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## Myopathy-causing Actin Mutations Promote Defects in Serum Response Factor Signaling

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short running title *SRF signaling in myopathy*

**Abbreviations**

AM- Actin myopathy

NEM- Nemaline Myopathy

IRM- Intranuclear Rod Myopathy

ACTA1- alpha skeletal muscle actin

MADS box – a transcription factor named after the genes in which the sequence element for recognition was first identified (MCM1, Agamous, Deficiens, SRF)

SRF- serum response factor

MAP kinase- mitogen activated protein kinase

Crm1- chromosome region maintenance 1 protein

Cyt D- cytochalasin D

Jasp- Jasplakinolide

LMB- leptomycin B

RPEL- amino acid RPXXXEL sequence motif

EGFP- enhanced green fluorescent protein

DMEM- Dulbecco's Modified Eagle's Medium

PBS- phosphate buffered saline

GST- Glutathione S transferase

## Abstract

Mutations in the gene encoding alpha skeletal muscle actin (ACTA1) account for around 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. Around 1/3 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 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. We explore the impact of myopathy mutations in actin on the control of the transcriptional response by serum response factor and we find that the majority of mutants examined have altered serum response factor signaling. We propose that altered serum response factor signaling could be a major factor in actin-based nemaline myopathy and that this area could be exploited to develop therapies for sufferers.

## Introduction

### *Mutations in skeletal muscle actin and actin myopathy*

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. Nemaline myopathy 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: AM actin myopathy, IRM intranuclear rod myopathy and NEM nemaline myopathy. NEM, AM and IRM are caused primarily by mutations in the muscle thin filament proteins, namely nebulin, alpha skeletal muscle actin (ACTA1),  $\alpha$ - and  $\beta$ - tropomyosin, troponin-2 and perhaps surprisingly, cofilin-2 [1], which is not a typical thin filament protein, reviewed in [2]. Mutations in ACTA1 account for around 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 localise to any specific region(s) of the actin molecule, but rather they are found throughout the 3D 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 mutants is 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]. Around half of the mutants tested thus far show a

reduced ability to co-polymerize with 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 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 box (Figure 1E). The CARG box is a 10 base pair sequence with the consensus CCW<sub>6</sub>GG [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 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 co-factors such as members of the ternary complex factor (TCF) proteins and controls transcription via MAP-kinase signaling [11]. Myocardin related transcription factors also interact with SRF in muscle-specific Rho signaling and actin assembly pathways that are independent of its control by TCF [12-14]. Overall, SRF is involved in many transcriptional signaling cascades and cooperates with other transcription factors to regulate fundamental elements of embryogenesis, including muscle differentiation and growth.

The co-factor for SRF activation, MAL, has three actin binding RPEL sequences and binds directly to unpolymerized (globular, or G-) actin (Figure 1E). Activation of actin assembly via serum stimulation leading to Rho GTPase activation causes MAL release from the G-actin as it polymerises 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 [15] and Figure 1E. 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 (serum response factor) pathway, we hypothesized that patients with actin mutations could have defects in SRF signaling 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 biological defects, to look for defects in SRF signaling. Based on our in vitro studies, we postulate that patients with actin mutations may have defects in SRF signaling that could lead to defects in muscle growth and development and we propose that this is a potential previously overlooked side-effect of actin mutation that should be explored further for possible therapeutic benefit in myopathy patients.

## Results

### *Actin myopathy mutations affect the serum response factor signaling pathway in C2C12 myoblasts*

When SRF signaling is activated by serum stimulation, cells show increased localization of MAL in the nucleus and enhanced transcription of SRF reporter genes [16] (Figure 1E). 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 wild type ACTA1 showed a dramatic relocalisation of MAL to the nucleus following serum stimulation, with 5% of resting cells and around 80% of stimulated cells showing nuclear MAL accumulation (Figure 1A and B). The luciferase activity in this assay increased around 2.5-fold for cells expressing wild-type ACTA1 upon serum stimulation (Figure 1 C and D). Cells expressing mutant actins displayed cytoplasmic or nuclear rods or aggregates as previously reported [6] (e.g. Fig. 1.A, 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 colocalisation between actin stress fibres, rods or aggregates and MAL, which localised 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 wild-type actin (Figure 1B). The luciferase reporter assay, which measures the level of SRF activation, also reflected this trend (Figure 1C and D) 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 the wild-type except one construct, the ACTA1-H40Y which had an approximately 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 (Figure 1B,C).

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 (Fig. 2 A), 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 [17]. We found 50% nuclear MAL accumulation caused by



ACTA1-G15S that does not change after treating cells with serum. In contrast, cells expressing ACTA1-H40Y show 60% and 25% nuclear MAL localised with and without serum, respectively, so ACTA1-H40Y appears to be somewhat more sensitive to serum stimulation than ACTA1-G15S but less than the wild type actin. It is interesting to note that ACTA1-G15R, a naturally occurring nemaline myopathy mutant, retains some serum sensitivity, unlike ACTA1-G15S (Figure 1). We treated the cells in 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 results in rapid cytoplasmic relocalization of MAL in serum treated wild type cells ([15] and Figure 1E). We did not find any change in the distribution of MAL after 40 min 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 depolymerisation 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 approximately 30% of cells show accumulation of MAL in the nucleus and this doesn't significantly change with addition of LMB for 5 or 15 minutes (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 cytochalasin D or jasplakinolide treatment*

We used Cytochalasin D (CytD) and jasplakinolide (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 wild type 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 actin filaments while CytD binds to barbed ends of filaments and prevents polymerization, as well as having some interactions with actin monomers and oligomers at higher concentrations [18]. Jasp induces nuclear MAL via stabilization of filaments and dissociation of actin monomer from MAL to allow faster nuclear import [13, 16]. 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 [16]. The actin myopathy-related mutations investigated in this study would not be predicted to alter the binding site of Jasp or CytD, based on their position in the 3D structure of ACTA1 [19]. We tested the mutant actin constructs using 15 min drug treatment, where significant effects of each drug on the actin cytoskeleton were observed (data not shown) (Fig. 3). All the mutants tested show a similar response to Jasp or CytD treatment with respect to the appearance of F-actin in phalloidin stained cells (data not shown). Cells expressing G15R, H40Y, I64N, and N115S mutant ACTA1 show lower activation of SRF in the presence of either drug, while ACTA1-R183G and ACTA1-D286G show higher SRF reporter gene activation than the wild type (Fig 3 A). Cells

expressing ACTA1- I136M, G182D and I357L show SRF overactivation while M132V and G268R have wild type 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, 9/12 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 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 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 to 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 the mutants tested could bind to the RPEL 2 domain. Two mutants, G268R and D286G bound with relatively higher affinity while H40Y showed lower affinity for RPEL 2 than the wild type. Thus these three mutants may show altered SRF activity due to a direct change in their affinity for MAL.

#### *Changes in levels of 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 wild-type 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 serum response factor activation of transcription [15] and Figure 1E. Since many myopathy mutants showed a lessened ability to copolymerize with wild-type actin in biochemical assays [6], we tested the effect of mutant actin expression on the G/F actin ratio in cells using a previously established flow cytometry sorting assay based on fluorescently labeled DNase-I binding to the G-actin and fluorescently labeled phalloidin binding to the F-actin as a readout of levels [20]. Of five mutants tested (G182D, I136M, G268R, I357L and D286G), we found that the G/F actin ratio was only significantly altered in G268R, with a 1.4-fold increase in the G-actin (Fig. 5). Thus, expression of mutant actins does not seem to generally cause a significant alteration in the balance between G/F actin, but may in some cases increase the G-actin pool, which could potentially contribute to low serum responsiveness.

### **Discussion**

Our 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



signaling could cause myopathy symptoms has precedent in the literature. Charvet and colleagues 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 myofibers, but not satellite cells (muscle stem cells) [21]. Mice showed severe myofiber hypotrophy, which resembled myopathy. SRF depletion altered the overall gene expression patterns of the mutant muscle, with upregulation of myogenin and downregulation of many of the genes controlled by SRF *in vitro* [21]. 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 myofibers 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 [21]. 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 modulates SRF transcription is emerging [15, 22]. 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 single filaments. However, since ACTA1-H40Y still retains around 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 MAL binding in an *in vitro* translation assay, indicating that lower affinity for MAL could be the cause for the observed higher than normal basal SRF activity of the mutant (Figure 1C). This is the opposite of G15S actin, which is reported to have a higher than normal affinity for MAL [17]. 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 and [15]). Nuclear export of actin promotes the low basal SRF activity of cells with wild type actin, as inhibiting CRM1 with leptomycin B causes an increase of nuclear actin-MAL complex (Figure 1E and [15, 20]). However, MAL did not accumulate in the nucleus in response to LMB in the R183G mutant expressing cells, indicating that the defect is likely to be due to reduced entry of MAL into the nucleus in R183G expressing cells. R183G actin expressing cells showed a relatively normal response to Jasp treatment, indicating that this actin is capable of polymerization induced by Jasp and thus nuclear accumulation of MAL. This mutant also had a relatively normal response to CytD, indicating perhaps that the normal serum-induced or signal-induced actin assembly pathway of this mutant is aberrant, but that drug stimulation can overcome this deficiency. ACTA1-R183G showed normal binding to the MAL RPEL domain *in vitro*, as well. Taken together, the data suggest that it could be worth investigating a possible aberrant interaction with the actin binding protein STARS, which mediates the dissociation of MAL-actin complexes in response to serum stimulation to promote nuclear MAL accumulation and SRF activation [12]. This also suggests that patients with ACTA1-R183G could likely have defective SRF responses.

Cells expressing ACTA1-G268R had increased levels of G-actin, perhaps leading to sequestration of MAL in the cytoplasm. ACTA1-G268R showed reduced ability to copolymerize with wild type actin in a previous study [6] and here we found that expression of ACTA1-G268R led to an increase in the G/F actin ratio in cells and a lower than normal serum response in an SRF reporter assay. ACTA1-G268R was not defective in binding to the MAL RPEL motifs, nor to other actin binding proteins, nor apparently was it unable to polymerize in response to addition of jasplakinolide to cells (Table 1). This mutant likely binds to MAL and sequesters it in the cytoplasm, thus dampening the SRF response, due to its relatively weaker ability to polymerize in response to signals that activate Rho.

Overall, most of the mutants that we tested showed a dampened SRF response pathway, with the majority having lower nuclear MAL accumulation and lower SRF reporter activity in response to serum stimulation. These mutants also mostly had previously shown actin co-polymerization defects in biochemical assays [6]. All of the mutants that we tested were capable of interaction with the MAL RPEL motif and indeed previous studies showed interaction with vitamin D binding protein, which interacts with a similar site to MAL in the hydrophobic cleft and close to the subdomain 3 ledge [23]. Significantly, 5 mutants (V163L, R183G, G268R, H40Y and D286G) showed low serum response in the SRF reporter assay but a significant increase in SRF activity upon treatment with Jasp or CytD, indicating that even though the actins conferred a defect in SRF responsiveness to serum, this could be overcome with drug treatment. Thus, we conclude that mutations which lead to reduced polymerization capacity in general might cause a dampened SRF response in myopathy patients and that perhaps the SRF response ought to be further investigated to see if enhancement SRF pathways via drug treatment could be of any benefit therapeutically.

Some of the mutants, V163L, I136M, G268R, H40Y and I64N have been reported to cause blebbing and eventual cell death when expressed in cultured myoblasts over several days and after the cells fuse to form myotubes [7]. It is also possible that the lowered SRF response in some of these mutants (all but I136M, which had a normal

SRF response) could be contributing to the cell death phenotype observed and that cell survival may be a major factor which is affected by SRF signaling.

The idea that patients with NEM have altered gene expression programmes is not new. A study of 10 patients with actin mutations leading to NEM showed that they had altered levels of several sarcomeric proteins by western blot analysis [24]. Some upregulated proteins (e.g.  $\gamma$ -filamin,  $\alpha$ -actinin, myotilin and desmin) may accumulate in the nemaline bodies deposited near the z-disks, while other proteins, such as nebulin were found to be reduced. Perhaps interestingly, desmin [25],  $\gamma$ -filamin and  $\alpha$ -actinin [8] are CARG box proteins and are thus likely to be regulated by SRF. The group of Beggs has done 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 [26]. However, they did not specify the nature of the mutation (e.g. actin versus other thin filament proteins) and they did not also highlight whether SRF responsive genes were specifically affected. Expression of ACTA1 in myoblasts changed the gene expression patterns for a number of muscle genes [27] and the authors pointed out that this may be through regulation of G/F actin ratios which will affect SRF signaling. 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.

## Materials and methods

### *Plasmids*

Expression vectors encoding C-terminal EGFP-tagged or N-terminal Myc-tagged wt or ACTA1 mutations were made with the Quick Change site directed mutagenesis kit (Stratagene, UK) as described previously [6]. The expression vectors for pEF-FLAG-MAL and the luciferase reporter SM22 promoter were reported previously [28, 29]. C-terminal EGFP-tagged ACTA1 G15S mutant derivative was made with pEGFP vector as template, a 5' primer containing XhoI and a 3' primer containing EcoRI site and appropriate primers.

### *Cell culture and transfection*

C2C12 cells were cultured at 37 °C in humidified 5% CO<sub>2</sub> incubator in a proliferation medium composed of Dulbecco's modified Eagle's medium (DMEM 41966, Gibco BRL, UK) supplemented with 10% fetal bovine serum (Sigma, UK). Transfections were made using Lipofectamine 2000 transfection reagent (Gibco BRL, UK) according to the manufacturer's protocol. Cells were generally used for experiments 48 hours after transfection.

### *Immunofluorescence and microscopy*

C2C12 cells were plated on glass coverslips than washed three times in PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Free aldehyde groups were blocked with 50 mM NH<sub>4</sub>Cl for 10 min and cells were permeabilized with PBS containing 0.1% Triton X-100 for 4 min. Cells were incubated with 9E10 anti-Myc antibody for 20 min at room temperature followed by Texas-red anti-mouse secondary antibody. For FLAG detection cells were blocked with 10% bovine serum albumin (BSA) and were incubated with 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 onto a slide with 5  $\mu$ l Mowiol (Calbiochem) plus Antifade.

Immunostained cells were examined with Zeiss microscope (Zeiss, Jena, Germany) using 63x 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 (Bridgewater, NJ) and processed using Photoshop software (Adobe, USA).

#### *Luciferase assay*

C2C12 cells ( $10 \times 10^5$  cells/well) were plated on 6-well plates and were cotransfected with encoding vectors of 3  $\mu$ g wt or mutants ACTA1, 3  $\mu$ g FLAG-MAL and 1  $\mu$ g luciferase reporter SM22. After 12h incubation the cells were serum starved for 24 hours and 20% serum was added for 4 hours. In case of drug treatment after 24 hours serum starving 2.5  $\mu$ M cytochalasin D (CytD), 0.5  $\mu$ M jasplakinolide (Jasp), 20 nM leptomycin B was added to the cells for the time specified separately. 1  $\mu$ M latrunculin B (LatB) was applied after 4 hours 20% serum treatment. Finally the cells were harvested and luciferase activities were measured according to the manufacturer's instructions. All the assays have been performed three times and the values have been normalized to the values obtained for wild type actin.

#### *GST-pulldown assay*

In vitro transcription translation reaction was used to express  $^{35}$ S labeled wt or mutant actin in reticulocyte lysate (Promega, UK) according to manufacturer's instructions. In the reactions 0.02  $\mu$ Ci  $^{35}$ S -methionine and 1  $\mu$ g DNA per 25  $\mu$ l reaction was used. After 1.5 hours incubation at 30°C the products were analyzed on sodium dodecyl sulfate (SDS) gel followed by autoradiography. The Pfam-defined RPEL2 and RPEL3 motifs (RPEL 2 domain) containing 85 amino acids from position 21 in MAL isoform 1 has been cloned into pGEX-4T-2 vector as template, a 5' primer containing the EcoRI and the 3' primer containing the XhoI site. The RPEL 2 domain protein has been expressed in BL21 cells than bound to GST-beads. 25  $\mu$ l of the *in vitro* transcription translation reaction of wt or mutant actin was centrifuged at 100,000 rpm for 20 min in a Beckman ultracentrifuge to remove aggregates. 5  $\mu$ l of the supernatant was taken for control. The supernatant then was incubated with 10  $\mu$ g of RPEL 2 domain bound to agarose beads for one hour at room temperature. The agarose beads were sedimented and the supernatant was removed. The agarose beads were washed three times then resuspended in 20  $\mu$ l PBS. The final pellets, supernatants and control were analyzed on 4-12% sodium dodecyl sulfate (SDS) gel followed by autoradiography. The band intensities were measured with ImageJ software and the values were normalized to the control. All mutants were analyzed three times. Statistical analysis was made at  $P < 0.05$  using the Student's t-test in Microsoft Excel.

#### *Flow cytometry*

$5 \times 10^5$  C2C12 cells were transfected with Myc-tagged ACTA1 constructs. Cells were pelleted at 300g for 5 minutes than resuspended in 100  $\mu$ l 4% paraformaldehyde for 10 min. Cells were washed with 1 ml FACS buffer (5% fetal calf serum, 0.02%  $\text{NaN}_3$ , 0.5% saponin (Invitrogen) in PBS) and sedimented. 9E10 anti-Myc primary antibody was applied for 30 min, followed by Marina blue conjugated anti-mouse secondary



antibody, Alexa fluor 488 deoxyribonuclease I and Texas Red-X phalloidin (Invitrogen) was added for 30 min. Cells were washed and sedimented between each step. The samples were analyzed with BD FACS Aria cell sorter system.  $10 \times 10^3$  blue positive cells were used for analysis and the green (530nm) and red (610nm) intensities were measured and the average values were calculated.

#### *Data Analysis and Statistics*

At least three independent experiments were performed for each assay. Error bars are standard deviations in all cases. The statistical analysis was made at  $P < 0.05$  with the unpaired Student's t-test using Microsoft Excel.

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#### **Figure legends**

**Figure 1 Cells expressing mutant actin show impaired subcellular MAL distribution and SRF activation.** (A) Representative immunofluorescence pictures of C2C12 cells cotransfected with EGFP-ACTA1 and mutants (wt-ACTA1, ACTA1-H40Y, ACTA1-R183G) or Myc-actin (green) and MAL (red) in response to serum stimulation. (B) Nuclear MAL accumulation in presence (black bars) or absence (white bars) of serum expressing wt or mutant EGFP-ACTA1 and MAL revealed by counting the cells observed in immunofluorescence (At least 100 cells were counted per experiment and three independent experiments were performed). Serum induced SM22 luciferase activation of C2C12 cell line expressing GFP- (C) or Myc-ACTA1 (D), MAL and SM22 luciferase reporter. Error bars are standard deviations 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 showing the relationship between actin assembly/disassembly and nuclear shuttling of MAL (summarized from [22, 15]). Arrow 1 shows the G-actin-MAL complex dissociating as actin polymerises 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 Jasplakinolide (Jasp) or cytochalasin D (CytD). Arrow 2 shows that when actin depolymerises, due to serum starvation or treatment with Latrunculin B (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 isn't known if 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 leptomycin B (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.

**Figure 2 Effect of mutants H40Y and R183G on nuclear MAL accumulation.**

C2C12 cells were tested for nuclear MAL after expressing MAL and wt, G15S or H40Y ACTA1 (A) or ACTA1-R183G (B) and treated with serum and latrunculin B (LatB, 1  $\mu$ M) or leptomycin B (LMB, 20 nM) as indicated. Wild type (wt) or G15S ACTA1 were used as comparisons for ACTA1-H40Y. At least 20 cells were counted per experiment and three independent experiments were performed per condition. Error bars are standard deviations.

**Figure 3 Different responses of myopathy mutants to disruption of the actin cytoskeleton**

SRF activation was monitored after jasplakinolide (white bars) or cytochalasin D (black bars) treatment. C2C12 cells were cotransfected with MAL, luciferase-reporter SM22 and GFP-actin (A) or Myc-actin (B). After serum starvation for 15 minutes, jasplakinolide (0.5  $\mu$ M) or cytochalasin D (2.5  $\mu$ M) treatment was applied. The luciferase activation is shown relative to cells expressing wt-ACTA1 in serum-starved conditions. Error bars are standard deviations from at least three independent experiments and asterisks label the values statistically different from the control value with significance at  $P < 0.05$  (unpaired Student's t-test).

**Figure 4 Actin binding to MAL-RPEL 2 domain is not greatly altered for selected mutants**

(A) The  $^{35}$ S-labelled wt or mutant actin from *in vitro* translation assays was incubated with GST-RPEL 2 domain bound to agarose beads and then sedimented. The pellet has been analyzed on SDS page and exposed to film and the band intensities were measured. (B) Error bars are standard deviations 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).

**Figure 5 Ratios of filamentous/monomeric actin in cells expressing selected mutants.**

C2C12 cells were transfected with myc-tagged ACTA1 constructs and fixed in formaldehyde and permeabilized. Myc was detected with 9E10 primary and Marina Blue secondary antibody, while Alexa 488 DNaseI and Texas Red phalloidin were used to detect G-actin and F-actin respectively. Blue myc-positive cells were used for analysis and the relative binding to Alexa 488 (G-actin) and Texas Red (F-actin) were compared. The relative ratio of G-actin/F-actin is shown in the bar graphs with the wild type ACTA1 expressing cells normalized to 1 and the error bars indicate the standard deviation from at least three independent experiments, while the asterisk denotes statistical significance at  $P < 0.05$  (unpaired Student's t-test).

**Table 1 Summary of results**

Categories of mutants are shown as from the study by Costa et al. [6] and we have added the new data from this study. Asterisks indicate data previously shown in Costa et al., 2004. nd= not done.

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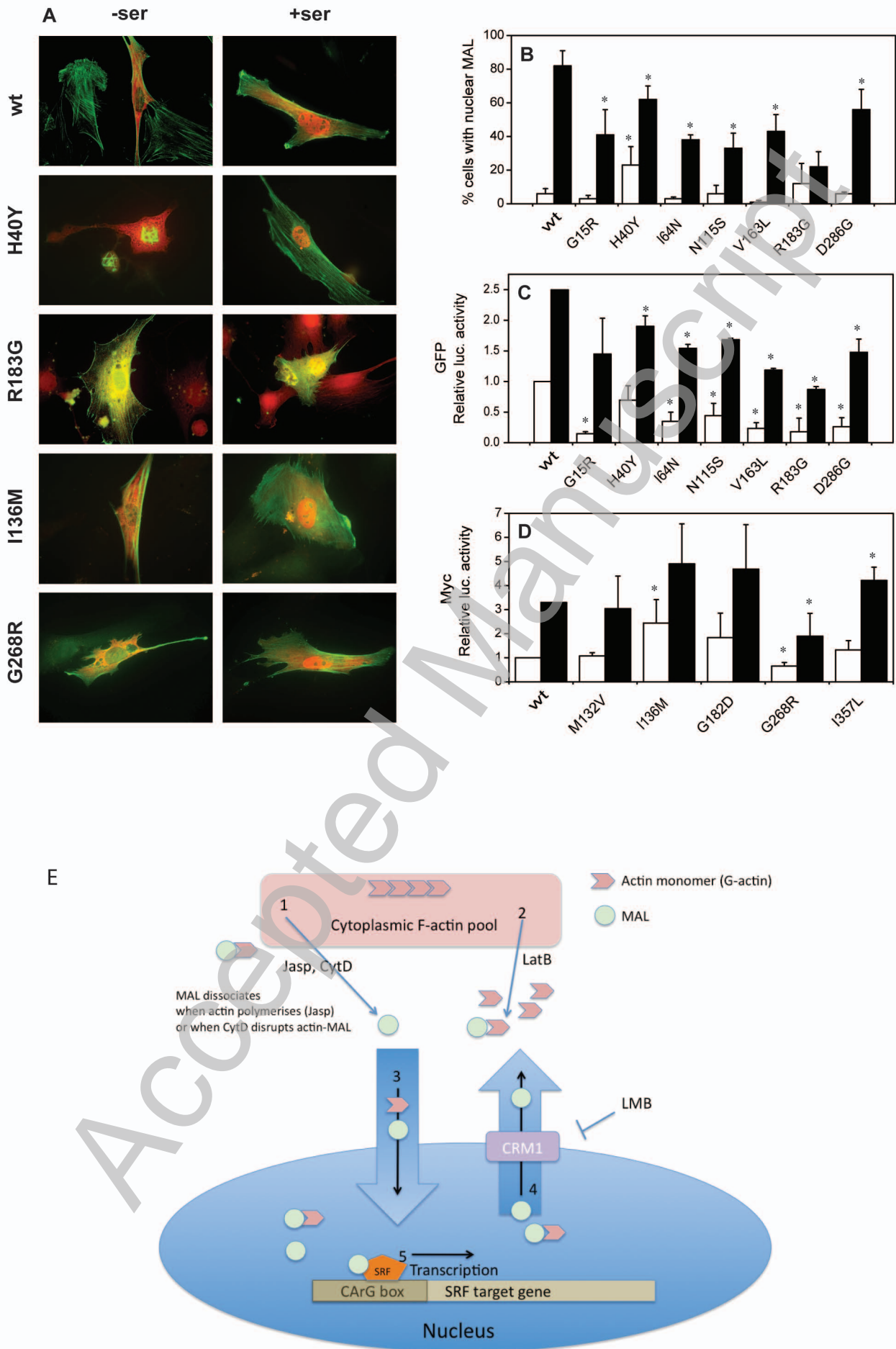


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Mutation	Nuclear MAL in serum	SRF activation serum response	SRF activation Jasp/CytD response	Binding to RPEL domain	G/F actin ratio in cells	Co-polymerization with actin*	Phenotype*
<b>Category II*- folds, binds to CAP</b>							
G15R	low	low	low/low	nd	nd	wt	AM
N115S	low	low	low/low	nd	nd	low	Mild and typical NEM
V163L	low	low	wt/wt	nd	nd	wt	AM, IRM
G182D	nd	wt	wt/high	wt	1	low	Typical NEM
<b>Category III*- copolymerization defect</b>							
I64N	low	low	low/low	nd	nd	low	Typical NEM
I136M	nd	wt	high/high	wt	1	low	Typical NEM
R183G	very low	very low	wt/wt	wt	nd	low	Severe NEM
G268R	nd	low	wt/wt	high	1.4	low	Severe NEM
I357L	nd	wt	wt/high	wt	1.2	low	Severe NEM, IRM
<b>Category IV*- no defect in vitro</b>							
H40Y	low	low	wt/low (modest)	low	nd	wt	Severe NEM, IRM
M132V	nd	wt	wt/wt	nd	nd	low	Mild NEM
D286G	low	low	high/high	high	1	low	Severe NEM

Figure 1.



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Figure 2.

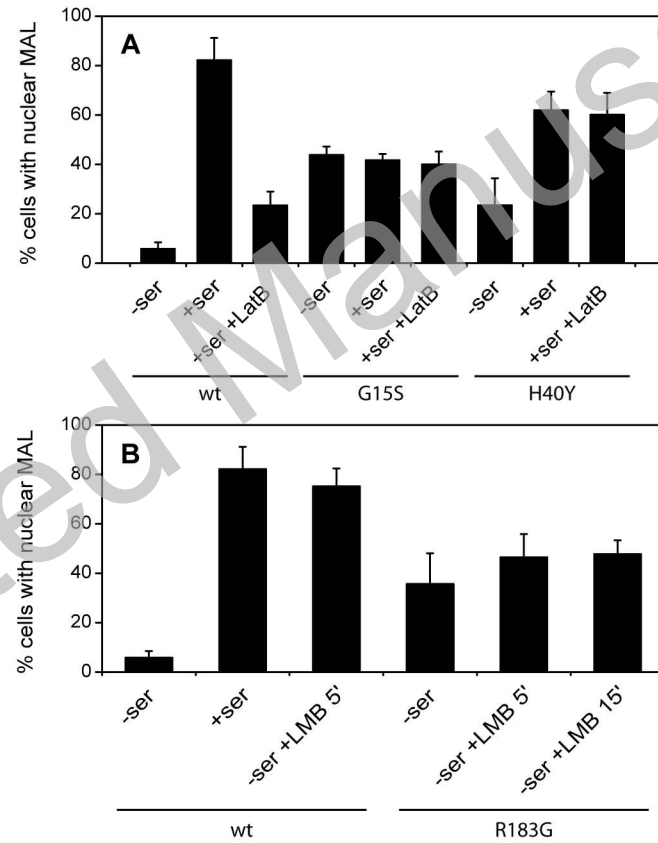


Figure 3.

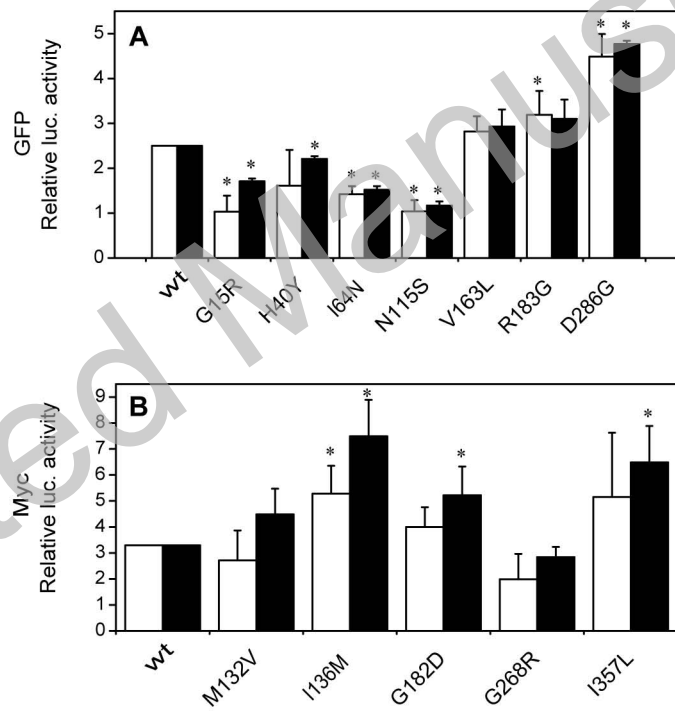




Figure 4.

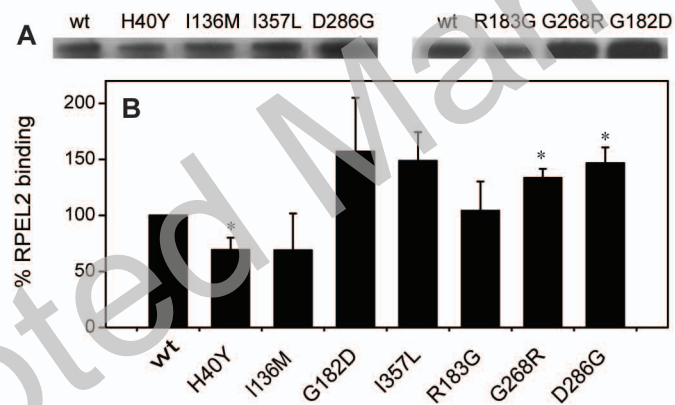


Figure 5

