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Transcription factor TEAD4 regulates expression of Myogenin and the unfolded protein response genes during C2C12 cell differentiation.

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Abstract.

The TEAD (1-4) transcription factors comprise the conserved TEA/ATTS DNA binding domain recognising the MCAT element in the promoters of muscle-specific genes. Despite extensive genetic analysis, the function of TEAD factors in muscle differentiation has proved elusive due to redundancy amongst the family members. Expression of the TEA/ATTS DNA binding domain that acts a dominant negative repressor of TEAD factors in C2C12 myoblasts inhibits their differentiation, while selective shRNA knockdown of TEAD4 results in abnormal differentiation characterised by the formation of shortened myotubes. Chromatin immunoprecipitation coupled to array hybridisation (ChIP-chip) shows that TEAD4 occupies 867 promoters including those of myogenic miRNAs. We show that TEAD factors directly induce Myogenin, CDKN1A and Caveolin 3 expression to promote myoblast differentiation. RNA-seq identifies a set of genes whose expression is strongly reduced upon TEAD4 knockdown amongst which are structural and regulatory proteins and those required for the unfolded protein response (UPR). In contrast, TEAD4 represses expression of the growth factor CTGF to promote differentiation. Together these results show that TEAD factor activity is essential for normal C2C12 cell differentiation and suggest a role for TEAD4 in regulating expression of the unfolded protein response genes.

Introduction.

The TEAD family of transcription factors was first identified through the purification and cloning of the gene encoding TEF-1 (TEAD1) as a factor binding to two degenerate motifs of the simian virus 40 (SV40) enhancer^{1,2}. The TEAD factors make a highly conserved family of eukaryotic DNA-binding proteins [³ (for review see^{4,5}). Four TEADs have been identified in mammals, each of which possess the TEA/ATTS binding domain derived from comparison of the mammalian proteins with their orthologues in yeast (TEC-1), *Aspergillus nidulans* (AbaA) and *Drosophilla* (scalloped)^{6,7}. The structure of the TEA/ATTS domain comprises a three-helix bundle with a homeodomain fold. TEAD factors bind to a consensus MCAT (5'-CATTCCA/T-3') element originally defined as the GT-II motif of the SV40 enhancer⁸. Most members of the family also bind cooperatively to tandemly repeated elements such as those found in the SV40 enhancer, the somatomammotropin (hCS)-B gene enhancer and the connective tissue growth factor (CTGF) proximal promoter^{1,9,10}.

Members of the mammalian TEAD family are expressed in a variety of tissues, with particularly prominent expression in the nervous system and muscle. A variety of *in-vitro*, cell based, knock-out and transgenic studies has partially elucidated the role of TEAD factors in the regulation of muscle-expressed genes¹¹⁻¹⁴. For example, cardiac troponin T, β -MHC and Myocardin, have been shown to have functional MCAT motifs in their regulatory regions⁵. TEAD4 may play a role in cardiac hypertrophy, characterized by increased cell size and reactivation of fetal cardiac genes¹⁵. Stimulation of α 1-adrenergic signalling has been shown to induce cardiac hypertrophy and activate transcription of the β -MHC and skeletal α -actin genes in a MCAT and TEAD-dependent manner in cultured neonatal rat cardiomyocytes^{16,17}. Similarly, cardiac muscle-specific overexpression of TEAD4 in transgenic mice has been shown to induce arrhythmias *in vivo*¹⁴.

TEAD factors and their cognate MCAT-binding sites are also involved in myofibroblast and smooth muscle differentiation through control of the myocardin and smooth muscle α -actin genes¹⁸. The smooth muscle α -actin gene is regulated by TEAD4 in myofibroblasts, but by TEAD1 in differentiated smooth muscle cells¹⁹. Similarly, the conserved tandem MCAT binding sites in the connective tissue growth factor (CTGF) proximal promoter are involved in TGF β -mediated activation of this gene¹⁰. CTGF is a critical factor inducing myofibroblast proliferation and matrix deposition.

TEAD4 is specifically expressed in developing skeletal muscle in mouse embryos³. Furthermore, chromatin immunoprecipitation (ChIP)-chip shows that TEAD4 is a direct target of the MYOD1 and MYOG transcription factors in C2C12 cell differentiation²⁰. Upregulation of TEAD4 by MYOD1 and MYOG during differentiation is proposed then to activate transcription of a downstream gene expression programme involving the muscle-specific structural genes described above. However despite these observations, mouse knockouts do not reveal any evident role for TEAD4 in muscle development. Knockout of TEAD4 rather leads to early preimplantation lethality due to lack of trophoctoderm specification²¹. Conditional TEAD4 inactivation subsequent to the specification of the trophoctoderm shows that TEAD4 is not required for post-implantation development perhaps due to redundancy with the other members of the family.

More recently it has been shown that TEAD factors mediate the control of cell and organ size via the hippo pathway in both *Drosophila* and mammalian cells^{22-24, 25, 26}. The C-terminal region of TEAD factors interacts with the YAP1 and TAZ/WWTR1 coactivators that are phosphorylated and inhibited by the Hippo tumor suppressor pathway^{27, 28}. TEAD factors are also required for YAP-induced cell growth, oncogenic transformation, and epithelial-mesenchymal transition^{24, 29}. Many of these events are correlated with the ability of TEAD4 to activate CTGF expression.

The above results suggest that TEADs in general and TEAD4 in particular are important regulators of muscle development, yet only a small number of target genes have been identified and it is still not clear what the specific roles for each TEAD factor may be. In addition, how can the contrasting roles of TEADs in proliferation and oncogenic transformation and in cell cycle arrest and differentiation in muscle be explained ?

To address these questions, we have made shRNA-mediated knockdown of TEAD4 or expression of the dominant negative TEAD4 DBD in C2C12 cells. We show that loss of TEAD function through expression of the DBD blocks C2C12 differentiation, while TEAD4 knockdown leads to the appearance of shortened myotubes. ChIP-chip experiments show that TEAD4 binds to the promoters of 867 genes and comparison with RNA-seq further identifies a novel set of genes directly and indirectly regulated by TEAD4. In contrast, TEAD4 knockdown leads to up-regulation of *Ctgf* and Cyclin D1 in C2C12 cells. Thus, in contrast to what is observed in breast cancer cells, TEAD4 represses CTGF expression to promote C2C12 cell differentiation.

Results.

TEAD factor activity is essential for C2C12 differentiation and TEAD4 plays a non-redundant role in myoblast fusion.

Analysis of public and our own Affymetrix gene expression data shows that undifferentiated and differentiated C2C12 cells express mRNA for all members of the TEAD family (data not shown). The expression of TEAD4 is strongly upregulated during differentiation, while that of the other TEAD factors is less affected (Fig. 1A and 1B lanes 1-4 and data not shown). To address the function of these factors in C2C12 cells, we therefore had to consider the potential redundancy between the closely related TEAD proteins. We chose to generate C2C12 cell populations stably expressing a Flag-tagged-TEAD4 DBD fused to a

nuclear localisation signal. The DBD is essentially identical in all members of the family, is not involved in coactivator interactions, and can be used as a dominant negative inhibitor of TEAD function³⁰. Expression of the isolated DBD (Fig. 1C, lane 2) leads to diminished up-regulation of TEAD4 during differentiation, as well as a loss of normal induction of MYOG, CDKN1A and strongly delayed and diminished β MHC expression (Fig. 1B). In agreement with these observations, the TEAD4 DBD almost completely inhibits differentiation, where only a few short fused myotubes are observed (Fig. 2A). In contrast, control cell populations selected after infection with the empty retroviral vector differentiated similarly to uninfected C2C12 cells (see appropriate panels in Fig. 2A). The activity of one or several TEAD factors is therefore essential for C2C12 cell differentiation.

As described in the introduction, one of the best candidates to have an important role in muscle differentiation is TEAD4. To address the role of this protein, we used lentiviral vectors expressing shRNA to make a stable knockdown of TEAD4. Compared to cell populations expressing a scrambled shRNA control sequence, the expression of two shRNAs, A and B, strongly diminished TEAD4 expression (Fig. 1A, and 1D, lanes 5-12). Knockdown was most efficient with ShB that almost completely represses TEAD4 expression. TEAD4 silencing by ShA or ShB led to the appearance of shortened myotubes compared to the scrambled ShSC control (Fig. 2A). This can be more clearly seen upon staining with antibody against β MHC (Fig. 2B). In the ShA and ShB knockdown cells, the majority of myotubes are significantly shorter than in the controls, with mainly 2-5 MYOD1 expressing nuclei per myotube (Fig. 2D). However, these cells showed β MHC expression indicating that they initiate differentiation, but failed to efficiently fuse to form longer myotubes. In addition, labelling with TEAD4 antibody revealed a small number of positively staining nuclei showing that knockdown was not complete in all cells and that residual expressing cells may be able undergo some fusion (Fig. 2C).

Dexamethasone (Dex) treatment has been shown to augment myoblast fusion^{31,32}. Treatment of control ShSC cells with Dex led to the formation of thickened myotubes that fused to form complex syncytial like structures (Supplemental Fig. 1A). TEAD4 expression was enhanced in the presence of Dex showing a more rapid induction (Supplemental Fig. 1B, lanes 1-8). Treatment of the ShB knockdown cells also augmented their fusion leading to myotubes that resembled those of the control ShSC cells in the absence of Dex. However, while addition of Dex indeed led to a partial rescue of the TEAD4 knock-down phenotype, the ShB cells failed to show the extensive fusion seen with the control cells.

Together these results show that TEAD factor activity is essential for proper myoblast differentiation and that TEAD4 promotes normal myoblast fusion during differentiation.

Identification of TEAD4 target genes.

To understand how TEAD4 regulates C2C12 cell differentiation, we performed chromatin immunoprecipitation coupled to array hybridisation (ChIP-chip). As current antibodies do not allow ChIP of TEAD4, we established C2C12 cells that stably express Flag-HA tagged (F-H-)TEAD4 (Fig. 1B lanes, 3 and 4). The level of exogenous tagged protein is comparable to the endogenous TEAD4 in undifferentiated cells, but is not up-regulated like the endogenous protein and hence makes up less of the TEAD4 population in differentiated cells (data not shown). Expression of exogenous tagged-TEAD4 did not affect myoblast proliferation, or differentiation (data not shown).

We first performed anti-Flag ChIP-qPCR on the C2C12 cells expressing the F-H-TEAD4 and control untagged cells differentiated for 5 days to assess F-H-TEAD4 occupancy of the MCAT motif of the skeletal muscle alpha 1 actin (*Acta1*) gene. This site shows clear enrichment in the cells expressing F-H-TEAD4 compared to control cells, while no enrichment is seen in either cell type at the control Protamine 1 (*Prm1*) promoter (Fig. 3A).

ChIPed DNA from three independent experiments was amplified and hybridised to the Agilent extended promoter array that comprises the region from around -5kb to +2kb of 17000 mouse promoters and peaks detected using the Agilent Chip-analytics programme and custom software as previously described [^{33,34} Materials and methods and see Supplemental text]. This analysis identified 867 promoters with at least one TEAD4 occupied site (Supplemental Table 1). Taking into account the divergent promoters a total of 929 genes are potentially regulated by TEAD4 in C2C12 cells. TEAD4 binding sites were almost equally distributed between the upstream promoter region and downstream of the transcription start site (TSS), but were enriched in the region close to the TSS. (Fig. 3B and C). TEAD4 was found to occupy sites at the regulatory regions of the *Myog*, stretch responsive muscle ankyrin repeat domain 2 (*Ankrd2*), Talin 1 (*Tln1*), Dysferlin (*Dysf*) Synaptogamin 8 (*Syt8*) and *Myod1* genes (Fig. 4 and Supplemental Tables 1 and 3).

TEAD4 also occupies sites at 17 miRNA genes including the muscle-enriched Mmu-mir-206 (Fig. 4A and Supplemental Table 1). TEAD4 occupies a site between Mmu-mir-1-1 and Mmu-mir-133a-2 and two sites located at the locus encoding mmu-mir-1-2 and 133a-1 (Supplemental Fig. 2). Like mmu-mir-206, these miRNAs play important roles in C2C12 cell differentiation ³⁵⁻³⁷. Likewise, TEAD4 occupies a site at the locus encoding mmu-mir-214 that promotes skeletal muscle differentiation ³⁸⁻⁴⁰. Occupancy of several of these sites was confirmed by ChIP-qPCR experiments (Fig. 3A).

Ontology analysis of the occupied genes revealed several potential functions for TEAD4. In addition to expected classes such as sarcomere, contractile fibre and cytoskeleton related to muscle differentiation, this analysis revealed a large number of genes involved in transcription regulation, cell cycle and the TRP53 signalling pathway including TRP53 itself as well as potential oncogenes and anti-oncogenes (Fig. 3D and Supplemental Table 2). While regulation of some of these genes may be associated with muscle differentiation, others may

reflect the known role of TEAD4 factors in oncogenic transformation and proliferation of non-muscle cells.

Analysis of the DNA sequences at the TEAD4 occupied peaks using the MEME programme [http://meme.nbcr.net/meme4_1/cgi-bin/meme.cgi⁴¹] identified a consensus MCAT motif (Fig. 4E) similar to that already described from *in vitro* studies. 475 MCAT motifs were found at 373 TEAD4 occupied sites (Supplemental Table 3). The TEAD4 occupied motifs at other sites are likely degenerate versions of this motif. At several promoters the TEAD4-occupied MCAT motif was conserved amongst mammals including humans. This is the case for the MCAT motifs at the *Myog* and *Myod1* promoters (located at -5237-5245 and -5002-5011 with respect to the TSS, respectively), as well as at several of the miRNAs (Fig. 4 and Supplemental Table 3). The conservation of these motifs highlights their potentially important role in regulating the genes promoting myogenic differentiation. At other promoters such as *Cdkn1a*, the murine MCAT motif (located at +485-493) is not conserved at the same location in human, but further analysis revealed the presence of MCAT motifs at other positions in the human promoters (Supplemental Table 3).

The MEME analysis also revealed more than 300 potential SP1 sites, 219 E-box motifs that are potential binding sites for the myogenic regulatory factors, 40 potential PITX2 sites, and 59 potential PAX binding sites (data not shown). We investigated the presence of motifs for Serum Response Factor (SRF) and MEF2 at the TEAD4 occupied loci and found only 18 and 26 sites respectively, showing that these motifs are not abundant at TEAD4 occupied loci.

We next compared our data with the CHIP-chip data on MYOG of Blais et al., where 137 MYOG-occupied promoters were identified²⁰. Of these, 21 are also occupied by TEAD4 (Supplemental Table 4) representing both muscle-specific genes such as *Acta1*, and 2, *Ankrd2* and Myomesin 2 (*Myom2*) and more generally expressed genes such as *Myc* or *Cdk5*.

Comparison with the MYOD1 and MEF2 ChIP-chip data from the same study that identified 126 and 28 occupied promoters respectively, shows that 13 promoters were commonly occupied by MYOD1 and TEAD4 and 12 by TEAD4 and MEF2. These shared targets again comprised both muscle-specific and generally expressed genes (Supplemental Table 4). Several promoters like those of *Acta1*, *Ing3* and *Myog* are occupied by all three factors.

Direct regulation of C2C12 cell differentiation genes by TEAD4

The ChIP analysis indicates that TEAD4 occupies a conserved MCAT motif in the *Myog* promoter (Fig. 4A) Immunoblot analysis shows that MYOG expression is normally stimulated between 1-3 days of differentiation and persists until day 7 (Fig. 1D, lanes 1-4). MYOG activation is reduced in the ShA cells, and almost completely repressed in the ShB cells (lanes 5-12) and in the cells expressing the DBD (Fig. 1C, lanes 5-8). Similar observations were made in RT-qPCR experiments where activation of *Myog* expression was strongly repressed in ShB cells (Fig. 5A). Therefore, TEAD4 binds to the *Myog* promoter and directly activates its expression in C2C12 cell differentiation. In contrast, TEAD4 knock-down does not affect MYOD1 expression in the ShB-expressing cells (Supplemental Fig. 3).

It has previously been shown that activation of *Cdkn1a* (p21^{cip1}) expression by MYOD1 is involved in cell cycle arrest during myogenic differentiation^{42,43}. ChIP-chip and ChIP-qPCR analysis shows that TEAD4 occupies a site in the first intron of the *Cdkn1a* gene (Fig. 3A, and Supplemental Fig. 4A). CDKN1A is strongly expressed in differentiating control cells with strongest expression observed at days 1 and 3, but its expression is strongly diminished in the ShB or DBD-expressing cells (Fig. 1C and D). Together, these observations indicate that both TEAD factors and MYOD1 are required for the normal and timely activation of *Myog* and *Cdkn1a* expression in C2C12 cell differentiation.

The ChIP-chip data shows that TEAD4 occupies the promoters of several muscle-enriched miRNAs whose expression is induced during differentiation and which have essential functions in the differentiation process (Fig. 3 and Supplemental Fig. 2). RT-qPCR experiments show that expression of Mmu-mir-206, Mir-1-2 and Mir-133a-1 are all significantly down-regulated in the ShB cells (Fig. 5B-D). TEAD4 is therefore required for the normal activation of these miRNAs during C2C12 cell differentiation.

The results presented above show that shRNA-mediated TEAD4 knockdown leads to shortened myotubes. To understand the basis of this phenotype, we looked for direct TEAD4 target genes that may be involved in the fusion process. The ChIP-chip results show that TEAD4 occupies sites in the promoters of the *Dysf*, *Tln1*, Caveolin 3, (*Cav3*), and Musculoskeletal, embryonic nuclear protein 1 (*Mustn1*) genes (Fig. 4D and E, and Supplemental Fig. 4 and data not shown) all of which have been shown to be involved in myoblast fusion⁴⁴⁻⁴⁸. RT-qPCR experiments in cells expressing the ShSC and ShB RNAs show that expression of *Cav3* is significantly reduced upon TEAD4 knockdown (Fig. 5E, while specific, but less pronounced effects are seen with the others such as *Tln1* (Fig. 5F). We also observed reduced CAV3 expression in immunoblots of the ShB and DBD expressing cells. (Fig 5G and H and Supplemental Fig. 1B). Together these results show that TEAD4 directly regulates CAV3 expression that contributes to normal myoblast fusion.

Novel TEAD4 regulated genes.

To identify additional genes that are deregulated upon TEAD4 knockdown, we performed mRNA-sequencing (mRNA-seq) on day 7 differentiated ShSc, ShA and ShB cells. Comparison of gene expression in the ShSc and the ShB cells identified 438 down-regulated and 518 up-regulated genes the vast majority of which showed similar deregulation in the

ShA cells (Supplemental Table 5). These genes are thus deregulated in differentiating C2C12 cells using two independent shRNA sequences to knockdown TEAD4. Comparison of the deregulated genes with those identified by ChIP-chip showed that at least 87 were direct targets whose regulatory regions are occupied by TEAD4 (Supplemental Table 5). For example, TEAD4 occupies a downstream intronic site of the myosin light chain 2 (*Myl2*) gene and expression of this gene is strongly reduced in ShA and ShB knockdown cells (Fig. 6A and B).

Amongst the more strongly down-regulated genes, are those encoding the endoplasmic reticulum (ER) chaperones and ER-associated degradation proteins that make up the unfolded protein response (UPR). RNA-seq indicates that expression of *Hspa5* (mBIP), *Syvn1* (HDR), *Ddit3* (CHOP), and its heterodimerisation partner *Cebpb*, *Sell1*, *Dnajc3*, *Gadd45a*, *Ppp1r15a* (GADD34) and *Chac1* are all strongly down-regulated upon TEAD4 knockdown (Fig. 6A and Supplemental Table 5). RT-qPCR indicates that *Ddit3* and *Syvn1* are strongly induced upon normal differentiation and confirms that this activation is lost in the ShA and ShB cells (Fig. 6B and data not shown). These genes are direct targets with TEAD4 occupied sites within their regulatory regions (Fig. 6A and Supplemental Table 5).

ER-stress and the UPR have been previously shown to be activated during several differentiation programmes including myogenic and keratinocyte differentiation^{49,50 51}. A consequence of ER stress is activation of IRE1 involved in splicing the *Xbp1* mRNA to generate an isoform encoding the XBP1 transcription factor^{52,53}. To determine if this splicing event took place in our differentiating C2C12 cells, we analysed the mRNA-seq reads that cover the splice junctions in exon 4 of the *Xbp1* mRNA in the ShSC, ShA and ShB cells. Quantification of the spliced and unspliced forms showed that around half of the *Xbp1* mRNA was spliced in the ShSC cells, while this splice form was strongly reduced or absent in the TEAD4 knockdown cells (Supplemental Fig. 5A). These results were confirmed by RT-PCR

generating products that distinguish the spliced and unspliced forms showing that the spliced form was present at day 7, but not day 1, in the ShSC cells, while no splice form was detected at any time in the TEAD4 knockdown cells (Supplemental Fig. 5B). These observations are indicative of activation of the ER stress response during normal C2C12 cell differentiation, while the incomplete differentiation of the TEAD4 knockdown cells is reflected in the diminished IRE1-mediated splicing of *Xbp1* mRNA.

The ER-stress induced UPR is mediated by the ATF6 and ATF4 transcription factors^{54 55}. RNA-seq shows that the expression of both ATF6 and ATF4 is strongly down-regulated, although they do not appear to be direct TEAD4 targets. Similarly, other UPR/ER stress response genes (*Herpud1*, *Bbc3* (PUMA), *Hsp90b1*, *Pdia4*; *Atp2a3*, *Ern1*, *Car6*, *Xbp1* and *Sdf2l1*) are down-regulated, but do not appear as direct TEAD4 target genes. Together, these observations show that TEAD4 knockdown leads to a diminished ER stress response in differentiating C2C12 cells. In addition, TEAD4 may participate in the normal activation of genes in the UPR pathway that occurs during this process.

The ChIP-chip and mRNA-seq data also show that TEAD4 directly regulates expression of *Nupr1* (Fig. 6C), encoding p8, a small HMG-related chromatin protein that is essential for cell cycle exit and *Myog* activation in differentiating C2C12 cells⁵⁶. We also noted that TEAD4 directly activates expression of interferon-related developmental regulator 1 (*Ifrd1*; Fig 6A and B), a transcriptional corepressor that interacts with mSin3B and histone deacetylase (HDAC) 1 to repress transcription⁵⁷. Mice lacking *Ifrd1* show progressive loss of muscle fibres and severely reduced muscle regeneration⁵⁸. Myogenic precursor cells from the mutant animals show severely reduced differentiation and myoblast fusion *in vitro*. The strongly reduced expression of the *Nupr1* and *Ifrd1* genes as well as the UPR upon TEAD4 knockdown may account for many of the observed differentiation defects suggesting that these are critical target genes mediating the functions of TEAD4. We further observed that

TEAD4 directly activates VEGFA expression in C2C12 cells (Fig. 6C) that has been reported to promote myogenic differentiation⁵⁹. TEAD4 regulation of VEGFA has previously been shown to involve IRF2BP2 and may also be important for the tumourigenic function of TEAD factors.⁶⁰

TEAD4 represses CTGF expression to promote myogenic differentiation.

As described in the introduction, TEAD factors interact with the YAP transcriptional activator to promote proliferation. For example, the TEAD-YAP1 complex activates proliferation in breast cancer cells through direct activation of *Ctgf*²⁴ and activates proliferation of embryonic neural precursors in mice through direct activation of *Ccnd1*⁶¹. ChIP-chip and ChIP-qPCR shows that TEAD4 occupies two sites in the *Ctgf* locus, one in the promoter¹⁰ and one intragenic, and two sites in the proximal and distal regions of the *Ccnd1* promoter in differentiated C2C12 cells (Figs. 7A and C and Supplemental Table 3). Analysis of the mRNA-seq data and RT-qPCR showed that while *Ccnd1* expression was down-regulated during the differentiation of ShSC cells, its expression remained elevated in the ShA and ShB cells (Fig. 7B and D). Similarly, *Ctgf* expression does not significantly change in differentiating ShSC cells, but is up-regulated in ShA and ShB cells. Furthermore, loss of TEAD4 also up-regulates expression of *Ect2* another growth promoting target gene (Supplemental Table 5 and data not shown). Thus while TEAD factors activate *Ctgf* and *Ccnd1* expression to promote proliferation in non-muscle cells, TEAD4 represses their expression in differentiating C2C12 cells.

Differential TEAD4 target site occupancy during differentiation.

The above data indicate that TEAD4 regulates genes that are both constitutively expressed (*Ctgf*, *Ccnd1*) or induced during differentiation. We next used ChIP-qPCR to investigate TEAD4 occupancy of a selected set of these genes in undifferentiated cells (day 0) and at

days 1, 3 and 5 of differentiation. TEAD4 occupies the distal site in the *Ccnd1* promoter in undifferentiated cells and occupancy does not strongly vary during differentiation (Fig. 8A). A similar profile is seen at the *Ifrd1* promoter, despite the fact that expression of this gene is low in undifferentiated cells and induced during differentiation (Fig. 8B). The *Myog* promoter shows significant occupancy in undifferentiated cells that further increases during differentiation (Fig. 8C). In contrast to the above, TEAD4 occupancy of *Cav3*, and *Nupr1* as well as the UPR genes *Ddit3*, *Synv1*, *Hspa5* and *Cebpb* is low in undifferentiated cells and strongly increases only between days 3 and 5 of differentiation (Fig. 8D-I).

Together these results show differential occupancy of TEAD4 target sites between those that are constitutively occupied like *Ccnd1*, and those of differentiation induced genes most of which are fully occupied only at later stages of differentiation. Interaction of TEAD4 with many of its cognate sites therefore seems to require either changes in chromatin structure and/or cofactors that are induced by differentiation.

Discussion.

The activity of TEAD factors and MYOD1 are required for proper initiation of myoblast differentiation.

Differentiation of myoblasts into myotubes is a complex process that requires cell cycle exit, activation of the genes encoding muscle-specific structural proteins that form the contractile fibre as well as the specialised cell junctions, fusion of the myoblasts to form multinucleate fibres and activation of genes involved in the neuromuscular junction. Previous studies have shown that this process is driven by myogenic regulatory factors such as MYOD1 that activates the *Myog* and *Mef2* genes which then together act to regulate downstream genes involved in the above processes^{20,62-64}. TEAD4 was considered as a downstream target of MYOD1 and MYOG required to regulate muscle structural genes. Our

present results show that TEAD factors not only regulate downstream targets, but they also play important roles in the initial events of the differentiation process.

TEAD4 occupies a conserved MCAT motif in the *Myog* promoter and expression of the TEAD-DBD or TEAD4 knockdown both lead to diminished *Myog* activation. Since diminished *Myog* activation cannot be ascribed to loss of MYOD1, we conclude that the activity of both MYOD1 and TEAD4 are required for normal *Myog* activation. TEAD4 occupies the *Myog* promoter in undifferentiated cells and occupancy increases during differentiation. A similar situation is observed for MYOD1 that shows low occupancy of the *Myog* promoter in myoblasts, but increased occupancy during differentiation⁶². The pre-bound MYOD1 et TEAD4 do not activate *Myog* transcription in undifferentiated cells as activation of this promoter further requires MEF2- and p38-dependent recruitment of the histone methyltransferases Ash2L and MLL to promote H3K4me3, and Six4 recruitment of the UTX histone demethylase to remove repressive marks⁶⁵⁻⁶⁷.

Blais et al have shown that MYOD1 and MYOG bind to the TEAD4 promoter²⁰, while we show here that TEAD4 binds the *Myog* promoter. Activation of *Myog* expression and differentiation therefore requires a positive feedback loop between the TEAD factors, MYOD1 and MYOG. Interestingly, it has been proposed that NUPR1 is present at the *Myog* promoter where it facilitates recruitment of MYOD1 and p300 to promote *Myog* expression⁵⁶. *Nupr1* expression is directly regulated by TEAD4 suggesting that TEAD4 acts directly by binding to the *Myog* promoter, but also indirectly through regulation of *Nupr1*.

Cdkn1a is activated in the initial stage of C2C12 cell differentiation where, like *Myog*, its expression is activated by MYOD1^{43,68}. We show here that CDKN1A expression is not properly induced when TEAD function is inhibited. We propose therefore that activity of both the TEAD and MYOD1 factors is required for normal activation of *Myog* and *Cdkn1a* expression and the proper initiation of myogenic differentiation.

Although our data describe the role of TEAD4 during differentiation, we have identified many TEAD4-occupied genes that are not muscle-specific and are not induced during differentiation. We did not however observe any changes in the proliferation of the TEAD4 knockdown myoblasts compared to controls. The role of TEAD4 in C2C12 cells therefore appears to be mainly related to differentiation where its expression is strongly up-regulated.

Regulation of UPR genes during myogenic differentiation.

ShRNA-mediated down-regulation of TEAD4 shows a weaker phenotypic effect than blocking all TEAD factors with the DBD and in agreement with this, expression of MYOG and CDKN1A is more strongly reduced in cells expressing the DBD than in those expressing the TEAD4 shRNAs. TEAD4 is therefore one of the major contributors to the differentiation process, but there is partial redundancy with other TEAD factors. Loss of TEAD4 leads to the appearance of shortened myotubes that may in part be accounted for by the strong down-regulation of *CAV3* expression, but also by the reduced expression of *Nupr1* and *Ifrd1* and/or more generally incomplete differentiation. Nevertheless, the expression of many other major muscle structural genes, as well as those involved in fusion such as *Dysferlin*, whose regulatory regions are occupied by TEAD4 is either mildly or unaffected by TEAD4 knockdown suggesting an extensive functional redundancy between the TEAD factors at these promoters.

In contrast, mRNA-seq identifies a specific set of direct target genes that are strongly down-regulated suggesting that TEAD4 has essential non-redundant functions at their promoters. Amongst the strongly repressed genes are essential components the UPR. The promoters of the genes encoding the DDIT3 and CEBPB activators are progressively occupied by TEAD4 and their expression induced during differentiation. In contrast, their activation is strongly diminished in the TEAD4 knockdown cells. Diminished activation of other UPR genes is likely due to indirect effects, for example *Bbc3* and *Car6* whose

expression is normally activated by DDIT3, or *Xbp1* that is regulated by MYOD1 and MYOG
69-71 51

A previous study⁵⁰ and the results of our RNA-seq experiments indicate that C2C12 cell differentiation is accompanied by ER stress and splicing of the *Xbp1* mRNA. The ATF4 and ATF6 factors, previously described mediators of the ER stress response, are expressed in normal differentiated C2C12 cells, but strongly down-regulated in cells lacking TEAD4. Although they do not appear to be direct targets, we cannot however rule out the possibility of TEAD4 sites at more distal regulatory elements of these genes through which it could directly regulate their expression. Together these results are consistent with the idea that normal C2C12 cell differentiation is accompanied by ER stress-induced activation of the UPR. The lack of UPR gene activation in the TEAD4 knockdown cells may reflect a requirement for this factor to activate their expression, but also the lack of a potent ER-stress response due to the generally altered differentiation of the shTEAD4 cells. Future experiments will determine whether TEAD4 is directly required for activation of the UPR genes either as a component of the ER stress response and/or in a differentiation-associated pathway.

TEAD4 plays an essential and non-redundant role in activating several other genes critical for myogenic differentiation. The expression of structural *Myl2* and *Myl6b* genes is strongly dependent on TEAD4, as is that of *Ankrd2* that is involved in the coordination of proliferation and apoptosis during myogenic differentiation⁷². TEAD4 is also an essential regulator of *Ifrd1* that plays an important role in maintaining muscle function and in regeneration⁷³. *Ifrd1* is further implicated in autosomal-dominant sensory/motor neuropathy with ataxia characterized primarily by progressive motor neuropathy [SMNA,⁷⁴].

TEAD4 is involved in cardiac hypertrophy, an adaptive response of the heart to increased workload and injury that occurs in a number of physio-pathological conditions such as hypertension, valvular disease, myocardial infarction, and cardiomyopathy^{5,75}. The exact

role of TEAD4 in cardiac hypertrophy is however as yet poorly characterised. Here we show that TEAD4 directly regulates expression of NUPR1 that is required for endothelin- and α -adrenergic agonist-induced cardiomyocyte hypertrophy⁷⁶. Moreover NUPR1 is a negative regulator cardiomyocyte autophagy, a cytoprotective pathway used to degrade and recycle cytoplasmic content. Dysfunctional autophagy has been linked to cardiomyopathies and hypertrophy⁷⁷. It is therefore possible that TEAD4 regulation of NUPR1 in cardiomyocytes may modulate cardiac hypertrophy.

Our observations demonstrate an important role for the TEAD factors in myoblast differentiation *in vitro*. This function has not been clearly demonstrated *in vivo* where post-implantation inactivation of TEAD4 gives rise to viable adult animals with no obvious phenotype^{21,78,79}. This may be explained by compensation by other TEAD factors during development in the animal model, that does not occur *in vitro* when an acute loss of TEAD4 expression is induced by shRNA silencing. TEAD factors clearly have redundant functions *in vivo* as while TEAD2 knockout mice have only a minor phenotype, TEAD1/TEAD2 double knockout mice have pleiotropic abnormalities much more severe than the loss of TEAD1 or TEAD2 alone^{14,80,81}.

Cell-specific regulation of proliferation genes by TEAD factors.

TEAD factors regulate key aspects of myoblast differentiation, in particular expression of the *Cdkn1a* gene required for cell cycle exit. These results contrast with the important role of TEAD factors in promoting cell proliferation via the Hippo signaling pathway by activation of target genes such as *Ctgf* or *Ccnd1*^{24,29,82}. It should be noted however that stable expression of the TEAD4-DBD that represses differentiation has no significant effect on C2C12 myoblast proliferation showing that TEAD factors are not essential for C2C12 cell proliferation. One possible mechanism to account for these

contrasting effects, would be if TEAD factors did not occupy the promoters of *Ctgf*, *Ccnd1* or other growth promoting genes in C2C12 cells. However, we observe TEAD4 occupancy of these loci in differentiated C2C12 cells. Nevertheless, in contrast to non-muscle cells, TEAD factors repress *Ctgf* expression during differentiation. This repression may be critical for the differentiation process as CTGF can inhibit C2C12 differentiation and even induce de-differentiation⁸³. These results suggest that differential regulation of CTGF by TEAD factors in muscle and in other cell types may play a key role in the transition from proliferation to cell cycle arrest.

This differential regulation of these growth promoting genes may be due to use of different cofactors in muscle and non-muscle cells. In C2C12 cells, YAP1 is phosphorylated and exported from the nucleus during differentiation⁸⁴. Moreover, expression of a non-phosphorable YAP1 mutant that remains nuclear inhibits differentiation. In contrast, the Vestigial (VGL) family of proteins may act as TEAD cofactors in muscle cells⁸⁵⁻⁸⁸. Exchange of YAP1 for VGL cofactors may therefore be an essential event in muscle differentiation and account for the differential regulation of growth promoting genes.

Materials and Methods.

C2C12 cell culture and differentiation.

C2C12 cells were cultured and differentiated under standard conditions as previously described⁸⁹. Differentiation was induced by switching to medium with 2% horse serum (Gibco). Day 1 is taken as 24 hours after switching to differentiation medium. C2C12 cell lines expressing Flag-HA tagged TEAD4 and the Flag tagged TEAD4-DBD were generated by infection with the corresponding pBABE retroviruses and infected cell populations were selected with continuous presence of puromycin as described³³. Cell populations expressing the ShRNAs were generated by infection with the appropriate pLKO.1 lentiviral vectors and

selection with continuous presence of puromycin. The TEAD4 shRNA sequences are, shA (5'-CCGGCCGCCAAATCTA TGACAAGTTCTCGAGAACTTGTCATAGATTTGGCGGTTTTTG-3'), and shB (5'-CCGGGCTGAAACACTTACCCGAGAACTCGAGTTCTCGGGTAAGTGTTTCAGCTTT TTG-3') and were ordered from Sigma-Aldrich. Control shRNA, pLKO.1-scramble shRNA (1864) was from Addgene

Immunoblots and immunofluorescence.

Total cell extracts were prepared by the freeze thaw technique as previously described (Mengus et al., 2005). Immunoblots were performed by standard techniques. The following antibodies were used. The following antibodies were used. TEAD4 (M01) from Abnova, β -MHC (MY-32) from Sigma-Aldrich, MYOG (F5D), CDKN1A (C-19) and MYOD1(C-20) from Santa-Cruz Biotechnology, β -TUB (AB21057) from Abcam, CAV3 (mouse monoclonal) from BD Transduction Laboratories, DYSF (mAb NCL-Hamlet-2) from Novocastra. Immunofluorescence was performed essentially as previously described⁹⁰. Briefly, cells were rinsed once in PBS and fixed in 4% paraformaldehyde. Slides were blocked with 3% serum in PBS with 0.1% Triton-X100. Primary antibodies were incubated overnight at 4°C and then washed 3 times with PBS 0.1% Tween-20. Slides were then incubated with fluorescent-labelled secondary antibodies, and after washing visualised by fluorescence microscopy. DNA was counterstained with Hoechst.

Chromatin immunoprecipitation.

ChIP experiments were performed according to standard protocols and are described in more detail in the Supplemental material. All ChIP was performed in triplicate and analysed by triplicate qPCR. For ChIP-chip, the total input chromatin and ChIPed material were hybridised to the extended promoter array from Agilent covering -5 kb to +2kb regions of around 17000 cellular promoters as previously described^{33,34}. Data were analysed with

ChIP Analytics from Agilent, further details are described in the Supplemental material. Flag ChIP was performed with Anti-Flag M2 Affinity Gel (SIGMA, A2220). Real-time PCR were performed on Roche Lightcycler using Roche SYBR Green mix. Primer sequences are shown in the Supplemental Table 6.

Bioinformatics analysis.

The 500 nucleotides under the highest scoring oligonucleotide at each TEAD4 binding site were analysed using the MEME programme (http://meme.nbcr.net/meme4_1/cgi-bin/meme.cgi)⁴¹). Comparisons with the previously described ChIP-chips from Blais et al.,²⁰ were performed with Excel using their Supplemental Tables. The presence of MCAT motif in human promoters was performed on the region from -5kb to + 2kb using a custom JAVA API application and allowing 1 mismatch compared to the consensus sequence outside of the 5'-TTCC'-3' core that was considered as invariable.

RNA extraction and quantitative real-time RT-PCR.

Total RNA was extracted using an RNeasy kit from Qiagen according to the manufacturer's instructions. 1 µg of RNA was reverse transcribed using AMV retrotranscriptase (Roche) using hexanucleotides. The final product was diluted 200 times and 5 µl were mixed with forward and reverse primers listed in Supplementary Table 3 (300 nM of each primer at final concentration) and 7.5 µl of SYBR Green master mix in total volume of 15µl. The real-time PCR reaction was performed using the LightCycler 1.5 system (Roche). Each cDNA sample was tested in triplicate. For quantification of gene expression changes, the δ Ct method was used to calculate relative fold changes normalized against beta-actin expression. Oligonucleotide primer sequences are listed in Supplemental table 6.

MicroRNAs were extracted using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. 1 µg of RNA was reverse transcribed using miScript reverse transcription kit (218061) from Qiagen. The real-time PCR was performed using miScript SYBR Green PCR Kit (218073) from Qiagen. Reverse transcription product was diluted 200 times and 5 µl were mixed with 1.5 µl universal primer (provided with miScript SYBR Green PCR Kit), 1.5 µl microRNA specific primer from Qiagen, 7.5 µl miScript SYBR Green PCR Kit. For quantification of gene expression changes, the δ Ct method was used to calculate relative fold changes normalized against small RNA U6 expression. The following primers were used: MiR-1-2 (MS00011004), miR-133a-1 (MS00007294) from Qiagen. For miR-206 and small RNA U6 the following primers were used: miR-206 (5'-TGGAATGTAAGGAAGTGTGTGG-3') and small RNA U6 (5'-CGCAAGGATGACACGCAAATTCGT-3').

mRNA-seq.

The mRNA-seq libraries were prepared following the Illumina protocol with some modifications. Briefly, mRNA was purified from total RNA using oligo-dT magnetic beads and fragmented using divalent cations at 95°C for 5 minutes. The cleaved mRNA fragments were reverse transcribed to cDNA using random primers. This was followed by second strand cDNA synthesis using Polymerase I and RNase H. The double strand cDNA fragments were blunted, phosphorylated and ligated to single-end adapter dimers followed by PCR amplification (30 sec at 98°C; [10 sec at 98°C, 30 sec at 65°C, 30 sec at 72°C] x 13 cycles; 5 min at 72°C). After PCR amplification, surplus PCR primers and dimer adapters were removed by purification using AMPure beads (Agencourt Biosciences Corporation). Size selection was performed by electrophoresis on a 2% agarose gel and DNA fragments in the range of ~250-350bp were excised and purified using QIAquick Gel Extraction Kit (Qiagen).

DNA libraries were checked for quality and quantified using 2100 Bioanalyzer (Agilent). The libraries were loaded in the flowcell at 6pM concentration and clusters generated and sequenced on the Illumina Genome Analyzer IIX as single-end 72 base reads. Image analysis and base calling were performed using the Illumina Pipeline version 1.6 and sequence reads mapped to reference genome mm9/NCBI37 using Tophat ⁹¹. Quantification of gene expression was done using Cufflinks ⁹² and annotations from Ensembl release 57. For each transcript the resulting FPKM were converted into raw read counts and these counts were added for each gene locus. Data normalization was performed with the method proposed by Anders et al. ⁹³ and implemented in the DESeq Bioconductor package.

Conflict of interest.

The authors declare no conflict of interest.

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Legends to Figures.

Figure 1. Expression TEAD factors in C2C12 differentiation. **A.** RT-qPCR of TEAD4 expression during days 1-7 of C2C12 cell differentiation in cells expressing the ShSC, ShA and ShB shRNAs. **B.** Immunoblot analysis of the expression of the indicated proteins during differentiation of control C2C12 cells and cells expressing the TEAD4-DBD. **C.** Immunoblot analysis of undifferentiated C2C12 cells stably expressing the Flag-tagged TEAD4-DBD or Flag-HA tagged full length TEAD4. Full length TEAD4 was detected using

anti-TEAD4 antibodies, while the TEAD4-DBD was detected with anti-Flag antibodies. **D.** Immunoblot analysis of the expression of the indicated proteins during differentiation of C2C12 cells expressing the indicated shRNAs.

Figure 2. Differentiation of C2C12 cells expressing the TEAD4-DBD or the TEAD4 shRNAs. **A.** Phase contrast images of unselected C2C12 cells or the indicated selected C2C12 cell populations at day 7 of differentiation. Magnification 20X. **B.** Macro-immunofluorescence of the indicated C2C12 cells at day 7 of differentiation using anti- β MHC antibody. Magnification X10 in upper two panels and X20 in lower panel. **C.** Immunofluorescence of the indicated C2C12 cells stained with Hoechst or labelled with anti-TEAD4 antibody. Magnification X20. **D.** Day 7 differentiated ShSC or ShB-expressing cells were stained with antibodies against MYOD1 to label nuclei and anti- β MHC to identify cells that had initiated differentiation. The results shown are the sum of two independent experiments. For the ShSC cells, 312 nuclei in 45 myotubes were counted in one experiment and 298 nuclei in 42 in the second. For ShB, 260 nuclei in 70 myotubes were counted in the first experiment and 283 nuclei in 85 myotubes in the second. The data represent the number of MYOD1 positive nuclei per β MHC-expressing cell.

Figure 3. ChIP-chip identification of TEAD4 occupied promoters. **A.** Anti-Flag ChIP-qPCR on control C2C12 cells and cells expressing Flag-HA tagged TEAD4. QPCR was performed on amplicons from the indicated promoters and expressed as the % input. **B.** Pie chart showing the location of the TEAD4 binding sites relative to the TSS using the Agilent array annotation. **C.** Location of the TEAD4 binding sites relative to the TSS. **D.** DAVID (<http://david.abcc.ncifcrf.gov/>) ontology analysis of the TEAD4 target genes. **E.** The MCAT consensus motif sequence identified by *de novo* Meme analysis of TEAD4 occupied sites.

Figure 4. Representative examples of TEAD4 promoter occupancy. **A-F.** Screenshots of the .Wig files in the UCSC browser of the triplicate anti-Flag ChIP-chips on the cells

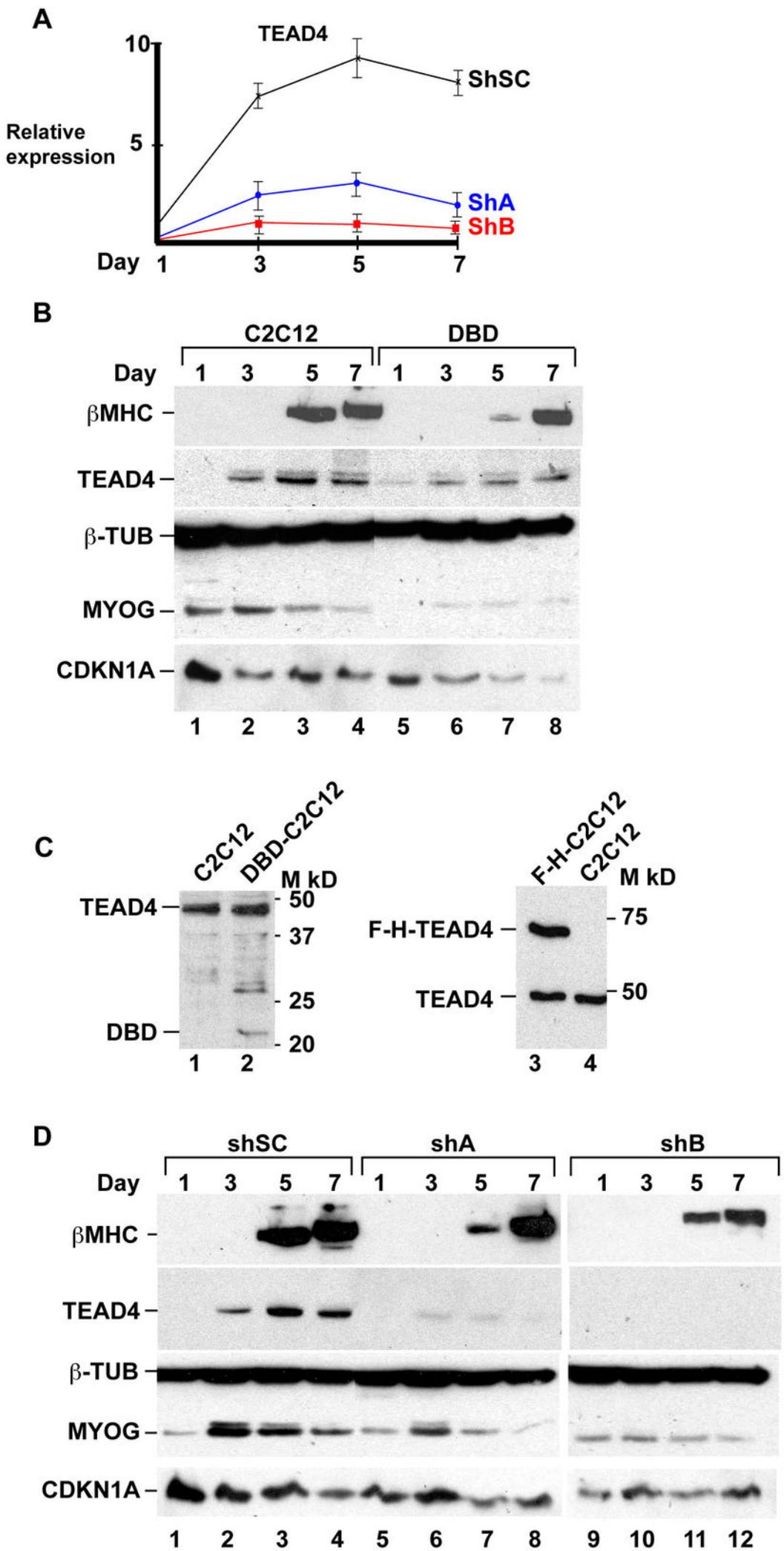
expressing the tagged TEAD4 (F-TEAD4) and the ChIP-chip on the un-tagged control cells (F-control) at the indicated promoters. The TEAD4 binding sites are indicated by the arrows. The values on the Y axis show the normalised IP/Input ratio. The sequences of the MCAT motifs at each peak in mouse rat and human are indicated, with the MCAT motif highlighted in bold. The locations of the TEAD4 binding sites relative to the TSS of the corresponding genes are: *Myog* -5237-5245, *mmu-mir-206* -848-856, *Ankrd2* 1275-1283, *Tln1* 4097-4105, *Dysf* -7031-7039, *Syt8* -4444-4452.

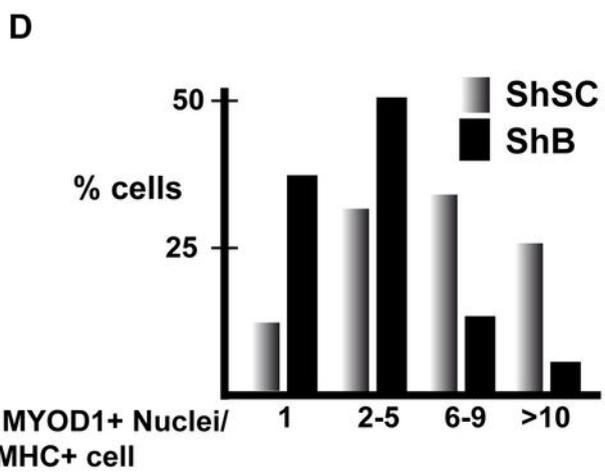
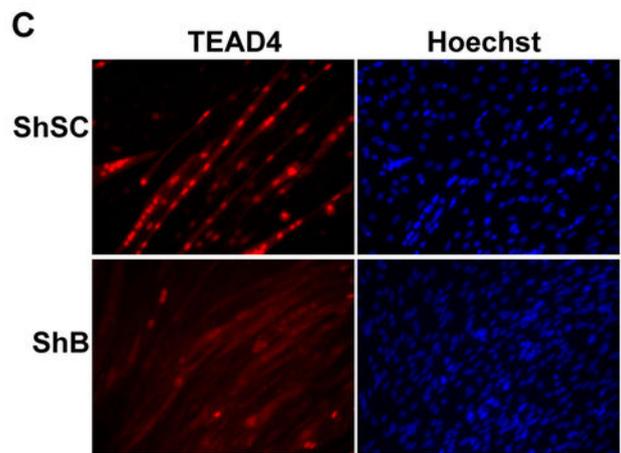
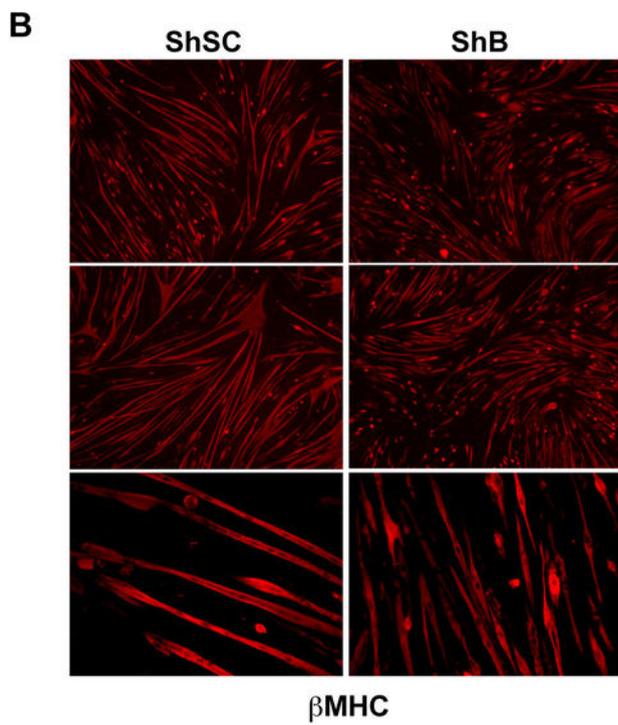
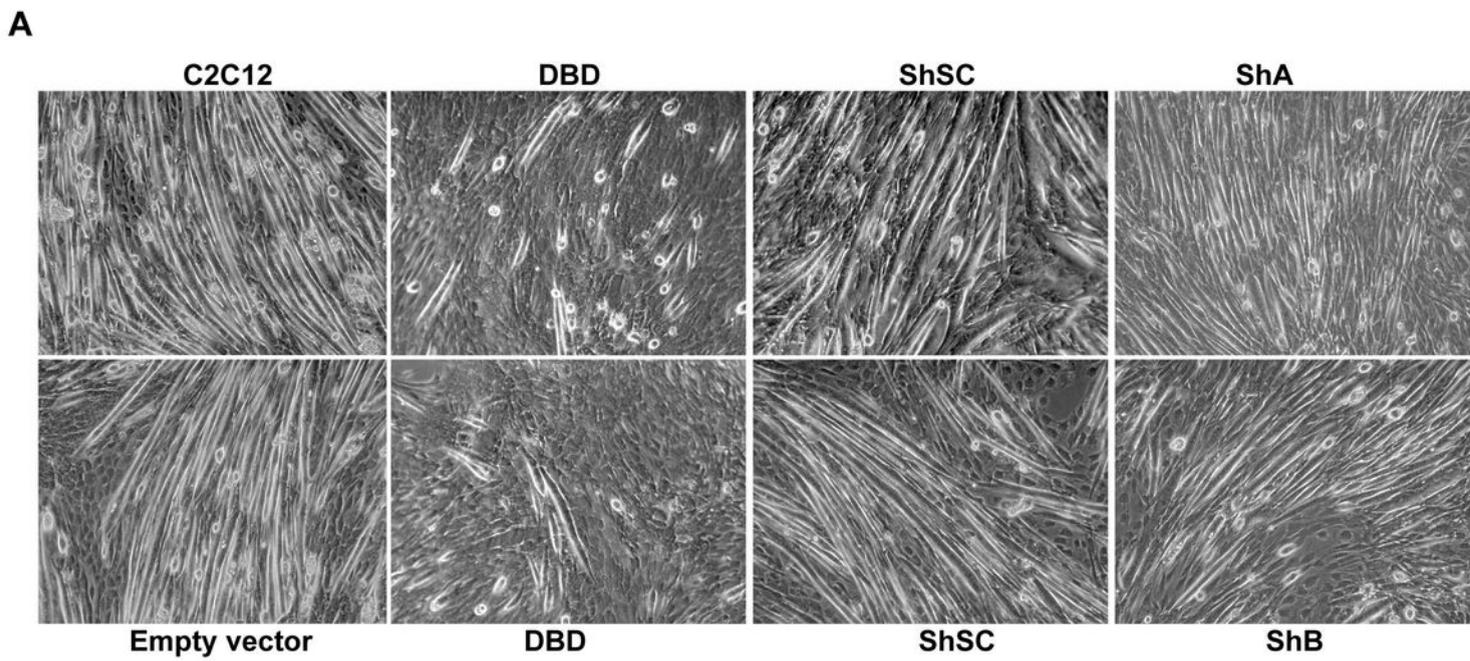
Figure 5. Changes in gene expression upon TEAD4 knock-down. **A-F.** RT-qPCR quantification of the expression of the indicated genes in control ShSC-expressing C2C12 cells and cells expressing ShB from day 1 to day 7 of differentiation. Error bars show standard deviation **G-H.** Immunoblot analysis of CAV3 expression during differentiation of the indicated cell lines.

Figure 6. TEAD4 regulation genes required for myoblast fusion. **A.** Screenshots of the .Wig files in the UCSC browser of the anti-Flag ChIP-chip and the mRNA-seq at the indicated promoters. **B-C.** RT-qPCR quantification of the indicated genes in the indicated cell lines. The locations of the TEAD4 binding sites relative to the TSS of the corresponding genes are: *Myl2*, 2121-2129, *Syvn1* 96-104, *Ddit3* -248-256, *Ifrd1* -284-292.

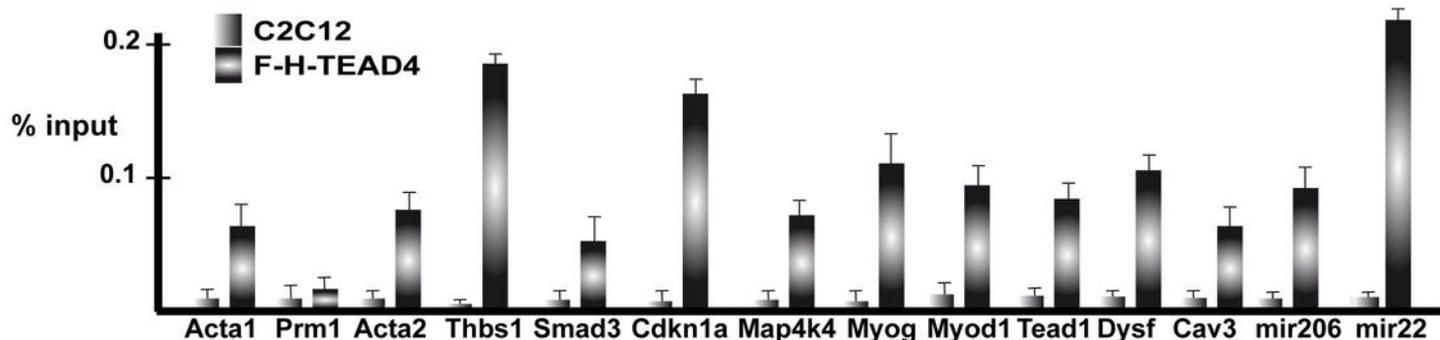
Figure 7. TEAD4 represses CTGF and CCND1 expression. **A-B.** Screenshots of the .Wig files in the UCSC browser of the anti-Flag ChIP-chip and the mRNA-seq at the *Ctgf* and *Ccnd1* promoters. **C.** Flag-ChIP-qPCR showing TEAD4 occupancy at the two sites in the *Ctgf* and *Ccnd1* promoters. **D.** RT-qPCR quantification of the indicated genes in the indicated cell lines. The locations of the TEAD4 binding sites relative to the TSS of the corresponding genes are: *Ctgf* downstream 1674-1682, *Ccnd1* distal -3737-3745, *Ccnd1* proximal -2075-2083.

Figure 8. Differential TEAD4 target site occupancy during differentiation. **A-I.** Anti-Flag ChIP-qPCR on control C2C12 cells and cells expressing Flag-HA tagged TEAD4 in the undifferentiated state (day 0) and at days 1, 3 and 5 of differentiation. QPCR was performed on amplicons spanning the TEAD4 occupied M-CAT motifs from the indicated promoters and expressed as the % input. The locations of the TEAD4 binding sites relative to the TSS of the corresponding genes are as described above and *Cav3* -54-62, *Nupr1* -2813-2821, *Hspa5* 346-354, *Cebpb* -3433-3441.

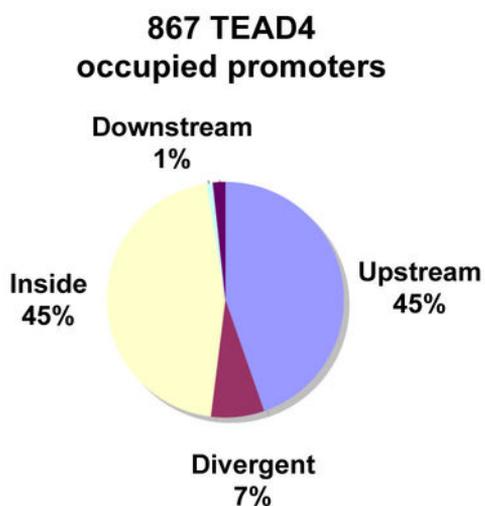




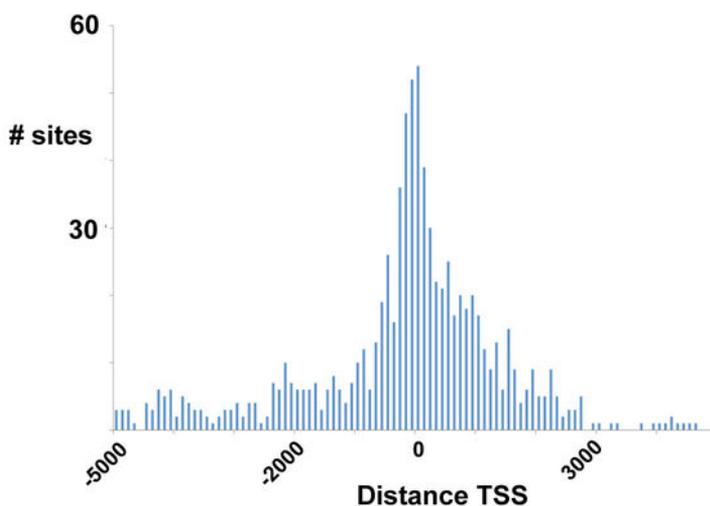
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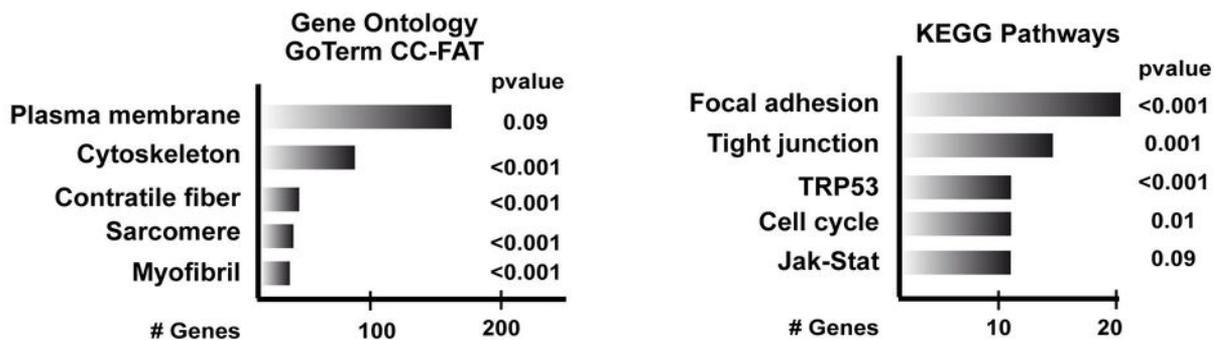
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D



E

