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The Hippo pathway member Yap plays a key role in influencing fate decisions in muscle satellite cells

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Summary

Satellite cells are the resident stem cells of skeletal muscle. Mitotically quiescent in mature muscle, they can be activated to proliferate and generate myoblasts to supply further myonuclei to hypertrophying or regenerating muscle fibres, or self-renew to maintain the resident stem cell pool. Here, we identify the transcriptional co-factor Yap as a novel regulator of satellite cell fate decisions. Yap expression increases during satellite cell activation and Yap remains highly expressed until after the differentiation versus self-renewal decision is made. Constitutive expression of Yap maintains Pax7⁺ and MyoD⁺ satellite cells and satellite cell-derived myoblasts, promotes proliferation but prevents differentiation. In contrast, Yap knockdown reduces the proliferation of satellite cell-derived myoblasts by ≈40%. Consistent with the cellular phenotype, microarrays show that Yap increases expression of genes associated with Yap inhibition, the cell cycle, ribosome biogenesis and that it represses several genes associated with angiotensin signalling. We also identify known regulators of satellite cell function such as BMP4, CD34 and Myf6 (Mrf4) as genes whose expression is dependent on Yap activity. Finally, we confirm in myoblasts that Yap binds to Tead transcription factors and co-activates MCAT elements which are enriched in the proximal promoters of Yap-responsive genes.

Key words: Satellite cells, Skeletal muscle, Yes-associated protein (Yap), Hippo pathway

Introduction

Yes-associated protein (Yap) is a transcriptional co-factor within the Hippo signal transduction pathway. Yap is phosphorylated at Ser127 and other residues by its upstream kinase Lats1/2 which in turn is phosphorylated and regulated by Mst1/2 and auxiliary proteins (Pan, 2010; Sudol and Harvey, 2010; Zhao et al., 2011). Yap can also be regulated independently of the Hippo pathway by α -catenin (Schlegelmilch et al., 2011), β -catenin (Heallen et al., 2011) or angiomotin (Chan et al., 2011; Oka et al., 2012). Yap, its paralogue Taz (transcriptional coactivator with PDZ-binding motif) and Vgll1-4 (vestigial-like, Vito, Tondue) all bind and co-activate Tead1-4 (TEA/ATTS domain/TEF/scalloped) transcription factors by forming protein complexes via specific Tead-co-factor binding domains (Halder et al., 1998; Mahoney et al., 2005; Mielcarek et al., 2002; Pobbati et al., 2012; Vassilev et al., 2001; Maeda et al., 2002). Co-activated Teads then regulate gene expression by binding via their ≈70 amino acid TEA/ATTS DNA-binding domain to so-called MCAT elements (muscle C, A and T; 5'-CATTC-3'). MCAT elements are located in the promoter or enhancer regions of key genes that either regulate early myogenesis (e.g. MyoD, Myf5, Mrf4), proliferation (e.g. cyclin D1), terminal differentiation (myogenin) or are expressed in terminally differentiated skeletal, cardiac or

smooth muscle (Benhaddou et al., 2012; Mizuno et al., 2012; Ribas et al., 2011; Yoshida, 2008). Whilst Yap is capable of co-activating a host of other transcription factors (Saucedo and Edgar, 2007), Yap and Tead mostly occupy the same promoters. A chip-on-chip analysis in MCF10A mammary epithelial cells revealed that Yap and Tead1 occupy the same promoters in <80% of the cases (Zhao et al., 2008) suggesting that Yap co-activation of Tead isoforms is the main mechanism by which Yap regulates gene expression.

The elements and function of the Hippo pathway were first identified during genetic screens for tumour suppressors in *Drosophila melanogaster*, where knockout of the inhibitory components upstream of the Yap homologue Yorkie resulted in overgrowth phenotypes (Harvey and Tapon, 2007). In 2007 two publications implicated the Hippo pathway in mammalian organ size control by overexpressing constitutively active hYAP1 S127A in the liver which triggered a ≈4-fold liver hypertrophy (Camargo et al., 2007; Dong et al., 2007). In addition to organ growth, Yap also regulates the identity and cell fate of several types of adult stem and/or progenitor cells. These include intestinal progenitors (Camargo et al., 2007), hepatic oval cells (Lee et al., 2010), neural stem cells (Cao et al., 2008) and epidermal stem cells (Schlegelmilch et al., 2011). In these stem

and progenitor cells Yap is generally required for their identity or 'stemness' (Camargo et al., 2007; Ramalho-Santos et al., 2002), promoting proliferation but inhibiting differentiation.

We have recently demonstrated that Yap and other members of the Hippo pathway are expressed in skeletal muscle and that Yap promotes proliferation but inhibits differentiation of C2C12 myoblasts (Watt et al., 2010). C2C12 myoblasts are an immortalised cell line, can fuse to form myotubes and are likely derived from muscle satellite cells, which are the resident stem cells of adult skeletal muscle (Mauro, 1961; Collins et al., 2005). The regulation of satellite cell fate can be studied in satellite cells cultured in their niche *ex vivo* (Collins and Zammit, 2009; Zammit et al., 2004). Quiescent satellite cells express the paired-box transcription factor Pax7 (Pax7⁺/MyoD⁻). When satellite cells become activated and re-enter the cell cycle, MyoD is expressed (Pax7⁺/MyoD⁺) in culture *ex vivo* or in response to injury and hypertrophic stimuli *in vivo* (Scharner and Zammit, 2011; Relaix and Zammit, 2012). MyoD binds to thousands of genes and induces chromatin modifications which presumably open up the chromatin for sequence-specific transcription factors (Cao et al., 2010). Activated satellite cells then proliferate and either differentiate (Pax7⁻/myogenin⁺) or self-renew and return to quiescence (Pax7⁺/MyoD⁻). Satellite cells are required for postnatal growth and muscle repair after injury (Lepper et al., 2011) but muscle hypertrophy can occur short term in muscle that is to more than 90% depleted of Pax7⁺ satellite cells (McCarthy et al., 2011). The proliferative and regenerative capacity of satellite cells is enormous: it has been estimated that one transplanted satellite cell (termed muscle stem cell in that paper due to the FACS-isolation method used) can differentiate and give rise to an estimated 20,000–80,000 progeny during repeated injury-regeneration cycles (Sacco et al., 2008). Also a purified, transplanted population of FACS-isolated skeletal muscle precursors (termed SMPs, which are likely largely composed of satellite cells) contributed with high efficiency to muscle fibres of dystrophin-deficient mdx mice (Cerletti et al., 2008).

Previous studies have shown that the activation, proliferation, differentiation and self-renewal of satellite cells is regulated by several signal transduction pathways including the Notch, Wnt and BMP pathways (Ono et al., 2011; Otto et al., 2008; Mourikis et al., 2012). Here, we demonstrate for the first time that the Hippo pathway member Yap plays a key role in satellite cell proliferation and fate. We show that Yap expression increases greatly during satellite cell activation and Yap remains elevated until after activated satellite cells either differentiate or self-renew. We report that constitutive Yap activity expands the pool of activated, Pax7 and MyoD-positive satellite cells and satellite cell-derived myoblasts but prevents their differentiation. Consistent with these observations, microarrays identify regulators of the cell cycle, ribosomal biogenesis and modulators of myogenic differentiation as genes that are targeted by Yap. Exploring the molecular mechanism by which Yap works, we found that Yap can bind Tead transcription factors and co-activate MCAT-elements in myoblasts.

Results

Yap is highly expressed in activated satellite cells

We first investigated the expression of Yap during myogenic cell fate progression of satellite cells using immunocytochemistry. To this end we cultured satellite cells *ex vivo* in their niche on muscle fibres isolated from mouse (*Mus musculus*) extensor digitorum

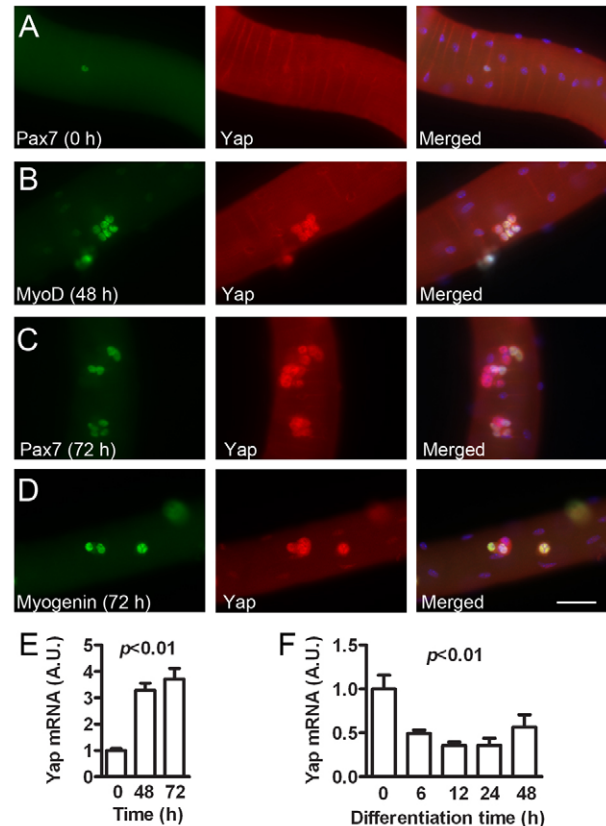


Fig. 1. Yap is highly expressed in activated satellite cells until after the differentiation versus self-renewal decision is made. (A–D) Sets of three example images in one row. (A) Low Yap expression in a Pax7⁺ satellite cell at 0 h of *ex vivo* culture. (B) Yap is highly expressed in all activated, MyoD⁺ satellite cells at 48 h. (C) Yap is highly expressed in satellite cells that still express Pax7 after 72 h and some adjacent cells. (D) Yap is also highly expressed in myogenin⁺, differentiating cells and again in some adjacent cells after 72 h. (E) Yap mRNA in satellite cells trypsin-digested from *ex vivo* cultured fibres increases significantly ≈ 2.6 -fold from 0 h to 48 h and is still highly expressed at 72 h (ANOVA, $P < 0.01$; $n = 3$ each; for MyoD expression, see supplementary material Fig. S1). (F) Yap mRNA decreases significantly by ≈ 2.3 – 3.2 -fold in plated satellite cell-derived myoblasts during differentiation (ANOVA, $P < 0.01$; $n = 4$ each apart from 48 h which is $n = 3$; mean \pm s.d.). Time points 6–48 h are all significantly different from 0 h (for Myf5 and myogenin expression, see supplementary material Fig. S1). Scale bar: 50 μ m.

longus muscle (Collins and Zammit, 2009; Zammit et al., 2004). In quiescent satellite cells (Pax7-positive cells at 0 h) the Yap signal was low and comparable to that seen in the adjacent fibre (Fig. 1A). During satellite cell activation, Yap protein increased and Yap was robustly expressed in activated, MyoD⁺ satellite cells (24 h and 48 h) (Fig. 1B). It remained highly expressed in clusters of satellite cells at 72 h, both in differentiating, myogenin-positive cells and also in Pax7-positive cells. The continued expression of Pax7 at 72 h in satellite cell progeny suggests that these cells are entering the self-renewal pathway (Zammit et al., 2004) (Fig. 1C,D).

To quantify Yap gene expression during satellite cell activation we removed satellite cells by trypsin digestion from *ex vivo* cultured muscle fibres at 0 h, 48 h and 72 h and measured Yap mRNA using quantitative RT-PCR. We found that Yap mRNA increased significantly ≈ 2.6 -fold from 0 h to 48 h to 72 h (Fig. 1E; supplementary material Fig. S1). The low level of

Yap in quiescent satellite cells and in muscle fibres implies that Yap is downregulated in both differentiating and self-renewing satellite cells. To obtain further evidence for the downregulation of Yap during differentiation we measured Yap mRNA in plated satellite cell-derived myoblasts that were triggered to differentiate by culture in mitogen-low differentiation medium. We observed a significant, ≈ 2.3 – 3.2 -fold decrease of Yap mRNA from 6 h to 48 h after differentiation was induced (Fig. 1F) and a significant, ≈ 1.6 – 1.9 -fold decrease of Yap protein (supplementary material Fig. S1).

Previous studies have demonstrated that Yap is controlled in part through phosphorylation at Ser127 which decreases its activity. In differentiating satellite cell-derived myoblasts, Yap Ser127 phosphorylation relative to total Yap increased significantly 2.1–2.5-fold after 24 h and 48 h of differentiation when compared to proliferating myoblasts, respectively (supplementary material Fig. S1). An increase of Yap Ser127 phosphorylation also occurs in differentiating C2C12 myoblasts although the fold-change of phosphorylation is lower in differentiating satellite cell-derived myoblasts (Watt et al., 2010). To summarise, Yap mRNA and protein increases during satellite cell activation and Yap remains elevated until activated cells differentiate or self-renew. During differentiation Yap mRNA and protein decreases whilst Yap Ser127 phosphorylation increases which together act to reduce the activity of Yap.

Yap expression and activity promotes proliferation of satellite cells and satellite cell-derived myoblasts

Yap has been previously shown to regulate the proliferation of several types of adult stem cells. To investigate whether Yap has a comparable function in satellite cells we carried out both gain- and

loss-of-function experiments. Yap was constitutively expressed in both plated satellite cell-derived myoblasts and muscle fibre-associated satellite cells via retroviral mediated delivery. For this wild-type hYAP1 and constitutively active hYAP1 S127A were cloned into modified pMSCV, where an IRES-eGFP marks infected cells. This allowed Yap dose-response analyses as hYap1 S127A increases the luciferase activity of an MCAT element-driven luciferase reporter more than wild-type hYAP1 which in turn increases luciferase activity more than the empty vector (Fig. 7A) which served as a negative control (Fig. 2A). Proliferation was assessed by ethynyldeoxyuridine (EdU) or iododeoxyuridine (IdU) incorporation and scored as the percentage of GFP-positive nuclei that additionally contained EdU or IdU, respectively. We found that constitutive expression of hYAP1 S127A but not wild-type hYAP1 significantly increased the proliferation of satellite cells cultured *ex vivo* by 75% when compared to the empty vector control (Fig. 2B,C,F). In plated satellite cell-derived myoblasts overexpression of wild-type hYAP1 significantly increased proliferation by 53% and overexpression of hYAP1 S127A increased proliferation by 79% against empty vector control, respectively (Fig. 2D,E,F). Enhanced proliferation signalling was supported by the finding that the concentration of the proliferation marker PCNA was higher in hYAP1 and hYAP1 S127A expressing cells than in the empty vector control (Fig. 2A).

Decreased Yap expression inhibits proliferation of satellite cell-derived myoblasts

To test whether Yap is required for normal proliferation we infected plated satellite cell-derived myoblasts with a lentivirus delivering either anti-Yap shRNA or scrambled shRNA as a

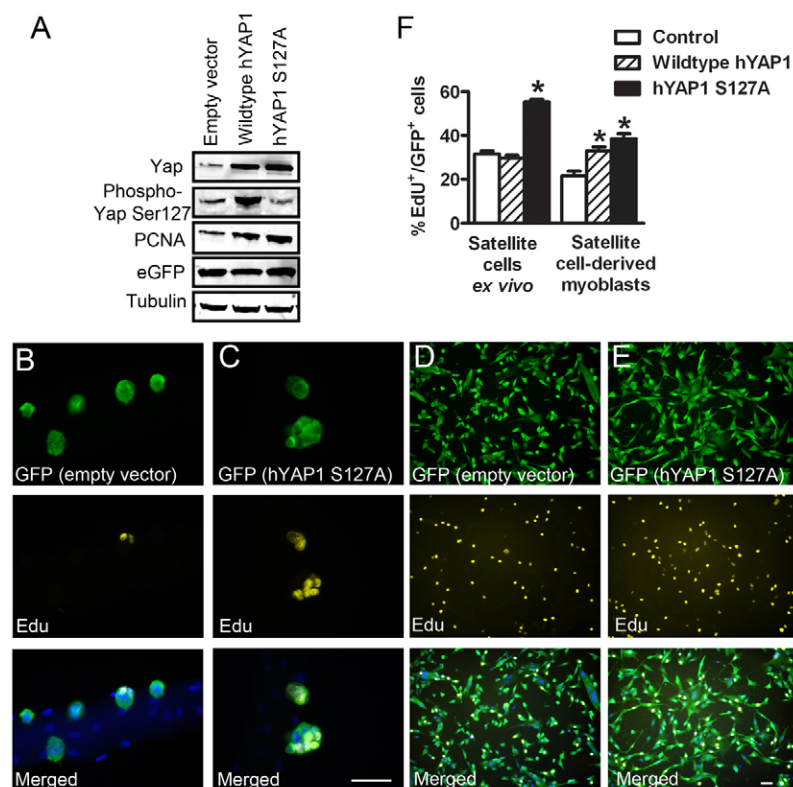


Fig. 2. Effect of retrovirus-mediated constitutive expression of wild-type hYAP1 or constitutively active hYAP1 S127A on the proliferation of satellite cells. (A) Western blot of Yap following retroviral delivery of hYAP1 and hYAP1 S127A compared to empty vector negative control. (B–E) show sets of representative images of EdU incorporation in muscle fibre-associated satellite cells (B,C) and plated satellite cell-derived myoblasts (D,E) following constitutive expression of hYAP1 S127A compared to control. (F) Quantification of the data ($n=3$ for satellite cells cultured *ex vivo*, ANOVA: $P=0.01$; $n=5$ for satellite cell-derived myoblasts; ANOVA: $P<0.01$; mean \pm s.d.). Scale bars both 50 μ m.

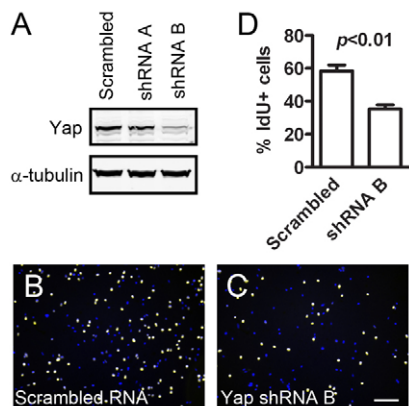


Fig. 3. Effect of Yap knockdown by lentiviral shRNA infection on the proliferation of satellite cell-derived myoblasts. (A) C2C12 myoblasts were infected with two anti-Yap shRNAs (shRNA A, shRNA B) and Yap protein was measured. shRNA B was chosen for subsequent experiments as it knocked down Yap protein by 72% in C2C12 myoblasts. (B) Effect of either scrambled RNA or (C) anti-Yap shRNA on IdU⁺ nuclei in satellite cell-derived myoblasts. (D) Anti-Yap shRNA B treatment reduces proliferation by ≈40% (*t*-test; mean±s.d.). Scale bar: 50 μm.

control. Efficient knockdown was first validated by western blot in C2C12 myoblasts (Fig. 3A). Infection of satellite cell-derived myoblasts with anti-Yap shRNA significantly reduced proliferation by ≈40% when compared to control myoblasts infected with a scrambled shRNA control (Fig. 3C,D). Together with the gain of function data these results show that high Yap activity promotes satellite cell proliferation whereas lowering the normal concentration of Yap in satellite cell-derived myoblasts decreases proliferation. This suggests that the normal increase of the Yap concentration during satellite cell activation drives the proliferation of activated satellite cells.

High Yap expression inhibits terminal differentiation

To investigate the effect of high levels of Yap expression and activity on the fate of satellite cells we again overexpressed either wild-type hYAP1 or hYAP1 S127A together with eGFP in satellite cells cultured in their niche *ex vivo* using retroviral infection. We then quantified the expression of the satellite cell fate markers Pax7, MyoD and myogenin in all eGFP-positive cells. After 72 h in culture, overexpression of wild-type hYAP1 and hYAP1 S127A significantly increased the number of Pax7-positive cells by 82% and 111% (Fig. 4A,B,E) but reduced differentiating, myogenin-positive cells significantly by 35% and 52%, respectively, when compared to empty vector controls (Fig. 4C,D,E). The number of MyoD-positive cells was unaffected (Fig. 4E; supplementary material Fig. S2). Together with the results shown in Figs 2 and 3 this suggests indirectly that high Yap expression and activity expands the pool of activated (Pax⁺, MyoD⁺) satellite cells but impairs differentiation.

High Yap expression and activity impairs myogenic differentiation

To specifically determine the effect of high levels of Yap expression and activity on myogenic differentiation we constitutively expressed wild-type hYAP1 or hYAP1 S127A in plated satellite cell-derived myoblasts and again compared them to empty vector negative controls. We induced differentiation by shifting from a mitogen-rich to a mitogen-low medium. In this model, wild-type hYAP1 and hYAP1 S127A overexpression reduced myogenic fusion (i.e. the percentage of nuclei found in myosin heavy chain-positive cells) significantly by 18% and 50% when compared to empty vector, respectively (Fig. 5A–D). Moreover, hYAP1 and hYAP1 S127A overexpression reduced the expression of the late differentiation regulator myogenin significantly by 21% and 38% but increased the percentage of Pax7-positive cells significantly by 42% and 85%, respectively (Fig. 5A–D). This confirms that high Yap expression and activity

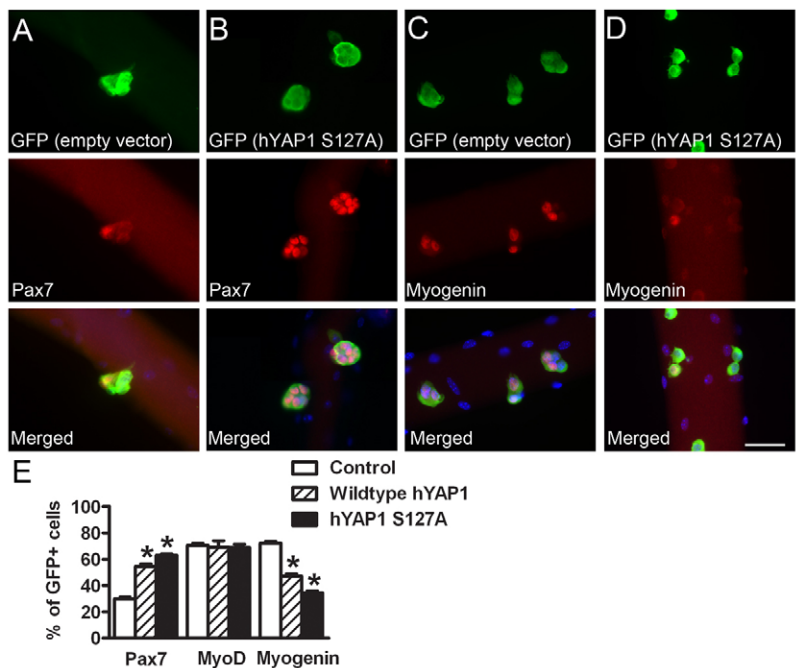


Fig. 4. Effect of retroviral overexpression of empty vector, wild-type hYAP1 (supplementary material Fig. S2) or constitutively active hYAP1 S127A on the expression of the cell fate markers Pax7, MyoD (supplementary material Fig. S2) and myogenin in satellite cells cultured in their niche *ex vivo*. (A–D) Sets of three images in a column. The images are examples for muscle fibres that were quantified. (E) Quantitative analysis. For Pax7 and myogenin, the differences between groups are significant (the fibres from *n*=3 muscles per group were quantified; ANOVA: *P*<0.01; mean±s.d.). Scale bar: 50 μm.

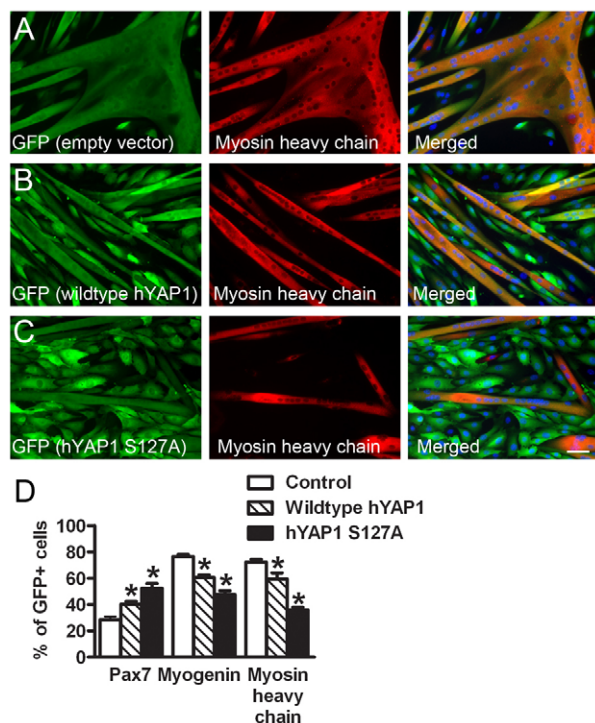


Fig. 5. Effect of retroviral overexpression of wild-type hYAP1 or constitutively active hYAP1 S127A on the expression of cell fate markers (Pax7 and MyoD) and markers of myogenic fusion (myosin heavy chain) in satellite cell-derived myoblasts cultured on Matrigel. (A–C) Sets of three images displayed in one row. The images show that increased levels of Yap activity (hYAP1 S127A > wild-type hYAP1 > empty vector) reduce the proportion of nuclei found in differentiated, myosin heavy chain-positive cells. Similar sets of example images for Pax7 and myogenin are shown in supplementary material Fig. S3. (D) Quantitative analysis. Increasing levels of Yap activity significantly increase Pax7 expression ($P < 0.01$), but decrease myogenin ($P < 0.01$) and myosin heavy chain expression (the fibres from $n = 3$ muscles per group were quantified; ANOVA: $P < 0.01$; mean \pm s.d.). Scale bar: 50 μ m.

prevents differentiation and indirectly suggests that it expands the pool of activated satellite cells.

Next we tested whether lentiviral shRNA knockdown of Yap affected myogenic differentiation. The data in Fig. 6 show that myogenic differentiation is not effected by a decreased concentration of Yap probably because Yap activity is already downregulated at this point so that a further reduction does not significantly affect differentiation.

Yap regulates the expression of genes associated with the cell cycle, ribosome biogenesis and terminal myogenic differentiation

In order to elucidate some of the mechanisms responsible for the phenotype induced by Yap we conducted an expression microarray analysis. Because high Yap activity inhibits terminal differentiation (Figs 4, 5) we chose short periods of hYAP1 S127A overexpression (20 h and 40 h of hYAP1 S127A induction by doxycycline) in order to identify especially those genes that are directly targeted by Yap and/or have a regulatory function rather than just comparing the transcriptome of differentiated myotubes versus myoblasts that have not differentiated because of high Yap activity. Myoblasts were

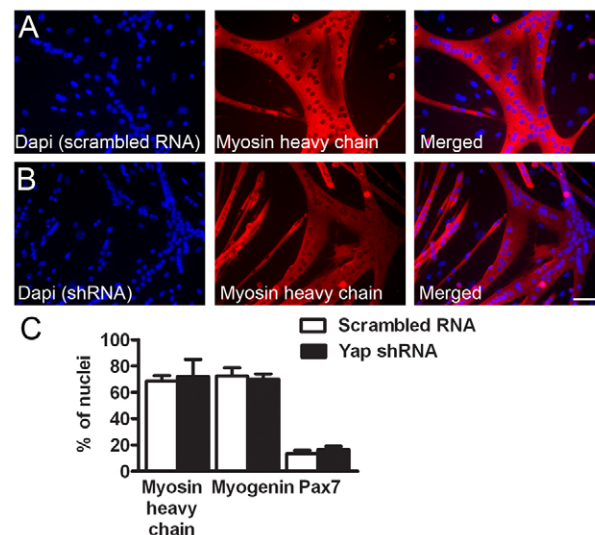


Fig. 6. No effect of lentivirus-mediated Yap knockdown on the differentiation of satellite cell-derived myoblasts. (A, B) Sets of three example images per row showing the effect of Yap knockdown by lentiviral delivered shRNA when compared to empty vector negative control. Similar sets of example images for Pax7 and myogenin are shown in supplementary material Fig. S4. (C) Quantification of the Yap knockdown experiment. The percentage of nuclei found in myosin heavy chain-positive myotubes or the percentage of nuclei that stained positive for myogenin and Pax7 was quantified (the fibres from $n = 3$ muscles per group were quantified; ANOVA: $P < 0.01$; mean \pm s.d.). Scale bar: 50 μ m.

obtained from Myf5-Cre Colla^{TetOP}-hYAP1 S127A/+ R26^{stop-RtTA/+} mice after purification by pre-plating. The overexpression of hYAP1 S127A was induced in Myf5-expressing myoblasts for 20 h or 40 h by doxycycline treatment and these myoblasts were compared to untreated myoblasts. Doxycycline treatment induced robust hYAP1 S127A overexpression (Fig. 7A). The numbers of genes that were upregulated or downregulated in response to 20 h, 40 h or both 20 h and 40 h doxycycline treatment are shown in Fig. 7B. The complete set of genes that responded to overexpression of hYAP1 S127A with a more than 1.3-fold change either up or down ($P < 0.05$) is shown in supplementary material Table S1.

To test in a non-biased way whether the genes upregulated by hYAP1 S127A overexpression were significantly associated with a particular GOBP (gene ontology biological process) we analysed our data set using Gene Set Enrichment Analyses (GSEA; see Materials and Methods). Consistent with the proliferation phenotype that is induced by high Yap activity, this analysis identified significant upregulation of genes that are linked to 17 gene ontology (GO) lists linked to the cell cycle (Fig. 7C; supplementary material Table S2). Additionally we found upregulation of genes linked to ribosomal biogenesis and rRNA metabolism and significant downregulation of genes linked to G-protein-linked signalling processes (Fig. 7C; supplementary material Table S2).

We also searched the list of genes that responded to hYAP1 S127A overexpression at both 20 h and 40 h by conducting a systematic Medline search using the additional search terms 'skeletal muscle', 'satellite cells' and 'myogenesis' as well as a Swissprot search for the gene name. We then compiled a selection of genes modulated by hYap1 S127A overexpression

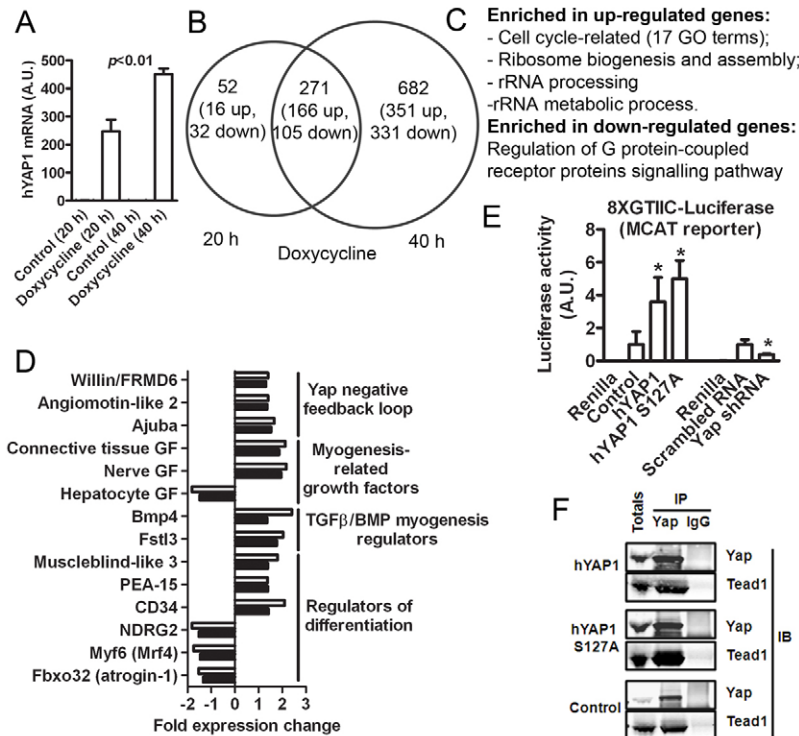


Fig. 7. Gene expression response to hYAP1 S127A overexpression in myoblasts, Yap binding to Tead1 and co-activation of MCAT elements by Yap. (A) Effect of 20 h and 40 h of doxycycline treatment on hYAP1 expression ($n=3$, ANOVA). (B) Venn diagram displaying the numbers of genes that changed their expression after 20 h, 40 h or both 20 h and 40 h of doxycycline treatment versus control (total; up, upregulated; down, downregulated). (C) Gene ontology (GO) terms enriched among 20 h and 40 h doxycycline-responsive genes that were either up- or downregulated. (D) Fold expression changes for individual genes after 20 h (black bars) and 40 h (white bars) of doxycycline treatment versus no doxycycline control. (E) Wild-type hYAP1 and constitutively active hYAP1 S127A increase the activity of an MCAT-driven luciferase promoter whilst Yap knockdown with shRNA (see Fig. 3 for the validation of the knockdown) reduces its activity in C2C12 myoblasts. (F) Yap binds to Tead1 in C2C12 myoblasts as demonstrated by immunoprecipitation. IP, immunoprecipitation; IB, immunoblot.

and whose known function might potentially explain important aspects of the observed pro-proliferation, anti-differentiation phenotype that is induced by high Yap activity (Fig. 7D).

Next we tested whether Yap could regulate gene expression by co-activating MCAT elements and by binding to Tead transcription factors in myoblasts as has been reported for other cell types. We found that hYAP1 and hYAP1 S127A overexpression significantly increased the activity of a luciferase reporter driven by 8 MCAT elements (8XGTTC-luciferase) by 3.6-fold and 5-fold, respectively. In contrast, knockdown of Yap by shRNA reduced the activity of the reporter by 61% (Fig. 7E). We also confirmed by immunoprecipitation that Yap can bind Tead1 in myoblasts (Fig. 7F). These experiments demonstrate that Yap can both activate MCAT elements and bind to Tead transcription factors in myoblasts. Because of this analysis and because Yap and Tead have been reported to bind in $\approx 80\%$ of the cases to the promoter of the same genes in MCF10A cells (Zhao et al., 2008) we tested for an overlap between genes upregulated by both 20 h and 40 h of doxycycline in our study and genes whose promoters were reported to be occupied by either Tead1 in adult skeletal muscle (Qiu et al., 2011) or by Tead4 in C2C12 myoblasts (Benhaddou et al., 2012) (supplementary material Table S3). These analyses reveal an overlap of 3 genes with the Tead1-occupied genes in adult skeletal muscle and of 29 genes with Tead4-occupied genes in C2C12 myoblasts. Because Yap is capable of co-activating MCAT elements in myoblasts (Fig. 7E), we also analysed the set of genes that responded to both 20 h and 40 h of doxycycline treatment for MCAT elements in the proximal promoter (-2 kb to $+2$ kb) relative to the transcription start site using the Transfac matrix for Tead1 (i.e. MCAT elements) (supplementary material Table S3). This analysis identified 13 genes (9.4%) with putative MCAT elements among

the 138 upregulated genes which represents a significant enrichment of MCAT elements ($P < 0.01$).

Discussion

Our data demonstrate for the first time an important function for the Hippo pathway member Yap in the regulation of satellite cell fate. Yap promotes the proliferation of activated satellite cells whilst inhibiting their differentiation. Consistent with the cellular phenotype, Yap regulates the expression of genes associated with the cell cycle, ribosome biogenesis, angiotensin signalling and myogenic differentiation.

Yap mRNA and protein increase during satellite cell activation and Yap remains highly expressed until after the differentiation versus self-renewal decision is made (Fig. 1). A similar decrease of Yap expression during differentiation has been reported for embryonal and neuronal stem cells (Lian et al., 2010; Zhang et al., 2012) suggesting that a decline of Yap expression during differentiation is not restricted to satellite cells. The increase of Yap during satellite cell activation and decline during differentiation is at least partially due to a decreased expression of Yap mRNA (Fig. 1E,F). In this study we have not tested whether some of the decline of Yap protein levels is due to Yap ubiquitination and degradation triggered by Ser381 phosphorylation (Zhao et al., 2010) as the Yap Ser381 antibody was not available to us.

Yap mRNA and protein not only declines during differentiation but the phosphorylation of the remaining Yap at Ser127 increases significantly at the onset of differentiation (supplementary material Fig. S1). As a consequence, Yap activity should be markedly decreased at the onset of differentiation as there is both less Yap protein and that there is less active due to increased, inhibitory Ser127 phosphorylation. In C2C12

myoblasts Ser127 phosphorylation increases <20-fold at the onset of differentiation but in contrast to satellite cell-derived myoblasts, the total concentration of Yap remains similar during differentiation (Watt et al., 2010) highlighting a difference between differentiating satellite cells and C2C12 myoblasts.

In activated satellite cells, high levels of Yap expression and activity promote proliferation (Fig. 2) whilst Yap knockdown reduces proliferation (Fig. 3). This is comparable to what we have previously reported for C2C12 myoblasts (Watt et al., 2010) and matches findings reported for other adult stem and progenitor cells such as intestinal stem cells (Camargo et al., 2007), hepatic oval cells (Lee et al., 2010), neural stem cells (Cao et al., 2008) and epidermal stem cells (Schlegelmilch et al., 2011). Thus Yap appears to be capable of generally expanding adult stem and progenitor cell populations. High Yap expression and activity also retains satellite cells in their activated state and prevents their differentiation (Figs 4, 5) as judged by the high expression of Pax7 and MyoD and decreased expression of myogenin in satellite cells where the activity of Yap has been increased by retroviral-mediated constitutive expression of hYAP1 S127A. A reduction of Yap protein by shRNA mediated knockdown at the onset of differentiation does not affect the progression of differentiation (Fig. 6) presumably because Yap expression and activity is already sufficiently downregulated. Thus, high Yap expression and activity expands the pool of activated satellite cells and prevents the differentiation of this cell population.

In order to identify the underlying molecular mechanisms, we conducted an expression microarray analysis in myoblasts where hYAP1 S127A expression was induced by 20 h and 40 h of doxycycline treatment versus untreated controls (Fig. 7A). We focussed our analyses on the 271 genes that were responsive to both 20 h and 40 h of doxycycline treatment. Additionally 52 and 682 genes responded only to either 20 h or 40 h of doxycycline treatment, respectively (Fig. 7B). We found that after 40 h the terminal differentiation markers Myh4 (myosin heavy chain IIb) and CKM (muscle creatine kinase) were significantly downregulated in the doxycycline treated myoblasts (supplementary material Table S1). This suggests that terminal differentiation had started after 40 h in the untreated myoblasts whilst hYAP1 S127A overexpression prevented this, resulting in a lower expression of terminal differentiation markers in the hYAP1 S127A overexpressing cells.

To determine whether the genes modulated by hYAP1 S127A overexpression were significantly enriched for a particular biological process, we performed GOBP (gene ontology biological process) and GSEA (gene set enrichment analysis) analyses for genes that responded to both 20 h and 40 h of doxycycline treatment. These analyses revealed significant upregulation of many cell cycle-related genes (Fig. 7C; supplementary material Table S2) which matches the pro-proliferation phenotype that is induced by high Yap activity (Figs 2, 3). The responsive cell cycle-related genes include cyclins, cyclin-related kinases, cell cycle checkpoint-regulating genes and genes that modulate other aspects of the cell cycle such as cytokinesis. There is emerging evidence that the promoters of at least some cell cycle-regulating genes are directly targeted by Yap (Mizuno et al., 2012). We also found an upregulation of genes associated with ribosome biogenesis and rRNA metabolism (supplementary material Table S2). This again matches the pro-proliferation phenotype induced by Yap because ribosome biogenesis is required for cell cycle

progression as ribosome stress will trigger cell-cycle arrest via a mechanism that involves p53 (Lempiäinen and Shore, 2009).

Interestingly, G-protein coupled receptor protein signalling genes were downregulated. This group includes ACE (angiotensin-converting enzyme), Bdkrb1 (bradykinin receptor, b1), RGS2/5 (regulator of G-protein signalling 2 and 5) and Gem (GTP binding protein; gene overexpressed in skeletal muscle) (supplementary material Table S2). These genes can be linked to the angiotensin pathway which has recently been associated with satellite cell function (Johnston et al., 2010). Recently it has been demonstrated that the Hippo pathway is regulated G-protein-coupled receptor signalling (Yu et al., 2012) and it is intriguing that high Yap activity transcriptionally inhibits several members of this pathway in myoblasts.

Next we compiled a list of candidate genes that were responsive to hYAP1 S127A overexpression and which could explain aspects of the phenotype induced by high Yap activity (Fig. 7D). Overexpression of hYAP1 S127A increased the expression of three Hippo signal transduction pathway genes at both 20 h and 40 h that have been reported to inhibit Yap activity. They are Willin/Frmd6 (Angus et al., 2012), angiomin-like 2 (amotl2) (Zhao et al., 2011) and ajuba (Das Thakur et al., 2010). After 40 h of doxycycline treatment another putative Yap inhibitor and Hippo pathway member, WWc2 [WW and C2 domain containing 2; kibra homologue (Yoshihama et al., 2012)], is additionally upregulated. The upregulation of these Yap inhibitors by high Yap activity is potentially important because Yap is an oncogene and this negative feedback loop might limit the intensity and duration of Yap signalling.

The next group of genes that is responsive to hYAP1 S127A overexpression are three growth factors that can be linked to satellite cells and/or myogenesis. Of these, connective tissue growth factor (CTGF) has been extensively characterised as a Yap/Tead-responsive gene by ChIP-Seq and Yap/Tead gain and loss-of-function experiments (Zhao et al., 2008). CTGF has three MCAT elements in the proximal promoter which was confirmed by our analysis (supplementary material Table S3). CTGF is highly expressed in dystrophic muscle (Sun et al., 2008) which contains a high proportion of activated and proliferating satellite cells (Pallafacchina et al., 2010). Thus the high expression of CTGF may be due to the high Yap activity in activated satellite cells (Fig. 1). Nerve growth factor (NGF) was shown to inhibit the differentiation of muscle-derived stem cells (Lavasani et al., 2006), which matches the function of Yap reported in this study. Hepatocyte growth factor (HGF) is downregulated by hYAP1 S127A overexpression. HGF was identified as a factor capable of activating satellite cells (Tatsumi et al., 1998) and thus the downregulation of HGF by Yap would be counterintuitive. However, a more recent study has suggested that high concentrations of HGF promote satellite cell quiescence (Yamada et al., 2010) and therefore the effect of the downregulation of HGF by Yap is difficult to interpret (Fig. 1).

TGF β and BMP signalling has been associated with muscle growth and satellite cell function. In our analysis BMP4 (bone morphogenetic protein 4) and Fstl3 (follistatin-related protein 3) increased their expression in response to hYAP1 S127A overexpression. Exogenous BMP4 was reported to promote satellite cell proliferation and inhibit differentiation (Ono et al., 2011) which is consistent with the function of Yap. Fstl3 inhibits myostatin via its N-terminal domain (Cash et al., 2012). Because myostatin inhibits satellite cell activation and re-entry into the

cell cycle (McCroskery et al., 2003), the increase expression of Fstl3 is consistent with satellite cell activation and proliferation due to myostatin inhibition by Fstl3.

Next we identified a diverse group of regulators of myogenesis and/or proliferation whose expression pattern and/or function matches the phenotype induced by hYAP1 S127A overexpression. Muscleblind-like 3 (MBNL3) is, like Yap, highly expressed in myoblasts but not in differentiated muscle and it inhibits differentiation of C212 myoblasts (Lee et al., 2008). PEA-15 (phosphoprotein enriched in astrocytes 15A) overexpression in myoblasts prevents, like high levels of Yap activity, their differentiation (Iovino et al., 2012). CD34 is a cell surface protein marker found, among others, on satellite cells. It has recently been reported that CD34 promotes re-entry into the cell cycle after injury (Alfaro et al., 2011; Beauchamp et al., 2000), which is consistent with the phenotype induced by Yap. In this group, several genes were downregulated by hYAP1 S127A overexpression. NDRG2 (N-myc downstream regulated gene 2) is highly expressed in differentiated myotubes and little in myoblasts but promotes proliferation (Foletta et al., 2009), which is not consistent with the pro-proliferation effect of Yap. However, the high expression of NDRG2 in post-mitotic myotubes and its positive effect on proliferation is contradictory. Like NDRG2, Myf6 (Mrf4) is repressed by hYAP1 S127A overexpression. Myf6 is a myogenic determination gene (Kassar-Duchossoy et al., 2004) whose expression increases, unlike that of MyoD and Myf5, late during myogenic differentiation (Tomczak et al., 2004). This involves an involvement in terminal differentiation. The repression of Myf6 by Yap may inhibit these effects. Fbox32 (atrogin-1; Mafbx) is a skeletal muscle atrophy-regulator (Sandri et al., 2004) and has been implicated in the degradation of MyoD (Tintignac et al., 2005). The downregulation of Fbox32 expression by high Yap activity in activated satellite cells may contribute to sustaining high levels of MyoD in activated satellite cells. Taken together, this list of candidate genes may represent important downstream targets of Yap that regulate aspects of the phenotype that is induced by high Yap activity in satellite cells and myoblasts.

In order to gain some insight into how Yap regulates gene expression we focussed on the hypothesis that Yap binds to Tead transcription factors to co-activate MCAT elements. We found that hYAP1 and hYAP1 S127A could activate an 8xGTIIc-luciferase reporter whereas knockdown of Yap by shRNA reduced it (Fig. 7E). The 8xGTIIc-luciferase reporter is described as a Yap/Taz-activity reporter (Dupont et al., 2011), which is driven by 8 MCAT elements obtained from the genome of the SV40 polyomavirus. Such MCAT elements are targeted by Tead transcription factors (Yoshida, 2008) and thus in our experiment Yap was most likely co-activating the MCAT elements of the reporter by binding to the Tead isoforms present in myoblasts. Next we performed a Yap-Tead1 immunoprecipitation experiment which confirmed that Yap can bind to Tead transcription factors in myoblasts (Fig. 7F). This is in line with previous results that have demonstrated that Yap can bind to all four Tead transcription factors in non muscle cells and that Yap and Tead1 occupy the same promoter in $\approx 80\%$ of the cases in MCF10A cells (Vassilev et al., 2001; Zhao et al., 2008).

Based on the ability of Yap to co-activate MCAT elements and to bind to Teads in myoblasts, we tested for an overlap between genes that were induced by both 20 h and 40 h of doxycycline

and genes whose promoters were reported to be occupied either by Tead1 in adult skeletal muscle (Qiu et al., 2011) or by Tead4 in C2C12 myoblasts (Benhaddou et al., 2012). This analysis revealed an overlap of 3 genes with the Tead1-occupied genes in adult skeletal muscle and of 29 genes with Tead4-occupied genes in C2C12 myoblasts (supplementary material Table S3). This is indirect evidence that Yap, Tead1 and Tead4 bind to and induce the expression of at least some genes. The low overlap with Tead1-occupied genes in adult skeletal muscle can be partially explained by the fact that only 138 genes were identified in the Tead1 ChIP-on-Chip analysis and that the analysis was performed on adult skeletal muscle rather than in myoblasts (Qiu et al., 2011). Unfortunately, no genome-wide Tead2 and Tead3 binding data are currently available for myoblasts which limits this analysis.

Next we probed for enrichment of MCAT elements (Transfac matrix for Tead1/Tef) in the proximal promoters (-2 kb to $+2$ kb relative to the transcription start site) of genes whose expression increased after 20 h and 40 h of doxycycline treatment. We found that 13 out of 138 genes (9.4%) had putative MCAT elements in their proximal promoters which represents a significant enrichment ($P < 0.01$) among the genes upregulated after both 20 h and 40 h of doxycycline treatment (supplementary material Table S3). Whilst this is a significant enrichment, it is still a low percentage. There are two possible explanations. First, some of the genes that responded to doxycycline treatment may only be indirectly regulated by Yap. Second, some genes that are directly targeted by Yap may have MCAT elements in enhancers that are located outside the proximal promoter. Evidence for distant enhancers comes from genome-wide searches for regulatory elements. Such studies have revealed for example that much of the tissue-specific regulation of gene expression depends on distant enhancers rather than proximal promoters (Cao et al., 2010; Heintzman et al., 2009).

In conclusion, Yap expression increases during satellite cell activation and Yap remains highly expressed until after the differentiation versus self-renewal decision is made. High Yap levels and activity expands the pool of activated satellite cells and prevents differentiation by activating genes associated with the cell cycle, ribosome biogenesis angiotensin signalling and genes that maintain the activated satellite cell or myoblast phenotype and/or inhibit differentiation.

Materials and Methods

Muscle fibre isolation and satellite cell culture

To study muscle fibre associated satellite cells *ex vivo*, viable single muscle fibres were isolated from mouse EDL muscles following collagenase I digestion and cultured in suspension as previously described (Collins and Zammit, 2009). Isolated fibres were maintained in muscle fibre medium (DMEM-glutamax with 10% v/v horse serum, 0.5% v/v chick embryo extract and pen-strep) for up to 72 h and then fixed in 4% PFA for 10 min at 0–72 h. For adherent satellite cell-derived myoblasts, freshly isolated muscle fibres were plated onto dishes coated with 1 mg ml^{-1} of Matrigel (BD Biosciences) and incubated in proliferation medium (DMEM-glutamax with 30% v/v foetal calf serum, 10% v/v horse serum, 1% v/v chick embryo extract, 10 ng ml^{-1} of basic fibroblast growth factor and pen-strep). After 72 h, muscle fibres were manually removed and re-seeded for further expansion. For differentiation assays satellite cell-derived myoblasts were seeded at high density and placed in differentiation medium (DMEM-glutamax with 2% v/v horse serum and pen strep) for at least 48 h. For assays involving immunofluorescent analysis satellite cells were seeded into 8 well chamber slides (Lab-tek) and fixed in 4% PFA for 10 min.

RNA extraction, cDNA synthesis and quantitative RT-PCR

The mRNA expression of genes from muscle fibre associated satellite cells was assessed using previously described protocols (Gnocchi et al., 2009). Briefly,

single muscle fibres were stripped of their satellite cells via digestion in 0.125% Trypsin-EDTA at 37°C for 15 min followed by gentle trituration to release cells. Fibre fragments were removed by passing medium through a 40 µm cell strainer (BD Falcon). Cells were collected by centrifugation and washed twice with PBS. The RNA from these cells or satellite cell-derived myoblasts was isolated using commercial reagents (RNeasy Qiagen or Trizol, Invitrogen) and reverse transcribed into cDNA using 100–400 ng of RNA (Quanti-Tect kit, Qiagen or iScript cDNA kit, Bio-Rad). For plated cultures, cells were washed twice in PBS and then RNA isolated using the same procedures as described above.

Quantitative RT-PCR was performed on an Mx3005P QPCR system (Stratagene) with MESA Blue qPCR MasterMix Plus and ROX reference dye (Eurogentec). Alternatively, a StepOnePlus™ RT-PCR system with TaqMan-based RT-PCR gene expression assays (Applied Biosystems) were used for mouse and human YAP1 (Mm00494249_m1, HS00902712_g1) and the multiplex-compatible Gapdh or 18S internal control probes (Applied Biosystems).

The following primers were designed using Primer blast (NCBI): *Yap1*, forward 5'-ACCCAAGGCTGGACCCTCGTT-3' and reverse 5'-AGCATTGTCTGTGCTGGATTGATA-3'; *MyoD*, forward 5'-AGCACTACAGTGGCGACTCA-3' and reverse 5'-GCTCCACTATGCTGGACAGG-3'; *Gapdh*, forward 5'-GTGAAG-GTCGGTGTGAACG-3' and reverse 5'-ATTGTATGTTAGTGGGGTCTCG-3'.

Expression was normalised to *Gapdh*. Gene expression relative to mRNA levels in control samples was calculated using the Pfaffl method (Pfaffl, 2001). Results show quantitative RT-PCR data from at least three independent experiments.

Microarray analysis

The Myf5-Cre mouse strain (B6.129S4-Myf5^{tm3(cre)Sor}/J) was obtained from Jax and crossed with Rosa26-Stop-RtTA Col1a TetOP-hYAP1 S127A mice (Schlegelmilch et al., 2011) to generate Myf5-Cre Col1a^{TetOP-hYAP1 S127A/+} R26^{Stop-RtTA/+} mice, in which Myf5-expressing cells express hYAP1 S127A upon doxycycline treatment. Primary myoblasts [also referred to as muscle-derived stem cells (MDSCs) or slowly adherent cells (SACs)] were isolated from the hind limb muscles of these mice using the preplating method described previously (Gharraibeh et al., 2008). Myoblasts were then cultured on 0.2% gelatin-coated surface in growth medium (Promocell, C-23160 containing 20% FBS) with 1 mg ml⁻¹ doxycycline to induce hYAP1 S127A overexpression or without doxycycline as control for 20 h or 40 h. Total RNA was isolated using RNeasy kit (Qiagen) and 5 µg of total RNA was used for analyses. Triplicate microarray analyses of hYAP1 S127A transgenic primary myoblasts were conducted at the Molecular Genetics Core Facility (Children's Hospital Boston) using Mouse Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA). The mean of expression data was determined to identify genes with altered levels of expression in the hYAP1 S127A overexpressing myoblasts (doxycycline-treated) versus non-treated at each time point (20 h and 40 h) using RMA16 normalisation algorithm with the Genespring 12 software (Agilent). An adjusted *p*-value cut off of 0.05 and a relative fold change cut off of 1.3 were used, and genes were classified by biological function based on Gene Ontology annotation [Genespring 12, Gene Set Enrichment Analysis (GSEA) at <http://www.broadinstitute.org/gsea/index.jsp>], National Center for Biotechnology Information gene descriptions or UniprotKB annotations. We additionally checked for overlap between genes identified as Tead1 (Qiu et al., 2011) or Tead4 targets (Benhaddou et al., 2012) and genes that responded to doxycycline treatment for both 20 h and 40 h using an overlap search (<http://jura.wi.mit.edu/bioc/tools/compare.php>).

Retroviral and lentiviral expression vectors and transduction methods

Wild type hYAP1 and a constitutively active mutant hYAP1 S127A cDNA were sub-cloned into a pMSCV-IRES-eGFP (Zammit et al., 2006) retroviral backbone from plasmid DNA (Addgene plasmids 17790 and 17791) creating pMSCV-hYAP1-IRES-eGFP and pMSCV-hYAP1 S127A-IRES-eGFP constructs. This system allows the expression of hYAP1 or hYAP1 S127A as a bi-cistronic message together with eGFP for the identification of infected cells. Empty vector pMSCV-IRES-eGFP was used as a negative control. Retroviruses were packaged in 293T cells using standard methods. To infect muscle fibre-associated satellite cells muscle fibres were cultured for 24 h following isolation and then incubated in diluted viral supernatant (1:5) until assayed. Plated satellite cells were infected by incubation with diluted viral supernatant (1:5) supplemented with polybrene (4 µg/ml) for 3–4 h before being rinsed in PBS and placed back into fresh proliferation medium. Lentiviral vectors (pLKO.1) containing anti-sense oligonucleotides targeted against mouse Yap mRNA (Lian et al., 2010) were used for Yap knockdown experiments. For virus production, lentiviral vectors were co-transfected with psPAX2 and pMD2.G packaging plasmids into 293T cells and viral supernatant was recovered using standard methods. C2C12 and plated satellite cells were infected via incubation in diluted viral supernatant (1:5) for 4 h. 24 h after infection, transduced cells were selected with medium supplemented with 2 µg ml⁻¹ of puromycin and assayed after at least 5 days.

Immunocytochemistry

Fixed single muscle fibres and plated satellite cells were permeabilised with 0.5% (v/v) Triton X-100 in PBS for 6 min and blocked with 10% (v/v) goat serum and

10% (v/v) swine serum in PBS for 30 min to reduce non-specific antigen binding. Primary antibodies were applied overnight at 4°C and consisted of rabbit anti-Yap (a gift from Maris Sudol), rabbit anti-phospho Yap Ser127 (Cell Signaling, no 9411), mouse anti-Pax7 (DSHB), mouse anti-MyoD (Dako, clone 5.8A), mouse anti-Myogenin (DSHB, clone FD5), mouse anti-MyHC (DSHB, clone MF20) and chicken anti-GFP (Abcam, ab13970). Species-specific fluorochrome-conjugated secondary antibodies were then applied for 2 h at room temperature. After that slides were mounted with Vectashield mounting medium which contains 100 ng ml⁻¹ of DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories).

Proliferation assays

Satellite cell proliferation was assessed by via EdU (5-ethynyl-2'-deoxyuridine) or IdU (Iododeoxyuridine) incorporation into DNA. Culture media were supplemented with 10 µM of either EdU or IdU and cells were pulsed for 2–4 h. For EdU assays, the Click-iT® EdU kit (Invitrogen) was used following the manufacturer's instructions. For IdU assays, cells were fixed in 4% PFA and stained using a mouse monoclonal anti-IdU antibody (ab8955, 1:100, Abcam, Cambridge, UK) diluted in 0.15 M NaCl containing DNase (Sigma-Aldrich).

Western blotting

Cells were lysed in RIPA buffer (Tris 50 mM, NaCl 150 mM, SDS 0.1%, Na-Deoxycholate 0.5% and Triton-X 100 1%) supplemented with 50 mM of NaF, 0.5 mM of sodium orthovanadate, 1 mM of EDTA and a protease inhibitor cocktail (Sigma). Whole cell lysates were then separated via SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed with the following primary antibodies overnight at 4°C: rabbit anti-Yap (a gift from Maris Sudol), rabbit anti-phospho Yap Ser127 (Cell Signaling, NO. 4911), mouse anti-MyHC (DSHB, clone MF20), mouse anti-α-tubulin (Sigma, clone B-5-1-2), mouse anti-PCNA (Cell Signaling, no. 2586), chicken anti-GFP (Abcam, ab13970) and Tead1. Primary antibodies were then visualised using species-specific Alexa Fluor 488/555 labelled secondary antibodies (Invitrogen). Bands were quantified using a Li-Cor Odyssey infrared imager.

Co-immunoprecipitation assays

Plated C2C12 myoblasts were lysed in immunoprecipitation (IP) lysis buffer (50 mM Tris HCl, 150 mM NaCl, 50 mM NaF, 1% Triton X-100) supplemented with 1 mM sodium orthovanadate, 0.5 mM EDTA and protease inhibitor cocktail (Sigma). Total cell lysates were homogenised and spun at 13,000 g for 15 minutes at 4°C. 500 µg of protein lysates were incubated with 15 µl protein G-agarose beads for 1 hour at 4°C to remove proteins that bind non-specifically. Each protein lysate was then incubated with ≈3 µg of Yap antibody (gift from Maris Sudol) or an equivalent amount of species matched IgG (rabbit) rotating overnight at 4°C. 50 µl of beads were then added to the lysate and antibody mixture and rotated for 2 h at 4°C. The beads were then recovered following centrifugation and washed 3 times in ice cold IP lysis buffer. The supernatant was removed using a 26 G needle to minimise bead loss. Beads were then resuspended in 30 µl 2× sample buffer and prepared for western blot analysis as above.

MCAT luciferase reporter assay

C2C12 cells were seeded into 6 well plates and transduced with control, hYAP1 wild-type and hYAP1 S127A retroviruses. Cells were then co-transfected with 8xGT10C-luciferase (MCAT-reporter) and Renilla construct using Lipofectamine LTX (Invitrogen) following the manufacturer's instructions. Twenty-four hours post transfection, cells were lysed and Firefly/Renilla luciferase activity quantified by luminescence using a Dual-Luciferase® Reporter Assay System (Promega) following the manufacturer's instructions.

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