Islr regulates canonical Wnt signaling-mediated skeletal muscle regeneration by stabilizing Dishevelled-2 and preventing autophagy

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Satellite cells are crucial for skeletal muscle regeneration, but the molecular mechanisms regulating satellite cells are not entirely understood. Here, we show that the immunoglobulin superfamily containing leucine-rich repeat (Islr), a newly identified marker for mesenchymal stem cells, stabilizes canonical Wnt signaling and promote skeletal muscle regeneration. Loss of Islr delays skeletal muscle regeneration in adult mice. In the absence of Islr, myoblasts fail to develop into mature myotubes due to defective differentiation. Islr interacts with Dishevelled-2 (Dvl2) to activate canonical Wnt signaling, consequently regulating the myogenic factor myogenin (MyoG). Furthermore, Islr stabilizes Dvl2 by reducing the level of LC3-labeled Dvl2 and preventing cells from undergoing autophagy. Together, our findings identify Islr as an important regulator for skeletal muscle regeneration.
Regeneration is critical to maintain the homeostasis of adult skeletal muscle in animals, and satellite cells (SCs) play an important role during this process. Adult skeletal muscle fails to regenerate by genetic ablation of SCs postnatally\(^1\,-^3\). Upon adult skeletal muscle injury, quiescent Pax7\(^+\) SCs are activated and give rise to a population of Pax7\(^+\)/Myf5\(^+\) committed SCs\(^4\,^5\). These progenitor cells proliferate into Pax7\(^+\)/MyoD\(^+\) myoblast cells\(^6\,^7\) and differentiate into the myogenin \(^+\) (MyoG\(^+\)) cells that fuse to form multinucleated myotubes\(^8\,^9\). SC differentiation is essential for skeletal muscle regeneration, and it is increasingly clear that SC differentiation is regulated by many signaling pathways. In particular, the canonical Wnt signaling pathway is important for promoting SC differentiation during skeletal muscle regeneration\(^10\,^11\). When Wnt ligands bind to Frizzled receptors and members of the low-density lipoprotein receptor-related protein (LRP) family, the canonical Wnt signaling is activated. The cytoplasmic part of Frizzled interacts with Disheveled-2 (Dvl2), facilitating interaction between Axin and the LRPs, which blocks the ubiquitination of \(\beta\)-catenin, which destroys the Disheveled-2 (Dvl2), facilitating interaction between Axin and the LRPs. The canonical Wnt signaling pathway is clear that SC differentiation is regulated by many signaling pathways. In particular, the canonical Wnt signaling pathway is important for promoting SC differentiation during skeletal muscle regeneration\(^10\,^11\). When Wnt ligands bind to Frizzled receptors and members of the low-density lipoprotein receptor-related protein (LRP) family, the canonical Wnt signaling is activated. The cytoplasmic part of Frizzled interacts with Disheveled-2 (Dvl2), facilitating interaction between Axin and the LRPs, which blocks the ubiquitination of \(\beta\)-catenin, which destroys the Disheveled-2 (Dvl2), facilitating interaction between Axin and the LRPs. The canonical Wnt signaling pathway is clear that SC differentiation is regulated by many signaling pathways.

Loss of Islr delays regeneration of adult skeletal muscle. In order to study the function of \(\text{Islr}\) in vivo, we crossed mice possessing the \(\text{Islr}^{\text{Exons1b-2loxP}}\) conditional allele with mice expressing a Cre protein driven in Myf5\(^+\) cells (conditional knockout \([\text{cKO}])\(^3\) (Fig. 2a). To evaluate the recombination efficiency at the protein level, we purified SCs of control (Ctrl) and \(\text{Islr}\text{ cKO}\) mice using FACS and found that \(\text{Islr}\) was not expressed at all in \(\text{Islr}\text{ cKO}\) mice (Fig. 2b). Furthermore, we injected CTX into the TA muscles of control and \(\text{Islr}\text{ cKO}\) mice and found that \(\text{Islr}\) was markedly deleted in most SCs of \(\text{Islr}\) cKO mice at 5 d postinjury (Fig. 2c, d).

The \(\text{Islr}\) cKO mice are fertile and healthy without any apparent change in gross phenotype under normal conditions. To understand the role of \(\text{Islr}\) in skeletal muscle regeneration, we histologically analyzed the TA muscles at 3.5, 5, and 14 d postinjury. At 3.5 d postinjury, control mice already displayed newly formed muscle fibers, whereas almost no muscle fiber regeneration was observed in \(\text{Islr}\text{ cKO}\) mice (Fig. 2e and Supplementary Fig. 2a). At 5 d postinjury, control mice had regenerated a large number of muscle fibers with centrally located myonuclei, whereas the diameters of the newly formed muscle fibers were very small in \(\text{Islr}\text{ cKO}\) mice (Fig. 2e and Supplementary Fig. 2a). At 14 d postinjury, control mice displayed tightly packed, well-formed muscle fibers, whereas \(\text{Islr}\) cKO mice had many small muscle fibers with single nuclei (Fig. 2e and Supplementary Fig. 2a). Five days after injury, the average cross-sectional area (CSA) of muscle fibers decreased by about 33\% in \(\text{Islr}\text{ cKO}\) mice compared to the control mice (Fig. 2f). In addition, the numbers of myofibers containing two or more centrally located nuclei were also significantly reduced in \(\text{Islr}\text{ cKO}\) mice (Fig. 2g).

Results
I slr is highly expressed in differentiated myogenic cells. The GEO database shows that \(\text{Islr}\) mRNA is highly expressed in \(\text{mdx}\) mice, thus we validated this information and found that \(\text{Islr}\) was indeed upregulated in \(\text{mdx}\) mice (Supplementary Fig. 1a, b), indicating that it might be involved in skeletal muscle regeneration. To examine the expression of \(\text{Islr}\) during skeletal muscle regeneration, the tibial anterior (TA) muscles were injured with an injection of cardiotoxin (CTX) and allowed to regenerate. Islr protein levels were higher in the injured than in the contralateral TA muscles (CTL) at 3 d postinjury (Supplementary Fig. 1c). The expression level of Islr increased between 3 and 5 day postinjury, which is a critical stage during which SCs participate in skeletal muscle regeneration (Supplementary Fig. 1d). To analyze the expression of \(\text{Islr}\) during myogenesis, we carried out immuno-histochemical staining for Islr, Pax7, and MyoG on serial sections of TA muscles. No Islr protein expression was detected in Pax7\(^+\) quiescent SCs (Fig. 1a). However, Islr protein was expressed in Pax7\(^+\) activated SCs and MyoG\(^+\) muscle progenitors (Fig. 1b, c).

A combination of cell surface markers (CD45\(^–\), CD31\(^–\), Sca1\(^–\), and a7-integrin\(^–\)) is widely used to purify SCs in skeletal muscle. Specifically, we isolated SCs from wild-type (WT) mice using fluorescence-activated cell sorting (FACS)\(^3\) (Fig. 1d). Although \(\text{Islr}\) mRNA was not significantly different between freshly isolated SCs and activated SCs cultured for 24 h, the mRNA and protein levels of Islr were higher in differentiating cells as compared to proliferating cells (Fig. 1e and Supplementary Fig. 1e).

To localize Islr expression in C2C12 cells in vitro, we generated an Islr expression vector in which green fluorescent protein (GFP) was inserted at the C-terminus of Islr (Islr-GFP). Islr co-localized with tubulin following plasmid transfection in C2C12 cells (Fig. 1f), indicating that Islr was expressed in the cytoplasm. Meanwhile, an expression vector fusing a Flag-tag after amino acid 18 (the leader peptide) of Islr was also constructed (L-Flag-Islr), yielding the same result as the Islr-GFP plasmid (Fig. 1g). Meanwhile, we found that endogenous Islr was mainly expressed in the cytoplasm of mature myotubes (Fig. 1h, i and Supplementary Fig. 1f). Furthermore, the expression of Islr gradually increased with the differentiation of C2C12 cells (Fig. 1j, k), which was consistent with the results of primary myoblasts derived from FACS-purified SCs. Taken together, we concluded that Islr was highly expressed in differentiated myogenic cells.
Fig. 1 Islr is upregulated during satellite cell differentiation and C2C12 differentiation. 

**a** Immunohistochemistry analysis of Islr and Pax7 in uninjured TA muscles of wild-type (WT) mice. Scale bar = 10 μm. 

**b** Immunohistochemistry analysis of Islr and Pax7 in injured TA muscles of WT mice at 5 d postinjury using serial sections. Scale bar = 10 μm. 

**c** Immunohistochemistry analysis of Islr and MyoG in injured TA muscles of WT mice at 5 d postinjury using serial sections. Scale bar = 10 μm. 

**d** Flow cytometry analysis of CD31, CD45, Sca1, and α7-integrin expression in whole muscle-derived cells. 

**e** Western blot analysis of Islr, Pax7, MyoG, and MyHC in satellite cells from WT mice at 4 d in proliferation medium (G4) or 2 d in differentiation medium (D2). 

**f** Immunofluorescence staining for tubulin in C2C12 cells transfected with the Islr-GFP plasmid. Scale bar = 25 μm. 

**g** Immunofluorescence staining for tubulin and Flag in C2C12 cells transfected with the leader peptide-Flag-Islr (L-Flag-Islr) plasmid. Scale bar = 25 μm. 

**h** Immunohistochemistry analysis of Islr in C2C12 cells cultured in differentiation medium for 5 d. Scale bar = 50 μm. 

**i** Immunohistochemistry analysis of Islr in primary myoblasts derived from FACS-purified satellite cells cultured in differentiation medium for 2 d. Scale bar = 50 μm. 

**j** Expression analysis of Islr in C2C12 cells cultured in either growth medium for 1 or 2 d (G1 and G2) or in differentiation medium for 1, 3, 5, or 7 d (D1, D3, D5, and D7) using quantitative real-time PCR (qRT-PCR). 

**k** Western blot analysis of Islr in C2C12 cells cultured in either growth medium for 2 d (G2) or in differentiation medium for 4 or 7 d (D4 and D7). Error bars represent the means ± s.d. ***P < 0.001; Student’s t test.
Islr regulates the differentiation potential of SCs. The higher expression of Islr in differentiated myogenic cells prompted us to investigate the importance of Islr in the process of SCs differentiation during skeletal muscle regeneration. There was a significant reduction in the numbers of eMyHC$^+$ muscle fibers in Islr cKO mice compared to control mice at 3.5 d postinjury. At 5 d postinjury, the size of eMyHC$^+$ fibers was significantly reduced in the regenerating TA muscles of Islr cKO mice. Statistical analysis showed that the surface area of eMyHC$^+$ muscle fibers was significantly decreased in Islr cKO mice (Fig. 3a). This suggested that the differentiation potential of SCs decreased. To further test this idea, we quantified the numbers of MyoG$^+$ cells and found a significant reduction of MyoG$^+$ cells in Islr cKO mice (Fig. 3b). Consistently, Western blotting showed that the protein levels of MyoG and eMyHC were significantly reduced in Islr cKO mice at 3.5 and 5 d postinjury (Fig. 3c, d).

To confirm the results in vitro, we isolated single myofibers from the extensor digitorum longus (EDL) muscles of control and Islr cKO mice. The number of MyoG$^+$ cells in the clusters associated with single myofibers was greatly reduced in Islr cKO mice compared to controls (Fig. 4a). Next, we analyzed FACS-purified SCs from control and Islr cKO mice. Although there was no significant difference during the proliferation period (Supplementary Fig. 2b), we found that the numbers of MyoG$^+$ cells were decreased in Islr cKO mice after inducing differentiation (Fig. 4b). Consistently, Western blotting showed that the protein level of MyoG was reduced in Islr cKO mice (Fig. 4c). Further, we found that the ability of primary myoblasts derived from FACS-purified SCs to fuse myotubes was significantly decreased, and the fusion index was decreased by about 40% in Islr cKO mice compared to controls (Fig. 4d). Statistical analysis showed that Islr cKO mice had fewer myotubes containing three or more nuclei (Fig. 4e), and the levels of MyHC...
protein were much lower in Islr cKO mice (Fig. 4f). To confirm these results, we established primary myoblasts of control and Islr cKO mice. Similarly, we observed that the differentiation of primary myoblasts was significantly decreased in Islr cKO mice (Supplementary Fig. 2c, d).

**SC-specific deletion of Islr impairs skeletal muscle regeneration.** The Myf5-Cre driver functions in the early embryonic development, and may cause compensatory mechanisms to influence the phenotype of Islr cKO mice. To address this concern, we crossed mice possessing the Islr<sup>fl/fl</sup> conditional allele with Pax7-CreER<sup>+</sup> mice to generate the Pax7-CreER<sup>+</sup>:Islr<sup>fl/fl</sup> mice. The mice were induced with tamoxifen (TAM) at the age of 10 weeks, followed by CTX damage (Fig. 5a). To evaluate the recombination efficiency at the protein level, we performed immunohistochemistry and Western blot experiments, and found that Islr was markedly deleted in the SCs of Pax7-CreER:Islr<sup>fl/fl</sup> mice at 5 d postinjury (Fig. 5b, c). To test whether Pax7-CreER: Islr<sup>fl/fl</sup> mice had a phenotype consistent with Islr cKO mice, we histologically analyzed the TA muscles at 5 and 14 d postinjury. At 5 d post-injury, Islr<sup>fl/fl</sup> mice had regenerated a large number of muscle fibers with centrally located myonuclei, whereas there were many blank areas in the TA muscles of the Pax7-CreER: Islr<sup>fl/fl</sup> mice (Fig. 5d and Supplementary Fig. 2e). Five days after injury, the average CSA of muscle fibers was significantly decreased in Pax7-
CreER:Islr<sup>fl/fl</sup> mice compared to the Islr<sup>fl/fl</sup> mice (Fig. 5e). In addition, the numbers of myofibers containing two or more centrally located nuclei were also significantly reduced in Pax7-CreER:Islr<sup>fl/fl</sup> mice (Fig. 5f). Meanwhile, the size of eMyHC<sup>+</sup> fibers was very small in the regenerating TA muscles of Pax7-CreER:Islr<sup>fl/fl</sup> mice (Fig. 5g). At 14 d postinjury, Islr<sup>fl/fl</sup> mice displayed tightly packed, well-formed muscle fibers, whereas Pax7-CreER:Islr<sup>fl/fl</sup> mice had many very small muscle fibers with single nuclei (Fig. 5d, h and Supplementary Fig. 2e). Overall, the phenotype of Pax7-CreER:Islr<sup>fl/fl</sup> mice was more severe, which demonstrated that Islr had a substantial effect on skeletal muscle regeneration.
Islr functioning by Dvl2-mediated canonical Wnt signaling.

In order to study the molecular mechanisms of Islr during differentiation, we generated Islr shRNA stable (shIslr) C2C12 cells. Islr mRNA was effectively knocked down in shIslr C2C12 cells compared to control shRNA (shCtrl) C2C12 cells (Supplementary Fig. 3a). The shCtrl and shIslr C2C12 cells were induced to differentiate, and the shIslr C2C12 cells failed to differentiate (Fig. 6a, b). Subsequently, we performed RNA sequencing (RNAseq) analysis on shCtrl and shIslr C2C12 cells after 2 d of culture in growth medium (G2) or 3 d of culture in differentiation medium (D3). There are 19 signaling pathways whose expression was modulated in both the G2 and D3, in which the Wnt signaling pathway was closely related to C2C12 cells differentiation (Supplementary Fig. 3b, c). Canonical Wnt pathway receptors and downstream target genes were significantly downregulated in shIslr C2C12 cells, and genes related to myogenic development were also significantly downregulated in shIslr C2C12 cells (Fig. 6c). The downregulation of Wnt target genes and myogenic genes were also confirmed by quantitative real-time PCR (qRT-PCR) (Fig. 6d). These results suggested that the differentiation phenotype of C2C12 cells might be associated with the perturbations of canonical Wnt signaling. Coincidentally, MyoG, a muscle differentiation marker, was reduced in the shIslr C2C12 cells (Fig. 6d). This is significant because MyoG was shown to be directly regulated by the canonical Wnt signaling pathway31,32.

Consistently, we found that the level of active β-catenin in the nucleus was significantly reduced during the differentiation of shIslr C2C12 cells (Fig. 6e). The TOP/FOP reporter assay also showed that the activity of the canonical Wnt signaling pathway was significantly decreased in the shIslr C2C12 cells (Fig. 6f). Dvl2 is an important component involved in canonical Wnt signaling38, and Western blotting showed that the protein levels
of Dvl2 were significantly decreased during the proliferation and differentiation of the shIslr C2C12 cells (Fig. 6g, h). Confirming the results obtained with C2C12 cells, we found that the protein levels of Dvl2 were significantly decreased during the differentiation of primary myoblasts from Islr cKO mice (Fig. 6i).

Wnt3a is a classical ligand of the canonical Wnt signaling pathway. Exogenous addition of Wnt3a can activate canonical Wnt signaling. Axin1 was degraded following the addition of Wnt3a into shCtrl C2C12 cells. However, Axin1 still accumulated in shIslr C2C12 cells (Fig. 7a). Meanwhile, the ability of β-catenin to enter the nucleus was significantly reduced in response to Wnt3a addition in shIslr C2C12 cells (Fig. 7b). This suggested that Wnt3a required Islr for its function. To confirm the results obtained with C2C12 cells, we injected Wnt3a into the TA muscles of control and Islr cKO mice after CTX injury, and found that Wnt3a could not effectively activate canonical Wnt signaling during skeletal muscle regeneration (Fig. 7c, d), which is consistent with the results of C2C12 cells. Furthermore, 1-azakenpaullone (1-AKP), which is known to activate the canonical Wnt signaling pathway independent of Dvl2\(^3\), reduced levels of Axin1 and GSK3β in shIslr C2C12 cells when added to the differentiation medium (Fig. 7e). As a consequence, MyHC was expressed in 1-AKP treated shIslr C2C12 cells (Fig. 7f). To confirm these results, we used another small-molecule Wnt...
agonist, CHIR-99021 (CHIR)\textsuperscript{34}, and obtained the same results (Supplementary Fig. 4a). We also checked the expression of MyoG in shCtrl and shIslr C2C12 cells. Not surprisingly, the numbers of MyoG\textsuperscript{+} cells were significantly decreased in shIslr C2C12 cells (Supplementary Fig. 4b); meanwhile, the protein levels of MyoG were also reduced (Supplementary Fig. 4c). We treated shCtrl and shIslr C2C12 cells with 1-AKP and CHIR, and found that both drugs rescued the MyoG\textsuperscript{+} cells (Fig. 7g, h). Meanwhile, we obtained the same results from primary myoblasts of control and Islr cKO mice that we established (Fig. 7i).
Cumulatively, 1-AKP and CHIR rescued the phenotype of the shIslr C2C12 cells and the primary myoblasts of Islr cKO mice, indicating that Islr might regulate C2C12 cell differentiation via the canonical Wnt signaling pathway.

Dvl2 complementation rescues the phenotype of Islr cKO mice. The aforementioned data suggested that the loss of Islr blocked the canonical Wnt signaling pathway via Dvl2 (Fig. 6g–i). In order to validate the results obtained from the C2C12 cells, we checked the expression of Dvl2 in TA muscles at 3 d postinjury and found that its expression pattern was consistent with that of Islr (Supplementary Fig. 5a). Meanwhile, Axin1 was also downregulated, suggesting activation of the canonical Wnt signaling pathway (Supplementary Fig. 5b). Consistently, Dvl2 was highly expressed and β-catenin translocated to nuclei in the cells where Islr was highly expressed at 5 d postinjury (Fig. 8a, b). These results demonstrated that Islr expression correlated with Dvl2. Subsequent results showed that Dvl2 could not be efficiently expressed in Islr cKO mice at 3.5 d postinjury (Fig. 8c). Meanwhile, primary myoblasts derived from FACS-purified SCs of Islr cKO mice expressed lower levels of Dvl2 and higher levels of Axin1 than control mice after 2 d of culture in differentiation medium. These results suggested that the canonical Wnt signaling pathway was significantly inhibited in Islr cKO mice (Fig. 8d). Based on the above results, we injected CHIR into the TA muscles of control and Islr cKO mice after CTX injury, and found that CHIR rescued the phenotype of Islr cKO mice (Fig. 8e, f). Cumulatively, these data suggested that Dvl2 might be modulated by Islr.

It has been reported that the inhibition of Dact1 upregulates the expression of Dvl2 and activates canonical Wnt signaling. Therefore, we generated Islr/Dact1 cKO double-mutant mice. Intriguingly, the expression level of Dvl2 was rescued in Islr/Dact1 cKO mice (Fig. 8g). Moreover, canonical Wnt signaling was reactivated in Islr/Dact1 cKO mice (Fig. 8h). As a consequence, the deletion of Dact1 rescued the phenotype of Islr cKO mice (Fig. 8i). The CSA of Islr/Dact1 cKO mice was significantly higher than in Islr cKO mice at 5 d postinjury (Fig. 8j). Meanwhile, the number of MyoG+ cells was restored in Islr cKO mice (Fig. 8k). Taken together, the in vivo and in vitro data supported the notion that Islr regulated canonical Wnt signaling via modulation of Dvl2.

Islr stabilizes Dvl2 by antagonizing the autophagy system. The autophagy system regulates canonical Wnt signaling via modulation of Dvl2 levels. We thus detected the occurrence of autophagy in C2C12 cells using a GFP-LC3 (a well-known marker of autophagosomes) plasmid. Overexpression of Dvl2-Flag increased the formation of GFP-LC3+ puncta in C2C12 cells. However, co-overexpression of Islr-HA and Dvl2-Flag decreased GFP-LC3+ puncta formation compared to overexpression of Dvl2-Flag alone (Fig. 9a). Moreover, co-overexpression of Islr-GFP and Dvl2-Flag decreased the formation of Dvl2-Flag+ puncta compared to overexpression of Dvl2-Flag alone in HEK293T cells (Supplementary Fig. 6a). We found that the autophagy markers LC3II and p62 were significantly increased and decreased, respectively, in shIslr C2C12 cells (Fig. 9b). Furthermore, overexpression of Dvl2-Flag in shIslr C2C12 cells displayed more GFP-LC3+ puncta but this was not observed in the Dact1 cKO mice. Meanwhile, GFP-LC3+ puncta were significantly reduced in Islr/Dact1 cKO mice (Fig. 9e), which confirmed the previous experiments performed in C2C12 cells. From above results, it was clear that the deletion of Islr increased the autophagy-mediated Dvl2 degradation. Therefore, we used bafilomycin A1 (BFA1) to treat primary myoblasts of control and Islr cKO mice that we established and found that Dvl2 and Wnt target genes Axin2 and Lef1 were rescued in primary myoblasts of Islr cKO mice (Fig. 9f). Meanwhile, the disruption of differentiation was rescued in the primary myoblasts of Islr cKO mice when BFA1 was added to the differentiation medium (Supplementary Fig. 6b). To further determine whether Islr interacted with Dvl2 to antagonize the autophagy system, we performed immunoprecipitation analysis and found that Islr-GFP interacted with Dvl2-Flag (Fig. 9g) and Supplementary Fig. 6c). Meanwhile, we also found that Islr interacted Dvl2 in differentiated primary myoblasts (Fig. 9h). Dvl2 is ubiquitinated before entering the phagophore. The ubiquitination level of Dvl2 was significantly increased in differentiated primary myoblasts of Islr cKO mice, indicating that Islr helped Dvl2 resist ubiquitination (Fig. 9i). Cumulatively, these results indicated that Islr stabilized Dvl2 protein by antagonizing the autophagy system.

Discussion
Our study found that Islr participated in regulating SC fate and skeletal muscle regeneration. The loss of Islr delayed skeletal muscle regeneration in Myf5-Cre:Islrfl/fl mice. Another SC-specific knockout (Pax7-CreERT:Islrfl/fl) mouse model confirmed these data with an even stronger phenotype. The SC differentiation capacity of Islr cKO mice was significantly decreased as a result of the inactivation of canonical Wnt signaling, in which Islr stabilized Dvl2 by antagonizing the autophagy system.
**Fig. 8** The ablation of Dact1 rescues the impaired skeletal muscle regeneration of Islr cKO mice by upregulating Dvl2. 

- **a** Immunohistochemistry analysis of Islr and Dvl2 in injured TA muscles of WT mice at 5 d postinjury using serial sections. Scale bar = 10 μm. 
- **b** Immunohistochemistry analysis of Islr and β-catenin in injured TA muscles of WT mice at 5 d postinjury using serial sections. Scale bar = 10 μm. 
- **c** Western blot analysis of Dvl2 protein levels in injured and CTL of control and Islr cKO mice at 3.5 d post injury. 
- **d** Western blot analysis of Dvl2 and Axin1 protein levels in isolated satellite cells of control and Islr cKO mice cultured for 2 d in differentiation medium. 
- **e** Intramuscular injection of CHIR or BSA at 2.5 d postinjury and H&E staining of injured TA muscles of control and Islr cKO mice at 5 d postinjury. N = 3 in each group. Scale bar = 100 μm. 
- **f** CSA of myofibers in (e). 
- **g** Western blot analysis of Dvl2 protein levels in injured TA muscles of control, Islr cKO, Dact1 cKO, and Islr/Dact1 cKO mice at 5 d postinjury. 
- **h** Western blot analysis of active β-catenin protein levels in injured TA muscles of control, Islr cKO, Dact1 cKO, and Islr/Dact1 cKO mice at 5 d postinjury. 
- **i** H&E staining of injured and contralateral TA muscles in control, Islr cKO, Dact1 cKO, and Islr/Dact1 cKO mice at 5 d postinjury. Control (CTL): N = 5; Islr cKO: N = 3; Dact1 cKO: N = 3; Islr/Dact1 cKO: N = 3. Scale bar = 50 μm. 
- **j** Immunofluorescence analysis of MyoG+ cells in TA muscles of control, Islr cKO, Dact1 cKO, and Islr/Dact1 cKO mice at 5 d postinjury. The numbers of MyoG+ cells per myofiber are shown on the right. Error bars represent the means ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001; Student’s t test.
Although we also found that Islr mRNA is expressed in quiescent SCs, similar to a recent report, we found that Islr protein was not expressed in Pax7+ quiescent SCs. Islr was expressed in Pax7+ activated SCs and more highly expressed in differentiated myogenic cells with the progression of myogenesis, suggesting a potential role for Islr in myogenic differentiation. Supporting this idea, we found that C2C12 cells stopped differentiating into myotubes when disrupting the expression of Islr, and that the ability of myoblasts to differentiate in Islr cKO mice was also significantly decreased, as evidenced by the reduction of MyoG in both in vivo and in vitro experiments. It is well-known that MyoG is a key factor required for SC differentiation and myocyte fusion. Skeletal muscle fails to develop normally in the absence of MyoG. Taken together, our findings suggest that Islr regulates the differentiation of SCs and C2C12 cells. Although Islr was downregulated during mesenchymal stem cell differentiation into bone and adipose tissues, there were many differences in the expressions of genes between these developing tissues and skeletal muscle. It is well-known that bone morphogenetic proteins are expressed in different patterns during bone formation and myogenic differentiation.

Many reports have shown that the differentiation of C2C12 cells is mainly regulated by canonical Wnt signaling which can directly regulate the expression of MyoD and MyoG. Kindlin 2...
directly binds the promoter of MyoG in conjunction with β-catenin and TCF4 to regulate C2C12 cell differentiation.32 Consistent with previous reports, we found that the activity of the canonical Wnt signaling pathway was significantly perturbed in shIslr C2C12 cells, resulting in defective differentiation.

Mechanistically, Islr interacted with and stabilized Dvl2 to activate the canonical Wnt signaling pathway. Dvl2 is an important factor required for canonical Wnt signaling,16,45 and a reduction in Dvl2 expression abrogates canonical Wnt signaling.46 Confirming this, activation of canonical Wnt signaling independent of Dvl2 was able to rescue the impaired skeletal muscle regeneration phenotype and increase the numbers of MyoG+ cells both in the shIslr C2C12 cells and primary myoblasts of Islr KO mice. Further, the deletion of Dact1 also rescued the impaired differentiation phenotype of Islr KO mice by upregulating Dvl2 in vivo. In brief, Islr regulated Dvl2-mediated canonical Wnt signaling and further regulated SC differentiation and skeletal muscle regeneration. Additionally, because Dvl2 has some functions in noncanonical Wnt signaling during skeletal muscle regeneration14, the potential effect of Islr on other members of the Wnt family, including noncanonical Wnt signaling warrant future investigations.

Autophagy is associated with many important signaling pathways such as Wnt, Shh (Sonie Hedgehog), transforming growth factor β, and fibroblast growth factor (FGF)47,48. A key factor is that LC3 targets Dvl2, thus regulating the activity of the canonical Wnt signaling pathway through the autophagy system.15 In our experiments, we found that Islr stabilized Dvl2 by inhibiting autophagy. GFP-LC3+/Dvl2-Flag+ puncta formed when the Dvl2-Flag plasmid was transfected into C2C12 cells. However, GFP-LC3+/Dvl2-Flag+ puncta could not form and Dvl2-Flag was uniformly dispersed in the cytoplasm when Dvl2-Flag and Islr-HA were co-transfected. These findings suggested that Islr antagonized the autophagy system, consequently protecting Dvl2 against degradation. Furthermore, we demonstrated that endogenous Dvl2 was more easily identified by LC3 both in shIslr C2C12 cells and Islr-deficient primary myoblasts derived from FACS-purified SCs when autophagy was induced.

Previous reports have shown that Dact1 can induce autophagy of Dvl2 and further inhibit canonical Wnt signaling.35,49. In agreement with this, we found that the deletion of Dact1 inhibited the formation of Dvl2+ autophagic cupules in Islr-deficient primary myoblasts derived from FACS-purified SCs. BFA1, an inhibitor of autophagy, rescued Dvl2 protein level in primary myoblasts of Islr KO mice. Reciprocal co-immunoprecipitation analysis demonstrated that Islr interacted with Dvl2 to antagonize the ubiquitination of Dvl2. It will be interesting to investigate how Islr affects the ubiquitination of Dvl2.

In conclusion, we found that Islr played an important role in the differentiation of SCs during skeletal muscle regeneration. At the molecular level, Islr protected Dvl2 against autophagy-mediated degradation, consequently activating the canonical Wnt signaling pathway (Fig. 10).

Methods

Animals. All experiments were performed in accordance with the China Agricultural University’s regulations for animal care and handling. The China Agricultural University Laboratory Animal Welfare and Animal Experimental Ethical Committee authorized the study and approved the protocol. We used the following mouse lines: Myf5-Cre, Pax7-CreER, Islrfl/fl, Dvl2Δfl/fl, Myf5-Cre and Pax7-CreER mice were provided by Dr. Dahai Zhu (State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and School of Basic Medicine, Peking University Medical College) and Dvl2fl/fl was provided by Dr. Yeguang Chen (State Key Laboratory of Biomembrane and Membrane Biotechnology, Tsinghua University). IslrKO mice were provided by Dr. Dahai Zhu (State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and School of Basic Medicine, Peking University Medical College) and Dvl2fl/fl was provided by Shanghai Biosensor Organism Science & Technology Development Co., Ltd. All mice were in the C57BL6 background, and their genotype was determined by PCR from tail DNA using the following primers: Myf5-Cre and Pax7-CreER forward: 5′-gcctgctacaggtgctatcc-3′, Myf5-Cre and Pax7-CreER reverse: 5′-caggtgttaaacaccactc-3′, IslrΔfl/fl forward: 5′-gcagcaaaaccttgaatcc-3′, IslrΔfl/fl reverse: 5′-cctgctcagtcagcttactc-3′, Dvl2ΔΔ forward: 5′-ttacccaggaatcacgcag-3′, Dvl2ΔΔ reverse: 5′-gcctgcttacaagactctg-3′. To induce Islr deletion in Pax7-CreER islrlfl mice, tamoxifen (TAM; Sigma) dissolved in corn oil (Sigma) was applied intraperitoneally on four subsequent days to 10-week-old mice at 40 mg/kg.

Skeletal muscle injury and paraffin sections preparation. To induce injury, 100 μl of cardiotoxin (CTX, 10 μM in PBS, Sigma) was injected into hindlimb TA muscles. All CTX injured mice were 10 weeks old. The TA muscles were harvested at 3.5, 5, and 14 d postinjury. The TA muscles were fixed with 4% paraformaldehyde for more than 72 h and then subjected to dehydration embedding. Finally, paraffin sections of the muscles were obtained at a thickness of 2–4 μm.

SC purification and culture. The hindlimb muscles were minced and digested with 0.2% type II collagenase (Sigma) and 2.5 U/ml dispase (Roche) for 1 h. Each sample was consecutively filtered through 70- and 40-μm cell strainers. The cell suspension was then centrifuged at 600g. Finally, the pellet was washed with FACS buffer (3% FBS in PBS) and stained with CD31-FITC (ebioscience, cat. no. 11-0311-82), CD45-FITC (ebioscience, cat. no. 11-0451-82), Sca1-PerCP (ebioscience, cat. no. 45-5981-82), and α7-integrin-APC (AbLab, cat. no. 67-0010-05) for 1 h at 4°C. We obtained SCs, followed by CD31+, CD45−, Sca1+, and α7-integrin− cells. FACS-purified SCs were cultured in 24-well plates (NUNC) in Dulbecco’s modified Eagle medium (DMEM; Gibco) with 20% FBS (Gibco) and basic FGF (bFGF; 5 ng/ml, Invitrogen). To induce the differentiation, proliferating cells were incubated with DMEM supplemented with 2% horse serum (HS; Gibco).

Primary myoblast purification and culture. Primary myoblasts of control and Islr KO mice were established by the method of differential adhesion. We cultured primary myoblasts in Ham’s F10 nutrient mixture medium (Gibco) containing 20% FBS and 5 ng/ml bFGF. To induce differentiation, primary myoblasts were incubated with DMEM supplemented with 2% HS.

Single myoblast isolation and culture. The EDL muscles were isolated and digested with 0.2% type I collagenase (Sigma) in DMEM for 1 h. Single myoblasts
were transferred to 48-well plates and incubated in DMEM supplemented with 10% HS, 5 ng/ml bFGF, and 0.5% chicken embryo extract (MP Biomedicals).

Plasmids and RNA interference. In order to construct a plasmid encoding Dvl2-Flag, the Flag-tag was added at the C-terminus of Dvl2. Dvl2-Flag was then cloned into the BamHI and EcoRI sites of the pcDNA3.1(+) vector (Invitrogen). In order to construct a plasmid encoding Islr-HA, the HA tag was added at the C-terminus of Islr. Islr-HA was then cloned into the BamHI and EcoRI sites of the pcDNA3.1(+) vector. In order to construct a plasmid encoding leader peptide-Flag-Islr, the Flag-tag was added after amino acid 18 (the leader peptide of Islr). The leader peptide-Flag-Islr was then cloned into the BamHI and EcoRI sites of the pcDNA3.1(+) vector. The GFP-LC3B plasmid was provided by Dr. Yeguang Chen (State Key Laboratory of Biombemembrane and Membrane Biotechnology, Tsinghua University). The TOPFlash/FOPFlash and Renilla luciferase expression plasmids were kept in our lab. Islr-GFP (MG206818), scrambled shRNA, and Islr shRNAs (TG512833) were purchased from OriGene. The sequences of Islr shRNAs were as follows: Islr shRNA1, 5'-cttacaggttgtctatctcagacgac-3'; Islr shRNA2, 5'-gcaatggaacaca- cactgcgctgactc-3'; Islr shRNA3, 5'-ggtctgtgtgcctgacccatgctg-3'; and Islr shRNA4, 5'-ggctgtcatacggcttgacagcagcag-3'.

Cell lines and Islr shRNA stable (shIslr) C2C12 cells. C2C12 and HEK293T cell lines were purchased from the Chinese Academy of Medical Sciences and the School of Basic Medicine at Peking Union Medical College. To obtain stably transfected C2C12 cells, we transfected C2C12 cells with scrambled shRNA plasmid or Islr shRNAs plasmid via electric transfection (Lonza). At 24 h after transfection, the cells were diluted and cultured with puromycin (2 μg/ml, Sigma) for resistance screening for 1 week. The clones were then transferred to a 96-well plate to expand the culture. Finally, transfection was confirmed by PCR, and the primers used were as follows: forward: 5'-ggaggg gcattctccat3', reverse: 5'-gcctccactgctgctgctg-3'. The efficacy of Islr shRNA interference was determined by quantitative real-time PCR (qRT-PCR) and Western blotting.

Immunofluorescence and live-cell staining. Muscle sections, single myofibers, and cultured cells were fixed in 4% paraformaldehyde and permeabilized for 10 min in 0.3% Triton X-100 in PBS. The samples were blocked in blocking buffer (Beyotime Biotechnology) for 2 h at room temperature. Primary antibodies were incubated in blocking buffer at 4 °C overnight. Subsequently, the samples were washed with PBS and stained with the appropriate fluorescently labeled secondary antibodies (Alexa Fluor 350, 488, or 594) for 1 h at room temperature. After washing with PBS, DAPI (Roche) was used to stain nuclei for 3 min. The primary antibodies used were as follows: mouse anti-Pax7 (Developmental Studies Hybridoma Bank [DSHB], 1:50), mouse anti-MyHC (DSHB, 1:500), mouse anti-MyoG (cat. no. ab1835, Abcam, 1:200), mouse anti-MyHC (cat. no. M44276, Sigma, 1:500), rabbit anti-laminin (cat. no. L3933, Sigma, 1:200), rabbit anti-Dvl2 (cat. no. ab22616, Abcam, 1:200), rabbit anti-β-tubulin (cat. no. ab6046, Abcam, 1:500), mouse anti-Flag (cat. no. F1804, Sigma, 1:500), and rabbit anti-HA (cat. no. ab9110, Abcam, 1:200). For live-cell staining, Dil (5 μM, Beyotime Biotechnology) and Hoechst 33342 (2 μg/ml, Beyotime Biotechnology) were applied for 20 min.

Immunohistochemistry. Muscle sections were fixed in 4% paraformaldehyde and permeabilized for 10 min in 0.3% Triton X-100 in PBS. The samples were treated with 3% H2O2 for 20 min and blocked in blocking buffer (ZSGB-BIO) for 2 h at room temperature. Primary antibodies were applied in blocking buffer at 4 °C overnight. Subsequently, the samples were washed with PBS and stained with the appropriate biotin-labeled secondary antibody (goat anti-rabbit or anti-mouse) for 1 h at room temperature. Finally, DAB (ZSGB-BIO) was used for color development. Samples were then washed with water, and hematoxylin was used to stain the nuclei for 5 min. The primary antibodies used were as follows: rabbit anti-Islr (cat. no. HPA050811, Sigma, 1:300), mouse anti-Pax7 (DSHB, 1:50), mouse anti-MyoG (cat. no. ab1835, Abcam, 1:200), rabbit anti-β-catenin (cat. no. 9562S, Cell Signaling Technology, 1:200), and rabbit anti-Dvl2 (cat. no. PA5-27965, Thermo Fisher, 1:200).

Plasmid transfection and luciferase reporter assay. Scrumbled shRNA, Islr shRNA, Dvl2-Flag, Islr-GFP, Islr-HA, or GFP-LC3B plasmids were transfected into C2C12 cells, HEK293T cells, or SCs using Lipofectamine 2000 (Invitrogen). The samples were then harvested between 24 and 36 h after transfection. In order to identify the activity of canonical Wnt signaling, the TOPFlash/FOPFlash expression plasmids with the Renilla luciferase expression plasmid were transfected when shCtrl and shIslr C2C12 cells were cultured after 2 d in the differentiation medium. After 24 h, the reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

**Fig. 10** Model of the role of Islr during adult skeletal muscle regeneration. Islr protects Dvl2 against autophagy-mediated degradation, resulting in the precise regulation of canonical Wnt signaling during skeletal muscle regeneration. Islr+/+: control mice; Islr−/−: Islr KO mice.
RNA-seq. The total RNA was extracted from shCtrl and shIsr C2C12 cells using an RNaseasy Mini Kit (Qiagen) and on-column DNase digestion (RNase-Free DNase Set, Qiagen) to avoid contamination by genomic DNA. The cDNA library construction, sequencing, and transcriptome data analysis were performed by Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China).

gRT-PCR analysis. Total RNA was prepared using TRIzol (Invitrogen). After DNase treatment, 2 μg of RNA was used for reverse-transcription, and qPCR was performed using the LightCycler 480 SYBR Green Master Mix (Roche) in an iCycler. 

Primary antibodies were applied in sealing fluid at 4 °C overnight. Subsequently, the PVDF membrane was washed with TBS and stained with the appropriate HRP-labeled secondary antibodies (goat anti rabbit or mouse) for 1 h at room temperature. After washing with TBS, the ECL Reagent (Milipore) was used, and the strips were on film. For immunoprecipitation analysis, the HEK293T cells were lysed with IP lysis buffer (Beyotime Biotechnology), and the total protein was immunoprecipitated with Flag or GFP antibody and protein G beads (Thermo Scientific, 1:1500), rabbit anti-GSK3β (cat. no. HPA050811, Sigma, 1:1000), rabbit anti-Axin1 (cat. no. 2087s, Cell Signaling Technology, 1:5000), rabbit anti-MyoG (cat. no. ab1835, Abcam, 1:1000), mouse anti-MyHC (cat. no. ab32604, Abcam, 1:5000), rabbit anti-GAPDH (cat. no. 2118s, Cell Signaling Technology, 1:1500), rabbit anti-Dvl2 (cat. no. ab22616, Abcam, 1:1000), rabbit anti-β-catenin (cat. no. 8814s, Cell Signaling Technology, 1:1000), rabbit anti-β-tubulin (cat. no. ab6046, Abcam, 1:5000), rabbit anti-MyHC (cat. no. M171, Abcam, 1:1500), mouse anti-Flag (cat. no. F1804, Sigma, 1:2000), and rabbit anti-GFP (cat. no. ab290, Abcam, 1:1500). Uncropped blots for Western blot analysis are shown in Supplementary Fig. 7.

Autophagy analysis. C2C12 cells or SCs were transfected with GFP-LC3B plasmid. After 24 h, cells were treated with rapamycin (4 μM, Sigma) for 6 h to induce autophagy, followed by immunofluorescence analysis.

Treatment with reagents in cell culture. In order to verify the activity of canonical Wnt signaling, shCtrl and shIsr C2C12 cells were treated with either Wnt3a (100 ng/ml, Peprotech) or saline for 24 h after 2 d in differentiation medium. The cell samples were then collected for Western blot analysis. In order to activate canonical Wnt signaling in shIsr C2C12 cells, the shIsr C2C12 cells were treated with either 1-azakena-pol (1-APK; 3 μM, Sigma) or CHIR-99021 (CHIR; 3 μM, Stemgent) during differentiation. Western blotting and immunofluorescence analysis were performed after 3 or 7 d of differentiation. To verify the activity of canonical Wnt signaling in vivo, the TA muscles of control and shIsr cKO mice were injected with Wnt3a (100 ng/ml, Peprotech) or PBS (0.1% BSA) at 1.5 d postinjury. The injured TA muscles were then collected for Western blot analysis at 4 d postinjury. To activate canonical Wnt signaling in vivo, the TA muscles of control and shIsr cKO mice were injected with CHIR-99021 (50 ng/ml, Stemgent) or PBS (0.1% BSA) at 2.5 d postinjury. The injured TA muscles were then collected for H&E analysis at 5 d postinjury. To inhibit autophagy, the primary myoblasts of cKO mice with or without the expression of GFP were collected for Western blot analysis.

Statistical analysis. All experiments included at least three biological replicates. The areas of fluorescent staining for muscle fibers and nuclei were determined using ImageJ software. Data were analyzed using Microsoft Excel. A two-tailed Student’s t-test was used to assess statistical significance.

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Author contributions
K.Z. designed and performed the experiments, analyzed the data, and wrote the manuscript. Y.Z., L.G., and M.L. carried out vector construction and mouse rearing. C.L., M.W., Y.S., M.G., T.W., Y.Y., C.L., and L.L. provided technical assistance. Q.L., Y.Z., Z.Y., F.W., and N.L. revised the manuscript. Q.M. guided the experiments, analyzed the results, revised the manuscript, and provided financial support. All the authors edited and approved the manuscript.

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