

**Title:** Membrane glucocorticoid receptors are localised in the extracellular matrix and signal through the MAPK pathway in mammalian skeletal muscle fibres.

**Authors:** Simona Boncompagni<sup>1</sup>, Lewis Arthurton<sup>2</sup>, Eugene Akujuru<sup>3</sup>, Timothy Pearson<sup>3</sup>, Dietmar Steverding<sup>3</sup>, Feliciano Protasi<sup>2</sup> and Gabriel Mutungi<sup>3</sup>

**Affiliations:** <sup>1</sup>CeSI - Center for Research on Ageing & DNICS - Dept. of Neuroscience, Imaging and Clinical Sciences; University G. d'Annunzio, I-66013 Chieti, Italy.

<sup>2</sup>School of Life Sciences, University Of Liverpool, Liverpool L69 7ZB.

<sup>3</sup>Department of Medicine, Norwich Medical School, University of East Anglia, Norwich, NR4 7TJ, UK

**Running title:** Glucocorticoid receptors in skeletal muscle

**Key words:** Cell signalling/Glucocorticoids/myogenesis/non-genomic actions/skeletal muscle

**Corresponding author:** Gabriel Mutungi, BioMedical Research Centre, Department of Medicine, Norwich Medical School, University of East Anglia, Norwich, NR4 7TJ, UK.

This is an Accepted Article that has been peer-reviewed and approved for publication in the *The Journal of Physiology*, but has yet to undergo copy-editing and proof correction. Please cite this article as an 'Accepted Article'; doi: 10.1113/JP270502 .

This article is protected by copyright. All rights reserved.

Telephone: 01603591323

e-mail: [g.mutungi@uea.ac.uk](mailto:g.mutungi@uea.ac.uk)

### **Key points summary**

- Many studies have previously suggested the existence of stress hormone receptors on the cell membrane of many cell types including skeletal muscle fibres. However, the exact localisation of these receptors and how they signal to the rest of the cell is poorly understood.
- In this study, we investigated the localisation and the mechanism(s) underlying the physiological functions of these receptors in mouse skeletal muscle cells.
- We found that the receptors were present throughout muscle development and that, in adult muscle fibres, they were localised in the extracellular matrix, satellite cells (muscle stem cells) and close to mitochondria.
- We also found that they signalled to the rest of the cell by activating enzymes called mitogen-activated protein kinases.
- From these results we suggest that, at physiological concentrations, stress hormones may be important in skeletal muscle differentiation, repair and regeneration.

**Abstract**

A number of studies have previously proposed the existence of glucocorticoid receptors on the plasma membrane of many cell types including skeletal muscle fibres. However, their exact localisation and the cellular signalling pathway(s) they utilise to communicate with the rest of the cell are still poorly understood. In this study, we investigated the localisation and the mechanism(s) underlying the non-genomic physiological functions of these receptors in mouse skeletal muscle cells. The results show that the receptors were localised in the cytoplasm in myoblasts, in the nucleus in myotubes and in the extracellular matrix, in satellite cells and in the proximity of mitochondria in adult muscle fibres. Also, they bound laminin in a glucocorticoid-dependent manner. Treating small skeletal muscle fibre bundles with the synthetic glucocorticoid, beclomethasone dipropionate, increased the phosphorylation (=activation) of extracellular-signal regulated kinase 1&2, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase. This occurred within 5min and depended on the fibre-type and the duration of the treatment. It was also abolished by the glucocorticoid receptor inhibitor, mifepristone, and a monoclonal antibody against the receptor. From these results we conclude that the non-genomic/non-canonical physiological functions of glucocorticoids, in adult skeletal muscle fibres are mediated by a glucocorticoid receptor localised in the extracellular matrix, in satellite cells and close to mitochondria and involve activation of the MAPK pathway.

## Abbreviations list

BDP, Becomethasone dipropionate; ERK1&2, Extracellular-signal regulated kinase 1&2; FAK, Focal adhesion kinase; FAKi-14, Focal adhesion kinase inhibitor 14; GC, Glucocorticoid; GR, Glucocorticoid receptor; cGR, Cytosolic glucocorticoid receptor; mGR, Membrane glucocorticoid receptor; JNK, c-Jun N-terminal kinase; MAPK, Mitogen-activated protein kinase; mTOR, Mammalian target of rapamycin; p38 MAPK, p38 mitogen-activated protein kinase.

## Introduction

Glucocorticoids (GCs, steroids) are the main stress hormones in the body. They are synthesised from cholesterol and released from cells in the zona fasciculata of the adrenal cortex. In healthy individuals, they are released in short irregular bursts that peak ~2hrs before the start of the day and whose plasma concentration rarely exceeds 250nM except during stress when it can be as high as 700nM (Krieger et al, 1971). GCs are essential for life and affect most tissues in the body including skeletal muscle. Indeed, skeletal muscle plays a key role in the stress response because it enables individuals to evade or to fight the stressor. Despite this, little is known about the physiological functions of GCs in skeletal muscle especially at the low concentrations normally found in plasma. Instead, what has been extensively studied and reported are the effects of treating laboratory animals, for long periods, with high doses of synthetic GCs (especially dexamethasone) on skeletal muscle function (Nava et al, 1996; van Balkom et al, 1997; Ma et al, 2003) and protein metabolism (Goldberg, 1969; Rannels & Jefferson, 1980; Wing & Goldberg, 1993). A lot is also known about the effects of lack of (Addison's disease) and excess of (Cushing syndrome) GCs on skeletal muscle mass and function.

GCs exert most of their physiological and pharmacological effects through an intracellular glucocorticoid receptor (GR, GCR, NR3C1). The GR belongs to the super family of ligand-activated nuclear transcription factors that include the steroid, thyroid and retinoic acid receptors (Kadmiel & Cidlowski, 2013). It is coded for by a single gene (the NR3C1 gene) that is spliced into two main isoforms, GR $\alpha$  (which is the active form) and GR $\beta$  (which is inactive and resides in the nucleus where it acts as a dominant negative inhibitor of GR $\alpha$ ) (Kadmiel & Cidlowski, 2013). GR $\alpha$  (henceforth referred to as GR) is expressed in most cells, including muscle fibres (=cells), where the inactive receptor is thought to reside in the cytosol (hence cytosolic GR, cGR). In this state, the receptor is maintained in its high affinity conformation and is protected from inactivation by chaperone molecules such as heat shock proteins (HSPs) e.g. HSP 90 and immunophilins e.g. FK506 binding protein (FKBP) 51 (Pratt and Toft, 2003). However, in a recent study we showed that, in adult mammalian skeletal muscle fibres, GRs were localised close to the surface of muscle fibres where they co-localised with laminin. We were also able to inhibit their effects on force using a monoclonal antibody against the receptor suggesting that they were easily accessible from the cell surface (Perez et al. 2013). Although a number of studies have previously alluded to the existence of membrane glucocorticoid receptors (mGRs) in other cell types including human lymphoma cells (Gametchu, 1993), peripheral blood mononuclear cells (Bartholome et al. 2004) and neuronal cells (Orchinik et al. 1991) their precise location is still unknown. It is also uncertain whether in skeletal muscle the GRs are localised close to the cell surface throughout myogenesis or just in adult muscle fibres.

In addition to their genomic actions, GCs also exert actions that occur within minutes and are relatively insensitive to inhibitors of transcription and translation (Stahn and Buttgeriet 2008; Perez et al. 2013). These non-canonical/non-genomic actions have been described in many cell types including HeLa cells (Bruna et al, 2003; Lasa et al, 2002); guinea pig neurones (Hua and Chen, 1989), human endometrial cells (Hafezi-Moghadam et al, 2002), human adenocarcinoma cells (Croxtall et al, 2000), rat thymocytes (Buttgeriet et al, 1997), airway vasculature (Alangari, 2010) and skeletal muscle (Perez et al, 2013). However, the cellular signal transduction events mediating them are still poorly understood. The receptor/mechanism underlying their actions is also controversial.

The primary aims of this study were two fold; (1) to investigate the localisation and physiological functions of the GR in mouse skeletal muscle cells and (2) to determine the cellular signal transduction events mediating their non-genomic physiological actions in adult mammalian skeletal muscle fibres.

### **Materials and methods**

***Small intact skeletal muscle fibre bundles.*** The experiments reported here were performed at room temperature (~20°C) using small skeletal muscle fibre bundles (~540±26µm in diameter) isolated from the extensor digitorum longus (EDL; a fast-twitch) or the soleus (slow-twitch) of adult male C57BL/6 mice. The mice were killed according to UK legislation (for a summary of the legislation see Drummond 2009) and all the experiments conformed to the University of East Anglia animal welfare committee guidelines. Throughout the experiments, the muscles and muscle fibre bundles were bathed in mammalian Ringer's solution with the following composition (in mM); 109 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 4 CaCl<sub>2</sub>, 24 NaHCO<sub>3</sub>, 1 NaHPO<sub>4</sub>, 10 sodium pyruvate plus 400mg l<sup>-1</sup> foetal bovine serum (FBS). The pH of the Ringer's solution

was maintained at  $\sim 7.42$  by continuously bubbling it with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. To avoid any confounding influence of the GCs present in the FBS, serum from the same batch was used in all the experiments.

**Cell culture.** C2C12 cells were grown on cover slips in growth media (Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% L-glutamine plus 10% FBS and 1% penicillin and streptomycin (all purchased from Sigma Aldrich, Poole, UK) for 3-4 days or until 90% confluence. Once 90% confluent, they were induced to differentiate by replacing the growth media with differentiation media i.e. growth media containing 2% horse serum instead of FBS. At the end of the experiment the cells were washed in phosphate buffered saline (PBS), air dried and permeabilised for 15min using Tris buffered saline with Tween® 20 (TBST). They were blocked for non-specific antibody binding for 10min using 1% BSA PBS. Finally, they were immunoblotted for the expression of the GR, laminin and double stranded DNA as described below.

**Determination of the effects of beclomethasone (Beclomethasone) dipropionate (BDP) on the MAPK pathway.** This experiment was performed as previously described in Hamdi & Mutungi (2010 and 2011). Briefly, the muscle fibre bundles were mounted horizontally between two stainless steel hooks in a Perspex muscle chamber (total volume  $\sim 40$ ml) with a glass bottom. Half of the bundles (controls) were treated for 5, 10, 30 and 60 minutes with the standard Ringer's solution plus 34nM ethanol (the vehicle used to dissolve BDP). The other half (experimental) was treated for the same period of time with the Ringer's solution containing 250nM BDP. At the end of the experiment, the fibre bundles were snap-frozen in liquid nitrogen and cytosolic proteins were extracted using NP40 lysis buffer. The proteins were immunoblotted for the activation/phosphorylation of c-Jun N-terminal kinase (JNK; also known as stress-activated protein kinase/SAPK);

extracellular signal-regulated kinase (ERK) 1&2) and p38 mitogen-activated protein kinase (MAPK) as described below.

***Determination of the receptor/mechanism mediating the effects of BDP in mammalian skeletal muscle fibres.*** To determine whether the effects of BDP on the MAPK pathway were mediated through the GR and whether the receptor was localised close to the fibre surface, another set of fast-twitch and slow-twitch bundles was divided into three groups. Group 1 (controls) were treated with the standard Ringer's solution plus the vehicle used to dissolve the GC (ethanol) and mifepristone (DMSO). Group 2 was pre-incubated for 10min in Ringer's solution containing 10µM mifepristone (RU486; a GR inhibitor) (Sigma-Aldrich, Gillingham, Dorset, UK); whereas, Group 3 was pre-incubated for the same period in a 1:5000 dilution of a monoclonal anti-GR (ab109022, Abcam, Cambridge, UK). The fibres in Group 2 were then treated for a further 30 minutes with the Ringer's solution containing 10µM mifepristone alone or mifepristone plus 250nM BDP; whereas, those in Group 3 were treated for 5 minutes with the Ringer's solution plus 1:5000 dilution of the anti-GR alone or 1:5000 dilution of the anti-GR plus 250nM BDP. To determine whether the effects of BDP were mediated via focal adhesion kinase (FAK), another set of fibres was pre-treated with 10µM FAK inhibitor (FAKi) 14 (Abcam, Cambridge, UK). They were then incubated for a further 10min in Ringer's solution containing the inhibitor alone or the inhibitor plus 250nM BDP. At the end of these experiments, the fibre bundles were frozen and cytosolic proteins were extracted using NP40 lysis buffer. They were then immunoblotted for the activation of ERK 1&2, JNK and p38 MAPK as described below.



**Immunoblotting.** To determine the effects of BDP on the activation/phosphorylation of ERK 1&2, JNK and p38 MAPK equal amounts of the proteins were immunoblotted as previously described in Perez et al. (2013). Briefly, 10µg of the proteins were separated by standard gel-electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked for non-specific anti-body binding using 5% milk. They were then immunoblotted for the expression of pERK 1&2, pJNK and pp38 MAPK using a 1:500 dilution of rabbit monoclonal antibodies (4377, 4671 and 9215, respectively) from Cell Signalling Technologies® (New England Biolabs, Herts, UK) and the expression of the GR using a 1:500 dilution of the monoclonal anti-GR from Abcam used in the experiment above (ab109022; Cambridge, UK). Finally, they were visualized using SuperSignal WestPico chemiluminescence substrate (Perbio Science UK Ltd, Cramlington, Northumberland) and exposure to film.

The following day, the membranes were stripped and re-probed with a pan-actin antibody (ab3280, Abcam, Cambridge, UK) to check for loading. In some experiments, the fibre bundles were fibre typed using monoclonal antibodies against fast and slow MyHCs (Sigma-Aldrich, Gillingham, Dorset, UK). All the blots were run in duplicate and each experiment was repeated at least twice.

**Immunocytochemistry.** The expression and localisation of the GR in myoblasts, myotubes and adult mammalian skeletal muscles fibre bundles was determined as previously described in Perez et al. (2013). Briefly, the EDL and soleus muscles isolated from adult male C57BL/6 mice were mounted perpendicularly on cryostat chucks using tissue Tek® OCT™ compound (Sakura Finetek UK Ltd, Berkshire, UK). They were snap frozen in liquid nitrogen cooled isopentane and 10µm thick serial sections were cut. Two of the sections and the two cover slips of myoblasts and myotubes (grown as described above) were blocked for non-specific antibody

labelling using 1% bovine serum albumin (BSA) dissolved in PBS. They were incubated at room temperature, for at least 1hr, with a 1:200 dilution of a monoclonal rat anti-laminin (MAB1905, Millipore Corporation, Billerica, MA, USA) and a 1:100 dilution of the monoclonal rabbit anti-GCR from Abcam (ab109022; Cambridge, UK). They were then washed five times in PBS and incubated in 1:500 dilution of species specific secondary antibodies conjugated to alexa fluorophores for 1hr. Finally, Hoechst 33342 (a double stranded DNA staining dye; Life Technologies, Paisley, UK) was added 5 minutes before the end of this incubation. Two more sections and cover slips (controls) were incubated with the primary or the secondary antibodies only. At the end of the incubation period, the sections and cover slips were washed 4 times in TBST, dried and mounted for viewing. Finally, they were visualised and photographed using an Axioplan 2 Imaging® microscope and AxioVison Release® 4.8 software (Carl Zeiss, Cambridge, UK). Like all the immunoblotting experiments, the immunocytochemistry was performed in duplicate and each experiment was repeated at least twice.

**Pull down assay.** To determine whether the GR was able to bind laminin, a modification of the pull-down assay described in De Beer et al. (1981) was used. Briefly, 250µg recombinant human merosin (laminin 2; laminin 211; Millipore (UK) limited, Hertfordshire, UK) the main laminin in skeletal muscle or 100µg recombinant mouse integrin  $\alpha 7\beta 1$  (the main integrins in mammalian skeletal muscle; R&D Systems, Abingdon, Oxford, UK) were coupled to cyanogen bromide (CNBr)-activated sepharose beads (Sigma-Aldrich, Gillingham, Dorset, UK), according to the manufacturer's instructions. The excess ligand was washed off with coupling buffer and all the unbound active groups were blocked using 0.2M glycine. 300µl of slow-twitch muscle lysate was added to the beads alone, the beads plus laminin or the

beads plus integrin and left over night at 4°C on a rotator. Slow-twitch muscle lysate was used because slow muscle expresses 4-5 times more GR than fast-twitch muscle (Perez et al, 2013). The following morning, the beads were washed two times with TBST and once in PBS. They were then mixed with 30-40µl elution buffer (Laemmli sample loading buffer) and left on a rotator for 1h. Finally, they were centrifuged at 2000g for 20sec and the supernatant was collected. 10µl of each supernatant was separated using standard gel-electrophoresis, transferred on to nitrocellulose membrane and finally immunoblotted for the GR using 1:500 dilution of the rabbit monoclonal anti-GR antibody from Abcam as described above.

***Immunofluorescence confocal microscopy (CM).*** Soleus muscles from 6 month old C57BL/6 mice were fixed in 2% paraformaldehyde in phosphate buffered saline (PBS) for 2 hours at room temperature. Small bundles were washed twice with PBS and blocked for 1 hour in PBS containing 1% BSA, 10% goat serum and 0.5% TritonX-100 (added to permeabilise the membrane). They were incubated overnight at 4°C in primary antibody and the following morning they were washed three times in PBS. They were incubated with the secondary antibody for one hour at room temperature before being mounted on coverslips with anti-bleach media (Slowfade Gold antifade reagent, Invitrogen). Primary antibodies were: anti-RYR1, 34C (dilution 1:30), Developmental Studies Hybridoma bank, University of Iowa; rabbit monoclonal anti-GR (dilution 1:100) from Abcam. Secondary antibodies were: Cy3-labeled goat anti-rabbit IgG (dilution 1:300) for single GR labelling; Cy5-labeled goat anti-mouse IgG (dilution 1:200) and Cy3-labeled goat anti-rabbit IgG for double labelling (dilution 1:300). Specimens were finally viewed using a scanning laser confocal microscope (LS510 META Carl Zeiss, Germany).

**Immunogold labelling electron microscopy (EM).** Soleus muscles were fixed for 20 min at room temperature in a fixative mixture containing 2% paraformaldehyde and 0.5% glutaraldehyde (in PBS buffer). Small bundles were permeabilised and blocked as described above. After incubation with the rabbit monoclonal anti-GR from Abcam, secondary antibodies conjugated with Nanogold® particles were applied for 2h at 4°C (dilution 1:100). Samples were then post-fixed with 1 % glutaraldehyde (in PBS buffer), at room temperature, and incubated with reagents to enhance the signal (Goldenhance™ EM Formulation - Nanoprobes, Yaphank NY 11980-9710) for 5 minutes. Fibre bundles were finally embedded and sectioned using standard EM protocols (Boncompagni et al. 2009). Ultrathin sections of ~40 nm were cut using a Leica Ultracut R microtome (Leica Microsystem, Austria) with a Diatome diamond knife (DiatomeLtd. CH-2501 Biel, Switzerland) and double-stained with uranyl acetate and lead citrate. Sections were viewed using a FP 505 Morgagni Series 268D electron microscope (FEI Company, Brno, Czech Republic), equipped with Megaview III digital camera and Soft Imaging System (Munster, Germany).

As the monoclonal antibody used in the immunocytochemistry, immunofluorescence confocal microscopy and Immunogold labelling electron microscopy has never been tested before for use in these techniques, the findings were confirmed using a polyclonal rabbit anti-GR (Abcam ab35780) that is recommended for use in immunocytochemistry.

### **Statistical analysis**

As mentioned above, all the immunoblotting experiments were run in duplicate and each experiment was repeated at least twice. To determine the expression of the GCR in the various muscles, the Western blots were digitised and the intensity of the various protein bands were analysed using Scion Image® from NIH and normalised to that of the loading control (actin). Statistical analysis of the data was performed using SigmaPlot® 11.2 (Systat Software Inc., London, UK). The data obtained from each fibre type under control and from the various experimental conditions were compared using a two-way ANOVA with Tukey's *post hoc* test and a  $P < 0.05$  was considered to be statistically significant.

### **Results**

The results in Fig.1 show the expression and localisation of GRs in myoblasts (Fig. 1A and 1B), myotubes (Fig. 1C and 1D) and adult muscle fibres (Fig. 1E and 1F). They confirm our previous findings in adult skeletal muscle fibres (Fig. 1E and 1F) but also show that the receptors were expressed throughout myogenesis and that their localisation, within the cell, changed with differentiation. Thus, they were localised in the cytoplasm in myoblasts (Fig 1A and 1B), in the nucleus in myotubes (Fig. 1C and 1D) and around the periphery of the muscle fibres, where they co-localised with laminin, in adult skeletal muscle fibres (Fig. 1E and 1F). Moreover, this shift in the localisation of GRs was accompanied by an increase in the expression of laminin (green staining in Fig 1D and 1F).

To visualise the precise localisation of the receptors, in adult mammalian skeletal muscle fibres, a combination of immunofluorescence confocal microscopy (CM) and immunogold-labelling electron microscopy (EM) was used. Since we have previously shown that the GR is expressed mainly in oxidative fibres (Perez et al. 2013), the soleus muscle which contains mostly oxidative fibres (i.e. type I and IIA fibres) was used in this experiment. The immunofluorescence CM confirmed some of our previous findings. For example, it showed that most receptors were localised in clusters on the fibre surface where they generated a narrow, but discontinuous band around the fibres (Fig.2A; white dotted square). In addition, it provided new evidence showing that the receptors were also localised in large quantities in satellite cells (white arrow, Fig. 2B) and to a lesser extent inside the muscle fibres where they formed a striated pattern. The immunogold labelling confirmed the CM data (Fig. 2C, D and E), and clearly demonstrated that the GR was quite abundant in the extracellular matrix (Fig. 2C) and in satellite cells (Fig. 2E). Gold particles were also found inside the fibres, preferentially but not exclusively, in the vicinity of mitochondria (Fig. 2F), that we know are specially localised at I bands (on both sides of Z lines) in adult fibres (Boncompagni et al. 2009). To determine the exact location of the GR signal inside the fibres, some of the fibres were double stained with an anti-GR and an antibody (anti-RYR1, 34C) that marks the position of  $\text{Ca}^{2+}$  release units (CRUs), or triads. In adult fibres, CRUs are specifically placed at the transition between A and I bands, where they form a very peculiar transverse double-cross striation on either side of the I band (Boncompagni et al., 2009a). They also contain the sarcoplasmic reticulum  $\text{Ca}^{2+}$  release channels, type-1 ryanodine receptors (RYR1). Double labelling for GRs and RYR1s (red and green, respectively in Fig. 2G) indicated that the GR staining, inside the fibres was exclusively localised at the

I-band, flanked on either side by the two green lines marking the position of the triads. These findings also suggested that some of the gold particles that were not at the I band may have been nonspecific (Fig. 2C and 2D). As the I band region of adult fibres is rich in mitochondria (Boncompagni et al. 2009), the results in Fig. 2G agree with those in Fig. 2E-F showing gold particles in the proximity of mitochondria (the dark and branched organelles).

To determine what tethers the GR in the extracellular matrix, laminin 2 (merosin, laminin 211) or integrins of the  $\alpha 7\beta 1$  type coupled to cyanogen bromide-activated sepharose beads were used to pull-down the GR from slow muscle lysates. As the results displayed in Fig. 3A show, only laminin coupled beads were able to bind and pull down the GR. Moreover, their ability to bind the receptor depended on the presence or absence of the ligand (GC). Thus, adding 500nM BDP to the muscle lysate, significantly reduced the amount of GR pulled down by the beads (Fig. 3B) suggesting that the GC was able to dissociate the GR from laminin.

To determine the cellular signalling pathways activated by physiological concentrations of GCs in adult skeletal muscle, small muscle fibre bundles isolated from the extensor digitorum longus (edl, a fast-twitch muscle) and soleus (a slow-twitch muscle) were treated with 250nM beclomethasone dipropionate (BDP, a synthetic GC widely used in the management of asthma). The phosphorylation (=activation) of JNK, ERK 1&2 and p38 MAPK was then determined. As the results displayed in Fig. 4 and Fig. 5 show, the effects of BDP on the phosphorylation of these kinases was complex and depended on the fibre type and the duration of the treatment. For example, treating the muscle fibre bundles with BDP for 5min increased the phosphorylation of p38 MAPK and ERK1&2 in the fast-twitch muscle fibres (Fig. 4A and C; Fig. 5A, D and E) and that of JNK in the slow-twitch fibres (Fig.

4B; Fig. 5B and C). Increasing the treatment time to 30min further enhanced the activation of p38MAPK in the fast-twitch fibres (Fig. 4A; Fig. 5A) and that of ERK 1&2 (Fig. 4C; Fig. 5D and E). However, it led to the activation of JNK in both fibre types (see Fig.4B and C; Fig. 5B and C). In contrast, prolonging the treatment duration to 60min led to a slight decrease in the phosphorylation of p38 MAPK and a marked decline in that of JNK in the fast-twitch fibres (Fig. 4A and 4B; Fig. 5A, B and C) but further enhanced that of JNK in the slow-twitch fibres (Fig. 4B; Fig. 5B and C). On the other hand, the phosphorylation of ERK 1&2 in the fast-twitch fibres remained elevated and was similar to that seen after the 30min treatment (Fig. 4C; Fig. 5D and E); whereas, in the slow-twitch fibres it was slightly reduced (Fig. 5D and E).

The findings in Fig.4 and Fig. 5 suggested that the activation of MAPKs by GCs in mammalian skeletal muscles was non-genomic (i.e. it occurred within 5 minutes). However, they did not tell us the receptor/mechanism underlying this effect. As the mechanisms underlying the non-genomic effects of GCs are still controversial (Lipworth 2000; Stahn & Buttgereit 2008; Perez et al, 2013), we investigated whether the activation of the various MAPKs by BDP were mediated through the GR or through another receptor/mechanism. As the results displayed in Fig. 6A and 6B show, pre-treating the muscle fibre bundles with 10 $\mu$ M mifepristone (RU486, a GR inhibitor), for 10min, completely abolished or significantly reduced the 30min BDP-induced activation of p38 MAPK and JNK suggesting that they were mediated by a GR. To determine whether they were mediated by a cGR or an mGR, the experiment above was repeated using the rabbit monoclonal anti-GR used in the immunocytochemistry and Western blotting experiment. The rationale behind this experiment was that the antibody was too large to cross the cell membrane and that it could only bind the GR and inhibit its effects, if the receptor was easily accessible



i.e. located close to the cell surface. As the results presented in Fig. 6C and 6D show, pre-treating the muscle fibre bundles with the antibody for 10min, completely abolished the BDP-induced activation of JNK and p38 MAPK in both fibre types suggesting that they were mediated by a GR that was easily accessible to the antibody (i.e. a cell surface GR). They also suggested that the antibody bound the GR close to the ligand binding site or caused a conformational change to the receptor that inhibited its activation of the MAPK pathway.

The results presented so far suggested that the effects of BDP on the activation of the MAPK pathway were non-genomic and were mediated by a GR located close to the cell surface. They also suggested that it may be bound to laminin. However, they did not tell us how the GC was able to activate the MAPKs. In mammalian skeletal muscle fibres, laminin is localised in the basal lamina (one of the two layers that form the basement membrane of skeletal muscles) and is thought to communicate with the rest of the cell through integrins (mainly integrin  $\alpha 7\beta 1$  in skeletal muscle) and dystroglycan (Gawlik & Durbeej, 2011). This led us to hypothesise that the GR signalled to the MAPK through the activation of focal adhesion kinase (FAK) by integrins (see Fig.10). To test this hypothesis, we pre-treated another set of muscle fibre bundles with the FAK specific inhibitor, FAK inhibitor 14. As the results displayed in Fig. 7 and Fig. 8 show, pre-treatment of the fibre bundles with the inhibitor completely abolished the GC-induced activation of p38 MAPK (Fig. 7A; Fig. 8A) and ERK 1 (p44; Fig. 7C; Fig. 8D and E). However, pre-treatment of the muscle bundles with the inhibitor alone augmented the activation of JNK (especially the 46kDa isoform) in the slow twitch fibres and returned to the level observed in the BDP-treated fibres when the bundles were treated with both the inhibitor and the GC (Fig. 7B; Fig. 8B and C).

## Discussion

### *Localisation of the GR in mouse skeletal muscle cells*

The results we report here, show for the first time, that GRs are expressed at all stages of myogenesis. However, their localisation changes with differentiation (Fig.1). They also revealed that, in adult mammalian skeletal muscle fibres, they were localised mostly in the extracellular matrix (Fig. 2A and C), in satellite cells (Fig. 2B and 2D) and in the I band region where they were closely associated with mitochondria. Although a number of studies have previously suggested that a membrane glucocorticoid receptor exists in many cell types (Orchinik et al. 1991; Gametchu, 1993; Bartholome et al. 2004; Perez et al. 2013) this is the first study to show that a GR localised close to the cell membrane exists. However, as our results show, it is localised in the extracellular matrix and in satellite cells and not in the plasma membrane as previous studies have suggested.

Adult mammalian skeletal muscle fibres, unlike most other cell types, are long, cylindrical, multinucleated cells whose nuclei are located close to the fibre surface. Their cytoplasm (=sarcolemma) is also packed with contractile proteins that are organised into striations consisting of highly regular repeats of thin (actin) and thick (myosin) filaments. Slow twitch fibres also contain large quantities of mitochondria that are mostly found at the I band region (Boncompagni et al. 2009) and under the sarcolemma (mostly in the proximity of capillaries). Here, we propose that this unique structural organisation of mammalian skeletal muscle fibres is responsible for the distinctive distribution and localisation of GRs in adult mammalian skeletal muscle fibres. We also hypothesise that, as myoblasts differentiate into myotubes, GRs migrate to the nucleus and as contractile proteins are laid down, the nucleus, the GRs and most other cell organelles are pushed to the periphery of the muscle

fibres. However, how the GRs get out of the muscle fibres and localises in the extracellular matrix is uncertain. Also, why and how the receptor localises in the proximity of mitochondria is also uncertain and further studies to investigate these uncertainties are necessary.

Another possibility is that GRs are bound to or closely associated with cytoskeletal proteins such as dystrophins, dystroglycans, utrophins and laminins. In adult mammalian skeletal muscle fibres, these proteins are important in the maintenance of cell shape, structure and function and are closely associated with the cell membrane (Gawlik & Durbeej, 2011). Indeed, GCs have been shown to increase the content of dystrophin and utrophin in both normal and dystrophic differentiating human skeletal muscle satellite cells (Sklar et al, 1991; Pasquini et al, 1995). In the present study, GRs were also found to be able to bind laminin (Fig.3). The reason why GC treatment increases the accumulation of these proteins is uncertain. Here we postulate that, in mammalian skeletal muscles, GCs and hence GRs may be important for the synthesis, recruitment and/or organisation of these extracellular matrix proteins. Indeed, the present findings show that myoblasts expressed little or no laminin (Fig. 1A and 1B) and laminin only started to appear in myotubes when the GRs translocated into the nucleus (Fig. 1C and 1D).

#### ***Effects of GCs on the MAPK pathway.***

Another novel finding in the present study is the observation that treating mammalian skeletal muscle fibre bundles with physiological concentrations of BDP increases the phosphorylation/activation of ERK 1&2, JNK and p38 MAPK in a fibre type- and duration-dependent manner (Figs 4 and 5). MAPKs are also activated in brain tissues of rats subjected to short periods of psychological stress (Gutierrez-Mecinas et al, 2011) as well as in hippocampal and PC12 (rat pheochromocytoma) cells treated

with low doses of corticosterone (Li et al, 2001; Qi et al, 2005). However, treatment of most other cell types, including HeLa cells, vascular endothelial cells, macrophages, mast cells and monocytes, with low doses of GCs has been shown to have the opposite effect i.e. it decreases MAPK activation (Franklin et al, 1997; Caelles et al, 1997; Gonzalez et al, 2000; Kassel et al, 2001; Imasato et al, 2002; Lasa et al, 2002; Bruna et al, 2003). Together these findings suggest that the effects of GCs on the MAPK pathway depend on the tissue that the cells are derived from and the physiological role of stress and MAPKs in that tissue. For example, in tissues such as the brain and skeletal muscle that are directly involved in the stress-response and where activation of MAPKs by short term stress is beneficial (Gutierrez et al, 2011, Perez, et al, 2013; present study), physiological concentrations of GCs increase the activation of MAPKs. Conversely, in tissues such as the immune system where activation of MAPKs by stress is detrimental to the long term survival of the organism, GCs inhibit their activation (Franklin et al, 1997; Caelles et al, 1997; Gonzalez et al, 2000; Bruna et al, 2003).

### ***Physiological functions of GCs***

In mammalian skeletal muscle cells, all three MAPKs (p38 MAPK, JNK and ERK) have been implicated in the regulation of myogenesis and the expression of skeletal muscle specific genes (Bennett & Tonks, 1997; Cuenda et al, 1999; Weston et al, 2003). Additionally, intense exercise (Widegren et al, 2000) and marathon running (Yu et al, 2001), both of which lead to fibre type switch, have been shown to induce the release of GCs and the activation of p38 and ERK 1&2 in the vastus lateralis portion of the quadriceps femoris muscle (note that the vastus lateralis consists mostly of fast-twitch muscle fibres). p38 MAPKs have also been shown to act as molecular switches in the activation of quiescent satellite cells (Jones et al, 2005).

Myogenesis is important in skeletal muscle development; whereas, satellite cell activation is essential for skeletal muscle repair/regeneration. The fact that GC treatment increases the activation of all three MAPKs in mouse skeletal muscle fibres (present study) and strenuous exercise activates p38 and ERK 1&2 in fast-twitch fibres, suggests that GCs may play an important role in both myogenesis and skeletal muscle repair/regeneration.. However, the exact role of GCs in myogenesis and satellite cell activation is uncertain and further studies to confirm this are necessary.

The GC-induced activation of ERK1&2 we report here may also be responsible for the GC-induced increase in maximum isometric force ( $P_o$ ) in slow-twitch muscle fibres previously reported by Perez and her colleagues (Perez et al. 2013). Here we speculate that the GC-induced activation of ERK1&2 leads to the phosphorylation of the regulatory light chains of myosin (RMLCs) and the potentiation of  $P_o$  as previously shown in Hamdi & Mutungi (2010) . Fast-twitch and slow-twitch muscle fibres express different isoforms of RMLCs that are phosphorylated at low stimulation frequencies in fast-twitch fibres and at high frequencies in slow-twitch fibres (Moor & Stull, 1984). The phosphorylation of RMLCs in fast-twitch fibres increases sub-maximal force (including twitch contraction); whereas, their function in slow-twitch muscles is unknown as their phosphorylation does not seem to affect twitch amplitude (Moore & Stull, 1984). Here we speculate that the phosphorylation of RMLCs in slow-twitch fibres leads to the potentiation maximum isometric force. However, the exact mechanism underlying the GC-induced increase in force is uncertain and further studies to confirm this are needed.

### ***Mechanism underlying the effects of GCs in skeletal muscle***

As mentioned above, in most cells, GCs inhibit the MAPK pathway. They do this either by up-regulating the synthesis and activation of proteins such as glucocorticoid-induced-leucine zipper, MAPK phosphatase-1 and annexin-1 (Gonzalez et al, 2000; Lasa et al, 2002) or by directly interacting with components of the MAPK pathway (Caelles et 1997; Bruna, 2003). The results reported here, suggest that the effects of GCs in mammalian skeletal muscle fibres are mediated through GRs localised close to the cell surface (Fig. 6) and that their stimulation leads to the activation of focal adhesion kinase (FAK, Fig. 9). Here we hypothesise that in mammalian skeletal muscle fibres, mGRs are bound to laminin and that their stimulation by GCs leads to a conformational change in laminin and the activation of FAK by integrins (Fig. 9). In mammalian skeletal muscles, laminin binds to and signals through both integrin  $\alpha7\beta1$  and dystroglycan (Gawlik & Durbeej, 2011). Therefore, we think that the conformational change in laminin activates integrin  $\alpha7\beta1$  in fast-twitch muscle fibres and dystroglycan in slow-twitch fibres (Fig. 9). We also think that integrins recruit and activate FAK leading eventually to the phosphorylation of p38 MAPK and ERK1&2; whereas, dystroglycans act slightly lower down the pathway and their stimulation leads to the activation of JNK. We also think that FAK has inhibitory effects on the dystroglycan pathway (Fig.9). However, the exact mechanism mediating the GC-induced increase in the activation of MAPKs in mammalian skeletal muscle fibres is uncertain and further studies to determine this are necessary.

## References

- Alangari AA (2010). Genomic and non-genomic actions of glucocorticoids in asthma. *Annals of Thoracic Medicine*, **5**, 133-139.
- Bartholome B, Spies CM, Gaber T, Schuchmann S, Berki T, Kunkel D, Bienert M, Radbruch A, Burmester GR, Lauster R, Scheffold A, & Buttgereit F (2004). Membrane glucocorticoid receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. *Faseb Journal* **18**, 70-80.
- Bennett AM & Tonks NK (1997). Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. *Science*, **278**, 1288-1291.
- Boncompagni S, Rossi AE, Micaroni M, Beznoussenko GV, Polishchuk RS, Dirksen RT, Protasi F (2009). Mitochondria are linked to calcium stores in striated muscle by developmentally regulated tethering structures. *Mol Biol Cell*, **20**, 1058-1067.
- Bruna A, Nicolas M, Munoz A, Kyriakis JM, & Caelles C (2003). Glucocorticoid receptor-JNK interaction mediates inhibition of the JNK pathway by glucocorticoids. *Embo Journal*, **22**, 6035-6044.
- Buttgereit F, Krauss S, & Brand MD (1997). Methylprednisolone inhibits uptake of Ca<sup>2+</sup> and Na<sup>+</sup> ions into concanavalin A-stimulated thymocytes. *Biochemical Journal*, **326**, 329-332.
- Caelles C, Gonzalez-Sancho JM, & Munoz A (1997). Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes & Development*, **11**, 3351-3364.

- Croxtall JD, Choudhary Q, & Flower RJ (2000). Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. *British Journal of Pharmacology*, **130**, 289-298.
- Cuenda A & Cohen P (1999). Stress-activated protein kinase-2 p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *Journal of Biological Chemistry*, **274**, 4341-4346.
- De Beer FC, Baltz ML, Holford S, Feinstein A, & Pepys MB (1981). Fibronectin and C4-Binding Protein Are Selectively Bound by Aggregated Amyloid-P Component. *Journal of Experimental Medicine*, **154**, 1134-1149.
- Drummond GB (2009). Reporting ethical matters in The Journal of Physiology: standards and advice. *Journal of Physiology-London* **587**, 713-719.
- Franklin CC & Kraft AS (1997). Conditional expression of the mitogen-activated protein kinase (MAPK) phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. *Journal of Biological Chemistry*, **272**, 16917-16923.
- Gametchu B, Watson CS, & Wu SH (1993). Use of Receptor Antibodies to Demonstrate Membrane Glucocorticoid Receptor in Cells from Human Leukemic Patients. *Faseb Journal* **7**, 1283-1292.
- Gawlik KI & Durbeej M (2011). Skeletal muscle laminin and MDC1A: pathogenesis and treatment strategies. *Skeletal muscle*, **1.9**, 1-13.
- Goldberg AL (1969). Protein Turnover in Skeletal Muscle .2. Effects of Denervation and Cortisone on Protein Catabolism in Skeletal Muscle. *Journal of Biological Chemistry*, **244**, 3223-3229.



- Gonzalez MV, Jimenez B, Berciano MT, Gonzalez-Sancho JM, Caelles C, Lafarga M, & Munoz A (2000). Glucocorticoids antagonize AP-1 by inhibiting the activation/phosphorylation of JNK without affecting its subcellular distribution. *Journal of Cell Biology*, **150**, 1199-1207.
- Gutierrez-Mecinas M, Trollope AF, Collins A, Morfett H, Hesketh SA, Kersante F, & Reul JM (2011). Long-lasting behavioral responses to stress involve a direct interaction of glucocorticoid receptors with ERK1/2-MSK1-Elk-1 signaling. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 13806-13811.
- Hafezi-Moghadam A, Simoncini T, Yang ZQ, Limbourg FP, Plumier JC, Rebsamen MC, Hsieh CM, Chui DS, Thomas KL, Prorock AJ, Laubach VE, Moskowitz MA, French BA, Ley K, & Liao JK (2002). Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nature Medicine*, **8**, 473-479.
- Hamdi M & Mutungi G (2010). Dihydrotestosterone activates the MAPK pathway and modulates maximum isometric force through the EGF receptor in isolated intact mouse skeletal muscle fibres. *Journal of Physiology-London*, **588**, 511-525.
- Hamdi M & Mutungi G (2011). Dihydrotestosterone stimulates amino acid uptake and the expression of LAT2 in mouse skeletal muscle fibres through an ERK1/2-dependent mechanism. *Journal of Physiology-London* **589**, 3623-3640.
- Hua SY & Chen YZ (1989). Membrane Receptor-Mediated Electrophysiological Effects of Glucocorticoid on Mammalian Neurons. *Endocrinology*, **124**, 687-691.

- Imasato A, sbouis-Mouthon C, Han JH, Kai H, Cato ACB, Akira S, & Li JD (2002). Inhibition of p38 MAPK by glucocorticoids via induction of MAPK phosphatase-1 enhances nontypeable Haemophilus influenzae-induced expression of toll-like receptor 2. *Journal of Biological Chemistry*, **277**, 47444-47450.
- Jones NC, Tyner KJ, Nibarger L, Stanley HM, Cornelison DDW, Fedorov YV, & Olwin BB (2005). The p38 alpha/beta MAPK functions as a molecular switch to activate the quiescent satellite cell. *Journal of Cell Biology*, **169**, 105-116.
- Kadmiel M & Cidlowski JA (2013). Glucocorticoid receptor signaling in health and disease. *Trends in Pharmacological Sciences*, **34**, 518-530.
- Kassel O, Sancono A, Kratzschmar J, Kreft B, Stassen M, & Cato ACB (2001). Glucocorticoids inhibit MAP kinase via increased expression & decreased degradation of MKP-1. *Embo Journal*, **20**, 7108-7116.
- Krieger DT, Allen W, Rizzo F, & Krieger HP (1971). Characterization of Normal Temporal Pattern of Plasma Corticosteroid Levels. *Journal of Clinical Endocrinology & Metabolism*, **32**, 266-284.
- Lasa M, Abraham SM, Boucheron C, Saklatvala J, & Clark AR (2002). Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. *Molecular and Cellular Biology*, **22**, 7802-7811.
- Li XY, Qiu J, Wang JW, Zhong YP, Zhu JQ, & Chen YZ (2001). Corticosterone-induced rapid phosphorylation of p38 and JNK mitogen-activated protein kinases in PC12 cells. *Febs Letters*, **492**, 210-214.
- Lipworth BJ (2000). Therapeutic implications of non-genomic glucocorticoid activity. *Lancet*, **356**, 87-89.

- Ma K, Mallidis C, Bhasin S, Mahabadi V, Artaza J, Gonzalez-Cadauid N, Arias J, & Salehian B (2003). Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. *American Journal of Physiology-Endocrinology and Metabolism*, **285**, E363-E371.
- Moore RL & Stull JT (1984). Myosin light chain phosphorylation in fast and slow skeletal-muscles *in situ*. *Am J Physiol* **247**, C462-C471.
- Nava S, GayanRamirez G, Rollier H, Bisschop A, Dom R, deBock V, & Decramer M (1996). Effects of acute steroid administration on ventilatory and peripheral muscles in rats. *American Journal of Respiratory and Critical Care Medicine*, **153**, 1888-1896.
- Orchinik M, Murray TF, & Moore FL (1991). A Corticosteroid Receptor in Neuronal Membranes. *Science* **252**, 1848-1851.
- Pasquini F, Guerin C, Blake D, Davies K, Karpati G, & Holland P (1995). The Effect of Glucocorticoids on the Accumulation of Utrophin by Cultured Normal and Dystrophic Human Skeletal-Muscle Satellite Cells. *Neuromuscular Disorders*, **5**, 105-114.
- Perez MH-A, Cormack J, Mallinson D, & Mutungi G (2013). A membrane glucocorticoid receptor mediates the rapid/non-genomic actions of glucocorticoids in mammalian skeletal muscle fibres. *Journal of Physiology-London*, **591**, 5171-5185.
- Pratt WB & Toft DO (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Experimental Biology and Medicine*, **228**, 111-133.

- Qi AQ, Qiu J, Xiao L, & Chen YZ (2005). Rapid activation of JNK and p38 by glucocorticoids in primary cultured hippocampal cells. *Journal of Neuroscience Research*, **80**, 510-517.
- Rannels SR & Jefferson LS (1980). Effects of Glucocorticoids on Muscle Protein-Turnover in Perfused Rat Hemicorpus. *American Journal of Physiology*, **238**, E564-E572.
- Sklar RM & Brown RH (1991). Methylprednisolone Increases Dystrophin Levels by Inhibiting Myotube Death During Myogenesis of Normal Human Muscle Invitro. *Journal of the Neurological Sciences*, **101**, 73-81.
- Stahn C & Buttgereit F (2008). Genomic and nongenomic effects of glucocorticoids. *Nature Clinical Practice Rheumatology*, **4**, 525-533.
- van Balkom RHH, vanderHeijden HFM, vanMoerkerk HTB, Veerkamp JH, Fransen JAM, Ginsel LA, Folgering HTM, vanHerwaarden CLA, & Dekhuijzen PNR (1996). Effects of different treatment regimens of methylprednisolone on rat diaphragm contractility, immunohistochemistry and biochemistry. *European Respiratory Journal*, **9**, 1217-1223.
- Weston AD, Sampaio AV, Ridgeway AG, & Underhill TM (2003). Inhibition of p38 MAPK signaling promotes late stages of myogenesis. *Journal of Cell Science*, **116**, 2885-2893.
- Widegren U, Wretman C, Lionikas A, Hedin G, & Henriksson J (2000). Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle. *Pflugers Archiv-European Journal of Physiology*, **441**, 317-322.

Wing SS & Goldberg AL (1993). Glucocorticoids Activate the ATP-Ubiquitin-Dependent Proteolytic System in Skeletal-Muscle During Fasting. *American Journal of Physiology*, **264**, E668-E676.

Yu M, Blomstrand E, Chibalin AV, Krook A, & Zierath JR (2001) Marathon running increases ERK1/2 and p38 MAP kinase signalling to downstream targets in human skeletal muscle. *Journal of Physiology-London*, **536**, 273-282.

### **Author contributions**

GM was responsible for the conception and design of the experiments. He dissected the small muscle fibre bundles and helped with the various treatments. He also did the immunocytochemistry experiments and wrote the manuscript. SB and FP did all the confocal and electron microscopy experiments including the immunogold labelling. LA did most of the Western blotting under the supervision of GM. He also helped with the treatment of the muscle fibre bundles with the various compounds. EK and LA did the pull down assays with the help of DS. TP performed all the C2C12 cell experiments.

### **Acknowledgements**

This study was funded by BBSRC grant BB/J01754X/1 to GM. LA and EA were Wellcome Trust and Society of Biology vacation students, respectively. This study was also supported by the following grants: a) Italian Telethon ONLUS Foundation GGP13213 (to FP); and RBF13A20K Future in Research 2013 MIUR (Italian Minister of University & Research) (to SB).

### **Conflict of interests**

None

Figure1

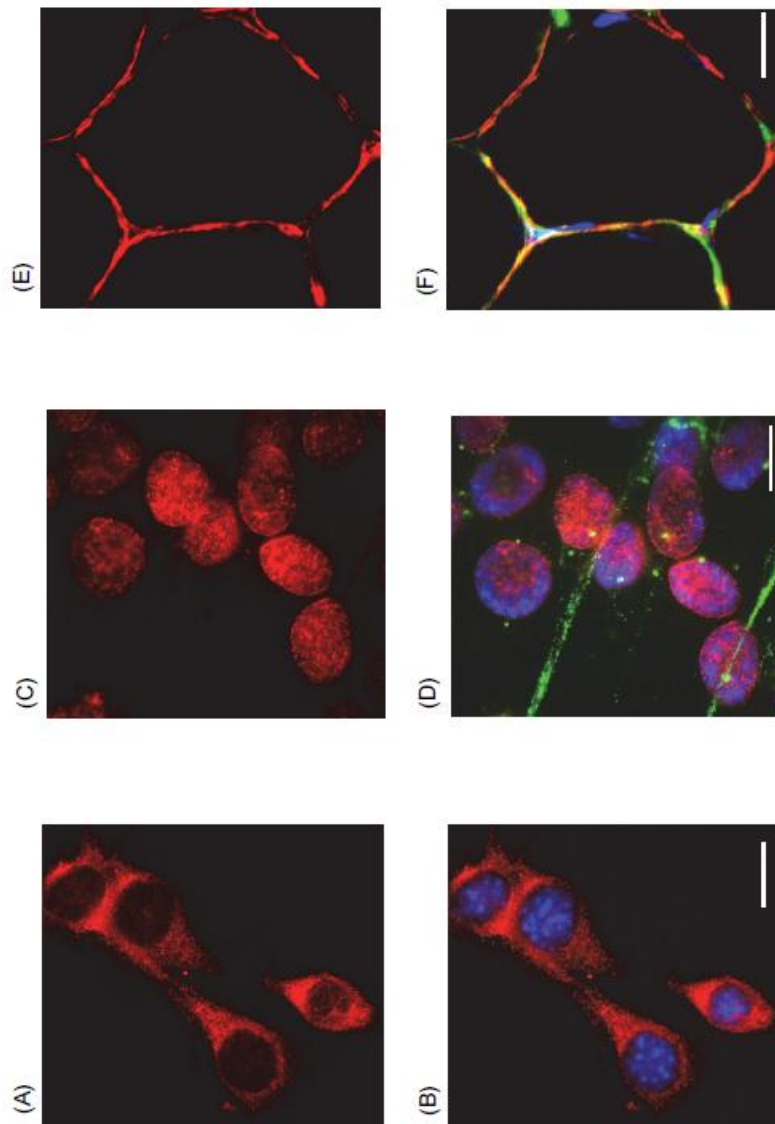


Figure 2

Figure 2

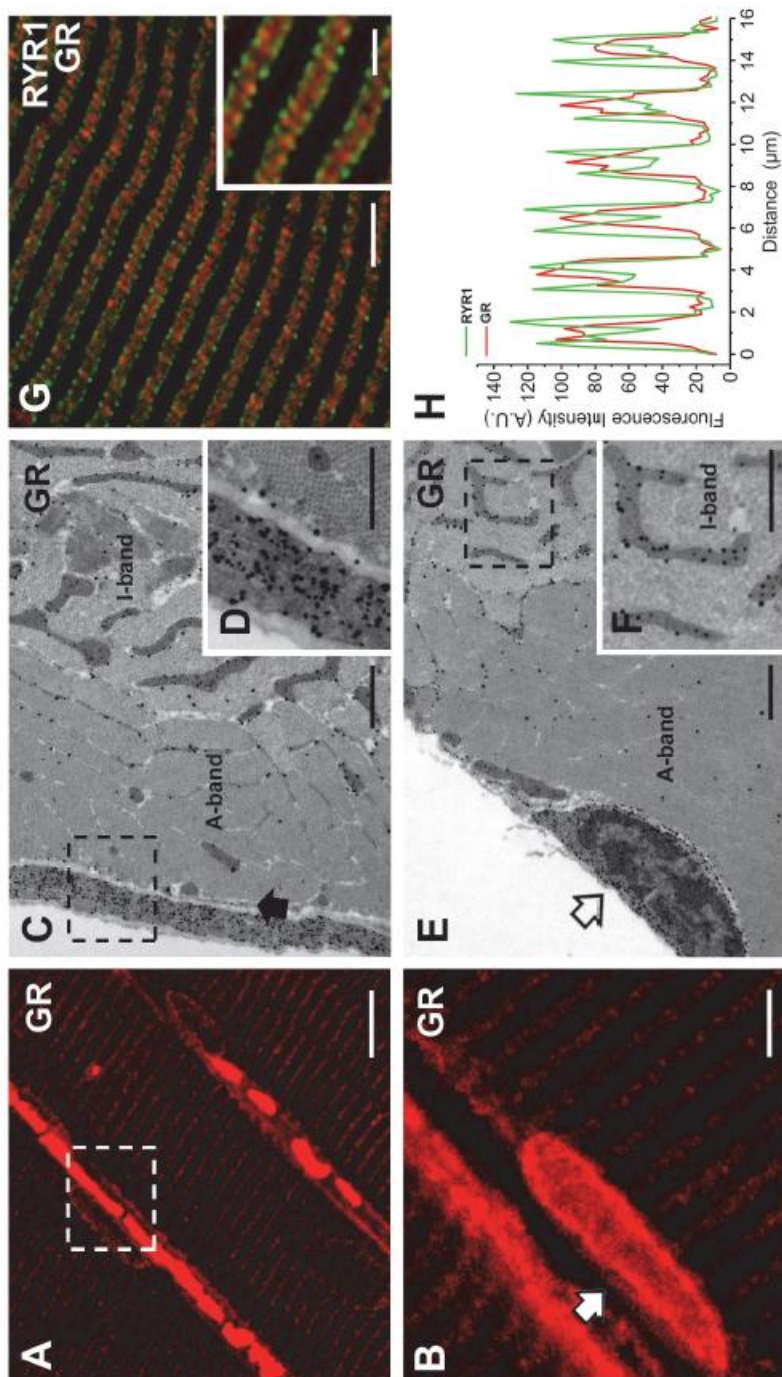


Figure3

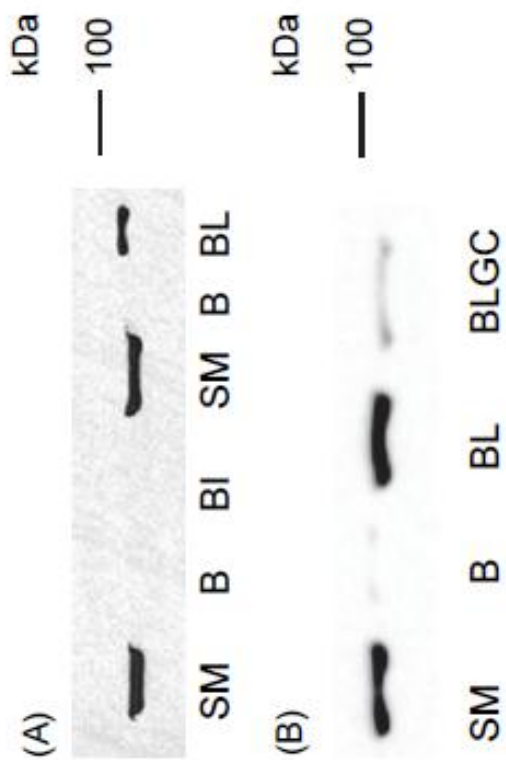




Figure 4

Figure 4

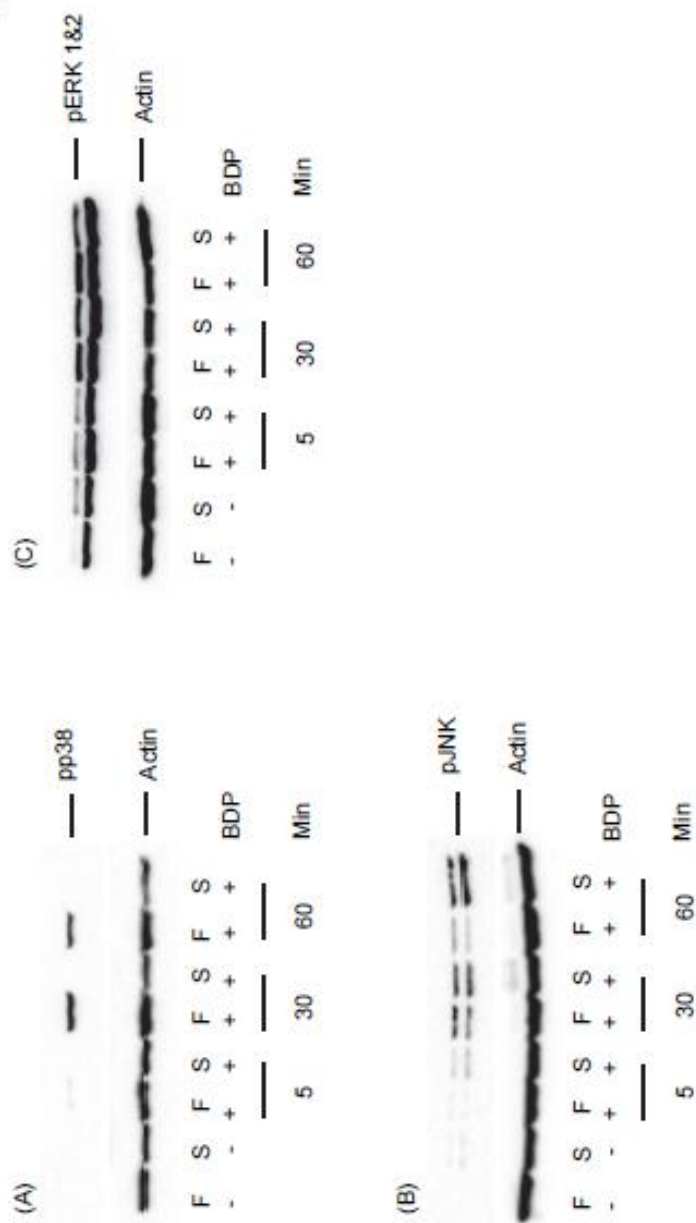


Figure 5

Figure 5

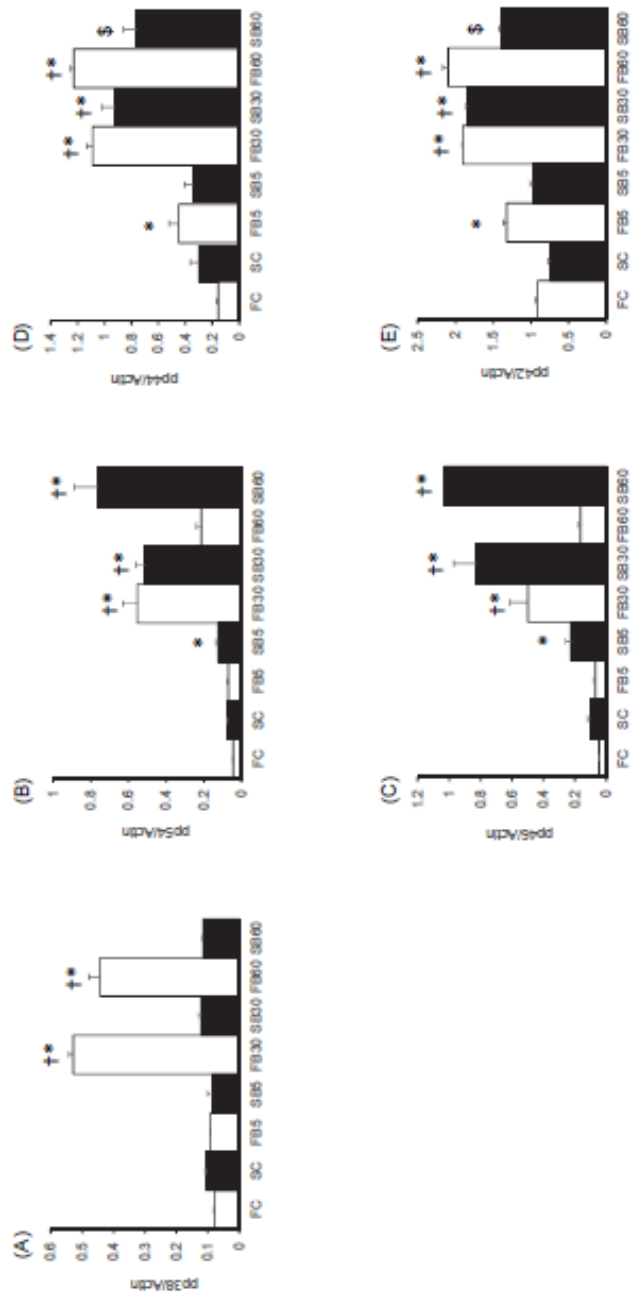


Figure 6

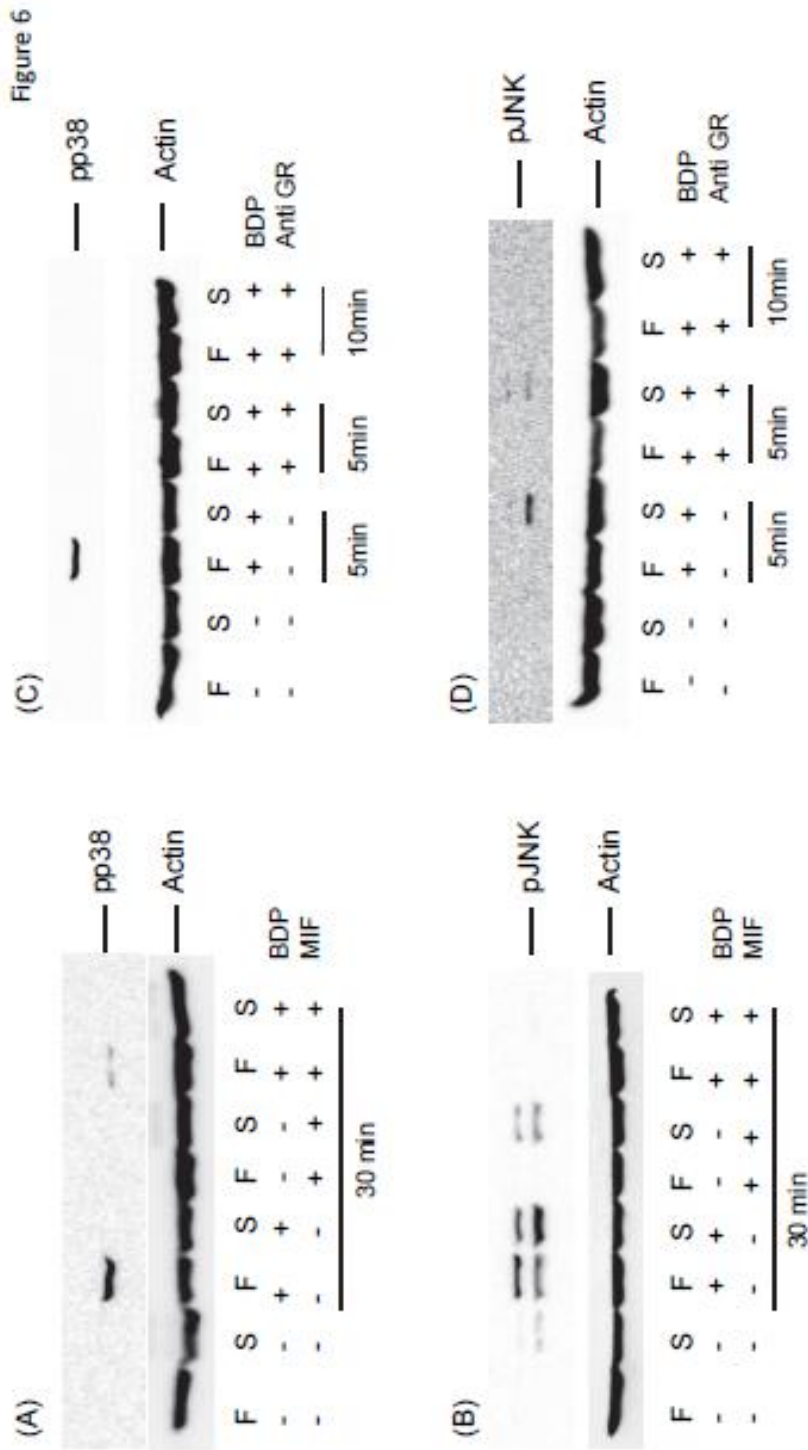


Figure 7

Figure 7

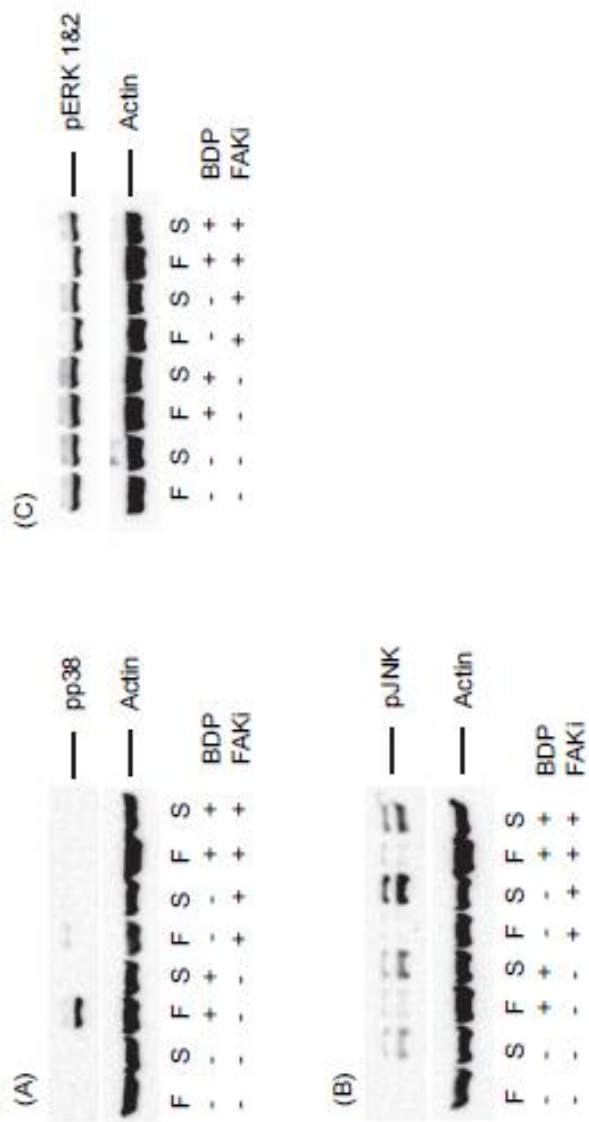


Figure 8

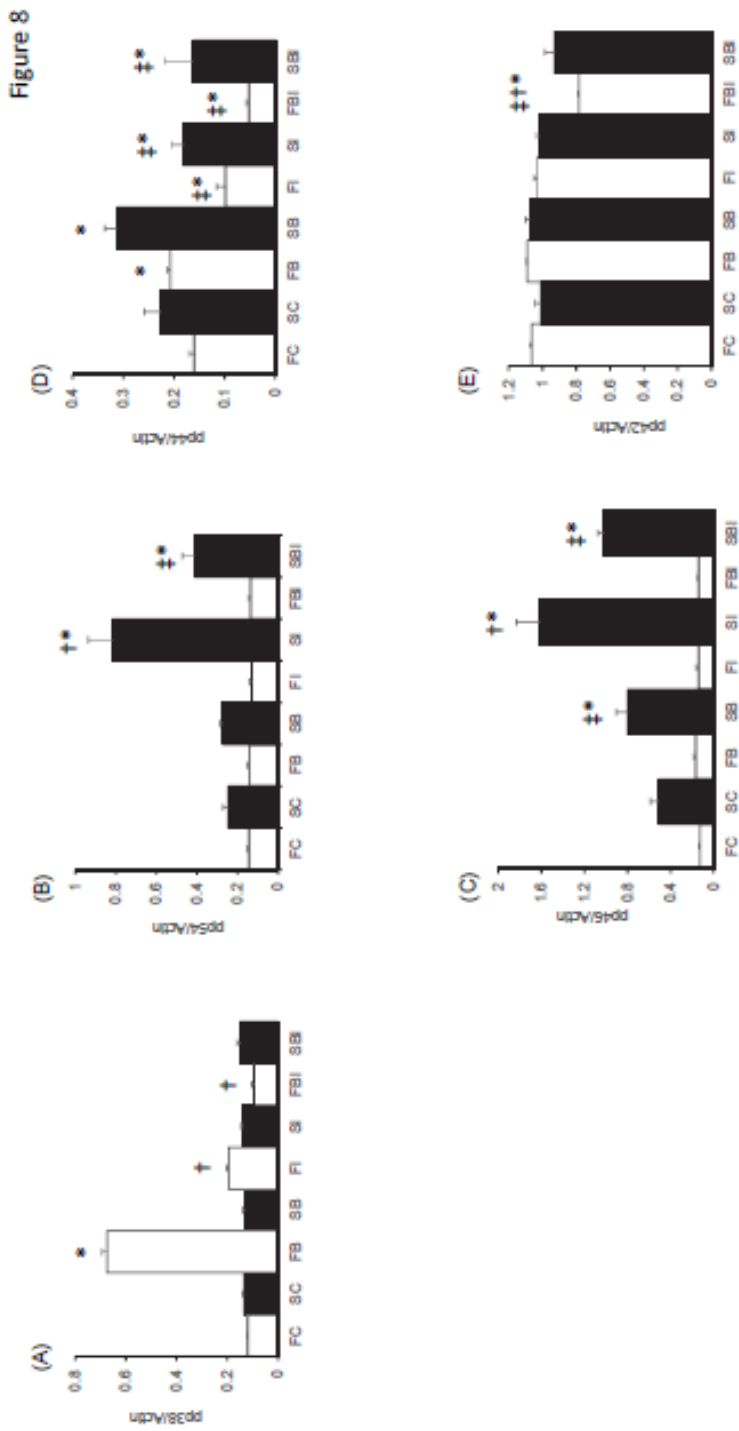


Figure 9

Figure 9

