Mutations in the gene encoding skeletal muscle α-actin (ACTA1) account for approx. 20% of patients with the muscular disorder nemaline myopathy. Nemaline myopathy is a muscular wasting disease similar to muscular dystrophy, but distinguished by deposits of actin and actin-associated proteins near the z-line of the sarcomere. Approx. one-third of the over 140 myopathy actin mutations have been characterized either biochemically or in cultured cells to determine their effects on the actin cytoskeleton. However, the actin defects causing myopathy are likely to be heterogeneous, with only a few common trends observed among the actin mutants, such as reduced polymerization capacity or an inability to fold properly. Notably, the transcriptional programme regulated by serum-response factor, which is instrumental in muscle development and maintenance, is directly controlled by the balance of actin assembly and disassembly in cells. In the present study, we explored the impact of myopathy mutations in actin on the control of the transcriptional response by serum-response factor and found that the majority of mutants examined have altered serum-response factor signalling. We propose that altered serum-response factor signalling could be a major factor in actin-based nemaline myopathy, and that this area could be exploited to develop therapies for sufferers.

Key words: actin, muscle, myopathy, nemaline myopathy, serum-response factor.

INTRODUCTION

NEM (nemaline myopathy) is a congenital neuromuscular disorder which results in muscle weakness and wasting similar to the more common and more well-studied disease muscular dystrophy. NEM is diagnosed partly by the presence in muscle biopsies of nemaline bodies, inclusions of sarcomere proteins deposited in the muscle tissue around the z-line. The appearance of the muscle biopsy specimens has been used to classify these myopathies further into three subclasses: (i) AM (actin myopathy), (ii) IRM (intranuclear rod myopathy), and (iii) NEM. NEM, AM and IRM are caused primarily by mutations in the muscle thin filament proteins, namely nebulin, skeletal muscle α-actin (ACTA1), α- and β-tropomyosin, troponin-2 and, perhaps surprisingly, coflin-2 [1], which is not a typical thin filament protein (reviewed in [2]). Mutations in ACTA1 account for approx. 20% of NEM patients and the mutations span more than 140 different residues of the ACTA1 protein [2,3]. The disease manifests itself in various forms, ranging from severe, where infants are dependent on a respirator from birth, to mild, where the symptoms present during childhood and patients have relatively normal motor milestones, but even the mild disease may be progressive [4].

The mutations in ACTA1 that cause NEM, AM or IRM do not obviously localize to any specific region(s) of the actin molecule, but rather they are found throughout the three-dimensional structure [2,3,5]. This may help to explain why, thus far, characterization of more than 30 different actin mutants that cause NEM, AM or IRM have not shown very clear correlations between the disease and any specific biochemical or cell biological defect of the mutant actin. It may also be that 30 mutations are still such a small proportion of the total number described that we need to analyse more mutations before a clear pattern emerges. With these caveats in mind, some interesting patterns have emerged, such as the observation that ACTA1-L94P and ACTA1-G259V, which cause severe recessive myopathy, show protein folding defects leading to lack of functional muscle actin [6]. Approx. half of the mutants tested thus far show a reduced ability to copolymerize with wt (wild-type) actin [2]. Many of the mutants also form aggregates and rods in the nucleus and cytoplasm of cells in culture that are reminiscent of those seen in patient biopsy samples [2]. However, whether these structures are related to nemaline bodies in more than just appearance remains to be explored. Expression of several different mutant actins leads to cell death with cell rounding and bleb formation, suggesting that apoptotic cell death may be a major feature of muscle wasting seen in NEM patients [7].

Muscle differentiation and development is regulated by multiple transcription factor families. SRF (serum-response factor) is a MADS [MCM1 (minichromosome maintenance 1), agamous, deficiens and SRF] box transcription factor, sharing a common DNA-binding motif with other MADS box proteins, that is highly expressed in skeletal muscles and controls growth and differentiation in multiple tissues. One of the main mechanisms of SRF-mediated activation is through recognition and binding with a DNA motif known as a CArG [CC(A+T-rich)GG] box (Figure 1E). The CArG box is a 10 bp sequence with the consensus CCwGG [8]. Several genes whose expression is regulated by SRF binding to CArG boxes in their promoter region have been identified. Many of these targets affect muscle growth and development, including ACTA1 itself, muscle creatine kinase, dystrophin, tropomyosin, myogenin, MyoD and myosin light chain 1/3 [9]. SRF also forms complexes with other transcription factors involved in muscle development, such as

Abbreviations used: AM, actin myopathy; CytD, cytochalasin D; DNaseI, deoxyribonuclease I; EGFP, enhanced green fluorescent protein; F-, filamentous; FCS, fetal calf serum; G-, globular; GST, glutathione transferase; IRM, intranuclear rod myopathy; Jasp, jasplakinolide; LatB, latrunculin B; LMB, leptomycin B; MADS, MCM1 (minichromosome maintenance 1), agamous, deficiens and serum-response factor; NEM, nemaline myopathy; SRF, serum-response factor; TCF, ternary complex factor; wt, wild-type.

1 Present address: Faculty of Medicine Department of Biophysics, University of Pécs, Szigiét ut 12, Pécs H-7624, Hungary
2 To whom correspondence should be addressed (email l.machesky@beatson.gla.ac.uk).
myogenin and MyoD [10]. SRF activates the expression of c-Fos, another transcription factor that regulates proliferation and differentiation [11]. At the promoter of c-fos, SRF interacts with other transcription cofactors, such as members of the TCF (ternary complex factor) proteins and controls transcription via MAPK (mitogen-activated protein kinase) signalling [11]. Myocardin-related transcription also interact with SRF in muscle-specific Rho signalling and actin assembly pathways that are independent of its control by TCF [12–14]. Overall, SRF is involved in many transcriptional signalling cascades and co-operates with other transcription factors to regulate fundamental elements of embryogenesis, including muscle differentiation and growth.

The cofactor for SRF activation, MAL, has three actin-binding RPEL sequences and binds directly to unpolymerized [G-(globular)] actin (Figure 1E). Activation of actin assembly via serum stimulation leading to Rho GTPase activation causes MAL release from the G-actin as it polymerizes and allows the released MAL to activate SRF-mediated transcription. Recent studies show that high rates of nuclear export via the CRM1 exporter maintain MAL in the cytoplasm of resting cells (Figure 1E) [15]. G-actin sequesters MAL in the nucleus and in the cytoplasm and prevents SRF activation (Figure 1E). Stimulation by serum blocks export of MAL and induces its accumulation in the nucleus. Simultaneous depletion of free nuclear and cytoplasmic actin via activation of actin assembly and also regulation of the MAL–actin interaction (by an unknown mechanism) leaves more free nuclear MAL that can form active complexes with SRF for transcription [15] (Figure 1E). It is not understood how actin regulates nuclear export of MAL or whether actin–MAL complexes travel together when going into and out of the nucleus [15].

To date, many myopathy-causing actin mutations have been found to affect actin assembly and cytoskeletal dynamics in cultured cells [2]. Since the actin cytoskeleton is intimately linked with the SRF pathway, we hypothesized that patients with actin mutations could have defects in SRF signalling, as well as in actin assembly and cytoskeletal function, in their muscle cells. We therefore investigated the effects of 12 mutants of ACTA1 that have previously exhibited a range of biochemical and cell-based mutagenesis kit (Stratagene) as described previously [6]. Overall, SRF is involved in many transcriptional signalling cascades and co-operates with other transcription factors to regulate fundamental elements of embryogenesis, including muscle differentiation and growth.

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**MATERIALS AND METHODS**

**Plasmids**

Expression vectors encoding C-terminal EGFP (enhanced green fluorescent protein)-tagged or N-terminal Myc-tagged wt or ACTA1 mutations were made with using the QuikChange** site-directed mutagenesis kit (Stratagene) as described previously [6]. The expression vectors for pEF-FLAG-MAL and the luciferase reporter SM22 promoter have been reported previously [16,17]. The C-terminal EGFP-tagged ACTA1-G15S mutant derivative was made with pEGFP vector as the template, a 5′ primer containing a XhoI site and a 3′ primer containing an EcoRI site and appropriate primers.

**Cell culture and transfection**

C2C12 cells were cultured at 37°C in a humidified 5% CO₂ incubator in a proliferation medium comprising DMEM (Dulbecco’s modified Eagle’s medium; Gibco BRL) supplemented with 10% FCS (fetal calf serum; Sigma). Transfections were made using Lipofectamine™ 2000 transfection reagent (Gibco BRL) according to the manufacturer’s protocol. Cells were generally used for experiments 48 h after transfection.

**Immunofluorescence and microscopy**

C2C12 cells were plated on to glass coverslips, then washed three times in PBS and fixed with 4% (w/v) paraformaldehyde for 10 min at room temperature (21°C). Free aldehyde groups were blocked with 50 mM ammonium chloride for 10 min and cells were permeabilized with PBS containing 0.1% Triton X-100 for 4 min. Cells were incubated with the 9E10 anti-Myc antibody for 20 min at room temperature, followed by Texas-Red-conjugated anti-mouse secondary antibody. For FLAG detection, cells were blocked with 10% BSA and were incubated with the M2 monoclonal anti-FLAG antibody for 2 h containing 3% BSA. Cells were washed with PBS for 5 min between each step. Finally, coverslips were washed three times in water and mounted on a slide with 5 μl of Mowiol (Calbiochem) plus Antifade.

Immunostained cells were examined with a Zeiss microscope using a 63× objective. At least 100 cells were counted in each independent experiment (or 20 cells in the case of control experiments) and statistics were calculated using three independent transfections. Images were recorded with a Hamamatsu C4880 camera and processed using Photoshop software (Adobe).

**Luciferase assay**

C2C12 cells (10⁵ cells/well) were plated in six-well plates and were co-transfected with encoding vectors of 3 μg of wt or ACTA1 mutants, 3 μg of FLAG–MAL and 1 μg of luciferase reporter SM22. After 12 h incubation, the cells were serum-starved for 24 h and then 20% serum was added for 4 h. In the case of drug treatment, after 24 h serum starvation, 2.5 μM CytD (cytochalasin D), 0.5 μM Jasp (jasplakinolide) or 20 nM LMB (leptomycin B) was added to the cells for the time specified. A 1 μM concentration of LatB (latrunculin B) was applied after 4 h of 20% serum treatment. Finally the cells were harvested and luciferase activities were measured according to the manufacturer’s instructions. All of the assays were performed three times and the values were normalized to the values obtained for wt actin.

**GST (glutathione transferase)-pull-down assay**

An in vitro transcription/translation reaction was used to express 35S-labelled wt or mutant actin in reticulocyte lysate (Promega) according to the manufacturer’s instructions. In the reactions, 0.02 μCi of [35S]methionine and 1 μg of DNA per 25 μl reaction was used. After 1.5 h of incubation at 30°C the products were analysed using SDS/PAGE, followed by autoradiography. The Pfam-defined RPEL 2 and RPEL 3 motifs (RPEL 2 domain) containing 85 amino acids from position 21 in MAL isoform 1 has been cloned into pGEX-4T-2 vector as a template, a 5′ primer containing the EcoRI site and the 3′ primer for the EcoRI site and appropriate primers.
then incubated with 10 μg of RPEL 2 domain bound to agarose beads for 1 h at room temperature. The agarose beads were sedimented and the supernatant was removed. The agarose beads were washed three times and then resuspended in 20 μl of PBS. The final pellets, supernatants and control were analysed using 4–12% SDS-PAGE, followed by autoradiography. The band intensities were measured with ImageJ software and the values were normalized to the control. All mutants were analysed three times. Values were considered statistically significant at \( P < 0.05 \), as measured using a Student’s \( t \) test in Microsoft Excel.

Flow cytometry

C2C12 cells (5×10⁵ cells) were transfected with Myc-tagged ACTA1 constructs. Cells were pelleted at 300 g for 5 min and then resuspended in 100 μl of 4% (w/v) paraformaldehyde for 10 min. Cells were washed with 1 ml of FACS buffer [5% FCS, 0.02% sodium azide and 0.5% saponin (Invitrogen) in PBS] and sedimented. The 9E10 anti-Myc primary antibody was applied for 30 min, followed by Marina-Blue-conjugated anti-mouse secondary antibody, Alexa Fluor® 488-conjugated DNase I (deoxyribonuclease I) and Texas-Red-X-conjugated phalloidin (Invitrogen) (for 30 min). Cells were washed and sedimented between each step. The samples were analysed with the BD Biosciences FACS Aria cell sorter system. Blue-positive cells (10⁵ cells) were used for analysis, and the green (530 nm) and red (610 nm) intensities were measured and the average values were calculated.

Data analysis and statistics

At least three independent experiments were performed for each assay. Values are means ± S.D. in all cases. Values were considered statistically significant at \( P < 0.05 \), as measured using an unpaired Student’s \( t \) test in Microsoft Excel.

RESULTS

AM mutations affect the SRF signalling pathway in C2C12 myoblasts

When SRF signalling was activated by serum stimulation, cells showed increased localization of MAL in the nucleus and enhanced transcription of SRF reporter genes (Figure 1E) [18]. We hypothesized that mutations in actin that cause myopathy might affect the SRF transcriptional programme either directly via altered interactions of the mutant G-actin with MAL, or indirectly via disruption of normal cellular actin dynamics. We monitored the nuclear accumulation of MAL in response to serum stimulation using an immunofluorescence microscopy assay, and we measured SRF activity in cells expressing mutant actins with a biochemical luciferase reporter assay [12]. Cells expressing only wt ACTA1 showed a dramatic relocalization of MAL to the nucleus following serum stimulation, with 5% of resting cells and approx. 80% of stimulated cells showing nuclear MAL accumulation (Figures 1A and 1B). The luciferase activity in this assay increased approx. 2.5-fold for cells expressing wt ACTA1 upon serum stimulation (Figures 1C and 1D). Cells expressing mutant actins displayed cytoplasmic or nuclear rods or aggregates as reported previously [6] (e.g. Figure 1A, ACTA1-H40Y green nuclear rods) as well as some localization of mutant actin to normal actin structures (e.g. Figure 1A and [6]). We did not generally find any co-localization between actin stress fibres, rods or aggregates and MAL, which localized evenly in the cytoplasm and the nucleus (Figure 1A). Cells expressing any of the mutant actins showed less nuclear accumulation of MAL in the presence of serum than cells expressing wt actin (Figure 1B). The luciferase reporter assay, which measures the level of SRF activation, also reflected this trend (Figures 1C and 1D) with only one mutant, ACTA1-I357L, showing slightly higher than normal levels of SRF activation. In the absence of serum, cells expressing most of the mutants had a similar level of SRF activation to that of the wt except for one construct, ACTA1-H40Y, which had an approx. 2-fold higher number of cells showing nuclear accumulation of MAL (Figure 1B), but still a slightly reduced or similar level of luciferase reporter activity (Figure 1C). The mutant ACTA1-R183G showed nearly no serum stimulation of nuclear accumulation of MAL, and only modest stimulation of luciferase SRF reporter activity (Figures 1B and 1C).

Since mutant ACTA1-H40Y showed an increased basal level of nuclear MAL accumulation and a somewhat reduced response to serum stimulation, and mutant ACTA1-R183G was severely blunted in serum responsiveness, we characterized these mutants further. We compared ACTA1-H40Y with ACTA1-G15S (Figure 2A), a mutant not associated with disease but previously shown to promote the nuclear accumulation of MAL in cultured cells and elevate basal activity of SRF, but not serum-responsive activity [19]. We found 50% nuclear MAL accumulation caused by ACTA1-G15S which does not change after treating cells with serum. In contrast, cells expressing ACTA1-H40Y showed 60% and 25% nuclear MAL localized with and without serum respectively, so ACTA1-H40Y appears to be somewhat more sensitive to serum stimulation than ACTA1-G15S, but less than the wt actin. It is interesting to note that ACTA1-G15R, a naturally occurring NEM mutant, retains some serum sensitivity, unlike ACTA1-G15S (Figure 1). We treated the cells in the presence of serum with the actin monomer-sequestering drug LatB to find out whether the nuclear MAL translocates back to the cytoplasm to interact with the higher concentration of G-actin induced by LatB. LatB treatment resulted in rapid cytoplasmic relocation of MAL in serum-treated wt cells ([15] and Figure 1E). We did not find any change in the distribution of MAL after 40 min of LatB treatment of actin ACTA1-H40Y cells (Figure 2A). Thus we conclude that ACTA1-H40Y mutant actin promotes a higher than normal accumulation of MAL in the nucleus, but does not increase SRF reporter activity, and is relatively insensitive to LatB treatment. Perhaps the LatB fails to induce depolymerization of ACTA1-H40Y and thus cells expressing ACTA1-H40Y fail to show cytoplasmic sequestration of MAL in response to LatB.

Since actin cycles through the nucleus and the MAL–actin complex is exported via the nuclear exporter CRM1 ([15] and Figure 1E), we tested the effect of inhibiting CRM1 on the nuclear localization of ACTA1-R183G to determine whether this mutant is preventing MAL accumulation in the nucleus or is causing more rapid export of MAL. We found that, without serum, approx. 30% of cells show accumulation of MAL in the nucleus and this does not significantly change with addition of LMB for 5 or 15 min (Figure 2B). Thus we conclude that actin R183G is inhibiting the import of MAL into the nucleus.

Response of cells expressing myopathy mutant ACTA1 to CytD or Jasp treatment

We used CytD and Jasp to further probe the defects in the SRF response that we observed with the myopathy mutant actins. CytD and Jasp both induce rapid nuclear accumulation of MAL in wt cells (Figure 1A) [15]. Both drugs are actin-specific and commonly used to alter cytoskeletal dynamics; however, they act in completely different ways: Jasp stabilizes
Figure 1  Cells expressing mutant actin show impaired subcellular MAL distribution and SRF activation

(A) Representative immunofluorescence pictures of C2C12 cells co-transfected with EGFP–ACTA1 and mutants (wt-ACTA1, ACTA1-H40Y and ACTA1-R183G) or Myc–actin (green) and MAL (red) in response to serum (ser) stimulation. (B) Nuclear MAL accumulation in the presence (black bars) or absence (white bars) of serum in cells expressing wt or mutant EGFP–ACTA1 and MAL revealed by counting the cells observed with immunofluorescence (at least 100 cells were counted per experiment and three independent experiments were performed). (C and D) Serum-induced SM22 luciferase activation of the C2C12 cell line expressing GFP– (C) or Myc– (D) ACTA1, MAL and SM22 luciferase reporter. Values are means ± S.D. from three independent experiments. Asterisks indicate significant difference from the control value at $P < 0.05$ in the unpaired Student’s $t$ test. (E) Schematic diagram showing the relationship between actin assembly/disassembly and nuclear shuttling of MAL (based on ideas proposed in [15,24]). Arrow 1 shows the G-actin–MAL complex dissociating as actin polymerizes and MAL is released. This free MAL then diffuses into the nucleus to interact with SRF and co-activate transcription. This is the reaction induced by serum and also by drug treatment with Jasp or CytD. Arrow 2 shows that when actin depolymerizes, due to serum starvation or treatment with LatB, actin monomer can sequester MAL in a complex that cannot participate in SRF co-activation. Arrow 3 shows that free MAL can enter the nucleus, where free MAL interacts with SRF and actin-bound MAL does not. It is not known whether actin–MAL complexes can enter the nucleus together, but there is actin in the nucleus and it can form complexes there with MAL. Arrow 4 shows that free MAL can be exported from the nucleus by the CRM1 transporter and that this reaction is inhibited by LMB. It is unclear whether actin–MAL complexes can be co-transported out of the nucleus. Arrow 5 shows that free nuclear MAL can interact with SRF and mediate transcription of CArG-box-containing target genes.
actin filaments, whereas CytD binds to barbed ends of filaments and prevents polymerization, as well as having some interactions with actin monomers and oligomers at higher concentrations [20]. Jasp induces nuclear MAL via stabilization of filaments and dissociation of actin monomer from MAL to allow faster nuclear import [13,18]. The mechanism by which CytD induces nuclear MAL accumulation is less clear, but it appears to disrupt the MAL–actin complex and promote MAL accumulation in the nucleus [18]. The AM-related mutations investigated in the present study would not be predicted to alter the binding site of Jasp or CytD, on the basis of their position in the three-dimensional structure of ACTA1 [21]. We tested the mutant actin constructs using 15 min of drug treatment (Figure 3) at a concentration where significant effects of each drug on the actin cytoskeleton were observed (results not shown). All of the mutants tested showed a similar response to Jasp or CytD treatment with respect to the appearance of F-actin (filamentous actin) in phalloidin-stained cells (results not shown). Cells expressing G15R, H40Y, I64N and N115S mutant ACTA1 show lower activation of SRF in the presence of either drug, whereas ACTA1-R183G and ACTA1-D286G show higher SRF reporter gene activation than the wt (Figure 3A). Cells expressing ACTA1-I136M, ACTA1-G182D and ACTA1-I357L show SRF overactivation, whereas M132V and G268R have wt-like activation. Thus we conclude that a subset of myopathy mutations induce significant alterations in SRF activity and that different mutants have different sensitivity to the actin-binding drugs Jasp and CytD. In general, nine out of 12 mutants showed a reasonable drug response, indicating that, whatever the cause of low SRF activity, it could be partially overcome with drug treatment to disrupt the MAL–actin interaction. This could represent an interesting opportunity for further studies on the effect of drug treatment on altered transcriptional programmes of myopathy muscles.

Interactions of mutant actin with the SRF RPEL 2 domain

One explanation for the altered SRF response in cells expressing mutant actins might be that mutations in actin cause altered binding to MAL. Mutants showing increased affinity for MAL would be expected to have an inhibitory effect on the nuclear translocation and/or on SRF activation once translocated (Figure 1E). Mutants showing a decreased affinity for MAL might be expected to promote increased basal levels of MAL activation and decreased overall serum responsiveness. We thus tested whether some of the myopathy mutants that showed altered SRF reporter responses showed altered binding capacity for the actin-binding RPEL domain of MAL. GST-tagged RPEL 2 domain containing RPEL 2 and 3 motifs bound to agarose beads were incubated with in vitro translated wt or mutant actin. Seven actin mutants which had impaired activation of the SRF response, MAL accumulation or drug response were tested (Figure 4). We found that all of the mutants tested could bind to the RPEL 2 domain. Two mutants, G268R and D286G bound with a relatively higher affinity, whereas H40Y showed lower affinity for RPEL 2 than the wt. Thus these three mutants may show altered SRF activity due to a direct change in their affinity for MAL.
significantly different from the control value with statistical significance at P < 0.05 (unpaired Student's t test). The pellet was analysed by SDS/PAGE and exposed to film, and the band intensities were measured. (B) Values are means ± S.D. from at least three independent experiments, and asterisks label the values significantly different from the control value with statistical significance at P < 0.05 (unpaired Student's t test).

Changes in levels of the G-actin/F-actin ratio in cells expressing mutant actins

Another possible explanation for how mutant actins might affect SRF activity is that their expression in the context of wt actin co-expression (as occurs in most myopathy patients) could alter the balance of polymerized F-actin and unpolymerized G-actin. Excessive G-actin might sequester MAL in the cytoplasm or nucleus and prevent SRF activation of transcription ([15] and Figure 1E). Since many myopathy mutants showed a lessened ability to co-polymerize with wt actin in biochemical assays [6], we tested the effect of mutant actin expression on the G-actin/F-actin ratio in cells using a previously established flow cytometry sorting assay based on fluorescently labelled DNaseI binding to the G-actin and fluorescently labelled phalloidin binding to the F-actin as a readout of levels [22]. Of five mutants tested (G182D, I136M, G268R, I357L and D286G), we found that the G-actin/F-actin ratio was only significantly altered in G268R, with a 1.4-fold increase in the G-actin (Figure 5). Thus expression of mutant actins does not seem to generally cause a significant alteration in the balance between G-actin/F-actin, but may, in some cases, increase the G-actin pool, which could potentially contribute to low serum responsiveness.

DISCUSSION

The present study suggests that the ability of mutant actins to activate SRF responses in developing muscle might be a major, yet thus far relatively unexplored, area for consideration in understanding actin-based myopathies. The idea that altered SRF signalling could cause myopathy symptoms has precedent in the literature. Charvet et al. [23] used a skeletal-muscle-specific deletion of SRF to show that mutant mice displayed growth retardation and loss of muscle mass when SRF was deleted in post-mitotic myofibres, but not satellite cells (muscle stem cells) [23]. Mice showed severe myofibre hypotrophy, which resembled myopathy. SRF deletion altered the overall gene expression patterns of the mutant muscle, with up-regulation of myogenin and down-regulation of many of the genes controlled by SRF in vitro [23]. This suggests to us that altered SRF responses in patients with actin-based myopathy could lead to altered gene expression programmes and a similar muscle degeneration phenotype. Strikingly, the authors described that SRF-depleted myofibres had extensive sarcomere disorder and they pointed out that they frequently observed “numerous fragments of Z-disks resembling rods that are observed in nemaline myopathies (NEM) and are thought to be accumulations of sarcomeric proteins.”

They pointed out that defects closely resembled the phenotype of patients with NEM caused by mutations in thin filament protein-encoding genes, and that several of the thin filament-encoding genes have CArG (SRF response) boxes in their promoters [23]. Thus mutant actin expression could alter major gene expression programmes in the muscle and possibly enhance the myopathy phenotype.

The control of SRF by actin is a complex cycle and is still an area of active investigation, but a molecular understanding of how actin dynamics modulate SRF transcription is emerging [15,24]. We explored the molecular mechanisms of effects of mutant actins on SRF stimulation for selected mutants that showed alterations in SRF responses. In particular, we showed that mutant ACTA1-H40Y, which conferred on cells a higher than normal basal level of SRF activity (Figure 1), showed low serum responsiveness and low serum-stimulated nuclear MAL accumulation. The inability of LatB to remove ACTA1-H40Y from the nucleus may be due to reduced binding of the drug to mutant actin or to the tendency of ACTA1-H40Y to form nuclear rods (see Figure 1A and [6]) that could be more resistant to LatB than are single filaments. However, since ACTA1-H40Y still retains approximately half of the wild-type level of serum responsiveness, it is only partially interfering with the ability of wild-type actin to respond in the SRF pathway and, in the absence of serum, it actually promotes SRF activity. Cells expressing this mutant retained relatively normal enhancement of SRF activity in response to Jasp and CytD (Figure 3), indicating that it did not have a strong dominant-negative effect on overall ability of cellular actin to polymerize (Jasp) or of CytD to promote nuclear MAL translocation and SRF activation. Interestingly, H40Y actin showed some reduction in SRF activity that they frequently observed "numerous fragments of Z-disks resembling rods that are observed in nemaline myopathies (NEM) and are thought to be accumulations of sarcomeric proteins."
a higher than normal affinity for MAL [19]. This is the first in vitro defect identified for H40Y actin to our knowledge, as it showed relatively normal responses in the actin dynamics assays we carried out previously [6], despite the appearance of nuclear rods. We conclude that patients with H40Y mutations are good candidates to test for altered SRF activity in their affected muscle tissue, since both basal and stimulated activity is affected by this mutant actin.

Another mutant that we chose for further study was R183G, which showed significantly reduced nuclear MAL accumulation and SRF serum responsiveness in expressing cells (Figure 1). Wild-type actin is normally shuttled out of the nucleus via a nuclear export signal and via the CRM1 exporter (Figure 2C) [15]. Nuclear export of actin promotes the low basal SRF activity of nuclear MAL, which is affected by SRF signalling.

Table 1 Summary of results

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Nuclear MAL in serum</th>
<th>SRF activation serum response</th>
<th>SRF activation Jasp/CytD response</th>
<th>Binding to RPEL domain</th>
<th>G-actin/F-actin ratio in cells</th>
<th>Co-polymerization with actin</th>
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<td>wt</td>
<td>wt</td>
<td>1</td>
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<td>Severe NEM</td>
</tr>
<tr>
<td>D375L</td>
<td>nd</td>
<td>wt</td>
<td>wt/high</td>
<td>wt</td>
<td>1.2</td>
<td>Low</td>
<td>Severe NEM, IRM</td>
</tr>
<tr>
<td>Category IV: no defect in vitro</td>
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<td></td>
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<td>Low</td>
<td>wt/low (modest)</td>
<td>Low</td>
<td>nd</td>
<td>wt</td>
<td>Severe NEM, IRM</td>
</tr>
<tr>
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<td>wt</td>
<td>wt/low</td>
<td>nd</td>
<td>nd</td>
<td>Low</td>
<td>Mild NEM</td>
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<tr>
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<td>Low</td>
<td>High</td>
<td>High</td>
<td>1</td>
<td>Low</td>
<td>Severe NEM</td>
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</table>

Whole actin in the G-actin/F-actin ratio in cells and a lower than normal affinity for MAL [19]. This is the first in vitro defect identified for H40Y actin to our knowledge, as it showed relatively normal responses in the actin dynamics assays we carried out previously [6], despite the appearance of nuclear rods. We conclude that patients with H40Y mutations are good candidates to test for altered SRF activity in their affected muscle tissue, since both basal and stimulated activity is affected by this mutant actin.

Another mutant that we chose for further study was R183G, which showed significantly reduced nuclear MAL accumulation and SRF serum responsiveness in expressing cells (Figure 1). Wild-type actin is normally shuttled out of the nucleus via a nuclear export signal and via the CRM1 exporter (Figure 2C) [15]. Nuclear export of actin promotes the low basal SRF activity of nuclear MAL, which is affected by SRF signalling.

Table 1 Summary of results

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Nuclear MAL in serum</th>
<th>SRF activation serum response</th>
<th>SRF activation Jasp/CytD response</th>
<th>Binding to RPEL domain</th>
<th>G-actin/F-actin ratio in cells</th>
<th>Co-polymerization with actin</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category II: folds, binds to CAP</td>
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<td>nd</td>
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<td>Mild and typical NEM</td>
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<td>wt</td>
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<td>AM, IRM</td>
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<td>wt</td>
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<td>1</td>
<td>Low</td>
<td>Severe NEM</td>
</tr>
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</table>

The idea that patients with NEM have altered gene expression programmes is not new. A study of ten patients with actin mutations leading to NEM showed that they had altered levels of several sarcomeric proteins by Western blot analysis [26]. Some up-regulated proteins (e.g. γ-filamin, α-actinin, myotilin and desmin) may accumulate in the nemaline bodies deposited near the z-disks, whereas other proteins, such as nebulin, were found to be reduced. Perhaps interestingly, desmin [27],
γ-filamin and α-actinin [8] are CArG box proteins and are thus likely to be regulated by SRF. Beggs and co-workers [28] have performed expression profiling of NEM muscle samples from patients and found that they have altered satellite (muscle stem cell) cell numbers and altered glycolytic enzyme transcription. However, they did not specify the nature of the mutation (e.g. actin compared with other thin filament proteins) and they did also not highlight whether SRF-responsive genes were specifically affected. Expression of ACTA1 in myoblasts changed the gene expression patterns for a number of muscle genes [29] and the authors pointed out that this may be through regulation of G-actin/F-actin ratios which will affect SRF signalling. Now that a comprehensive CArG-ome of CArG-box-containing SRF-responsive genes has been defined at least by one group [8], it will be interesting to check whether there is a trend in any particular actin mutations of myopathy patients to correlate with altered expression of CArG-ome genes.

AUTHOR CONTRIBUTION
Balazs Visegrady performed the experiments. Laura Machesky and Balazs Visegrady conceived the study, interpreted the results and wrote the manuscript together.

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