that forced expression of Fzd1 in myogenic cells from young mice can decrease these differentiated cells (Fig.5), suggesting that high levels of Fzd1 in mSCs and myogenic cells may be involved in age-related suppression of myogenic differentiation, possibly through the regulation of a late phase of differentiation. Further studies will be required to clarify the role of Fzd1 in age-related myogenic differentiation.

Collectively, our present findings reveal that Fzd1 plays a critical role in age-related activation of Wnt/β-catenin signaling and inhibition of myogenic differentiation *in vitro* presumably by acting as a receptor for Wnt3a. However, it remains unclear how expression of *Fzd1* is regulated in mSCs and myogenic cells from aged mice during *in vitro* differentiation, and whether or not Wnt3a is indeed acting as a ligand for Fzd1 during myogenic differentiation in aged mice. Further study will be required to clarify these issues.

Experimental procedures

Mice

Male mice (an ICR background) at 2 – 3 months old (young) and > 22 months old (aged) were used. Muscle injury experiments were performed using cardiotoxin (Ctx; Sigma-Aldrich, St Louis, MO, USA). Ctx [2.5 μl of 10 μM Ctx / body weight (g)] was injected into the TA muscle unilaterally. Ctx-treated mice were allowed to recover for

12 days post-injury. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee (Permission number: P121005-R2) and conducted at Institute for Experimental Animals, Kobe University Graduate School of Medicine according to the Kobe University Animal Experimentation Regulations.

Glycogen content measurement

Glycogen was extracted from frozen TA muscle tissues and hydrolyzed by H_2SO_4 as previously reported (Doi *et al.* 2010). The hydrolyzed free glucose concentration was measured using Glucose C-II test Wako (Wako Pure Chemical Industries, Osaka, Japan) and normalized by weight of each TA muscle.

Isolation of mSCs and primary myogenic cells

The TA muscles were digested with collagenase type II at 37 °C for 90 min with occasional trituration and passed through a 40 μm ϕ nylon mesh. The resultant cell suspensions were washed with ice-cold PBS containing 2% fetal bovine serum (FBS), and mSCs were isolated by using positive selection for SM/C-2.6 and negative selection of CD31, CD45 and Sca-1 by FACS with Moflo-XDP and Summit ver. 5.3 (Beckman Coulter, Miami, FL, USA) as previously reported (Fukada *et al.* 2004; Segawa *et al.* 2008). For isolation of myogenic cells, the cells obtained from the TA muscles were cultured in DMEM supplemented with 20 % FBS (Growth medium; GM) for 2 hrs, and then non-adherent cells were harvested and replated in fresh GM. This process was repeated 3 times and almost all cells obtained were myogenic cells (Conboy & Rando 2002).

Cell culture and transfection

C2C12 cells, a myogenic cell line, and primary myogenic cells were maintained in GM at 37 °C under a humidified 5 % CO_2 atmosphere. Differentiation of these myogenic cells were induced by replacing GM with DMEM/F12 supplemented with 2 % horse serum (differentiation media; DM) on collagen-coated dish at about 90% confluency. Cells were transfected with the plasmid vectors (pEGFP-N3-Fzd1 or pEGFP-N3) or siRNA oligonucleotides (Sigma-Aldrich, *Control*, SIC-001s/as; *Fzd1#1*, mixture of Mm_Fzd1_8562s/as and Mm_Fzd1_8564s/as; *Fzd1#2*, mixture of Mm_Fzd1_8565s/as and Mm_Fzd1_8566s/as) by electroporation and Lipofectamine 2000 (Life Technologies Inc., Carlsbad, CA, USA), respectively. To establish C2C12 cells stably expressing Fzd1-GFP or GFP, cells transfected with pEGFP-N3-*Fzd1* or pEGFP-N3 were selected with 400 μg/ml G418 for 2 weeks and GFP-expressing cells were sorted by FACS.

Real-time quantitative RT-PCR

Total RNA was isolated from the TA muscles using Isogen (Nippon Gene Co., Ltd.,

Tokyo, Japan), and reverse-transcribed using the PrimeScript RT reagent kit (TAKARA Bio Inc., Shiga, Japan). Expression levels of the respective genes of interest were measured by real-time quantitative PCR using a LightCycler 480 (Roche Diagnostics K.K., Tokyo, Japan). The sequences of the primer pairs are indicated in Table S1. The amount of mRNA was normalized relative to that of 18S ribosomal RNA.

Immunofluorescence microscopic analysis

Cells were fixed and stained with DAPI and anti-Myhc (MAB4470, R&D Systems, Abingdon, UK) and anti-GFP antibodies (AB290, Abcam Inc., Cambridge, MA, USA). For measurement of cross-sectional area of myofibers, ten μm cryosections of the TA muscles were stained by DAPI and anti-laminin antibody (L9393, Sigma-Aldrich). Fluorescent images were obtained using a laser scanning confocal imaging system (LSM710; Carl Zeiss MicroImaging, Thornwood, NY, USA) and processed using ZEN 2011 (Carl Zeiss MicroImaging). For quantification of myofiber diameter, the cross-sectional area of myofibers in 6 randomly chosen microscopic fields was measured using Image J (National Institutes of Health, Bethesda, MD, USA). For quantification of the percentage of nuclei belonging to Myhc-positive cells, nuclei in 6 randomly chosen microscopic fields was counted using Image J.

Luciferase reporter assay

TOPflash and FOPflash reporter vectors (containing wild-type and mutated TCF/LEF-response elements, respectively) were co-transfected with pRL-*TK* (Promega KK, Tokyo, Japan) into C2C12 cells. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega KK). The transcriptional activities were normalized relative to Renilla luciferase activities.

Western blotting

Cells were solubilized with ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Nonidet-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM p-Amidinophenylmethanesulfonyl fluoride (p-APMSF), 10 mM dithiothreitol (DTT)]. Proteins were separated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Milford, MA, USA). Membranes were immunoblotted with the respective antibodies as follows; anti-GFP (AB290, Abcam), anti-β-catenin (610154, BD Biosciences, San Jose, CA, USA), anti-active β-catenin (8E7, Millipore), anti-LRP6 (ab75358, Abcam), anti-phospho-LRP6 (#2568, Cell Signaling Technology, Beverly, MA, USA), anti-Fzd1 (BAM11201, Abcam) and anti-α/β-tubulin (#2148, Cell signaling Technology). Immunoreactive bands were visualized using the ECL detection system.

Acknowledgements

We are grateful to Dr. Tohru Tezuka (The University of Tokyo) for providing C2C12 cells and critical information about culture methods. We thank Drs. Motoi Kanagawa and Tatsushi Toda (Kobe University) for providing critical information about mouse model for muscle damage and mSCs isolation. This work was supported by a grant-in-aid for Scientific Research (B) (Y.M.), for Scientific Research on Innovative Areas (Y.M. and K.Y.), for Scientific Research (C) (M.N.), for Young Scientists (B) (M.E.) and for Global Center of Excellence Program (Y.M.) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. This work was also supported by Takeda Science Foundation (K.Y.). R.D. was supported by the Japan Society for the Promotion of Science (JSPS) Research Fellowships for Young Scientists.

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Figure Legends

Figure 1. Delayed muscle regeneration following cardiotoxin (Ctx)-induced muscle damage in aged mice. (A) Measurement of body weight and TA muscle weight of young (blue bars) and aged (red bars) mice. (B) Measurement of glycogen content in TA muscles from young (blue bar) and aged (red bar) mice. (C) Real-time RT-PCR analyses of *Axin2* expression in TA muscles (uninjured and injured) from young (blue bars) and aged mice (red bars) at day 12 after Ctx-treatment. (D, E) Cross sections of TA muscles (uninjured and injured) from young and aged mice at day 12 after Ctx-treatment were stained with DAPI (blue) and anti-laminin (red). Images are representative of three independent experiments (D). Scale bars: 50 μm. The cross-sectional area of myofibers was measured (E). Data are expressed as the percentages of myofibers with the following cross-sectional areas: < 500 μm^2 (blue); 500-1500 μm^2 (red); 1500-2500 μm^2 (green); > 2500 μm^2 (purple). Values are means ± SEM (n = 3-6). **P* < 0.05 compared with values from TA muscles of young mice (Student's t test).

Figure 2. Age-related high expression of *Fzd1* gene in mSCs isolated from TA muscles. (A) FACS profiles displaying the percentage of mSCs (positive selection for SM/C-2.6 and negative selection for CD31, CD45 and Sca-1) from TA muscles of young and aged mice. (B) Quantification of the percentage of mSCs in TA muscles from young (blue

bar) and aged (red bar) mice. (C) Real-time RT-PCR analysis of *Pax7* expression between unsorting cells (UCs) and mSCs from young (blue bar) and aged (red bar) mice. (D) Expression of *Wnt* receptor genes (*Fzds* and *Lrps*) in mSCs. Values are means ± SEM (n=3). *P < 0.05 compared with values from mSCs of young mice (Student's t test).

Figure 3. Age-related expression of *Fzd1* genes and its associated activation of Wnt/β-catenin signal in myogenic cells during differentiation. (A) Primary myogenic cells isolated from TA muscles of young (blue bars) and aged (red bars) mice were cultured in DM to induce differentiation for 0, 3, and 7 days. Expression levels of the indicated genes were quantified by real-time RT-PCR. Values are means ± SEM (n=3). *P < 0.05 compared with values from myogenic cells of young mice (Student's t test). (B) Primary myogenic cells cultured in DM for 3 days were fixed and stained with DAPI (blue) and anti-Myhc (red). Images are representative of three independent experiments. Scale bars: 100 μm. Percentage of the number of nuclei belonging to Myhc-positive cells in 6 randomly chosen microscopic fields was counted. Values are means ± SEM (n=6). *P < 0.05 compared with values from myogenic cells of young mice (Student's t test).

Figure 4. Involvement of Fzd1 in activation of Wnt/β-catenin signal in myogenic cells. (A) C2C12 cells were transfected with the indicated *siRNAs*. Thirty six hrs after transfection, media were replaced with serum-depleted DM, where cells were cultured for 12 hrs. Subsequently, media were replaced with DM in the presence or absence of Wnt3a (100 ng/ml), and cultured for 24 hrs. Cell lysates were prepared from the respective cells, and subjected to western blotting with anti-Fzd1 or anti-tubulin antibodies as indicated. Expression of *Fzd1* and *Axin2* were quantified at mRNA levels by real-time RT-PCR. Values are means ± SEM (n=3). *P < 0.05 compared with values from *Control siRNA* transfected cells without Wnt3a (ANOVA followed by Tukey's method). (B) C2C12 cells stably expressing GFP and Fzd1-GFP, respectively, were stimulated with or without Wnt3a (5 ng/ml) for 12 hrs. Cell lysates were prepared from the respective cells, and subjected to western blotting with the antibodies as indicated. (C) FOPflash and TOPflash reporter vectors were co-transfected with pRL-TK into two independent stable clones of C2C12 cells expressing GFP (GFP-C1 and GFP-C2) and Fzd1-GFP (Fzd1-GFP-C1 and Fzd1-GFP-C2), respectively. Cells were stimulated with Wnt3a (5 ng/ml) for 24 hrs. The transcriptional activities were normalized relative to Renilla luciferase activities (Relative luciferase units, RLU). Values are means ± SEM

(n=4). *$P < 0.05$ compared with values from non-stimulated cells (Student's t test).

Figure 5. Association of Fzd1 expression with age-related delay in myogenesis. (A, B) Primary myogenic cells from aged mice were transfected with the indicated *siRNAs*. Fourty eight hrs after transfection, media were replaced with DM. At 3 days after differentiation, the respective cells were harvested for western blot analysis with anti-Fzd1 or anti-tubulin antibodies as indicated (A) or fixed and stained with DAPI (blue) and anti-Myhc (red) (B). Images are representative of two independent experiments. Scale bars: 100 μm. Percentage of the number of nuclei belonging to Myhc-positive cells in 6 randomly chosen microscopic fields was counted. Values are means ± SEM (n=6). *$P < 0.05$ compared with values from *Control siRNA* transfected cells (ANOVA followed by Tukey's method).(C) Primary myogenic cells from young mice were transfected with the indicated expression vectors. Fourty eight hrs after transfection, media were replaced with DM. The respective cells were fixed at 10 days after differentiation and stained with DAPI (blue) and anti-GFP (green) and anti-Myhc (red) antibodies. Images are representative of two independent experiments. Scale bars: 100 μm. Percentage of the number of nuclei belonging to Myhc-positive cells in 6 randomly chosen microscopic fields was counted. Values are means ± SEM (n=6). *$P <$ 0.05 compared with values from GFP expressing cells (Student's t test).

Fig.1

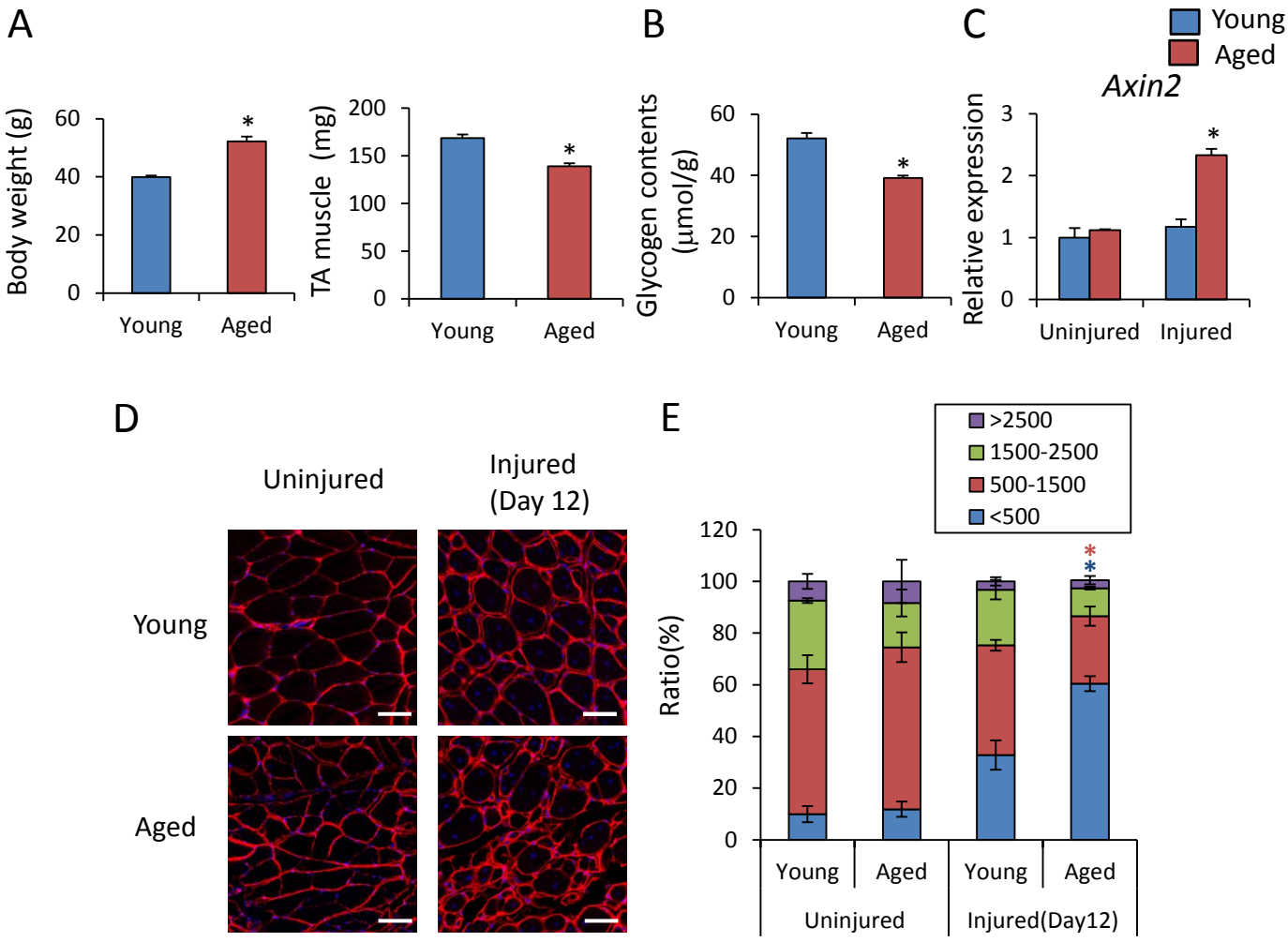

A
Body weight (g)
60
40
20
0
*
Young
Aged
TA muscle (mg)
200
150
100
50
0
*
Young
Aged
B
Glycogen contents (μmol/g)
60
40
20
0
*
Young
Aged
C
Young
Aged
Axin2
Relative expression
3
2
1
0
*
Uninjured
Injured
D
Uninjured
Injured (Day 12)
Young
Aged
E
>2500
1500-2500
500-1500
<500
Ratio(%)
120
100
80
60
40
20
0
*
*
Young
Aged
Young
Aged
Uninjured
Injured(Day12)

Fig.2

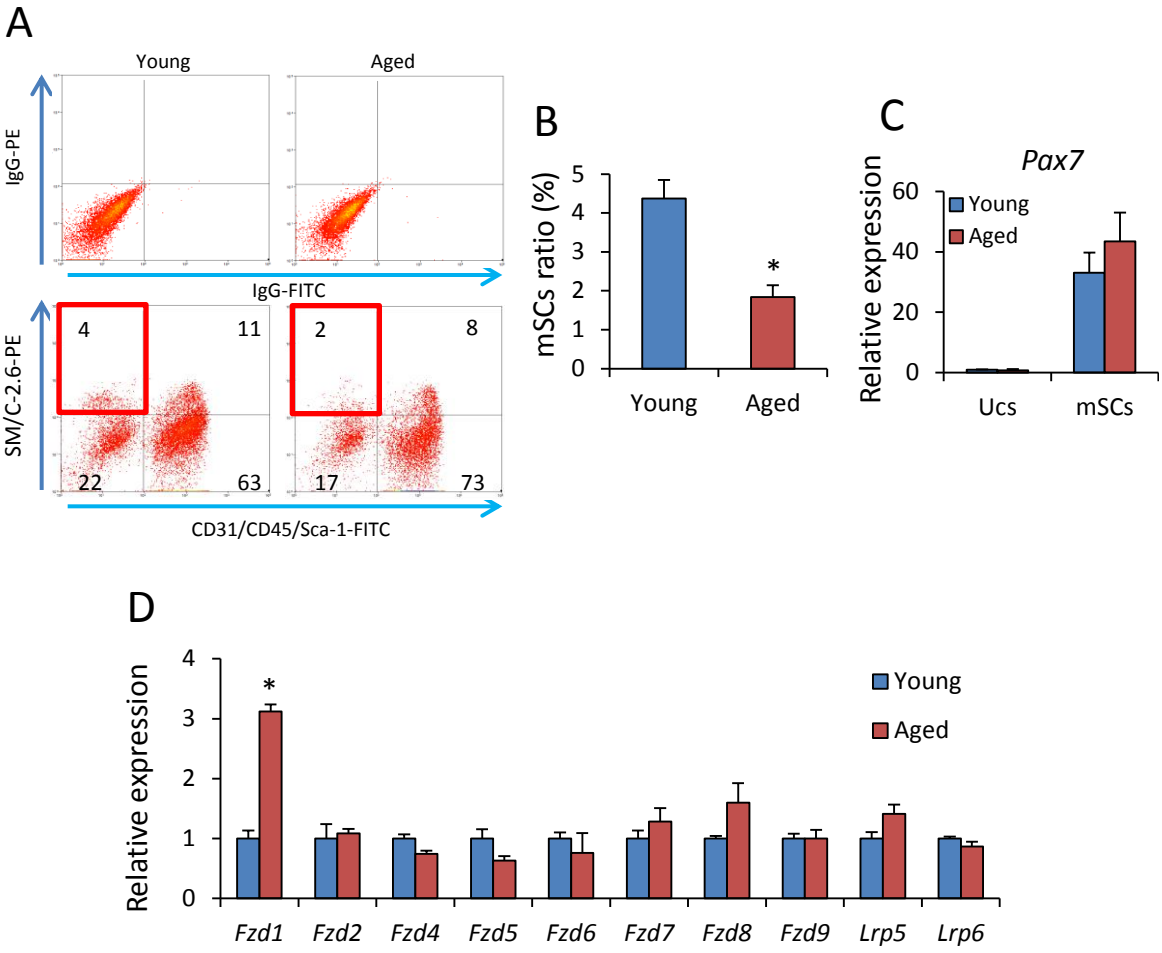
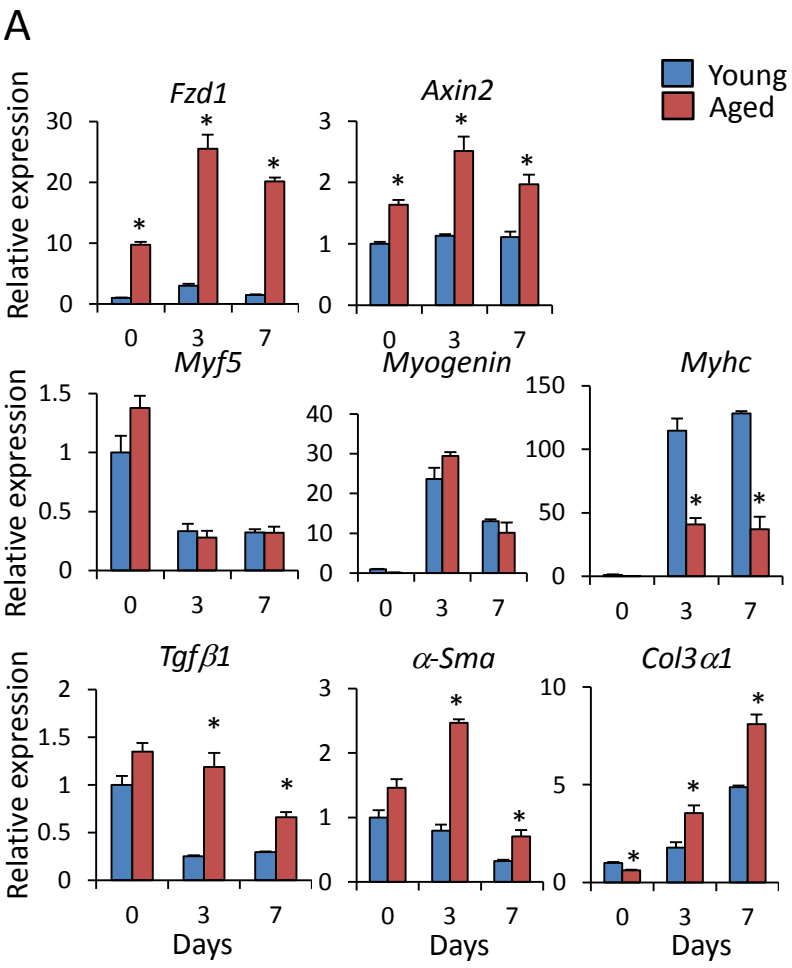

A
Young
Aged
IgG-PE
IgG-FITC
SM/C-2.6-PE
4
11
2
8
22
63
17
73
CD31/CD45/Sca-1-FITC
B
mSCs ratio (%)
5
4
3
2
1
0
*
Young
Aged
C
Pax7
Relative expression
60
40
20
0
Young
Aged
Ucs
mSCs
D
Relative expression
4
3
2
1
0
*
Young
Aged
Fzd1
Fzd2
Fzd4
Fzd5
Fzd6
Fzd7
Fzd8
Fzd9
Lrp5
Lrp6

Fig.3


A
Young
Aged
Fzd1
Axin2
Myf5
Myogenin
Myhc
Tgfβ1
α-Sma
Col3α1
Relative expression
Days

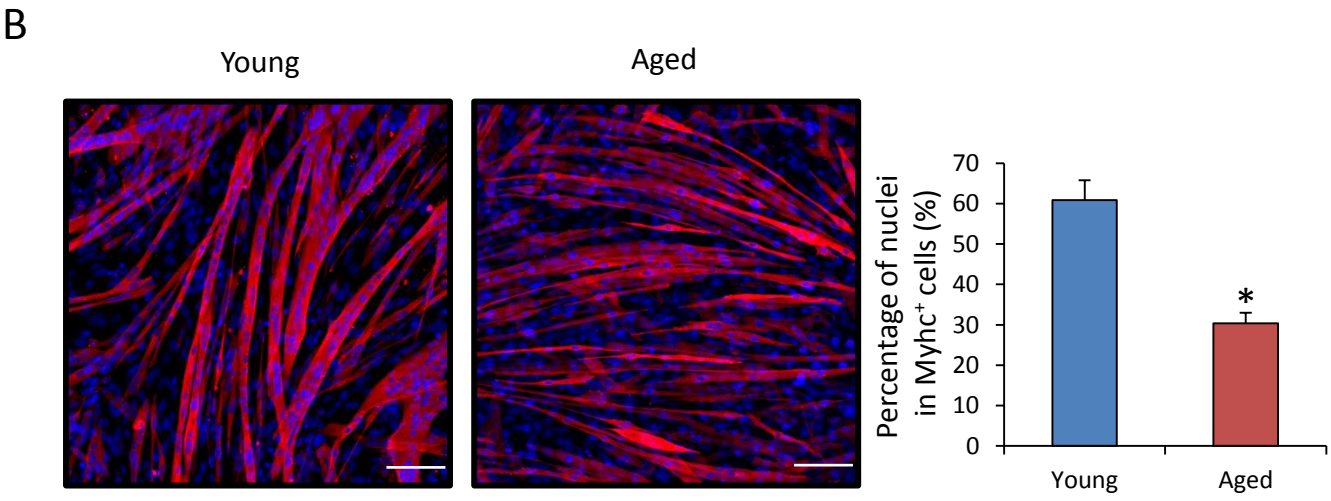

B
Young
Aged
Percentage of nuclei in Myhc+ cells (%)
Young
Aged

Fig.4

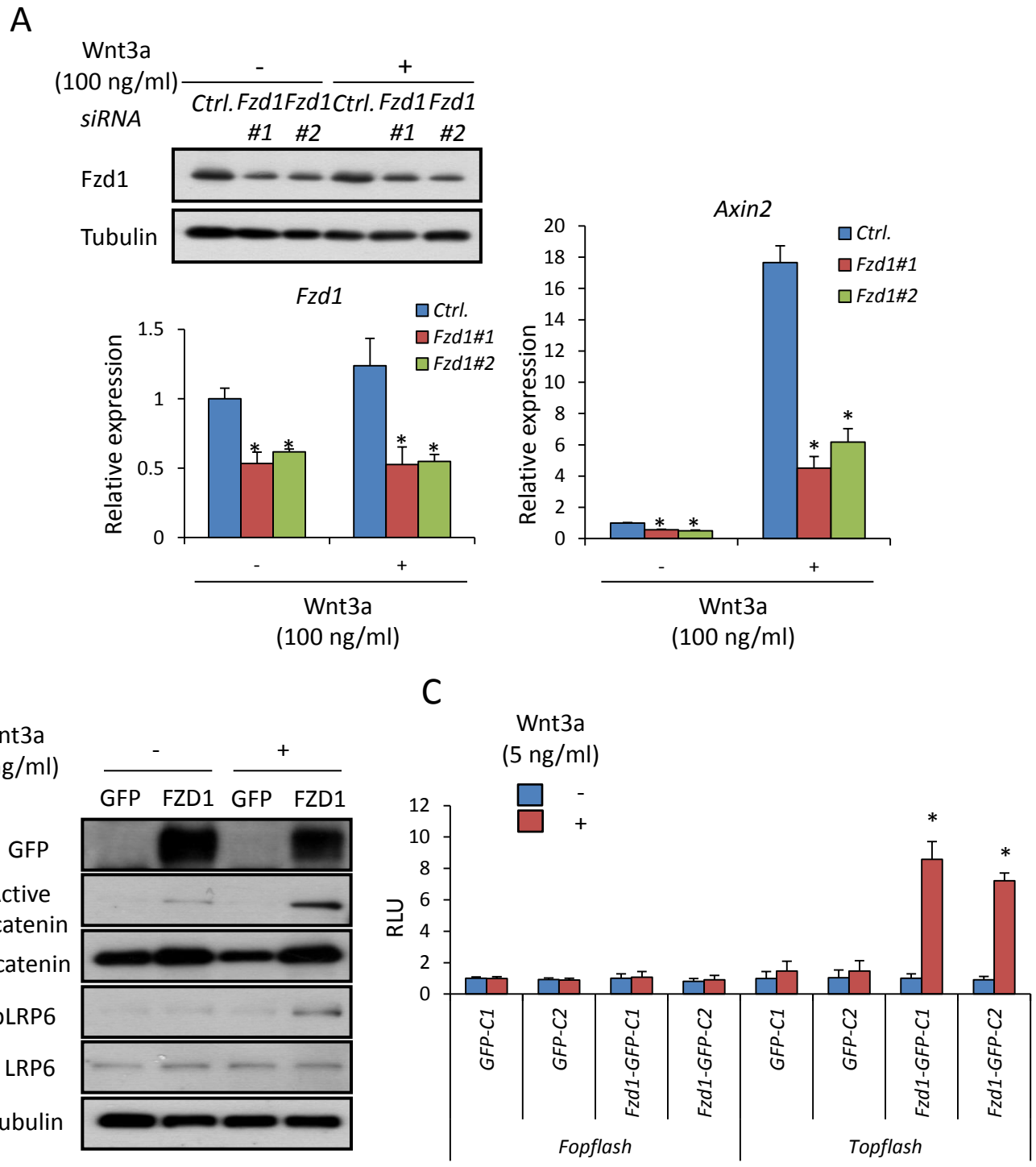
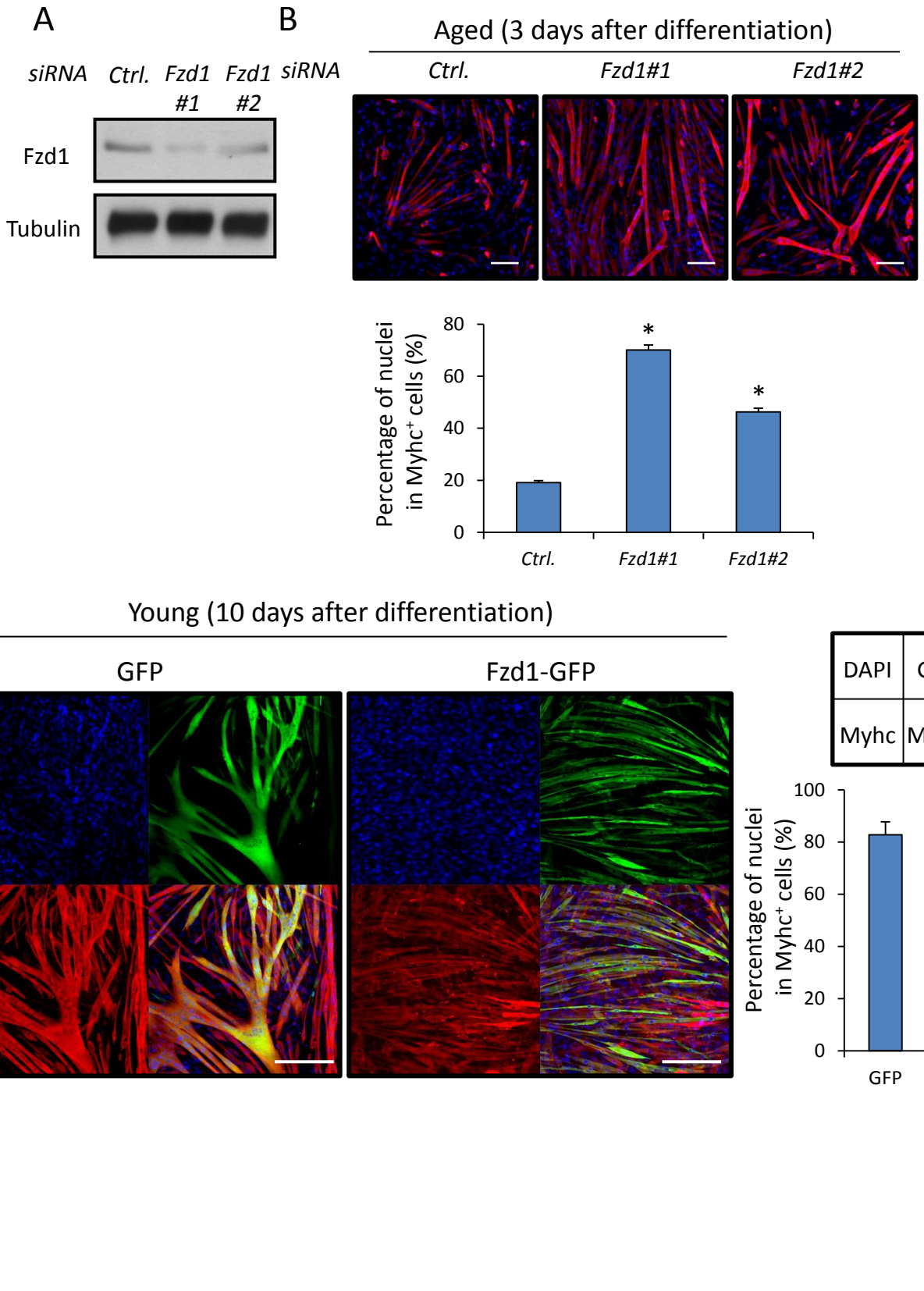

A
Wnt3a
(100 ng/ml)
-
+
siRNA
Ctrl.
Fzd1 #1
Fzd1 #2
Ctrl.
Fzd1 #1
Fzd1 #2
Fzd1
Tubulin
Fzd1
Ctrl.
Fzd1#1
Fzd1#2
Relative expression
1.5
1
0.5
0
Wnt3a
(100 ng/ml)
Axin2
Ctrl.
Fzd1#1
Fzd1#2
Relative expression
20
18
16
14
12
10
8
6
4
2
0
Wnt3a
(100 ng/ml)
B
Wnt3a
(5 ng/ml)
GFP
FZD1
GFP
FZD1
GFP
Active β-catenin
β-catenin
pLRP6
LRP6
Tubulin
C
Wnt3a
(5 ng/ml)
RLU
12
10
8
6
4
2
0
GFP-C1
GFP-C2
Fzd1-GFP-C1
Fzd1-GFP-C2
GFP-C1
GFP-C2
Fzd1-GFP-C1
Fzd1-GFP-C2
Fopflash
Topflash

Fig.5


A
siRNA
Ctrl.
Fzd1 #1
Fzd1 #2
Fzd1
Tubulin
B
siRNA
Aged (3 days after differentiation)
Ctrl.
Fzd1#1
Fzd1#2
Percentage of nuclei in Myhc+ cells (%)
80
60
40
20
0
*
*
Ctrl.
Fzd1#1
Fzd1#2
C
Young (10 days after differentiation)
GFP
Fzd1-GFP
DAPI
GFP
Myhc
Merge
Percentage of nuclei in Myhc+ cells (%)
100
80
60
40
20
0
*
GFP
Fzd1-GFP

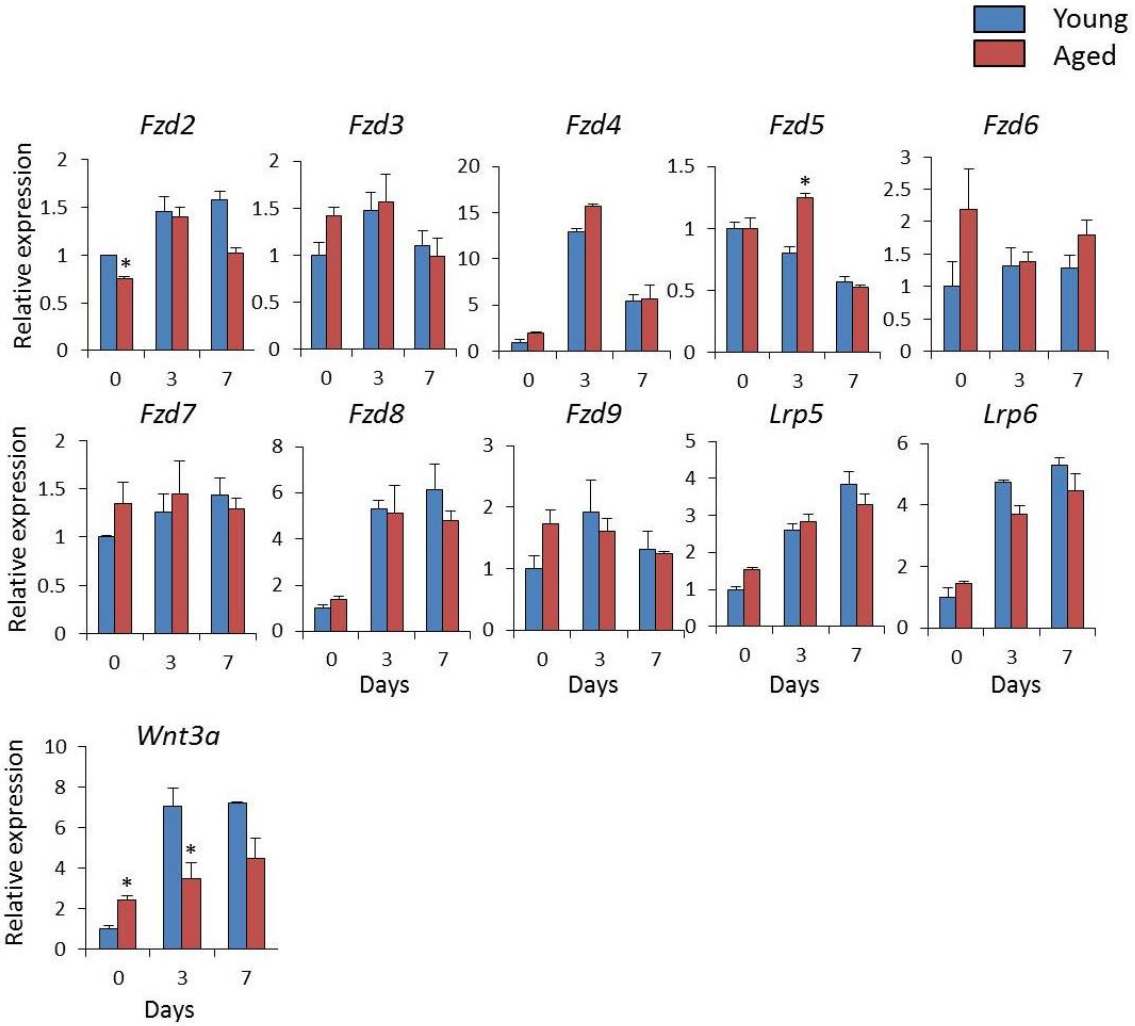


Fig. S1. Expression profiles of *Fzd* and *Wnt3a* genes in myogenic cells during differentiation. Primary myogenic cells isolated from TA muscles of young (blue bars) and aged (red bars) mice were cultured in DM to induce differentiation for 0, 3, and 7 days. Expression levels of the indicated genes were quantified by real-time RT-PCR. Values are means ± SEM (n=3). **P* < 0.05 compared with values from myogenic cells of young mice (Student's t test).

Table S1. Primer sequences utilized for real-time quantitative RT-PCR

Gene	Forward sequences	Reverse sequences
18s	5'-CGATAACGAACGAGACTCTG-3'	5'-GACATCTAAGGGCATCACAG-3'
Pax7	5'-CTGGATGAGGGCTCAGATGT-3'	5'-GGTTAGCTCCTGCCTGCTTA-3'
Myf-5	5'-TGAAGGATGGACATGACGGACG-3'	5'-TTGTGTGCTCCGAAGGCTGCTA-3'
Myogenin	5'-TGCCGTGGGCATGTAAGGT-3'	5'-TGCGCAGGATCTCCACTTTAG-3'
Myhc	5'-AATCAAAGGTCAAGGCCTACAA-3'	5'-GAATTTGGCCAGGTTGACAT-3'
Tgfβ1	5'-CTAATGGTGGACCGCAACAAC-3'	5'-CACTGCTTCCCGAATGTCTGA-3'
α-Sma	5'-AGACAGCTATGTGGGGGATG-3'	5'-AGTGGTGCCAGATCTTTTCC-3'
Col3a1	5'-AGGCTGAAGGAAACAGCAAA-3'	5'-TAGTCTCATTGCCTTGCGTG-3'
Wnt3a	5'-CCCTTTCCAGTCCTGGTGTA-3'	5'-CTTGAAGAAGGGGTGCAGAG-3'
Wnt8a	5'-ACGGTGGAATTGTCCTGAGCATG-3'	5'-GATGGCAGCAGAGCGGATGG-3'
Wnt8b	5'-TTGGGACCGTTGGAATTGCC-3'	5'-AGTCATCACAGCCACAGTTGTC-3'
Wnt10b	5'-TTCTCTCGGGATTTCTTGGATTC-3'	5'-TGCACTTCCGCTTCAGGTTTTC-3'
Axin2	5'-GGGTTCTGAAATTCATAGACTAAGA-3'G	5'-CGACTGTTCAATAAATATCAGTAA-3'GG
Cyclind1	5'-TGGAACTGTTCTGGTGAAC-3'	5'-TTCACATCTGTGGCACAGAG-3'
Fzd1	5'-CACCTGGATAGGCATCTGGT-3'	5'-CAGAAAGCCAGCGATGTAGG-3'
Fzd2	5'-CCGACGGCTCTATGTTCTTC-3'	5'-TAGCAGCCGGACAGAAAGAT-3'
Fzd3	5'-AGCGTGCCTATAGCGAGTGT-3'	5'-TCTCTGGGACACCAAAAACC-3'
Fzd4	5'-CTGCAGCATGCCTAATGAGA-3'	5'-CGTCTGCCTAGATGCAATCA-3'
Fzd5	5'-TCTTGTCTGCGTGCTACCTG-3'	5'-GGCCATGCCAAAGAAATAGA-3'
Fzd6	5'-TGTTGGGCTGTCTCTCCTCT-3'	5'-TCTCCCAGGTGATCCTGTTC-3'
Fzd7	5'-CCATCCTCTTCATGGTGCTT-3'	5'-TGGCCAAAATGGTGATTGTC-3'
Fzd8	5'-CTGTTCCGAATCCGTTCAGT-3'	5'-CGGTTGTGCTGCTCATAGAA-3'
Fzd9	5'-AGAGCAACCATGTACTGCTG-3'	5'-GCTCTGCCAAGTCTGAAAAG-3'
Fzd10	5'-GTGGAGAAGAGCGAATCTTG-3'	5'-AGCACATGGAGAGGAAGATG-3'
Lrp5	5'-CAGGTGCTTGTGTGGAGAGA-3'	5'-CATGTTGGTGTCCAGGTCAG-3'
Lrp6	5'-GGTGTCAAAGAAGCCTCTGC-3'	5'-ACCTCAATGCGATTTGTTCC-3'