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C6ORF32 is upregulated during muscle cell differentiation and induces the formation of cellular filopodia.

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Abstract

We have identified a gene by microarray analysis that is located on chromosome 6 (*c6orf32*), whose expression is increased during human fetal myoblast differentiation. The protein encoded by *c6orf32* is expressed both in myogenic and non-myogenic primary cells isolated from 18-week old human fetal skeletal muscle. Immunofluorescent staining indicated that C6ORF32 localizes to the cellular cytoskeleton and filopodia, and often displays polarized expression within the cell. mRNA knockdown experiments in the C2C12 murine myoblast cell line demonstrated that cells lacking *c6orf32* exhibit a myogenic differentiation defect, characterized by a decrease in the expression of myogenin and myosin heavy chain (MHC) proteins, whereas MyoD1 was unaltered. In contrast, overexpression of *c6orf32* in C2C12 or HEK293 cells (a non-muscle cell line) promoted formation of long membrane protrusions (filopodia). Analysis of serial deletion mutants demonstrated that amino acids 55-113 of C6ORF32 are likely involved in filopodia formation. These results indicate that C6ORF32 is a novel protein likely to play multiple functions, including promoting myogenic cell differentiation, cytoskeletal rearrangement and filopodia formation.

Keywords

Myoblast; Differentiation; Filopodia; *c6orf32* (gene); C6ORF32 (protein); C2C12

Introduction

Mononuclear muscle progenitors can be efficiently propagated *ex vivo* and induced to differentiate to form multinucleated myotubes (Yablonka-Reuveni, 2004). These processes are tightly regulated and require the expression of specific proteins at precise times. Many of the fundamental regulators that orchestrate myogenic differentiation have been elucidated and include the myogenic transcription factors *MyoD1, Myf5, MRF4* and *myogenin* (Charge and Rudnicki, 2004;Berkes and Tapscott, 2005). Many of these events are recapitulated *in vivo* in response to exercise-induced damage or after muscle trauma (Shi and Garry, 2006). For instance, muscle regeneration is mediated by the activation of satellite cells, which are also defined as tissue-specific stem cells (Wagers and Conboy, 2005). Satellite cells proliferate after acute muscle injury prior to undergoing post-mitotic myogenic differentiation and syncitial fusion to form new myofibers and restore muscle activity. This process is reminiscent of the events that occur during muscle development in embryogenesis (Pownall et al., 2002;Parker et al., 2003). Thus, the mechanisms involved in muscle cell differentiation that lead to myofiber formation can be dissected using both *in vitro* and *in vivo* models.

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Understanding these mechanisms may have broader clinical implication, as it could highlight new cues on how to improve the efficacy of cell transplantation as a potential therapy for a variety of muscle diseases. Some of the key proteins involved in muscle cell differentiation and myofiber formation are relatively well studied in *Drosophila* (Abmayr et al., 2003;Taylor, 2003;Chen and Olson, 2004;Abmayr and Kocherlakota, 2006). In this model system, transmembrane proteins such as Dumbfounded *(Duf)* and Roughest *(Rst)* expressed in founder cells, and Sticks and stones *(Sns)*, expressed in fusion-competent cells, are involved in the recognition steps during muscle formation (Bour et al., 2000;Ruiz-Gomez et al., 2000;Strunkelnberg et al., 2001). Cells lacking both *Duf* and *Rst* do not fuse, while overexpression of either gene can attract fusion-competent myoblasts (Dworak et al., 2001;Galletta et al., 2004). When fusion-competent cells recognize and contact founder cells, proteins such as Antisocial, Loner and Myoblast city transduce signals from the cell surface to the cytoskeleton (Erickson et al., 1997;Chen and Olson, 2001;Rau et al., 2001;Chen et al., 2003). One important family of such signal transduction proteins is the Rho family of guanine nucleotide exchange factors (GEF) (Bryan et al., 2005a;Bryan et al., 2005b). These proteins are involved in a number of diverse functions, including cytoskeletal rearrangement, formation of focal adhesions, cell polarity, cell cycle progression and neurite outgrowth (Etienne-Manneville and Hall, 2002). In vertebrates, several additional proteins have been identified as regulators of myogenic differentiation and syncitial fusion. These include cell surface proteins such as metalloproteinase/disintegrins (Yagami-Hiromasa et al., 1995), IL-4 and mannose receptors (Horsley et al., 2003;Jansen and Pavlath, 2006), intracellular proteins such as calpain and nitric oxide (Lee et al., 1994;Balcerzak et al., 1995;Barnoy et al., 1998;Dulong et al., 2004) and transcription factors such as Foxo1a (Bois and Grosveld, 2003;Bois et al., 2005). However, these are likely to represent only a fraction of the proteins that regulate these complex biological processes.

To identify novel candidate genes, we performed microarray analyses on human fetal primary muscle cultures harvested at three distinct stages of myogenic differentiation: myoblasts, early myotubes and late myotubes (Cerletti et al., 2006). It was found that *c6orf32* is one of the genes upregulated early during muscle cell differentiation, when myotubes have a maximum of 5 nuclei. High levels of *c6orf32* mRNA are maintained when myotubes are larger in size (up to 15 myonuclei) (Cerletti et al., 2006). *C6orf32* is also known as *pl48*, or *diff48* (NCBI, AceView). *Pl48* (a short isoform of *c6orf32*) was isolated by subtractive hybridization between cDNAs derived from undifferentiated human cytotrophoblast and differentiating cytotrophoblast (Dakour et al., 1997). The level of *pl48* mRNA increases during cytotrophoblast differentiation and it has been suggested that it may play a role in the formation of non-mitotic multinucleated syncytium during placental differentiation (Dakour et al., 1997). PL48 contains a number of putative functional motifs, including a potential transmembrane domain, myristylation signals, and several phosphorylation sites, suggesting multiple regulatory functions; however, none of these putative motifs have been further studied or tested for function (Dakour et al., 1997).

To gain insight on the function of *c6orf32* (a longer isoform of *pl48*) in muscle cells, we first confirmed that this protein was expressed upon human fetal myoblast differentiation, when muscle cells become confluent and begin to fuse. The protein was localized at the cell membrane, in the cytoplasm and also in cellular filopodia associated with cytoskeletal proteins. We then studied the consequences of modulating the expression of *c6orf32 in vitro*, both via mRNA knockdown and via over-expression in muscle cells. mRNA knockdown experiments in C2C12 murine myoblasts demonstrated that reduced *c6orf32* expression significantly decreased fusion of mononuclear cells into multinucleated myotubes. This apparent fusion defect appears to be a secondary effect due to altered myoblast differentiation, as demonstrated by reduced expression of myogenin. In contrast, over-expression of *c6orf32* promotes formation of filopodia both in muscle and in non-muscle cells. Deletion mutant studies

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indicated that amino acids 55-113 of C6ORF32 are likely involved in the formation of filopodia. Together, these results suggest that just like described for GEFs, C6ORF32 may play multiple roles during muscle cell differentiation by mediating filopodia formation, cytoskeletal rearrangement, and by influencing either directly or indirectly the progression of myogenic differentiation.

Materials and Methods

Cloning of c6orf32

C6orf32 cDNA was amplified by RT-PCR from total RNA of human primary fetal muscle cells harvested after 5 days in differentiation medium. The primer set used for amplifying the *c6orf32* cDNA was as follows: *c6orf32* ISO1, ATGTTGGTAGGATCCCAGTC (forward) and CAGCTGTTAGGCAGTTAACCTGTA (reverse); *c6orf32* ISO2, ATGTTGGTAGGATCCCAGTC (forward) and CTACTTTTTTAGAATATCATC (reverse). Amplified *c6orf32* cDNA was inserted into pCR4-TOPO and sequenced. To obtain a V5-tagged C6ORF32 protein, *c6orf32* was PCR amplified and inserted into pCDNA3.1/V5-His-TOPO (Invitrogen). The plasmids encoding the N-terminal and C-terminal Flag-tagged C6ORF32 were generated by PCR amplification using a primer encoding the flag sequence (GACTACAAAGACGATGACGACAAG). The plasmids encoding truncated C6ORF32 proteins were generated by PCR amplification of the relevant regions and were verified by sequence analyses.

Cell culture

HEK293 cells were maintained in DMEM supplemented with 10% (v/v) fetal calf serum, penicillin/streptomycin (50 units/ml) and L-glutamine (2 mM). Human fetal primary myoblasts were isolated and maintained as previously described (Pavlath and Gussoni, 2005). Murine C2C12 cells were maintained in DMEM containing 20% (v/v) fetal calf serum supplemented with penicillin, streptomycin and L-glutamine. For HEK293 cells, the indicated plasmids were transfected using lipofectamine 2000^{TM} (Invitrogen) (2μl lipofectamine/μg DNA) in suspension. The culture medium was replaced in the dishes 6–8 hours after transfection. Approximately 20–24 hour post-transfection cells were immunostained or visualized by phase microscopy. For C2C12 cells, the indicated plasmids were transfected using the Amaxa electroporation system for C2C12 cells (Nucleofector V solution, Amaxa). The culture medium was replaced in the dishes 10–14 hours after transfection. Approximately 20 hours post-transfection cells were immunostained.

Immunostaining

HEK293 or C2C12 cells were fixed in cold methanol for 2 minutes, blocked with PBS containing 1% BSA for 1 hour and incubated with rabbit anti-V5 antibody (1:500, Sigma) or with rabbit anti-Flag antibody (1:100, Sigma) overnight at 4°C. Primary human fetal muscle cells were fixed in 4% paraformaldehyde containing 0.3% Triton X-100 for 10 minutes, blocked with PBS containing 1% BSA for 1 hour and incubated overnight at 4°C with one of the following primary antibodies: anti-desmin 1:500 (Epitomics), anti-C6ORF32 1:400 (Abnova), anti-MyoD 1:200 (BD Pharmingen), anti-myogenin 1:100 (DakoCytomation), anti-myosin heavy chain (MF20, 1:30, Developmental Studies Hybridoma Bank, University of Iowa) and anti-dystrophin 1:1,500 (Byers et al., 1993;Lidov et al., 1993). After three washes with PBS, cells were incubated with the appropriate secondary antibody conjugated with FITC, Alexa 488 or Rhodamine. For co-immunostaining of phalloidin with C6ORF32, phalloidin-Alexa 568 (Invitrogen) was reconstituted as per manufacturer's instruction, diluted 1:50 in PBS and added together with the secondary antibody. Cells were visualized using a Nikon Eclipse E-1000 microscope, photographed using a Hamamatsu digital camera, and images were acquired using OpenLab software version 3.1.5 (Improvision). For the C6ORF32 Flag-tagged

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overexpression and deletion mutant analyses, cells were visualized using a Nikon Eclipse TE2000-S, photographed using a Nikon 7.4 Slider camera, and images were acquired using Spot software version 4.1.1 (Diagnostic Instruments).

Quantitative RT-PCR

Total RNA was isolated from human fetal tissue using the Qiagen RNA isolation kit, and cDNA was reversed-transcribed using the Superscript RT kit (Invitrogen), according to the manufacturer's instructions. PCR primer sequences for human *c6orf32* were selected as follows: TTTCTGCCCCTGATAGTTGG (forward) and CATGTCCTCCACGTCAAATG (reverse), which amplify a 207 bp product. PCR amplification was performed using the SYBR Green PCR master mix Kit (Applied Biosystems) for 46 cycles at 95°C for 15 sec and 60°C for 1 min. The end product of the RT-PCR reactions were electrophoresed on 1.7% agarose gel and DNA was visualized by ethidium bromide staining. The PCR product was confirmed by sequence analysis and using the Sequence Detector v1.7a software. All quantitations were normalized to the endogenous control *gapdh*, which was amplified under the same conditions in separate tubes. Human *gapdh* primer sequences are as follow: GAAGGTGAAGGTCGGAGTC (forward) and GAAGATGGTGATGGGATTTC (reverse).

Immunoblotting

Cells were lysed in 50mM Tris-Cl, pH 7.5, 150mM NaCl, 1% Triton X-100, 1X protease inhibitor cocktail (Invitrogen). Total proteins were separated on 4–12% polyacrylamide NuPAGE Bis-Tris precast gels (Invitrogen) and blotted on nitrocellulose membrane (Invitrogen). The membrane was incubated with anti-C6ORF32 antibody (1:1000 dilution, Abnova, Taiwan), followed by peroxidase conjugated affinity purified goat anti-mouse IgG (Vector). Signals were detected using the ECL kit (Amersham). In vitro *RNA interference*

Retroviral constructs encoding small interfering RNAs targeting mouse *c6orf32* (accession number AL513014) were obtained or synthesized according to manufacturer's instructions (BD Biosciences). To generate hairpin siRNA, 67-bp oligonucleotides complementary to different target sequences of mouse *c6orf32* cDNA were synthesized (Dharmacon) (536: TGACCTAGACAAGCAAATT; 612: CTCTATGAAGCCTATTGTA) and inserted into the pSIREN-retroQ vector (BD Biosciences).

Retroviral vector stocks were produced by transient transfection using the ProFection Mammalian Transfection Systems-calcium phosphate kit (Promega). Briefly, 4µg of VSV-G (envelope) and 7µg of appropriate vector were transfected by calcium phosphate onto 293GP cells at 70% confluency in 6 cm dishes overnight. The following morning the cell medium was replaced with 2.5 ml of medium and the viral supernatants harvested 24–30 hours later.

For retroviral infection of C2C12, cells were plated into 12-well plates and infected overnight with 4µg/ml of polybrene and 300µl of viral supernatant. Each infection was performed in duplicate and the following day the culture medium was replaced. Proliferation medium was switched to low serum (2% horse serum) differentiation medium when cells were approximately 90% confluent. Myotube formation was examined on the fourth day after addition of differentiation medium. The fusion index (FI) was calculated by dividing the number of nuclei contained within myotubes by the total number of nuclei in a given field. A total of 6 randomly chosen fields (3 for each replica infection) were visually counted per each sample. To quantify the level of *c6orf32* gene expression in C2C12 cells, real-time quantitative RT-PCR was performed on C2C12 RNA isolated from each culture using the SYBR Green PCR master mix Kit (Applied Biosystems). PCR primers for mouse *c6orf32* GGGCTTGATGAGTACCTGGA (forward), and GAAGGCTTGCTTCATTTTGC (reverse) and for *gapdh* as an internal control: *gapdh* AACTTTGGCATTGTGGAAGG (forward), and

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ACACATTGGGGGTAGGAACA (reverse). PCR amplification was performed for 55 cycles at 95°C for 15 sec and 60°C for 1 min.

Results

Expression of c6orf32 mRNA during differentiation of human fetal myoblasts

Microarray studies interrogating genes regulated during differentiation and fusion of human fetal myoblasts indicated that the level of *c6orf32* expression is increased during the early steps of these processes (Cerletti et al., 2006). Quantitative real-time PCR was performed to confirm these results. Total RNA was isolated from human fetal muscle cells at each stage of differentiation (Fig. 1A). We have defined myoblasts as mononuclear cells, early myotubes as cells containing between 2–5 myonuclei, and late myotubes as cells containing up to 15 myonuclei. Real-time quantitative RT-PCR demonstrated that expression of *c6orf32* mRNA increased approximately 16-fold in both early and late myotubes (Fig. 1B, b, c) compared to the levels detected in mononuclear cells (Figure 1B, a). Figure 1C illustrates the PCR products at ‘plateau’ levels, indicating that the primers specifically amplified one product, which was confirmed by sequence analysis to be *c6orf32*.

Localization of native C6ORF32 during human fetal myoblast fusion

In cultures of primary human fetal skeletal muscle cells maintained under proliferation conditions, no expression of C6ORF32 was detected (data not shown). When cells were allowed to make contact and cultures were switched to differentation medium, C6ORF32 expression was detected at the cell membrane and it also appeared associated with the cell cytoskeleton, often localized to one side of the cell (Figure 1D–F, arrows). In rare cells undergoing mitosis, C6ORF32 localized to the cytoplasm (Figure 1G, H open arrowhead) and spread to filopodia-like protrusions that resembled cellular anchorage points (Figure 1H, arrow). C6ORF32 protein was also detected in long cellular filopodia (Figure 1I–M). Simulataneous detection of C6ORF32 expression with F-actin suggested co-localization of these proteins (Figure 1N–Q, arrows), although C6ORF32 also appeared present in cytoplasmic regions negative for F-actin expression (Figure 1O, Q). To investigate whether cells positive for C6ORF32 in human fetal primary muscle cultures were myogenic, co-immunostaining of C6ORF32 and desmin or dystrophin were performed during a time-course differentiation study (Figure 2). In mononuclear cells prior to fusion, co-localization of C6ORF32 protein with desmin demonstrated that C6ORF32 is expressed both in myogenic (Figure 2A, B) and non-myogenic cells (Figure 2C). Cells expressing C6ORF32 were mononuclear and often located adjacent to multinucleated myotubes, which were positive for dystrophin (Figure 2D–H). Co-expression of dystrophin and C6ORF32 was also noted in cells very closely located to one another that may have been undergoing fusion (Figure 2I, J, arrowheads). To estimate the percentage of C6ORF32-positive cells at different timepoints during myogenic differentiation, the number of cells positive for C6ORF32 over the total number of nuclei in 5 random microscopic fields per each sample were visually counted at days −1, +3 and +5 (day 0 is when differentiation medium was added to the cultures). C6ORF32-positive cells were approximately 5% at day −1 (70% of which stained positive for desmin), and increased to 18.6% at day 3 and to 43.3% at day 5 of differentiation.

Expression of multiple C6ORF32 isoforms in human fetal myoblasts

C6orf32 is an open reading frame putatively encoding 12 alternatively spliced forms (NCBI, AceView). To analyze whether multiple isoforms of this protein are expressed in human fetal muscle cells under differentiation *in vitro*, western blot analysis was performed (Figure 3A). Several isoforms of C6ORF32 protein were detected in human fetal muscle cells during fusion, and the amount of some of these isoforms increased during differentiation (Figure 3A, black arrowheads).

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Two known alternatively spliced forms of *c6orf32* were amplified by RT-PCR from human fetal muscle cells (Iso 1 and Iso 2). Primers were designed according to the nucleotide sequences of AB002384 (1068 aa), which encodes the longest known isoform, and NM_015864 (Iso 2, 591 aa). As shown in Figure 3A, Iso 1 (1018 aa), an isoform known to be expressed in brain (NCBI, AceView), and Iso 2 are expressed during differentiation of human fetal primary myoblasts. C6ORF32 Iso 2 (591 aa) is also present in muscle and is 50 amino acids longer at its N-terminus than PL48, which is expressed in placenta (Figure 3B) (Dakour et al., 1997).

Effect of c6orf32 *mRNA knockdown during muscle cell differentiation*

To investigate the function of *c6orf32* during myoblast differentiation, its expression was inhibited via *in vitro* RNA silencing (RNAi). Similarly to our findings in primary human fetal muscle cells, the expression of mouse *c6orf32* mRNA in C2C12 cells increases approximately 6-fold after 4 days in differentiation medium (data not shown). Two small interfering RNAs (siRNA 536, 612) were generated using oligonucleotides specific to the mouse *c6orf32* sequence and tested on the mouse myogenic cell line C2C12, while a retrovirus encoding the *lacZ* gene was used as control for retroviral infection (Figure 4). C2C12 cells were infected with the retroviruses two days before switching the cultures from proliferation to differentiation medium (Figure 4A). *C6orf32* mRNA expression was analyzed at day 0, while cell fusion was assessed after 4 days in differentiation medium. The efficacy of each siRNA to down-regulate *c6orf32* mRNA expression was assessed via quantitative real-time RT-PCR (Figure 4B). PCR amplifications demonstrated that siRNA 536 and 612 down-regulate mouse *c6orf32* mRNA to 23% and 19% of normal levels, respectively (Figure 4B). *Gapdh* expression in each cell line was also measured as an internal control for non-specific inhibition of the siRNAs, which was not detected. C2C12 cultures infected with retroviruses containing the siRNAs or the control *lacZ* gene were also assessed for cell fusion and expression of the myogenic differentiation marker myosin heavy chain (MHC) (Figure 4C). Cells infected with *c6orf32* siRNAs showed decreased fusion compared to the control culture infected with *lacZ* (Figure 4C). In particular, C2C12 cells infected with the retrovirus encoding siRNA 536 exhibited severe reduction of myotube formation (Figure 4C). The fusion index was determined by counting the number of nuclei within myotubes, divided by the total number of nuclei. These numbers were obtained for 6 random fields in each sample, and numbers were obtained from 4 independent experiments (Figure 4D). Results demonstrated that siRNA 536 and 612 significantly decreased myotube formation compared to control retrovirus-infected cells (Figure 4D).

To determine whether the observed decreased fusion of C2C12 could be a secondary effect due to a defect in the progression of muscle cell differentiation, expression of MyoD1, a master regulator that is expressed in proliferating muscle cells (Lassar et al., 1989) and myogenin, an early marker of myogenic differentiation (Wright et al., 1989) were assessed by immunofluorescence in the cultures infected with retroviruses containing siRNA oligo 536 and 612 (Figure 5A). After 4 days in differentiation medium, the expression of MyoD1 in C2C12 cells treated with *c6orf32* siRNA 536 and 612 was not significantly different from control cultures infected with *LacZ* (Figure 5A). In contrast, expression of myogenin was significantly decreased in C2C12 cells infected with siRNA 536 and 612, suggesting that myogenic differentiation was affected by reduced expression of *c6orf32* (Figure 5A, B). These results indicate that *c6orf32* expression may play a role in early myogenic differentiation and the decreased muscle cell fusion observed after RNA knockdown is a secondary effect due to impaired or slowed differentiation.

Over-expression and localization of tagged C6ORF32 in muscle and non-muscle cells

Inhibition of expression of *c6orf32* mRNA *in vitro* in C2C12 cells demonstrated that down-regulation of this gene results in decreased myogenic differentiation. To gain insight on the

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putative role of this protein, two *c6orf32* constructs were over-expressed in the myoblast cell line C2C12 and in the human-derived kidney cell line, HEK293 (Figure 6). In the constructs, a Flag-tag was placed at either the N- or the C- terminus of *c6orf32*-ISO2 by inserting the appropriate synthetic oligonucleotide into the primer sequence when amplifying *c6orf32*-ISO2 by PCR (Figure 6A). Both constructs were sequenced to confirm the changes yielded to an in-frame product. C2C12 cells (Figure 6B) and HEK293 cells (Figure 6C) were transfected with the plasmids in the presence of lipofectamine. Approximately 20–24 hours post-transfection cells were stained with an anti-Flag antibody and protein expression was detected in both the cytoplasm and filopodia of transfected cells (Figure 6B, C, white arrows). Phase contrast images demonstrated that cells transfected with *c6orf32*-ISO2 plasmids presented long filopodia compared to control HEK293 or C2C12 cells transfected with GFP (Figure 6B, C). These results were also seen in C2C12 cells transfected with *c6orf32*-ISO1 (data not shown). Therefore, overexpression of *c6orf32* in both muscle and non-muscle cells induces formation of filopodia and supports our initial findings in human muscle primary cells, where C6ORF32 was detected in the cytoplasm and cellular filopodia (Figure 1).

To determine the domain of C6ORF32 that promotes the formation of filopodia, serial in frame deletion mutants of *c6orf32* tagged with V5 were constructed and transfected into C2C12 cells (Figure 7B). Cells were examined 20 hours after transfection by immunostaining with a rabbit polyclonal anti-V5 antibody (Figure 7A). Results demonstrated that the N-terminal region (55-113aa) is important for filopodia formation (Figure 7A, Table 1). The percentage of cells with filopodia in C2C12 cells transfected with plasmids encoding mutant forms of *c6orf32* was quantified (Table 1). Transfection with plasmids deleting 53 amino acids from the N-terminus (Figure 7A, N-D54) or deleting 110 amino acids from the C-terminus (Figure 7A, C-D110) did not cause a loss of filopodia formation. However, after transfection with plasmid D113, in which 113 amino acids are deleted from the N-terminal portion of C6ORF32, the percentage of transfected cells exhibiting filopodia decreased from 48% to 10% (Figure 7A, Table 1). Similar results were also obtained using HEK293 cells (data not shown). These results suggest that amino acids 55–113 in C6ORF32 are likely involved in the formation of cell filopodia.

Discussion

Cellular differentiation and fusion are essential steps in muscle development. During these processes, myoblasts undergo changes in expression of myogenic regulatory factors prior to recognizing, adhering and fusing with each other to form myotubes. Several proteins have been identified that are involved in vertebrate muscle cell differentiation and fusion (Lee et al., 1994;Balcerzak et al., 1995;Yagami-Hiromasa et al., 1995;Barnoy et al., 1998;Gorza and Vitadello, 2000;Bois and Grosveld, 2003;Horsley et al., 2003;Dulong et al., 2004). However, despite these reports, detailed understanding of these processes is unknown; therefore, it is likely that several key proteins have yet to be identified. Using microarrays, we sought for genes regulated during fusion of human fetal myoblast *in vitro* (Cerletti et al., 2006). Among the ones that appeared to increase in expression during ‘early’ fusion (2–5 myonuclei) was a gene named *c6orf32*. Expression of *c6orf32* was confirmed to be upregulated during human myoblast differentiation at both the mRNA and protein levels. Localization of C6ORF32 by immunofluorescence in primary human myoblast cultures demonstrated this protein localizes to the cell membrane and in filopodia-like structures. C6ORF32 is expressed in mononuclear cells, not in myotubes, upon induction of differentiation and cellular fusion. Co-staining with the myoblast–specific marker desmin indicated that both myogenic and non-myogenic cells within human primary muscle cultures express this protein. Mononuclear cells expressing C6ORF32 often displayed localization of the protein to one side of the membrane, adjacent to a multinucleated myotube.

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To elucidate the function of C6ORF32 during muscle cell differentiation, down-regulation and over-expression of this protein were studied *in vitro*. RNAi experiments performed on the murine muscle cell line C2C12 demonstrated that down-regulation of C6ORF32 mRNA caused a significant decrease in myoblast fusion. To investigate the step at which C6ORF32 downregulation affects myoblast fusion, cultures of C6ORF32 RNA knockdown were analyzed for expression of myogenic markers, such as MyoD, myogenin and MHC. Results indicated that down-regulation of *c6orf32* correlated with reduced levels of myogenin and MHC, both markers of myogenic differentiation, while the levels of MyoD1, expressed in proliferating myoblasts, were unaltered. These results suggest that impaired cell fusion due to decreased C6ORF32 expression is likely a secondary effect due to altered myogenic differentiation.

Over-expression of *c6orf32* studies in both muscle and non-muscle cells showed increased formation of filopodia-like structures, which supported the localization studies performed on primary human muscle cells. Short filopodia are commonly found in motile cells, such as fibroblasts and keratinocytes (Salas-Vidal and Lomeli, 2004), while longer thin filopodia have been implicated in crucial morphogenetic events during development, including muscle development (Tanaka-Matakatsu et al., 1996;Jacinto et al., 2000;Fulga and Rorth, 2002;Ritzenthaler and Chiba, 2003). Filopodia are formed by actin polymerization, which is regulated by the Rho family of small guanosine triphosphatase (Rho GTPase) such as RhoA, Rac1 and Cdc42 (Hall, 1998;Miki et al., 1998). Signaling through Rho GTPase is mediated by the intracellular proteins WASP and Arp2/3, which result in actin assembly (Snapper et al., 2001). In *Drosophila*, it has been reported that many intracellular proteins involved in myoblast fusion have a role in regulating the actin cytoskeleton (Chen and Olson, 2005). We therefore speculate that C6ORF32 may be a protein involved in cytoskeletal rearrangement during muscle cell differentiation that leads to cell fusion. In the overexpression studies, we identified that amino acids 55-113 are necessary to promote formation of cellular filopodia. Upon overexpression, it was observed that multiple filopodia were induced in HEK293 or C2C12 cells. These results suggest that upregulation of C6ORF32 may be in response to signals that induce cellular movement, including the migration of myogenic cells towards the site where fusion will occur.

In conclusion, we have found that C6ORF32 is a previously unrecognized protein upregulated during human fetal muscle cell differentiation. Downregulation of its expression *in vitro* causes a decrease in differentiation and fusion, while its upregulation promotes formation of cellular filopodia, suggesting a role of C6ORF32 in the cytoskeletal rearrangement during fusion of myoblasts into multinucleated myotubes. Future studies will address whether C6ORF32 is a novel GEF factor by dissecting its putative multiple roles and by defining their precise links to myogenic cell differentiation.

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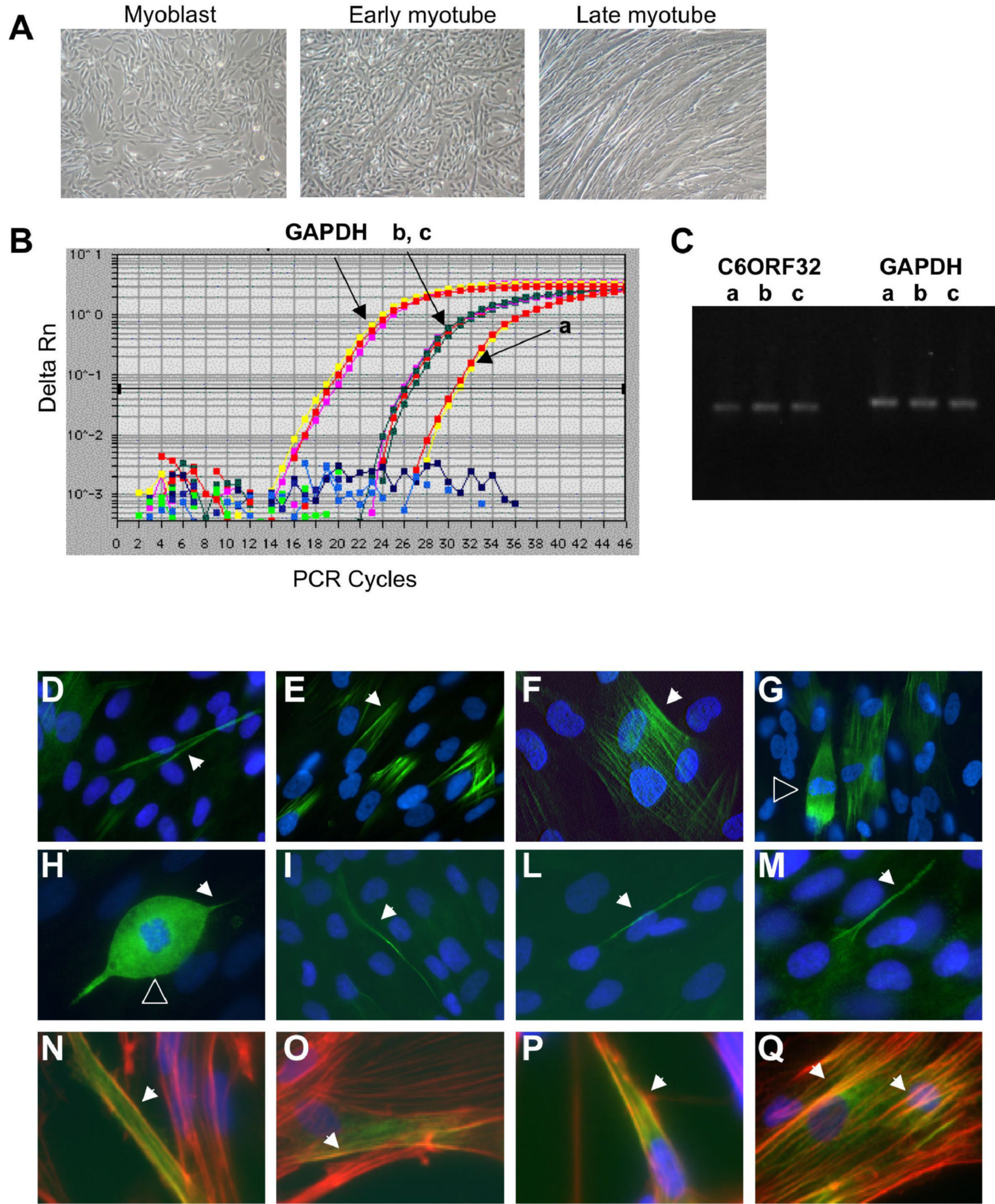

Figure 1.

C6ORF32 mRNA is upregulated upon differentiation of human fetal myoblasts. (A) Phase contrast images of cultured human fetal myoblasts harvested for RT-PCR analyses. (B) Quantitative RT-PCR graph illustrating increased amounts of C6ORF32 mRNA during human fetal myoblast differentiation. a, myoblasts; b, early myotubes; c, late myotubes. Each PCR reaction was performed in duplicate and GAPDH was used as an internal control. (C) The end products (plateau levels) of the RT-PCR reactions were run on an agarose gel, purified and sequenced to confirm their specificity. (D–M) Immunofluorescence detection of C6ORF32 protein (green) in differentiating human primary fetal myoblast cultures. Expression was detected in the cytoskeleton (E–G), rare cells undergoing mitosis (G, H open arrowheads) and

in cellular filopodia (I–M, arrows). (N–Q) Localization of C6ORF32 (green) and phalloidin (red, which binds to F-actin). C6ORF32 appears to localize with F-actin (yellow, arrows), but it is also expressed in the cytoplasm. Nuclei are counterstained in blue with DAPI.

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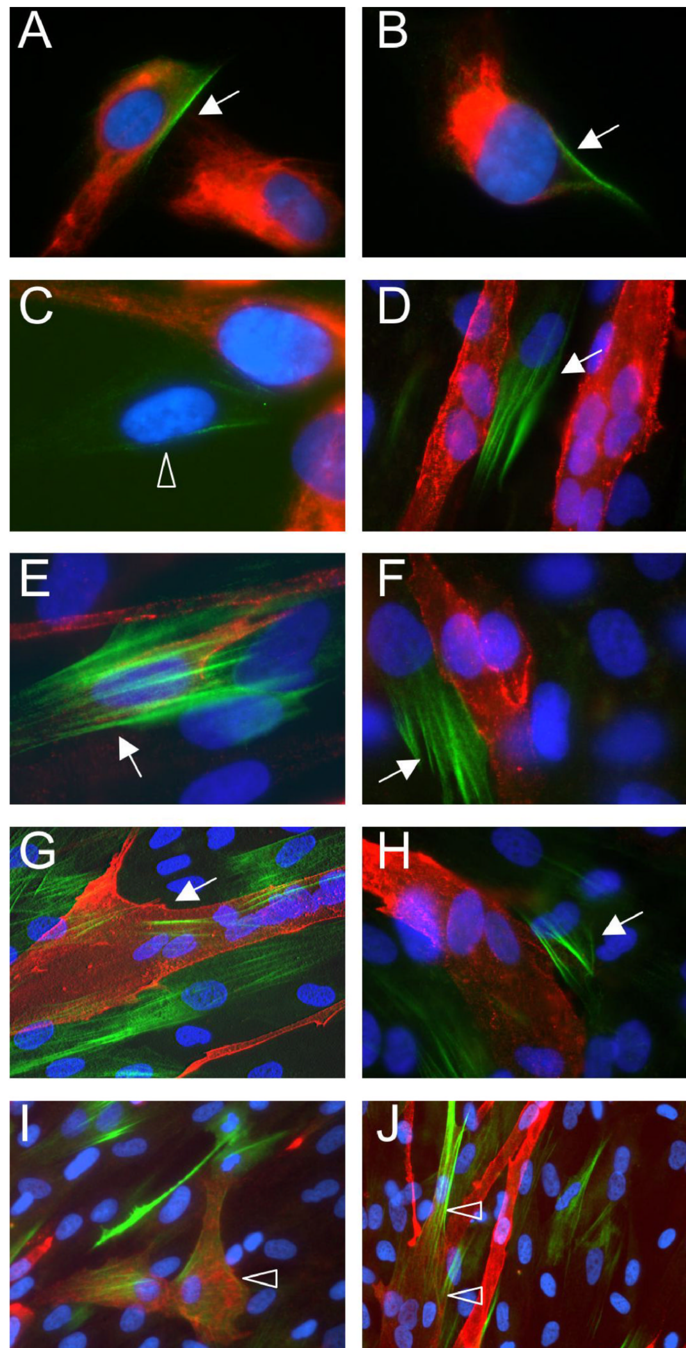

Figure 2.

Expression of C6ORF32 protein during human fetal myoblast fusion. (A–C) At day -1, cells were co-stained for desmin (red) and C6ORF32 (green). Nuclei are stained in blue with DAPI. C6ORF32 is detected both in myogenic (A, B) and non-myogenic cells (C, open arrowhead). (D–J) Human fetal myoblasts were induced to differentiate in low serum medium (added at day 0) for 5 days. Cultures were co-stained for dystrophin (red) and C6ORF32 (green). C6ORF32-positive mononuclear cells were adjacent to dystrophin-positive myotubes (D–H, arrows). (I–J) Co-detection of dystrophin and C6ORF32 was seen is rare cells that appeared to be undergoing fusion (open arrowheads).

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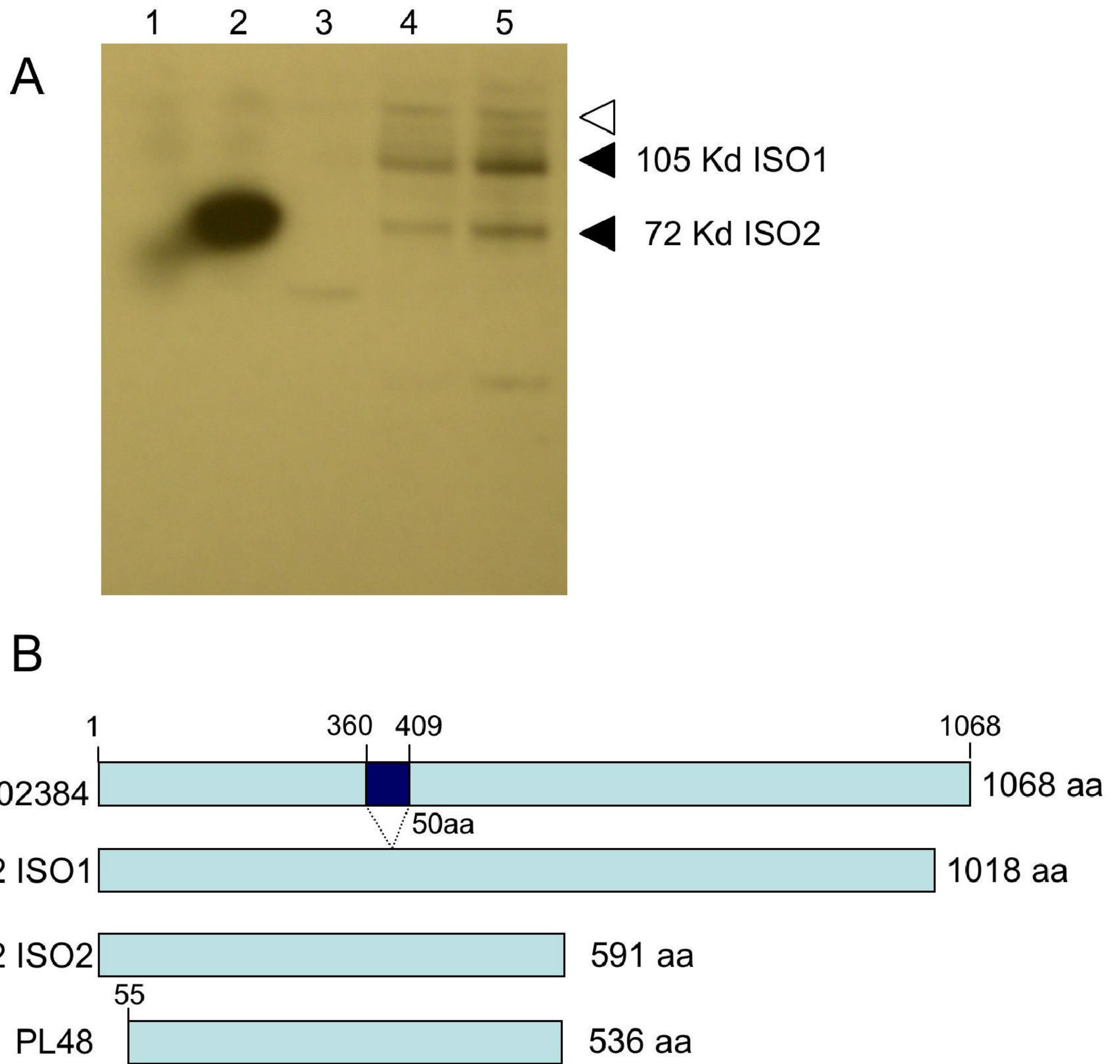

Figure 3.

Multiple isoforms of C6ORF32 protein are upregulated during human muscle cell differentiation. (A) Western blot using mouse anti-C6ORF32 monoclonal antibody. Lane 1, 20 μg of cell lysate from mock-transfected HEK293 cells; lane 2, 10 μg of cell lysate from HEK293 cells transfected with C6ORF32 ISO2-expressing plasmid; lane 3, 45 μg of cell lysate from human primary myoblast cells two days prior to fusion (day −2); lane 4, 45 μg of cell lysate from human primary myoblast cells just before switching medium into differentiation medium (day 0); Lane 5, 45 μg of cell lysate from human primary myoblast cells 1 day after switching medium into differentiation medium (day +1). Black arrowheads point at putative C6ORF32 isoforms that are upregulated during fusion, the white arrowhead points to a band that does not increase during differentiation. (B) Schematics of C6ORF32 ISO1 and ISO2 proteins compared to the known AB002384 and PL48. C6ORF32 ISO2 is a truncated form of C6ORF32 ISO1 protein.

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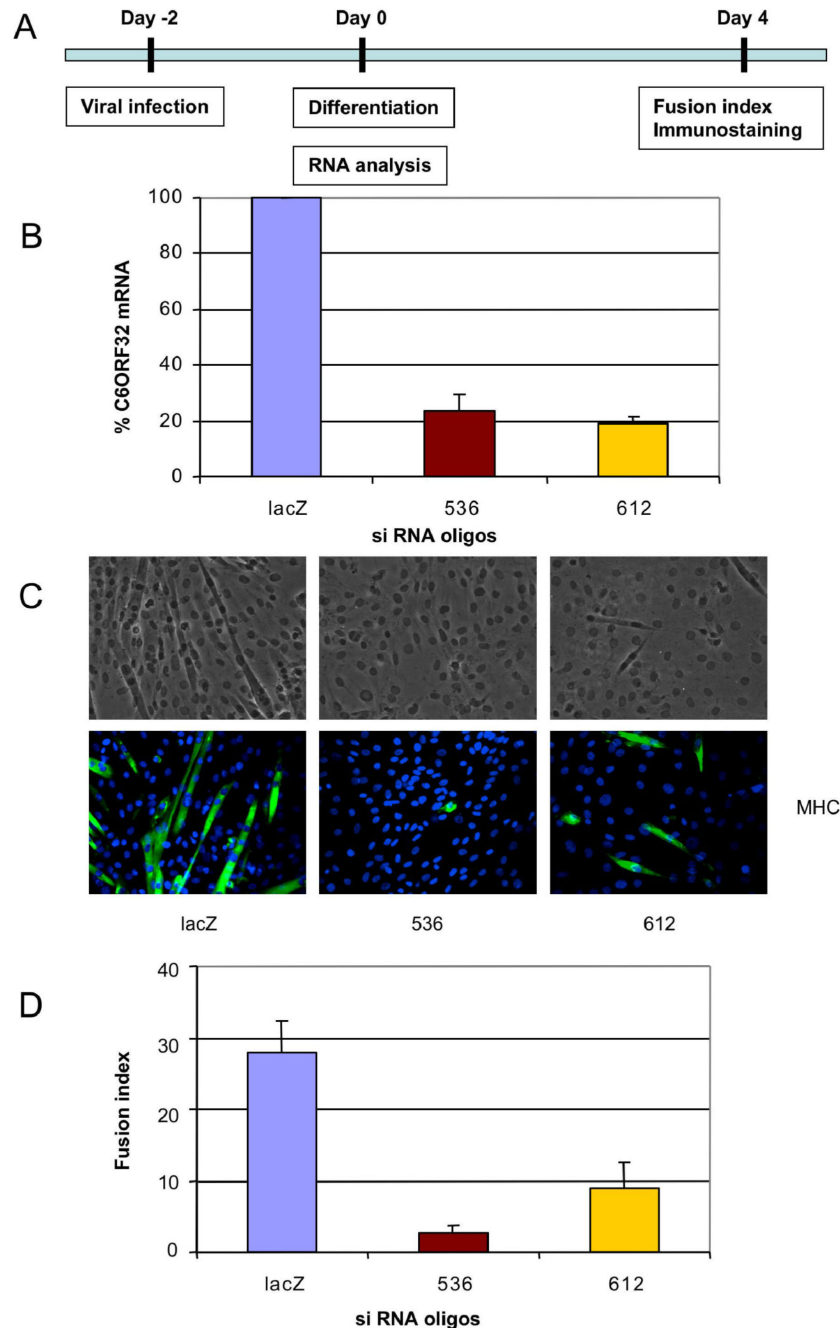

Figure 4.

mRNA knockdown of *c6orf32* in C2C12 cells infected with retroviruses containing two distinct siRNA oligos. (A) Schematic representation of RNA interference experiment. (B) Quantitative real-time RT-PCR was performed to estimate the amount of *c6orf32* mRNA after treatment with each siRNA, which was approximately 20% of normal levels. (C) mRNA knockdown of *c6orf32* slows myotube formation in C2C12 cells, as represented by phase contrast and by immunostaining of the cultures for myosin heavy chain (MHC, green). Note that the cultures with decreased *c6orf32* mRNA expression have also low numbers of MHC-positive cells. (D) Fusion index of C2C12 cultures treated with control or siRNA for *c6orf32*. Both oligo 536 and 612 exhibited significant decrease in myotube formation compared to control cultures.

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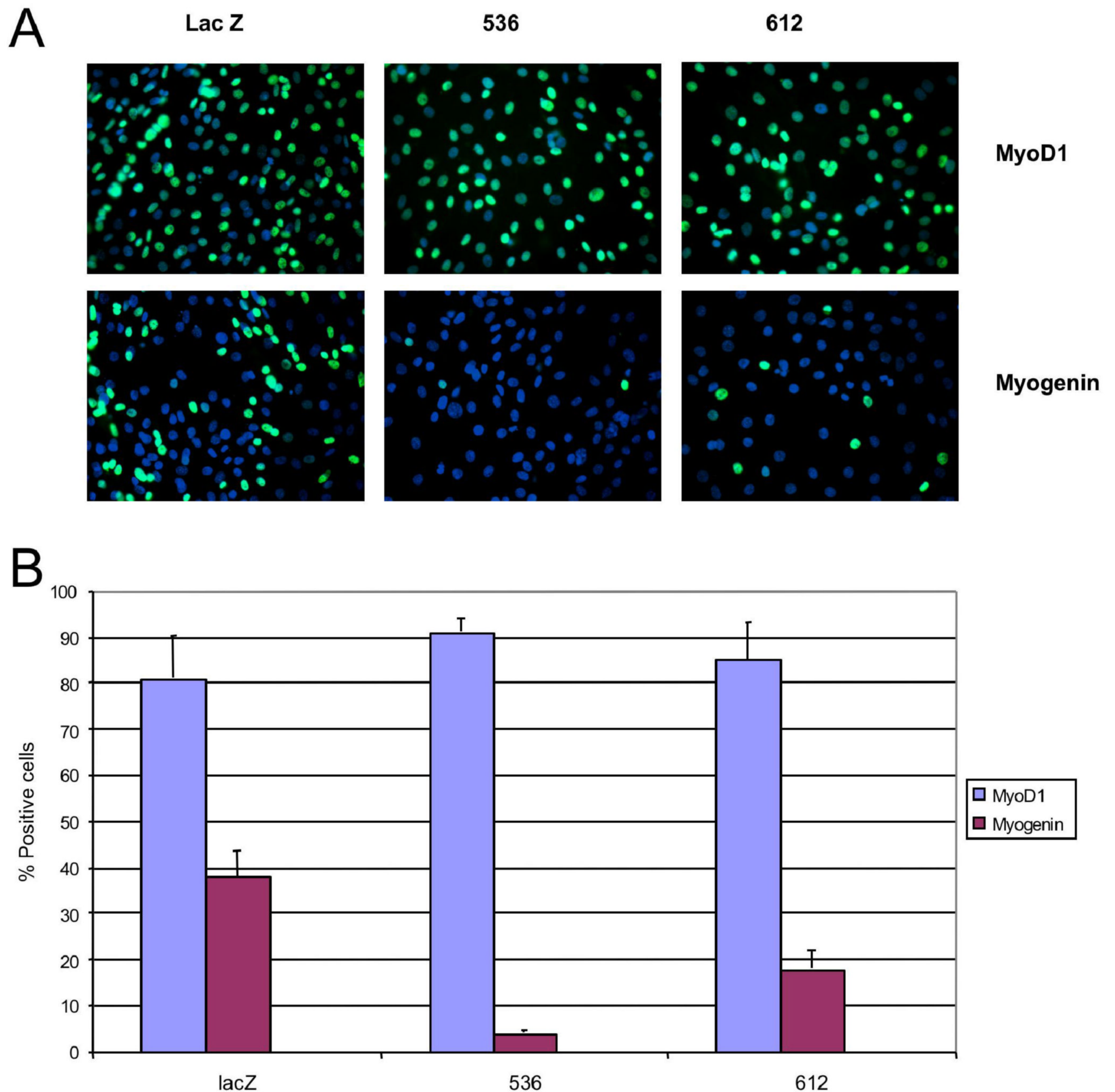

Figure 5.
Expression of MyoD1 and myogenin in C2C12 cells after knockdown of *c6orf32* mRNA. (A) C2C12 cells at day 4 in differentiation medium were immunostained with MyoD1 or myogenin (green) and nuclei were counterstained with DAPI (blue). (B) Quantitation of MyoD1$^+$ and myogenin$^+$ cells demonstrate that the percentage of MyoD1$^+$ cells is not affected after treatment with siRNA oligos 536 or 612 compared to the control culture, while the expression of the early myogenic differentiation marker myogenin is significantly decreased in cultures with reduced *c6orf32* mRNA expression.

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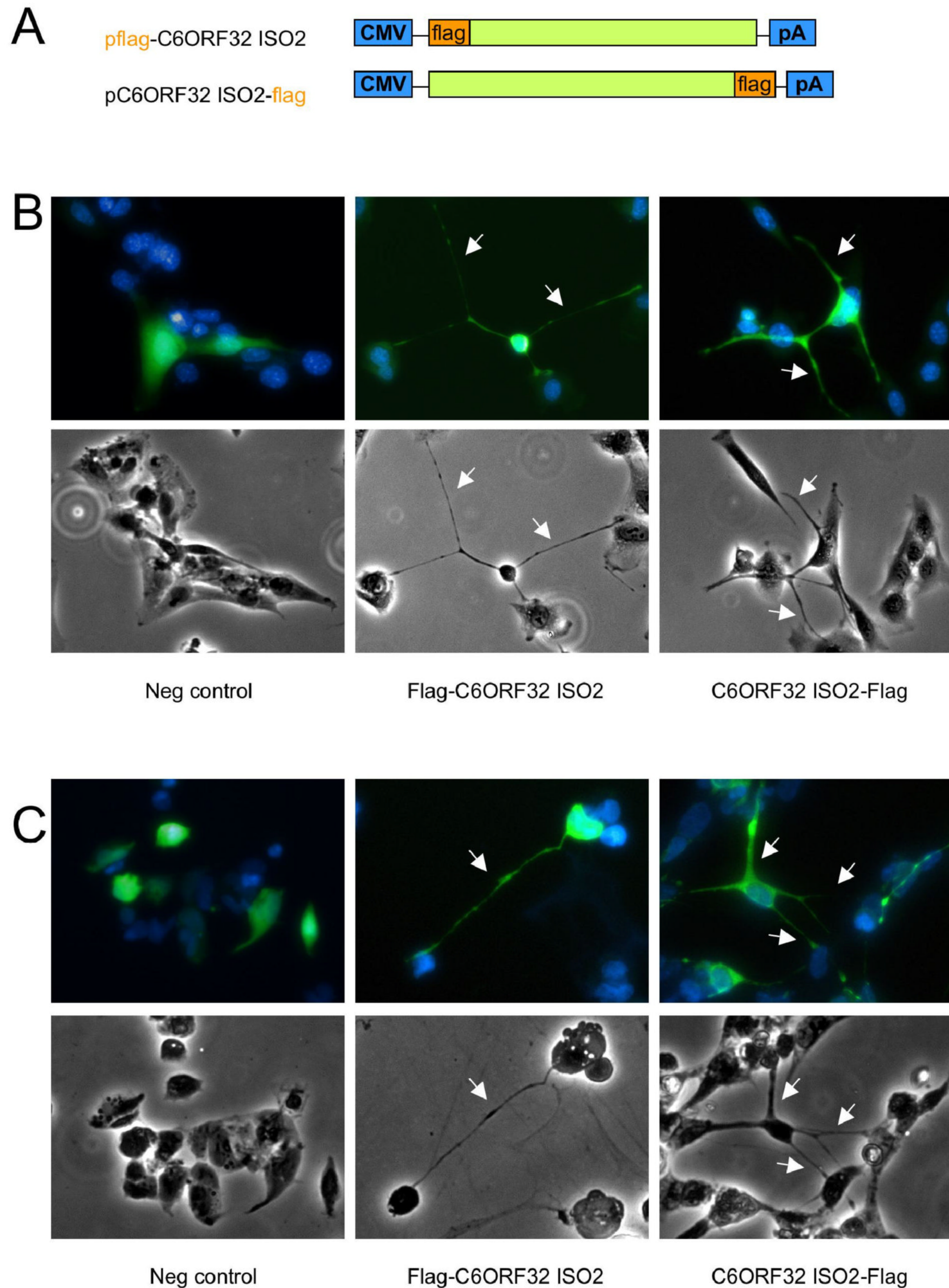

Figure 6.

Over-expression of C6ORF32 in murine C2C12 (B) and HEK293 cells (C). (A) Schematic representation of the constructs used for transfection. CMV is the human cytomegalovirus enhancer/promoter and pA is the BGH polyadenylation signal. Protein expression in cells transfected with pFlag-*c6orf32*-ISO2 or p*c6orf32*-ISO2-Flag was detected using an anti-flag antibody (green) and nuclei were counterstained with DAPI (blue). Images demonstrate the presence of long filopodia in transfected C2C12 (B) and HEK293 cells (C), but not with the control plasmid encoding for GFP.

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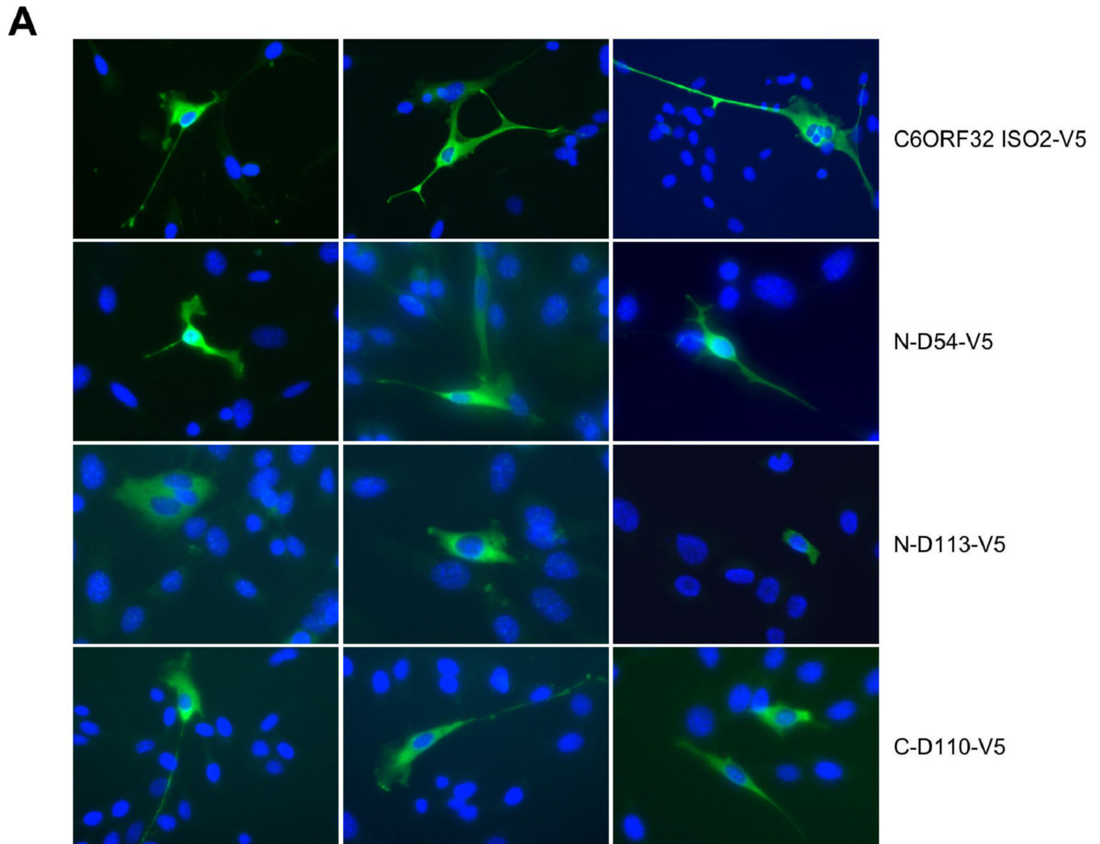

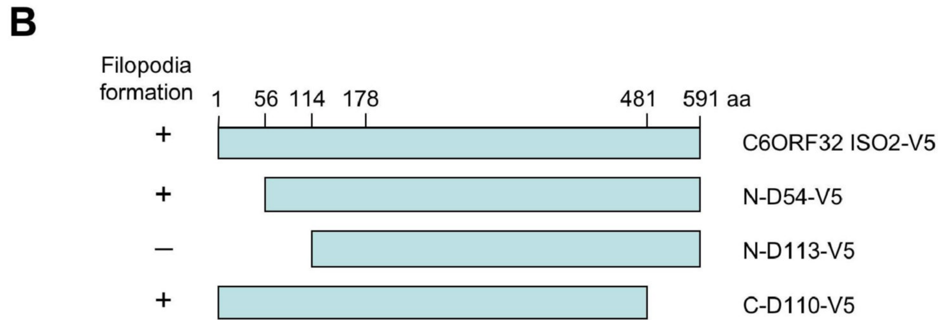

Figure 7.

The N-terminus domain of C6ORF32 (aa 55-113) is important for formation of cellular filopodia. (A) C2C12 cells were transfected with each designated plasmid and immunostained with anti-V5 polyclonal antibody (green), cell nuclei were counterstained with DAPI (blue). Cells transfected with N-D113-V5 exhibited decreased number of filopodia. (B) Schematic of deletion mutants constructed to determine the region of C6ORF32 responsible for the formation of filopodia.

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Table 1

Percentage of C2C12 cells with filopodia after transfection of C6ORF32 deletion mutants.

Plasmid	Total (n)	+Filopodia (n)	−Filopodia (n)	%Filopodia
pC6ORF32 ISO2	605	295	310	48
N-D54	131	62	69	47
N-D113	185	20	165	11
C-D110	398	192	206	48

C2C12 cells were transfected with each plasmid as specified and immunostained with polyclonal anti-V5 antibody 20 hours after transfection. The overall transfection efficiency for mutants D54 and D113 was lower than in cells transfected with C6ORF32-Iso2 and C-D110. The percentage of filopodia was calculated by dividing the number of transfected cells exhibiting filopodia (+filopodia) by the total number of C2C12 transfected cells.