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Yap is a novel regulator of C2C12 myogenesis

Kevin I. Watt [a], Robert Judson [a], Paul Medlow [c], Kenneth Reid [a], Tobias B. Kurth [b], Jatin G. Burniston [d], Aivaras Ratkevicius [a], Cosimo De Bari [b], Henning Wackerhage [a,*]

[a] Molecular Exercise Physiology, Bone & Musculoskeletal Research Programme, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, United Kingdom
[b] Regenerative Medicine, Bone & Musculoskeletal Research Programme, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, United Kingdom
[c] Sports and Exercise Research Institute, University of Ulster, Shore Road, Newtonabby, Country Antrim BT37 0QB, United Kingdom
[d] Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Henry Cotton Campus, 15-21 Webster Street, Liverpool L3 2ET, United Kingdom

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ABSTRACT

The expression, regulation and function of mammalian Hippo pathway members in skeletal muscle is largely unknown. The aim of this study was thus to test the hypothesis that core members of the mammalian Hippo pathway are expressed in skeletal muscle and that the transcriptional co-factor Yap, a core member of the Hippo pathway, regulates C2C12 myogenesis. We found that the major components of the mammalian Hippo pathway including Yap are all expressed in skeletal muscles, C2C12 myoblasts and myotubes. In C2C12 myoblasts, Yap Ser127 phosphorylation is low and Yap localises to nuclei. Upon differentiation, Yap Ser127 phosphorylation increases ≈20-fold and Yap translocates from the nucleus to the cytosol. To test whether the observed increase of Yap Ser127 phosphorylation is required for differentiation we overexpressed hYAP1 S127A, a mutant that can not be phosphorylated at Ser127, in C2C12 myoblasts. We found that overexpression of hYAP S127A prevented myotube formation, whereas the overexpression of wildtype hYAP1 or empty vector had no effect. In addition, more hYAP1 S127A overexpressing cells progressed through the S phase of the cell cycle and the expression of MRFs (myogenin, Myf5), Mef2c and cell cycle regulators (p21, cyclin D1) was significantly changed when compared to wildtype hYAP1 and empty vector overexpressing cells. This data suggests that the phosphorylation of Yap at Ser127 leads to a changed expression of MRFs and cell cycle regulators and is required for C2C12 myoblasts to differentiate into myotubes.

Introduction

During myogenesis precursor cells first commit to the myogenic lineage in a process known as determination before withdrawing from the cell cycle and differentiating to form multinucleated muscle fibres [1]. Myogenic regulation also plays a role postnatally in satellite cells during repair from muscle injury and in response to resistance exercise [2]. Myogenesis is regulated by a plethora of transcription factors and signal transduction pathways. The myogenic regulatory factor (MRF) family of transcription factors (Myf5, MyoD, Mrf4 and myogenin) are major transcriptional regulators of myogenesis [3,4]. Previous studies using knock out mice have shown that MyoD, Mrf4 and Myf5 regulate myogenic determination, whereas myogenin, MyoD and Mrf4 regulate the terminal differentiation of myoblasts [1,3,4]. The myocyte enhancer factor 2 (MEF2) family of transcription factors can also regulate myogenesis by physically interacting with and by inducing the expression of MRF and other muscle-specific genes [5]. The aforementioned transcription factors and other cell functions during myogenesis are additionally regulated by signalling pathways such as the mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR), sonic hedgehog, Notch, transforming growth factor beta (TGFβ Smad (from Sma and mothers against DPP homologue) and wingless (Wnt) signalling pathways [6,7].

The Hippo signal transduction pathway is a pathway whose regulation and function has not yet been studied in skeletal muscle. It

Abbreviations: bHLH, helix-loop-helix transcription factors; cdk, cyclin dependent kinase; DMEM, Dulbecco's modified Eagle's medium; Frmd6, FIRM domain containing 6; IdU, iododeoxyuridine; LAP, liver associated protein; Lats1/2, large tumour suppressor kinase; MEF2, myocyte enhancer factor 2; MRF, myogenic regulatory factors; Mrf4, myogenic regulatory factor 4; Mst1/2, mammalian Ste20-like kinase; mTOR, mammalian target of rapamycin; Myf5, myogenic factor 5; MyHC, myosin heavy chain; MyoD, myogenic determination factor D; Nf2, neurofibromatosis 2; Pax, paired box; p38, p38 mitogen-activated protein kinase; Smad, from Sma and mothers against DPP homologue; TGFβ, transforming growth factor beta; Wnt, from Wg (wingless) and Int; Yap, yes-associated protein (hYAP indicates human YAP, whereas Yap indicates Yap in other species)

* Corresponding author. Address: Health Sciences Building, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, United Kingdom.
E-mail address: h.wackerhage@abdn.ac.uk (H. Wackerhage).

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controls events such as proliferation, apoptosis, differentiation, organ size and tumorigenesis in *Drosophila melanogaster* and some mammalian tissues [8,9]. A kinase cascade forms the core of the Hippo pathway: Mammalian Ste20-like 1/2 kinase (Mst1/2) first phosphorylates large tumour suppressor 1/2 kinase (Lats1/2) which in turn phosphorylates the transcriptional co-activator Yes-associated protein (Yap) at five serine residues [10]. The key residue Ser127 is part of a conserved 14-3-3 binding motif [11] and phosphorylation of Ser127 causes 14-3-3 proteins to sequester Yap in the cytosol [12,13], whereas unphosphorylated Yap localises to the nucleus [11].

More recent studies have begun to characterise the function of Yap in vertebrates and mammals *in vivo*. Conditional overexpression of human YAP (hYAP) isoforms under the control of a liver-specific promoter led to a 4–5-fold increase in liver size due to increased proliferation [14,15]. Overexpression of constitutively active hYAP S127A expanded multipotent progenitor cells in the crypt compartment of the intestine and these cells could not undergo terminal differentiation until hYAP1 S127A overexpression was stopped [15]. In the chick neural tube Yap overexpression similarly stimulated proliferation but inhibited differentiation of neural progenitor cells [16].

Taken together these results suggest that the Hippo pathway can regulate the proliferation and terminal differentiation of cells in several tissues and organs which is relevant for their development, repair and size. Little is known about the expression, regulation and function of Hippo pathway members in skeletal muscle cells. The aim of this study was thus to examine the expression of core members of the Hippo pathway in skeletal muscle and to investigate the regulation and function of the transcriptional co-factor Yap during C2C12 myogenesis.

Materials and methods

Chemicals and materials. All chemicals and materials were purchased from Sigma–Aldrich, Poole, United Kingdom unless otherwise indicated.

C2C12 cell culture. C2C12 myoblasts (Health Protection Agency Culture Collections, Salisbury, United Kingdom) were cultured in growth medium (Dulbecco's modified Eagle's medium (DMEM) with 4 mM glutamine and 10% vol/vol foetal calf serum). At ≈90% confluency, medium was changed to differentiation medium (DMEM, 4 mM glutamine and 2% vol/vol horse serum) to induce differentiation. Cells were maintained in differentiation medium for up to 96 h.

Protein extraction and Western blotting. Gastrocnemius, tibialis anterior and soleus muscles were excised from C57BL/6 mice and suspended in lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 1% vol/vol Triton X-100, pH 7.5) supplemented with protease inhibitor cocktail, 10 mM β-glycerophosphate, 50 mM NaF and 0.5 mM sodium orthovanadate. C2C12 cells were washed in ice-cold PBS and lysed in lysis buffer. Skeletal muscles and C2C12 lysates were homogenised on ice and then centrifuged at 13,000*g* for 10 min. The protein concentration was measured using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Northumberland, United Kingdom). The supernatant was then diluted in 3× Laemmli SDS buffer and heated for 5 min at 95 °C.

For Western blotting, protein extracts were separated on 10–12% SDS–PAGE gels and then transferred to PVDF membranes. Membranes were blocked with 5% vol/vol skimmed milk powder in Tris buffered saline supplemented with 0.1% Tween-20 and incubated overnight with primary antibodies, followed by HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (1:2000; Cell Signaling Technologies, Massachusetts, USA), before detection using enhanced chemiluminescence (ECL) detection reagent (Amersham Biosciences, Buckinghamshire, United Kingdom). Primary antibodies for the detection of phosphorylated Yap Ser127 (1:1000; #4911) and β-actin (1:1000; #4967) were from Cell Signaling Technologies and the antibody for total Yap (1:500; #sc-15407) was from Santa Cruz (California, USA).

RNA extraction, end point and real-time reverse transcriptase PCR. Total RNA was extracted from organs of a male, 4 month old C57BL/6 mouse or C2C12 cells using TRIzol according to the manufacturers protocol (Invitrogen, Paisley, UK) and purified using a RNeasy® minikit (Qiagen, Sussex, UK). Reverse transcription was performed using 2 μg of RNA with the Superscript II® reverse transcriptase kit (Invitrogen) and random primers (Invitrogen) according to the manufacturers recommendation.

End point PCR was performed over 35 cycles of denaturation (95 °C for 30 s), annealing (55–65 °C for 45 s) and extension (72 °C for 30 s), using oligonucleotides (Sigma–Aldrich) for Frmd6, Nf2, ww45, Mst1, Mst2, Mob1, Lats1, Lats2 and Yap (primer sequences are listed in the Supplementary data in Table S1). Post PCR products were visualised using ethidium bromide electrophoresis. Quantitative RT-PCR was performed on a Roche Lightcycler 480 (Roche, Hertfordshire, UK). PCR primers (Sigma–Aldrich) and

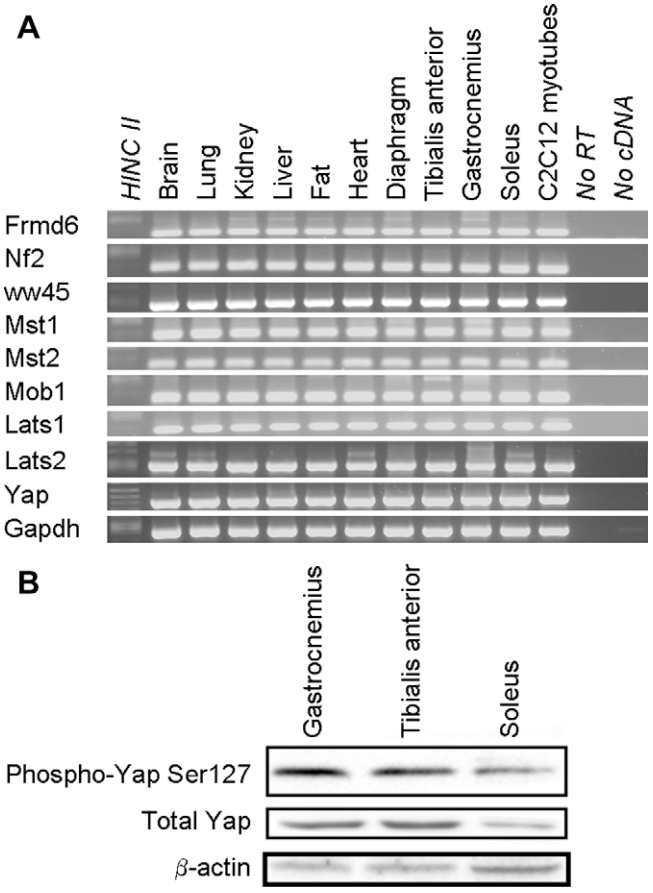

Fig. 1. Core components of the mammalian Hippo pathway including Yap are ubiquitously expressed in a range of mouse tissues including skeletal muscle. (A) mRNA expression using RT-PCR of mammalian Hippo pathway members in a range of mouse tissues from a 4 month old C57BL/6 male mouse and in C2C12 myotubes. Firm domain containing protein 6 (Frmd6) and neurofibromatosis 2 (Nf2) are thought represent the mammalian homologues of Merlin and Expanded, two upstream Hippo pathway proteins identified in *Drosophila melanogaster*. ww45 and Mob1 are proteins that have been proposed to interact with the Mst1/2 and Lats1/2 kinases in mammals. The primers used to amplify Yap amplify all isoforms because they do not not flank the WW domains of Yap. (B) Yap and Yap that is phosphorylated at Ser127 protein compared to β-actin in three different mouse skeletal muscles. Image is representative of two further independent replicates.

Taqman fluorogenic probes were designed for hYAP and mouse p21, CyclinD1, Myf5, myogenin, Mef2c, dysferlin, dystrophin and muscle creatine kinase (primer sequences are listed in the Supplementary data in Table S2) using the Roche Universal Probe Library. Following an initial denaturation step of 95 °C for 5 min, quantitative RT-PCR was performed over 45 cycles of 95 °C for 10 s and 60 °C for 30 s. All quantification of mRNA was normalised to mouse Gapdh (Applied Biosystems, California, USA) as an internal control using multiplexing assays. Quantification was corrected for efficiency by use of a standard curve created by the serial dilution of cDNA.

Transfection of C2C12 cells with YAP plasmids. DNA plasmids that encode wildtype human YAP1 (hYAP1) or a mutant protein that can no longer be phosphorylated at Ser127 (hYAP1 S127A) were obtained from Addgene (www.addgene.org) deposited by the lab of Dr. Marius Sudol (Geisinger Clinic, USA). The hYAP1 isoform used contains one WW domain. C2C12 myoblasts were seeded at 150,000 per well of a six well plate and transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Empty vector plasmids were used as negative controls. Upon confluency cells were differentiated as described for up to 96 h.

IdU incorporation assay. After 48 h in differentiation medium C2C12 cells were incubated with 20 μM of IdU (Iododeoxyuridine) for 2 h and fixed with 4% paraformaldehyde. A mouse monoclonal anti IdU antibody (ab8955, 1:100, Abcam, Cambridge, UK) diluted in 0.15 M NaCl containing DNAse (Sigma–Aldrich) was used to detect IdU incorporation. IdU positive nuclei were quantified by counting a minimum of 1000 nuclei using ImageJ software (www.rsbweb.nih.gov/ij/) from nine images per treatment. IdU positive nuclei have been calculated as a percentage of all nuclei.

Immunocytochemistry of C2C12 cells. C2C12 myoblasts were seeded at 60,000 cells per well of a 24 well plate and differentiated for up to 96 h as described above. Cells were fixed with 4% paraformaldehyde (PFA) in PBS at the indicated time points. Autofluorescence was quenched with PBS containing 50 mM NH_4Cl and permeabilised with 0.2% Triton X-100 in PBS. Cells were blocked for 30 min with blocking reagent (1% BSA in PBS containing 0.2% Triton X-100) and incubated with antibody against total YAP (1:25; #4912 Cell Signaling Technologies) or total myosin heavy chain (myosin heavy chain) (1:5, #18-0105, Invitrogen) overnight at 4 °C. Goat anti-rabbit IgG antibody (Calbiotech, California, USA) was used as a negative control. Cells were incubated with either goat anti-mouse 488 or goat anti-rabbit 594 Alex-Fluor conjugated secondary antibodies (both 1:200; Invitrogen) for 45 min and counterstained with DAPI (Invitrogen) prior to visualisation.

Statistical analysis. Data are presented as mean ± SD and were analysed using Prism version 5.02 for windows. Data in Figs. 4 and S3 were analysed using a two factorial ANOVA (factors were

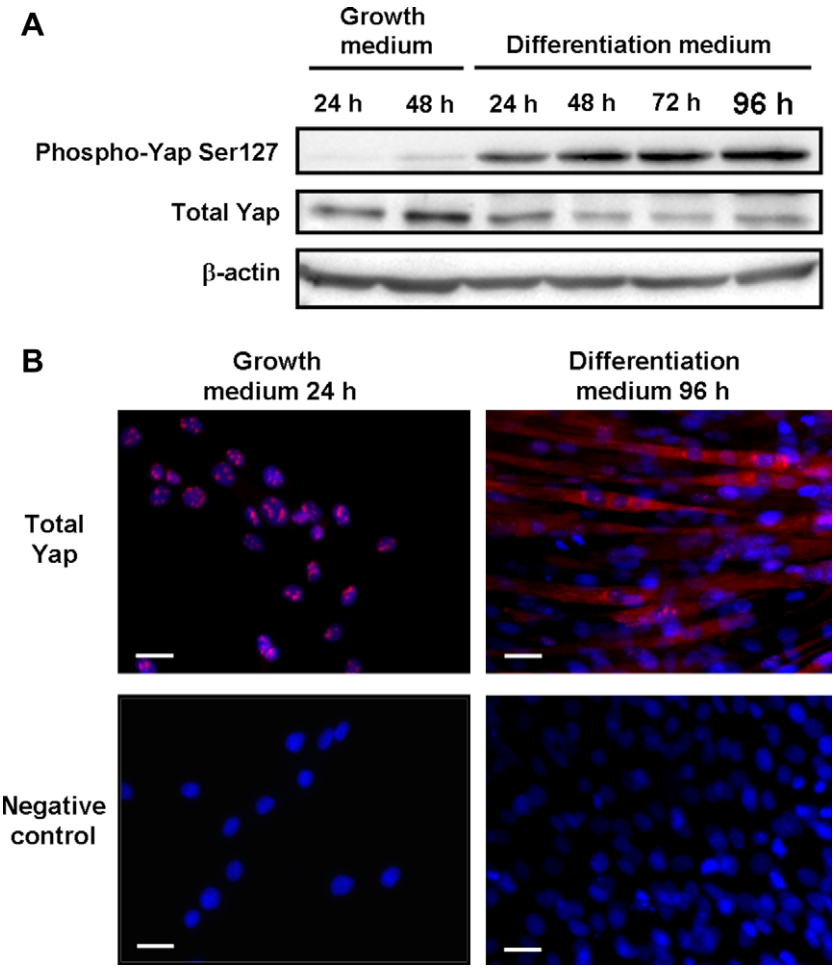

Fig. 2. Yap Ser127 phosphorylation increases during C2C12 myogenesis and translocates from the nucleus to the cytoplasm. (A) Levels of Yap phosphorylated at Ser127, total Yap and β-actin throughout C2C12 myogenesis. Image is representative of two further independent replicates; the statistical analysis and quantification is shown in Fig. S1b. (B) Localisation of total Yap (red) in subconfluent C2C12 myoblasts (upper left image) cultured in growth medium for 24 h and in C2C12 myotubes (upper right image) after 96 h of differentiation. Isotype negative controls for each time point are also shown (lower images). Nuclei counterstained with DAPI (blue). White lines represent 20 μm. (For interpretation of colour mentioned in this figure, the reader is referred to the web version of this article.)

treatment and time) and a Bonferroni *post hoc* analysis. Quantified results of the data presented in Figs. 3D and S1a, 2 in the Supplementary data were analysed using a one factorial ANOVA and a Bonferroni *post hoc* analysis. $p < 0.05$ was considered significant for all analyses.

Results

We first tested whether core members of the Hippo pathway are expressed in C2C12 myoblasts, myotubes and mouse skeletal muscles. Fig. 1A shows that core components of the mammalian Hippo pathway are ubiquitously expressed in all tissues studied at the mRNA level. Figs. 1B and 2A additionally show that the transcriptional co-factor Yap, a core member of the Hippo pathway, and Yap phosphorylated at Ser127 are detectable in mouse skeletal muscles, C2C12 myoblasts and myotubes as proteins.

After determining the expression of Hippo pathway members in skeletal muscle we examined the regulation and function of Yap. We first monitored the localisation and phosphorylation of Yap at Ser127 during myogenesis for which we used the C2C12 mouse myoblast cell line as an *in vitro* model (Fig. 2). During C2C12 myogenesis, myoblasts first proliferate to confluency, withdraw from the cell cycle and then differentiate into myotubes upon a shift to differentiation medium containing lower concentrations of mitogens. We found that Yap Ser127 phosphorylation is low in myoblasts but after 24 h in differentiation medium Ser127 phosphorylation is ≈20-fold increased and after 96 h it is ≈28-fold higher than in myoblasts, respectively (Fig. 2A, see also Fig. S1a in the Supplementary data for a quantification). Changes in the phosphorylation of Yap during differentiation are accompanied by the gradual translocation of Yap from distinct loci in the nucleus of myoblasts to the cytoplasm of myotubes (Fig. 2B; see Fig. S2b in the Supplementary data for additional images).

To test whether the observed increase of Yap Ser127 phosphorylation is required for C2C12 differentiation we overexpressed hYAP1 S127A in C2C12 myoblasts and induced differentiation by a change to differentiation medium. The hYAP1 S127A mutant can not be phosphorylated at residue Ser127 allowing us to increase the concentration of unphosphorylated Yap at a time when Ser127 phosphorylation of endogenous Yap would normally be increased ≈20-fold or more and when the concentration of unphosphorylated Yap would consequently be decreased. Fig. 3 shows that cells transfected with wildtype hYAP1 and empty vector differentiate as normal, forming multinucleated myotubes that are positive for myosin heavy chain. In contrast, hYAP1 S127A overexpressing cells do not form myotubes and are negative for myosin heavy chain (Fig. 3A and C). hYAP1 S127 overexpression also increases the percentage of IdU positive nuclei after 48 h of differentiation by ≈3-fold (15.1 ± 1.3%) compared to cells transfected with wildtype hYAP1 (5.2 ± 0.9%) and empty vector (5.3 ± 0.6%; Fig. 3D; $p < 0.05$). IdU incorporation into nuclei marks cells that have progressed through the S phase of the cell cycle suggesting that hYAP1 S127A overexpression increases the proportion of cells within the cell cycle. Furthermore, the expression of the terminal differentiation markers dysferlin, muscle creatine kinase, and dystrophin was significantly lower by 79 ± 4.0%, 52 ± 4.2% and 96 ± 1.6% in cells that were transfected with hYAP1 S127A compared to empty vector transfected cells, respectively (see Fig. S2 for the full data set, Supplementary data; these results were obtained using quantitative RT-PCR). These findings suggest that phosphorylation of Yap at Ser127 is essential for cell cycle withdrawal, the expression of late differentiation markers and differentiation of C2C12 myoblasts into myotubes.

We next aimed to identify regulatory genes whose expression is directly or indirectly dependent on Yap phosphorylation. For this we analysed the expression of myogenic transcription factors Myf5, myogenin and Mef2c and the cell cycle regulators p21 and

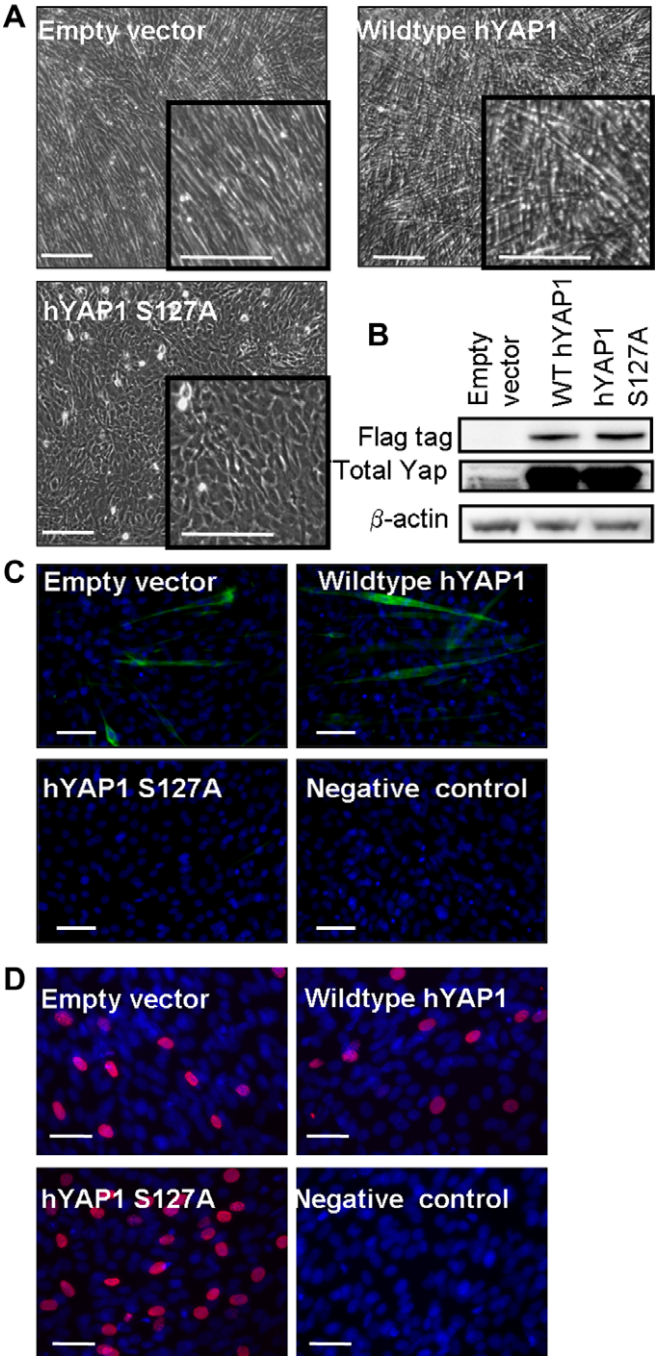

Fig. 3. Overexpression of hYAP1 S127A impairs C2C12 differentiation. (A) Phase contrast images of cells that were transfected either with empty vector, wildtype hYAP1 or hYAP1 S127A and differentiated for 96 h. Images were taken at 200 times magnification, inserts show magnified detail (white bars are proportionally enlarged). Images representative of two further independent replicates. (B) Levels of Flag, total Yap and β-actin in the cells transfected with empty vector, wildtype hYAP1 or hYAP1 S127A and differentiated for 96 h. (C) Total myosin heavy chain (green) with nuclear DAPI counterstain (blue) in cells transfected with empty vector, wildtype hYAP1 or hYAP1 S127A and differentiated for 96 h. Isotype negative controls for each condition are also shown. White lines represent 100 μm. (D) Representative images for cells that were either in or progressed through the S phase of the cell cycle as indicated by IdU labelling (red) after 48 h in differentiation medium. Nuclei were counterstained with DAPI (blue). Isotype negative controls for each condition are also shown. White lines represent 100 μm. (For interpretation of colour mentioned in this figure, the reader is referred to the web version of this article.)

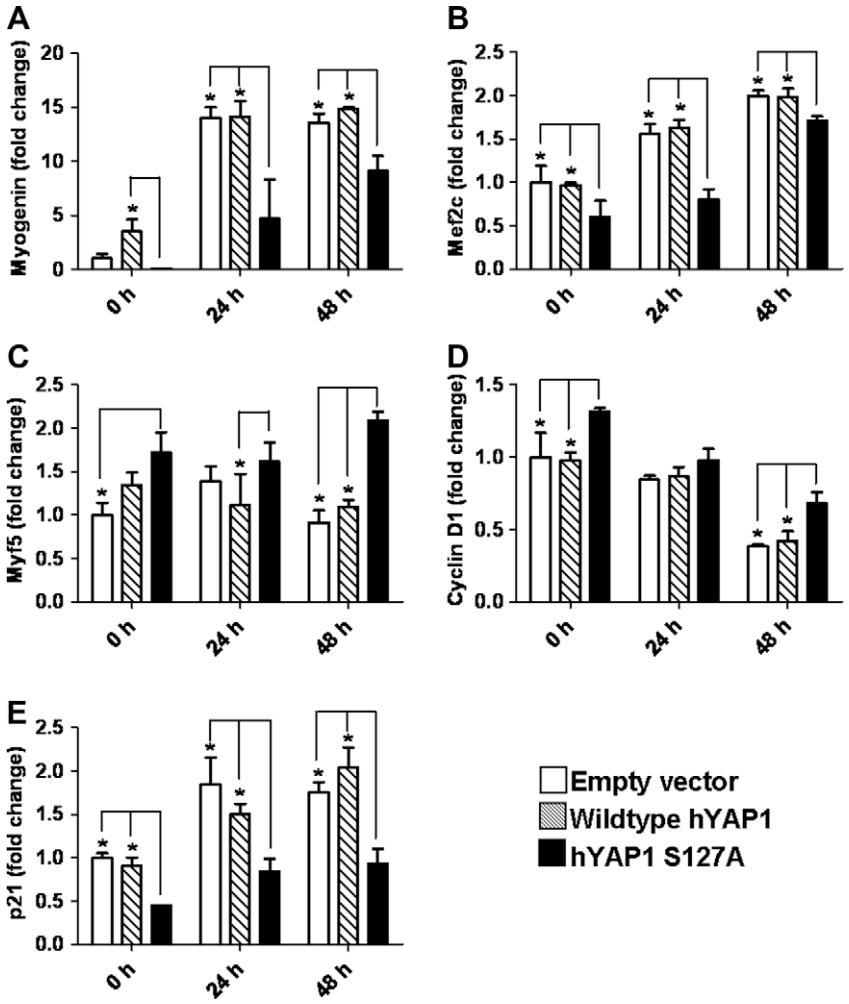

Fig. 4. hYAP1 S127A changes the expression of the MRFs, MEFs and cell cycle regulators. (A) Myogenin expression; (B) Mef2c expression; (C) Myf5 expression; (D) Cyclin D1 expression; p21 expression (E). Data normalised to the value for the empty vector transfected cells at 0 h and are expressed as a fold change relative to this. These results were obtained using quantitative RT-PCR. * Significantly different from hYAP1 S127A transfected cells ($p < 0.05$).

cyclinD1 during 0–48 h of differentiation in C2C12 myoblasts transfected with hYAP1 S127A, wildtype hYAP and empty vector (Fig. 4; see Fig. S3 in the Supplementary data for the levels of hYAP construct expression). We found that the expression of Myf5 and cyclinD1 remained significantly elevated upon induction of differentiation in cells that were overexpressing hYAP1 S127A compared to cells transfected with wildtype hYAP and empty vector (Fig. 4C and D). Furthermore, the expression of key myogenic differentiation transcription factors myogenin, Mef2c and the cell cycle inhibitor p21 were significantly decreased at all time points in cells transfected with hYAP S127A when compared to controls (Fig. 4A, B and E; note that there was no significant difference for myogenin between empty vector and hYAP1 S127A at 0 h).

Discussion

The organ-specific expression of members of the mammalian Hippo pathway has been previously demonstrated for individual genes such as Yap [17,18]. The data presented here (Fig. 1) extends these findings and provides a more detailed overview of the mRNA expression of the core mammalian Hippo pathway genes. All the major genes of the Hippo pathway are expressed in adult skeletal muscles with both a faster (tibialis anterior, gastronemius) and slower (soleus, diaphragm) phenotype as well as in C2C12 myoblasts and myotubes (Figs. 1 and 2A). The expression of the Hippo pathway in skeletal muscle suggests that the pathway may have a function in this tissue. As Yap has previously been reported to promote proliferation and inhibit differentiation of neural [16] and intestine progenitors [15] we decided to study Yap during C2C12 myogenesis in order to test whether it had a similar function in skeletal muscle.

At the onset of C2C12 differentiation Yap Ser127 phosphorylation increased ≈20-fold and Yap increasingly translocates from the nucleus to the cytosol (Fig. 2). The likely mechanism is that the Yap Ser127 residue is phosphorylated by Lats1/2 [10,14,19] and that Ser127 phosphorylated Yap is then sequestered by 14-3-3 binding proteins in the cytosol [11–13]. Our data suggest that Yap Ser127 phosphorylation is an essential differentiation event because the overexpression of a hYAP1 S127A mutant, which can not be phosphorylated at residue 127, prevents myotube formation and the expression of late differentiation markers (Fig. 3 and Fig. S2 in Supplementary data). The effect of overexpressing hYAP1 S127A is similar in C2C12 cells than in neural [16] and intestine progenitor cells [15] because hYAP1 S127A prevents differentiation in all three cell types. hYAP1 S127A overexpression also increases the percentage of proliferating myoblasts (Fig. 3D) which is again similar to the effect observed in both neural and intestine progenitors [15,16]. These data suggest that unphosphorylated Yap promotes the proliferation but inhibits the differentiation of progenitor cells in several tissues.

Yap functions as a transcriptional co-activator and has been reported to bind to at least ten transcription factors [11]. Thus the observed Yap translocation to the cytosol (Fig. 2B) implies that Yap can at that stage no longer co-regulate nuclear transcription factors and the genes that depend on such regulation. To identify genes via which hYAP1 S127A overexpression might directly or indirectly prevent cell cycle withdrawal and differentiation, we measured the expression of known MRF and cell cycle regulators in hYAP1 S127A, wildtype hYAP1 or empty vector transfected cells from 0 to 48 h after the onset of differentiation. This time window represents a critical period where key cell cycle and myogenic regulators change their expression during C2C12 differentiation [20].

We found that hYAP1 S127A overexpression significantly reduced the expression of myogenin and Mef2c compared to controls (Fig. 4A and B). This finding is consistent with an inhibition of differentiation because an upregulation of these genes is required for the differentiation of both C2C12 cells [4] and skeletal muscle in mice [3,21]. In contrast, hYAP1 S127A overexpression significantly increased Myf5 expression especially at 48 h (Fig. 4C). Myf5 is regulated in a cell cycle specific fashion in C2C12 myoblasts [22] and has been shown to promote myoblast proliferation [23]. Thus the increase of Myf5 is consistent with the hypothesis that hYAP1 S127A prevents cell cycle withdrawal. The elevated expression of cyclin D1 and decreased expression of p21 in hYAP1 S127A overexpressing cells (Fig. 4D and E) is equally consistent with the hypothesis that hYAP1 S127A prevents cell cycle withdrawal because cyclin D1 promotes G1-S phase cell cycle progression, whereas p21 promotes cell cycle withdrawal [24]. Cyclin D1 expression is also dependent on Yap in neural and intestine progenitors [15,16], showing that Yap can regulate the same gene in different tissues. Taken together, these data suggest that the ≈20-fold increase in Yap Ser127 phosphorylation at the onset of differentiation affects the expression of MRF and cell cycle regulators in a way that allows differentiation to proceed. In contrast, if hYAP1 S127A is overexpressed cell cycle withdrawal and differentiation are inhibited.

To summarise, all of the identified members of the core mammalian Hippo pathway are expressed in skeletal muscle *in vitro* and *in vivo*. Yap is unphosphorylated and localises to the nucleus in C2C12 myoblasts. At the onset of differentiation Yap becomes phosphorylated at Ser127 and translocates from the nucleus to the cytoplasm. Ser127 phosphorylation is required for terminal differentiation of C2C12 cells because if the concentration of Yap that can not be phosphorylated at Ser127 is increased by overexpressing a hYAP1 S127A mutant then myoblasts withdraw less from the cell cycle and do not differentiate into myotubes (see Fig. S4 for a schematic drawing). We therefore identify Yap as a novel regulator of C2C12 myogenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.034.

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