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Actin-organizing protein palladin modulates C2C12 cell fate determination

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ABSTRACT

Background: Cell confluency and serum deprivation promote the transition of C2C12 myoblasts into myocytes and subsequence fusion into myotubes. However, despite all myoblasts undergoing the same serum deprivation trigger, their responses vary: whether they become founder myocytes, remain proliferative, or evolve into fusion-competent myocytes remains unclear. We have previously shown that depletion of the scaffolding protein palladin in myoblasts inhibits cell migration and promotes premature muscle differentiation, pointing to its potential significance in muscle development and the necessity for a more in-depth examination of its function in cellular heterogeneity.

Methods and results: Here, we showed that the subcellular localization of palladin might contribute to founder-fate cell decision in the early differentiation process. Depleting palladin in C2C12 myoblasts depleted integrin-β3 plasma membrane localization of and focal adhesion formation at the early stage of myogenesis, decreased kindlin-2 and metavinculin expression during the myotube maturation process, leading to the inability of myocytes to fuse into preexisting mature myotubes. This aligns with previous findings where early differentiation into nascent myotubes occurred but compromised maturation. In contrast, wildtype C2C12 overexpressing the 140-kDa palladin isoform developed a polarized morphology with star-like structures toward other myoblasts. However, this behaviour was not observed in palladin-depleted cells, where the 140-kDa palladin overexpression could not recover cell migration capacity, suggesting other palladin isoforms are also needed to establish cell polarity.

Conclusion: Our study identifies a counter-intuitive role for palladin in regulating myoblast-to-myocyte cell fate decisions and impacting their ability to form mature multinucleated myotubes by influencing cell signalling pathways and cytoskeletal organization, necessary for skeletal muscle regeneration and repair studies.

1. Introduction

Skeletal muscle is the largest tissue mass in the body and is indispensable for locomotion and metabolism regulation [1,2]. Diminished muscle mass is a critical factor in morbidity and mortality, highlighting the importance of myogenesis research in health maintenance and muscle quality [3]. Regeneration of an adult lost limb involves restoring multiple tissue types, with muscle regeneration critical. In adults, satellite cells, which are skeletal muscle stem cells on the myofiber surface, support muscle repair by proliferating and fusing to existing fibers after injury. Since muscle fiber number is set at birth, satellite cell fusion contributes to muscle regeneration through fiber augmentation [4] rather than de novo muscle fibers. Thus, the differentiation process plays a more pivotal role in forming new muscle fibers needed for regenerating large muscle masses [5,6]. Indeed, satellite cell proliferation alone cannot produce functional muscle fibers without activating differentiation factors that differentiate myoblasts into myocytes. For instance, mice lacking Pax7, which depletes satellite cells, still show muscle regeneration through other myogenic progenitor cells [5]. Conversely, MyoD- and myogenin-null mice, despite having functional satellite cells,

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exhibit severe muscle defects and compromised muscle development [6,7]. Moreover, newly differentiated myocytes contribute to improved innervation, integration into the existing muscle framework, and vascularization of regenerating muscle tissue, leading to better functional recovery. Thus, understanding and promoting myoblast differentiation is fundamental for advancing regenerative medicine for limb loss.

During embryogenesis, skeletal muscles originate from paraxial mesoderm, forming somites that differentiate into dermomyotome, generating dermis and skeletal muscle cells, and sclerotome, forming skeletal elements. Muscle precursor cells in the dermomyotome initiate two waves of myoblast formation, which differentiate and fuse into primary and secondary myotubes and myofibers [8]. The basic–helix-loop-helix MRFs Myf-5, MyoD, myogenin, and MRF4 are transcriptional activators of skeletal muscle genes, expressed in unique but overlapping patterns in muscle precursors and their descendants [9]. Myf-5 is the earliest MRF expressed in somites, appearing about two days before MyoD [10]. Both Myf-5 and MyoD are essential for developing epaxial (e.g., back muscles) and hypaxial (e.g., limb and body wall muscles) musculature, co-expressing in most myogenic cells [11]. While Myf-5 [12] or MyoD [13] null mice develop normal myofibers, double mutants lack muscle fibers and myoblasts [7]. Proliferative MyoD and/or Myf5-positive myoblasts then induce p21(Waf1/Cip1) expression, promoting cell cycle exit and differentiation into myocytes that express “late” MRFs, myogenin and MRF4. Myogenin null mice display an almost total absence of myofibers, but myoblasts appear normal [14,15], suggesting that myogenin is necessary for terminal differentiation. MRF4 null mice exhibit phenotypes indicating a late role in myogenesis [16], positioning Myf-5 and MyoD upstream of myogenin and MRF4. Finally, mononucleated myocytes fuse to form nascent and then mature multinucleated myofibers, expressing muscle-specific genes like myosin heavy chain (MHC) and muscle creatine kinase (MCK), which mature into contracting muscle fibers. Some myoblasts remain undifferentiated, becoming quiescent satellite cells on the myofiber surface by day E17. It is widely accepted that muscle regeneration after injury mirrors embryonic myogenesis in two stages: differentiated myoblasts fuse to form nascent myotubes with few nuclei, and additional myoblasts subsequently integrate into these nascent myotubes, resulting in mature myofibers that increase in size and express contractile proteins.

The C2C12 myoblasts, established in 1977, are primary *in vitro* models for studying muscle formation and have been pivotal in muscular dystrophy research. C2C12 myoblasts are cultured on cell culture dishes until they are near confluence and differentiate into myotubes upon serum withdrawal [17]. Intriguingly, despite exposure to identical mitogen-poor conditions, myoblasts exhibit heterogeneous differentiation: some initiate the formation of myotubes ("founder" myocytes) that recruit the other cells for further fusion and maturation, while others retain proliferative and fusion capabilities. In addition, it is important to note that confluent C2C12 cultures also actively differentiate, even in a mitogen-rich medium, implicating a role for mechanotransduction in myogenesis. This disparity poses fundamental questions about the mechanisms driving such heterogeneity and the associated signalling pathways—answers that could advance our understanding of myogenesis.

Our earlier work in the C2C12 myoblast differentiation revealed that palladin, a cytoskeletal-associated protein, plays a key role in myoblast differentiation. Loss of palladin accelerates the differentiation of myoblasts into nascent myocytes but impairs the subsequent maturation into mature myotube [18,19].

The palladin gene, which is conserved between mice and humans, spans ~400 kb on mouse chromosome 8B3.3 and human chromosome 4q32.3. Palladin is widely expressed in various cells and tissues and enriches actin-associated structures such as focal adhesions (FA), membrane ruffles, and podosomes that regulate human and animal cell morphology [20–23]. Previous reports have identified the involvement of palladin in cell migration and adhesion via its ability to organize actin bundles [21,23–26]. Moreover, palladin interacts with many proteins in mechanosensitive structures, such as FA [26] and stress fibers [21,23], and modulates the mechanosensitivity of tumor fibroblasts [27]. At least four major isoforms have been transcribed from different promoters: 200-kDa, 140-kDa, and 90–92 kDa doublets. Alternative splicing produces additional, less-characterized variants [21]. All major isoforms feature three C-terminal IgCAM (immunoglobulin domain Cell Adhesion Molecule subfamily) domains for binding ezrin and F-actin [22,28]. Up to three proline-rich regions, depending on the isoform, mediate interactions with profilin and Mena/VASP proteins [29]. The 90–92 kDa isoforms are ubiquitously expressed across species and tissues [23]. The 140-kDa isoform includes a fourth IgCAM domain and additional proline-rich sequences, interacts with the actin-binding protein LIM- and SH3-domain containing protein (Lasp-1), and is mainly expressed during development in various tissues, with minimal adult tissue expression [30]. The 200-kDa isoform, the longest palladin, includes a fifth IgCAM domain and is expressed in the developing hearts of chickens and mice [23,31].

Palladin is involved in striated muscle development. Palladin belongs to the myotilin/myopalladin family, which is restricted and expressed in striated muscle [21]. The Ig-domains of Palladin is most homologous to the sarcomeric Z-disk protein myotilin [25]. Ablation of palladin in the adult heart results in dilated cardiomyopathy with intercalated disc abnormalities [32]. A decrease of palladin mRNA is found in atrophying skeletal muscle due to food deprivation [33]. During embryogenesis, each of these isoforms is expressed in a unique but overlapping pattern in skeletal muscle precursors and their descendants. 140-kDa palladin mRNA peaks between E11.5-E13.5, while 200- and 90-kDa isoforms peak between E13.5-E15.5 [31], reminiscing primary and secondary waves of myoblast differentiation. Interestingly, the decreasing of 140-kDa palladin mRNA after E13.5 evokes its absence is required for myoblast-to-myocyte transition. At the protein level, the appearance of 140-kDa palladin at E15 [23] suggests its necessary role in myogenesis. In addition, migrating myoblasts of the hypaxial myotome, which invade the limb bud mesenchyme, is also positive for the 140-kDa isoform [31]. The expression of 140-kDa isoform decreases as somites differentiate into dermomyotome and sclerotome [31]. The 200-kDa palladin is highly homologous to the 145-kDa sarcomeric protein myopalladin (MYPN), both binding to α-actinin, nebulin, and PDZ-LIM proteins within the Z-disk of skeletal muscle. In undifferentiated C2C12 myoblasts, the 140-kDa isoform level is high, while 200-kDa is nearly undetectable [19,31]. During the differentiation process, the expression levels of 140-kDa palladin declines and 200-kDa increases [34,35]. Knockdown of palladin in C2C12 cells upregulates p21 and Mef2C mRNAs [19], essential factors for myoblast differentiation and muscle maturation. Importantly, several putative myogenic transcription factors are found in palladin isoform promoter regions [35]. These observations underscore the importance of palladin in striated muscle, especially skeletal muscle development, although its precise function remains elusive.

2. Materials and methods

2.1. Cell culture, lentivirus-mediated shRNA silencing, and cell transfection

Culture cells were grown or differentiated at 37 °C in a humidified incubator containing 5 % CO_2 as described elsewhere [18,19,36]. shRNA-mediated knockdown of palladin and overexpression of palladin isoforms in C2C12 myoblasts were performed as previously described [19]. The pool stable C2C12 palladin knockdown myoblasts are referred to as shPalld1, shPalld2, and shLuc (shCtrl).

2.2. Western blotting (WB)

C2C12 cell cultures were collected at specified times, and proteins

were extracted using RIPA buffer (Cell Signaling Technology, USA) with a protease and phosphatase inhibitor cocktail (Sigma-Aldrich, USA). Equal amounts (50 μg) of protein were resolved on SDS-PAGE, electrophoretically transferred to nitrocellulose membranes (Bio-Rad), blocked with 5 % skim milk/TBS and incubated with appropriate primary antibodies at 4 °C overnight, and secondary antibodies for 1 h at room temperature (RT) before exposure using ECL immunoblotting kit (GE Healthcare, USA). Antibodies used were: palladin (1:2000, ProteinTech, 10853-1-AP), vinculin (1:1000, abcam, ab18058), integrin-β3 (1:1000, abcam, ab75872), ILK (1:2000, BD Transduction Laboratories™, 611803), kindlin-1 (1:1000, abcam, ab68041), kindlin-2 (1:1000, Cell Signaling Technology, #13562), GAPDH (1:5000, Novus Biologicals, NB300–221SS), horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, 1:5000).

2.3. Immunofluorescence (IF) microscopy

Cell cultured on 22 × 22 mm glass coverslips were fixed with 4 % paraformaldehyde for 15 min, rinsed in phosphate-buffered saline (PBS), permeabilized with 0.2 % (v/v) Triton X-100 for 15 min, and blocked with 5 % BSA in PBS. Subsequently, samples were incubated overnight at 4 °C with primary antibodies, followed by fluorochrome-conjugated secondary antibodies for 1 h at RT. Cell nuclei were counterstained with DAPI (1:10000, Invitrogen™) for 10 s. Antibodies used were: sarcomeric-α-actinin (1:500, Genetex, GTX29465), palladin (1:400, ProteinTech, 10853-1-AP), vinculin (1:100, abcam, ab18058), integrin-β3 (1:250, abcam, ab75872), integrin-β1 (1:200, abcam, ab52971), ILK (1:100, BD Transduction Laboratories™, 611803), heavy chain cardiac Myosin (1:800, Genetex, GTX20015), Rhodamine-phalloidin (1:400, Invitrogen™, R415), and AlexaFluor-488 goat anti-rabbit IgG and AlexaFluor-568 goat anti-mouse IgG (1:400, Invitrogen™). Samples were then mounted and examined with a 63 × oil immersion objective lenses on an Eclipse T*i* epifluorescence microscope (Nikon Inc., Japan). Images were processed using an NIS-element software.

2.4. Cell adhesion assay

Myoblasts were trypsinized, enumerated, and allowed to adhere for 1 h at 37 °C on glass coverslips in growth medium (GM). Cells were washed with PBS and fixed with 4 % paraformaldehyde. Adherent cells were then probed with palladin, integrin-β3, vinculin, and ILK antibodies for the evaluation of FA formation, and DAPI for adhesion rate quantification.

2.5. Cell migration assay

Cell migration was performed as previously described [19,34].

2.6. Quantification of nascent and focal adhesions and adhesion rate

Adhesion dynamics at the leading edge were quantified by background subtraction and pixel intensity analyses using ImageJ software. The adhesion rate was quantified by quantifying the average number of nuclei (stained with DAPI) from 10 random 10 × microscope fields (Nikon Inc., Japan).

2.7. The BacMam system-mediated nuclei labeling

Control myoblasts were transduced with Invitrogen™CellLight™Nucleus-RFP BacMam2.0 (red), while palladin knockdown myoblasts were transduced with Invitrogen™CellLight™Nucleus-GFP, BacMam2.0 (green) (Thermo Fisher Scientific) 24h before their differentiation induction, according to the manufacturer's instructions, to visualize nuclei.

2.8. Statistical analysis

Results for individual cell experiments were replicated in at least three independent experiments ($n \geq 3$), each performed with triplicate samples, and are presented as the mean ± SEM. Data were analyzed using an unpaired two-tailed Student's *t*-test for two-group comparisons. GraphPad Prism version 7.0 was used for analysis. The values of $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***) were considered statistically significant.

3. Results

3.1. Subcellular localization of palladin in early myoblast differentiation

Our studies revealed that palladin deficiency in C2C12 myoblasts impedes cellular migration while accelerating premature differentiation [18,19]. This observation led us to hypothesize that myoblasts with diminished palladin levels might migrate less and stabilize, potentially acting as the initial cells ("founder" myocytes) to establish a niche for myotube differentiation. To elucidate the role of palladin in muscle cell function, we first examined its colocalization with sarcomeric-α-actinin during the early stages of murine myoblast differentiation, a process initiated by cellular contact and subsequent differentiation.

We identified two distinct patterns of palladin subcellular distribution in mononuclear myoblasts. Specifically, myogenic myoblasts expressing striated sarcomeric-α-actinin exhibited punctate palladin expression in the cytoplasm, with minimal peri-membranous presence (Supplementary Fig. 1A). On the other hand, in C2C12 cells lacking prominent striated sarcomeric-α-actinin staining, palladin was predominantly localized at the cell membrane and extended into filopodia-like structures that seemed like anchoring points for the cells (Supplementary Fig. 1A, inset). These patterns persisted consistently throughout a 48-h differentiation timeline (Supplementary Figs. 1B and C). Cells with palladin at the membrane were usually mononuclear and positioned adjacent to multinucleated myotubes (Supplementary Fig. 1C, inset). These observations imply a potential role for palladin's subcellular localization in directing myoblast differentiation, possibly through interactions between the actin cytoskeleton and the extracellular matrix.

3.2. Integrin-β3 recruitment to nascent focal adhesions is impaired by palladin depletion

The involvement of palladin in mechano-signalling and its importance for cell migration have been previously demonstrated [18,24,27]. Our previous work demonstrates that palladin knockdown in myoblasts leads to a marked decrease in cellular migration [18]. This reduction in migration could be attributed to compromised cell adhesion, potentially due to impaired mechano-transduction consequent to palladin depletion. To examine the role of palladin in forming FA, we utilized lentivirus-mediated shRNA to knockdown palladin in C2C12. C2C12 cells were transfected with either a control non-targeting sequence (shLuc, hereafter shCtrl) or palladin-targeting sequences (shPalld1 and shPalld2, collectively termed KD), resulting in a significant 70 % reduction in endogenous palladin levels (Supplementary Fig. 2).

Given the integral role of integrin in activating inside-out signalling via FAs, we examined the formation of integrin-dependent FAs in the context of palladin depletion. Palladin KD and control myoblasts were subjected to trypsinization and subsequently replating onto glass coverslips in GM. We observed no significant difference in initial cell adhesion (60 min post-spreading) between the groups (Supplementary Fig. 3). Interestingly, while Ctrl myoblasts spread out and formed clusters of integrin-β3 with vinculin at the nascent adhesions at the cell periphery (Fig. 1A, Supplementary Fig. 4A), the KD cells displayed a notable absence of integrin-β3 in vinculin-containing nascent adhesions (Fig. 1A, inset A″). Intriguingly, this effect appeared specific to integrin-β3, as actin polymerization and integrin-β1 clustering remained

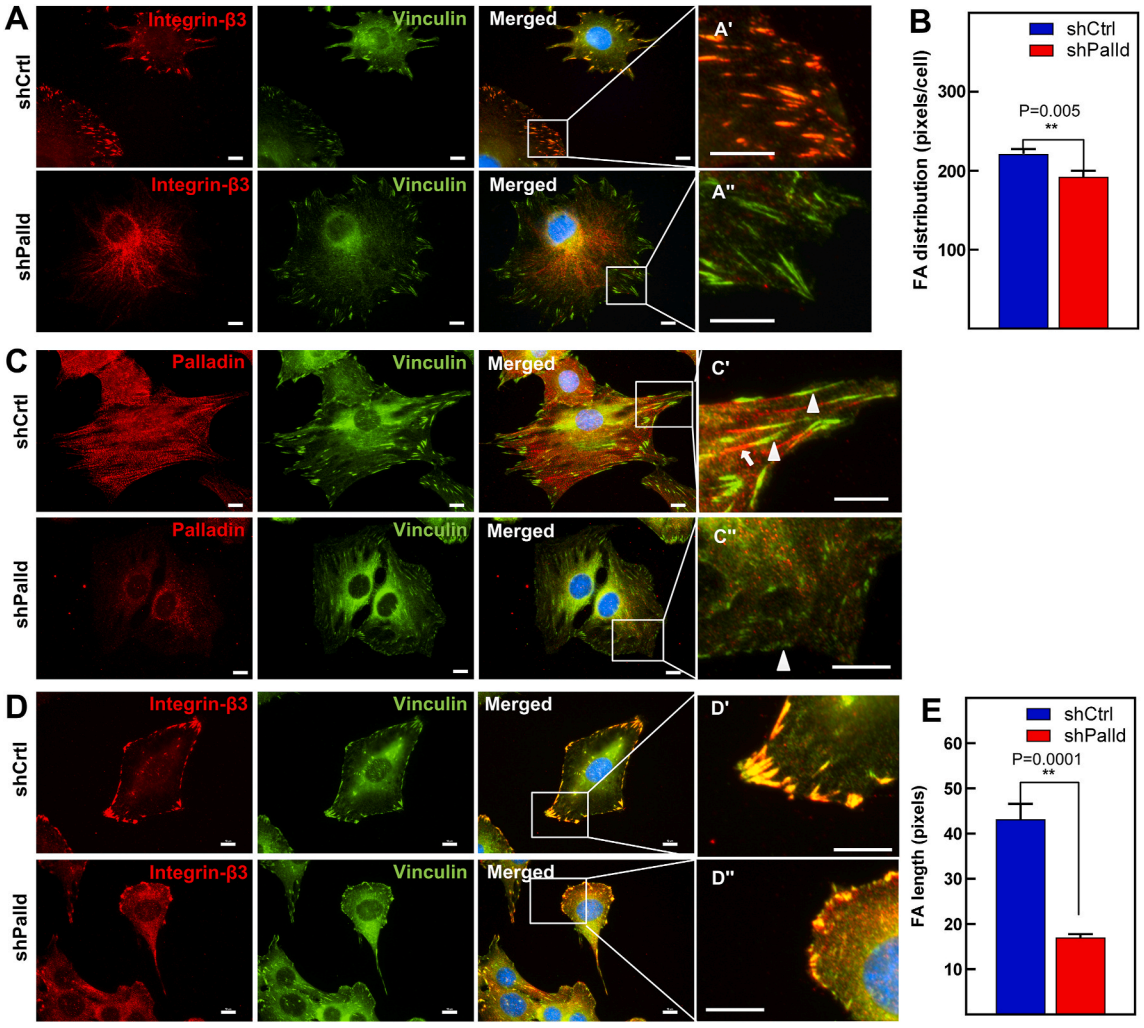

Fig. 1. Integrin-β3 fails to localize to nascent adhesion in palladin knockdown C2C12 cells (**A**) Palladin-depleted C2C12 myoblasts (upper panels) and control cells (lower panels) were allowed to adhere for 60 min. Cells were fixed and co-stained with antibodies to integrin-β3 (red), vinculin (green) to mark FA, and DAPI (blue). The merged image shows the co-localization of vinculin and integrin-β3 in control cells (yellow). Note that vinculin-containing adhesions in palladin-knockdown cells lack integrin-β3. Inset, enlarged area of peripheral adhesion staining. (**B**) FA numbers from (A) were quantified using ImageJ. n = 50 cells for each condition from three independent biological replicates. (**C, D**) Palladin-depleted C2C12 myoblasts (upper panels) and control cells (lower panels) were cultured in growth media for 24 h. Cells were fixed and co-stained with antibodies to (**C**) palladin (red) or (**D**) integrin-β3 (red), vinculin (green), and DAPI (blue). Note that palladin is less expressed in KD cells. Palladin is expressed in the vicinity site of vinculin (**C'**, arrow). Inset, enlarged area of peripheral adhesion staining. (**E**) Quantification of FA size in D. Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

unaffected by palladin knockdown (Supplementary Figs. 4B and C). These results collectively suggest a crucial role for palladin in the recruitment of integrin-β3 to cell membrane during the early stages of myoblast adhesion, thereby underscoring its critical function in cell-matrix interactions during myoblast differentiation.

3.3. Palladin-depleted myoblasts form smaller focal adhesions

Our aforementioned results reinforced the speculation that palladin is essential for integrin-β3 trafficking to FAs. Given that integrin-β3 is known to bind vinculin within FAs, we explored the effects of palladin depletion on the positioning of integrin-β3 in mature adhesions using immunofluorescence. Notably, at 60 min post-spreading on glass coverslips, KD myoblasts formed significantly fewer nascent FAs than Ctrl cells (Fig. 1B). After a 24-h adhesion and growth, Ctrl myoblasts displayed palladin close to vinculin (Fig. 1C, C′, arrow), and more mature adhesion sites were observed where vinculin and integrin-β3 colocalized (Fig. 1D, D′). Conversely, while palladin depletion did not hinder the localization of integrin-β3 to vinculin-positive mature adhesion sites after 24-h growth (Fig. 1D, D″), it resulted in the formation of smaller FAs in KD cells compared to Ctrl (Fig. 1E). Further analysis showed that palladin-KD cells had fewer actin stress fibers, despite normal integrin-linked kinase (ILK)-containing adhesions (Supplementary Fig. 5). These findings underscore the significant role of palladin in determining the appropriate size and quantity of FAs.

3.4. Palladin deficiency promotes founder cell role in early myoblast differentiation

Given the above-mentioned reduction in FAs in the KD myoblasts, we speculated a link to the accelerated myogenic differentiation we had previously documented [19]. We first assessed the number of FAs during the early differentiation (Fig. 2A). Unexpectedly, we found no significant difference in FA numbers between Ctrl and KD myoblasts after 24 h of exposure to differentiation media (DM) (Fig. 2B). However, a notable morphological change was observed; while both groups were predominantly round-shaped in GM, KD myoblasts turned to more spindle-shaped myocytes after 24 h in DM, compared to Ctrl cells (Fig. 2A–C). This result supports previous findings that palladin depletion expedites the myoblast-to-myocyte transition.

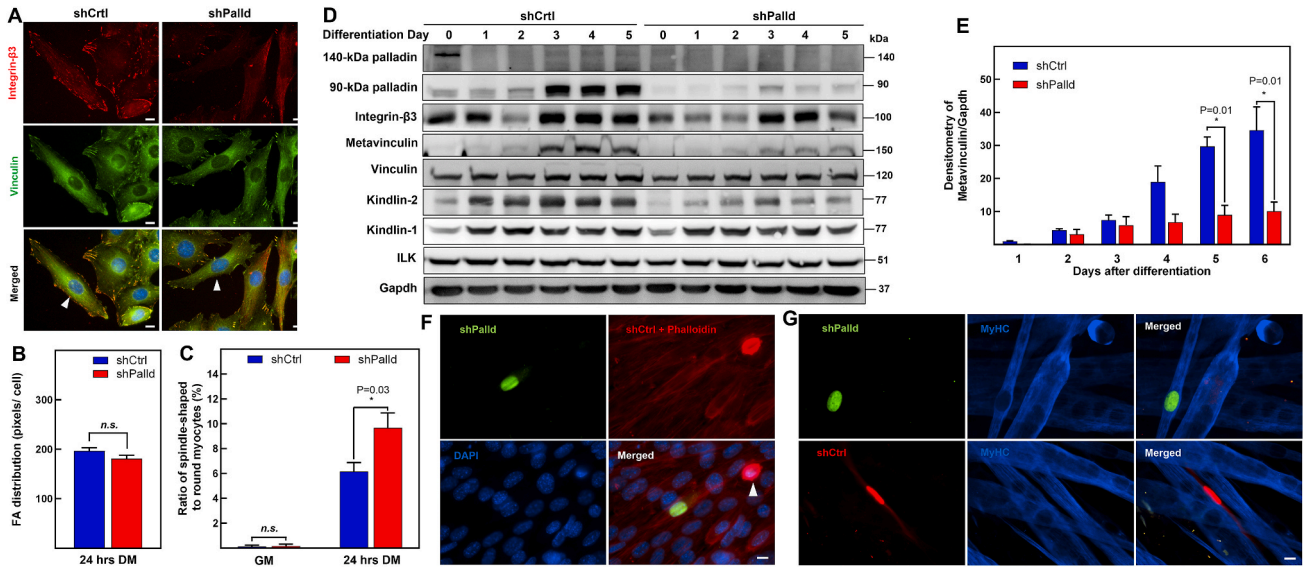

Fig. 2. Myoblasts lacking palladin prone to become founder myocytes. (**A-C**) Palladin-depleted C2C12 myoblasts (right) and control cells (left) were differentiated in differentiating media for 24 h. Cells were (**A**) fixed and co-stained with antibodies to integrin-β3 (red), vinculin (green), and DAPI (blue) and quantified for (**B**) FA or (**C**) early differentiated myocyte (spindle-shape cells). (**D**) The immunoblot analyses of the adhesion proteins in palladin-knockdown and control cells during myogenesis (Day 0 to Day 5). (**E**) Quantification of metavinculin from (**D**) were shown. Gapdh served as a loading control. (**F-G**) Palladin-KD myoblasts were infected with CellLight Nucleus, BacMam 2.0 as green nuclei, and Ctrl as red nuclei cells, and mixed at 1:1 ratio. After (**F**) 48 h of differentiation, cells were stained with Rhodamine phalloidin (red). After (**G**) 96 h of differentiation, cells were stained with Myosin heavy chain (blue). Arrow indicates the binding of Ctrl cells (red) to an established myotube, as evidenced by spindle-shape or myosin-heavy chain staining. Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

We then investigated if this morphology was associated with altered FA- and palladin-associated protein expression, especially focusing on adhesome proteins. Immunoblot analysis of integrin-β3, vinculin, Ilk, and kindlin-1 showed similar expression levels in KD and Ctrl cells throughout the 5-day differentiation period (Fig. 2D). Interestingly, a significant decrease in metavinculin expression, a muscle-specific vinculin variant critical for force transduction at adhesion sites [37], was observed in KD cells from day 4 of differentiation (Fig. 2D and E). In addition, a notable reduction in kindlin-2, a co-activator of integrin-β3 [38], was also observed in KD cells as early as day 2 of differentiation (Fig. 2D). These data suggest palladin depletion results in interfering with the expression of some adhesome proteins during myogenesis, which might obstruct further cell fusion.

To explore the differentiation dynamics, we employed a Bacman system to label the nuclei of KD and Ctrl myoblasts differentially. After 48 h of differentiation, cells lacking palladin (marked with green nuclei) displayed characteristics of early differentiation with spindle-shaped, multinucleated myotubes and distinct grooved nuclei (Supplementary Fig. 6). In contrast, the Ctrl cells (identified by red nuclei) mainly showed smooth nuclei and were less likely to form multinucleated myotubes (Supplementary Fig. 7). Interestingly, some Ctrl cells, but not KD cells, were often found adjacent to nascent myotubes, indicating a potential defect in adherence capacities to mature myotubes of KD cells (Supplementary Fig. 7, inset).

To further assess their fusion abilities, we co-cultured labelled myoblasts from both groups in a mixed 1:1 ratio. Palladin-depleted cells predominantly formed spindle-shaped myotubes within 48 h, whereas Ctrl cells formed similar structures or maintained a round shape, approaching nascent myotubes for fusion (Fig. 2F, arrowhead). At 96 h, most KD cells appeared as thin myotubes with fewer myonuclei (Fig. 2G, upper). Interestingly, Ctrl cells were observed binding to established myotubes at this stage, marked by myosin heavy chain staining (Fig. 2G, lower), a phenomenon not observed in KD cells (Fig. 2G, Supplementary Fig. 8). These findings strengthen our previous findings that palladin loss accelerates differentiation in myoblasts and diminishes their ability to adhere to mature myotubes, underscoring its significance in myogenesis.

3.5. Overexpression of 140-kDa palladin modulates directional cell interaction

In C2C12 myoblast cultured under proliferative conditions, we observed a high expression of the 140-kDa palladin isoform (Fig. 2D, shCtrl). At this stage, myoblasts are fully capable of migration. This migratory ability diminished upon myotube formation, which coincided with a reduction in 140-kDa palladin and an increase in the 90- and 200-kDa isoforms. To elucidate the distinct roles of these palladin isoforms in cellular interaction, we transfected Ctrl cells for overexpression and KD cells for rescue experiments with GFP-tagged 90, 140, or 200-kDa palladin constructs. Subsequently, we observed the resulting cell behaviours using ILK immunofluorescence.

Cells expressing GFP-90-kDa palladin exhibited evenly distributed, stress fiber-like patterns around the cell membrane, interacting randomly with adjacent cells along their lateral or leading edges (Fig. 3A, Supplementary Fig. 9, upper line). On the other hand, while overexpression of the 140-kDa palladin isoform resulted in the formation of compacted star-like structures (Fig. 3A, arrowheads), as previously documented [30], a distinctive behaviour was observed in Ctrl cells overexpressing GFP-140-kDa palladin. These cells exhibited directional interactions, characterized by a specific polarity of star-like GFP-140-kDa palladin structures inside cells. Specifically, the side of cells accumulated more star-like GFP-140-kDa palladin structures that were oriented towards or in contact with non-overexpressing cells. Notwithstanding, the polarized interaction pattern was absent in KD cells, where other cells towards or in contact with the overexpressed cell randomly (Fig. 3, Supplementary Fig. 9, arrowheads). Cells overexpressing GFP-200-kDa palladin showed patterns of expression and interaction that appeared to be a combination of the behaviours observed with overexpression of both GFP-90 and 140-kDa palladin (Fig. 3A, Supplementary Fig. 9, lower line).

We speculated that the absence of polarity caused by 140-kDa palladin overexpression in KD cells results from lacking other palladin isoforms. Since polarity is closely related to cell migration, we examined whether migration behavior depends on 140-kDa palladin

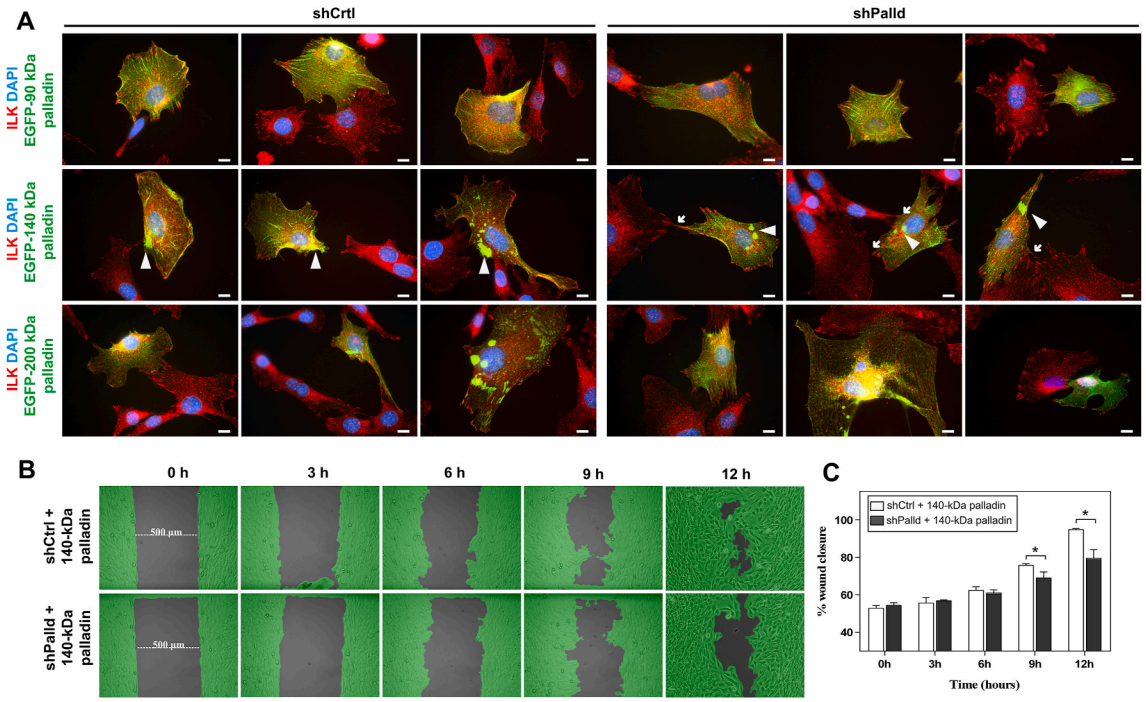

Fig. 3. 140-kDa palladin accumulation grants cell polarity and promotes cell migration (**A**) Palladin-depleted C2C12 myoblasts (right) and control myoblasts (left) were transiently transfected with the GFP-90-kDa, GFP-140-kDa, or GFP-200-kDa palladin constructs in GM for 24 h. Cells were fixed and co-stained with antibodies to ILK (red). The GFP-90-kDa palladin protein displayed an evenly striated, stress fiber-like structure in the cell membrane and randomly interacted with other cells at lateral or leading edges of the cells in both Ctrl and palladin-depleted cells. GFP-140-kDa palladin-expressing cells showed directional cell locomotion, in which the abundant expression of GFP-140 kDa palladin toward or in contact with other non-expressing cells only happened in Ctrl cells. The C2C12 GFP-200-kDa palladin-expressing cells showed expressing and interacting patterns similar to a mixture of both GFP-90 and 140-kDa palladin-expressing cells. Both GFP-140 kDa and GFP-200 kDa palladin display star-like expression patterns. Scale bars, 10 μm. (**B–C**) The wound-healing migration of 140-kDa palladin transient transfectants of Ctrl or KD myoblasts were recorded with an optical microscope at the indicated time points and subjected to statistical analysis (n = 3 per group). The results are represented as the percentage of cell-covered area (B) and quantification (C). Scale bar is 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

overexpression. Results indicated that overexpression of 140-kDa palladin in KD cells, which lack 140-kDa palladin polarity, did not restore cell migration capacity compared to Ctrl cells with polarized 140-kDa palladin (Fig. 3B and C). These observations collectively indicate that the 140-kDa palladin isoform is essential for directional cell interaction. This finding suggests a role for 140-kDa palladin isoform in establishing myoblast polarity and facilitating interaction or migration, but the presence of other palladin isoforms is also necessary.

4. Discussion

The current study expands on the multifaceted role of palladin during skeletal muscle cell differentiation process, particularly highlighting the distinct functions of its isoforms in the differentiation and interaction dynamics of myoblasts. Our data provide insights into the cellular mechanics underlying myogenesis and reinforce the critical roles of cytoskeletal proteins in myogenesis.

We demonstrated that palladin depletion leads to an impairment of in-time integrin-β3 recruitment to nascent FAs and subsequently, a reduction in FA formation, which is concomitant with premature differentiation of myoblasts into myocytes. These results provide insight into the mechanotransduction disturbances that might underlie the migration defects observed. The decrease in metavinculin expression, together with the diminished fusion capacity of palladin-depleted myocytes at later differentiation stages aligns with our previous findings and further supports the concept that palladin is involved in the maturation and functional specialization of muscle cells, potentially by orchestrating the assembly of adhesion complexes necessary for cell fusion and force transduction.

The observed high expression of the 140-kDa palladin isoform in proliferating C2C12 myoblasts aligns with its role in facilitating robust cell migration. This is in stark contrast to the diminished migratory capacity concurrent with myotube formation, where a decrease in 140-kDa palladin and an increase in the 90- and 200-kDa isoforms were noted. The shift in palladin isoform expression suggests a complex regulatory mechanism where different isoforms play distinct roles at various stages of muscle cell development. The overexpression experiments further underscored the unique contribution of the 140-kDa isoform to directional cell interaction and polarity establishment, highlighting the role of palladin isoform in cell-cell communication and migration. The specific absence of this polarized interaction pattern in palladin-deficient cells suggests the necessity of other palladin isoform for normal myoblast behavior in this context.

This study suggests future research into modulating palladin isoforms to control myogenesis, with potential implications for treating myopathies and age-related muscle decline. Further work is needed to uncover the molecular details of palladin's role and validate these findings in vivo, highlighting its importance in muscle regeneration strategies.

5. Conclusions

Our study reveals critical insights into the distinct functions of palladin isoforms in myoblast differentiation. The 140-kDa isoform emerges as a key regulator of cell migration and interaction, while palladin, in general, plays a pivotal role in myoblast differentiation and focal adhesion dynamics. Our study not only underscores the importance of palladin in myoblast differentiation and fusion but also

highlights the potential of palladin as a regulatory molecule that influences skeletal muscle cell fate decisions, offering promising prospects for muscle disease research and regenerative medicine.

CRediT authorship contribution statement

Ngoc Uyen Nhi Nguyen: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ching-Cheng Hsu:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Shah R. Ali:** Writing – review & editing. **Hao-Ven Wang:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101762.

References

[1] M. Daczewska, Differentiation of skeletal muscles, Semin. Cell Dev. Biol. 104 (2020) 1–2, https://doi.org/10.1016/j.semcdb.2020.05.008.
[2] P. Li, A. Liu, C. Liu, Z. Qu, W. Xiao, J. Huang, Z. Liu, S. Zhang, Role and mechanism of catechin in skeletal muscle cell differentiation, J. Nutr. Biochem. 74 (2019) 108225, https://doi.org/10.1016/j.jnutbio.2019.108225.
[3] D.J. Wilkinson, M. Piasecki, P.J. Atherton, The age-related loss of skeletal muscle mass and function: Measurement and physiology of muscle fibre atrophy and muscle fibre loss in humans, Ageing Res. Rev. 47 (2018) 123–132, https://doi.org/10.1016/j.arr.2018.07.005.
[4] H. Yin, F. Price, M.A. Rudnicki, Satellite cells and the muscle stem cell niche, Physiol. Rev. 93 (2013) 23–67, https://doi.org/10.1152/physrev.00043.2011.
[5] C. Lepper, S.J. Conway, C.M. Fan, Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements, Nature 460 (2009) 627–631, https://doi.org/10.1038/nature08209.
[6] P. Seale, L.A. Sabourin, A. Girgis-Gabardo, A. Mansouri, P. Gruss, M.A. Rudnicki, Pax7 is required for the specification of myogenic satellite cells, Cell 102 (2000) 777–786, https://doi.org/10.1016/s0092-8674(00)00066-0.
[7] M.A. Rudnicki, P.N. Schnegelsberg, R.H. Stead, T. Braun, H.H. Arnold, R. Jaenisch, MyoD or Myf-5 is required for the formation of skeletal muscle, Cell 75 (1993) 1351–1359, https://doi.org/10.1016/0092-8674(93)90621-v.
[8] B. Swierczek, M.A. Ciemerych, K. Archacka, From pluripotency to myogenesis: a multistep process in the dish, J. Muscle Res. Cell Motil. 36 (2015) 363–375, https://doi.org/10.1007/s10974-015-9436-y.
[9] H. Weintraub, R. Davis, S. Tapscott, M. Thayer, M. Krause, R. Benezra, T. K. Blackwell, D. Turner, R. Rupp, S. Hollenberg, et al., The myoD gene family: nodal point during specification of the muscle cell lineage, Science 251 (1991) 761–766, https://doi.org/10.1126/science.1846704.
[10] S. Tajbakhsh, D. Rocancourt, M. Buckingham, Muscle progenitor cells failing to respond to positional cues adopt non-myogenic fates in myf-5 null mice, Nature 384 (1996) 266–270, https://doi.org/10.1038/384266a0.
[11] G. Cossu, R. Kelly, S. Tajbakhsh, S. Di Donna, E. Vivarelli, M. Buckingham, Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm, Development 122 (1996) 429–437, https://doi.org/10.1242/dev.122.2.429.
[12] T. Braun, M.A. Rudnicki, H.H. Arnold, R. Jaenisch, Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death, Cell 71 (1992) 369–382, https://doi.org/10.1016/0092-8674(92)90507-9.
[13] M.A. Rudnicki, T. Braun, S. Hinuma, R. Jaenisch, Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development, Cell 71 (1992) 383–390, https://doi.org/10.1016/0092-8674(92)90508-a.
[14] P. Hasty, A. Bradley, J.H. Morris, D.G. Edmondson, J.M. Venuti, E.N. Olson, W. H. Klein, Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene, Nature 364 (1993) 501–506, https://doi.org/10.1038/364501a0.
[15] Y. Nabeshima, K. Hanaoka, M. Hayasaka, E. Esumi, S. Li, I. Nonaka, Y. Nabeshima, Myogenin gene disruption results in perinatal lethality because of severe muscle defect, Nature 364 (1993) 532–535, https://doi.org/10.1038/364532a0.
[16] E.N. Olson, H.H. Arnold, P.W. Rigby, B.J. Wold, Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4, Cell 85 (1996) 1–4, https://doi.org/10.1016/s0092-8674(00)81073-9.
[17] D. Yaffe, O. Saxel, A myogenic cell line with altered serum requirements for differentiation, Differentiation 7 (1977) 159–166, https://doi.org/10.1111/j.1432-0436.1977.tb01507.x.
[18] N.U. Nguyen, V.R. Liang, H.V. Wang, Actin-associated protein palladin is required for migration behavior and differentiation potential of C2C12 myoblast cells, Biochem. Biophys. Res. Commun. (2014), https://doi.org/10.1016/j.bbrc.2014.08.143.
[19] N.U. Nguyen, H.V. Wang, Dual roles of palladin protein in in vitro myogenesis: inhibition of early induction but promotion of myotube maturation, PLoS One 10 (2015) e0124762, https://doi.org/10.1371/journal.pone.0124762.
[20] S. Goicoechea, D. Arneman, A. Disanza, R. Garcia-Mata, G. Scita, C.A. Otey, Palladin binds to Eps8 and enhances the formation of dorsal ruffles and podosomes in vascular smooth muscle cells, J. Cell Sci. 119 (2006) 3316–3324, https://doi.org/10.1242/jcs.03076.
[21] C.A. Otey, A. Rachlin, M. Moza, D. Arneman, O. Carpen, The palladin/myotilin/myopalladin family of actin-associated scaffolds, Int. Rev. Cytol. 246 (2005) 31–58, https://doi.org/10.1016/S0074-7696(05)46002-7.
[22] O.M. Mykkanen, M. Gronholm, M. Ronty, M. Lalowski, P. Salmikangas, H. Suila, O. Carpen, Characterization of human palladin, a microfilament-associated protein, Mol. Biol. Cell 12 (2001) 3060–3073, https://doi.org/10.1091/mbc.12.10.3060.
[23] M.M. Parast, C.A. Otey, Characterization of palladin, a novel protein localized to stress fibers and cell adhesions, J. Cell Biol. 150 (2000) 643–656, https://doi.org/10.1083/jcb.150.3.643.
[24] S.M. Goicoechea, D. Arneman, C.A. Otey, The role of palladin in actin organization and cell motility, Eur. J. Cell Biol. 87 (2008) 517–525, https://doi.org/10.1016/j.ejcb.2008.01.010.
[25] S.M. Goicoechea, B. Bednarski, R. Garcia-Mata, H. Prentice-Dunn, H.J. Kim, C. A. Otey, Palladin contributes to invasive motility in human breast cancer cells, Oncogene 28 (2009) 587–598, https://doi.org/10.1038/onc.2008.408.
[26] P.N. Tay, P. Tan, Y. Lan, C.H. Leung, M. Laban, T.C. Tan, H. Ni, J. Manikandan, S. B. Rashid, B. Yan, C.T. Yap, L.H. Lim, Y.C. Lim, S.C. Hooi, Palladin, an actin-associated protein, is required for adherens junction formation and intercellular adhesion in HCT116 colorectal cancer cells, Int. J. Oncol. 37 (2010) 909–926.
[27] M. Azatov, S.M. Goicoechea, C.A. Otey, A. Upadhyaya, The actin crosslinking protein palladin modulates force generation and mechanosensitivity of tumor associated fibroblasts, Sci. Rep. 6 (2016) 28805, https://doi.org/10.1038/srep28805.
[28] R.D. Dixon, D.K. Arneman, A.S. Rachlin, N.R. Sundaresan, M.J. Costello, S. L. Campbell, C.A. Otey, Palladin is an actin cross-linking protein that uses immunoglobulin-like domains to bind filamentous actin, J. Biol. Chem. 283 (2008) 6222–6231, https://doi.org/10.1074/jbc.M707694200.
[29] M. Boukhelifa, M.M. Parast, J.E. Bear, F.B. Gertler, C.A. Otey, Palladin is a novel binding partner for Ena/VASP family members, Cell Motil Cytoskeleton 58 (2004) 17–29, https://doi.org/10.1002/cm.10173.
[30] A.S. Rachlin, C.A. Otey, Identification of palladin isoforms and characterization of an isoform-specific interaction between Lasp-1 and palladin, J. Cell Sci. 119 (2006) 995–1004, https://doi.org/10.1242/jcs.02825.
[31] H.V. Wang, M. Moser, Comparative expression analysis of the murine palladin isoforms, Dev Dyn 237 (2008) 3342–3351, https://doi.org/10.1002/dvdy.21755.
[32] G. Mastrototaro, P. Carullo, J. Zhang, B. Scellini, N. Piroddi, S. Nemska, M. C. Filomena, S. Serio, C.A. Otey, C. Tesi, F. Emrich, W.A. Linke, C. Poggesi, S. Boncompagni, M.L. Bang, Ablation of palladin in adult heart causes dilated cardiomyopathy associated with intercalated disc abnormalities, Elife 12 (2023), https://doi.org/10.7554/eLife.78629.
[33] R.T. Jagoe, S.H. Lecker, M. Gomes, A.L. Goldberg, Patterns of gene expression in atrophying skeletal muscles: response to food deprivation, FASEB J 16 (2002) 1697–1712, https://doi.org/10.1096/fj.02-0312com.
[34] N.U. Nguyen, V.R. Liang, H.V. Wang, Actin-associated protein palladin is required for migration behavior and differentiation potential of C2C12 myoblast cells, Biochem. Biophys. Res. Commun. 452 (2014) 728–733, https://doi.org/10.1016/j.bbrc.2014.08.143.
[35] B.E.F. Ouali, T.Y. Liu, C.Y. Lu, P.Y. Cheng, C.L. Huang, C.C. Li, Y.C. Chiang, H. V. Wang, Cloning and promoter analysis of palladin 90-kDa, 140-kDa, and 200-kDa isoforms involved in skeletal muscle cell maturation, BMC Res. Notes 13 (2020) 321, https://doi.org/10.1186/s13104-020-05152-9.

[36] N.-U.-N. Nguyen, T.-Y. Liu, H.-V. Wang, Timing appearance and integration of actin-organizing palladin protein in dynamic myofibril assembly, bioRxiv (2016) 047183, https://doi.org/10.1101/047183.
[37] V. Kanoldt, C. Kluger, C. Barz, A.L. Schweizer, D. Ramanujam, L. Windgasse, S. Engelhardt, A. Chrostek-Grashoff, C. Grashoff, Metavinculin modulates force transduction in cell adhesion sites, Nat. Commun. 11 (2020) 6403, https://doi.org/10.1038/s41467-020-20125-z.
[38] Y.Q. Ma, J. Qin, C. Wu, E.F. Plow, Kindlin-2 (Mig-2): a co-activator of beta3 integrins, J. Cell Biol. 181 (2008) 439–446, https://doi.org/10.1083/jcb.200710196.