Requirement for PINCH in Skeletal Myoblast Differentiation

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Abstract

Background: Skeletal muscle is composed of bundles of myofibers ensheathed by extracellular matrix networks. Malformation of skeletal muscle during embryonic development results in congenital myopathies. Disease mechanisms of congenital myopathies remain unclear. PINCH, an adaptor of focal adhesion complex, plays essential roles in multiple cellular processes and organogenesis. Elucidation of the molecular mechanisms underlying skeletal myogenesis will offer new insights into pathogenesis of myopathies.

Methods: We generated muscle-specific PINCH knock-out mice to study the functional role of PINCH in skeletal myogenesis. Histologic and Transmission Electron Microscopy analysis demonstrated that Impaired myogenic differentiation and maturation in mice with PINCH1 being ablated in skeletal muscle progenitors, and Ablation of PINCH1 and PINCH2 resulted in reduced size of muscle fibers and impaired multinucleation; Cell culture and immunostaining showed that defects in myoblast fusion and cytoskeleton assembly in PINCH double mutant mice; Western blotting showed that defects in expression of cytoskeleton proteins and proteins involved in myogenesis in DMUT skeletal muscles.

Results: Double ablation of PINCH1 and PINCH2 resulted in early postnatal lethality with reduced size of skeletal muscles and detachment of diaphragm muscles from the body wall. Myofibers of PINCH mutant myofibers failed to undergo multinucleation and exhibited disrupted sarcomere structures. The mutant myoblasts in culture were able to adhere to newly formed myotubes, but impeded in cell fusion and subsequent sarcomere genesis and cytoskeleton organization. Consistent with this, expression of integrin β1 and some cytoskeleton proteins, and phosphorylation of ERK and AKT were significantly reduced in PINCH mutants. Expression of MRF4, the most highly expressed myogenic factor at late stages of myogenesis, was abolished in PINCH mutants, that could contribute to observed phenotypes. In addition, mice with PINCH1 being ablated in myogenic progenitors exhibited only mild centronuclear myopathic changes, suggesting a compensatory role of PINCH2 in myogenic differentiation, indicating a critical role of PINCH proteins in myogenic differentiation.

Conclusion: Our results demonstrated an essential role of PINCH in skeletal myogenic differentiation.

Background

Skeletal muscle is composed of bundles of myofibers ensheathed by extracellular matrix networks, and is responsible for force generation, contraction and body movement. Malformation of skeletal muscle during embryonic development results in congenital myopathies, a heterogeneous group of primary muscle disorders characterized by muscle weakness and a number of myopathic changes in muscle biopsies, including nemaline rods, central core, multi-mini core and centralized nuclei[1–4]. Disease mechanisms of congenital myopathies remain unclear. Further elucidation of the molecular mechanisms underlying skeletal myogenesis will offer new insights into pathogenesis of myopathies and is critical to the development of novel therapies.

Skeletal myogenesis is a multistep process regulated by distinct signaling pathways (e.g. Wnt, FGF and Notch pathways) and a network of myogenic transcription factors[5–8]. Pax3 and Pax7, via their downstream myogenic regulatory factors (MRF5, MyoD), are required for the specification and determination of myogenic progenitors and myoblasts[9, 10]. Myoblasts initiate the expression of other two myogenic factors MRF4 and MyoG1 that promote muscle differentiation by upregulation of genes encoding muscle-specific contractile and structure proteins. Mononucleated myoblasts eventually exit the cell cycle and fuse to form multinucleated myofibers[11, 12]. The mature myofibers are filled with well-aligned myofibrils made up of sarcomeres, which in turn are interconnected with the cell membrane (sarcolemma), nucleus and organelles via actin filament networks. One of the features of mature myofibers is the peripheral positioning of nucleus underneath the cell membrane. Nuclear shape and position of myofibers are controlled by coordinated action of both actin and microtubule-based cytoskeleton systems and by the interaction of cytoskeleton and nucleoskeleton. This unique cytoskeletal architecture of muscle cells is essential for mechanical sensing and transduction, gene expression and contractile function. However, molecular mechanisms regulating cytoskeletal assembly and integrity of skeletal muscle are not fully understood.

Integrin signaling pathways mediate cell-matrix interactions and play essential roles in multiple cellular processes and during embryonic development. Integrin-focal adhesion complex mediate the crosstalk between extracellular matrix (ECM) and intracellular signaling pathways and cytoskeleton, and is essential for ECM deposition and cytoskeleton organization and remodeling, and functions as a key mechanical sensor and anchorage sites for cell adhesion and migration. Integrin pathways play an important role in muscle development and function, and disruptions of integrin-mediated adhesion in skeletal muscles result in myopathies in humans and mice[13]. Receptors of β1 Integrin family are required for the fusion of mononucleated myoblasts and subsequent assembly of cytoskeleton[14]. Blocking integrin α6β1 binding to laminin inhibits myotome formation, and mutations in the Integrin α7 gene cause congenital myopathy in both patients and mice[15–17]. Muscle-specific ablation of ILK or Talin1 similarly causes progressive muscular dystrophy with detachment of myotendinous junctions[18, 19].

PINCH is a key component of focal adhesion in complex with ILK and Parvin, and plays a critical role in mediating Integrin signaling[20, 21]. Genetic studies have revealed essential roles of PINCH during embryonic development and in morphogenesis of multiple tissues and organs, similar with that of ILK and β1-Integrin[22–25]. In C. elegans, loss of UNC-97 (PINCH) results in impaired muscular adhesion junctions and body wall muscle deformity[26, 27]. Similarly, mutation of PINCH in muscle cells of drosophila leads to muscle detachment[28]. Myocardial-specific ablation of PINCH1 in mice causes dilated cardiomyopathy with disrupted sarcomere structures, aberrant expression of cytoskeleton-associated proteins and reduced Akt phosphorylation and activation[24]. So far, role of PINCH in skeletal myogenesis in mammals remains unclear.

In this study, we ablated PINCH1 and PINCH2 specifically in skeletal muscle progenitor cells using MyoD-Cre mice. PINCH mutant mice exhibited congenital skeletal myopathy of variable severity. Ablation of

PINCH1 resulted in postnatal growth retardation, and hypoplastic and centrally nucleated myofibers. Double ablation of PINCH1 and PINCH2 caused early postnatal lethality and defects in myoblast fusion and subsequent myofibril assembly. Western blot and immunostaining revealed reduced phosphorylation and activation of AKT and ERK1/2 and a significant reduction in the expression of proteins associated with muscle cytoskeleton and contraction. We found that MRF4, the most highly expressed myogenic factor at late stages of myogenesis, was abolished in PINCH mutant muscles, that could contribute to impaired terminal differentiation of muscle fibers.

Materials And Methods

Animals

To generate skeletal muscle-specific PINCH1 knock-out mice (MyoD-Cre; PINCH1$^{f/f}$; PINCH2$^{+/+}$) with C57/B6 background, we crossed cross MyoD-Cre; PINCH1$^{f/+}$; PINCH2$^{+/+}$ males with PINCH1$^{f/f}$; PINCH2$^{+/+}$ females on Rosa26-LacZ background in order to trace Cre lineage. Muscle-specific PINCH1 and PINCH2 double knock-out mice (MyoD-Cre; PINCH$^{f/f}$; PINCH2$^{-/-}$) were produced by crossing Myo-Cre; PINCH1$^{f/+}$; PINCH2$^{-/-}$ males with PINCH1$^{f/f}$; PINCH2$^{-/-}$ females. Mice were housed in Laboratory Animal Facility at Tongji University. All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals (Ministry of Health, China, 1998) and monitored by the Institutional Animal Care and Use Committee of Tongji University School of Medicine.

Histologic analysis

Mice were sacrificed and muscle tissues were dissected and fixed overnight in 4% paraformaldehyde at 4°C, and paraffin embedded. Sections were cut at 10 μm from paraffin blocks and stained with hematoxylin and eosin.

Transmission Electron Microscopy (TEM)

The quadriceps muscles were processed for electron microscopy analysis as described[24]. Briefly, the quadriceps muscles of P1 DMUT and control mice were fixed in 2.5% glutaraldehyde in PBS overnight at 4°C, and then immersed in 1% osmium tetroxide for 1h at room temperature. After fixation, the samples were dehydrated in graduated alcohols, and embedded with epoxy resin. Ultrathin sections longitudinally to the myofibrils were performed and stained with toluidine blue. Then, the sections were contrasted with uranyl acetate and lead citrate and subsequently examined in electron microscope.

Western blotting

For protein extraction, mice in P1 were sacrificed and dissected. Protein was extracted and prepared using the Protein Extraction kit (Keygen KGP250). The following antibodies were used: β1 integrins (1:500, Sigma-Aldrich), ILK (1:1000, Sigma-Aldrich), Talin (1:100, Abcam), Cadherin (1:1000, Sigma-Aldrich), Vinculin (1:2000, Sigma-Aldrich), Akt (1:200, Cell Signaling,), Phospho-Akt (1:500, Cell Signaling), Erk1/2

(1:250, Cell Signaling), P-Erk1/2 (1:800, Cell Signaling), MRF4 (1:200, Invitrogen) , Myosin (1: 200; Abcam), Titin (1:250, Novus Biologicals), Desmin (1: 80; Abcam). Western Blot was performed as described. Relative expression levels of proteins were quantified by ImageJ.

Cell culture and immunostaining

Myoblast fusion and immunostaining experiments were carried out with primary myoblasts collected from hindlimb muscle as described[29]. The antibodies were used as list above.

Statistical Analysis

Data are presented as mean ±SEM. Student t-test was used for 2-group comparisons. Differences were considered statistically significant at P<0.05.

Results

Impaired myogenic differentiation and maturation in mice with PINCH1 being ablated in skeletal muscle progenitors

To investigate the specific role of PINCH in skeletal muscle development, we generated a mouse line in which PINCH1 was deleted in skeletal muscle progenitor cells using MyoD-Cre[30]. PINCH1 mutant mice (MyoD-Cre; PINCH1$^{f/f}$, SMUT) were born at expected mendelian ratio and appeared normal at birth. But with postnatal development, SMUT mice exhibited mild growth retardation with shorter body length and lower body weight compared to control littermates (Fig. 1A, B). PINCH1 mutants appeared less active, otherwise fertile and lived a normal lifespan.

The skeletal muscles are composed of bundles of multinucleated myofibers, each of which is filled with myofibrils with its nuclei placed at the periphery underneath sarcolemma[31, 32]. To investigate if there were any morphological change in SMUT skeletal muscles, we dissected the quadriceps muscles from 2-month adult SMUT mice and control littermates and performed H&E (Hematoxylin-Eosin staining) histological analyses. We found that ablation of PINCH1 in skeletal muscle progenitors resulted in a significant myopathic change. In longitudinal (Fig. 1C, D) and cross (Fig. 1F, G) sections, myofibers of control samples were compact and the nuclei were spindle-like and evenly located immediately below the plasma membrane (Fig. 1C, D). However, SMUT myofibers were loosely packed and significantly hypoplastic and variable in size, and there was a significant decrease in average cross area of SMUT myofibers compared to controls (Fig. 1C-G). Nuclei of SMUT myofibers exhibited a more rounded morphology and centrally localized, a feature of myopathies (Fig. 1D, arrow). The number of nuclei and percentage of centrally localized nuclei in SMUT myofibers were significantly increased compared to the control (Fig. 1H, I). These results suggested that deletion of PINCH1 in skeletal muscle progenitors leads to impaired terminal differentiation and maturation of multinucleated myofibers.

Ablation of PINCH1 and PINCH2 resulted in reduced size of muscle fibers and impaired multinucleation

Two PINCH isoforms (PINCH1 and 2) are expressed in mammals with partial functional redundancy[23, 24]. Relative mild phenotype in PINCH1 mutants could be attributed in part to the compensatory role by PINCH2. To examine this possibility, we generated a muscle-specific PINCH1 and PINCH2 double knockout mouse line (MyoD-Cre;PINCH1$^{f/f}$; PINCH2$^{-/-}$, DMUT). A majority of DMUT mice would die within 48 hours after birth, and all remaining mice die within 7-10 days (Fig. 2A). Neonatal DMUT pups appeared weak, slim with an empty stomach, suggesting defects in sucking and motor function. We found a marked reduction in the size of muscles in the back and thighs (Fig. 2A, B, boxed) in DMUT pups compared to control littermates. The diaphragm muscles of DMUT pups appeared shorter, and in most cases were readily detached from body wall (Fig. 2C-E, arrow).

During myogenesis, mononucleated myoblasts fuse to form multinucleated muscle cells that further differentiate to become mature myofibers. We examined the H&E-stained longitudinal sections of thigh and diaphragm muscles from postnatal day 1 (P1) DMUT mice and littermate controls. The control muscles were composed of elongated, cylindrical, well-aligned and compact myofibers that were filled with striated myofibrils, whereas the mutant myofibers were markedly reduced in size and disconnected from each other (Fig. 2F-J). Many mutant muscle cells appeared spindle-shaped without a clear striated pattern (Fig. 2G). Although both control and DMUT myofibers were multinucleated, quantitative analysis revealed a significant reduction in the number of nuclei in PINCH mutant myofibers, suggesting impaired myoblast fusion (Fig. 2K). To examine whether decreased nuclear number in PINCH mutant myofibers could result from a change in nuclear division, we performed immunostaining with a mitosis-specific antibody to phosphohistone H3 (Ph3), and observed instead a significant increase in mitotic nuclei in P1 DMUT muscle fibers compared to that in controls (Fig. 2L). In addition, significantly increased apoptosis was also observed in DMUT muscle cells (Fig. 2M).

MyoD is expressed early during myogenesis in myogenic progenitors and undifferentiated myoblasts and required for muscle lineage commitment. To determine whether PINCHs play a role in early myogenesis, we traced skeletal muscle lineage (MyoD-Cre+) by β-Galactosidase (β-gal) staining of E13.5 DMUT and control mice (on Rosa-lacZ background). We found that the overall shape and size of somites and muscle primordial blocks of the head, body and limbs were comparable between PINCH mutant and control mice (Fig. 2N-Q), suggesting that PINCH is dispensable for early stage myogenesis, but it is required for subsequent differentiation and maturation of muscle cells.

Defects in myoblast fusion and cytoskeleton assembly in PINCH double mutant mice

To further investigate the role of PINCH in sarcomere genesis, we examined the ultrastructure of muscle fibers by transmission electron microscopy (TEM). Control myofibers of the limb (Fig. 3A) and diaphragm (Fig. 3C) exhibited a typical striated pattern and well-organized sarcomere structures, including Z- and M-lines and I and A bands, and well-packed actin and myosin myofilaments (Fig. 3A-D). However, PINCH mutant limb myofibers largely lost their striated appearance with disorganized and truncated myofibrils. Sarcomere structures were severely disrupted, and only residual Z-lines and loosely packed myofilaments were visible (Fig. 3B, arrow). Sarcomere structure of PINCH mutant diaphragm myofibers appeared to be

preserved, however, the myofibrils were markedly thinner and mal-aligned (Fig. 3D). Consistent with this, α-actinin immunostaining of hindlimb muscles showed that, compared to the controls, the myofibrils of the mutant myofibers were markedly thinner and lost their striated pattern (Fig. 3E, F), suggesting PINCH is essential for assembly of myofibril in vivo.

We further analyzed myoblast fusion and cytoskeleton assembly in vitro. Myoblasts were isolated from hindlimb of control and DMUT mice at E18.5 and cultured in 10% Matrigel-coated dishes. After 3 days in culture, cells were fixed and co-immunostained with antibody to cadherins and a-actinin. We found that the majority of control myoblasts were fused, elongated and become multinucleated myotubes with clear striated appearance (Fig. 3J). However, a majority of DMUT myoblasts remained mono- or binucleated, and were markedly smaller and spindle-like with no clear striations (Fig. 3H), even with extended culture time, which, however, resulted in excessive cell death in mutant cultures. Quantification revealed a significant reduction in the number of nuclei in mutant myofibers (Fig. 3I). However, we frequently observed cells that were aggregated (Fig. 3H yellow arrow) or aligned and fused to preexisting primary myofibers (Fig. 3H red arrow), suggesting those might be the cells in the process of cell fusion. In addition, expression of cadherins, known to regulate cell-cell adhesion and myoblast fusion, were correctly localized at the sites of cell contact and fusion, despite its level of expression was reduced (Fig. 3J, K, arrow, boxed inlet 1 and 2), suggesting PINCH may play a direct role in regulating myoblast fusion.

Defects in expression of cytoskeleton proteins and proteins involved in myogenesis in DMUT skeletal muscles

Integrin signaling pathway regulates cytoskeleton organization and is required for skeletal myogenesis[33]. Therefore, we analyzed the expression of proteins of integrin pathway and cytoskeleton, and of those involved in late stage myogenesis. DMUT and control myoblasts from E18.5 embryos were cultured for 48-72 hours to avoid excessive cell death. Cells were fixed and immunostained with antibodies to desmin, myosin, talin and vinculin to reveal the structures of cytoskeleton. DMUT muscle cells were markedly smaller and remained a myoblast-shape compared to controls, suggesting defects in cell spreading and cytoskeleton remodeling. In control muscle cells, expression of desmin, myosin and talin were cytoskeleton associated, and vinculin was concentrated at focal adhesion sites. In DMUT muscle cells, however, expression of myosin and talin were downregulated and aggregated. Vinculin was expressed in a pattern similar with that in control cells, although its expression was notably downregulated. These results suggested impaired assembly and organization of cytoskeleton, but relatively normal adhesion of PINCH mutant cells. Consistent with this observation, western blot analysis of P1 mouse skeletal muscles revealed a significant decrease in the expression of a number of cytoskeleton-associated proteins, including β1-integrin, talin, cadherin, vinculin, myosin and Titin. Interestingly, expression of ILK, known to interact with PINCH in focal adhesion complex, was slightly but significantly upregulated in DMUT, which might compensate partially for the loss of PINCH in cell adhesion. In DMUT skeletal muscles, phosphorylation of AKT and ERK1/2, the downstream mediators of integrin-focal adhesion, were significantly reduced, and total AKT is also slightly decreased. MRF4 is the predominant myogenic factor at late stages of myogenesis and is required for myoblast fusion and

cytoskeleton gene expression. Mutation of MYF4 is associated with centronuclear myopathy in humans. MRF4 expression was abolished in DMUT muscles that might contribute in part to observed defects in myoblast fusion and cytoskeleton organization.

Discussion

Skeletal muscle development is a multistep process involving complex cell-cell and cell-ECM interactions[27, 28, 34-37]. Progenitors that committed to skeletal myogenic fate delaminate and migrate to form the primordial muscle groups of the body. A large number of ECM molecules and their receptors (integrins and dystrophin) are expressed and play important roles in developing skeletal muscles. Despite their critical roles in cell adhesion and migration, initial muscle formation appears not to be disturbed in their absence. We found that initial formation of muscle primordial masses in PINCH mutant mice were not disturbed. β1 integrin is the predominant beta subunit of integrin receptors, in its absence, however, migration and proliferation of myoblasts and initial muscle formation appear to be normal. Deletion of ILK leads to progressive myopathy at adult stages. Similarly, β1 integrin, ILK and PINCH are not required for the initial formation of myocardium. Instead, they are essential for the perinatal myocardial remodeling and maturation.

One of the unique features of skeletal myogenic differentiation is the fusion of mononucleated myoblasts to form multinucleated myotubes that further differentiate to become mature myofibers. Myoblast fusion is composed of several coordinated cellular processes including myoblast migration, adhesion, elongation, cell-cell recognition, alignment and membrane fusion, and it is tightly controlled by a large number of genes and signaling pathways, including those involved in regulation of cytoskeleton dynamics, cell-ECM and cell-cell interactions. We found that, in PINCH mutant muscles, many of muscle cells remained a myoblast shape, and the size of myofibers and the number of myofiber nuclei were significantly decreased. Consistent with these in vivo observations, PINCH mutant myoblasts in culture were defective in spreading and elongation and appeared smaller. However, the mutant myoblasts were able to aggregate and align to primary myotubes, but impeded at cell fusion. Expression of a number of cell adhesion molecules, such as N-cadherin and vinculin, was properly confined to the adhesion sites of PINCH mutant myoblasts, although the level of their expression was significantly reduced. These results have suggested a critical role of PINCHs in various aspects of myoblast fusion. Myoblast fusion defect in PINCH mutants closely resembles that observed in β1 integrin mutants. Similarly, β1 integrin deficient myoblasts are able to aggregate and express N-cadherin at cell adhesion sites correctly, but stalled at cell membrane fusion. These results suggest that PINCH and β1 integrin regulate myoblast fusion by overlapping genetic pathways. However, one notable difference in myoblast fusion between PINCH and β1 integrin-regulated pathways is that, in β1 integrin mutants, expression of the tetraspanin CD9, which is known to complex with β1 integrin and play a role in myoblast fusion, is abolished. But the expression of myogenic lineage markers is not changed, suggesting fusion defect in β1 integrin mutant myoblasts is not caused by defects in cell differentiation[38]. However, in PINCH mutants, expression of MRF4 is diminished, suggesting impaired myogenic differentiation may contribute to defective cell fusion in PINCH mutants. These results are consistent with the notion that multiple pathways are involved

myoblast fusion. In the future, it would be interesting to investigate the potential crosstalk between CD9- and PINCH-mediated pathways in regulating myoblast fusion.

Our study has revealed a critical requirement for PINCH in regulating the assembly of the muscle fiber cytoskeleton. PINCH mutant myofibrils were markedly thinner and disorganized, and sarcomere structures were severely disrupted. Expression of a number of cytoskeleton-associated proteins (β1-integrin, talin, cadherin, vinculin, myosin and Titin) were significant reduced. This defect is reminiscent of that observed in β1 integrin mutant muscles. Similarly, previous studies have shown that myocardial-specific ablation of PINCH1, β1-integrin and ILK in mice causes dilated cardiomyopathy with disrupted sarcomere structures and aberrant expression of cytoskeleton-associated proteins.

Muscle-specific ablation of PINCH1 caused only growth retardation and mild myopathy changes, likely due to the compensatory role by PINCH2 in skeletal muscles. PINCH1 mutants exhibited hypoplastic myofibers, split myofibers and increased central nuclei. Previous studies have shown that in many cases PINCH acts in the focal adhesion complex in the same genetic pathway as β1 Integrin and ILK[22-25]. In Drosophila and Caenorhabditis elegans, ablation of PINCH, ILK and β1 integrin results in similar skeletal muscle phenotypes[27, 28, 34-37]. In contrast to that of β1 Integrin and PINCHs, ablation of ILK in mouse skeletal muscle only resulted in progressive myopathy at adult stages, a phenotype similar with that observed in PINCH1 mutants, suggesting an additional mechanism may have evolved in skeletal myogenesis in mice that acts redundantly with ILK in binding integrins, which may warrant future study. In this context, it is relevant to note that talin and α-actinin, for instance, can bind to β1 integrins, thus link integrins to actin cytoskeleton in cell adhesions[39].

Skeletal muscle development is critically regulated by a family of myogenic regulatory factors, among which, MRF4 is the most highly expressed MRF in muscles after birth[40, 41]. MRF4 plays an important role in myoblast fusion and maturation and muscle mass maintenance[42]. Genetic studies have revealed that ablation of MRF4c leads the myopathy with disorganized and significantly reduced myofibrils[42, 43]. In human, mutation of MRF4 has been associated with myopathy and Becker muscular dystrophy[44]. Our study has suggested that PINCH is required for expression of MRF4, which in turn promotes late-stage skeletal muscle differentiation and maturation.

Conclusion

In conclusion, our results demonstrated an essential role of PINCH in skeletal myogenic differentiation. Ablation of PINCH1 and PINCH2 in skeletal muscle progenitors resulted in impaired myoblast fusion and cytoskeleton organization. Expression of proteins involved in integrin pathway and cytoskeleton were significantly decreased in PINCH mutant skeletal muscles. Reduced expression of MRF4 might contribute to observed phenotypes in PINCH mutants.

Abbreviations

ECM - extracellular matrix

PH3 - phosphohistone H3

TEM - Transmission Electron Microscopy

PINCH - Particularly Interesting New Cysteine- Histidine Rich Protein

MRF4 - Myogenic regulatory factor 4

ILK - integrin-linked kinase

P1 - postnatal day 1

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

Declarations

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Author information

Siyi Xie, Chushan Fang and Yujie Gao contributed equally to this work.

Authors' contributions

SY.X., CS. F. and YJ.G. carried out experiments and analyzed the data. LN.L. and J.Y. carried out experiments, YF.S. and XQ.L. conceived and planned experiments, supervised the project, analyzed the data, wrote the manuscript and provided financial support. All authors have read and agreed to the published version of the manuscript.

Ethics declarations

Ethics approval and consent to participate

All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals (Ministry of Health, China, 1998) and monitored by the Institutional Animal Care and Use Committee of Tongji University School of Medicine(TJAA10320101).

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

References

1. Mercuri E, Muntoni F. Muscular dystrophies. The Lancet. 2013;381(9869):845–60.

2. Mercuri E, Muntoni F. The ever-expanding spectrum of congenital muscular dystrophies. Ann Neurol. 2012;72(1):9–17.

3. Jungbluth H, et al. Congenital myopathies: disorders of excitation-contraction coupling and muscle contraction. Nat Rev Neurol. 2018;14(3):151–67.

4. Vasli N, et al. Recessive mutations in the kinase ZAK cause a congenital myopathy with fibre type disproportion. Brain. 2017;140(1):37–48.

5. Bryson-Richardson RJ, Currie PD. The genetics of vertebrate myogenesis. Nat Rev Genet. 2008;9(8):632–46.

6. Bober E, et al. The muscle regulatory gene, Myf-6, has a biphasic pattern of expression during early mouse development. J Cell Biol. 1991;113(6):1255–65.

7. Braun TG, et al. MyoD expression marks the onset of skeletal myogenesis in Myf-5 mutant mice. Development. 1994;120(11):3083–92.

8. Kassar-Duchossoy L, et al. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature. 2004;431(7007):466.

9. Daston G, et al. Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. Development. 1996;122(3):1017–27.

10. Seale P, et al. Pax7 is required for the specification of myogenic satellite cells. Cell. 2000;102(6):777–86.

11. Du. and J. S., Positive and Negative Regulation of Muscle Cell Identity by Members of the hedgehog and TGF-beta Gene Families. J Cell Biol. 1997;139(1):145–56.

12. Tajbakhsh S, et al. Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. Development. 1998;125(21):4155–62.

13. Sparrow JC, Sch?Ck F. The initial steps of myofibril assembly: integrins pave the way. Nat Rev Mol Cell Biol. 2009;10(4):293–8.

14. Schwander M, et al. Beta1 integrins regulate myoblast fusion and sarcomere assembly. Dev Cell. 2003;4(5):673–85.

15. Hirabayashi S, et al. Mutations in the integrin alpha7 gene cause congenital myopathy. Nat Genet. 1998;19(1):94–7.

16. Bajanca. and F., Integrin α6β1-laminin interactions regulate early myotome formation in the mouse embryo. Development. 2006;133(9):1635–44.

17. Rooney JE, et al. Severe muscular dystrophy in mice that lack dystrophin and alpha7 integrin. J Cell Sci. 2006;119(Pt 11):2185–95.

18. Wang HV, et al., *Integrin-Linked Kinase Stabilizes Myotendinous Junctions and Protects Muscle from Stress-Induced Damage*. Journal of Cell Biology, 2008. 180(5): p. 1037–1049.

19. Conti FJ, et al. Progressive myopathy and defects in the maintenance of myotendinous junctions in mice that lack talin 1 in skeletal muscle. Development. 2008;135(11):2043–53.

20. Attila, et al. PINCH2 is a new five LIM domain protein, homologous to PINCHand localized to focal adhesions⬚. Experimental Cell Research; 2003.

21. Xu H, Cao H, Xiao G. *Signaling via PINCH: functions, binding partners and implications in human diseases*. Other, 2016. 594(1).

22. Legate KR, et al. ILK, PINCH and parvin: the tIPP of integrin signalling. Nat Rev Mol Cell Biol. 2006;7(1):20–31.

23. Liang X, Sun Y, Chen J. Particularly interesting cysteine- and histidine-rich protein in cardiac development and remodeling. J Investig Med. 2009;57(8):842–8.

24. Liang X, et al. Targeted ablation of PINCH1 and PINCH2 from murine myocardium results in dilated cardiomyopathy and early postnatal lethality. Circulation. 2009;120(7):568–76.

25. Liang X, et al. PINCH1 plays an essential role in early murine embryonic development but is dispensable in ventricular cardiomyocytes. Mol Cell Biol. 2005;25(8):3056–62.

26. Oliver H, et al., *A Conserved LIM Protein That Affects Muscular Adherens Junction Integrity and Mechanosensory Function in Caenorhabditis elegans*. Journal of Cell Biology, 1999.

27. Norman KR, et al. UNC-97/PINCH is involved in the assembly of integrin cell adhesion complexes in Caenorhabditis elegans body wall muscle. Dev Biol. 2007;309(1):45–55.

28. Clark KA, Mcgrail M, Beckerle MC. *Analysis of PINCH function in Drosophila demonstrates its requirement in integrin-dependent cellular processes*. Development, 2003.

29. Olivier, et al. Normal innervation and differentiation of X-linked myotubular myopathy muscle cells in a nerve-muscle coculture system. Neuromuscular Disorders; 2001.

30. Chen JC, et al. MyoD-cre transgenic mice: a model for conditional mutagenesis and lineage tracing of skeletal muscle. Genesis. 2005;41(3):116–21.

31. Chal J, Pourquie O. Making muscle: skeletal myogenesis in vivo and in vitro. Development. 2017;144(12):2104–22.

32. Roman W, Gomes ER. Nuclear positioning in skeletal muscle. Semin Cell Dev Biol. 2018;82:51–6.

33. Gheyara AL, et al. Deletion of integrin-linked kinase from skeletal muscles of mice resembles muscular dystrophy due to alpha 7 beta 1-integrin deficiency. Am J Pathol. 2007;171(6):1966–77.

34. Zervas CG, Gregory SL, Brown NH. Drosophila integrin-linked kinase is required at sites of integrin adhesion to link the cytoskeleton to the plasma membrane. J Cell Biol. 2001;152(5):1007–18.

35. Mackinnon AC, et al. C. elegans PAT-4/ILK functions as an adaptor protein within integrin adhesion complexes. Curr Biol. 2002;12(10):787–97.

36. Beumer KJ, et al. A role for PS integrins in morphological growth and synaptic function at the postembryonic neuromuscular junction of Drosophila. Development. 1999;126(24):5833.

37. Lee M, et al. Roles for βpat-3 Integrins in Development and Function ofCaenorhabditis elegans Muscles and Gonads. J Biol Chem. 2001;276(39):36404.

38. Madaro L, et al. PKCθ signaling is required for myoblast fusion by regulating the expression of caveolin-3 and β1D integrin upstream focal adhesion kinase. Molecular Biology of the Cell; 2011.

39. Ellis SJ, et al., Talin autoinhibition is required for morphogenesis. Current biology: CB, 2013. 23(18).

40. Zammit PS. Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. Semin Cell Dev Biol. 2017;72:19–32.

41. Weintraub H, et al. The myoD gene family: nodal point during specification of the muscle cell lineage. Science. 1991;251(4995):761–6.

42. Patapoutian A, et al. Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. Development. 1995;121(10):3347.

43. Arnold HH, Winter B. Muscle differentiation: more complexity to the network of myogenic regulators. Curr Opin Genet Dev. 1998;8(5):539–44.

44. Kerst B, et al. Heterozygous myogenic factor 6 mutation associated with myopathy and severe course of Becker muscular dystrophy. Neuromuscul Disord. 2001;10(8):572–7.
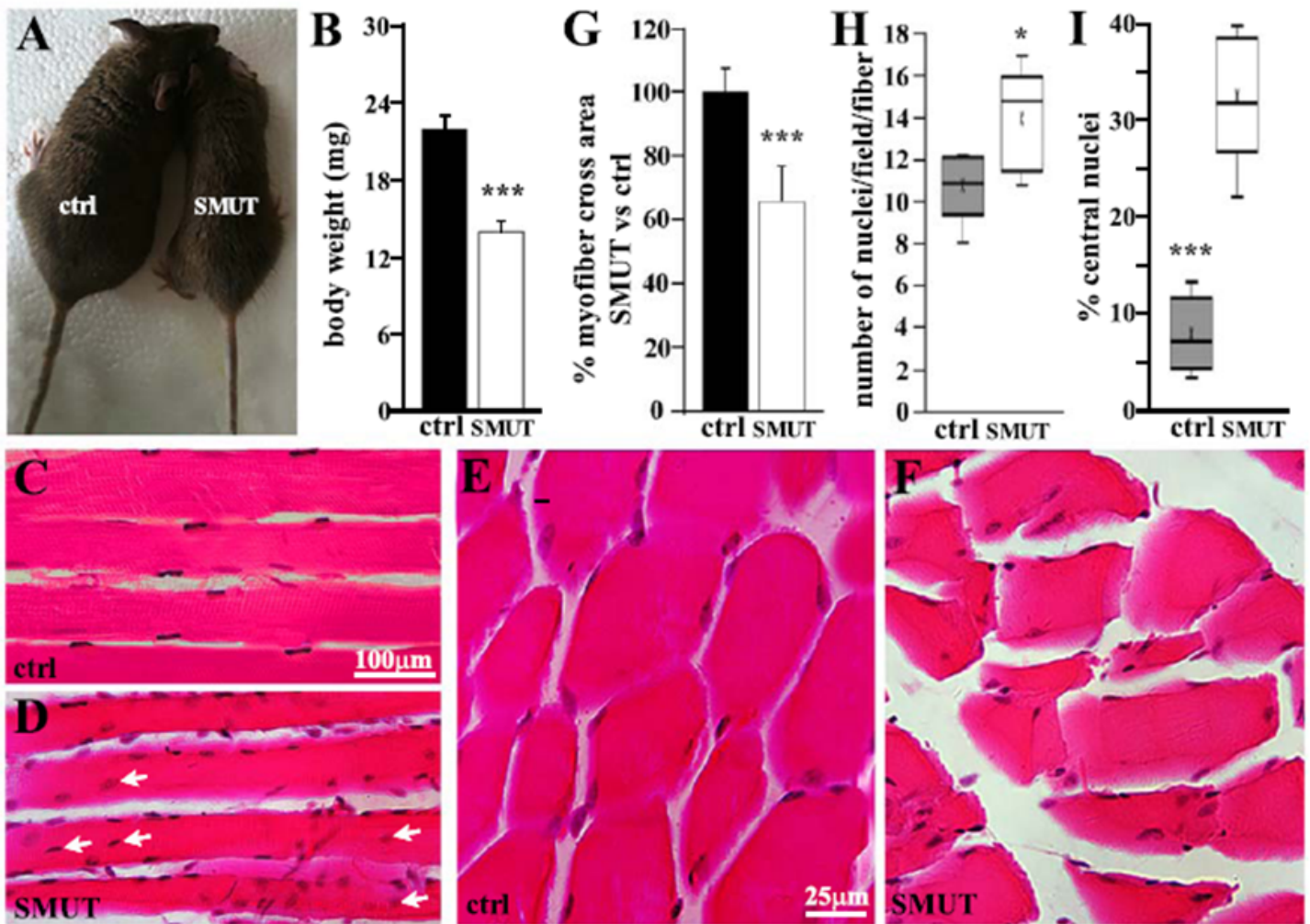
Figures

Figure 1

Single ablation of PINCH1 in skeletal muscle progenitors caused myopathic changes. (A, B) At postnatal 12 weeks, MyoD-Cre; PINCH1f/-(SMUT) mice exhibited significant growth retardation with reduced height and body weight compared to control littermates (ctrl). (n = 11, p < 0.01, 2-tailed t-test). (C-G) H&E staining of sagittal (C, D) and transversal sections (E, F) of quadriceps femoris of SMUT and controls. (G) Quantification of average area of myofibrils in SMUT as percentage of ctrl; (H) Quantification of number of nuclei per myofiber per section area; (I) Quantification of number of central nuclei expressed as percentage of total number of nuclei per fiber per section area. (n = 16, Error bars represent ±SD, *p<0.05, ***p<0.001, 2-tailed t-test).
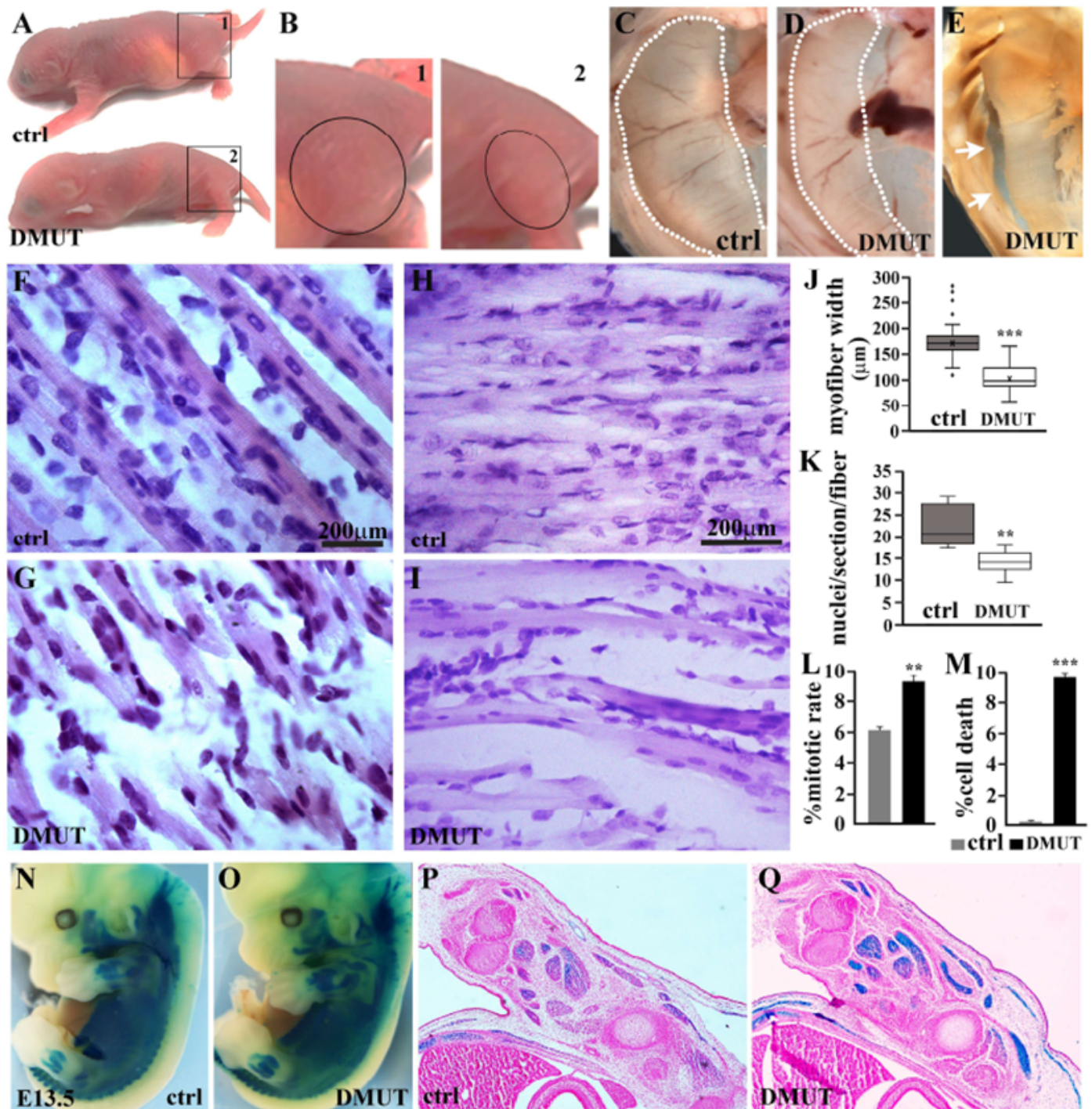
Fig. 2

Figure 2

Ablation of PINCH1 and PINCH2 results in reduced size of muscle fibers and impaired multinucleation (A, B) A majority of PINCH1 and PINCH2 mutant mice (DMUT) died within 48 hrs, while all remaining mice would survive no more than 7-10 days. DMUT appeared to be slim with markedly reduced muscle masses compared to littermate control (ctrl). (C-E) Wholemount view of DMUT and control diaphragm muscles, showing marked hypotrophy (D) and detachment (E, arrow) of DMUT diaphragm muscles. (F, G) H&E-

stained longitudinal sections of thigh (F, G) and diaphragm (H, I) muscles from postnatal day 1 (P1) DMUT mice and littermate controls. (J-M) Quantification of myofiber width (J), number of nuclei (K), mitotic nuclei (L) and cell death (M). (n = 12, Error bars represent ±SD, **p<0.01, ***p<0.001, 2-tailed t-test). (N-Q) Wholemount and section β-gal staining of E13.5 DMUT and control mice staining showing overall shape and size of somites and muscle primordial groups were comparable between DMUT and control mice.
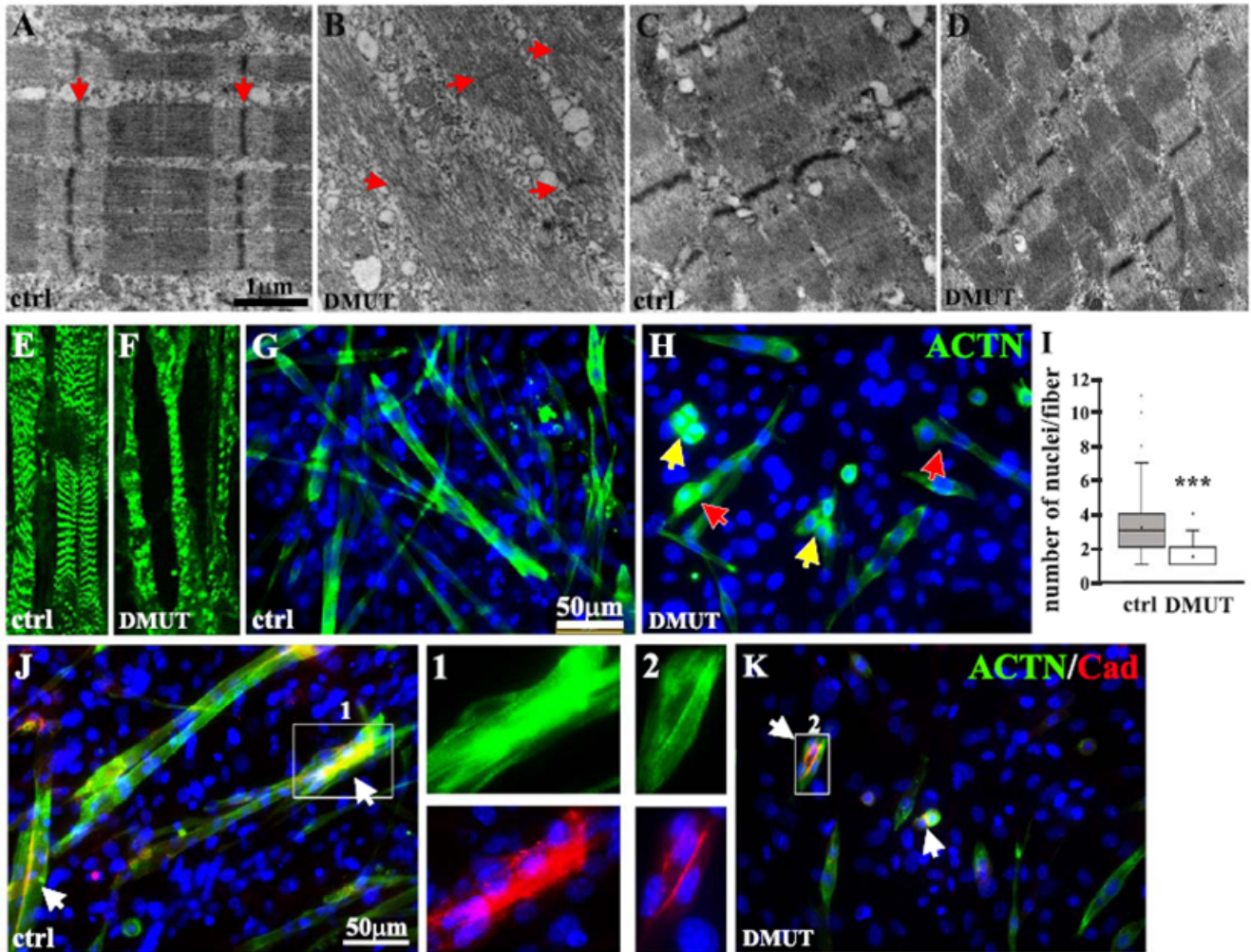

Fig. 3

Figure 3

Defects in myoblast fusion and cytoskeleton assembly in PINCH double mutant mice (A-D) Transmission electron microscopy (TEM) analysis of the limb (A, B) and diaphragm (C, D) of DMUT and control muscles. Red arrow in A and B showing Z-line. (E, F) α-actinin immunostaining of hindlimb muscles showing defective assembly of myofibrils in DMUT mice at P1. (G-I) α-actinin immunostaining of myoblasts from hindlimb muscles of DMUT and control mice at E18.5, showing that a majority of DMUT myoblasts remained mono- or binucleated, and were markedly smaller and spindle-like with no clear striations (yellow arrow indicating aggregated cells; red arrow indicating myoblasts aligned to newly

formed myofibers). Quantification of the number of nuclei in DMUT and control myofibers (I). (n = 8, Error bars represent ±SD, ***p<0.001, 2-tailed t-test). (J-K) α-actinin and N-cad co-immunostaining showing expression of N-cadherin at the sites of cell fusion (arrow, boxed inlet 1 and 2).
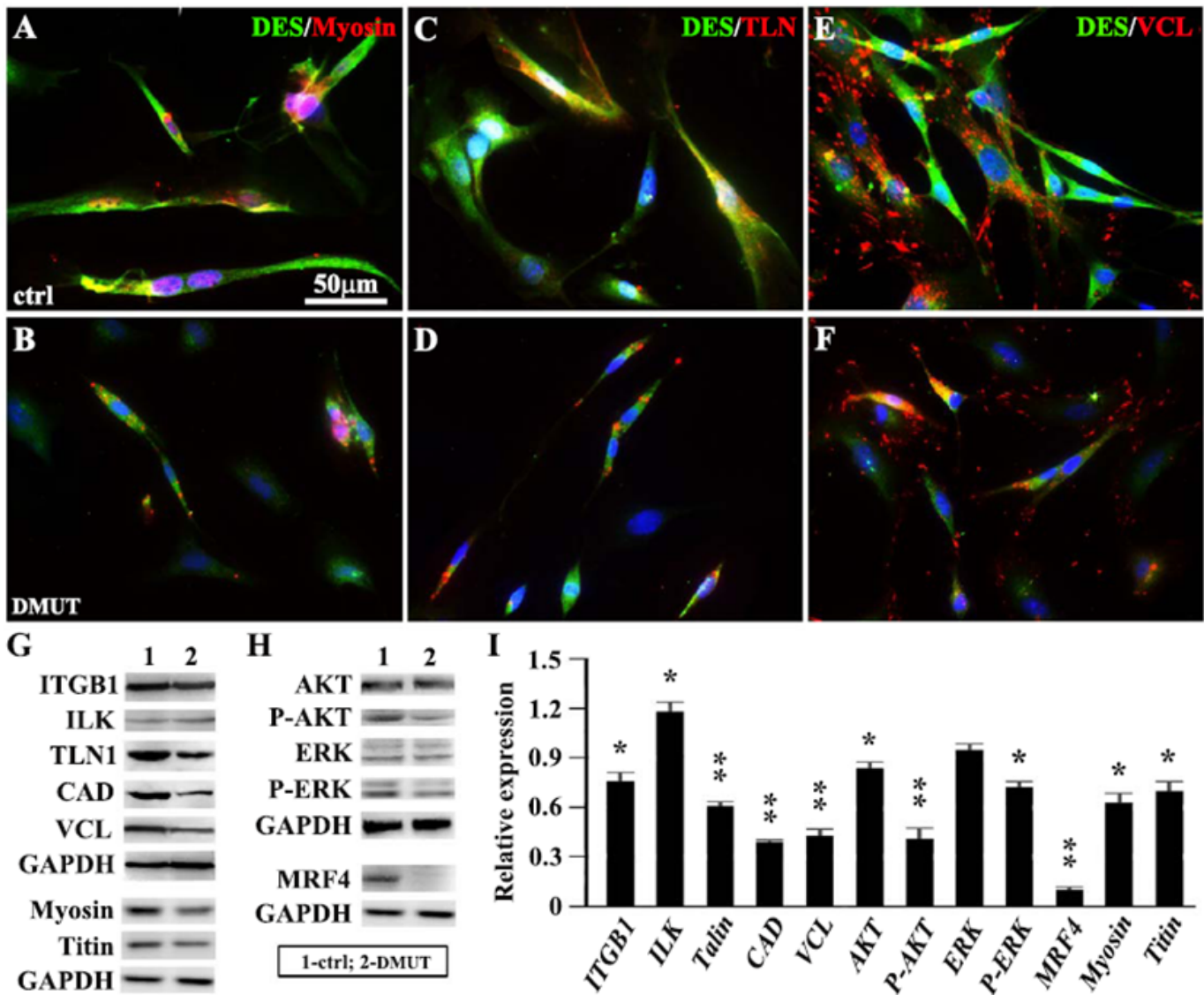

Fig. 4

Figure 4

Defects in expression of cytoskeleton proteins and proteins involved in myogenesis in DMUT skeletal muscles (A-F) DMUT and control myoblasts from E18.5 embryos were cultured for 48-72 hours and co-immunostained to show expression of desmin (DES), Myosin, Talin (TLN) and Vinculin (VCL) during myoblast fusion and cytoskeleton assembly. (G, H) Western blot of protein lysates from DMUT and control quadriceps at P1. GAPDH was used as control. (I) The quantitative assessment of western blot bands (n=3, *P<0.05, **P<0.01, 2-tailed t-test, Error bars represent ±SD).