

# Filamin C regulates skeletal muscle atrophy by stabilizing dishevelled-2 to inhibit autophagy and mitophagy

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**FilaminC (Flnc) is a member of the actin binding protein family, which is preferentially expressed in the cardiac and skeletal muscle tissues. Although it is known to interact with proteins associated with myofibrillar myopathy, its unique role in skeletal muscle remains largely unknown. In this study, we identify the biological functions of Flnc *in vitro* and *in vivo* using chicken primary myoblast cells and animal models, respectively. From the results, we observe that the growth rate and mass of the skeletal muscle of fast-growing chickens (broilers) were significantly higher than those in slow-growing chickens (layers). Furthermore, we find that the expression of Flnc in the skeletal muscle of broilers was higher than that in the layers. Our results indicated that Flnc was highly expressed in the skeletal muscle, especially in the skeletal muscle of broilers than in layers. This suggests that Flnc plays a positive regulatory role in myoblast development. Flnc knockdown resulted in muscle atrophy, whereas the overexpression of Flnc promotes muscle hypertrophy *in vivo* in an animal model. We also found that Flnc interacted with dishevelled-2 (Dvl2), activated the wnt/ $\beta$ -catenin signaling pathway, and controlled skeletal muscle development. Flnc also antagonized the LC3-mediated autophagy system by decreasing Dvl2 ubiquitination. Moreover, Flnc knockdown activated and significantly increased mitophagy. In summary, these results indicate that the absence of Flnc induces autophagy or mitophagy and regulates muscle atrophy.**

## INTRODUCTION

The skeletal muscle is a form of striated muscle tissue, which accounts for approximately 40%–60% of the body weight of adult animals.<sup>1</sup> The skeletal muscle consists of multinucleated cells called muscle fibers, which are formed by the coalescence of myoblasts.<sup>2</sup> The study on skeletal muscle is of great interest in the areas of morbidity and mortality associated with muscular dystrophy and myopathy. The skeletal muscle also contributes significantly to the economic value of animals used for meat.<sup>3</sup> Therefore, proper investigation on the regulatory mechanisms underlying the growth and development of the

skeletal muscle to improve human health and animal husbandry is crucial. Chicken is regarded as a suitable research model for studying skeletal muscle development in vertebrates, since galline developmental anatomy is similar to that of other amniotes, including humans.<sup>4</sup> Fast-growing chickens (broilers) and slow-growing chickens (layers) are two types of breeds that have been developed through long periods of genetic selection. The growth and development of the skeletal muscle between these two breeds of chickens differs significantly. For instance, in broilers, the growth rate of skeletal muscle is five times higher than that of the laying hens at the age of 6 weeks. Due to the differences in the genetic make-up and genome levels of both layer hens and broilers, this therefore makes them suitable models for studying skeletal muscle growth and development.

Filamin proteins are actin binding proteins,<sup>5</sup> containing three family members, including Flna, Flnb, and Flnc. Flna and Flnb are typically expressed in all tissues, whereas Flnc is mainly expressed in the myocardium and skeletal muscle.<sup>6,7</sup> Flnc, a Z-band-associated 2,725 amino acid protein, contains one N-terminal actin binding domain and 24 serine-type immune globulin repeats, and can bind to several Z-disk proteins, including myotilin (Myot) and myozenins (Myoz).<sup>8,9</sup> Dalkilic and Kunkel found Flnc genes among approximately 30 disease-associated genes in various forms of muscular dystrophy.<sup>10</sup> Juo et al. also reported that Flnc interacts with the muscle-specific protein HSPB7 and that Flnc deficiency could lead to progressive skeletal muscle myopathy.<sup>11</sup> The study by Ruparelia et al. reported that

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impaired autophagy and protein dysfunction could cause Flnc myofibrillar myopathy,<sup>12</sup> which suggested that Flnc may help regulate skeletal muscle development by modulating autophagy.

Autophagy is a process of bulk degradation by which cytoplasmic proteins and eliminating defective or damaged organelles are recycled back through the formation of double membranous vesicles (autophagosomes),<sup>13</sup> followed by fusion with lysosomes to be degraded by lysosomal acid hydrolases and proteases.<sup>14</sup> Skeletal muscle autophagy regulates muscle homeostasis. Excessive autophagy could reduce muscle mass due to continuous removal of cellular components. Reduction in autophagy leads to chronic loss of muscle mass due to cell damage or aging.<sup>14,15</sup> Disorders of autophagy are thought to be associated with many forms of inherited muscular dystrophy, including Duchenne muscular dystrophy, Ullrich congenital muscular dystrophy, and Bethlem myopathy.<sup>16</sup> Imbalances in autophagy homeostasis are also believed to play a vital role in various myopathies with inclusions or mitochondrial abnormalities. To maintain metabolic homeostasis in the cell, damaged mitochondria and their accumulated toxicity, must be cleared through a selective autophagy mechanism known as mitophagy.<sup>17</sup>

Flnc is a cytoskeleton-associated protein that binds Z-disk proteins to activate downstream signaling cascades. However, the role of Flnc in autophagy and mitophagy remains poorly understood. In this study, we determined the biological impact of Flnc on skeletal muscle growth and development *in vitro* and *in vivo* using primary myoblast cells and animal model (chicken chest muscle), respectively. These studies showed that Flnc is a potential therapeutic target for increasing skeletal muscle mass during skeletal myopathy.

## RESULTS

### Flnc is upregulated in hypertrophic broilers and plays a role in skeletal muscle differentiation

The results obtained in this study showed that the growth rates of broilers and layers differed significantly (Figure S1A). The body weight, pectoral muscle weight, pectoral muscle percentage, and muscle fiber area of broilers were much higher than those of the layers (Figures S1B–S1F). To screen for candidate genes that regulate muscle development, we determined the chest muscle transcriptomes of both Ross 308 broilers and White leghorn layers. Of the 12,744 expressed transcripts, 869 had expression profiles that were significantly different between the two breeds, such as Stac3, Klf5, MyoG, Nexn, Myot, and MyH7, which are signature genes that regulate muscle development (Figure 1A). We then validated the gene characteristics of skeletal muscle development using qPCR (Figure 1B). Transcriptome sequencing indicated that Flnc expression in fast-growing chickens was 50-fold higher than that in slow-growing chickens. Subsequently, we found that Flnc was mainly expressed in skeletal muscle and cardiac muscle (Figure 1C). In addition, Flnc plays an important role in maintaining the structural integrity of cardiac and skeletal muscles. Therefore, we speculate that Flnc plays an important role in the growth and development of skeletal muscles. We then collected chicken primary myoblasts to study the function of Flnc. Primary

myoblasts were identified using MyoD immunofluorescence staining (Figure 1D). qPCR analysis indicated that Flnc mRNA expression was higher in chicken myotubes than in chicken myoblasts (Figure 1E). Using qPCR and western blotting, we also found that Flnc expression increased gradually during the differentiation of myoblasts into myotubes (Figures 1F and 1G).

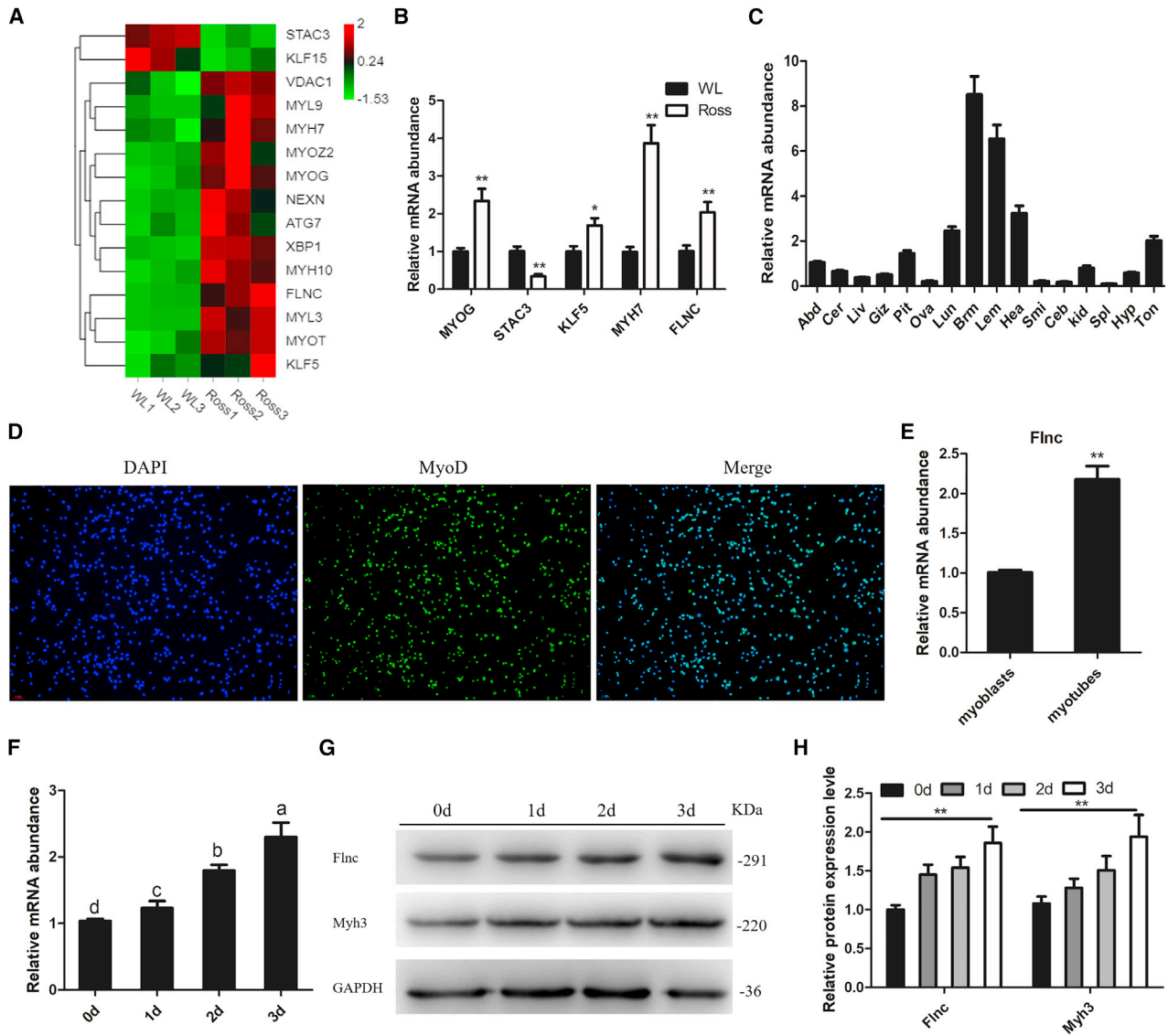
### Flnc promotes myoblast differentiation

To explore the role of Flnc during myogenic differentiation, we transfected Flnc siRNA to reduce Flnc mRNA and protein expression in chicken myoblasts (Figures 2A–2C). We measured mRNA expression for three myogenic marker genes: myogenin (MyoG), myosin heavy chain 3 (MyH3), and myoglobin (Mb); expression of the three genes was significantly reduced in Flnc-silenced cells (Figure 2D). Immunofluorescence staining revealed that Flnc silencing restrained myoblast differentiation and decreased the total area of myotubes (Figures 2E and 2F). Western blotting also showed that MyoG and MyH3 protein expression decreased significantly in Flnc-silenced cells (Figures 2G and 2H). We also determined the effect of Flnc overexpression on myoblast differentiation. We achieved Flnc overexpression by transfecting cells with the Flnc-overexpressing plasmids. In contrast, Flnc overexpression significantly increased myoblast differentiation, myogenic gene expression, and myotube area (Figures 3A–3H). To confirm these results, we established control, Flnc-silenced, and Flnc-overexpressing C2C12 mouse myoblast cell lines. Similarly, we observed that the differentiation of Flnc-silenced C2C12 cells was significantly decreased (Figures S2A–S2F), while the differentiation of Flnc-overexpressing C2C12 cells was increased (Figures S3A–S3F). Then, we examined the effects of Flnc on cell proliferation and apoptosis. The results showed that Flnc silencing promoted proliferation and inhibited apoptosis, while Flnc overexpression inhibited proliferation and promoted apoptosis (Figures S4A–S4C and S5A–S5F). This suggests that Flnc may reduce myoblast number and affects myogenic differentiation.

### Flnc could rescue skeletal muscle atrophy

Next, we investigated the influence of Flnc on atrophy-related genes in primary myotubes. We found that Flnc interference significantly increased expression of atrogin-1 and MuRF1 mRNA and atrogin-1 protein (Figures 4A–4C). In contrast, atrogin-1 and MuRF1 mRNA expression and atrogin-1 protein levels were decreased significantly after Flnc overexpression (Figures 4D–4F). It is known that synthetic glucocorticoid dexamethasone overdose induces muscle atrophy.<sup>18</sup> Therefore, we silenced and overexpressed Flnc *in vitro* in dexamethasone-induced myotube atrophy. Expression of atrogin-1 protein was further enhanced after dexamethasone treatment of Flnc-silenced cells (Figures 4G and 4H). However, Flnc overexpression reduced atrogin-1 expression after dexamethasone treatment (Figures 4I and 4J). These results indicate that Flnc may help regulate the expression of muscle atrophy-related genes and remedy dexamethasone-induced myotube atrophy.

Furthermore, we tested the effect of Flnc on muscle development *in vivo*, and found that Flnc mRNA expression was significantly

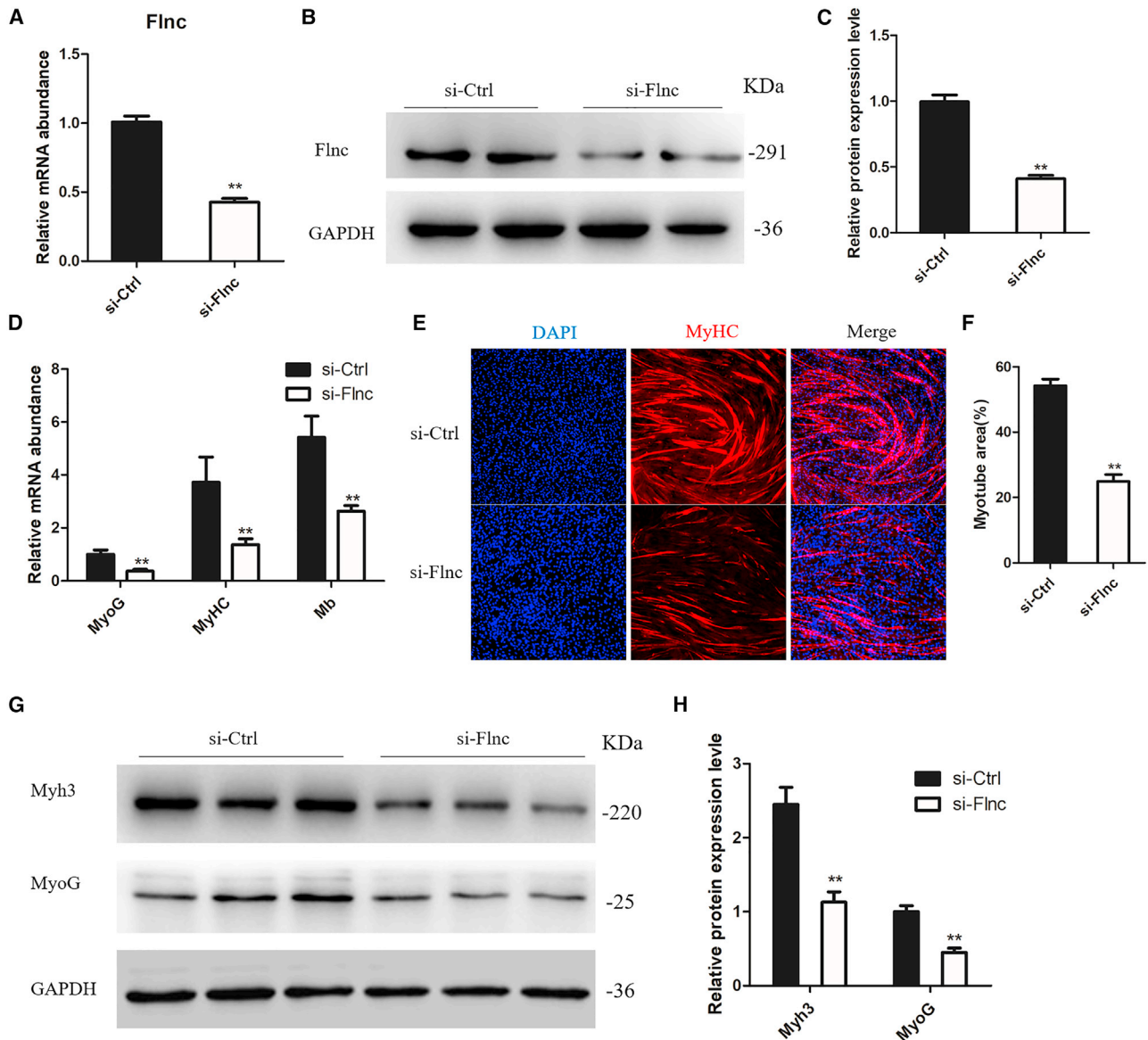


**Figure 1. The role of Flnc in skeletal muscle differentiation**

(A) Heatmaps of differentially expressed mRNAs in the chest muscles of broilers and layers. WL, White leghorn layer; Ross, Ross 308 broiler. (B) qPCR analysis of the expression levels of representative differential genes in broilers and layers. GAPDH was used as an internal control. n = 3 chickens in each group. (C) RNA expression levels of Flnc in 16 different tissues of broilers. Abd, abdominal fat; Cer, cerebrum; Liv, liver; Giz, gizzard; Pit, pituitary; Ova, ovary; Lun, lung; Brm, breast muscle; Lem, leg muscle; Hea, heart; Smi, small intestine; Ceb, cerebellum; Kid, kidney; Spl, spleen; Hyp, hypothalamus; Ton, tongue; n = 3 chickens in each group. (D) MyoD immunostaining of primary chicken myoblasts. (E) Relative expression levels of Flnc mRNA in chicken myoblasts and myotubes. (F) The expression level of Flnc after myoblasts were differentiated into myotubes for 0, 1, 2, and 3 days. Bars not sharing the same letter labels are different, p < 0.05. (G and H) The protein expression levels of Flnc and Myh3 after myoblasts were differentiated into myotubes for 0, 1, 2, and 3 days. Data are expressed as mean ± SEM (n = 3 independent cell cultures). \*p < 0.05, \*\*p < 0.01.

increased in breast muscle after lentivirus-mediated Flnc overexpression, and decreased in Flnc knockdown vector-treated muscle (Figures 5A and 5B). The mass of Flnc-silenced chickens was also markedly lower than that of the controls at days 7 and 9 (Figure 5C). Chicken body mass was higher at day 9 after injection with Flnc-overexpressing lentivirus vector (Figure 5D). The chest muscle fiber diameter and muscle fiber cross-sectional area (CSA) were decreased in Flnc knockdown groups when compared with controls. However, both were increased in

Flnc-overexpressing chickens (Figures 5E–5H). Hematoxylin and eosin staining indicated that muscle fibers in the Flnc knockdown chickens were abnormal and the fiber CSA was reduced (Figure 5I). Muscle fiber CSA had increased significantly in Flnc-overexpressing chickens (Figure 5J). Similarly, Flnc silencing enhanced atrogen-1 expression, while overexpression reduced atrogen-1 expression *in vitro* (Figures 5K–5N). These results suggest that Flnc may play a role in regulating muscle atrophy and hypertrophy.



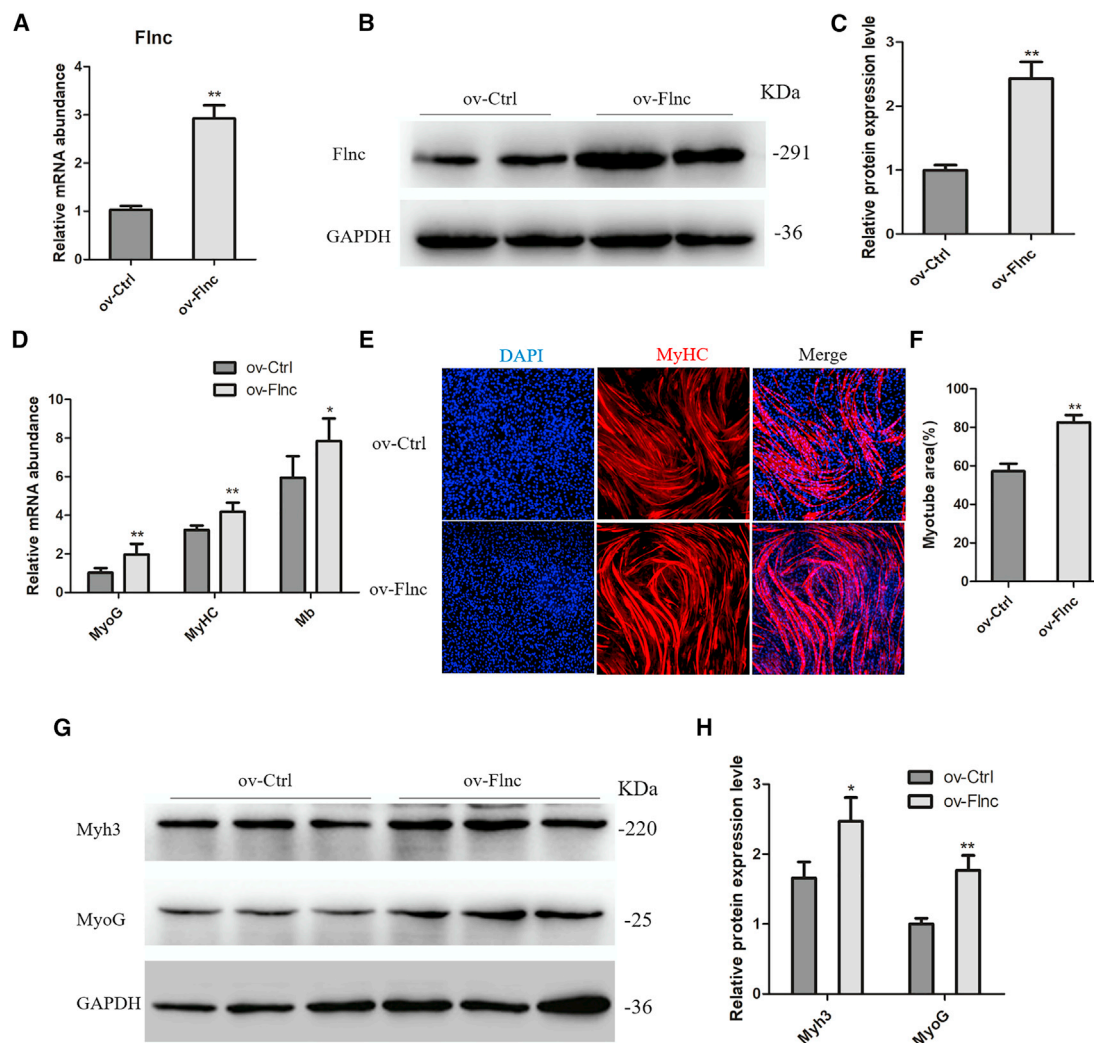
**Figure 2. Effect of FlnC knockdown on differentiation of myoblasts**

(A) Relative expression levels of FlnC mRNA in cells transfected with FlnC siRNA and control siRNA. (B and C) Western blot of FlnC expression protein levels in cells transfected with FlnC siRNA and control siRNA. (D) The mRNA expression levels of MyoG, MyHC, and Mb in FlnC-silenced and control cells. (E) Immunofluorescence staining for MyHC in FlnC-silenced and control cells after 3 days in differentiation medium. (F) The area of myotubes in FlnC-silenced and control cells. (G and H) Western blot analysis of Myh3 and MyoG protein levels in FlnC-silenced and control cells. Data are expressed as mean  $\pm$  SEM ( $n = 3$  independent cell cultures). \* $p < 0.05$ , \*\* $p < 0.01$ .

### FlnC functions through the Wnt/ $\beta$ -catenin signaling pathway mediated by Dvl-2

RNA sequencing was used to investigate the molecular mechanisms underlying the role of FlnC in myoblast differentiation by analyzing FlnC-silenced and control cells after 3 days of differentiation. The expression of myogenesis-related genes was significantly decreased during myoblast differentiation, which was inhibited by FlnC siRNA (Figure 6A). Meanwhile, qPCR confirmed that mRNA expression was significantly downregulated in myogenic and Wnt signaling

pathway target genes (Figure 6B). GO functional annotation analysis of differentially expressed genes (DEGs), identified genes involved in muscle cell regulation and development, muscle tissue morphogenesis, and muscle fiber development (Figure 6C). KEGG pathway analysis indicated that the DEGs were clearly involved in the MAPK and wnt signaling pathways. However, the Wnt signaling pathway is closely involved in muscle cell differentiation (Figure 6D).<sup>19</sup> To detect the regulatory effect of FlnC on Wnt signaling, we determined the expression of Dvl2 and  $\beta$ -catenin proteins, the key proteins of the



**Figure 3. Effect of Flnc overexpression on differentiation of myoblasts**

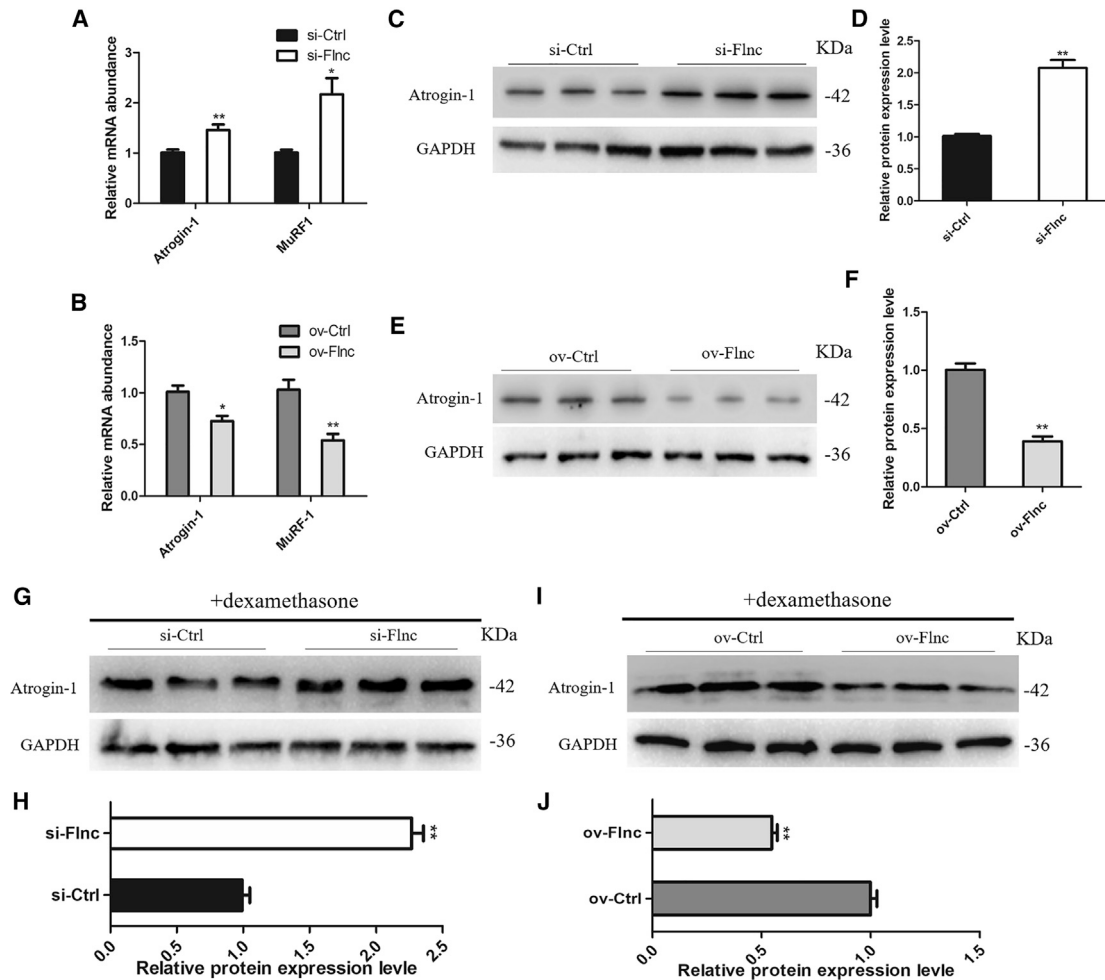
(A) Flnc mRNA transfected with Flnc-overexpressing and empty vector pcDNA3.1 plasmid. (B and C) Flnc protein levels in Flnc-overexpressing and empty vector pcDNA3.1 plasmid. (D) The mRNA expression levels of MyoG, MyH3, and Mb in Flnc-overexpressing and control cells. (E) Immunofluorescence staining of MyHC in Flnc-overexpressing and control cells after 3 days in differentiation medium. (F) The area of myotubes in Flnc-silenced and control cells. (G and H) Western blot analysis expression protein levels of MyH3 and MyoG in Flnc-overexpressing and control cells. Data are expressed as mean  $\pm$  SEM ( $n = 3$  independent cell cultures). \* $p < 0.05$ , \*\* $p < 0.01$ .

wnt pathway,<sup>20</sup> and found that Dvl2 abundance decreased in Flnc-silenced cells (Figures 6E and 6F). Moreover,  $\beta$ -catenin's ability to enter the nucleus was reduced significantly after Flnc silencing (Figures 6G and 6H). Subsequently, LiCl was used to explore the effect of Flnc on Wnt/ $\beta$ -catenin signaling, as LiCl activates the Wnt/ $\beta$ -catenin pathway.<sup>21</sup> The results showed that the expression of Axin1 was reduced after LiCl treatment, although Axin1 was still amassed in Flnc-silenced cells treated with LiCl (Figure 7A). Nucleus  $\beta$ -catenin protein was increased dramatically in LiCl-treated cells but, in Flnc knockdown cells, nuclear  $\beta$ -catenin expression was lower than in control cells (Figure 7B). Upon addition of wnt3a, another Wnt signaling pathway activator,<sup>22</sup> Wnt signaling was inhibited in Flnc-silenced cells (Figures 7C and 7D). 1-Azakenpauullone (1-AKP) can activate

Wnt/ $\beta$ -catenin signaling independent of Dvl2 participation.<sup>23</sup> The results showed that MyH3 was increased in Flnc-silenced cells treated with 1-AKP when compared with the untreated Flnc-silenced cells (Figures 7E and 7F). Western blot analysis indicated that protein levels of Axin1 and GSK3 $\beta$  were reduced in Flnc-silenced cells after incubation with 1-AKP (Figures 7G and 7H). This suggests that the regulation of Wnt signaling by Flnc requires the mediation of Dvl2.

#### Dvl2 complementation can rescue myogenesis damaged by Flnc silencing

The disheveled binding antagonist of  $\beta$ -catenin 1 (Dact1) is known to co-regulate Wnt signaling by antagonizing Dvl2 during mammalian development.<sup>24</sup> Here, we found that Dact1 silencing significantly



**Figure 4. Effects of FlnC on skeletal muscle atrophy**

(A and B) The mRNA expression levels of atrogin-1 and MuRF1 in FlnC-silenced, FlnC-overexpressing, and control cells. (C–F) The expression protein levels of atrogin-1 in FlnC-silenced, FlnC-overexpressing, and control cells. (G–J) The expression protein levels of atrogin-1 in FlnC-silenced, FlnC-overexpressing, and control cells after treatment with dexamethasone for 24 h to induce atrophy of myotubes. Data are expressed as mean  $\pm$  SEM ( $n = 3$  independent cell cultures). \* $p < 0.05$ , \*\* $p < 0.01$ .

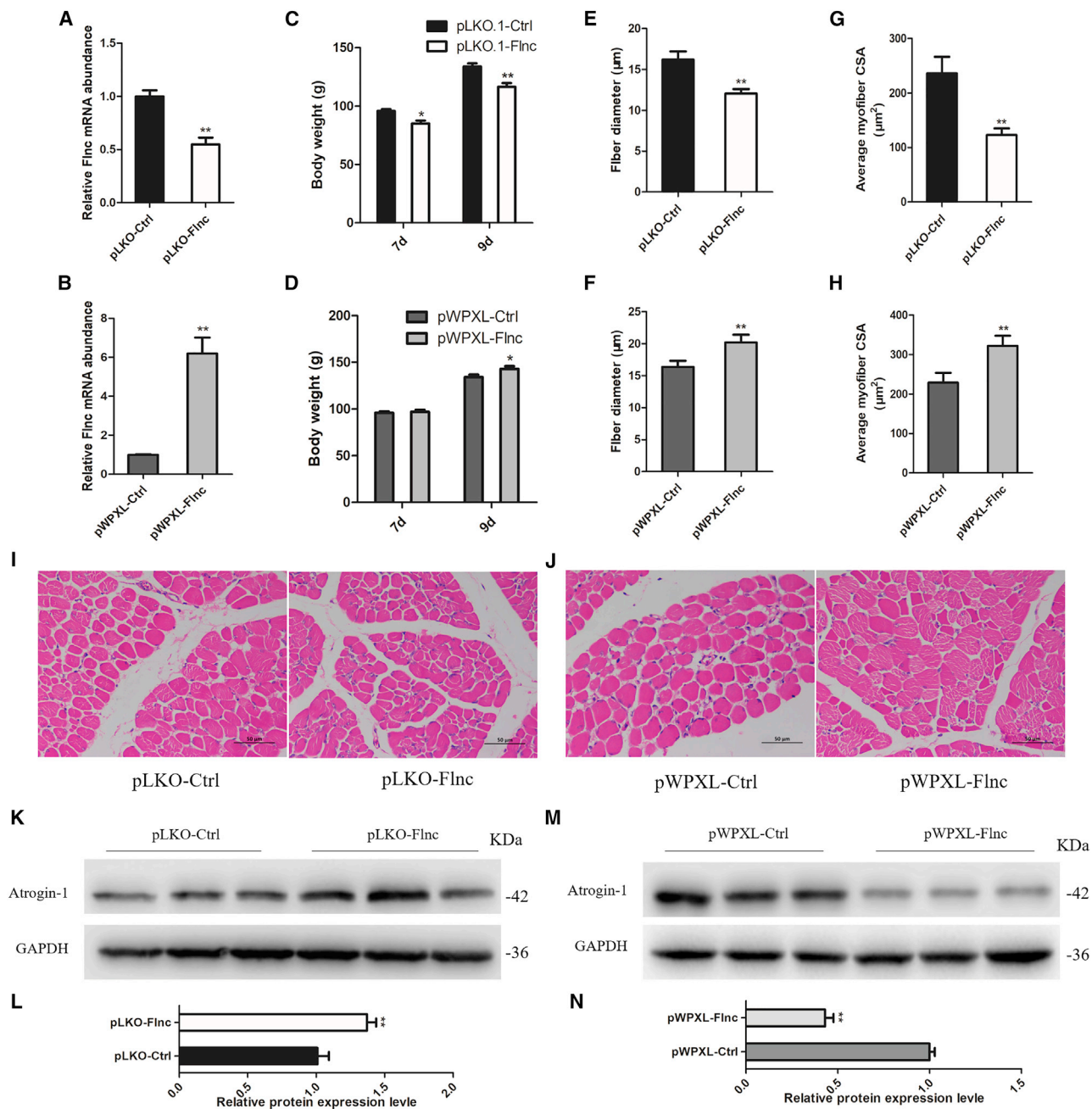
increased Dvl2 and MyH3 expression in chicken myoblast (Figures 8A and 8B). We found Dact1 silencing significantly increased Dvl2 expression, although Dvl2 was inhibited when both FlnC and Dact1 were silenced when compared with Dact1-silenced cells (Figure 8C). MyH3 immunofluorescence showed that Dact1 silencing could rescue myotubes in FlnC-silenced cells (Figures 8D and 8E). Thus, Dvl2 complementation can rescue the normal phenotype in myotubes injury induced by FlnC silencing. These results suggest that FlnC regulates Wnt/ $\beta$ -catenin signaling via mediation by Dvl2.

#### FlnC inhibits autophagy by binding to Dvl2

Autophagy negatively regulates Wnt signaling by modulating disheveled degradation,<sup>25</sup> and may be involved in FlnC-regulated Wnt/ $\beta$ -catenin signaling. Autophagy marker genes, including autophagy-related 5 (ATG5), autophagy-related 7 (ATG7), and Beclin-1, were significantly increased when FlnC was silenced (Figure 9A). Western

blot results indicated that the levels of autophagy-related proteins, such as LC3B and Beclin-1, were increased, whereas P62 was significantly decreased in the FlnC-silenced cells (Figures 9B and 9C). Moreover, in the chicken myoblasts, we constructed a stable tandem mRFP-GFP-LC3 and found that FlnC silencing-induced autophagic flux (Figures 9D and 9E). Electron microscopy indicated that the number of autophagosomes increased significantly after FlnC silencing, accompanied by a proliferation of mitochondria (Figures 9F–9H). In addition, we confirmed that FlnC silencing promoted autophagy in C2C12 myoblasts (Figures S6A–S6C).

Furthermore, we found that the transfection of Dvl-2-Flag significantly exacerbated LC3 puncta in the DF1 cells. After co-transfection with Dvl-2-Flag and FlnC-HA, LC3 fluorescence intensity was significantly reduced (Figures 10A and 10B). The results with FlnC-silenced and control cells with autophagy inducer Rap and inhibitor 3MA,

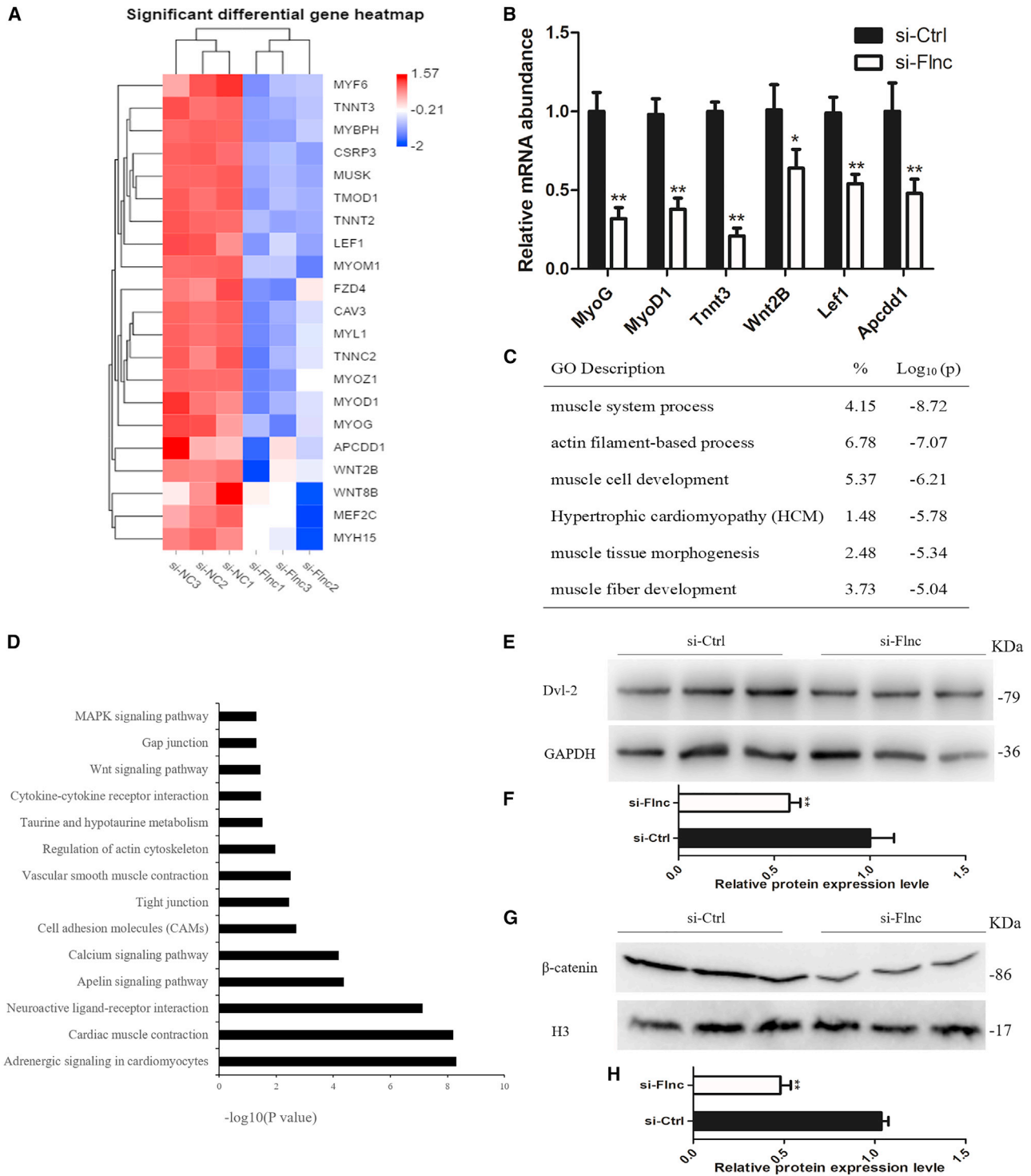


**Figure 5. Finc regulates muscle mass and mean muscle fiber *in vivo***

(A and B) Expression analysis of expression levels of Finc mRNA in lentivirus-mediated Finc knockdown and Finc-overexpressing chest muscle using qPCR. (C and D) Chicken body weight; (E and F) chest muscle fiber diameter; (G and H) breast muscle fiber cross-section, and (I and J) hematoxylin and eosin staining after injecting lentivirus-mediated pLKO-Finc or pLKO-Ctrl, pWPXL-Finc, and pWPXL-Ctrl into the chicken breast muscle. (K–N) Atrogin-1 protein expression in lentivirus-mediated Finc knockdown and Finc-overexpressing chest muscle.  $n = 15$  chickens in each group. Data are expressed as means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ .

showed that GFP-LC3<sup>+</sup> puncta were significantly increased in Finc-silenced cells treated with Rap. However, there was no significant difference between the 3MA-induced Finc-silenced cells when compared with the controls (Figures 10C and 10D). Furthermore, Finc-silenced myoblasts and the control cells were treated with

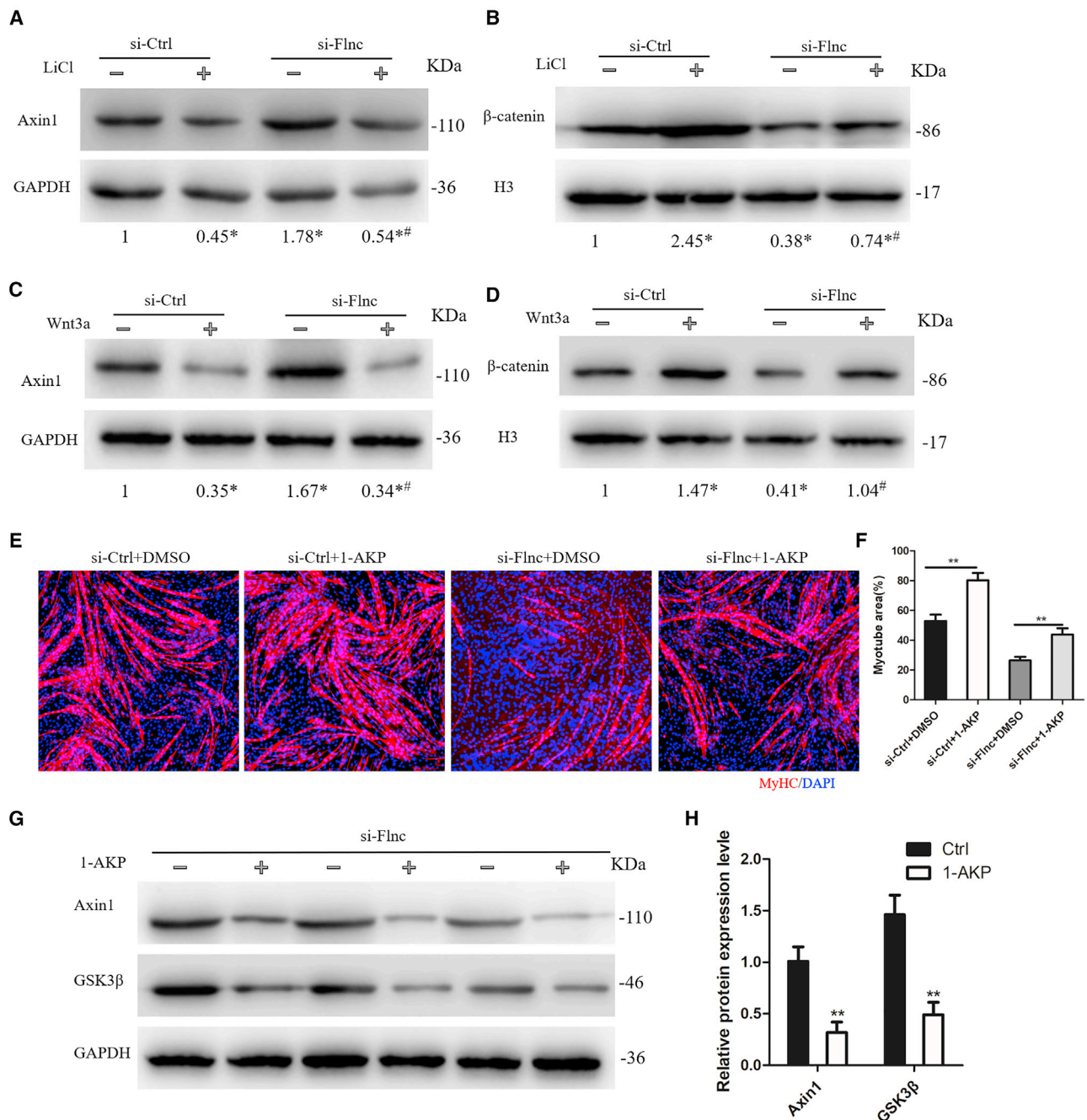
3MA, and the results showed that Dvl2 increased in the Finc-silenced myoblasts (Figure 10E). Immunoprecipitation experiments were performed to detect the interaction between Finc-HA and Dvl2-Flag (Figure 10F). Furthermore, the immunoprecipitation analysis showed that endogenous Finc interacts with Dvl2 (Figures 10G and S7A).



**Figure 6. Knockdown of Flncl suppresses Wnt/β-catenin signaling**

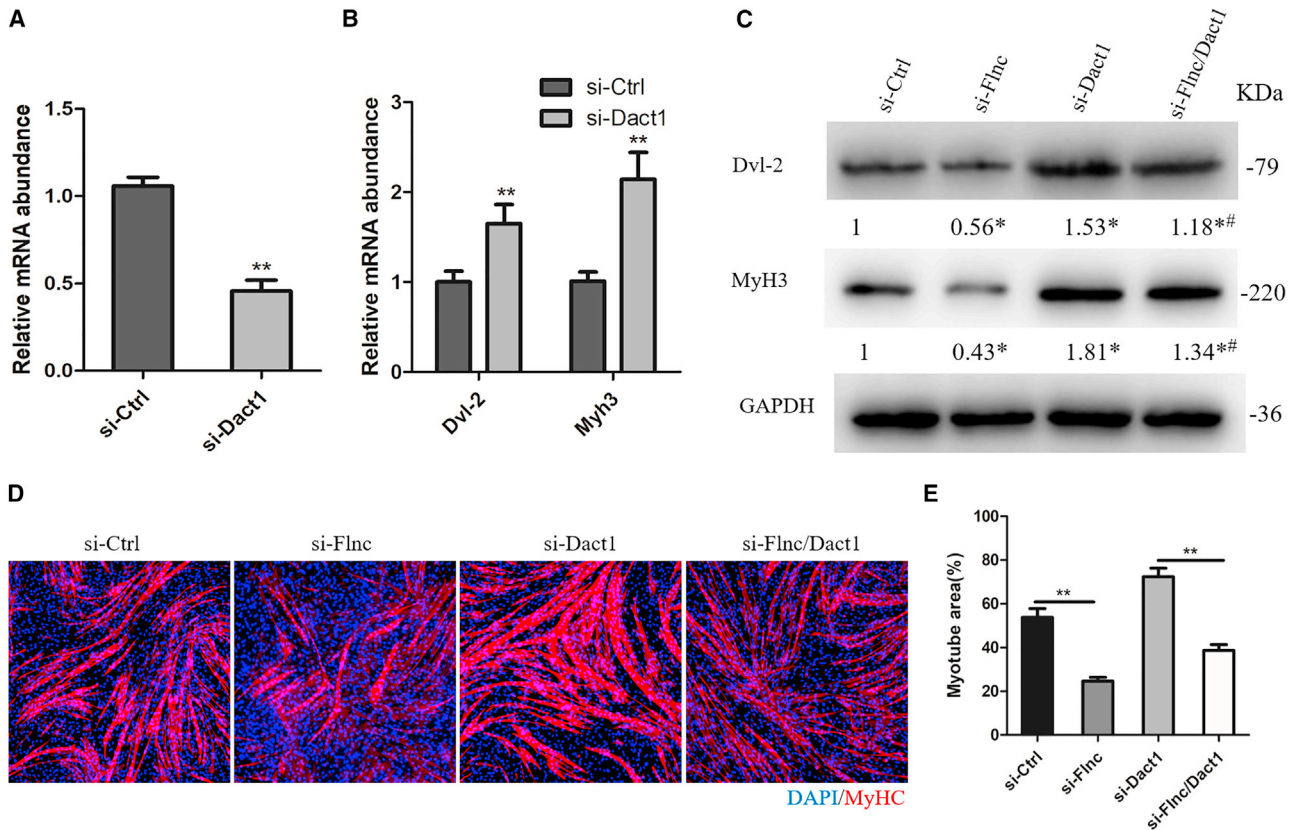
(A) Grading clustering and heatmap of gene expression levels associated with marker myogenesis in chicken primary myoblast cells transfected with control siRNA or Flncl siRNA for 48 h after induction of differentiation. (B) The expression levels of myogenic marker genes and Wnt target genes in Flncl-silenced and control cells. (C) Functional annotation associated with genes that were suppressed in Flncl-silenced cells. (D) Enrichment analysis of pathways with significantly differentially expressed genes in control and Flncl-silenced cells. (E and F) Western blot analysis of Dvl-2 expression protein levels in Flncl-silenced and control cells. (G and H) Western blot analysis of β-catenin expression protein levels in the nucleus of control and Flncl-silenced cells. Data are expressed as mean ± SEM (n = 3 independent cell cultures). \*p < 0.05, \*\*p < 0.01.





**Figure 7. FlnC functions via the Dvl2-mediated wnt/β-catenin signaling pathway**

(A) Western blot analysis of Axin1 expression protein levels in FlnC-silenced and control cells treated with LiCl or DMSO for 24 h. \*Significantly different from control cells. \*\*Significantly different from FlnC-silenced cells. Significance was set at  $p < 0.05$ . (B) Western blot analysis of β-catenin expression protein levels in nuclear lysates extracted from FlnC-silenced and control cells treated with LiCl or DMSO for 24 h. \*Significantly different from control cells. \*\*Significantly different from FlnC-silenced cells. Significance was set at  $p < 0.05$ . (C) Western blot analysis of Axin1 expression protein levels in FlnC-silenced and control cells treated with wnt3a or DMSO for 24 h. \*Significantly different from control cells. \*\*Significantly different from FlnC-silenced cells. Significance was set at  $p < 0.05$ . (D) Western blot analysis of β-catenin expression protein levels in nuclear lysates extracted from FlnC-silenced and control cells treated with wnt3a or DMSO for 24 h. \*Significantly different from control cells. \*\*Significantly different from FlnC-silenced cells. Significance was set at  $p < 0.05$ . (E) Immunofluorescence staining of MyHC in control and FlnC-silenced cells treated with 1-AKP or DMSO in differentiation medium for 48 h. (F) Myotube area (%) was determined in control and FlnC-silenced cells treated with 1-AKP or DMSO in differentiation medium for 48 h. (G and H) The expression protein levels of Axin1 and GSK3β in FlnC-silenced cells treated with 1-AKP or DMSO after 3 days in differentiation medium. Data are expressed as mean  $\pm$  SEM ( $n = 3$  independent cell cultures). \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 8. Myogenesis disruption caused by Flnc silencing can be rescued by Dvl2 complementation**

(A) Relative expression of Dact1 mRNA levels in cells transfected with Dact1 siRNA and control cells. (B) Relative expression levels of Dvl-2 and MyHC in Dact1-silenced and control cells. (C) Western blot analysis of Dvl-2 and MyHC protein levels in transfected Flnc siRNA, Dact1 siRNA, control siRNA, and co-transposition Flnc and Dact1 siRNA cells. \*Significantly different from control cells. \*\*Significantly different from Dact1 silenced cells. Significance was set at  $p < 0.05$ . (D) Immunofluorescence staining of MyHC in transfected Flnc siRNA, Dact1 siRNA, control siRNA, and co-transposition Flnc and Dact1 siRNA cells. (E) Myotube area (%) was determined in transfected Flnc siRNA, Dact1 siRNA, control siRNA, and co-transposition Flnc and Dact1 siRNA cells. Data are expressed as mean  $\pm$  SEM ( $n = 3$  independent cell cultures). \* $p < 0.05$ , \*\* $p < 0.01$ .

Moreover, we found that the ubiquitination of Dvl2 was significantly increased in the Flnc-silenced cells (Figures 10H and 10I), suggesting that Flnc can prevent Dvl2 ubiquitination and also inhibit correct formation of autophagosomes. These results indicate that Flnc stabilizes the Dvl2-mediated Wnt/ $\beta$ -catenin signaling pathway to antagonize autophagy.

#### Flnc silencing-induced autophagy mediates muscle atrophy by mitochondrial clearance

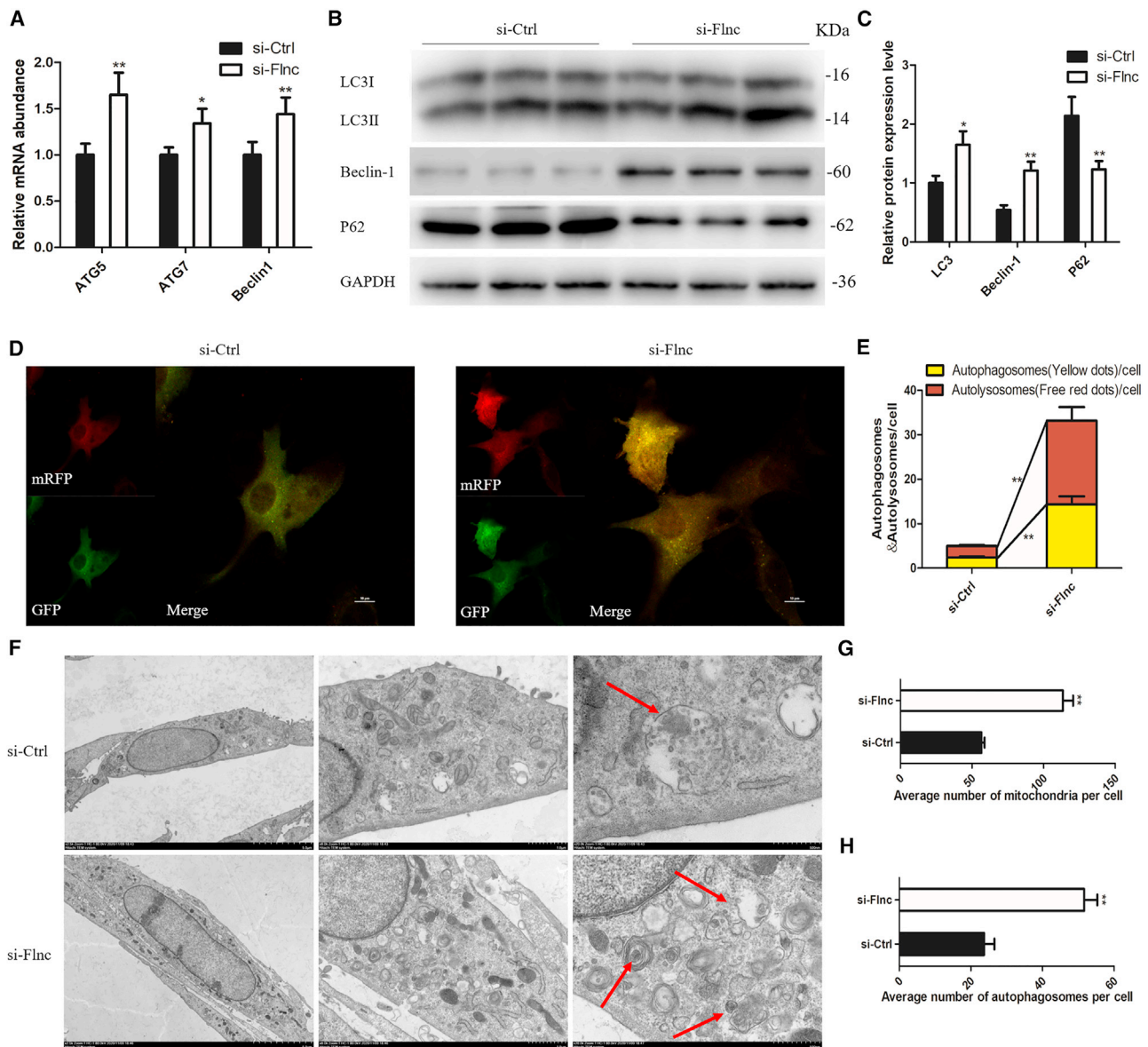
We observed increased autophagosomes together with large numbers of mitochondria after Flnc silencing using electron microscopy (Figure 9G). We then investigated whether mitochondrial autophagy (mitophagy) is involved in muscle atrophy induced by Flnc silencing, and found that the potential of mitochondrial membrane decreased 24 h after Flnc silencing (Figures 11A). In addition, oxygen consumption and ATP production were dramatically increased in Flnc-silenced cells compared with controls (Figures 11B and 11C). We further assessed the role of Flnc in mitochondrial fusion. Flnc silencing increased the expression of mitochondrial fission protein dynamin-

1-like protein (Figures 11D and 11E). We also investigated whether autophagy and mitochondrial fragmentation are related to selective mitochondrial degradation. Our results indicated that Flnc silencing markedly promoted lysosome-mitochondria colocalization compared with controls, and mitophagy was also increased in cells damaged by dexamethasone treatment (Figure 11F). This suggests that muscle atrophy induced by Flnc silencing may be mediated by mitophagy.

#### DISCUSSION

Skeletal muscle atrophy causes many chronic diseases or inherent complications, including cancer, diabetes, chronic heart failure, and cystic fibrosis.<sup>26,27</sup> Thus, identifying new mechanisms to regulate skeletal muscle growth and development may generate potent targeted therapies to prevent myopathy.

Flnc is a member of the filamin family, which plays a key role in cell signaling, transcription, organ development, and cytoskeleton formation.<sup>28</sup> Homozygous mutations of filamin A and B lead to aneurysms

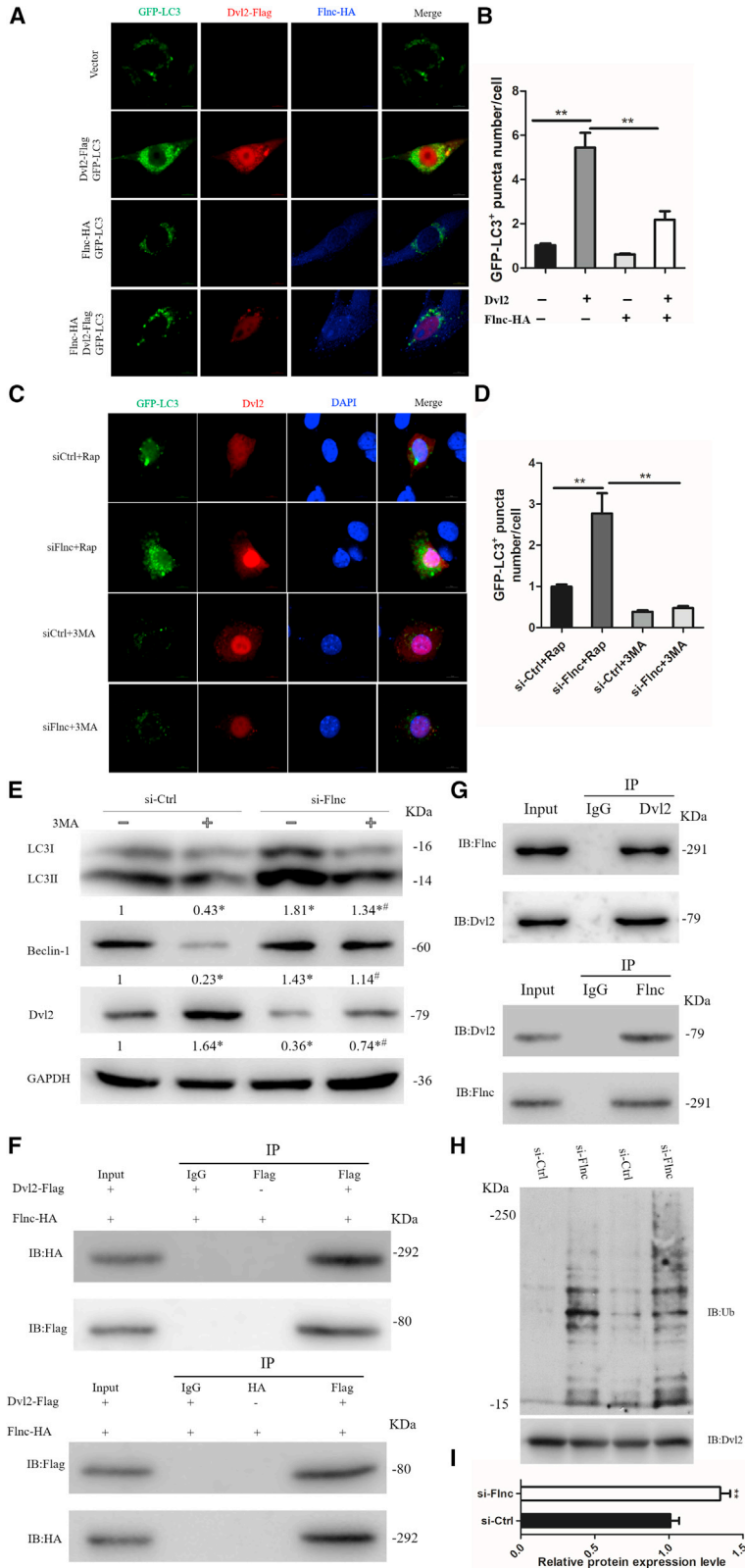


**Figure 9. Effects of FlnC silencing on autophagy**

(A) Relative mRNA expression levels of ATG5, ATG7, and Beclin-1 in FlnC-silenced and control cells. (B and C) Western blot analysis of LC3B, Beclin-1, and P62 protein levels in FlnC-silenced and control cells. (D and E) The autophagosomes of FlnC-silenced and control cells were observed by confocal microscopy. Scale bar, 10  $\mu$ m. Green fluorescence, lysosome; red fluorescence, autolysosomes; yellow fluorescence, autophagosomes. (F) Autophagosomes were observed by transmission electron microscopy in FlnC-silenced and control cells. Red arrow: autophagosomes (magnification from left to right:  $\times 2,500$ ,  $\times 8,000$ , and  $\times 20,000$ ). (G) The average number of mitochondria in FlnC-silenced and control cells was observed by electron microscopy. (H) The average number of autophagosomes in FlnC-silenced and control cells was observed by electron microscopy. Data are expressed as mean  $\pm$  SEM (n = 3 independent cell cultures). \*p < 0.05, \*\*p < 0.01.

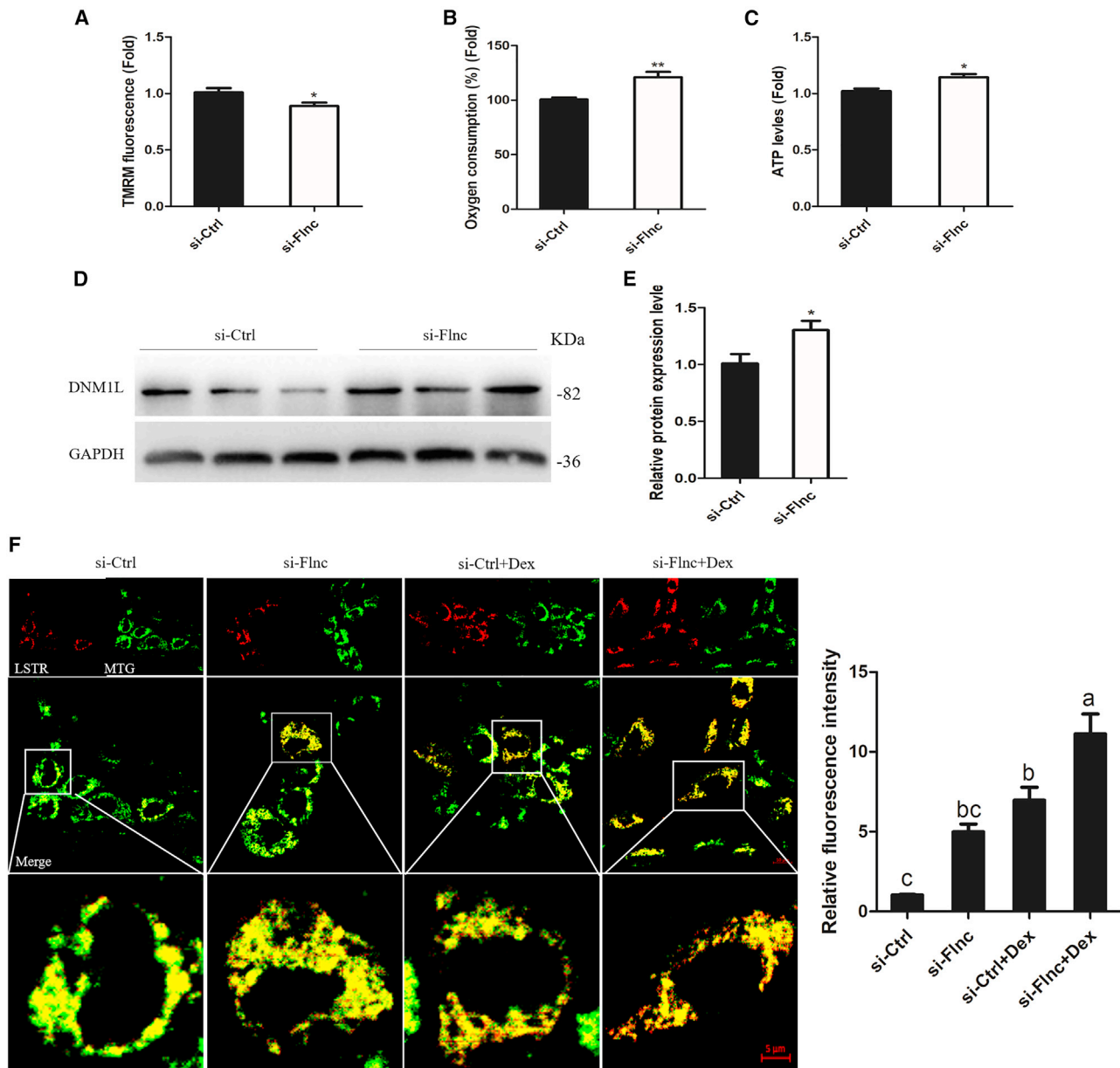
and cardiac defects, aberrant upgrowth of the brain, cardiovascular system, and skeleton.<sup>29,30</sup> FlnC also has important functions as other affiliates of the filamin family. Studies have shown that mutations in the N-terminal actin binding region of FlnC can result in distal myopathy.<sup>31</sup> Chen et al., reported that mutations in the FlnC gene result in myofibrillar myopathy with lower motor neuron syndrome.<sup>32</sup> In addition, FlnC is a highly dynamic protein involved in rapid myofibrillar

microdamage.<sup>33</sup> In this study, we found that FlnC plays a vital role in promoting the fusion of muscle cells and the safeguarding of muscle mass, strengthening the hypothesis that FlnC participates in muscle development, and the identification of the likely cause of myofibrillar myopathies associated with FlnC mutation. Muscular dystrophy is the most common skeletal muscle disease, which has a severe impact on human health and animal production. We found that



**Figure 10. Finc interacts with Dvl2 to antagonize autophagy**

(A) Immunofluorescence analysis of GFP-LC3<sup>+</sup> puncta in cells transfected with GFP-LC3 and Dvl2-Flag plasmids, GFP-LC3, and Finc-HA plasmid or GFP-LC3, DVL2-Flag, and Finc-HA plasmids. White arrow, GFP-LC3<sup>+</sup> puncta. (B) Histogram represents the number of GFP-LC3<sup>+</sup> puncta numbers on the right. (C) Immunofluorescence analysis of GFP-LC3<sup>+</sup> puncta of Finc-silenced and control cells after treatment with Rap and 3MA. (D) The histogram represents the number of GFP-LC3<sup>+</sup> puncta on the right. (E) Western blot analysis of LC3B, Beclin-1, and Dvl2 protein levels in Finc-silenced and control cells treated with 3MA or untreated. \*Significantly different from control cells. \*\*Significantly different from Finc-silenced cells. Significance was set at  $p < 0.05$ . (F) Reciprocal co-immunoprecipitation analysis of HA-tagged Finc and Flag-tagged Dvl2 in DF1 cells. (G) Co-immunoprecipitation analysis of Finc and Dvl2 after primary myoblasts were cultured in differentiation medium for 72 h. (H and I) Western blot analysis of the ubiquitination levels of immunoprecipitated Dvl2 in Finc-silenced and control cells. Data are expressed as mean  $\pm$  SEM ( $n = 3$  independent cell cultures). \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 11. FlnC silencing induces autophagy and mediates muscle atrophy through mitochondrial clearance**

(A) Mitochondrial membrane potentials of FlnC-silenced cells and control cells were determined using tetramethylrhodamine (TMRM) fluorescence. (B) Oxygen consumption of FlnC-silenced cells and control cells were measured. (C) ATP levels in FlnC-silenced cells and control cells. (D and E) Western blot analysis of dynamin-1-like (DNM1L) protein levels in FlnC-silenced and control cells. (F) Mitochondrial (MTG) and lysosome (LSTR) colocalization revealed mitochondrial autophagy levels in the dexamethasone-treated or untreated FlnC-silenced and control cells. The figure on the right shows the quantification of colocalization between the mitochondria and lysosomes. MTG, mitochondria; LSTR, lysosome. Bars not sharing the same letter labels are different,  $p < 0.05$ ; data are expressed as mean  $\pm$  SEM ( $n = 3$  independent cell cultures). \* $p < 0.05$ , \*\* $p < 0.01$ .

FlnC silencing significantly reduced skeletal muscle mass and muscle fiber size, while overexpression increased muscle fiber hypertrophy. This was consistent with the study of Dalkilic et al., who identified FlnC as a muscular dystrophy gene.<sup>10</sup> Beatham et al. found that FlnC could interact with KY protein in muscular dystrophy and is distributed abnormally in KY-deficient mouse muscle fibers.<sup>34</sup> These

findings will provide a better understanding of the pathological mechanisms of FlnC-associated myofibromyopathies.

While FlnC is vital for maintaining the structural integrity of cardiac and skeletal muscle, its regulation of the signaling pathway has not been reported previously. We found that FlnC interacted with Dvl-2

to activate the wnt/ $\beta$ -catenin signaling pathway. It is known that the Wnt/ $\beta$ -catenin signaling pathway is essential for embryo development and adult tissue homeostasis and skeletal muscle regeneration.<sup>35</sup> In addition, activation of the Wnt/ $\beta$ -catenin signaling pathway enhances the expression of myogenic factor 5 (MYF5), a basic-helix-loop-helix myogenic determination factor. This promotes myogenic differentiation and myotubule formation.<sup>36</sup> Dvl2 is a vital component of the Wnt signaling pathway. We found that Flnc requires Dvl2 to regulate the Wnt/ $\beta$ -catenin pathway. Dact1 is an inhibitor of the Wnt signaling pathway, and suppressing Dact1 upregulates Dvl2 expression, activating the Wnt/ $\beta$ -catenin signaling pathway.<sup>37</sup> The study showed that the expression of MyH3 and Dvl-2 was rescued by silencing Flnc in Dact1 knockdown cells. In brief, Flnc regulates the Dvl2-mediated Wnt/ $\beta$ -catenin signaling pathway and controls myoblast differentiation and skeletal muscle development. Since Dvl2 has a complex role in canonical Wnt signaling during skeletal muscle development, the effect of Flnc on other members of the disheveled family requires further study. Studied have shown that Dvl2 can shuttle between the nucleus and cytoplasm.<sup>38,39</sup> Under normal circumstances, Dvl2 is mainly expressed in the cytoplasm, with little expression in the nucleus. Itoh et al. found that treatment of mammalian cells cultured with Wnt3a resulted in the accumulation of endogenous Dvl2 proteins in the nucleus.<sup>40</sup> This may be due to the Dvl2 nuclear localization required for its function in the Wnt/ $\beta$ -catenin signaling pathway. In our study, we found that Dvl2-Flag immunostaining showed nuclear localization, which may be related to the Wnt/ $\beta$ -catenin signaling pathway. In addition, nuclear localization of Dvl2 may indirectly affect the stability of  $\beta$ -catenin by regulating the protein interaction that sequesters  $\beta$ -catenin in the nucleus, thus preventing its cytoplasmic degradation. However, the regulatory mechanism of Dvl2 in the nucleus requires further study.

Disheveled is the hub of wnt signaling and plays a decisive role in the switch of wnt signaling pathways. Studies have shown that ubiquitinated Dvl2 binds to P62, and then forms a complex with LC3 to create autophagosomes, which selectively enter autophagy through the lysosomal degradation pathway.<sup>41</sup> Sharma et al. found that Malin could control the wnt signaling pathway by degrading Dvl2, and enhance K48 and K63-linkage ubiquitination of Dvl2, which also leads to its degradation via proteasome cleavage and autophagy.<sup>42</sup> In addition, the wnt signaling pathway is activated by the inhibition of autophagy, suggesting that Dvl2 is closely involved in the correct formation of autophagosomes through ubiquitination. Our results confirm the interaction between Flnc and Dvl2, which may be an important reason for the involvement of Flnc in autophagy regulation. The Wnt signal antagonist Dapper1 increases the degradation of Disheveled2 by promoting ubiquitination and aggregation-induced autophagy.<sup>43</sup> We also confirmed that Flnc reduced ubiquitinated Dvl2 by stabilizing free Dvl2, thereby inhibiting autophagosome formation and antagonizing autophagy.

Autophagy plays a significant role in skeletal muscle homeostasis and muscular dystrophy. The integrity of skeletal muscles is damaged

when the balance of autophagy is disrupted.<sup>44</sup> Excessive activation of autophagy aggravates skeletal muscle mass loss in the catabolic state. In hyperautophagy X-linked myopathy, progressive sarco-plasmic accumulation of autosomes filled with non-degraded fragments leads to skeletal muscle atrophy and weakness.<sup>45</sup> Pagano et al. found that enhanced autophagy is a likely factor underlying age-related skeletal muscle atrophy.<sup>46</sup> Excessive autophagy is a crucial cause of skeletal muscle atrophy. Zhang et al. found that Islr controls the canonical wnt signaling pathway by inhibiting Dvl2 ubiquitination and thus mediates skeletal muscle regeneration.<sup>47</sup> These results suggest that Dvl2 ubiquitination is a significant hub of autophagy and the Wnt signaling pathway. Flnc has an important role in regulating muscle integrity by stabilizing Dvl2 to maintain the autophagy system and homeostasis of skeletal muscle. This is further evidence that Flnc maintains muscle development and structural maintenance of skeletal muscles.

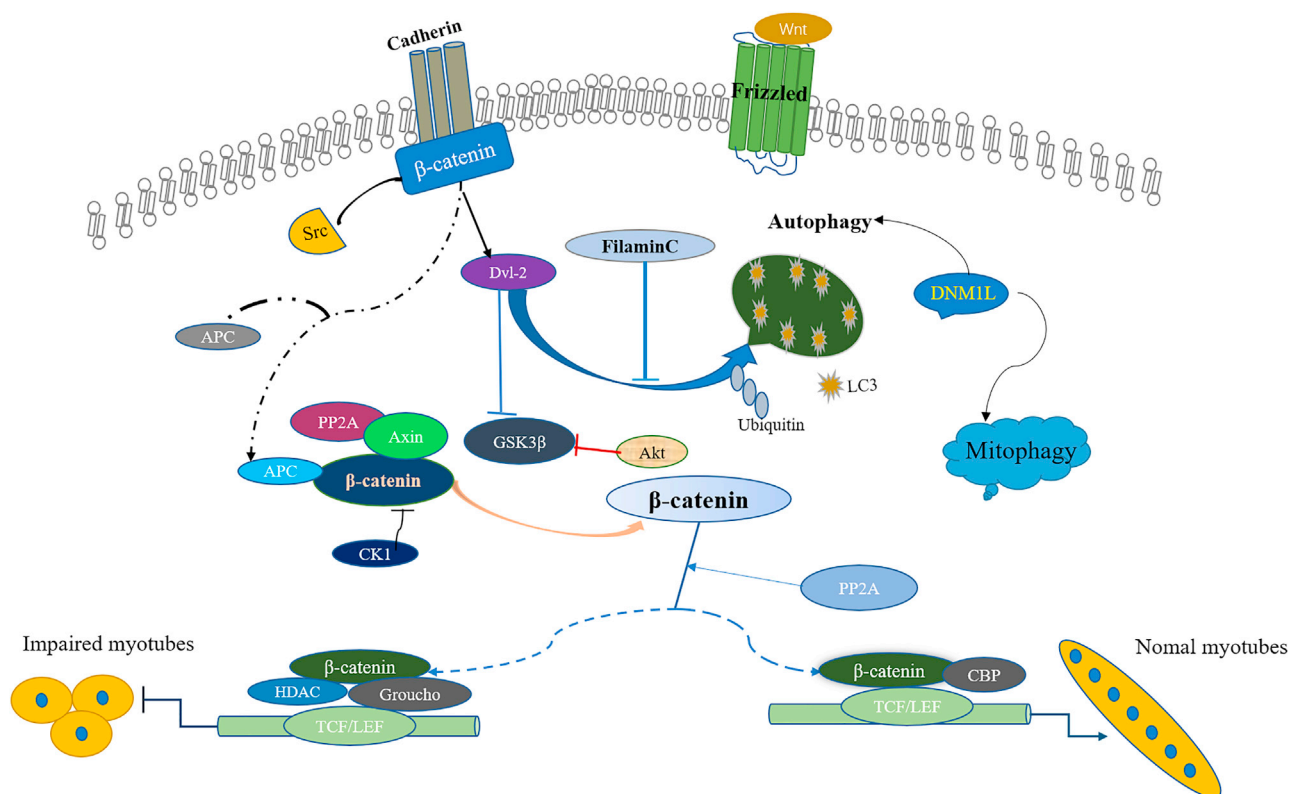
Skeletal muscle mitochondria are necessary to provide the energy required for muscle contraction. In response to such activity, mitochondria generation is upregulated to satisfy the energy needs of muscle cells.<sup>48</sup> Mitophagy helps autophagosomes to degrade mitochondria, thus contributing to the maintenance of mitochondrial quality.<sup>49</sup> Troncoso et al. found that dexamethasone-induced autophagy mediates muscle atrophy through mitochondrial clearance.<sup>50</sup> Kang and Li found that overexpression of PGC-1 $\alpha$  could suppress the activation of mitophagy in the atrophying muscle induced by immobilization.<sup>51</sup> Mitophagy can accelerate mitochondrial fragmentation, which exacerbates mitochondria degradation in lysosomes and promotes muscle atrophy.<sup>52</sup> We also found that Flnc silencing significantly increased mitochondrial protein fragmentation, mitochondrial activity, and mitophagy, suggesting that mitophagy was activated by Flnc silencing.

In conclusion, Flnc plays crucial roles in myogenesis and the maintenance of muscle mass (Figure 12). In addition, myotube damage and myofiber defects in *in vitro* and *in vivo* models suggest a novel function for Flnc in maintaining myofiber structure. At the molecular level, we found that Flnc maintained normal muscle development by inhibiting autophagy and protecting Dvl2 from ubiquitination. Since autophagy involves a variety of pathological processes, a better comprehending of the regulatory role of Flnc in autophagy is important for the treatment of both skeletal muscle diseases and other related diseases.

## MATERIALS AND METHODS

### Animals

Fast-growing chickens (Ross 308 broiler) and slow-growing chickens (White leghorn layer) were used in this experiment. The experimental chickens were provided by the poultry breeding farm at Sichuan Agricultural University (Ya'an, Sichuan, China). The experimental procedures were approved by the Animal Ethics Committee of Sichuan Agricultural University (approval no. 201810200710), and all methods follow relevant guidelines and regulations.



**Figure 12. Model of the role of FlnC in skeletal muscle atrophy**

FlnC stabilizes dishevelled-2 to avoid ubiquitination and autophagy-mediated degradation, thereby accurately regulating wnt/ $\beta$ -catenin signaling during skeletal muscle development.

### Cell culture

Fetal myoblasts were isolated from 11-day-old embryonic Ross 308 broiler muscles.<sup>53</sup> Single cells are released from the muscle by collagenase and trypsin digestion and further enriched with myogenic cells by Percoll density centrifugation, as described in previous publications. The cells were grown on a 2% gelatin-coated culture plate using our standard culture medium for myoblast cultures containing Dulbecco's modified Eagle medium (Sigma, St. Louis, MO, USA), 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 5% chicken embryo extract, and 0.5% penicillin and streptomycin (Solarbio, Beijing, China) at 37°C under 5% CO<sub>2</sub> in air. When the cell density reached 80%, complete medium was replaced with differentiation medium containing 2% horse serum (Gibco) to achieve myotube differentiation.

### FlnC knockdown and overexpression

To interfere with FlnC mRNA and protein expression, siRNA was constructed to directly target FlnC. FlnC siRNA and negative control siRNA were designed and purchased from Sangon Biotech (Sangon Biotech, Shanghai, China). FlnC siRNA sequences were sense: GGGAAUACGAUGUCAACAUTT; antisense: AUGUUGACAUCGUAUCCCTT. To overexpress FlnC, chicken myoblasts were stably transfected with a pcDNA3.1 expression vector encoding chicken FlnC-HA (Sangon Biotech). siRNA and overexpressed plasmids

were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### RNA isolation and real-time qPCR

The cells were washed twice with PBS, TRIzol reagent (Takara, Tokyo, Japan) was added to each culture dish, and the cells were scraped and collected in a 1.5 mL Eppendorf tube. The RNA extraction protocol was based on previous reports. Approximately 1  $\mu$ g of RNA was reverse transcribed, and cDNA was synthesized using the Takara reverse transcription kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. qPCR was performed on the Bio-Rad CFX Connect Real-Time System using the SYBR Green kit (Takara). All PCR reactions were repeated three times. PCR amplification was performed as follows: 95°C for 3 min and 40 cycles each at 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s using a Bio-Rad sequence detector. The mean normalized cycle threshold ( $\Delta$ Ct) was used for the statistical analysis of real-time PCR results.<sup>54</sup> All amplicon primers were designed using the NCBI primer Design Center. The primers in the study are listed in Table S1.

### Treatment protocols and antibodies

To verify the activity of Wnt/ $\beta$ -catenin signaling, FlnC-silenced and control cells were treated with 10 mM LiCl (Sigma) and 200 ng

wnt3a (Sigma) for 24 h in differentiation medium. To verify activity of wnt signaling, Flnc-silenced cells were treated with 1-AKP (Selleck, Houston, TX, USA) or DMSO for 48 h. The following primary antibodies were used: anti-MyHC (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-MyoD (Santa Cruze, CA, USA), anti-MyoG (Biorbyt, Cambridge, UK); Flnc (ZenBio, Chengdu, China); GSK3 $\beta$  (ABclonal, Wuhan, China); Axin1 (Cell Signaling Technology, Boston, MA, USA);  $\beta$ -catenin (Cell Signaling Technology); Anti-Dvl2 (Abcam, Cambridge, UK); anti-Ubiquitin (Abcam); anti-Axin1 (Bioss, Beijing, China); anti-H3 (ABclonal, Wuhan, China); and GAPDH (ZenBio). The following secondary antibodies were used: mouse anti-rabbit horseradish peroxidase (HRP) and goat anti-mouse HRP from ZenBio Biology (ZenBio).

#### Western blot and immunoprecipitation assays

The cell total protein and nuclear protein were extracted using a kit from BestBio (Shanghai, China), the protein extraction procedure was according to the manufacturer's instructions. Protein concentration was determined using a bicinchoninic acid assay kit (BestBio), and 5 $\times$  loading buffer was added to the lysates and desaturated in boiling water at 100°C for 10 min. A total of 20  $\mu$ g of protein was protein electrophoresed on SDS-PAGE gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked in 5% skimmed milk at room temperature for 2 h, and then incubated with a primary antibody at 4°C overnight. The next day, the PVDF membrane was washed with Tris-buffered saline Tween (TBST) and incubated with HRP-labeled secondary antibodies at room temperature for 1 h. After washing with TBST, the relevant proteins were visualized using an enhanced chemiluminescence reagent. The captured images were analyzed using ImageJ software (National Health Institute, Bethesda, MD, USA). For the immunoprecipitation assay, the cells were lysed with IP lysis solution and the total proteins were immunoprecipitated with protein G beads. Immuno-complexes were washed three times with IP lysis buffer, samples were electrophoresed on 8% SDS-PAGE gel and western blotted with appropriate antibodies.

#### Immunofluorescence and confocal microscopy

Cells were plated on glass cover slides in culture medium, washed with PBS after the culture medium was removed, and fixed with 4% paraformaldehyde for 10 min. The cells were then washed three times with PBS and permeabilized with 0.1% Triton X-100 for 20 min. After washing with PBS, the cells were incubated with a primary antibody at 4°C. After primary antibody incubation and washing, the cells were incubated with fluorescent secondary antibodies at room temperature for 1 h in the dark. The cells were then washed three times with TBST and the fluorescence intensity was observed using an LSM 510 confocal microscope (Zeiss, Milan, Italy).

#### Lentivirus production and transduction

A mixture of lentivirus-mediated Flnc overexpression vector (pWPXL-Flnc), lentivirus-mediated Flnc knockdown vector (pLKO-Flnc), and lentivirus-mediated control vector were synthesized by RiboBio Biotechnology (RiboBio, GuangZhou, China). Chicks (1-day-old

Ross 308 broiler) were infected with lentivirus (pWPXL-Flnc, pWPXL control, pLKO-Flnc, and pLKO-control; n = 15) into the chest muscle by direct injection. After 9 days of feeding, the breast muscles were removed, and a standard blade was used to take a 4 mm section according to the designated anatomical marks. Protein and RNA were extracted for subsequent western blotting and qPCR analyses.

#### Histological analysis

Chicken chest muscle tissue was extracted with using surgical blades and fixed with 4% paraformaldehyde. The tissues were fixed and embedded in paraffin, sliced, stained with hematoxylin and eosin, and the images were analyzed.

#### Mitochondrial transmembrane potential and ROS production

The cells were loaded with 200 nM tetramethylrhodamine (Sigma), or 25 nM dihydrorhodamine-123 (Invitrogen) for 30 min according to the manufacturer's instructions, respectively. Fluorescence was detected using a FACScan system using flow cytometry.

#### RNA sequencing

Total RNA was extracted from Flnc siRNA, control siRNA cells, and Ross 308 broiler and White leghorn layer chest muscle, according to the experimental steps described above, and cDNA library construction, sequencing, and transcriptome data analysis were performed by Shanghai Personalbio (Shanghai, China). Sequencing and bioinformatics analyses were conducted in strict accordance with the company's standard operating procedures, which are available online (<http://www.personalbio.cn/>).

#### Statistical analysis

The data were statistically analyzed using the non-parametric Kruskal-Wallis ANOVA on ranks followed by Dunn's test or the Student-Newman-Keuls test. For comparison of the means of two groups, Student's t test or non-parametric Mann-Whitney U test were used. When the p value is <0.05, the difference was considered significant. All data are expressed as mean  $\pm$  standard error of the mean (SEM).

#### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2021.11.022>.

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#### AUTHOR CONTRIBUTIONS

Shunshun.Han. and Can.Cui. conceived the study. Shunshun.Han., Xiyu.Zhao., Qing.Zhu., and Yao. Zhang designed the study. Yun.Zhang, Jing.Zhao., Xiaoxu.Shen., and Haorong.He. performed the experiments. Shunshun.Han., Jianping.Wang., Menggen.Ma., and Diyan.Li. analyzed the data. Shunshun.Han. and Huadong.Yin. wrote the manuscript. All authors reviewed and edited the manuscript.



## DECLARATION OF INTERESTS

The authors declare no competing interests.

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