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Dp71, utrophin and β-dystroglycan expression and distribution in PC12/L6 cell co-cultures.

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RUNNING TITLE

Dp71 in PC12-L6 co-cultures

Abstract

Dystrophin Dp71 is the most ubiquitous and highest expressed dystrophin isoform in brain, however, Dp71 function and those specific for its spliced d- and ab- isoforms remains undetermined. To study Dp71, utrophin and β-dystroglycan in cell-to-cell interactions, we first established a co-culture model using PC12 cells and L6 myotubes. Confocal microscopy assays of these co-cultures, in which PC12 cells are differentiated in the presence of L6 myotubes, showed that the Dp71d isoform accumulates in PC12 nuclei, Golgi-complex- and endoplasmic reticulum-like structures, being depleted from neurites and cytoplasm, while Dp71ab concentrates at neurite tips and cytoplasm and colocalizes with β-dystroglycan, utrophin, synaptophysin and acetylcholine receptors. Evidences suggest Dp71ab isoform unlike Dp71d, may take part in neurite-related processes. This is the first work on the role of dystrophins as well as members of the DAP complex in a cell-line based co-culturing system, which may prove useful in determining protein associations in a more controlled environment than ex-vivo systems.

Keywords: β-dystroglycan, dystrophin Dp71, L6, PC12, utrophin

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ABBREVIATIONS

α-DG: α-Dystroglycan.

β-DG: β- Dystroglycan.

ACh: Acetylcholine.

AChE: Acetylcholinesterase.

AChR: Acetylcholine Receptor.

araC: Cytosine Arabinoside.

CK: Creatine Kinase

DMD: Duchenne Muscular Dystrophy.

D-MEM: Dulbecco-modified Eagle Medium.

DAP: Dystrophin Associated Protein.

Dp: Dystrophin.

DRP: Dystrophin-Related Protein, utrophin.

ERG: Electroretinogram.

FBS: Fetal Bovine Serum

HS: Horse Serum.

ILM: Inner Limiting Membrane.

sLN: s-Laminin.

MASC: Myotube Associated Specificity Component.

NGF: Neural Growth Factor.

NMJ: Neuromuscular Junction.

NOS: Nitric Oxide Synthase.

OPL: Outer Plexiform Layer.

PAGE-SDS: Poly-Acrylamide Gel Electrophoresis under denaturing conditions (with Sodium Dodecyl Sulphate).

Up: Utrophin.

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Introduction

The Duchenne muscular dystrophy (DMD) gene is the largest known to date, encoding for a family of proteins known as dystrophins (Dp), with molecular weights ranging from 30 kDa up to 427 kDa [34]. While the exact role for the different dystrophin isoforms remains unclear, current opinion proposes that dystrophins are important for cell structure maintenance, at both the intracellular and extracellular level. Dystrophin has an autosomal protein homologue, named utrophin (Up). At least 4 different Up products have been detected [7, 48, 69, 74]: Up395, Up140, Up110 -G-utrophin- and Up80. Up overexpression can overcome, to some extent, the Dp-deficient phenotype [61]; however, Dp and Up seem to have different roles since they are often restricted to distinct subcellular locations, and their interactions with dystrophin associated proteins and others vary in affinity and origin.

The dystrophin associated proteins (DAP) complex is a large group of proteins inserted into the cell membrane that can interact directly or indirectly with dystrophin [21, 39, 75] and to some extent with utrophin [71]. The DAP complex components vary in a tissue-specific manner and comprise mainly sarcoglycans – α, β, γ, δ, ε and ζ-, dystroglycans – α and β-, sarcospan and syntrophin, most of which are transmembranal proteins [2, 20, 56]. There are other proteins that interact with DAP, and thus, DAP complex activity and function depends largely on the proteins associated to it. Its absence can induce, or be a consequence of, muscular dystrophy as well [18, 55]. Furthermore, at least one DAP, β-dystroglycan, has been found to be involved in myoneural synapses. [13, 40].

The interaction between the membrane components can respond to different requirements, as seen at the neuromuscular junction (NMJ) [29, 60]. In these cases, dystrophin and utrophin are proposed to be an anchor for surface receptor complexes in the cell membrane using DAP and other membrane proteins as coupling links, contributing to surface receptor clustering and

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stabilization at specific sites [57]. While most of the information on the role of dystrophins and DAP in synapse formation and stabilization has been gathered from the post-synaptic region of the neuromuscular junction [3, 9, 26, 29, 33, 36, 49], the pre-synaptic part is poorly understood. Few elements have been described as indispensable for NMJ formation comprising the terminal part of the motor neuron that innervates the muscle fiber. Studies have focused mostly on factors liberated at the tip of the axon (agrin) [17, 22], and the neurotransmitter (acetylcholine, ACh) release pattern upon stimulation and degradation.

Few approaches have elucidated the role of dystrophins and DAP in pre-synaptic activity, but interesting results have arisen in studies of the retina. It has been found that dystrophin isoforms Dp427, Dp260 and Dp71, as well as utrophin, show distinct subcellular localization. Dp427 and Dp260 seem to be more related to the outer plexiform layer (OPL) in mouse retina, whereas Dp71 is localized to the inner limiting membrane (ILM) and retinal blood vessels [35]. Moreover, Dp71 is required for proper localization of the Kir4.1 potassium channel and water pore aquaporin 4 (AQP4) to the endfeet of Müller glial cells in murine retina [12, 14]. Interestingly, not only AQP4 subcellular location but its expression is also affected in Dp71null mice, indicating that Dp71 is not only involved in protein localization, it is also directly or indirectly involved in protein stability or synthesis.

Dp71 expression depends upon a very complex mechanism of posttranscriptional control since its mRNA has several alternative splicing sites. Differential splicing of exons 71 through 74, and 78 [42, 44, 62], leads to multiple Dp71 isoforms, with different protein-protein affinities and subcellular localizations. Of these isoforms, our group has identified those expressed in PC12 cells; isoforms Dp71 a and c, which share the same carboxyl-terminus as dystrophin, and are also known as d-dystrophins, and isoform ab, that possess a novel C-terminal sequence in which the last 13 hydrophilic amino acids are replaced by 31 hydrophobic amino acids, and that is formerly known

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as f-dystrophin [44, 50] (Genbank Accession Numbers AY326947, AY326948 and AY326949, respectively).

Interestingly, subcellular localization of ab- and d- dystrophins is very different, at least in PC12 cells, HeLa, C2C12 and N1E115 [28, 50]. D-dystrophin (Dp71d) is highly concentrated in PC12 cell nuclei upon differentiation, being found also in cytoplasm and neurites, while f-dystrophin (Dp71ab) is absent from nuclei and seems to accumulate in aggregates near neurite tips and varicosities, colocalizing with synaptic-like microvesicle protein synaptophysin. Using anti-sense technology, it has been shown that inhibition of Dp71 expression lead to diminished neural growth factor (NGF)-induced neurite outgrowth [1] and cell adhesion disturbances [19].

All of these results suggest that, although Dp71 shares some domains with the rest of the Dp molecules, its role in cell function is clearly different. These findings, along with the results indicating that Dp71 seems to be linked to Kir4.1 and AQP4 localization, and the limited knowledge of the presynaptic end of the NMJ, as well as PC12 cells expressing mainly Dp71 isoforms [10, 28, 50], make PC12 cells a very interesting model to test the possible role for Dp71 in NMJ formation, at the presynaptic end. In this work, we addressed the problem by identifying the subcellular localization of Dp71 isoforms, utrophin and β-dystroglycan in a co-culture model based upon the differentiation of PC12 cells in the presence of L6 myotubes, similar to that described by Schubert [68], where PC12 cells were found able to develop electrically functional synapses when co-cultured with L6 myotubes. Additionally, possible interactions between Dp71 isoforms and AChR in co-cultured cells were investigated.

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Materials and Methods

Cell culture. PC12 cells were cultured in 75 sq. cm flasks, using Dulbecco Modified Eagle Medium (D-MEM) (Invitrogen, California, USA) supplemented with 10% Horse Serum (HS) (Invitrogen, California, USA), 5% Fetal Bovine Serum (FBS) (Invitrogen, California, USA), and antibiotic-antimycotic (Invitrogen, California, USA) at a final concentration of 100 U of penicillin, 100 micrograms of streptomycin and 250 nanograms of fungizone (amphotericin B) per mililiter. L6 rat myoblasts (CRL-1458, ATCC, Virginia, USA) were grown in 75 sq. cm flasks in D-MEM medium with 10% FBS and 1 mM MEM sodium pyruvate (Invitrogen, California, USA), but for all L6 culture and co-culture assays, 100 U of penicillin and 100 micrograms of streptomycin per ml was used instead of antibiotic-antimycotic, since fungizone, present in the antibiotic-antimycotic mixture, is known to affect L6 cells morphology and myotube formation [43]. Myotube formation was achieved by allowing L6 myoblasts to reach confluence and switching medium to D-MEM 1% HS; after 7 days in this medium, differentiation was assessed by morphology, RT-PCR of rat myogenin mRNA and creatine kinase (CK) activity (Creatine Kinase (NAC) Assay, Diagnostic Chemicals Limited, Prince Edward Island, Canada). All cells were incubated at 37ºC in a humidified incubator with a 5% CO_2 atmosphere.

Protein crude and total RNA extracts. L6 cells were grown as described above and allowed to reach about 60% confluence. For myoblast assays, cells were harvested at this point, and for myotube assays, cells were cultured until confluence was achieved; then, medium was replaced by D-MEM 1% HS, incubation continued for 7 days and then cytosine arabinoside (araC) (Sigma, Missouri, USA) was added at a final concentration of 0.5 microgram/ml to prevent myoblast proliferation. After culturing for 3-4 additional days, araC was removed and cells were incubated for 1 more day and collected. Cells were then divided into two groups: the first group

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was centrifuged and pellets were resuspended in 250mM Tris 1mM EDTA pH 8.0, chilled-thawed cyclically 7 times for 5 minutes each, quantified using Bradford assay and used as protein crude extracts: the second group was treated for total RNA extraction using the RNAid kit (Bio 101, OHIO, USA). For undifferentiated PC12 crude extracts, cells were grown as described above, and collected upon reaching 50% confluence, and chilled-thawed as for L6 cells.

Nuclear and cytoplasmic extract preparations. Nuclear extracts were prepared as follows [67]: PC12 cells were grown as for crude extracts described above, and then placed on ice. All procedures were performed at 4ºC. Cells were washed twice with cold TD buffer (NaCl 137 mM, KCl 5 mM, Na2HPO4 0.7 mM, Trizma-Base 25 mM, pH 7.4), scraped and transferred to a 1.5 ml microcentrifuge tube. Cells were centrifuged at low speed for 3 minutes and the supernatant was discarded. The pellet was resuspended in 400 ul of Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 1X Complete protease inhibitors (Roche, Basel, Switzerland)) and incubated on ice for 15 minutes, then 25 ul of 10% Nonidet NP-40 (Fluka) detergent was added, the mixture vortexed vigorously for 30 seconds, pelleted by centrifugation for 30 seconds, and supernatant was used as cytoplasmic extract. The pellet was then resuspended in 50 ul of buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 1X Complete protease inhibitors (Roche, Basel, Switzerland)), vigorously rocked on a shaking platform for 20 minutes, pelleted by centrifugation for 5 minutes, and supernatant containing nuclear extracts was collected and tested by Western blot.

Western blot assays. To determine the pattern of expression of dystrophins, utrophin and β-dystroglycan in L6 cells, Western blot assays were performed with 50 μg of crude protein extracts using rat brain and rat or mouse muscle extracts as control. Antibodies used for Western

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blot were H5A3, a monoclonal panspecific anti-dystrophin and anti-utrophin antibody; H4, polyclonal antibody that detects d-dystrophins; 5F3, monoclonal Ab that detects specifically the ab-Dystrophins C-terminus [24]; K7, polyclonal for utrophin detection; Jaf or LG5, both of polyclonal origin, were used for β-DG assays. Monoclonal anti-α actin antibody was a kind gift of Dr. Manuel Hernandez, while monoclonal mouse anti-AChR α subunit antibody (RDI-ACHERAabm) was purchased from Research Diagnostics Inc (Massachusetts, USA). Finally, synaptophysin polyclonal antibody was purchased from DAKO (Denmark). Western blots were carried out on nitrocellulose Hybond-N (Amersham, New Jersey, USA) membranes and treated with the ECL Western blot kit (Amersham, New Jersey, USA) as described by the manufacturer.

RT-PCR assays. For RT-PCR assays, 2 micrograms of total RNA were subject to cDNA synthesis using random primers Kit and M-MLV retrotranscriptase (Invitrogen, California, USA), and for each reaction 5 microliters of cDNA were added to a final volume of 50 microliters of a mixture containing 1.5 mM MgCl2, 1.5 U of *Taq* DNA Polymerase (Invitrogen, California, USA), 0.2mM of each dNTP, and 20 pmol of the appropiate oligonucleotide set. Oligonucleotide sequences, reaction conditions and product sizes are summarized in table 1. PCR was performed using a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, California, USA) and PCR products were subjected to electrophoresis in 1% agarose gels.

Differentiation of PC12 cells in the presence of L6 myotubes. L6 myoblasts were plated on coverslips that were previously treated overnight at 37°C with 1 microgram/ml of poly-L-lysine (Sigma, Missouri, USA); cells were cultured until local confluence was obtained and then the medium was replaced by 1% HS D-MEM. After 7 days, cytosine arabinoside (araC) (Sigma, Missouri, USA) was added to the medium at a final concentration of 0.5 μg/ml to avoid myoblast proliferation, and cells were incubated for 3 more days; araC was later removed from the medium

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and about 10000 dissociated PC12 cells were placed on each coverslip. Co-cultures were kept for 7 days in 1% HS D-MEM to which NGF (Invitrogen, California, USA) was added at a final concentration of 100 ng per ml, and were subsequentially used for immunofluorescence assays.

Indirect immunofluorescence assays. Coverslips previously treated overnight at 37°C with 1 microgram/ml of poly-L-lysine (Sigma, Missouri, USA), containing either L6 myotubes, differentiated PC12 cells, or co-cultures of differentiated PC12 cells and myotubes were subject to the following treatment. Coverslips were rinsed in cytoskeleton buffer (CB), consisting of 10mM Methyl Sulphonic Acid (MES), 150 mM NaCl, 5mM MgCl2, 5mM EGTA pH 7.0, 0.9 mg/ml Glucose, and then fixed and permeabilized for 5 minutes in 3% paraformaldehyde (PFA)-0.5% Triton X-100 in CB, washed 3 times for 5 minutes each in CB, and then fixed in 3% PFA for 20 minutes. They were then washed 3 times for 5 minutes in PBS (29mM Na_2HPO_4, 8.5mM NaH_2PO_4, NaCl 154mM, pH 7.2) and blocked with 0.5% gelatin for 20 minutes. Slips were rinsed in PBS and primary antibodies were added at the proper dilution. Incubation times varied depending on the source of the primary antibody, but were generally 30-minute periods at room temperature, except for 5F3 antibody, which was incubated overnight at 4°C. For some assays, isospecific LG5 antibody was used instead of Jaf. Secondary antibodies were Alexa 488 linked donkey anti-mouse (Molecular Probes), and CY3 linked goat anti-rabbit (Amersham, New Jersey, USA). For AChR detection, anti-AChR and Alexa 594 linked α-bungarotoxin (BTx) (Molecular Probes, Invitrogen, California, USA) at a concentration of 3 micrograms/ml, were used indistinctively, since signal detected with either one was identical (data not shown). All preparations were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and analyzed using a Bio-Rad 1024 (Bio-Rad Laboratories, California, USA) confocal microscope, and Confocal Assistance 4.0 software (Todd Clark Brelje, University of Minnesota).

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Results

Expression of dystrophin, utrophin and β-DG in L6 cells. Since dystrophin, utrophin and β-dystroglycan expression pattern in L6 cells had not been documented, Western blot as well as RT-PCR assays were performed. By using the dystrophin-utrophin panspecific antibody H5A3 (Fig. 1A), it was revealed that L6 cells expressed either dystophin and utrophin isoforms, or both, which resembles previous work [64]. A band corresponding to 140 kDa was shown to increase in expression 6-fold as a result of L6 myotube formation (Fig. 1A, lanes 4 and 5), while an approximately 400 kDa isoform, detected by H5A3, decreased to almost 50%. A 30 kDa isoform that was absent in brain, muscle and undifferentiated PC12 cells (lanes 1 through 3), was present at the same levels in myoblasts and myotubes (lanes 4 and 5). In these cells, Dp71 was not detected by H5A3 under assay conditions. Using the anti-utrophin (K7) antibody (Fig. 1B), bands corresponding to Up395 and Up140 were revealed, both in myoblasts and myotubes. H4, a d-dystrophin specific antibody, showed 116 and 71 kDa bands in myoblasts and an increase in the latter in myotubes (Fig. 1C lanes 4 and 5); Dp140d was low and Dp427 was undetected in myoblasts but both were detected in myotubes, with a notorious high upregulation of Dp427. A band that migrated at about 180 kDa (p180), which has not been previously described, increased in expression in myotubes. 5F3 antibody detection of ab-isoforms (Fig 1D), showed very low levels of expression of Dp71ab in myoblasts (lane 3) and even lower in myotubes (lane 4). As for β-DG a strong 48 kDa band was found when using LG5 to reveal β-dystroglycan in myoblasts and in myotubes (data not shown).

RT-PCR analysis showed that, as expected, Dp427 mRNA expression increased when myoblasts differentiated into myotubes (Fig. 2A lanes 5 and 6). Dp260 mRNA as well as DP140 mRNA were not expressed in L6 cells, only in rat eye (Fig. 2B lane 4), brain and eye (Fig. 2C lanes 2 and

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4), respectively. Dp116 180 bp PCR product (Fig. 2D) was detected low in brain and muscle (lanes 2 and 3), but clearly detected in eye, myoblasts and myotubes (lanes 4-6). Finally, Dp71 was detected in all samples, including rat muscle (Fig. 2E lanes 2-6). All proper controls yielded negative amplification, except the control using Dp116 oligonucleotides, which showed a band at approximaterly 850 bp; this band also appeared in PCR amplifications (upper band in Fig. 2D lanes 2-6), which is the expected size that corresponds to chromosomal amplification [8].

Co-cultures of differentiated PC12 cells and L6 myotubes. In order to obtain co-cultures, L6 cells were differentiated by confluence and low-serum medium, as indicated in Materials and Methods. Differentiation was confirmed by visual assessment, and metabolically using creatine phosphokinase activity and myogenin mRNA expression as differentiation markers. L6 myotubes exhibited a 20-fold increase of activity of creatine kinase as compared to myoblasts (mean 19.79 ± 0.02), while myogenin mRNA expression was only detected and at high levels in differentiated L6 cells, not in myoblasts (data not shown). Prior to culturing PC12 cells in the presence of L6 myotubes, PC12 cells were grown in D-MEM; under these conditions, the cells were responsive to NGF exhibiting large neurites in a great number of cells. PC12 cells differentiated well in the presence of L6 myotubes; very long neurites, ranging up to 0.5 mm or more in length, arise from PC12 cells body (Fig. 3) reaching the myotubes surface. PC12 cells also showed low branching levels, and their neurites seemed to be directed towards L6 cells. These data suggest that PC12 cells differentiated in the presence of L6 myotubes can form NMJ-like structures in a well-oriented manner.

Subcellular localization of dystrophins, utrophin, β-dystroglycan and AChR in PC12 and L6 cells. Prior to co-culture testing, subcellular localization of ab- and d-dystrophins, β-

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dystroglycan, utrophin and AChR was determined in separate cultures of undifferentiated and differentiated PC12 cells, as well as of L6 myoblasts and myotubes, by indirect immunofluorescence using the antibodies 5F3, H4, K7, LG5 and anti-AChR, respectively.

For PC12, the only DMD gene products that have been detected are the Dp71 isoforms [50,64]; thus, 5F3 and H4 antibodies showed that Dp71ab and Dp71d distribution in differentiated cells differed greatly; while Dp71ab was located in cytoplasm, neurites and was absent almost entirely from cell nucleus, Dp71d was heavily concentrated in the nucleus, as previously described [50] (Fig. 4A). Meanwhile, utrophin showed a similar localization pattern as Dp71d (Fig. 4B).

Since we were interested in determining whether cell-to-cell contacts between distal tips of the PC12 neurites and sarcolemma of L6 myotubes might resemble neuromuscular junctions , either functionally or structurally, we tried to identify AChR clusters in L6 sarcolemma upon neurite contact stimulation. Thus, we initially tested AChR detection in PC12 cells, and we found a strong positive signal for AChR-α-1 subunit in PC12 cell nuclei besides a signal along cytoplasm and neurites (Fig. 4C). To confirm these results, a Western blot assay was performed using nuclear and cytoplasmic extracts of PC12; Figure 5 shows the corresponding AChR-α1 subunit band present in the nucleus (lane 4).

We analyzed the subcellular localization of d- and ab-dystrophin, β-dystroglycan and AChR in L6 myotubes (Fig. 6A and B), and found that d-Dys behaved in a similar way to d-Dp71 in PC12 cells, concentrating in nuclei and almost absent in the cytoplasm, while ab-dystrophin was located throughout the cytoplasm and sarcolemma. Also, d- and ab-dystrophins in L6 myotubes (Fig. 6B), as seen in PC12 cells, seemed to be mutually exclusive since Dp71d was concentrated in nucleus, endoplasmic reticulum- and Golgi complex-like structures, while Dp71ab was located in cytoplasm, neurites and membrane, and depleted from nucleus. β-dystroglycan accumulated in

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the nucleus and was also present in the cytoplasm and sarcolemma. AChR was located all over the L6 myotube surface (Fig. 6A).

Distribution of dystrophins in L6 and PC12 co-cultures. The ab- and d-dystrophins detected in PC12 cells in co-cultures showed a strong difference in localization. d-dystrophins were confined mainly in to the nucleus and a structure that resembled the endoplasmic reticulum- and Golgi complex-like structures, and were almost entirely absent from neurites, while ab-dystrophins were located in the cytoplasm, membrane, neurite, varicosities and concentrated in neurite growth cones (Fig. 7A). This distribution of Dp71d contrasted with previous assays using PC12 cells monocultures, where Dp71d was found along neurites [50].

Utrophin and β-dystroglycan (Fig. 7B and C respectively) signal correlated to a high degree with Dp71ab, along cytoplasm, neurites and membrane, but unlike Dp71ab, β-dystroglycan and, to some extent, utrophin, accumulated in PC12 cell nuclei. To determine the possibility that ab-dystrophins might take part in synaptic-like cell-signaling from PC12 cells, synaptophysin localization was compared to ab-dystrophins (Fig. 7D), showing that ab-dystrophins do co-localize with synaptophysin, with a high accumulation inside the tip of the neurite growth cone. Furthermore, ab-dystrophins colocalized with synaptophysin in the inside of the growth cone, and were surrounded by a synaptophysin signal.

When ab- and d-dystrophin location was compared to that of AChR (Fig. 8), it became evident that ab-dystophins and AChR co-localized in the PC12 cytoplasm, membrane and neurites (Fig. 8A, right panel), but not in the nucleus, where ab-dystrophins were almost absent. As for d-dystrophins, they colocalized in most of cytoplasm and nucleus, but were depleted from neurites (Fig. 8B, right panel).

Finally, due to the high-level signals and unexpected subcellular distribution of AChR in PC12 cells in co-culture, we analyzed the colocalization of AChR with synaptophysin (Fig. 9A), β-dystroglycan (Fig. 9B) and utrophin (Fig. 9C) . AChR was distributed in cytoplasm and along neurites, while concentrating in the distal tips and varicosities. In the contacts of the neurite growth cones with L6 myotubes sarcolemma, there seemed to be an accumulation of AChR signal, suggesting that these formed a NMJ-like structure (Fig. 9, arrows). AChR colocalized highly with synaptophysin and β-dystroglycan, but not with utrophin, codistributing mainly at the distal tip of the neurite, and to a much lower extent along the neurites.

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Discussion

The use of cell lines as models for studying dystrophins and DAP functions allow us to analyze the role for individual elements that would be harder to test in *ex vivo* experiments [15]. The drawback of using cell lines as model for dissection of cell processes is that cell-to-cell interactions are often missing and this misleads research since such systems may not respond naturally. This is the main reason why the model PC12/L6 co-culture gains relevance, since we established conditions for testing dystrophins and DAP in a less isolated environment: one in which cells can interact with others in ways that resemble more accurately actual conditions. Co-culturing has been shown to modify cell behaviour, as shown when PC12 cells were co-cultured with rat myoblasts obtained *ex vivo*, yielding higher levels of myoblast differentiation into myotubes, and longer-lasting cell stability [16].

Since PC12 cells exhibit mainly activity of only one of the promoters of the DMD gene, the Dp71 promoter [50,64], it has become an excellent model for understanding the role for Dp71 in neuronal function and development. NGF responsiveness [1] and surface attachment [19] have been found impaired in Dp71-deficient PC12 cells, linking its activity to cell differentiation processes. Pillers [58] used several strains of mice lacking different Dp isoforms, and found that Dp260 and Dp71 isoforms seem to be related with synapse defects, leading to an abnormal electroretinogram (ERG) when absent. This evidence, as well as all data linking Dp71 to synaptic vesicles in brain [38], neurite growth cone and varicosities in PC12 cells [50], its relations with AQP4 and Kir4.1 subcellular localization in Glial Muller cells, the absence of presynaptic information regarding dystophin-utrophin-DAP role in synapse formation, and the partial evidence on Dp71 function, led us to an attempt to test whether Dp71 is related to NMJ formation in a cell-line model that expresses almost no other dystrophin isoforms than Dp71.

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To start, we determined the dystrophin isoforms that are present in L6 myoblasts and myotubes. Using Western blots, we were able to identify, as expected, an important increase in Dp427 when L6 cells differentiated into myotubes (Fig. 1C), a 140kDa isoform that increased 6-fold when detected by H5A3, and a decrement in Dp116 after L6 differentiation, detected by H4 antibody. RT-PCR assays confirmed Dp116 mRNA expression in myotubes, the increment in Dp427 mRNA expression as well as a slight decrement in Dp71 mRNA expression during L6 differentiation, but no Dp140 mRNA was detected that could account for the 6-fold increase in the isoform detected by Western blot analysis. All RT-PCR products were sequenced (data not shown). We can speculate that mRNAs that are used for the expression of the Dp140 isoform detected by Western blot did not contain the regions where one – most probably Dp140U oligonucleotide - or both oligonucleotides bind together during the PCR reaction. H5A3 antibody can detect both dystrophins and utrophins, and RT-PCR did not confirm that this isoform was Dp140d; nevertheless, H4 Western blot assays strongly suggest it might be Dp140. If the 140 kDa isoform detected is indeed a Dp140, it would be interesting to study it further, since Dp140 has been described mainly in the kidney and central nervous system [45, 46, 47], its deficiency has only been associated with cognitive impairment [5, 6], and information regarding its role in cell function is not yet clear. Thus, further analysis of the 140 kDa isoform detected by H5A3 and H4 antibodies would be informative in elucidating the nature of this protein and its role in L6 differentiation.

We believe this is the first report of Dp116 expression in a muscle cell line, confirmed both by Western blot and RT-PCR. Further studies would be interesting in order to elucidate the mechanisms of control of Dp116, since in humans it has been described only in peripheral nerves, brain and Schwann cells [8, 23, 51] but RT-PCR assays of Tokarz [70] showed Dp116 mRNA was present in all mouse tissues but liver. Controversially, it has recently been found that transgenic

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expression of Dp116 displaced utrophin in muscle of dystrophin-deficient mdx (4cv) mice [37], inducing a more severe dystrophic phenotype. We also found the expression of two other proteins, p30 and p180, detected by H5A3 and H4 antibodies, respectively; p30 may be the result of protein degradation or a short product of the dystrophin gene. More interesting is the case of p180, which was detected by the d-dystrophin specific H4 antibody. This product does not correspond in size with any other Dp isoform described so far, and increased from absent to very evident when L6 differentiated into myotubes. Since this protein has not been previously described, its identity and characterization might help complete the model for dystrophin-utrophin-DAP and DG complexes in rat muscle.

This evidence indicated that L6 cells show a similar pattern of expression of dystrophin isoforms as other muscle cells, with the exception of Dp116 and Dp140d, for which we found no other report of expression in a muscle cell line or ex-vivo muscle cells upon differentiation, and the 180 and 30 kDa isoforms, that would require further identification.

For utrophins, by Western blot analysis we found only Up395 and Up140 proteins. For Up395, previous studies indicated that its expression is moderately increased in C2 muscle cell line differentiation [30] and up-regulated in regenerating muscle [27], which corresponds only in part with what we found in our Western blot assays. Here we found that Up395 is clearly up-regulated upon L6 cell differentiation, and resembles previous evidence with PC12 cells, in which utrophin expression is increased when PC12 cells are induced to differentiate with NGF [64]. Regarding Up140, we believe this is the first report of such a utrophin isoform in a muscle cell line. The Up140 isoform has been previously described in several human and rodent tissues, both fetal and adult [74], but it has not yet been reported in a rodent cell line. Up140 could be an interesting target for further investigations since, despite being a Dp140 sequence analogue, it is expressed in all tissues tested so far, while Dp140 is more restricted to central nervous system and kidney.

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When we established the co-culturing conditions, it was observed that PC12 NGF-induced differentiation was initiated as early as the third day after NGF exposure and to a greater extent in co-cultures than monocultures, including low-branching and long-extension neurites directed towards L6 myotubes. However, these findings are not so unexpected since it has been shown that co-culturing may provide factors other than NGF that can promote differentiation; this was the case when skeletal myotubes were cultured in the presence of PC12 cells which showed a better and longer-lasting state of differentiation than those without [16]. Furthermore, cell-to-cell contacts between distal termini of PC12 cells and L6 myotube sarcolemma appear to be NMJ-like structures, similar to those described by Schubert [68].

PC12 cells were found to be AChR positive in the immunofluorescence assays, which is consistent with previous reports indicating that AChR may have an autostimulatory effect or serve as a rectification mechanism on PC12 cells [4, 11, 32, 65, 52, 53]. However, more interesting is our finding of AChR in PC12 cell nuclei (Fig. 4C and 5), an unexpected result since AChR is a transmembrane protein complex that forms a Ca+ channel and is stimulated by contact with ACh. This is its known function: the influx of Ca+ into the muscle cell as a result of the stimulus induced by ACh released from synaptic vesicles. The antibody we used is targeted to the α-1 subunit of the AChR, the same subunit BTx binds to, and is able to detect mouse, rat and human AChR. Since our system allows us to detect only two of the 5 subunits that comprise the AChR complex, it remains to be proved that all other subunits are present, and that the AChR is functional. However, it is known that the α-1-subunit contains the acetylcholine binding site [25, 41, 54] and is located near the BTx binding site [66].

Previous evidence of Western blots using 5F3 and H4 shows no other detectable signal than Dp71; thus, the ab- and d-dystrophins detected in our assays correspond mainly to Dp71ab and

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Dp71d, respectively. Dp71d in PC12 cells, as expected, does not colocalize with Dp71ab, and this the first report confirming the differential localization of these isoforms in the same sample preparations, using wild-type dystrophins instead of recombinant constructs or parts thereof. Thus, it has been confirmed that localization seems to be mutually exclusive, but it becomes much more evident when co-cultures are used. Earlier reports [50] indicated that in PC12 cells differentiated in monocultures, Dp71d seems to be located partially, and at reduced levels, in the cytoplasm and neurites. However, when co-cultured with L6 myotubes it is absent from these structures, remaining only in the nucleus and a structure that resembles endoplasmic reticulum- and Golgi complex-like structures, and being depleted from cytoplasm. Such data suggest that cell-to-cell signaling might take place between PC12 cells and L6 myotubes, causing rearrangements in subcellular location.

Colocalization of Dp71ab with utrophin and β-dystroglycan in PC12 cells in co-culture resembles previous reports [50], where Dp71ab colocalizes to a great extent with both proteins, throughout the cytoplasm and neurites, while Dp71d only colocalizes in the nucleus, but to a lower extent. This suggests that Dp71ab might be interacting with DAP complex at the sarcolemma. Utrophin subcellular distribution in co-cultured PC12 cells is similar to previous results [50,64] showing utrophin localized in the cytoplasm, nucleus and neurites (Fig. 7B), and it concentrates in the distal tips of the neurites where there is contact with L6 sarcolemma (Fig. 9C, merge, magnification), colocalizing highly with AChR in this region. This evidence clearly suggests utrophin may play a role on the establishment of such cell-to-cell interactions, on the neurite side. Considering that utrophin has been proved to be related to NMJ formation on the postsynaptic side[29] mainly by interacting with rapsyn through β-DG, by stabilizing AChR clusters in the

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synapse, there is a possibility that its role in the co-cultured PC12 cells might also be related to the AChR we found at the tip of the neurites.

To elucidate the probable function of Dp71 in NMJ-related processes in our co-culture model, we used the presynaptic vesicle protein marker synaptophysin [72]. Synaptophysin has been reported in PC12 cells as a synaptic vesicle protein [50, 73] and in our model it colocalizes to a great extent with Dp71ab in neurites and cytoplasm, and being absent from the nucleus. Interestingly, the Dp71ab signal seems to be surrounded by the synaptophysin signal (Fig. 7D), suggesting that Dp71ab might be located on the inner side of the neurite, while synaptophysin is attached to neurite tips, at the membrane level.

In order to obtain information that the model might resemble a NMJ, we tested for AChR, and found a positive signal both in PC12 and in L6 cells. Dp71d and AChR colocalization is confined almost entirely to nucleus and to a structure that resembles the endoplasmic reticulum and Golgi complex in co-cultures (Fig. 8 B). In spite of the fact that AChR glycosylation and endoplasmic reticulum-Golgi presence of AChR has been previously reported [31, 59, 63], this is the first report we are aware of where such subcellular localization is determined in PC12 cells. However, in PC12 cells it was evident that Dp71ab and AChR colocalize almost entirely along neurites, membrane and cytoplasm but not in the nucleus, both in monocultures as in co-cultures (Fig. 4C and 8A). When synaptophysin localization is compared to AChR (Fig. 9A) colocalization is almost complete, along all cytoplasm, neurites, growth cones and varicosities. All this evidence suggest that Dp71ab, as well as utrophin, synaptophysin and AChR in PC12 cells might interact and take part in PC12 processes, such as neurite elongation, branching or maturation, or synaptic microvesicle transport along the neurites.

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The questions raised above require further analysis in order to determine whether Dp71 isoforms, as well as utrophin and β dystroglycan, are related to PC12 cell differentiation, and to assess their part in intercellular communications processes.

In this paper, we have found differences between previous reports of subcellular localization of Dp71 isoforms in monocultures and the results of our co-cultures with L6 myotubes, where Dp71ab seems to be related to neurite processes and to synaptic vesicle positioning in neurite growth cones. We think such differences support the hypothesis that Dp71 may be involved in cell-to-cell interactions, at least under the conditions tested in this work. These findings might help clarify neurological alterations present in DMD patients, as well as the poorly understood role of Dp71.

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Table 1. Primers, reaction conditions and product size for reverse transcriptase polymerase chain reactions (RT-PCR).

mRNA	**Primers**	**Sequence**	**Reaction Conditions**	**Product size**
Dp427	Dp427-5 Dp427-3	5´-AGC ACA AGG AGA GAT TTC AAA GGA-3´ 5´-CCA TTT GCA GAT ATT TGC CCA TCG-3´	94ºC 5 min, once, 94ºC 30 sec, 55ºC 30 sec, 72ºC 30 sec, 30 cycles, 72ºC 7 min once.	547 bp
Dp260	Dp260-5 Dp260-3	5´-CTG AAT GAG TGC CCG AAA GCT-3´ 5´-TCA ATG AAT TCA AGC GAC TCC-3´	94ºC 5 min, once, 94ºC 30 sec, 55ºC 30 sec, 72ºC 30 sec, 30 cycles, 72ºC 7 min once.	141 bp
Dp140	R140U R140L	5´-GAG GCA TTG CTG ACT GTT CT-3´ 5´-TTA GCT GTT CCT CAT CTC CA-3´	94ºC 5 min, once, 94ºC 30 sec, 55ºC 30 sec, 72ºC 30 sec, 30 cycles, 72ºC 7 min once.	367 bp
Dp116	Dp116 U Dp116 L	5´- CTA TGC AAC AGG ATC AGT GC -3´ 5´- ACA GGG GTG CTT CGT CTG AA -3´	94ºC 5 min, once, 94ºC 30 sec, 55ºC 30 sec, 72ºC 30 sec, 30 cycles, 72ºC 7 min once.	180 bp
Dp71	Primer 5 247	5´- CTG ATA GAA TTC TGA CTG CCT GTG AAA TCC TTA C -3´ 5´- TCT GCA GGA TAT CCA TGG GCT GAT C -3´	94ºC 5 min, once, 94ºC 30 sec, 55ºC 30 sec, 72ºC 30 sec, 30 cycles, 72ºC 7 min once.	282 bp
Utrophin	Utr 1 Utr 2	5´-ATC GGT GTG CCT TTC TCT GAC TTG G-3´ 5´-CTC AAT CTG GGC ACT CTC CTC ATC C-3´	94ºC 3 min, once, 94ºC 1 min, 62ºC 1 min, 72ºC 1 min, 30 cycles, 72ºC 3 min once.	129 bp
β-DG	Dg1 Dg2	5´-GTG GTG GAA TGG ACC AAC AA-3´ 5´-AGC AAT GAT TCC AGC AAT GA-3´	94ºC 3 min, once, 94ºC 30 sec, 58ºC 30 sec, 72ºC 1 min, 30 cycles, 72ºC 3 min once.	340 bp
Actin	Actin-1 Actin-2	5´-TTG TCA CCA ACT GGG ACG-3´ 5´-GAT CTT GAT CTT CAT GGT GCT AGG-3´	94ºC 5 min, once, 94ºC 30 sec, 55ºC 30 sec, 72ºC 30 sec, 30 cycles, 72ºC 7 min once.	764 bp
Myogenin	RATMYO5 RATMYO3	5´-AGT GAA TGC AAC TCC CAC AGC-3´ 5´-GTG GCG TCT GAC ACC AAC TCA-3´	94ºC 3 min, once, 93ºC 40 sec, 58ºC 30 sec, 72ºC 30 sec, 23 cycles, 72ºC 5 min once.	254 bp

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Fig. 1. Expression of dystrophin and utrophin in L6 myoblasts and myotubes. Western blots were performed as indicated in materials and methods, using 3-10% gradient PAGE-SDS (A , B and C) or 10% (D) gels, and H5A3 pan-dystrophin-utrophin (A), K7 anti-utrophin (B), H4 anti-d- (C) and 5F3 anti-ab-dystrophin (D) antibodies. A, B and C. Lanes: 1, rat brain; 2, rat muscle; 3, undifferentiated PC12 cells; 4, L6 myoblasts; and, 5, L6 myotubes. D. Lanes: 1, rat brain; 2, mouse muscle; 3, L6 myoblasts; and, 4, L6 myotubes. Proteins detected by the antibodies are indicated by arrows. Actin was used as a loading control. Molecular weights are shown on the left.

Fig. 2. RT-PCR using oligonucleotides for Dp427 (A), Dp260 (B), Dp140 (C), Dp116 (D) and Dp71 (E). Total RNA was subjected to RT-PCR assays with oligonucleotides Dp427-5 and Dp427-3 for Dp427, Dp260-5 and Dp260-3 for Dp260, Dp140-5 and Dp140-3 for Dp140, Dp116U and Dp116L for Dp116 and Primer 5 and 247 for Dp71. Expected product sizes are identified with arrows, and are 547 bp for Dp427, 141 bp for Dp260, 367 bp for Dp140, 180 bp for Dp116 and 282 for Dp71. Lanes 1 through 6 are, respectively, molecular size markers, rat brain, muscle and eye, L6 myoblasts and myotubes. RT-PCR assays were performed as described in material and methods. Actin (E, bottom) was used as an amplification control. Molecular sizes are indicated on the left.

Fig. 3. Co-cultures of PC12 cells differentiated in the presence of L6 myotubes. PC12 cells were layered on coverslips containing L6 myotubes and were differentiated with NGF, as described in materials and methods. Arrows indicate contacts between PC12 neurite distal growth cone and L6 myotube sarcolemma. Size bar (100 μm) is shown at the bottom.

Fig. 4. Indirect immunofluorescence of differentiated PC12 cells. PC12 cells attached to coverslips and cultured for 7 days with NGF, were treated with primary antibodies against ab-

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dystrophins (mainly Dp71ab, left) and d-dystrophins (mainly Dp71d) or utrophin (middle), as described in material and methods. Left images correspond to the green channel, as middle images correspond to the red channel. Merge images are shown on the right. A. Subcellular distribution of Dp71ab (green) and Dp71d (red). B. Localization of Dp71ab (green) and utrophin (red). C. Localization of Dp71 (green) and AChR (red) in differentiated PC12 cells. Size bar (100 μm) is shown at the bottom (C, merge).

Fig. 5. AChR is present in PC12 nuclear extracts. Western blots of PC12 nuclear and cytoplasmic extracts was performed using 100 μg of nuclear, cytoplasmic and crude extracts in 10% PAGE-SDS gels, transferred to Hybond-N filters and treated with ECL Western blot kit, as specified by manufacturer. The 49 kDa band corresponding to α-subunit of AChR is indicated on the left. Lanes: 1, nuclear extracts; 2, cytoplasmic extracts; 3, crude extracts; and 4, brain crude extracts.

Fig. 6. Distribution of dystrophins, β-DG and AChR in differentiated L6 cells. Coverslips containing L6 differentiated cells were subject to indirect immunofluorescence assays to identify the subcellular localization of d- and ab-dystrophins, as well as β-DG and AChR. A. Distribution of d-dystrophins (upper left), ab-dystrophins (upper right), β-dystroglycan (lower left) and AChR (lower right). B. Colocalization of ab- (green) and d-(red) dystrophins in L6 cells. Size bars (100 μm) for each section are shown (A, AChR, bottom right; B, merge, bottom right).

Fig. 7. Subcellular localization of dystrophins, utrophin, β-dystroglycan and synaptophysin in co-cultures of PC12 and L6 cells. Coverslips containing co-cultures of PC12 cells differentiated in the presence of L6 myotubes, were co-treated with 5F3 and H4, K7, LG5 or anti-synaptophysin antibodies. A. Colocalization of ab- and d-dystophins. d- and ab- dystrophins were tested for colocalization in PC12-L6 co-cultures. B. Subcellular localization of ab-dystrophins and utrophin. C. Localization of ab-dystrophins and β-dystrolycan. D. Colocalization of ab-

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dystrophins and synaptophysin. For all purposes, ab-dystrophin image corresponds to the green channel (left), while H4, K7, LG5 and anti-AChR detection correspond to the red channel (middle). Merge images are on the right. Inserts (bottom right, merge) correspond to 2X magnifications of the area indicated by squares in the merge image. Size bar (100 μm) is shown at the bottom (D, merge).

Fig. 8. Colocalization of ab- (A, left) and d-dystrophins (B, left) with AChR (middle) in co-cultured PC12 cells. When codetecting ab-dystrophins and AChR, Alexa 594 linked α-bungarotoxin was used to identify AChR, and for d-dystrophins, the anti-AChR antibody was the choice. For homogeneity purposes, in the merge images (right), channels have been switched to show AChR in red, either with anti-AChR or α-bungarotoxin, while d- and ab-dystrophins are in green. Size bar (100 μm) is shown at the bottom (B, merge).

Fig. 9. Coimmunofluorescence of AChR with synaptophysin, β-dystroglycan and utrophin in co-cultures of PC12 and L6 cells. AChR localization, in green, was assessed altogether with synaptophysin (A), β-dystroglycan (B) and utrophin (C), all of the latter in red. Merge images are shown on the right. Inserts (bottom right, merge) correspond to 2X magnifications of the area indicated by squares in the merge image. Size bar (100 μm) is shown at the bottom (C, merge).

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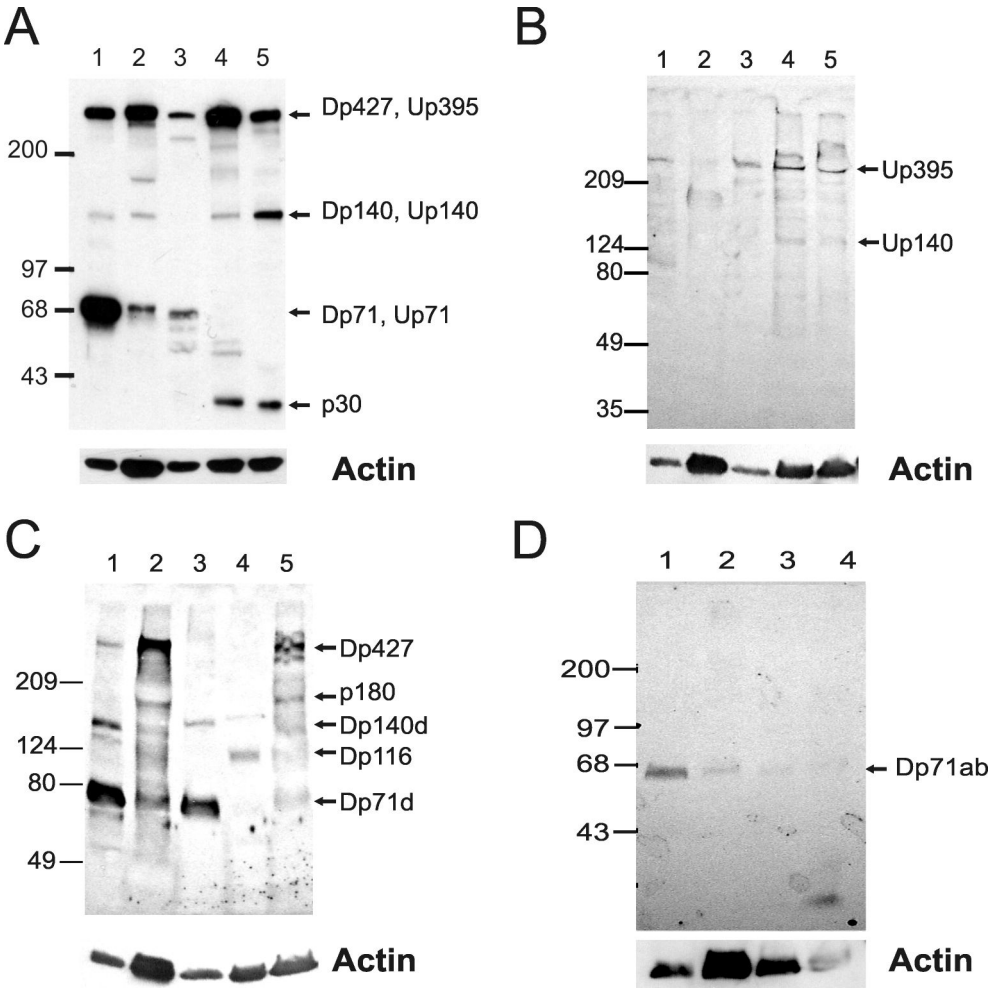

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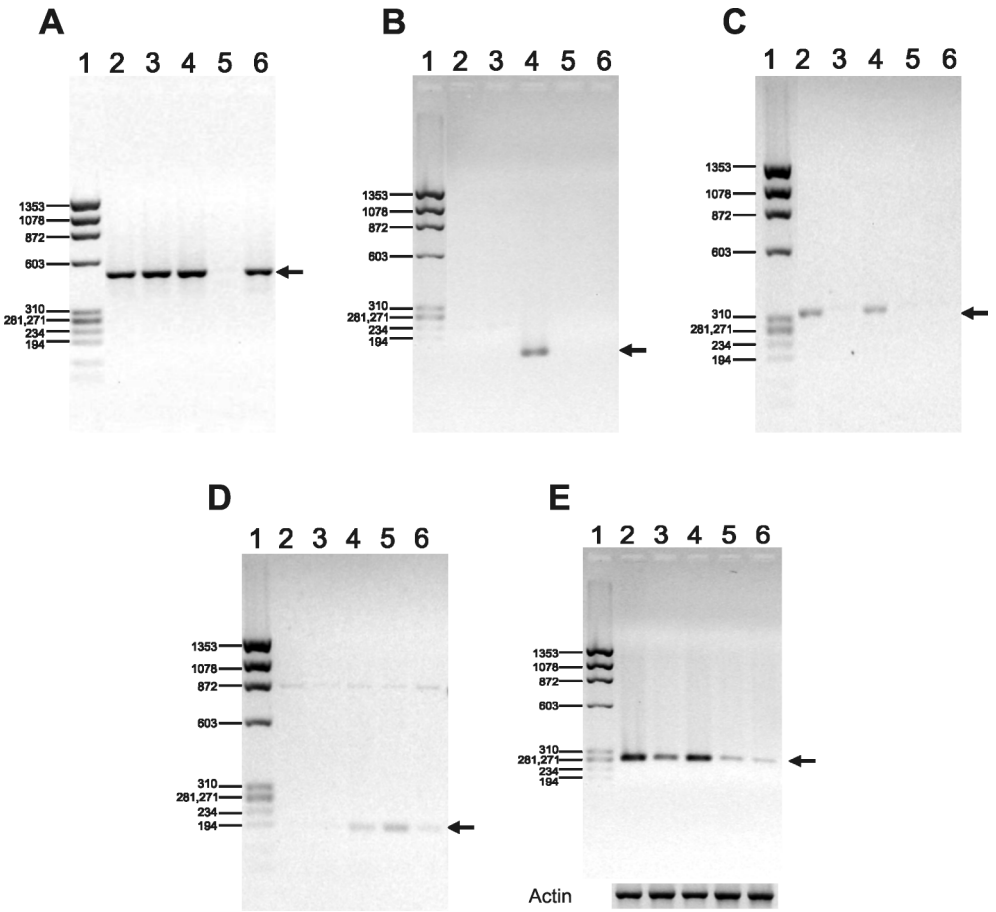

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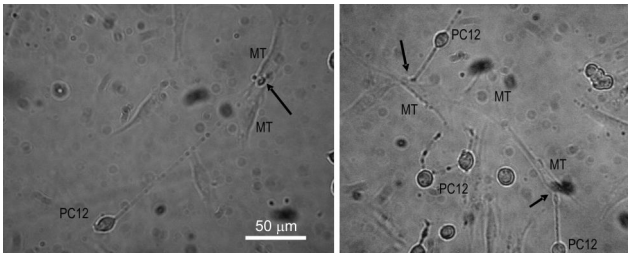

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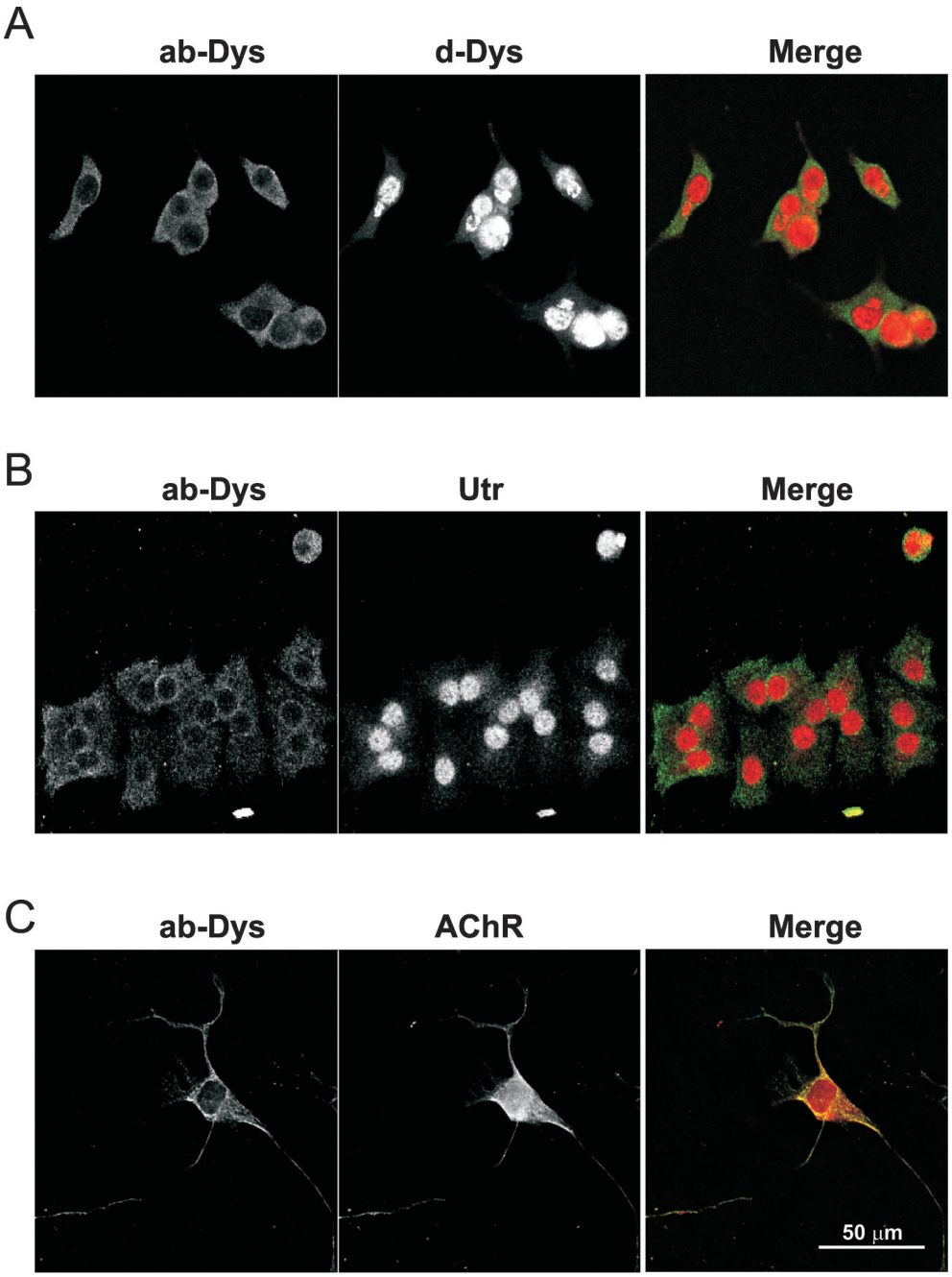

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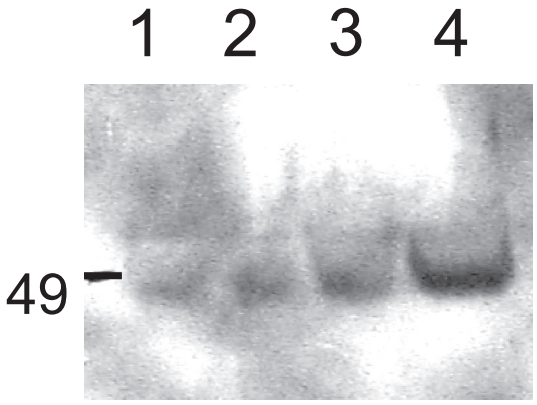

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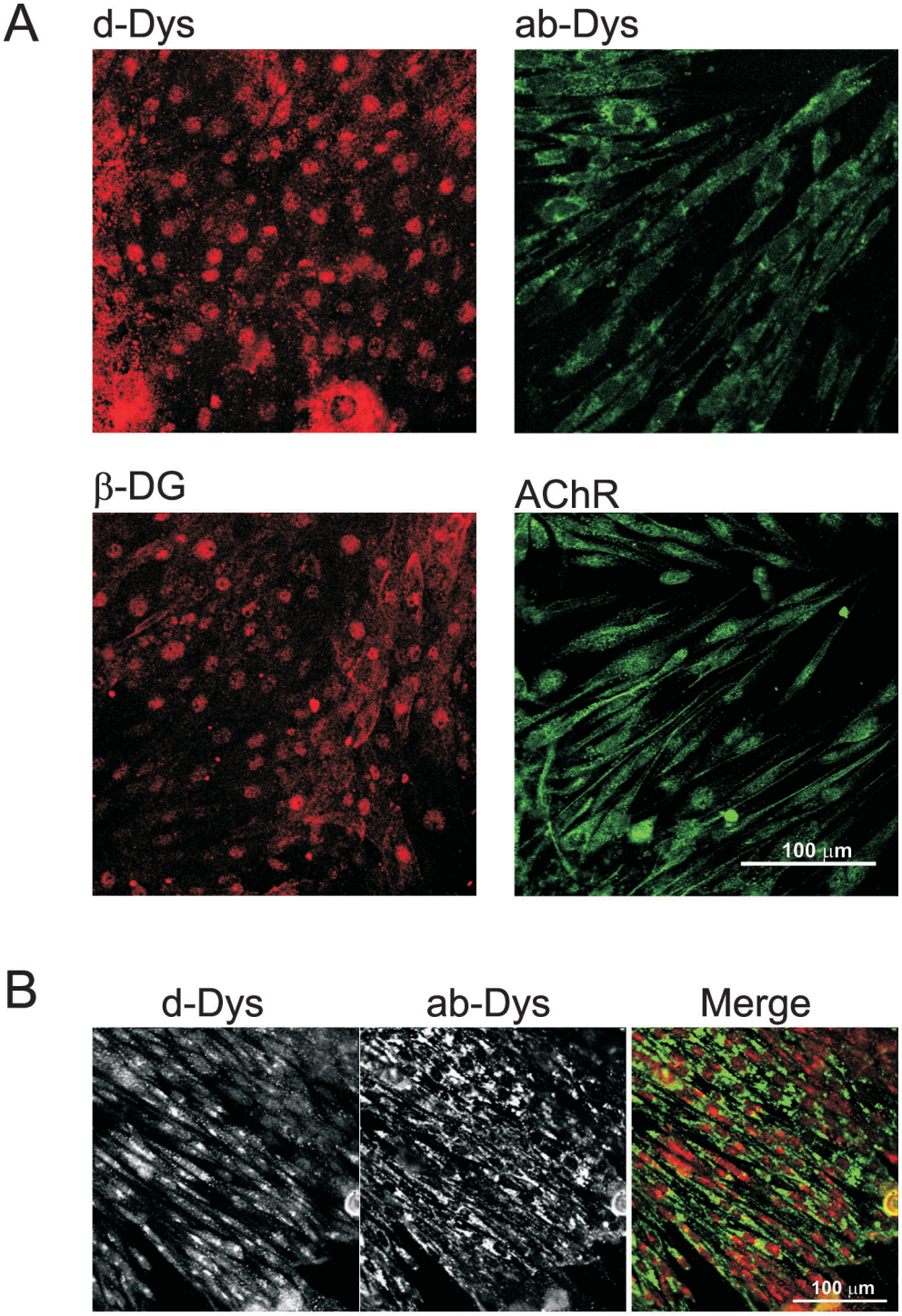

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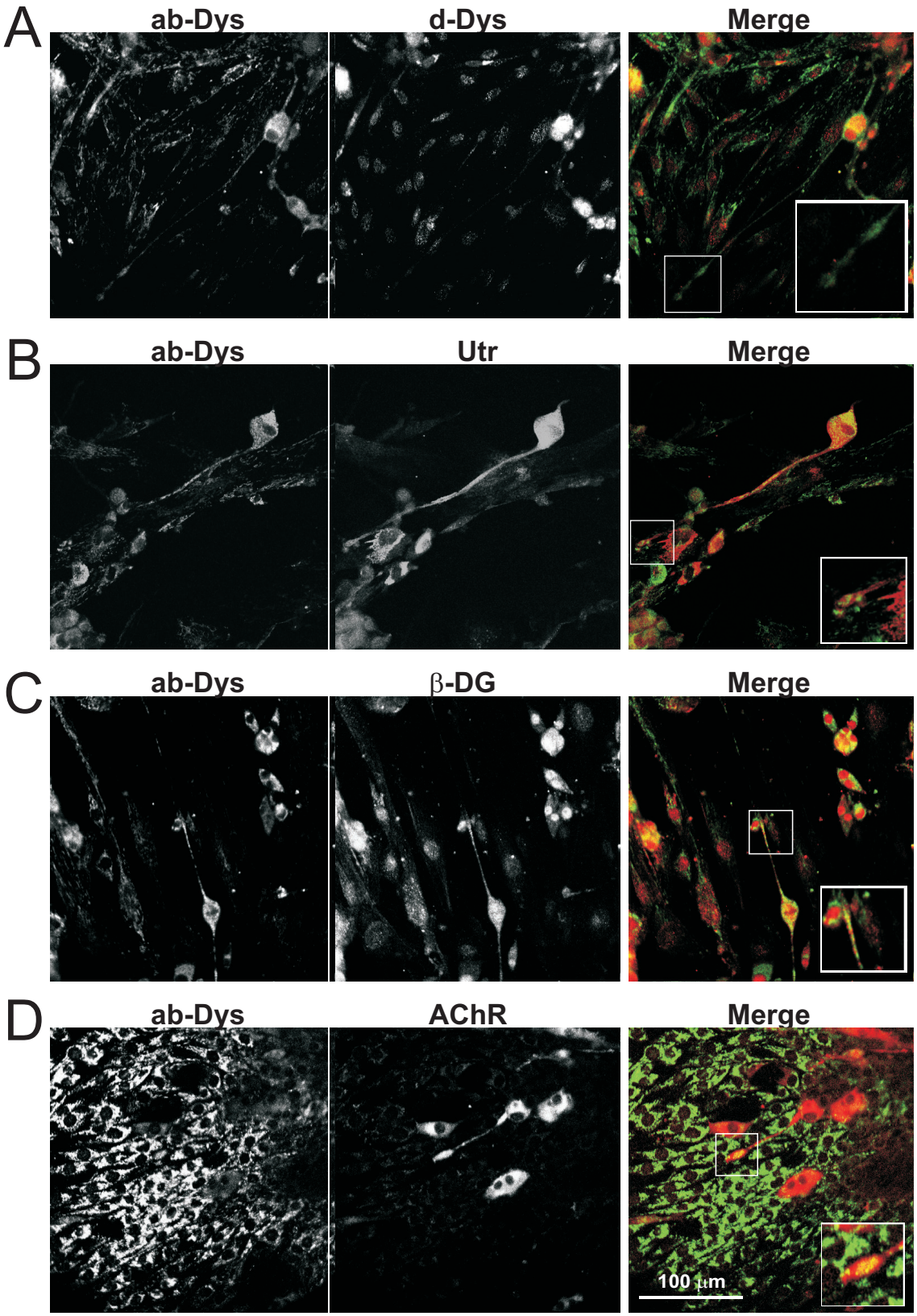

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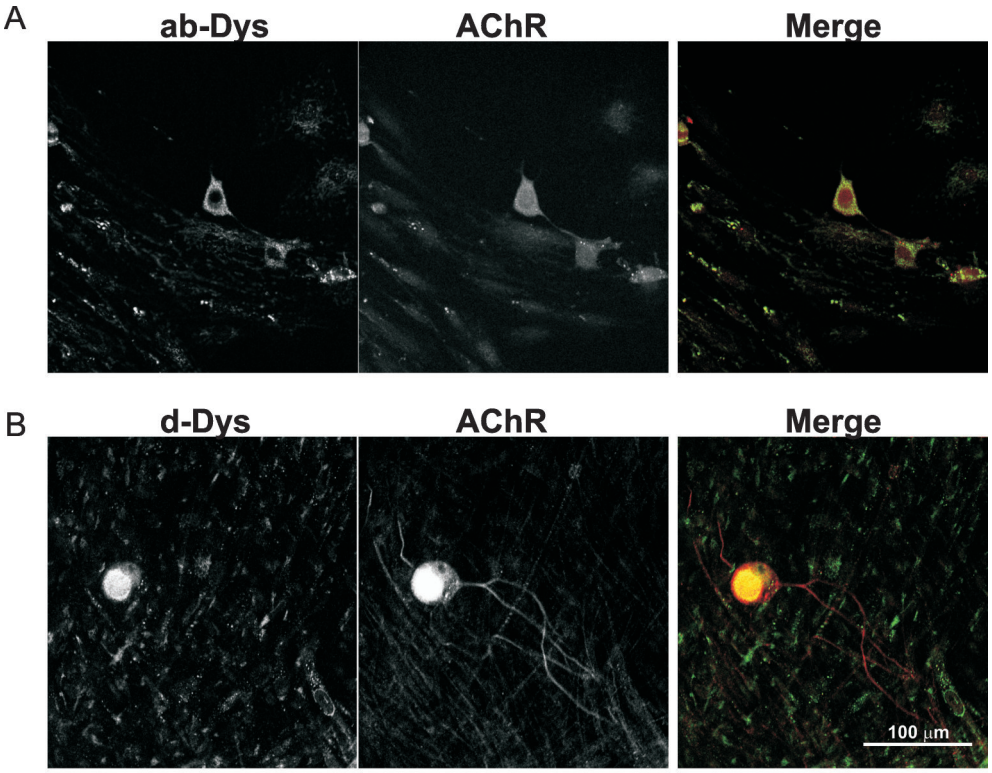
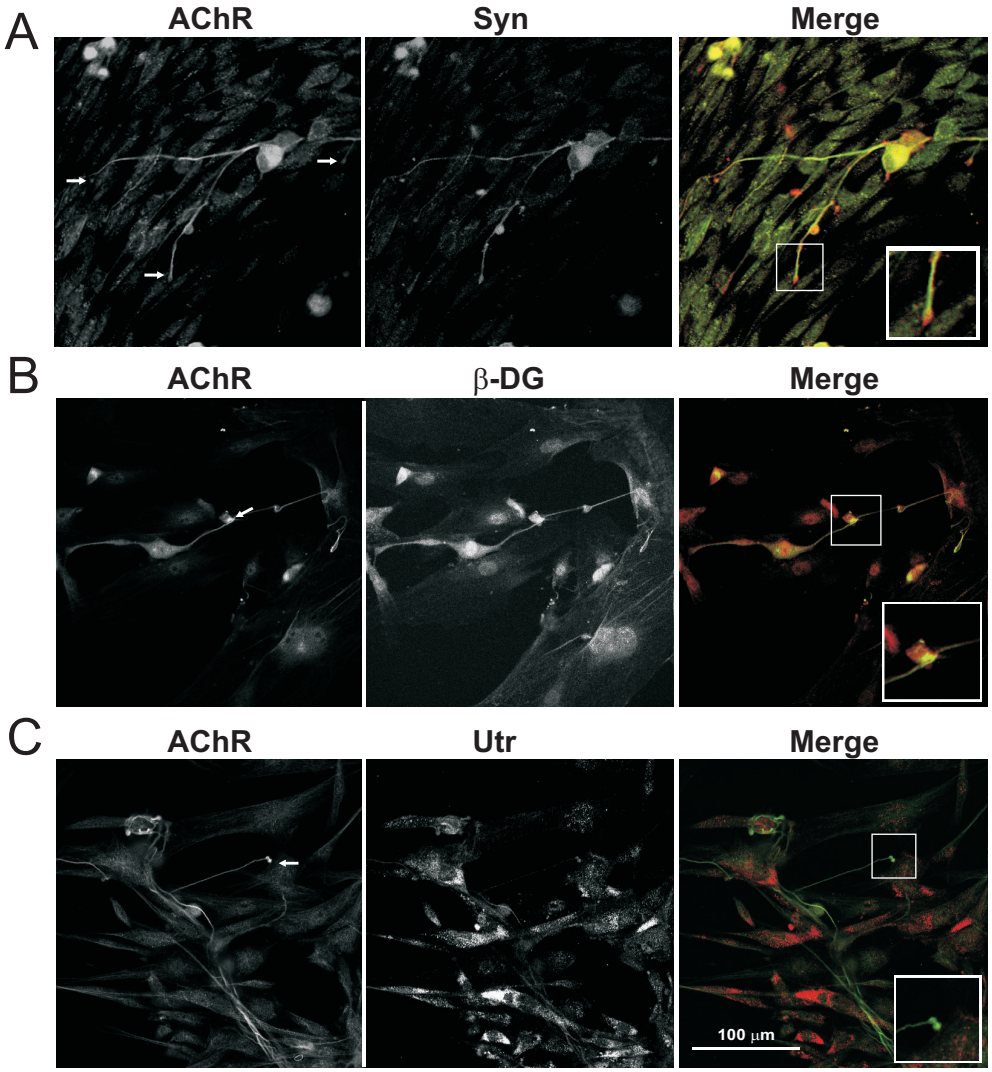
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