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## $\alpha$ -Skeletal Muscle Actin Mutants Causing Different Congenital Myopathies Induce Similar Cytoskeletal Defects in Cell Line Cultures

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Central core disease (CCD), congenital fibre type disproportion (CFTD), and nemaline myopathy (NM) are earlyonset clinically heterogeneous congenital myopathies, characterized by generalized muscle weakness and hypotonia. All three diseases are associated with  $\alpha$ -skeletal muscle actin mutations. We biochemically characterized the CCD and CFTD causing actin mutants and show that all mutants fold correctly and are stable. Expression studies in fibroblasts, myoblasts, and myotubes show that these mutants incorporate in filamentous structures. However they do not intercalate between the nascent z-lines in differentiating muscle cell cultures. We also show that the distribution of mitochondria and of the ryanodine receptors, and calcium release properties from ryanodine receptors, are unchanged in myotubes expressing the CCD causing mutants. CFTD causing mutants induce partly similar phenotypes as NM associated ones, such as rods and thickened actin fibers in cell culture. Our results suggest that molecular mechanisms behind CFTD and NM may be partly related. *Cell Motil. Cytoskeleton* 2009. © 2009 Wiley-Liss, Inc.

**Key words:**  $\alpha$ -actin mutation; central core disease; congenital fibre type disproportion; nemaline myopathy; muscle cells

Drieke Vandamme and Heidi Rommelaere contributed equally to this work.

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## INTRODUCTION

Central core disease (CCD) and congenital fibre type disproportion (CFTD) are earlyonset, clinically heterogeneous congenital myopathies, characterized by generalized muscle weakness and hypotonia. CCD is associated with areas devoid of oxidative enzyme activity (core lesions) and shows type I fiber predominance. CFTD is also associated with type I fiber predominance, but the main hallmark in patients is that the mean diameter of type I fibers (slow twitch) is at least 12% smaller than of type II fibers (fast twitch) [Brooke, 1973; Romero et al., 2005].

Ninety-percent of CCD patients have a mutation in the ryanodine I receptor (RyR1), which is a calcium-release channel of the sarcoplasmic reticulum with important functions for  $\text{Ca}^{2+}$  homeostasis [Zhang et al., 1993]. Next to that, mutations in selenoprotein N-1 (*SEPN1*) and myosin heavy chain-7 have also been described [Fananapazir et al., 1993; Ferreira et al., 2002]. *SEPN1* mutations are also associated with CFTD in recessive cases [Clarke et al., 2005], but for most of the CFTD cases, the genetic basis is still unknown. Remarkably, some dominant CCD and CFTD cases are associated with mutations in the  $\alpha$ -skeletal muscle actin gene (*ACTA1*) [Kaindl et al., 2004; Laing et al., 2004].

Another congenital myopathy resulting from mutations in  $\alpha$ -skeletal muscle actin (further referred to as  $\alpha$ -actin) is nemaline myopathy (NM). This disease is characterized by muscle weakness and hypotonia as well, but is defined by the presence of nemaline bodies or rods in muscle biopsies of patients. Over 100 *ACTA1* mutations leading to NM have been identified until now and they account for about 20% of the NM cases [Wallgren-Pettersson and Laing, 2006]. The other genetic loci associated with NM are also coding for components of skeletal muscle thin filaments (e.g. troponin and tropomyosin, for a review see [Clarkson et al., 2004]. The impact of this disease on the patients can be very different, the types range from mild, with minor muscle weakness and long-time survival, to severe, with lethality soon after birth. The clinical severity correlates poorly with the muscle pathology of the patients [Ryan et al., 2003] of which biopsies show various phenotypes. Based on this NM caused by *ACTA1* mutations is subdivided in classes [Sparrow et al., 2003]; intranuclear rod myopathy is characterized by intranuclear inclusions and NM by sarcoplasmic nemaline bodies. Another muscle disease: actin myopathy is distinguished by thin filamentous inclusions in areas which normally would be part of the myofibrillar lattice, but no nemaline bodies are found in muscle biopsies. However, more than one phenotype can be caused by the same *ACTA1* mutation [Nowak et al., 1999].

It is not surprising that mutations in  $\alpha$ -actin are causing diseases, since actin is an essential protein in muscle contraction. It also matures via a special folding pathway, involving the chaperones prefoldin and cytosolic chaperone containing TCP-1 (CCT). CCT assists in the folding of actin, in an ATP-dependent manner and releases it as an active monomer [Gao et al., 1992; Vainberg et al., 1998; Neirynck et al., 2006]. In addition actin has an absolute requirement for ATP to remain stable [Estes et al., 1992]. Once folded, actin can polymerize into filaments, which in muscle form the thin filaments that interact with several (muscle specific) actin-binding proteins e.g.  $\alpha$ -actinin, troponin, and myosin, thereby contributing to contractile force generation [Gordon et al., 2000].

In a previous analysis of 19 NM causing mutants of  $\alpha$ -actin, we found that the mutations can lead to three different biochemical defects in actin: folding defects, defective ATP-binding resulting in unstable proteins and reduced copolymerisation capacity [Costa et al., 2004]. Additionally, we could reproduce the typical hallmarks of NM, rods and aggregates, in cultured fibroblasts. Here, we conducted similar assays with the  $\alpha$ -actin mutants D1Y and E334A, associated with CCD, and L221P, D292V, and P332S, associated with CFTD. We show that all mutants fold correctly and are stable. Expression in cultured fibroblasts and myoblasts shows they are all capable of integrating in nonmuscle specific actin filaments, yet they behave aberrant in myotubes. Additionally, we observed similar cellular phenotypes as are induced by NM associated  $\alpha$ -actin mutants, when expressed in fibroblasts [Costa et al., 2004], or in muscle cells [Bathe et al., 2007] (Rommelaere et al., in preparation). These data show that these diseases, which are very different at the histological level, may have related or common molecular causes.

## MATERIALS AND METHODS

### Construction and Biochemical Analysis of the $\alpha$ -Actin CCD and CFTD Causing Mutants

*ACTA1* mutations were made with the QuikChange site directed mutagenesis kit (Stratagene) according to the manufacturer's instructions, using the human *ACTA1* cDNA sequence in pcDNA3.1 (Invitrogen) as a template and appropriate primers. N-terminal myc-tagged wild type actin and actin mutants were made by PCR using wild type actin, respectively the actin mutants in the pcDNA3.1 vector as template, a 5' primer containing the myc-sequence preceded by a HindIII site and a 3' primer containing an XbaI site. These fragments were ligated into HindIII-XbaI digested pcDNA3.1. Constructs were sequenced to verify the complete *ACTA1* coding

sequence and correct introduction of the desired mutation. Expression of actin mutants, band shift assays with ABPs, copolymerization assays were performed as described in [Costa et al., 2004; Rommelaere et al., 2004]. Briefly,  $\alpha$ -skeletal muscle actins were expressed as  $^{35}\text{S}$ -labeled proteins by in vitro translation reactions in reticulocyte lysates (MP Biochemicals). Reaction products were analysed on denaturing tricine gels [Schagger and von Jagow, 1987] and on nondenaturing polyacrylamide gels [Safer, 1989] with ATP, followed by autoradiography. To monitor cyclase associated protein (CAP) binding, ATP was omitted from the native gels. The amount of  $^{35}\text{S}$ -actin bound to CAP was quantified by phosphor imaging (Typhoon 9200 variable mode imager; Amersham Biosciences) and the Image Quant software package. As a reference the folding compromised NM mutant L94P and unstable NM mutant G182D were included. For the band-shift assays, a solution of actin binding proteins (ABP) was added. Final concentration of each ABP was 8  $\mu\text{M}$  for DNaseI, 4.8  $\mu\text{M}$  for Vitamin D binding protein (VDBP). Binding capacity was assessed qualitatively; reduced binding means only a part of the population of the mutant molecules shifted, and part remained unshifted.

### Cell Culture and Transfection

NIH3T3 fibroblasts were grown on glass coverslips, in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 1% antibiotics (penicillin-G, 100  $\mu\text{g}/\text{ml}$  and streptomycin, 100  $\mu\text{g}/\text{ml}$ , Invitrogen). After 24 h cells were transfected with lipofectamin 2000 (Invitrogen), according to manufacturer's protocol. Coverslips were mounted for immunofluorescence after 24 h of transfection. Sol8 myogenic cells [Mulle et al., 1988] were seeded in 12 well plates containing thermanox coverslips (Nunc). Myoblasts were grown in DMEM/HamF-12 medium (Invitrogen) (50:50) supplemented with 10% fetal calf serum (Invitrogen), 1% L-glutamine, and 1% antibiotics (penicillin-G, 100 i.u./ml and streptomycin, 100  $\mu\text{g}/\text{ml}$ , Invitrogen). After 24 h the cells were transfected using jetPEI (Qbiogene) or nucleofected, using cell line nucleofector kit V (Amaxa biosystems) with the pcDNA3.1 vectors encoding N-terminally myc-tagged  $\alpha$ -skeletal muscle actin (wild type or mutant). To study transfected myoblasts, coverslips were mounted for immunofluorescence after 48 h of transfection. To promote fusion (F) of myoblasts into myotubes, the growth medium was replaced by a differentiation medium (DMEM supplemented with 2% heat-inactivated horse serum (Invitrogen), 10  $\mu\text{g}/\text{ml}$  insulin (Sigma), 1% L-glutamine and 1% antibiotics, following 2–3 days of proliferation (37°C, 5%  $\text{CO}_2$ , water saturated air) to

around 80% of confluence. Myotubes were immunostained on day F + 4 or F + 5.

### Immunological Staining

The cultured cells were fixed in TBS (20 mM Tris base, 154 mM NaCl, 2 mM EGTA, 2 mM  $\text{MgCl}_2$ , pH 7.5)/4% paraformaldehyde and permeabilized with TBS/0.5% Triton X-100. Samples were then incubated for 1 h with primary antibodies in TBS/1%BSA. To visualise the mutant actins we used a polyclonal anti-myc antibody (Abcam) or a mouse anti-myc antibody (Cell Signalling technologies). To visualise z-bodies we used a monoclonal antibody against  $\alpha$ -actinin (Sigma), for visualisation of mitochondria a mouse anti-cytochrome C antibody (invitrogen), a mouse anti-apoptosis inducing factor (AIF) antibody (Santa Cruz) or rabbit anti-endonuclease G (Endo G) antibody (Abcam). Ryanodine receptors were visualized with a goat polyclonal anti RyR1 antibody (Sigma). After washing in TBS, the cells were incubated for 30 min in TBS/1% BSA with alexa 488-conjugated goat anti-mouse, alexa 594-conjugated anti-rabbit or alexa 594-donkey anti-goat secondary antibodies, or alexa 488-phalloidin (Molecular Probes). Samples were mounted using Vectashield mounting medium (Vector).

### Cytofluorescence Analysis

The immunolabelled samples were examined using an Olympus IX71 epifluorescence microscope and images were acquired using a cooled Spot Camera (Diagnostic Instruments) and Analysis software (Soft Imaging Systems). Confocal images were acquired by means of confocal laser scanning microscopy using an Olympus confocal system equipped with an argon-krypton gas laser. The excitation wavelength of 488 and 543 nm were respectively used for acquisition of images in green channel (520 nm) and in red channel (600 nm). For statistics of stress fibre incorporation and diffuse cytoplasmic myc-actin staining, pictures were visually inspected and blindly scored by a second person.

### Ratiometric $\text{Ca}^{2+}$ Measurement

Myotubes, plated on glass coverslips, were briefly rinsed with a standard external solution (1.8 mM  $\text{Ca}^{2+}$  solution) (130 mM NaCl, 5.4 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgCl}_2$ , 10 mM Hepes, 5.6 mM D-glucose, pH 7.4 with NaOH), incubated for 30 min at room temperature and then 15 min at 37°C in the same solution supplemented with 3  $\mu\text{M}$  (final concentration) Fura-2 AM (FluoProbes). After loading, cells were washed with standard external solution (1.8 mM  $\text{Ca}^{2+}$  solution), before measurement of calcium responses. Interrogated cells were perfused by means of a home-made gravity



microperfusion device. Fura-2 loaded cells were excited by alternating 340/380 nm light with a CAIRN monochromator (Cairn Research Limited) and emission fluorescence was monitored at 510 nm using a CCD camera (Photonic Science Limited) coupled to an Olympus IX70

inverted microscope ( $\times 40$  water immersion fluorescence objective). The variation of fluorescence ratio (340/380) was recorded with the Imaging Workbench 4.0 (IW 4.0) software (Indec BioSystems).

## RESULTS

### CCD and CFTD Causing $\alpha$ -Actin Mutants are Correctly Folded and Stable

In this study, we investigated  $\alpha$ -actin mutants causing either CCD ( $\alpha$ -actin D1Y and E334A) or CFTD ( $\alpha$ -actin L221P, D292V, and P332S). All five  $\alpha$ -actin mutations lie on the surface of the actin monomer, but do not cluster together (Fig. 1a, [Kabsch et al., 1990]). Using similar approaches as in our previous studies on a set of  $\alpha$ -actin mutants causing NM [Costa et al., 2004; Rommelaere et al., 2003, 2004], we monitored the folding capacity and stability of these mutant actin molecules. Analysis on native gels shows that they all fold properly as none of the mutants significantly accumulates on CCT (Fig. 1b). Additionally, in the absence of ATP in the native gels, none of the mutants shows an increased binding to CAP, compared to WT  $\alpha$ -actin, suggesting ATP-binding is not compromised and that these mutants are stable. They appear correctly folded since they migrate at a position similar to monomeric actin in gels with ATP (Fig. 1b) and can bind to DNaseI and VDBP that contact opposing phases of the actin molecule [Kabsch et al., 1990; Otterbein et al., 2001] (Table I).

### CCD and CFTD $\alpha$ -Actin Mutants Show Actin Stress Fiber Incorporation, but Act L221P and P332S also Induce Diffuse Cytoplasmic myc-actin Staining in Fibroblasts

Since expression of myc-tagged  $\alpha$ -actin mutants associated with NM in NIH3T3 fibroblasts induces a range of defects in the actin cytoskeleton [Costa et al., 2004], we investigated if expression of myc-tagged  $\alpha$ -actin mutants causing CCD and CFTD induce similar cytoskeletal defects. Therefore, we used fluorescent phalloidin to probe for actin filaments and to test for integration of myc-tagged  $\alpha$ -actin mutants into cellular

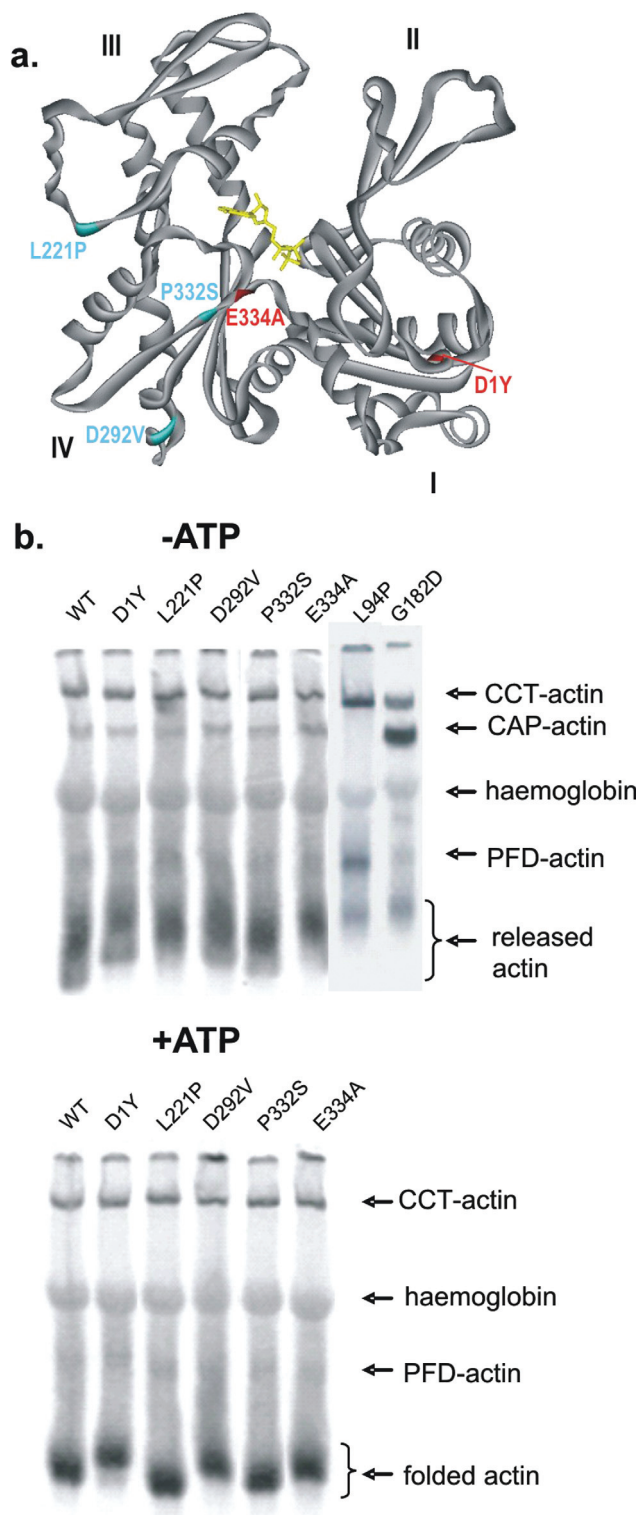


Fig. 1. (a) Location of the studied  $\alpha$ -actin mutations on the 3-Dimensional representation of the actin molecule (Kabsch et al., 1990). Mutants in blue are causing CFTD, mutants in red are causing CCD. I–IV indicate the actin subdomains. ATP is in yellow. The PDB structure 1ATN was displayed by ViewerLite. (b) Native gel analysis of the CCD and CFTD causing  $\alpha$ -actin mutants. Autoradiograms of native gel analysis in the absence or presence of ATP of  $^{35}\text{S}$ -labeled  $\alpha$ -actin and  $\alpha$ -actin mutants produced in *in vitro* transcription translation reactions in reticulocyte lysate, which endogenously contains CAP [McCormack et al., 2001; Neirynck et al., 2004] and prefoldin and CCT [Gao et al., 1992; Rommelaere et al., 1993; Vainberg et al., 1998].

**TABLE I. Summary of the Biochemical Characterization of Seven Myopathy Causing  $\alpha$ -Skeletal Muscle Actin Mutants**

Mutant	Phenotype	Folding	CAP	ABP	Copol	In actin structure
WT		+	+	+	++	
D1Y	CCD	+	+	+	++	N-terminus
L221P	CFTD <sup>†</sup>	+	+	+	++	Surface
D292V	CFTD <sup>†</sup>	+	+	+	++	Surface
P332S	CFTD	+	+	+	+	Surface
E334A	CCD	+	+	+	++	Surface

The properties investigated here are folding, binding to cyclase associated protein (CAP), binding to the actin binding proteins (ABP), DNAseI, and Vitamin D binding protein in band shift assays and copolymerisation with actin in cells (copol). The position in the actin structure according to Kabsch et al. (Kabsch et al., 1990) is also indicated, in addition to the patient's phenotype as presented in the reference.

<sup>†</sup> = lethal, CCD = Central core disease, CFTD = congenital fibre type disproportion.

F-actin containing structures. We use NIH3T3 fibroblasts because these cells show a pronounced staining of F-actin structures with fluorescent phalloidin. We did not observe many differences between WT and mutant  $\alpha$ -actin with respect to incorporation in actin fibers. All CCD and CFTD causing  $\alpha$ -actin mutants are able to incorporate in stress fibers in the majority of NIH3T3 fibroblasts (Figs. 2a and 2b and Table II). Intriguingly, next to proper incorporation in filamentous structures we also observed a diffuse cytoplasmic myc-actin staining in some of the mutant transfected cells. We counted these cells, to rule out the effect of different expression levels of the mutant, and noticed that the majority of the fibroblasts expressing the CFTD causing mutants  $\alpha$ -actin L221P and P332S (resp. 90 and 85%) display this phenotype (Fig. 2b), whereas the other mutant and WT-expressing cells were similar. Additionally, we monitored the behavior of the mutants in the same way in myoblasts, which are physiologically more relevant. Myc-tagged WT  $\alpha$ -actin incorporates into the actin cytoskeleton of myoblasts and myc staining of L221P, D292V, and P332S is comparable (Fig. 2c).

### Two CFTD Associated $\alpha$ -Actin Mutants D292V and L221P Induce Nemaline Rods and Aggregates in Fibroblasts

Two  $\alpha$ -actin mutants induce phenotypes that are typical for NM.  $\alpha$ -actin D292V induces long thin rods (Fig. 3) in a significant part of the transfected fibroblast population. These rods do not stain with phalloidin and are in a few cases intra- or perinuclear, but in most cases they are present in the periphery of cells. In addition, this mutant also induces cytoplasmic aggregate formation (Fig. 3). Similar phalloidin negative aggregates and rods are also present in fibroblasts expressing  $\alpha$ -actin L221P, but only in a minority of the population (data not

shown). In myoblasts, however, these mutants did not induce these phenotypes (Fig. 2c).

### CCD and CFTD Causing $\alpha$ -Actin Mutants Exhibit Disturbed Localization in Nascent Myofilaments

To monitor the behavior of the CCD and CFTD causing  $\alpha$ -actin mutant in differentiating muscle cells, we transfected myoblasts with the  $\alpha$ -actin mutants and allowed them to fuse into myotubes during 5 days. The myotubes can be identified by their elongated shape and the presence of multiple nuclei. They contain premyofibrils, which have  $\alpha$ -actinin rich z-bodies fused to short actin filaments. Later on, these z-bodies move towards the centre of the myotube, align and fuse with formation of the z-disc [Sanger et al., 2002; Lloyd et al., 2004]. Confocal images reveal the expected striated  $\alpha$ -actinin staining or troponin-T pattern for WT actin expressing cells (Fig. 4a). We could see the  $\alpha$ -actinin striations in all cells expressing mutants, yet  $\alpha$ -actin D1Y, E334A (CFTD), and P332S (CCD) show few or no intercalation with the  $\alpha$ -actinin striation in the differentiating sarcomers (Figs. 4b and 4c). Expression of L221P and D292V (CFTD) resulted in another phenotype; thick curved actin fibers, that were only partly decorated with  $\alpha$ -actinin (Fig. 4c). This phenotype was also observed for NM mutants D154N (Fig. 4d) and G15R [Bathe et al., 2007]. Strikingly, expression of  $\alpha$ -actin L221P and D292V induced  $\alpha$ -actin rod formation, which contained  $\alpha$ -actinin.

### Localization of Mitochondria and Ryanodine Receptor 1 Appears Normal in Myotubes Expressing CCD Causing $\alpha$ -Actin Mutants

The major histological phenotype of CCD in patient muscle biopsies is the existence of areas devoid of oxidative enzyme activity. The lack of oxidative enzyme activity suggests that in CCD patients, the location or activity of mitochondria in muscle cells may be affected.

We therefore investigated the distribution of mitochondria in myotubes, expressing CCD causing  $\alpha$ -actin mutants D1Y and E334A. We performed stainings for cytochrome c, a marker for mitochondria, on differentiated myotubes, in combination with myc-staining to identify the transfected cells (Fig. 5). Differentiating muscle cells increase the number of mitochondria in order to cope with the high energy demands [Rochard et al., 2000; Hood et al., 2006] and this is reflected in a much more abundant dot-like cytoplasmic cytochrome C staining in myotubes, compared to myoblasts or fibroblasts. However, we observed no differences in staining pattern between transfected WT and mutant  $\alpha$ -actin expressing myotubes. These results were confirmed by stainings with two other mitochondrial proteins, endonuclease G (Fig. 5) and AIF (not shown). In addition we did not observe dif-

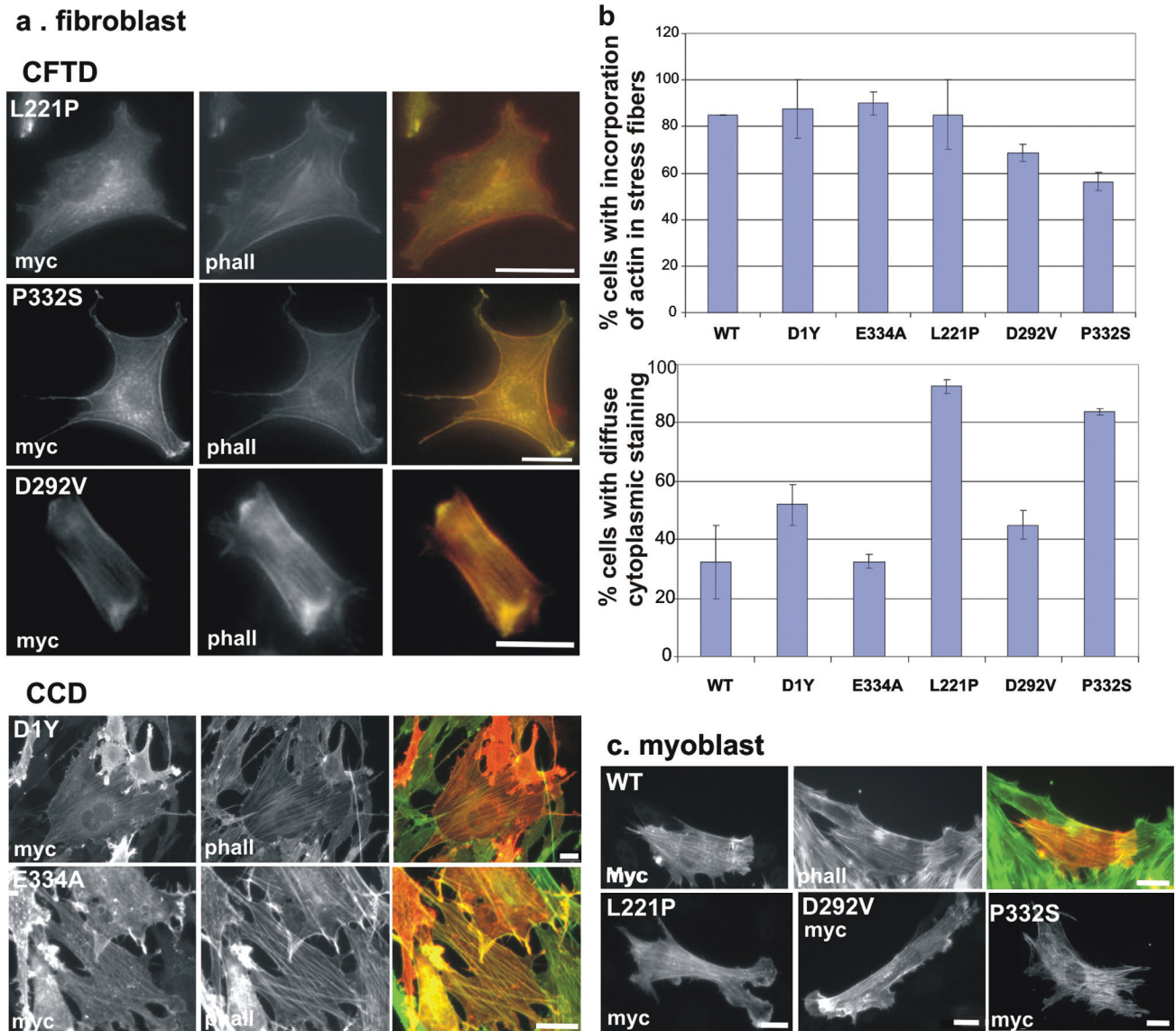


Fig. 2.  $\alpha$ -actin mutants causing CCD and CFTD show normal actin stress fiber incorporation in fibroblasts and myoblasts, but two CFTD mutants show in addition diffuse cytoplasmic myc-staining. (a) Myc staining of NIH3T3 fibroblasts expressing CFTD causing myc-tagged mutants  $\alpha$ -act L221P, D292V, and P332S and NIH3T3 fibroblasts expressing CCD causing myc-tagged mutants  $\alpha$ -act D1Y or  $\alpha$ -act E334A. (b) Graphs of counts of fibroblasts which have myc-actin

incorporation in stress fibers, and fibroblasts which show diffuse cytoplasmic myc staining,  $n = 20$  cells, error bars show SEM between two independent counts of the same experiment by different persons. (c) Myoblast expressing WT myc- $\alpha$ -actin, and myc-tagged act L221P, D292V, and P332S. In (a) and (c) Myc is in red and phalloidin in green on all merged pictures. Scale bars are 20  $\mu$ m.

fuse cytoplasmic cytochrome C, endo G, or AIF staining, which indicates that mitochondria are not disintegrating nor losing their inner membrane potential.

Most CCD patients have mutations in the RyR1 resulting in some cases to a modification of the  $\text{Ca}^{2+}$  release complex arrangement with depletion of RyR1 from the cores [Herasse et al., 2007]. Since in photoreceptor cells RyR1 channels seem to be positioned on specific sites of the endoplasmic reticulum via interaction with the F-actin system [Baumann, 2001; Wang

et al., 2002] we investigated if the expressed CCD causing  $\alpha$ -actin mutants D1Y and E334A possibly induce a mislocalization of this calcium channel on the sarcoplasmic reticulum.

Therefore we localized the RYRs in myotubes expressing the CCD causing  $\alpha$ -actin mutants by immunofluorescence staining of the Ryanodine Receptor (Fig. 6). We observed no differences in staining pattern between untransfected, WT, and mutant  $\alpha$ -actin expressing myotubes. RyR1 staining appears as intracytoplasmic



**TABLE II. Summary of the Cellular Phenotypes we Observed Upon Expression of Seven Myopathy Causing  $\alpha$ -Skeletal Muscle Actin Mutants**

Mutant	Phenotype	Biochemical phenotype	Cell line	Cellular phenotype
WT	–	Normal	Fibroblast Myocyte Myotube	Normal Normal Normal
D1Y	CCD	Normal	Fibroblast Myocyte Myotube	Normal Normal No incorporation in nascent sarcomeres
E334A	CCD	Normal	Fibroblast Myocyte Myotube	Normal Normal No incorporation in nascent sarcomeres
L221P	CFTD <sup>†</sup>	Normal	Fibroblast  Myocyte Myotube	Phalloidin negative nemaline rods and aggregates in the cytoplasm diffuse cytoplasmic myc-actin staining Normal Thick curved actin fibers
D292V	CFTD <sup>†</sup>	Normal	Fibroblast Myocyte Myotube	Phalloidin negative nemaline rods and aggregates in the cytoplasm Normal Thick curved actin fibers
P332S	CFTD	Normal	Fibroblast Myocyte Myotube	Diffuse cytoplasmic myc-actin staining Normal No incorporation in nascent sarcomeres

mic dots, sometimes aligned along the longitudinal axis (Figs. 6a–6d). This distribution of RyR clusters is classical in developing myotubes and is consistent with the location of the sarcoplasmic reticulum along the myofibrils [Castellani et al., 1996; Flucher et al., 1993, 1994].

Variations in intracellular free  $\text{Ca}^{2+}$  concentration were measured in myotubes loaded with the fluorescent calcium probe Fura-2. Myotubes were stimulated with caffeine in order to directly activate the RyR and to analyze calcium release responses in cells expressing  $\alpha$ -actin mutants. The expression of both D1Y and E334A  $\alpha$ -actin mutants did not prevent the calcium response to caffeine stimulation, and the calcium signals were not changed compared to control cells (Figs. 6e and 6f). This showed that calcium release properties and RyR1 functions are not altered by the presence of the  $\alpha$ -actin mutants causing CCD.

## DISCUSSION

### CFTD and CCD Causing $\alpha$ -Actin Mutants are Correctly Folded and Stable

In this study we investigated two  $\alpha$ -actin mutants causing CCD and three  $\alpha$ -actin mutants implicated in CFTD. Using our in vitro folding and band-shift assay, we showed that all five mutants fold correctly, are stable and bind to the ABPs DNaseI and VDBP. For the CFTD mutants, our biochemical data complement the results reported by Clarke et al. [Clarke et al., 2007]. These authors showed, using immunoprecipitation, that mutants  $\alpha$ -actin L221P, D292V, P332S incorporate in filaments and that  $\alpha$ -actin D292V and  $\alpha$ -actin P332S

function in an in vitro motility assay [Clarke et al., 2007]. This is consistent with our observation that the mutants are folded to a functional conformation, given they interact with the ABPs tested. However for  $\alpha$ -actin D292V the interaction with tropomyosin seems to be particularly disrupted [Clarke et al., 2007].

### CCD Causing $\alpha$ -Actin Mutants Incorporate Normally in Actin Structures and do not Affect the Distribution of Mitochondria or Ryanodine Receptors in Cultured Myotubes

The two  $\alpha$ -actin mutants associated with CCD incorporate normally in nonmuscle specific actin structures in cultured fibroblasts and undifferentiated Sol8 myoblasts. On the other hand, we observed no or little incorporation of these mutants between the nascent z-lines in differentiating myotubes, suggesting these mutants are not included in muscle specific structures, i.e. those forming sarcomeres. So the structural defects of the cytoskeleton are clearly muscle specific. A summary of the cellular phenotypes we observed for the mutants causing CCD is given in Table II.

The molecular mechanism behind core myopathies has not yet been unraveled. In patients, the CCD causing  $\alpha$ -actin mutants induce core lesions, which differ in size, number and appearance. The muscle biopsies of the family affected with the D1Y mutation show broad, unstructured, central cores that do not run along the entire long axis of the muscle, which are typical CCD cores, whereas those of the family expressing the E334A  $\alpha$ -actin mutant show minicores, and a single muscle fiber



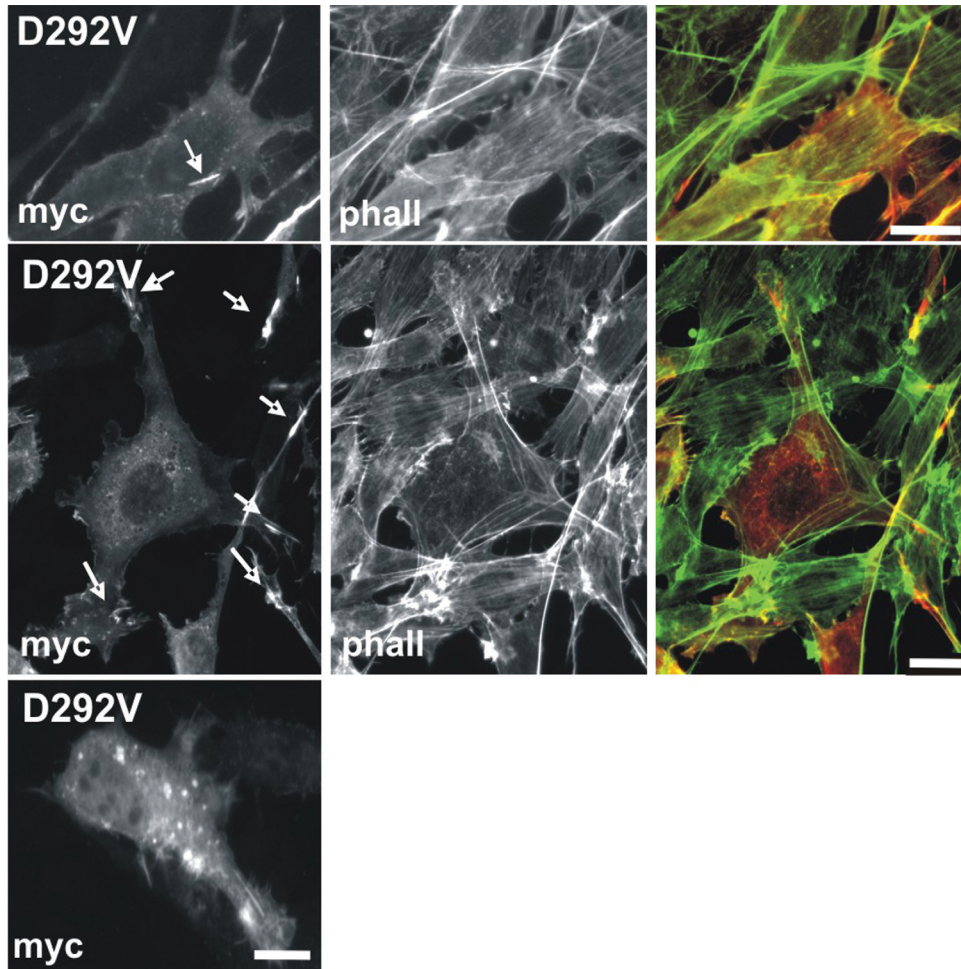


Fig. 3. CFTD associated  $\alpha$ -actin mutant D292V induces nemaline rods (upper and middle panel) and displays aggregate formation (lower panel) in NIH 3T3 fibroblasts. Arrows indicate rods.

contains multiple cores [Kaindl et al., 2004]. However, by performing cytochrome c, endonuclease G, or AIF stainings, we could not detect defects in the distribution of mitochondria in early myotubes expressing the CCD causing  $\alpha$ -actin mutants. The stainings further indicate that mitochondria are not disintegrating, nor losing their inner membrane potential at this stage of differentiation. We can not rule out that formation of cores can only be observed in more mature muscle cells, as this may be a long term physiopathologic process or an adaptation to more general metabolic alterations.

In 90% of the patients with CCD, mutations in RyR1 occur. This is a sarcoplasmic reticulum channel,

which mediates  $\text{Ca}^{2+}$  release during excitation–contraction coupling. Two models exist to explain the consequences of these mutations. The first model predicts that the RyR1 mutations lead to a sustained calcium leak. The second model suggests a loss of functional connection between RyR1 and the dihydropyridine receptor DHPR- $\alpha 1$  in the T-tubuli, leading to excitation–contraction uncoupling [Herasse et al., 2007]. Our immunostainings on RyR1 showed no specific phenotypes for the CCD causing  $\alpha$ -actin mutants in myotubes after 5 days of differentiation. The localization of RyRs clusters along the longitudinal axis of the cell is consistent with the classical observed distribution in differentiating myo-

Fig. 4. In myotubes, CCD causing mutants do not incorporate between nascent Z-lines in differentiating myotubes, CFTD causing mutants induce curved thickened actin fibers. (a) myc-staining and troponin-T staining or  $\alpha$ -actinin staining of Sol8 myotubes expressing WT  $\alpha$ -actin, (b–d)  $\alpha$ -actinin staining and myc-staining of Sol8 myotubes expressing  $\alpha$ -actin D1Y and E334A, (c)  $\alpha$ -actin L221P, D292V and P332S, and (d)  $\alpha$ -actin D154N. Scale bars are 20  $\mu\text{m}$ . Underneath the pictures of  $\alpha$ -actin WT, D1Y, L221P, and D292V expressing cells are zoomed-in pictures of the yellow square regions.



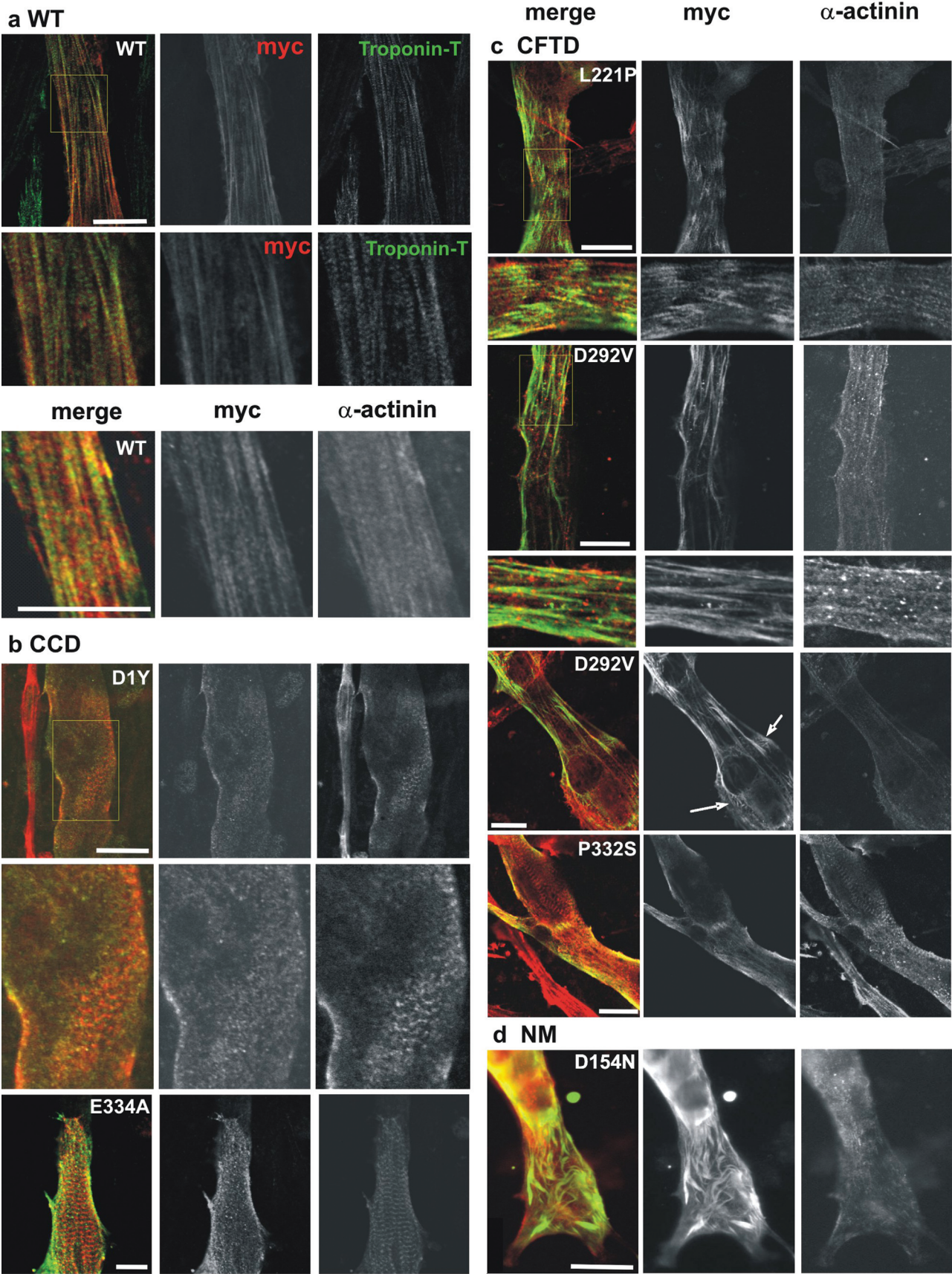


Figure 4.

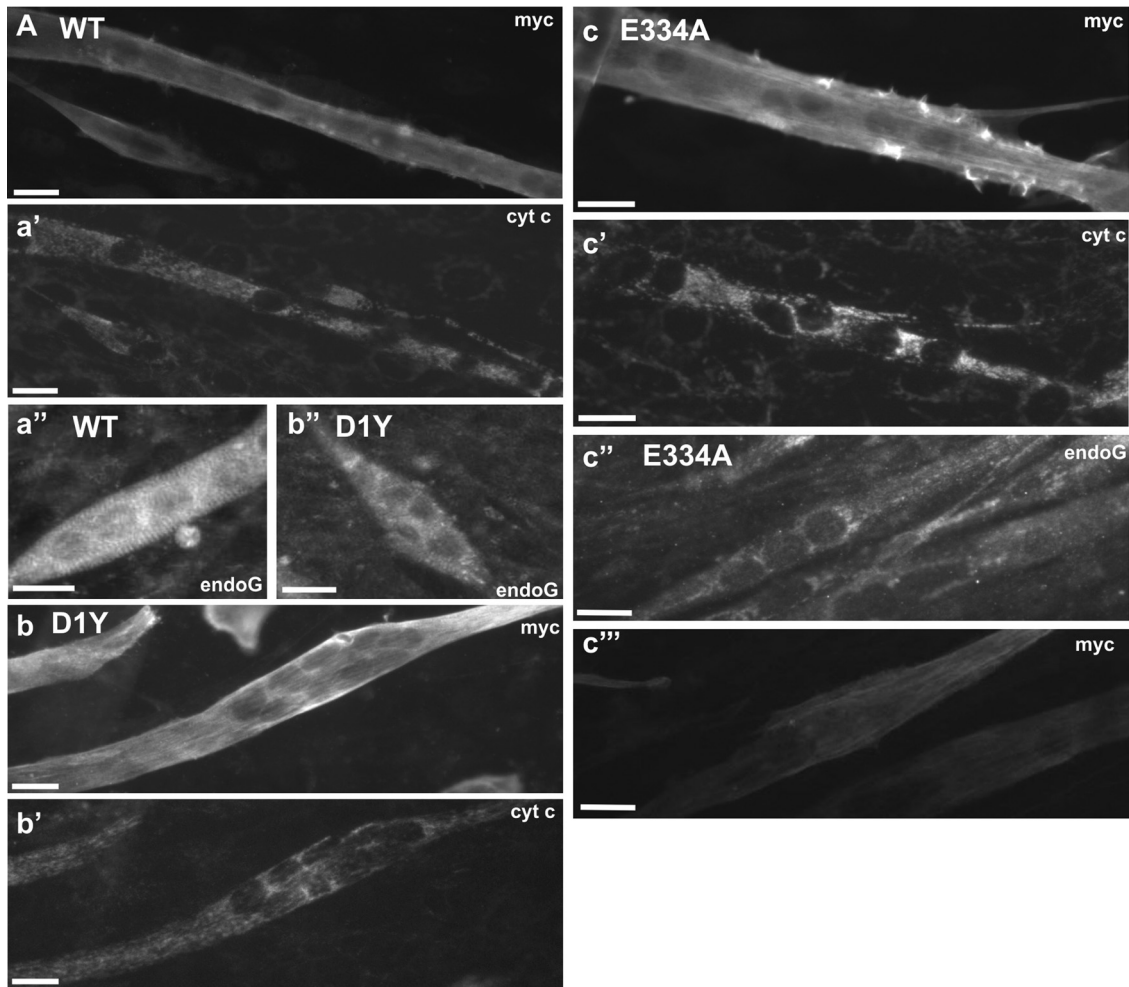


Fig. 5. Localization of mitochondria appears normal in myotubes expressing CCD causing  $\alpha$ -actin mutants. (a–c) Myc-staining of Sol8 myotubes expressing WT  $\alpha$ -actin (a),  $\alpha$ -actin D1Y (b), and  $\alpha$ -actin E334A (c). (a'–c') Corresponding stainings of mitochondria with an anti-cytochrome C antibody. (a''–c'') Stainings of mitochondria of myc- $\alpha$ -actin expressing myotubes with an anti-endonuclease G antibody (c'') and a corresponding myc-actin staining of (c'''). Scale bars are 20  $\mu$ m.

tubes and is not markedly different between untransfected, WT or mutant expressing myotubes [Yuan et al., 1991]. The expression of  $\alpha$ -actin mutants causing CCD did also not alter the calcium release properties from RyR1 in myotubes suggesting that there are no direct relations between physiopathologic pathways due to RyR1 mutations and alterations due to  $\alpha$ -actin mutants.

#### In Cultured Cells Some CFTD Causing $\alpha$ -Actin Mutants Display Partly Similar Phenotypes as NM Causing Mutants Suggesting Molecular Mechanisms of CFTD and NM may be Partly Related

Expression of the N-terminally myc-tagged CFTD causing  $\alpha$ -actin mutants in cultured fibroblasts and undifferentiated Sol8 myoblasts reveals that all the mutants are able to incorporate in nonmuscle specific actin fila-

mentous structures. However, respectively, 90 and 85% of fibroblasts expressing  $\alpha$ -actin L221P and P332S show additionally a diffuse cytoplasmic localization of the myc-tagged actin, suggesting these mutants do not fully incorporate in actin filaments.

A phenotype which is similar to our previous observations expressing NM causing actin mutants, is that mutants  $\alpha$ -actin D292V and, to a lesser extent,  $\alpha$ -actin L221P induce aggregate and/or rod formation when expressed in fibroblasts. A small amount of the phalloidin negative rods in  $\alpha$ -actin D292V expressing fibroblasts appears intranuclear or perinuclear, but the majority of the rods is seen in long cell extensions of the spread cells. These rods in the cell extensions are specific for this mutant and, to our knowledge, not earlier described for NM causing  $\alpha$ -actin mutants. The lack of phalloidin staining of the rods does not indicate they



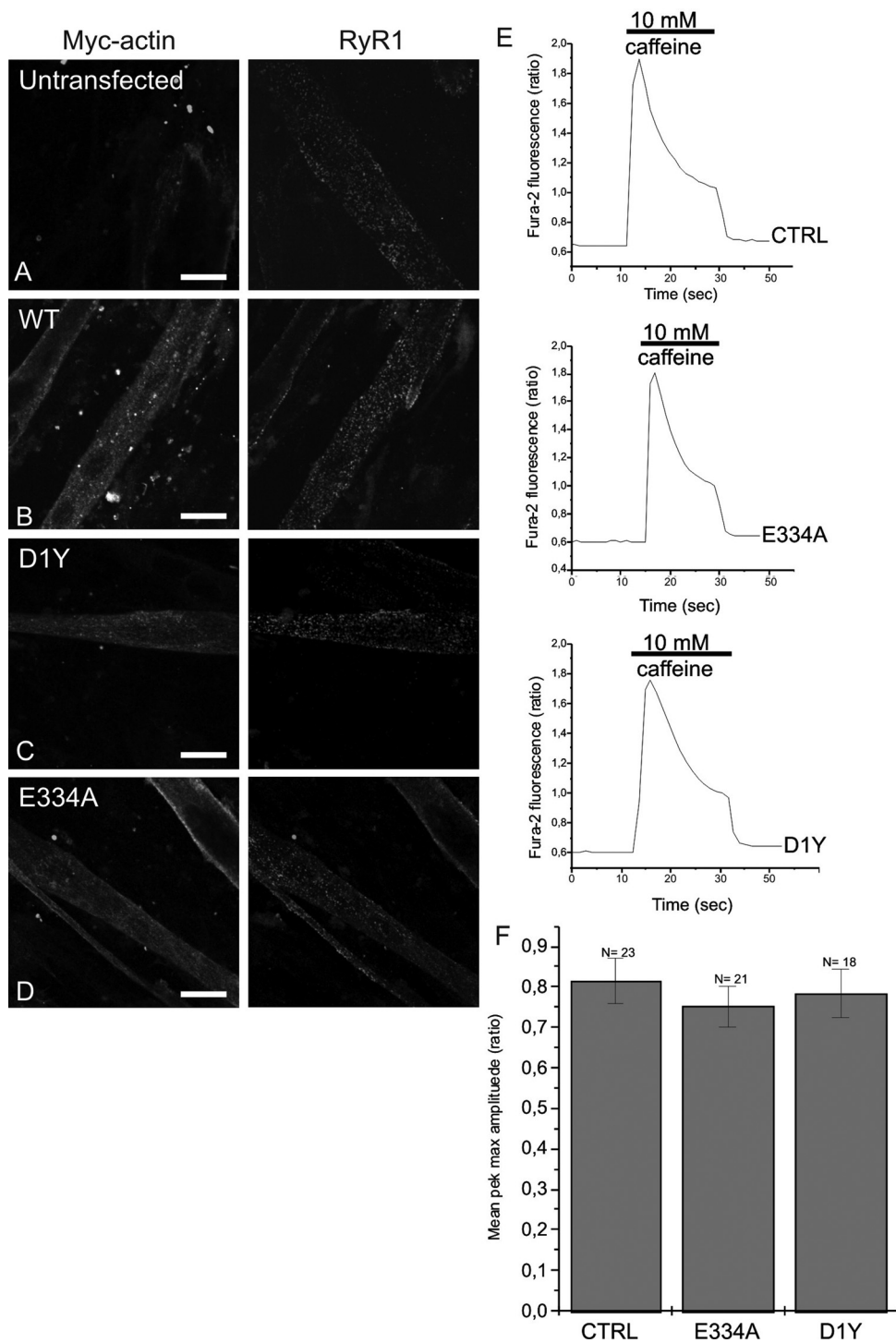


Fig. 6. Localization of RyR1 and calcium release properties were normal in myotubes expressing CCD causing  $\alpha$ -actin mutants. (a–d) Myc stainings and corresponding RyR1 stainings of untransfected Sol8 myotubes (a) or Sol8 myotubes expressing WT  $\alpha$ -actin (b),  $\alpha$ -actin D1Y (c), and  $\alpha$ -actin E334A (d). Scale bars are 20  $\mu$ m. (e) Representative examples of intracellular calcium transients in Sol8 cells induced by 10 mM caffeine in control myotubes or in myotubes trans-

fected with the  $\alpha$ -actin mutant D1Y or E334A. Calcium transients were recorded with the fluorescent probe Fura-2/AM in F + 4 myotubes. Bold lines correspond to the superfusion of caffeine. (f) Histogram of calcium transient parameters induced by 10 mM caffeine. Histograms correspond to variations in amplitude of Fura-2 fluorescence ratio. The bar graphs correspond to the mean  $\pm$  sem. *n* corresponds to the number of experiments.

contain monomeric actin, but it may mean that the filaments in the rods are decorated with an actin binding protein that occludes the phalloidin binding sites. For instance it is well known that cofilin decorated filaments are phalloidin negative [Minamide et al., 2000]. A summary of the cellular phenotypes we observed for the CFTD causing  $\alpha$ -actin mutants is given in Table II.

The fact that CFTD causing  $\alpha$ -actin mutants L221P and D292V induce the main characteristics of NM in fibroblasts, i.e. actin aggregates, and in the case of  $\alpha$ -actin D292V rods, is perhaps surprising. However, Clarke et al. made a similar observation for C-terminally GFP-tagged  $\alpha$ -actin D292V, in C2C12 myoblasts [Clarke et al., 2007], but not for  $\alpha$ -actin L221P. This difference with our observations can be due to the use of different tags as we previously described that the type and position of the tags can influence observed phenotypes [Rommelaere et al., 2003]. Alternatively the difference may be due to the used cell lines, since we recently observed that, for certain NM causing  $\alpha$ -actin mutants, rod formation is more abundant in fibroblasts [Costa et al., 2004] than in muscle cells (Rommelaere et al., in preparation). Additionally, in Sol8 myotubes, some of these mutants induce the formation of thickened actin fibers, which are sometimes curved. These cells also have no striated  $\alpha$ -actinin pattern suggesting sarcomere formation is also affected. Incidentally such fibers also appear in some differentiated Sol8 myotubes expressing NM causing  $\alpha$ -actin mutants, e.g. G15R [Bathe et al., 2007] and D154N (Fig. 4d). Nevertheless the observation that some CFTD causing mutants display similar phenotypes as NM causing  $\alpha$ -actin mutants suggests that in some cases the underlying molecular mechanisms of CFTD and NM may be (partly) common.

### A Relation Between NM, CCD and CFTD?

Our observation of similar phenotypes of CFTD and NM associated mutants in cultured cells is in line with the fact that some  $\alpha$ -actin mutations associated with NM affect size and distribution of muscle fiber types, which is characteristic for CFTD [Goebel et al., 1997; Schroder et al., 2004; Hutchinson et al., 2006]. On the other hand the coexistence of rods and core lesions in patients suffering from NM caused by  $\alpha$ -actin mutations has been described [Jungbluth et al., 2001]. This suggests that CCD and NM may also share partly common dysfunctions of the actin cytoskeleton. Mutations in the *cofilin-2* gene also induce a myopathy where both, nemaline bodies and minicores, are observed [Agrawal et al., 2004]. Additionally patients suffering from CCD caused by mutations in the RyR1 can present nemaline rods in their muscle biopsies [Monnier et al., 2000; Scacheri et al., 2000]. It is also striking that there is one case where CFTD and minicores have been found in one and

the same family. This suggests a common, or similar, mechanism behind CCD and CFTD pathogenesis [Jongpiputvanich et al., 1995]. Taken together, it seems that NM, CFTD, and CCD may have related defects. This leads us to the question: what could be the underlying molecular mechanism of NM, CFTD, and CCD associated with  $\alpha$ -actin mutations? For the  $\alpha$ -act D292V CFTD causing mutant, Clarke et al. suggested a defect in tropomyosin binding [Clarke et al., 2007]. This links to  $\text{Ca}^{2+}$  sensitivity as the tropomyosin function is under control of this ion via the troponin complex [Gordon et al., 2000]. Although the possibility of altered  $\text{Ca}^{2+}$  regulation has not been studied in NM, it has been earlier suggested that muscle weakness can be caused by a reduced  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus in this disease [Michele et al., 1999; Gommans et al., 2003] and the idea is reinforced by the fact that NM can also be caused by mutations in tropomyosin and troponin [Clarkson et al., 2004]. CCD can of course be considered as a disease where  $\text{Ca}^{2+}$  homeostasis is affected, given that the majority of CCD cases are caused by mutations in RyR1. Although expression of both D1Y and E334A  $\alpha$ -actin mutants did not alter the calcium response of cells, we cannot rule out a reduced  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus here as well.

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