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ETUDE DU ROLE DU FACTEUR DE TRANSCRIPTION TEAD4 DANS LE CONTROLE DE LA DIFFERENCIATION MYOGENIQUE DES MYOBLASTES C2C12 *IN VITRO*

Le jury d'examen est composé de

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-French summary-

La famille des facteurs de transcription TEAD/TEF a été identifiée dans mon laboratoire d'accueil grâce à la purification et au clonage du premier facteur TEF (transcriptional enhancer factor) des mammifères, TEF1 (TEAD1). TEAD1, se lie aux séquences GT-II et Sph de l'enhancer du virus SV40 dont il régule la transcription des promoteurs précoce et tardif. Des études ultérieures ont montré que les facteurs TEAD forment une famille hautement conservée de 4 facteurs de transcription [TEAD1 (TEF1), TEAD2 (TEF4), TEAD3 (TEF5), et TEAD4 (TEF3)]. Tous comprennent un domaine de liaison à l'ADN (DBD) appelé domaine TEA très conservé pendant l'évolution et identique à plus de 95% dans les 4 membres murins de la famille. Le domaine TEA est aussi appelé domaine ATTS du fait de sa conservation dans les facteurs de transcription ABAA, TEC1, TEF1 et Scalloped présents respectivement chez *Aspergillus Nidulans*, la levure (*S. cerivisiae*), les mammifères et *Drosophila melanogaster*. La structure tridimensionnelle du domaine TEA/ATTS comprend trois hélices α formant un repliement de type homéodomaine. Les facteurs TEAD se lient à la séquence d'ADN MCAT (5'-CATTCCT/A-3 ') présente dans les promoteurs des genes des muscles cardiaque, squelettiques et lisses, le placenta et la crête neurale. Récemment, il a été démontré que TEAD1 se lie faiblement aux sites riches en A/T des promoteurs des gènes musculaires, élargissant ainsi le répertoire des promoteurs potentiellement régulés par la famille TEAD.

Chez les mammifères, les membres de la famille TEAD jouent des rôles divers dans la physiopathologie du muscle. Il a été démontré que les gènes codant pour la Troponine T cardiaque, la chaine lourde de la myosine bêta (β-MHC) et la Myocardine, présentent des motifs MCAT fonctionnels dans leurs régions régulatrices. TEAD4 pourait jouer un rôle dans l'hypertrophie cardiaque, caractérisée par une augmentation de la taille cellulaire et la réactivation des gènes cardiaques fœtaux. Il a été montré que l'activation de la signalisation α1-adrénergique induit une hypertrophie cardiaque et active la transcription des gènes de la β-MHC et de l'α-actine squelettique de façon dépendante de la séquence MCAT et des facteurs TEAD dans des cardiomyocytes de rats nouveaux-nées en culture. De plus, dans des souris transgéniques, la surexpression spécifique de TEAD4 dans le muscle cardiaque provoque des arythmies.

D'autres preuves d'un rôle de TEAD4 dans la différenciation musculaire chez la souris proviennent de l'observation qu'il est exprimé spécifiquement dans le muscle squelettique au cours du développement embryonnaire et est induit au cours de la différenciation des myoblastes C2C12 *in vitro*. En outre, l'immunoprécipitation de la chromatine couplée à l'hybridation sur puce (ChIP-chip) montre que TEAD4 est une cible directe des facteurs de

transcription MYOD1 et Myogenin (MYOG) dans la différenciation des cellules C2C12. Il a été proposé alors que MYOD1 et MYOG régulent positivement TEAD4 au cours de la différenciation des cellules C2C12 pour activer la transcription d'un programme d'expression génique impliquant les gènes de la structure de la fibre musculaire. Cependant, malgré ces observations, les souris où l'expression de TEAD4 a été invalidée ne présentent pas de déficit évident dans le développement musculaire. L'invalidation de TEAD4 conduit plutôt à une létalité préimplantatoire de l'embryon en raison de l'absence de spécification du trophectoderme. L'inactivation conditionnelle de TEAD4 après la spécification du trophectoderme montre que TEAD4 n'est pas nécessaire pour le développement postimplantatoire peut-être en raison de la redondance avec les autres membres de la famille.

Plus récemment il a été montré que les facteurs TEAD sont impliqués dans le contrôle de la taille des cellules et des organes via la voie de signalisation du suppresseur de tumeurs Hippo chez la drosophile et la souris. Les facteurs TEAD interagissent avec les coactivateurs YAP1 et TAZ/WWTR1 qui sont phosphorylés et inhibés par la voie Hippo. Les facteurs TEAD sont également nécessaires pour la fonction de YAP dans l'induction de la croissance cellulaire, la transformation oncogénique, et la transition épithélio-mésenchymateuse. Bon nombre de ces événements sont en corrélation avec la capacité de TEAD4 d'activer l'expression du facteur de croissance du tissu conjonctif (CTGF).

L'objectif de mon projet est de comprendre le rôle des facteurs TEAD dans la différenciation des myoblastes C2C12 qui expriment plusieurs membres de cette famille. Nous avons généré des cellules C2C12 exprimant le DBD de TEAD4 qui agit comme un répresseur dominant négatif de tous les membres de la famille. L'expression du DBD inhibe presque complètement la différenciation en myotubes. Seul un petit nombre de myotubes courts est observé. Pour déterminer plus spécifiquement le rôle de TEAD4 dans ce processus, nous avons réalisé des expériences de perte de fonction par interférence ARN avec des « short hairpin RNA » (shRNAs) dans les cellules C2C12. La perte d'expression de TEAD4 conduit à une différenciation anormale caractérisée par la génération de myotubes raccourcis avec un faible nombre de noyaux par myotube. Ces résultats montrent que la famille des facteurs TEAD est essentielle pour la différenciation des cellules C2C12 et que TEAD4 joue un rôle spécifique et non redondant dans la fusion des myoblastes pour former des myotubes correctement dimensionnés.

Pour comprendre le mécanisme d'action de TEAD4, nous avons généré des lignées C2C12 qui expriment TEAD4 étiqueté Flag-HA et effectué des expériences de ChIP-chip. Nous avons identifié 864 promoteurs occupés par TEAD4 dans les cellules différenciées,

notamment ceux de *Myod1*, *Myog*, des miRNAs spécifiques du muscle miR-206, miR-1 et miR-133a, ainsi qu'un ensemble de gènes structuraux impliqués dans les fibres contractiles, le sarcomère et la jonction neuromusculaire. Pour déterminer quels gènes sont régulés par TEAD4, nous avons réalisé des RT-qPCR et des immunoblots. Nos résultats indiquent que TEAD4 est essentiel pour l'induction de l'expression *Myog*, *Cdkn1a* (p21) et miR-206 lors de la différenciation. En plus, TEAD4 régule directement l'expression de la dysferline et la cavéoline 3 qui sont nécessaires à la fusion des myoblastes. Ces résultats montrent que TEAD4 joue un rôle important dans le contrôle de la différenciation des myoblastes C2C12 en myotubes. TEAD4 forme une boucle de régulation avec *Myod1* et *Myog* et régule directement des gènes impliqués dans la sortie du cycle cellulaire et la fusion des myoblastes.

Dans la transformation oncogénique (par exemple dans les cellules du cancer du sein MCF7) les facteurs TEAD se lient à un site conservé dans le promoteur du CTGF et active son expression pour stimuler la prolifération. Lors de la différenciation des cellules C2C12, l'expression du CTGF reste quasiment stable, mais elle augmente dans les cellules C2C12 exprimant le shRNA contre TEAD4. Ces résultats montrent que dans les cellules MCF7, les TEADs agissent pour stimuler directement l'expression du CTGF, tandis que dans les cellules C2C12, TEAD4 réprime directement cette expression. Une observation similaire a été faite avec le gène codant pour le coactivateur YAP1 dont le promoteur est directement lié par TEAD4 dans les cellules C2C12. L'expression de YAP1 est normalement réprimée au cours de la différenciation, alors qu'elle est induite lors de la perte d'expression de TEAD4. Ces résultats suggèrent un modèle pour comprendre comment la voie TEAD-YAP1 peut stimuler la prolifération des cellules cancéreuses en activant l'expression du CTGF tandis que dans la différenciation des cellules C2C12, TEAD4 réprime l'expression de CTGF et YAP1. La régulation différentielle de ces gènes par la famille TEAD peut jouer un rôle important dans la décision entre la prolifération ou la sortie du cycle cellulaire. La base moléculaire de cette régulation différentielle reste à déterminer.

Une interaction de TEAD4 avec des cofacteurs distincts dans le muscle et les cellules non-musculaires pourrait fournir une explication de la régulation différentielle du CTGF. Afin de tester cette hypothèse, nous avons effectué une série préliminaire d'études de protéomiques en purifiant TEAD4 étiqueté à partir de cellules C2C12. Nos résultats montrent que TEAD4 interagit avec ses cofacteurs WWTR1 et YAP1 dans les cellules C2C12. Nous avons également identifié RBBP4 comme un nouveau partenaire de TEAD4 qui peut servir à faciliter son interaction avec la chromatine.

-English summary-

The TEAD/TEF family of transcription factors was first identified in my host laboratory through the purification and cloning of its first mammalian member, TEF1 (transcription enhancer factor 1), as a factor binding to the GT-IIC and Sph enhansons of the SV40 enhancer where it regulates transcription from early and late promoters. Subsequent studies have shown that the TEAD factors make a highly conserved family of 4 [TEAD1 (TEF1), TEAD2 (TEF4), TEAD3 (TEF5), and TEAD4 (TEF3)] transcription factors sharing a highly conserved DNA binding domain (DBD) called the TEA domain. The TEAD factors are more 95% identical in the TEA domain. The TEA domain is also referred to as the ATTS domain as it is conserved in the Aspergillus Nidulans, yeast (S.cerivisiae), mammalian and Drosophila Melanogaster transcription factors AbaA, TEC1, TEF1, and Scalloped respectively. The three dimensional (3D) structure of the TEA/ATTS domain comprises a three helix bundle with a homeodomain fold. TEAD factors bind to the MCAT DNA sequence motif (5'-CATTCCT/A-3') present in promoters of cardiac, skeletal and smooth muscle, placenta, and neural crest genes. Recently, it has been shown that TEAD1 binds weakly to A/T rich binding sites in muscle promoters, expanding the repertoire of promoters that are potentially regulated by the TEAD family.

Members of the mammalian TEAD family play diverse roles in muscle physiopathology. cardiac troponin T, β-myosin heavy chain (β-MHC) and Myocardin have been shown to have functional MCAT motifs in their regulatory regions. TEAD4 may play a role in cardiac hypertrophy, characterized by increased cell size and reactivation of the foetal cardiac genes. Stimulation of α1-adrenergic signaling has been shown to induce cardiac hypertrophy and activate transcription of the β-MHC gene and the skeletal α-actin gene in a MCAT and TEAD-dependent manner in cultured neonatal rat cardiomyocytes. Similarly, cardiac muscle-specific overexpression of TEAD4 in transgenic mice has been shown to induce arrhythmias *in vivo*.

Additional evidence for a role of TEAD4 in muscle differentiation comes from the observation that it is specifically expressed in developing skeletal muscle in mouse embryos and is up regulated in differentiating C2C12 myoblasts. Furthermore, chromatin immunoprecipitation coupled to array hybridisation (ChIP-chip) shows that TEAD4 is a direct target of the MYOD1 and Myogenin (MYOG) transcription factors in C2C12 cell differentiation. Upregulation of TEAD4 by MYOD1 and MYOG during differentiation is proposed to activate transcription of a downstream gene expression programme involving the muscle-specific structural genes. 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 trophectoderm specification. Conditional TEAD4 inactivation subsequent to the specification of the trophectoderm 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 tumour suppressor pathway in both *Drosophila* and mammalian cells. TEAD factors interact with the YAP1 and TAZ/WWTR1 coactivators that are phosphorylated and inhibited by the Hippo pathway. TEAD factors are also required for YAP-induced cell growth, oncogenic transformation, and epithelial-mesenchymal transition. Many of these events are correlated with the ability of TEAD4 to activate expression of connective tissue growth factor (CTGF).

The aim of my project is to understand the role of TEAD factors in C2C12 myoblast differentiation. C2C12 cells express several members of the TEAD family. We generated C2C12 cells expressing the TEAD DNA binding domain (DBD) that acts as a dominant negative repressor of all the members of the family. Expression of the DBD almost completely inhibited differentiation into myotubes where only a small number of short tubes could be observed. To address more specifically the role of TEAD4 in this process, we performed shRNA-mediated knockdown in C2C12 cells. Loss of TEAD4 leads to abnormal differentiation characterised by the generation of shortened myotubes with a lower number of nuclei per tube. These results show that the TEAD family of factors are essential for C2C12 cell differentiation and that TEAD4 plays a specific and non-redundant role in fusion of the myoblasts to form correctly sized myotubes.

To understand the mechanism of action of TEAD4, we generated C2C12 lines that expressed Flag-HA tagged TEAD4 and performed ChIP-chip experiments. TEAD4 was found to occupy 864 promoters in differentiated cells, notably those of *Myod1*, *Myogenin*, muscle cell miRNAs miR-206, miR-1 and miR-133a, as well as as a set of structural genes involved in contractile fibers, sarcomeres and the neuromuscular junction. To determine which genes are regulated by TEAD4, we performed RT-qPCR and western blot analysis. Our results indicate that TEAD4 is essential for the induction of *MYOG*, *Cdkn1a* (p21) and miR-206 expression during differentiation. Morevover, TEAD4 directly regulates expression of the Dysferlin and Caveolin 3 genes that are required for myoblast fusion. These results show that TEAD4 plays an important role in the control C2C12 differnciation into myotubes. Additionally TEAD4 forms a regulatory loop with MYOD1 and MYOG and direct regulation of genes involved in cell cycle exit and fusion.

In oncogenic transformation (for example in MCF7 breast cancer cells) TEAD factors bind to a conserved site in the CTGF promoter and activate its expression to stimulate proliferation. In differentiating C2C12 cells, CTGF expression shows little change in expression. However, in TEAD4 knockdown C2C12 cells CTGF is significantly induced. These results show that in MCF7 cells, TEADs act to directly stimulate CTGF expression, while in C2C12 cells, TEAD4 directly represses its expression. A similar observation was made with the gene encoding the YAP1 coactivator whose promoter is directly bound by TEAD4 in C2C12 cells. YAP1 expression is normally repressed during differentiation, while in the TEAD4 knockdown cells it is up-regulated. These results provide a model to understand how TEAD-YAP1 pathway can stimulate proliferation in cancer cells by activating CTGF expression while in differentiating C2C12 cells TEAD4 represses the expression of both CTGF and YAP1. The different regulation of these genes by the TEAD family may play an important role in the proliferation vs cell cycle exit decision. The molecular basis of this differential regulation remains to be determined.

One explanation for the differential regulation maybe through the interaction of TEAD4 with distinct cofactors in muscle and non-muscle cells. To begin to address this issue we have performed a preliminary series of proteomics experiments by purification of tagged TEAD4 from differentiated C2C12 cells. Our results show that TEAD4 co-purifies with its previously described WWTR1 and YAP1 cofactors in C2C12 cells and we also identify RBBP4 as a novel TEAD4 partner that may mediate TEAD4 interaction with chromatin.

-Abbreviations-

3D	three dimensional
ACh	Acetylcholin
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AER	apical ectodermal ridge
AMPK	AMP activated protein kinase K
BAC	bacterial artificial chromosome
bHLH	basic helix loop helix
BMP	bone morphogenetic protein
Cadh15	M-Cadherin
CaMK	Calmodulin dependent kinase
CaN	Calcineurin
CDX2	Caudal type homeobox 2
CHGNA1	Acetylcholin receptor a1
ChIP	chromatin immunoprecipitation
CHRNG	Acetylcholine receptor g
cTNT	cardiac Troponin T
DAP	Dystrophin-associated protein
DAPC	Dystrophin-associated protein complex
DBD	DNA binding ddomain
E	embryonic day
ECM	extracellular matrix
EHD2	Eps15 homology domain protein
ELC	essential light chain
EMT	epithelial-mesenchymal transition
EOMES	Eomesodermin
ERRα	Estrogen-related receptor a
ES	embryonic stem
ESC	embryonic stem cell
FGF	fibroblast growth factor
FOXA2	Forkhead box A2 gene
FTL1	Folistatin-like 1
GFP	green fluorescent protein
Gja1	gap junction protein, alpha 1
Gja5	gap junction protein, alpha 5
hCS-B	human chorionic somatomammotropin-B
HDAC	histone deacetylase protein
LCD	light chain domain
LGMD2B	limb girdle muscular dystrophy type 2B
LMM	light meromesin
LPM	lateral plate mesoderm
MASC	muscle associated specific component
MBC	myoblast city
MCAT	muscle CAT
MCK	muscle creatine kinase

MD	motro domain
MEF2	myocyte enhabcer factor 2
MHC	Myosin heavy chain
miRNA	microRNA
MLC	Myosin light chain
MRFs	myogenic regulatory factors
MST	mammalian ste20 lilke
MTJ	myotendinous junction
MuSK	muscle skeletal receptor tyrosine kinase
Mustn1	muscule, skeletal, embryonic nuclear protein 1
MYF5	myogenic factor 5
MYOD1	myogenic differentiation factor 1
MYOG	Myogenin
NATA	N-Acetyl Tryptophanamide
NC	Notochord
NFAT	nuclear factor of activated T cells
NLS	nuclear localisation signal
NMJ	neuromuscular junction
nPTB	polypyrimidine tract-binding protein
NRF	nuclear respiratory factor
NT	neural tube
nt	Nucleotide
PAX3	Paired box gene 3
PCR	polymerase chain reaction
PGC1-a	Peroxysome proliferator-activated receptor-g coactivator-1a
PP1	protein phosphatase 1
Pu/Py	polypurine/polypyrimidine
Pura	Purine-rich binding protein-a
Purb	Purine-rich binding protein-b
qPCR	quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RLC	regulatory light chain
SBP	Streptavidine binding peptide
SE	surface ectoderm
shh	Sonic Hedghog
siRNA	small interfering RNA
SM	smooth muscle
SNP	single nucleotide polymorphism
SR	sarcoplasmic reticulum
SRF	serum response factor
SSBP	single stranded DNA binding protein
SV40	simian virus 40
TA	transcriptioanl activation
TAF4b	TATA box binding protein(TBP)-associated protein 4b
TAg	T antigen
TAZ	transcriptional coactivator with PDZ-binding motif
TB	TEAD binding domain
TEAD	TEA domain transcription factor

TEF	transcription enhancer factor
Tm	Tropomyosin
Tn	Troponin
TNFa	tumour necrosis factor-alpha
TS	trophoblast stem
Tspan29	Tetraspanin CD9
UTR	untranslated region
UTRN	Utrophin
VGCC	voltage-gated calcium channel
VGCC	voltage-gated calcium channel
VGKC	voltage-gated potassium channel
VGLL	Vestigial like
WWTR1	WW-domain containing transcription regulator 1
YAP1	Yes/src associated protein 1
YBD	YAP1 binding domain

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INTRODUCTION

I. Skeletal muscle formation.

I.1. Somitogenesis.

During vertebrate embryogenesis, skeletal muscle arises in the embryo from the somites. The somites are masses of mesoderm distributed along the two sites of the neural tube and notochord. The somites, differentiate ventrally into the sclerotome and dorsally into the dermomyotome. The sclerotome contributes to the cartilage and bone of the vertebral column and ribs. The dermomyotome forms the overlaying derm of the back and the skeletal muscle of the body and limbs. The somite can be divided into epaxial and hypaxial domains according to an anatomical division of the body. The epaxial dermomyotome is adjacent to the neural tube and notochord and forms the deep back muscles. The hypaxial dermomyotome is localized ventrolaterally and gives rise to limb muscles and to the rest of the musculature of the body [Figure 1] (Buckingham et al., 2003).

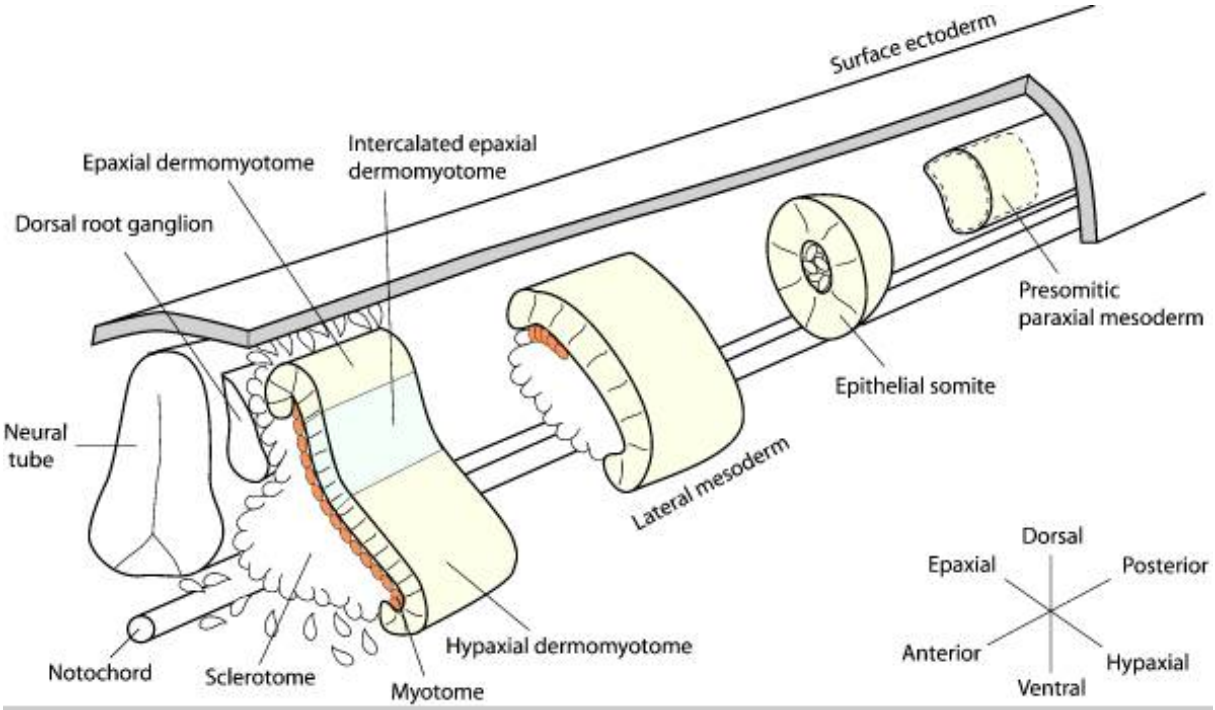

Figure 1. Schematic representation of somitogenesis.

(from Buckingham et al 2003)

Muscle progenitor cells, the premoyogenic cells, delaminate from the hypaxial dermomyotome in response to signals from the adjacent lateral plate mesoderm. The premoyogenic cells then migrate into the limb field were they proliferate, express myogenic determination factors and subsequently differentiate into skeletal muscle.

I.2. Moyogenic compartimentalisation of the somites.

The somites are initially formed as epithelial spheres that bud off the anterior end of the paraxial mesoderm. Somatic budding occurs sequentially in an anterior to posterior direction on either side of the neural tube (Parker et al., 2003). Within hours after somite epithelialisation, dorsoventral orientation becomes established by the formation of the epithelial dermomyotome dorsally and the mesenchymal sclerotome ventrally [Figure 1].

The interplay of diffusible signals secreted by neighbouring tissues induces the determination of the dermomoyotome (Cossu and Borello, 1999). Axial structures such as the notochord and the neural tube (Brand-Saberi et al., 1993 ; Pourquie et al., 1993) (Fan and Tessier-Lavigne, 1994) secrete signalling molecules such as sonic hedgehog (Shh) and Wnts that promote premoyogenic cell determination in epaxial dermomyotome (Fan and Tessier-Lavigne, 1994 ; Munsterberg et al., 1995). Hypaxial dermomyotome is subject to the effects of Wnt signalling from surface ectoderm (SE), in addition to negative Bone Morphogenetic Protein (Bmp) signalling from the nearby lateral plate mesoderm. [Figure 2]

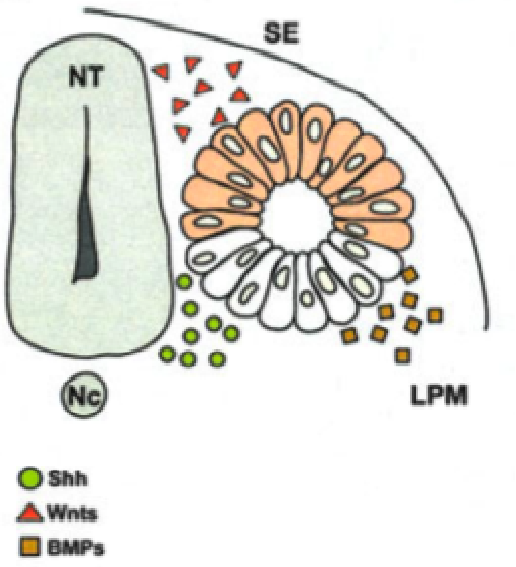

Figure 2. Dorsoventral organisation of the epithelial somite by Sonic Hedgehog, Wnts, and Bone Morphogenetic Proteins (BMPs). Shh signals (green circles) are secreted by notochord (NC) and the floor plate of the neural tube (NT). Wnts signals (red triangles) are secreted by the dorsal NT and the surface ectoderme (SE). BMPs (brown squares) are secreted by the lateral plate mesoderm (LPM).

(from Huh et al. 2005).

I.3. Delamination and migration of premyogenic cells.

Both delamination and migration depend on the expression of C-MET, a tyrosine kinase receptor, and the PAX3 transcription factor in premoyogenic cells. C-MET interacts with its ligand "scatter factor", also known as hepatocyte growth factor, produced by lateral plate mesoderm (Dietrich et al., 1999). In mutant mouse embryos which lack functional C-MET, skeletal muscle is absent from the limbs (Bladt et al., 1995). Transcription of the *Cmet* gene depends on PAX3 (Epstein et al., 1996). *Pax3* mutant mice also have no limb muscle and cells do not delaminate from the hypaxial dermomyotome (Tajbakhsh et al., 1997). PAX3 is initially expressed in hypaxial dermomyotome and is required for the epithelial mesenchymal-transformation of skeletal muscle precursor cells. Another transcription factor, LBX1 which is also regulated by PAX3 (Gross et al., 2000; Mennerich et al., 1998), is also implicated in the migration of the cells from the somite. In *Lbx1* mutant embryos, muscle progenitor cells delaminate from the dermomyotome, but remain in the vicinity of the somites where they can adopt other cell fates (Schafer and Braun, 1999). [Figure 3]

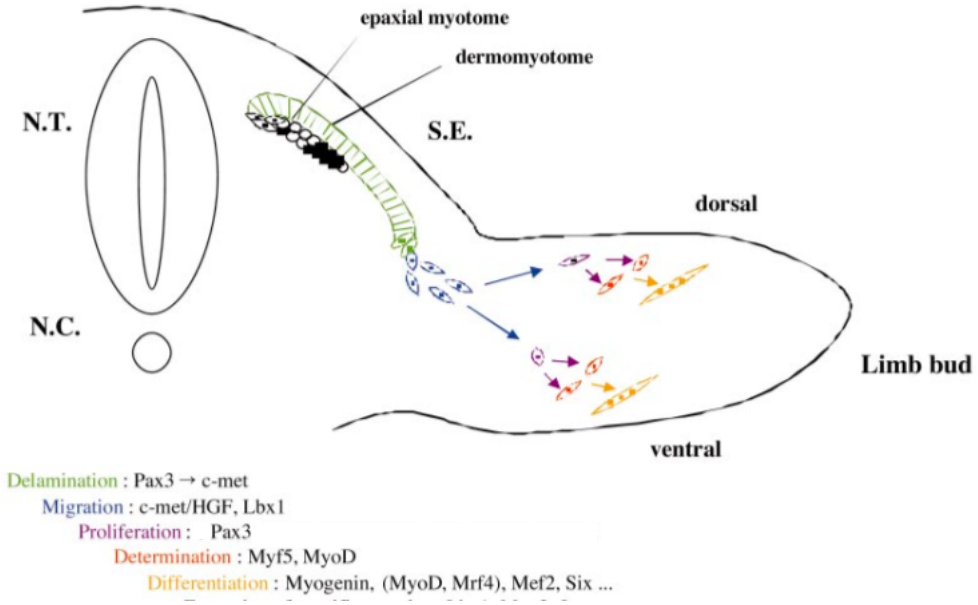

Figure 3. Schematic representation of skeletal muscle formation in the limb, with the different stages and the factors potentially involved in each stage. NC, notochord; NT, neural tube; SE, surface ectoderm.

(from Buckingham et al 2003).

The molecular signals that control delamination and migration processes are well characterized. Scatter factor and members of the Fibroblast Growth Factor (FGF) family have been shown to be major players. Both FGF and scatter factor can provoke delamination of the

lateral dermomyotome when applied ectopically into interlimb flank mesenchyme (Brand-Saberi et al., 1996 ; Heymann et al., 1996). FGF signalling appears to be upstream of scatter factor. Indeed, FGF signalling is able to induce ectopic scatter factor expression. In addition to inducing delamination, both scatter factor and FGFs act as a chemotactic source promoting migration towards limb bud (Itoh et al., 1996 ; Takayama et al., 1996; Webb et al., 1997).

I.4. Expression of myogenic factors, proliferation, differentiation in the limb.

During early development and migration, signals from the lateral plate mesoderm inhibit differentiation. The growth factor BMP4 has been shown to be a key inhibitory molecule, but FGFs and scatter factor also play a role (Pourquie et al., 1996). Once within the limb bud, the premoyogenic cells switch off *Pax3* and *Lbx1* expression and express myogenic regulatory factors (MRFs) (Gross et al., 2000; Uchiyama et al., 2000). These factors form a family of four basic-Helix-loop-Helix (bHLH) transcription factors: MYF5, MYOD1, MRF4 and Myogenin (MYOG). MYF5 and MYOD1 are expressed first and mark the initial onset to myogenic commitment whereas MRF4 and MYOG are expressed later. Genetic knock out of MRFs in mice clearly demonstrates that they are essential for myogenic differentiation. In double MYF5/MYOD1 mutants no skeletal muscle forms because the precursor myoblast population is absent (Rudnicki et al., 1993). In the absence of these factors, cells in the somite, which would normally become myoblasts, do not locate correctly to sites of myogenesis and adopt other cell fates (Tajbakhsh et al., 1996), thus demonstrating the role of MYOD1 and MYF5 as myogenic determination factors. Similarly, the MYOG null mouse has severe muscle defects. In this case, the myoblasts form but are unable to undergo terminal differentiation (Hasty et al., 1993 ; Nabeshima et al., 1993).

Differentiation starts in the proximal mesenchyme and then progresses distally as the limb bud develops and elongates. This elongation process is under the control of apical ectodermal ridge (AER), a region of thickened ectoderm at tip of the limb, which expresses a number of FGFs including FGF2, 4 and 8, which repress myogenic differentiation (Robson and Hughes, 1996). In addition to the AER, dorsal signals from the ectoderm are implicated in the control of myogenic differentiation through BMP signalling [Figure 4].

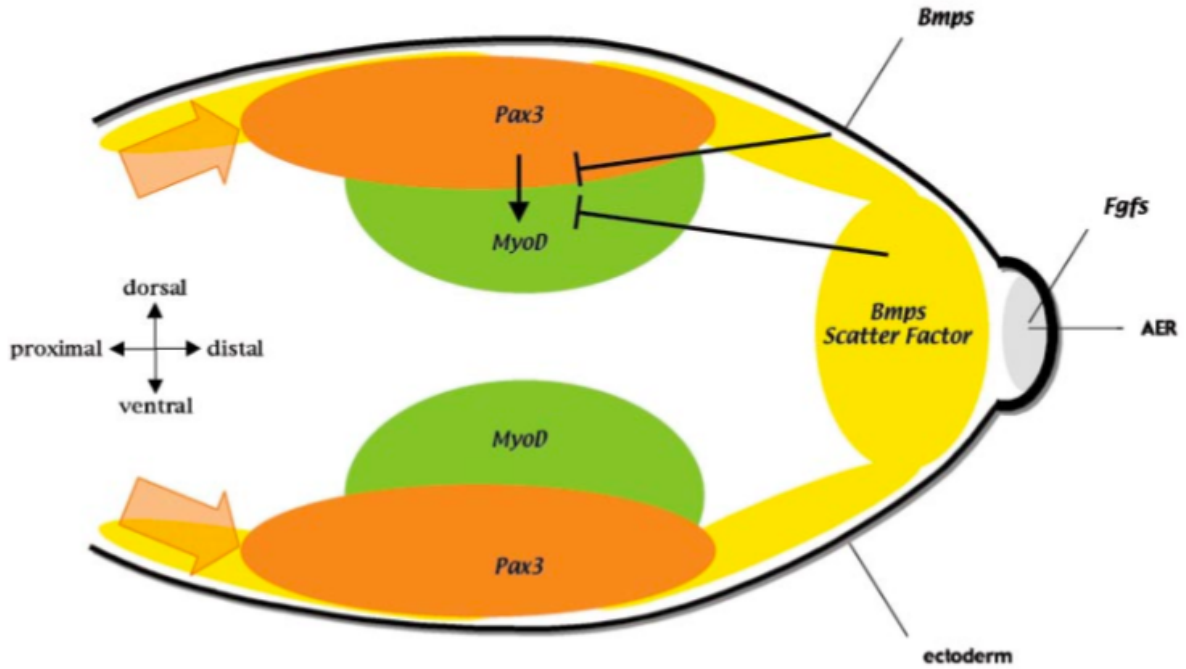

Figure 4. Signals that control migration and differentiation of the limb muscle precursors. The premoyogenic cells (expressing Pax3 and shown in orange migrate distally towards the AER (grey) which expresses FGFs and regulates scatter factor expression in the underlying mesenchyme (yellow). Once within the limb bud, a subpopulation of premoyogenic cells in the proximal limb bug starts to differentiate, switching on the expression of the myogenic regulatory genes, Myf5 and Myod1 (green). Cells committed to myogenesis are found towards the centre of the limb bud whilst the proliferative Pax3-expressing cells are found closer to the ectoderm. BMP signalling from the ectoderm and underlying mesenchyme, together with scatter factor in the mesenchyme (yellow) and FGFs in the AER, repress myogenic differentiation.

(From Francis-west et al. 2003).

Before skeletal muscle forms, the muscle precursor cells undergo extensive proliferation in dorsal and ventral mesenchyme and form two blocks that extend distally during limb overgrowth. Ectoderm signals, through BMP2 and BMP4, maintain PAX3 expression in cells that form a proliferative muscle-precursor pool. The proliferative myogenic cells are localised in the sub-ectodermal layer of the growing limb bud while the differentiating cells expressing MYOD1 are found more in the mesenchymal core. Muscle growth is dependent upon maintaining a balance between undifferentiated proliferative cells and differentiated cells. Removal of ectoderm inhibits muscle growth by depriving precursor cells of the proliferative signals and results in premature differentiation (Amthor et al., 1998). Furthermore, in the absence of ectoderm signals, progenitor cells down regulate PAX3 and up-regulate MYOD1 and cells begin to differentiate.

Within the limb field, the differentiation programme is marked by the expression of the MRFs. MYF5 is detected first and is soon followed by MYOD1. MRF4 is then transiently expressed and this is followed by MYOG expression. Subsequently myoblasts terminally differentiate and express slow or fast myosin heavy chain (MHC) isoforms, which determine the muscle fibre type. The MHC expressing myoblasts fuse into multinucleated myotubes and assemble to form the muscle fibre [Figure 5](Francis-West et al., 2003).

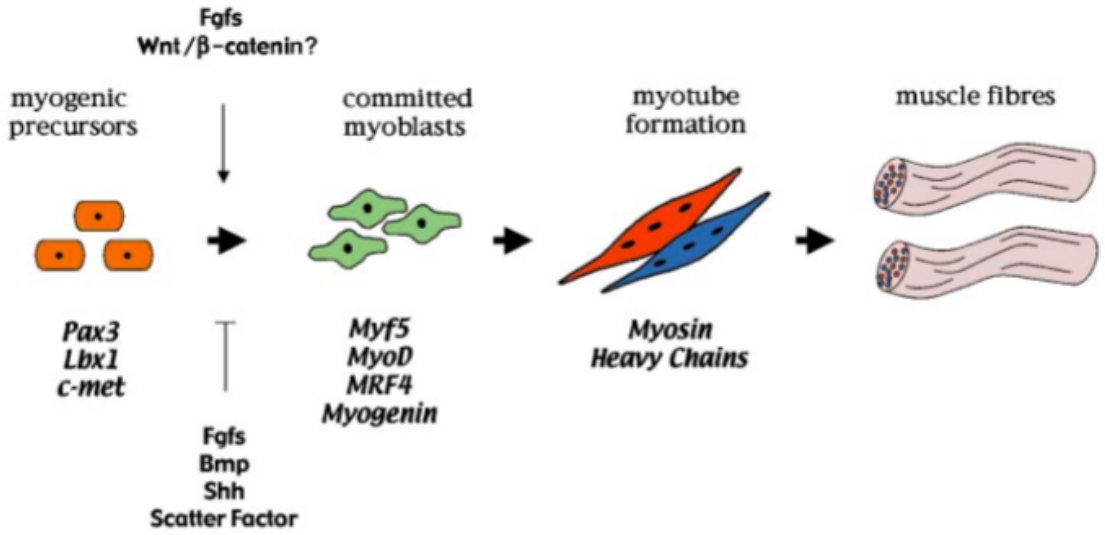

Figure 5. The regulation of myogenesis in the developing limb.

(from Francis-West et al, 2003)

I.5. Myoblast fusion.

The formation and growth of multinucleated myofibres from mononucleated myoblasts occur through a process called myoblast fusion. The cellular events of myoblast fusion are well characterized. Nevertheless the molecular mechanisms of plasma membrane fusion are less well understood.

I.5.1. Cellular processes of myoblast fusion.

Myoblast fusion is an ordered set of specific cellular events: recognition, adhesion, alignment, and membrane union (Horsley and Pavlath, 2004). Through a sequence of cellular interactions that are protein and calcium dependent, myoblasts recognize and adhere to one another during myotube formation (Knudsen and Horwitz, 1977). Following adhesion, alignment occurs through the parallel apposition of the membranes of elongated myoblasts with myotubes or other myoblasts (Wakelam, 1985). Membrane union then occurs between the aligned plasma membranes in small areas of cytoplasmic continuity. The pairs of plasma

membranes then undergo vesiculation in the fusion area (Lipton and Konigsberg, 1972 ; Rash and Fambrough, 1973), resulting in the formation of a single multinucleated cell.

I.5.2. Factors that regulate myoblast fusion.

Several intracellular, membrane associated, and transmembrane proteins as well as extracellular proteins and secreted factors have been found to regulate myoblast fusion (for a review see (Horsley and Pavlath, 2004; Rochlin et al.)). In this chapter I will focus on the role of Caveolin 3 and Dysferlin, the α7β1 integrin complex and Mustn1 muscle, skeletal, embryonic nuclear protein 1).

I.5.2.1. Caveolin 3 and Dysferlin in myoblast fusion.

Caveolin 3 is the principal structural component of caveolae in skeletal muscle cells. Caveolae are invaginations in the plasma membrane involved in vesicular trafficking and signal transduction. Interestingly, vesicular trafficking has been implicated in the process of myoblast fusion (Doberstein et al., 1997; Doherty et al., 2008). Caveolin 3 is the muscle specific member of the Caveolin family (Song et al., 1996). The expression of Caveolin 3 is induced during the differentiation of skeletal myoblasts and is localized to the sarcolemma where it forms a complex with Dystrophin and its associated glycoproteins (Song et al., 1996). Myoblasts lacking Caveolin 3 differentiate but do not fuse, implicating this protein in the fusion process (Galbiati et al., 1999).

Dysferlin is a 230 kDa, membrane-anchored protein that is abundantly expressed in skeletal and cardiac muscle (Anderson et al., 1999). A role for Dysferlin in membrane fusion was initially suggested by its homology to the Caenorhabditis elegans protein FER1, which is essential for vesicle fusion to the sperm plasma membrane (Achanzar and Ward, 1997). Dysferlin, like FER1, contains multiple C2 domains that are found in a number of membrane-associated proteins, including those involved in membrane fusion events, such as the synaptotagmins. The first C2 domain of Dysferlin binds to negatively charged phospholipids in a calcium-sensitive manner (Davis et al., 2002). Recently Dysferlin knockdown has been shown to lead to reduced efficiency of myoblast fusion (Belanto et al.). The loss of Dysferlin does not completely block the fusion process, but rather renders it less efficient. This could be because of functional compensation with Myoferlin.

Myoferlin is another transmembrane protein of the ferlin family that is expressed in skeletal muscle and induced during C2C12 differentiation. Myoferlin and Dysferlin amino acid sequences are 74% similar and both contain six cytoplasmic C2 domains (Doherty et al.,

2005). The first C2 domain of both proteins binds negatively charged phospholipids in the presence of calcium (Davis et al., 2002) and both proteins play redundant roles in myoblast fusion (Doherty et al., 2005). It has been shown that the endocytic recycling protein Eps15 homology domain protein (EHD2) interacts with Myoferlin to regulate myoblast fusion (Doherty et al., 2008). Introduction of dominant negative EHD2 into myoblasts leads to the sequestration of Myoferlin and inhibition of myoblast fusion. This interaction constitutes a molecular overlap between the endocytic recycling pathway and the machinery that regulates myoblast membrane fusion. Doherty et al. proposed a model in which Myoferlin and EHD2 are required for recycling transmembrane proteins implicated in myoblast fusion and myogenesis [Figure 6] (Doherty et al., 2008).

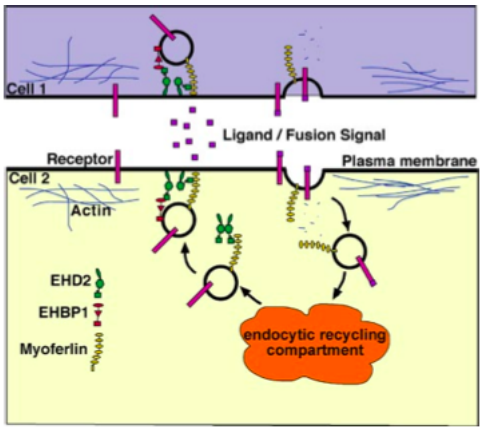

Figure 6. Model for the role of myoferlin and EHD2 in vesicle cycling and myoblast fusion. Many cell surface, membrane-bound receptors undergo recycling after endocytosis. These receptors and their bound ligand are internalized, shuttled to the endocytic recycling compartment, and then are recycled back to the plasma membrane where they can function in another round of ligand binding. EHD2 and myoferlin are implicated in vesicle cycling. It is hypothesized that improper endocytic recycling of receptors involved in propagating myoblast fusion signals could cause myoblast fusion defects due to an interruption in the receptor signaling cascade.(From Doherty et al. 2008)

Dysferlin associates with Caveolin 3 in the sarcolemma of normal muscle where they function together in membrane repair (Matsuda et al., 2001; Wallace and McNally, 2009). Caveolin 3 is required for localisation of Dysferlin to the sarcolemma. In fact, Dysferlin accumulates in the Golgi apparatus of Caveolin 3-null cells (Hernandez-Deviez et al., 2006). Recently, Caveolin 3 has been implicated in maintaining normal levels of Dysferlin at the muscle membrane by preventing its endocytosis as well (Hernandez-Deviez et al., 2008).

I.5.2.2. Molecular mechanisms of myoblast fusion: the role of Caveolin 3 and Dysferlin.

In normal muscle, membrane patches enriched in Dysferlin can be detected in response to sarcolemma injuries (Bansal et al., 2003). Dysferlin-null muscle cells are deficient in calcium-dependent membrane repair (Bansal et al., 2003; Lennon et al., 2003). In addition, electron micrographs of muscles Dysferlin-null mice show an accumulation of vesicles at the sarcolemma (Bansal et al., 2003 ; Ho et al., 2004). A model has been proposed in which Dysferlin, serves to recruit vesicles to the site of membrane disruption to generate the enriched patch [Figure 7] (Wallace and McNally, 2009).

Mutations in the Dysferlin gene cause limb girdle muscular dystrophy type 2B (LGMD2B) (Bashir et al., 1998) and Miyoshi myopathy (Liu et al., 1998).

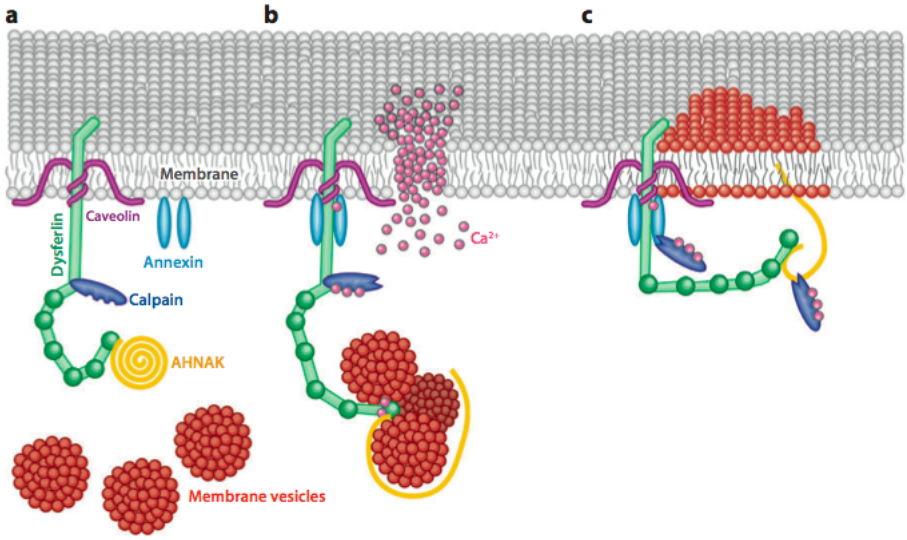

Figure 7.A model for Dysferlin-mediated membrane repair. (a) Dysferlin (green) is localized at the sarcolemma as part of a membrane repair complex. Caveolin 3 (purple), a muscle-specific Caveolin, interacts with Dysferlin. Dysferlin also interacts with Desmoyokin (AHNAK) (yellow) and Calpain (dark blue), a calcium-activated protease. (b) Tears in the sarcolemma result in an influx of calcium (pink spheres), which activates and alters the binding properties of proteins in the membrane repair complex. Annexins (light blue) bind Dysferlin and phospholipids with higher affinity in the presence of calcium, the C2A domain of Dysferlin binds phospholipids in a calcium-dependent manner, and calpains are activated. These interactions are thought to encourage the recruitment of internal vesicle structures (red). (c) Within seconds of activation, membrane lesions are resealed, calcium concentrations are normalized, and the repair complex is deactivated. (from Wallace et al. 2009)

Interestingly, in electron micrographs, intracellular vesicles are seen at sites of membrane contact during fusion in cultured chick myoblasts (Kalderon and Gilula, 1979), and in *Drosophila* embryos (Doberstein et al., 1997). In *Drosophila* myoblasts, these vesicles, called prefusion complexes, are juxtaposed within each of the apposed cells at high density to create electron-dense areas of dual plasma membrane. The dual plasma membrane region undergo vesiculation resulting in cytoplasmic continuity between the apposing cells [Figure

8] (Doberstein et al., 1997).

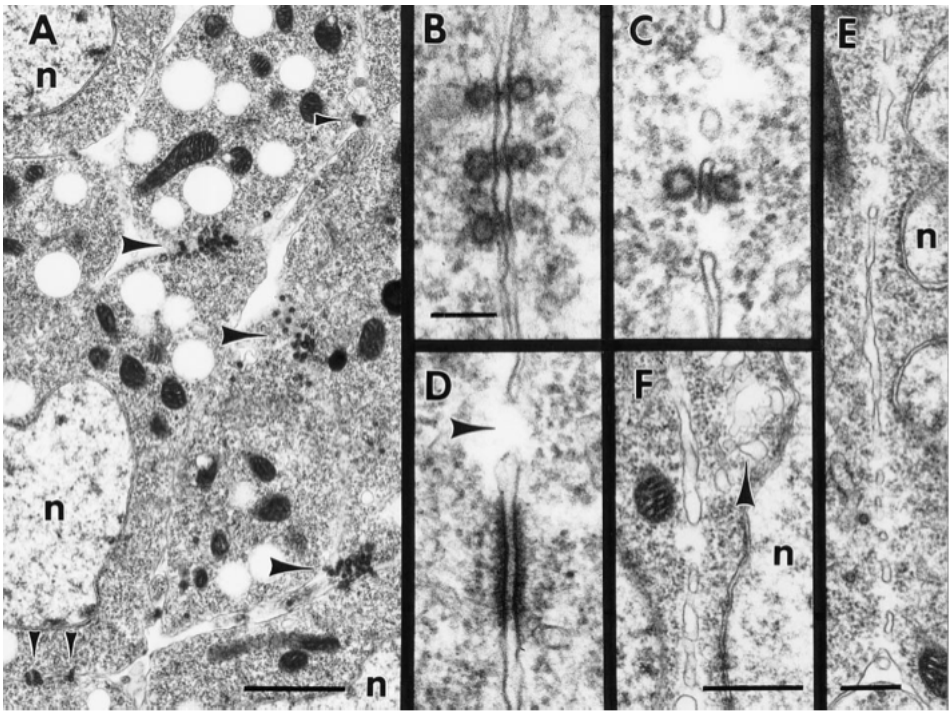

Figure 8.Ultrastructure of intermediate steps in myoblast fusion. Electron micrographs of wild-type myoblast fusion in early stage 13 embryos. All stages of the fusion process occur simultaneously in various parts of the developing musculature. (A) Myoblasts in early stage of fusion. Note prefusion complexes at points of cell–cell contact (arrowheads); n indicates myoblast nuclei. (B) Three sets of paired vesicles. Note electron-dense material in the extracellular space between pairs of vesicles. (C) Paired vesicles oriented across a vesiculating pair of plasma membranes. (D) An electron-dense plaque near a region of actively fusing membrane; note fusion pore (arrow). (E) Fusion pores in a vesiculating plasma membrane. The cytoplasm within and beneath the pore is free of staining material such as ribosomes. (F) Later stage vesiculating plasma membrane. The membrane sacs have increased in width and a group of irregular clear vesicles is present (arrowhead). Bars: (A) 1μm; (B–D) 100 nm; (E) 250 μm; (F) 500 μm. (from Doberstain et al. 1997)

The initial alignment of vesicles across the electron-dense, dual plasma membrane is thought to be highly critical. *Drosophila* (myoblast city) *mbc* mutants show neither vesicle alignment nor an electron-dense membrane, and, in this model, myoblasts completely fail to fuse (Doberstein et al., 1997).

I.5.2.3. The α7β1 integrin complex and myoblast fusion.

Recent work has implicated the α7β1 integrin complex to myoblast fusion (Schwander et al., 2003 ; Quach et al., 2009). β1 integrin heterodimerises with α7-integrin to form the α7β1 integrin multimeric protein complex, that localize to costameres and myotendinous junctions (MTJ) of skeletal muscle cell and link the extracellular matrix (ECM) component to

the actin cytoskeleton in muscle cells (Wallace and McNally, 2009). The extracellular domain of integrins binds directly to ECM (Brakebusch and Fassler, 2003) while the intracellular domain associates with proteins such as Talins to mediate binding to the actin cytoskeleton. The cytoplasmic domain of β1 integrin interacts with Talin 1, which interacts with F-actin, establishing a link between β1 integrin and the cytoskeleton (Nayal et al., 2004).

Ablation of the murine β1 integrin subunit gene, which leads to loss of all αβ1 integrins, causes defects in myoblast fusion. In fact, β1 integrin-deficient myoblasts adhere to each other, but plasma membrane breakdown is defective. The integrin-associated tetraspanin CD9 (*Tspan29*) gene that regulates cell fusion is no longer expressed at the cell surface of αβ1 integrin deficient myoblasts, suggesting that α7β1 integrins regulate the formation of a protein complex important for fusion (Schwander et al., 2003).

Talin 1 and Talin 2 have been shown to be required for mediating β1 integrin functions in myoblast fusion (Conti et al., 2009). Inactivation of Talin 1 in skeletal muscle leads to a progressive myopathy, caused by mechanical failure of MTJs (Conti et al., 2008). This phenotype resembles the defect observed in mice with a mutation in the gene encoding the integrin α7 subunit (Mayer et al., 1997 ; Miosge et al., 1999), suggesting that Talin1 mediates α7β1 integrins functions at MTJs. The Talin 1 deficient mice did not show the defects in myoblast fusion and sarcomere assembly that have been observed in β1 integrin deficient mice (Schwander et al., 2003). This may be due to functional compensation between Talin 1 and 2. Indeed, it has been shown that Talin 1 and Talin 2 have redundant functions in integrin-mediated attachment of fibroblasts in culture (Zhang et al., 2008c).

I.5.2.4. MUSTN1 and myoblast fusion.

Mustn1 (Mustang, or muscle, skeletal, embryonic nuclear protein 1) encodes a small 82 amino acid nuclear protein that is expressed uniquely in murine adult skeletal muscle and tendon (Lombardo et al., 2004). *Mustn1* was recently shown to be required to myoblast fusion (Liu et al.). *Mustn1*-silenced myoblasts elongated poorly and were mononucleated with decrease in the expression of myofusion markers Calpain 1, Caveolin 3, and Cadherin 15 (M-cadherin; Cadh15) as well as the MRFs, MYOD1 and Myogenin. Further studies are needed to elucidate the role of mustn1 in the process of myoblast fusion.

II. Skeletal muscle structure.

II.1. Anatomical organisation of skeletal muscle.

Skeletal muscle consists of a heterogeneous population of multinucleated, striated myofibres held together by connective tissue. The connective tissue, which surrounds both individual myofibres and bundles of them, is rich in blood vessels and innervation. At the end of the muscle fibre, the connective tissue extends as a tendon that attaches the muscle to the skeleton [Figure 8].

The skeletal muscle is composed of several layers of connective tissues:

- The endomysium surrounds each muscle fibre.
- The fascicle includes several muscle fibres together into a bundle.
- The perimysium assembles several fascicles.
- The epimysium covers the whole muscle.

Skeletal muscle is innervated and highly vascularised by penetration of blood vessels into the epimysium with branches into the peri- and endomysium. The terminal branches of motor neurons lie in troughs on the surface of the muscle cell, where the plasma membrane is highly folded. Each fibre is innervated by a single axon.

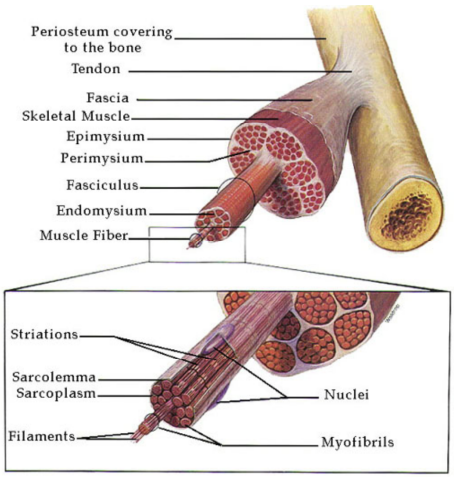

Figure 8. The relationship between muscle fibres and the connective tissues of the tendon, epimysium, perimysium, and endomysium. Close-up shows an expended view of a single muscle fibre. (Fig. 12.2 from Fox, S.I. Human physiology, 4th Ed. Wm.C Brown, publ.)

II.2. Cellular organisation of skeletal muscle.

Myofibres, also called muscle cells, are roughly cylindrical, with diameters between 10 and 100 µm, but up to a few centimetres long. Each cell is embedded in a basal lamina of collagen and large glycoproteins. Between the fibre and the basal lamina numerous satellite cells are important in the growth and repair of the fibre. Myofibres contain up to thousand nuclei derived from the fusion of myoblasts in fetal and postnatal life. Most of the myofibre nuclei are located peripherally beneath the sarcolemma. The fibres are further composed of

myofibrils, membranes, and cytoskeletal network, which anchor the contractile fibrils to the sarcolemma.

II.2.1. Satellite cells.

Satellite cells are adult skeletal muscle stem cells located at the periphery of skeletal myofibres. Satellite cells are quiescent and under the appropriate conditions they proliferate for both self-renewal and differentiation into myoblasts and myotubes.

II.2.1.1. Embryonic origin of satellite cells.

Early experiments using quail-chick chimeras suggested that satellite cells derive from the somite (Armand et al., 1983). Recent experiments support this work define satellite cells as a population of undifferentiated stem cells originating from the dorsal part of the somite: the dermomyotome. Using green fluorescent protein (GFP) labelled cells in conjunction with quail-chick grafting Gros et al. showed that embryonic muscle progenitors and satellite cells share a common origin that can be traced back to the dermomyotome (Gros et al., 2005). Similarly Relaix et al. identified a population of myogenic progenitor cells originating from the somite and expressing the Pax3 and Pax7 transcription factors, but no skeletal muscle specific markers that are able to contribute to the satellite cell pool (Kassar-Duchossoy et al., 2005; Relaix et al., 2005).

II.2.1.2. Histological characterisation of adult muscle satellite cells.

Satellite cells were first described by Mauro et al, based on their morphological characteristics (Mauro, 1961). Muscle satellite cells can easily be identified by electron microscopy due to their distinct location between the plasma membrane of muscle fibre and the basal lamina (Mauro, 1961). They have an increased nuclear to cytoplasmic ratio, a reduced organelle content, and smaller nuclei than those of the myotube [Figure 9] (Charge and Rudnicki, 2004).

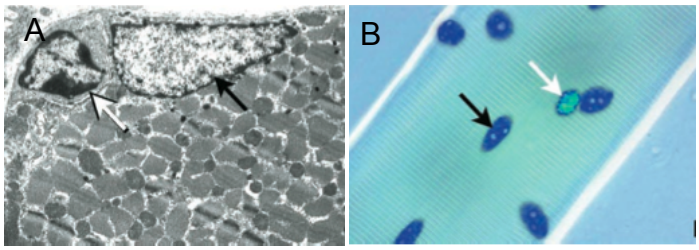

Figure 9. Morphological characteristics of adult mammalian satellite cell nuclei and myofibre nuclei (myonuclei). A. Muscle satellite cell nuclei (white arrow) can be distinguished from myonuclei (black arrow) by their abundant heterochromatin reflecting their mitotic quiescence. B. Muscle satellite cells are present on myofibres isolated by mild enzymatic digestion and are characterized by their high levels of Pax7 expression as demonstrated by immunocytochemistry (white arrow) compared with myonuclei (black arrow).
(from charge, 2004)

Satellite cells are present in all skeletal muscles and are associated with all muscle fibre types, albeit with unequal distribution. High numbers of satellite cells are found associated with slow muscle fibres compared with fast fibres within the same muscle (Gibson and Schultz, 1982). The satellite cell population varies also with age. At birth the number of satellite cells is the highest in decreases with age (Gibson and Schultz, 1983).

II.2.1.3 Muscle satellite cell function: muscle repair.

In the adult, satellite cells are mitotically quiescent, but they can be activated upon mechanical trauma, muscle injury or during degenerative diseases. Moreover, when transplanted into regenerating muscle, satellite cells contribute to new muscle fibre formation and contribute to satellite cell population for later rounds of regeneration (Heslop et al., 2001).

Upon exposure to signals from a damaged environment, satellite cells are first activated to exit their quiescent state to start proliferating. At the molecular level, activated satellite cells are characterized by the rapid up-regulation of MYF5 and MYOD1 (Cooper et al., 1999). Satellite cells then proliferate, express MYOG and MRF4, begin their terminal differentiation programme and fuse to each other to form new myofibres or to damaged myofibres for repair (Smith et al., 1994). During the course of muscle regeneration a subset of satellite cells reenters the quiescent state to replenish the satellite cell pool for subsequent muscle repair [Figure 10] .

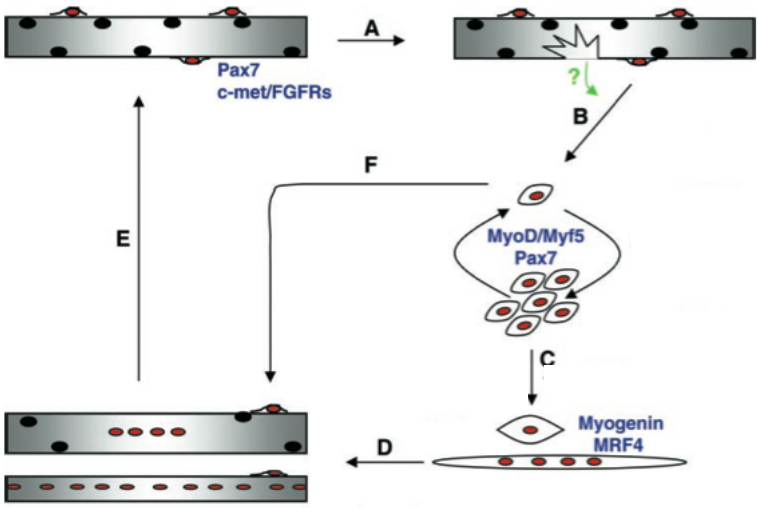

Figure 10. Schematic representation of the molecular events regulating muscle satellite cell activation during skeletal muscle regeneration. Following damage to the myofibre (A), quiescent satellite cells are activated to enter the cell cycle and proliferate, allowing for expansion of the myogenic cell population (B). Activated satellite cells are characterized by high expression of the MRFs MYOD1 and MYF5. The proliferative phase is followed by terminal differentiation (C) and fusion of myoblasts to damaged myofibres for repair or to each other for new myofibre formation (D). Myoblast terminal differentiation is characterized by the up-regulation of the MRFs MYOG and MRF4. Finally, repaired or new myofibres grow to resemble original myofibres (E). During the course of muscle regeneration, a subset of myoblasts reenters the quiescent state to re- plenish the satellite cell pool for subsequent muscle repair. (Modified from Charge, 2004)

Satellite cell self-renewal is a necessary process without which recurrent muscle regeneration would rapidly lead to the depletion of the satellite cell pool. The balance between self-renewal and differentiation is crucial for stem cell maintenance and tissue homeostasis. Recent advances have revealed a critical role for asymmetric division in satellite cell self-renewal *in vivo* and *in vitro*. First, asymmetric cosegregation of older and younger DNA strands into different daughter cells was documented in a proportion of satellite cells during muscle growth and regeneration (Shinin et al., 2006). Second, Numb-mediated asymmetric divisions have been observed during satellite cell proliferation (Holowacz et al., 2006). Third, Kuang et al. have demonstrated that a noncommitted Pax7+/Myf5- satellite cell can asymmetrically generate a self-renewal Pax7+/Myf5- and a committed Pax7+/Myf5+ daughter cell *in vivo* (Kuang et al., 2007). Together, these data support the notion that satellite cells divide asymmetrically to give rise to self-renewing satellite cells and to committed differentiating daughter cells to maintain the homeostatis of skeletal muscle tissue.

II.2.1.4. *In vitro* model of myogenic differentiation.

C2C12 myoblasts, the most common *in vitro* model of myogenic differentiation used to date, were originally derived from satellite cells (Yaffe and Saxel, 1977). C2C12 cells are a subclone of C2 myoblasts, which spontaneously differentiate in culture after serum removal and provide a useful experimental model to study myogenesis and muscle differentiation.

The temporal events that occur during C2C12 differentiation are well characterised [Figure 11] Andres, 1996 #735}.

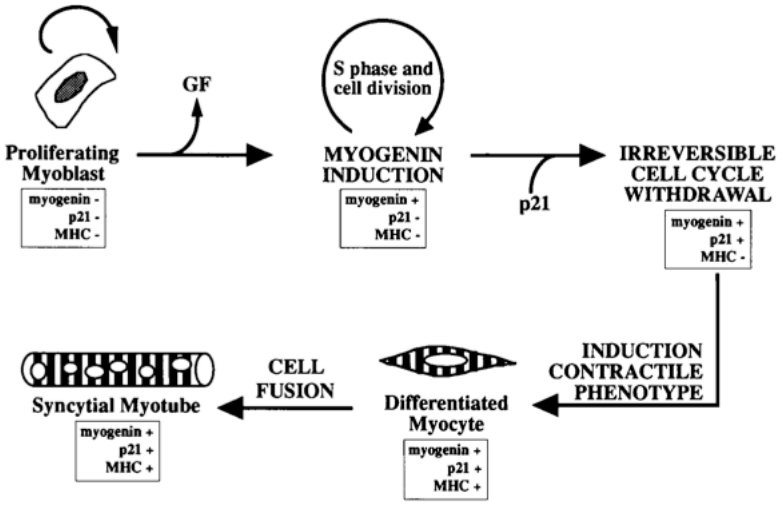

Figure 11. Model for the myogenic differentiation of C2C12 cells. This model shows the temporal relationship between myogenin expression and the subsequent myogenic events, but does not imply any causal relationship. The phenotype associated with each of the stages of myoblasts and differentiating myocytes is boxed. Myoblasts induce myogenin expression after removal of growth factors and remain capable of replicating DNA. Subsequently, myogenin positive myocytes withdraw from the cell cycle, then phenotypically differentiate, and finally fuse to form multinucleated syncytial myotybes.(From Andrés et al. 1996)

II.2.2. The myofibre.

II.2.2.1. The myofibrils.

Myofibrils are bundles of highly organized filaments that extend the length of the cell and are composed of two types of filaments: thick Myosin filaments (about 15 nm in diameter) and thin Actin filaments (about 7 nm in diameter). Each myofibril is organized as a chain of contractile units called sarcomeres. This organisation is largely responsible for the striated banding pattern observed by light and electron microscopy (Gregorio and Antin, 2000).

II.2.2.2. The sarcomere.

The sarcomere is both the structural and the functional unit of skeletal muscle. The sarcomere, which is approximately 2.3 μm long, consists of several distinct regions,

distinguishable by electron microscopy as dark and light regions. Sarcomeres are delineated at their ends by the Z disc where thin Actin filaments of opposite directions are linked together by α-Actinin dimers (Luther, 2000). Within each sarcomere a dark band, called the A band, alternates with a light band, called the I band. These bands correspond to the presence or absence of Myosin filaments. The I bands contain only thin Actin filaments, whereas the A bands contain thick Myosin filaments. The Myosin and Actin filaments overlap in peripheral regions of the A band, whereas a middle region, called the H zone, contains only Myosin. The Actin filaments are attached to the Z disc. The Myosin filaments are anchored at the M line in the middle of the sarcomere. Thick filaments are connected to giant Titin molecules (3000kDa) expanding to half of a sarcomere, from Z-line to M-line (Luther, 2009). Titin is thought to function as a spring and a ruler defining sarcomere length after muscle contraction (Gautel et al., 1999). Nebulin (800 kDa) runs along the thin filaments and overlaps in the Z-disc [Figure 12].

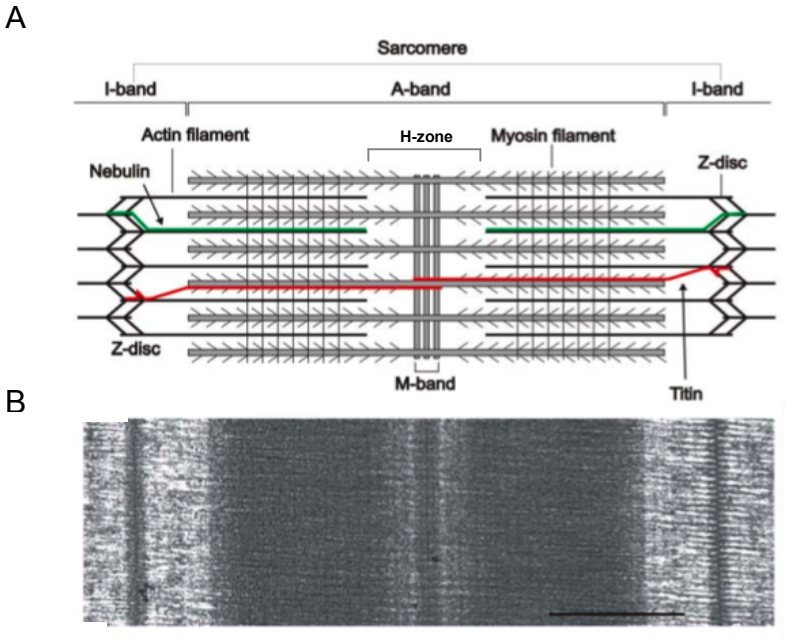

Figure 12. Striated muscle sarcomere. A. Schematic diagram showing the main components of the sarcomere. Electron micrography of a longitudinal section of fish white (fast) muscle showing details of the sarcomere. (modified from Luther, 2009)

II.2.2.2.1. Thick filaments.

The major component of the thick filaments is Myosin II. Myosin II or "conventional Myosin" is a hexameric molecule composed of a pair of heavy chains, and two copies of two light chains (Craig and Woodhead, 2006).

- The heavy chains: heavy chain is an elongated protein consisting of an amino-terminal globular head domain (also called subfragment 1 or S1) which harbors Actin binding and motor activities, followed by a long alpha-helical coiled-coil tail (tail domain), which mediates heavy chain dimerisation. The tail is divided into distal light meromesin (LMM) responsible for self-association and more soluble proximal subfragment 2 (S2), which acts as a flexible link between LMM and the heads [Figure 8].
- The light chains: the two light chains are referred to as the essential light chain (ELC) and the regulatory light chain (RLC). Phosphorylation of the RLC regulates the activity of entire molecule [Figure 13].

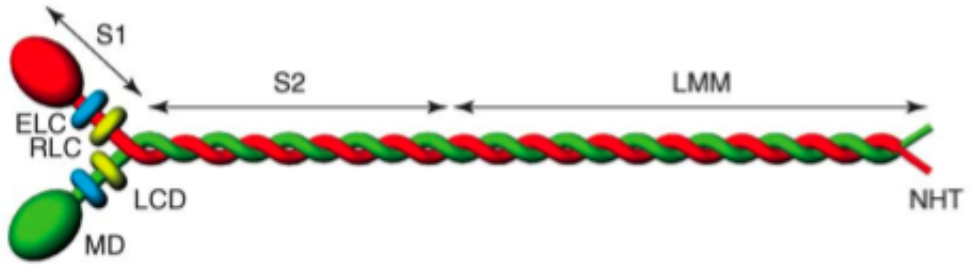

Figure 13. Schematic representation of the Myosin molecule.
Heads (S1) comprise the motor domain (MD) and light chain domain (LCD), which contains the essential light chain (ELC, blue) and the regulatory light chain (RLC, yellow). The tail is a coiled-coil formed by the C-terminal halves of each heavy chain (red and green). (From Craig et al, 2006)

Thick filaments have a bipolar organisation formed by the association of Myosin tails running approximately parallel to the filament axis with head of the molecule pointing away from the filament centre. Hence, the middle of a thick filament is devoid of heads of the molecule.

II.2.2.2.2. Thin filaments.

Thin filaments are composed essentially of Actin and two regulatory proteins: Tropomyosin and Troponin [Figure 14]. Actin polymerizes spontaneously to form the backbone of the thin filament, called F-Actin, which has a two-stranded helical structure. Each Tropomyosin molecule is bound to Troponin, which is a complex of three polypeptides: Troponin C (Ca^{2+}-binding), Troponin I (inhibitory of Actomyosin interaction), and Troponin T (Tropomyosin-binding). When the concentration of Ca^{2+} is low, the complex of the Troponins with Tropomyosin blocks the interaction of Actin and Myosin, so that the muscle does not contract. At high concentrations, Ca^{2+} binding to Troponin C shifts the position of the complex, relieving this inhibition and allowing contraction to proceed (Gordon et al., 2000).

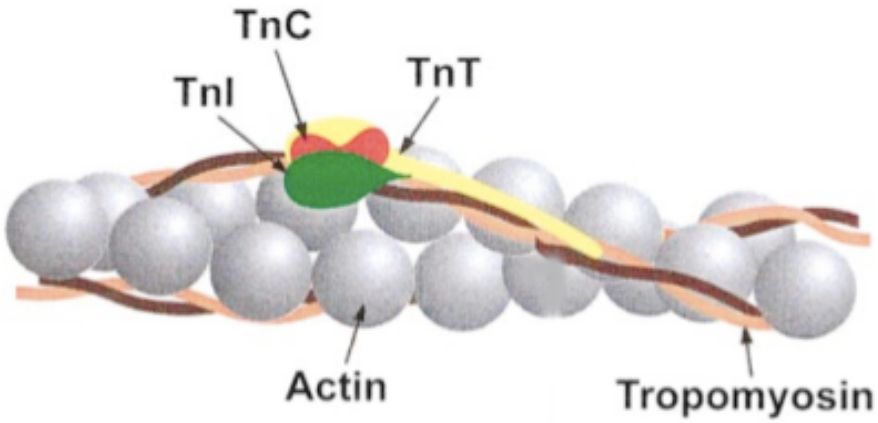

Figure 14. A model of the molecular arrangement of Troponin (Tn), Tropomyosin (Tm), and Actin. TnC: Troponin C. Tn I: Troponin I. TnT: Troponin T.
(Modified from Gordon et al. 2000)

II.2.2.3. The non-contractile cytoskeleton.

The non-contractile cytoskeleton can be sub-divided into: the intra-sarcomeric cytoskeleton, the peri-sarcomeric cytoskeleton, and the sub-sarcolemmal cytoskeleton. The non-contractile cytoskeleton provides linkage and mechanical coordination between adjacent myofibrils and links the contractile apparatus to the sarcolemma and indirectly to the extracellular matrix (Berthier and Blaineau, 1997).

II.2.2.3.1. The intra-sarcomeric cytoskeleton.

The intra-sarcomeric cytoskeleton is composed essentially of Titin and Nebulin, which act as linear blueprints or rulers for thick and thin filaments respectively. Titin spans half of the sarcomere from Z disc to M band and interacts with numerous sarcomeric proteins along its length. For example, the C terminus of Titin interacts with Myosin and M band proteins, such as Myomesin, to facilitate incorporation of Myosin thick filaments into the sarcomere. Nebulin is a large linear side-binding protein (700kDa) of the Actin thin filament, and the length of Nebulin corresponds to the length of thin filaments from Z disc to pointed end of the filaments (Ferrari et al., 2006).

II.2.2.3.2. The peri-sarcomeric cytoskeleton.

Peri-sarcomeric cytoskeleton is essentially formed by desmin-containing intermediate filaments and provides linkage and mechanical coordination between adjacent myofibrils. Desmin filaments laterally interlink Z-discs, perhaps via their interaction with Nebulin and integrate the myofibrils with the sarcolemma, nuclei, mitochondria and, possibly microtubules (Clark et al., 2002). Other components of the intermediate filament are Vimentin, Nestin,

Synemin and Paranemin. Nevertheless, their role in peri-sarcomeric cytoskeleton is less established (Clark et al., 2002).

II.2.2.3.3. The sub-sarcolemmal cytoskeleton.

The sub-sarcolemmal cytoskeleton is thought to provide a linkage between the peripheral myofibrils and the sarcolemma and indirect connection to the extracellular matrix (ECM). It has important physiological roles in stabilizing the sarcolemma and transmitting the force of contraction to the ECM. It can be divided into junctional and non-junctional domains. The junctional sub-sarcolemmal cytoskleton is found in connection with myotendinous junction (MTJ), neuromuscular junction (NMJ) and costameres (the costameres are repetitive sub-sarcolemmal units present all along the sarcolemma outside the MTJ and the NMJ).

Three types of cytoskeletal systems are found in junctional domains of the sub-sarcolemmal cytoskeleton :

- The focal adhesion-type membrane cytoskeleton is the main system providing cellular attachment to the ECM through integrins. Integrins are transmembrane receptors consisting of a large extracellular domain, transmembrane domain and a short cytoplasmic domain. The transmembrane domain mediates interactions with ECM proteins such as collagens, laminin, and fibronectin while the cytoplasmic domain interacts with Actin-associated cytoskeletal proteins such as Talin (Horwitz et al., 1986) and α-Actinin (Pavalko et al., 1991). Other components of the focal adhesion-type membrane cytoskeleton are Vinculin, Tensin and Paxilin (Berthier and Blaineau, 1997).
- Spectrin-based membrane cytoskeleton: this macromolecular structure is composed of Spectrins, Actin and associated proteins such as Ankyrin and γ-Actin. The different components of the Spectrin-based membrane cytoskeleton are present, all together or not, at various sarcolemmal sites (NMJ, costameres). This system plays a role in membrane stabilisation and may be important in organizing heterogeneous membrane domains by preventing free diffusion of intrinsic membrane proteins (Berthier and Blaineau, 1997).
- Dystrophin based membrane cytoskeleton: this system is composed essentially of Dystrophin and Dystrophin-associated proteins (DAP). Among DAPs are Caveolin 3 and Syntrophins. DAP are proteins and glycoproteins which mediate sarcolemmal anchorage of Dystrophin. This system plays a role in stabilizing the sarcolemma and maintaining its integrity during skeletal muscle contraction (Menke and Jockusch, 1991) [Figure 15].

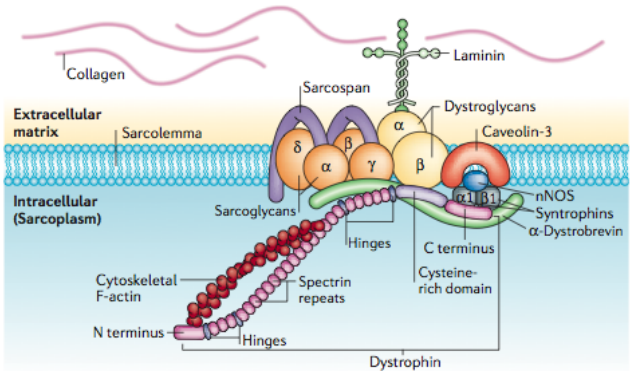

Figure 15. Dystrophin binds to the dystrophin-associated protein complex (DAPC) at the sarcolemma. Dystrophin, which is localized at the sarcolemma, has a long central rod domain made up of spectrin repeats, which are interspersed with hinge regions. The C terminus is preceded by a cysteine-rich domain. Dystrophin binds to the Dystrophin-associated protein complex (DAPC) through its C terminus. The DAPC is comprised of sarcoplasmic proteins (α-Dystrobrevin, Syntrophins and neuronal nitric oxide synthase (nNOS)), transmembrane proteins (β-Dystroglycan, the Sarcoglycans, Caveolin 3 and Sarcospan) and extracellular proteins (α-Dystroglycan and Laminin). Many members of the DAPC are also associated with muscular dystrophy, either owing to mutations in the genes that encode them (for example, α-, β-, γ- and δ-Sarcoglycan, Laminin or Caveolin 3), or through mutant binding partners (for example, nNOS, Syntrophin, α-Dystrobrevin, α-Dystroglycan, β-Dystroglycan or Sarcospan). The N terminus of Dystrophin binds to the cytoskeleton through filamentous F-Actin. Therefore, the DAPC provides a strong mechanical link between the intracellular cytoskeleton and the extracellular matrix. Loss of sarcolemmal integrity is thought to occur when a mutant protein of the DAPC is expressed, resulting in muscle fibres that are more susceptible to damage.

(from Davies et al. 2006)

II.2.2.4. Sarcoplasmic reticulum and T-tubule.

The sarcoplasmic reticulum (SR) is a specialized endoplasmic reticulum that stores calcium ions needed for muscle contraction. The release of Ca^{2+} from the SR occurs upon nerve impulses from motor neurones.

T-tubule (or transverse tubule) is a deep invagination of the sarcolemma that is perpendicular to the length of muscle cell [Figure 16]. The contact zone between T-tubules and SR is called triad. The triad plays an important role in calcium release from SR.

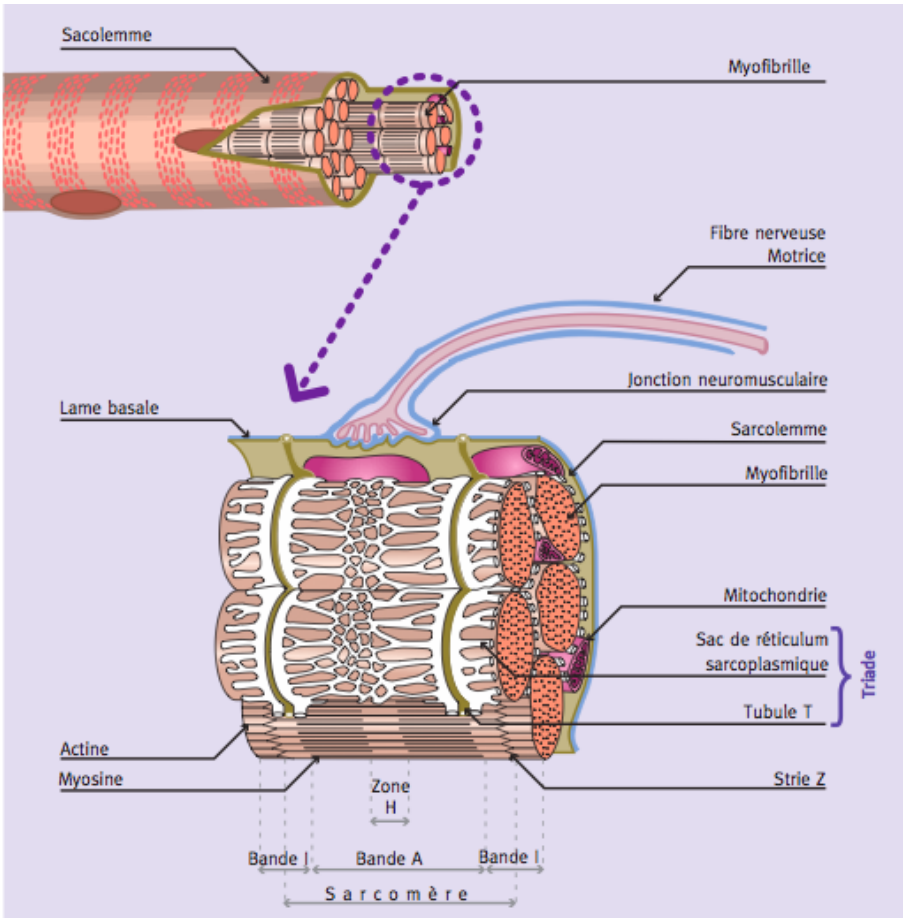

Figure 16. Cellular organization of muscle fibre
From the library of the AFM (association française contre les myopathies).

II.2.2.5. Neuromuscular junction.

The neuromuscular junction (NMJ) is the synapse or junction of the terminal axon of a motor neuron with the motor end plate (MEP). The MEP is a highly-excitable region of muscle fibre plasma membrane responsible for initiation of action potentials across the muscle's surface, ultimately causing muscle contraction.

The arrival of action potential to the presynaptic terminal neuron leads to the opening of voltage-gated calcium channels (VGCC) in the presynaptic membrane. The calcium influx causes Acetylcholine (ACh) containing vesicles to fuse with the presynaptic neuron's cell membrane emptying its contents in the synaptic cleft. The postsynaptic sarcolemma contains

acetylcholine receptors (AChR). The binding of Ach to its receptors leads to the opening of cation channel. Cations, mainly Na^+ enter the muscle cell causing membrane depolarization and the creation of end plate potential (EPP). The resulting muscle action potential spreads across the surface of the muscle fibre into T-tubules, eliciting the release of calcium from the SR, thus initiating muscle contraction (Hill, 2003; Liyanage et al., 2002).

The Ach is then hydrolysed in the synaptic cleft by the Acetylcholinesterase (AchE) enzyme. Choline is transported into the terminal nerve by a high-affinity choline transporter. ACh is resynthesized from choline by Choline Acetyltransferase. [Figure 17]

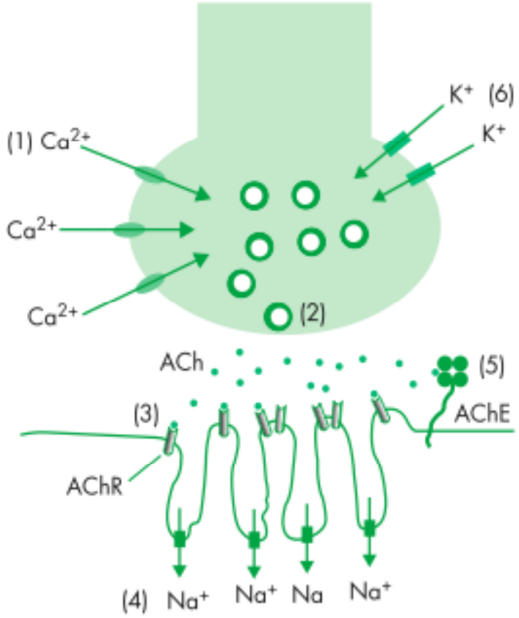

Figure 17. Diagrammatic representation of neuromuscular transmission. (1) Action potential arriving at nerve terminal triggers opening of voltage gated calcium channels (VGCCs) and entry of calcium. (2) Rise in intracellular calcium triggers release of packets of acetylcholine (ACh). (3) Interaction of ACh with ACh receptors (AChR) depolarises post-synaptic membrane. (4) Voltage gated sodium channels (VGSCs) open, triggering muscle action potential. (5) ACh esterase (AChE) breaks ACh into acetyl and choline, which are taken up by the nerve terminal to be reformed into ACh. (6) Opening of voltage gated potassium channels (VGKCs) repolarises nerve terminal.
(from Hill, 2005)

Neuromuscular junction associated proteins play important roles in neuromuscular transmission and in signal transduction (Liyanage et al., 2002). Some features of the molecular architecture of the post-synaptic membrane are shown in [Figure 18].

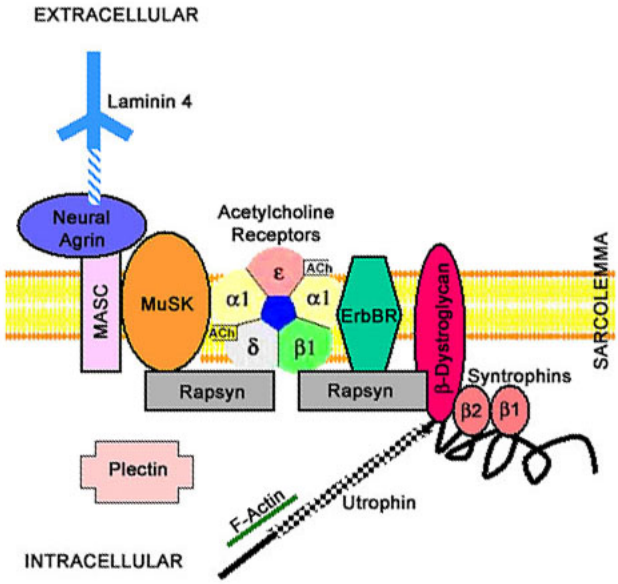

Figure 18. Molecular organization of the postsynaptic plasma membrane. Agrin, secreted by the motor nerve terminal, interacts with Muscle skeletal receptor tyrosine kinase (MuSK),via the muscle associated specific component (MASC), and leads to Rapsyn-dependent clustering of both AcetylCholine receptors (AChR) and ERBB2/3 receptors. The neuromuscular junction (NMJ) is linked to the cytoskeleton through utrophin, syntrophin β1 and β2, and β-dystroglycan. (From Liyanage et al. 2002)

II.3. Muscle fibre types.

Skeletal muscle is a complex, versatile tissue composed of a large variety of functionally diverse fibre types. The overall properties of a muscle largely result from a combination of the individual properties of its different fibre types and their proportions. Muscle fibre types can be delineated according to the major Myosin heavy chain (MHC) isoforms found in a single cell (Pette and Staron, 2000). Several following pure fibre types exist:

- Type I: with MHCIβ isoform, which is a slow twitch fibre.

  And three fast twitch fibre types:
- Type IIA: with MHCIIa isoform.
- Type IIB: with MHCIIb isoform.
- Type IID: with MHCIId isoform.

The expression of specific pairs of these major MHC isoforms results in the formation of hybrid fibres, which can be subdivided based on the predominant MHC isoform.

Muscle fibres are dynamic structures and their fibre type composition changes under various conditions. Increased neuromuscular activity, mechanical loading, and hypothyroidism are conditions that induce fast-to-slow transitions, whereas reduced

neuromuscular activity, mechanical unloading, and hyperthyroidism cause transitions in the slow-to-fast direction. Fibre type transition follows a general scheme of sequential and reversible transitions:

MHCIβ ↔ *MHCIIa* ↔ *MHCIId* ↔ *MHCIIb*

These fibre types differ according to their molecular, metabolic, structural, and contractile properties. The fast-twitch and slow-twitch fibres express different isoforms, and frequently different concentrations of most of the myofibrillar proteins, of the membrane proteins mediating calcium release and removal for excitation–contraction coupling and of many metabolic enzymes (Pette and Staron, 1997). Slow muscle fibres have oxidative metabolism with high amount of mitochondria while fast muscle fibres have glycolytic metabolism with low mitochondria content (Peter et al., 1972).

Fibre type-specific programmes of gene expression are not restricted to the MHC isoforms, but exist for many other muscle proteins (Pette and Staron, 1997) (Schiaffino and Reggiani, 1996). For example, fibre type-specific isoforms exist for the essential and regulatory Myosin light chains (MLC), the three Troponin subunits, Tropomyosin, α-Actinin, and various Ca^{2+}-regulatory proteins (e.g., sarcoplasmic reticulum Ca^{2+}-ATPase, Calsequestrin, and the α-subunit of the dihydropyridine receptor)

III. Signaling pathways and fibre types plasticity in adult skeletal muscle.

III.1. Calcineurin/NFAT.

Calcineurin is a heterodimeric phosphatase composed of a catalytic subunit A and a regulatory subunit B that binds calcium. During muscle contraction, calcium is released from SR and binds the regulatory subunit B. Calcium binding activates Calcineurin that subsequently dephosphorylates the transcription factor NFAT (nuclear factor of activated T cells). The activated NFAT is then translocated into the nucleus, where it upregulates the expression of genes implicated in slow muscle fibre (MHCI, Myoglobin, slow Troponin…) [Figure 19] (Liu et al., 2005b).

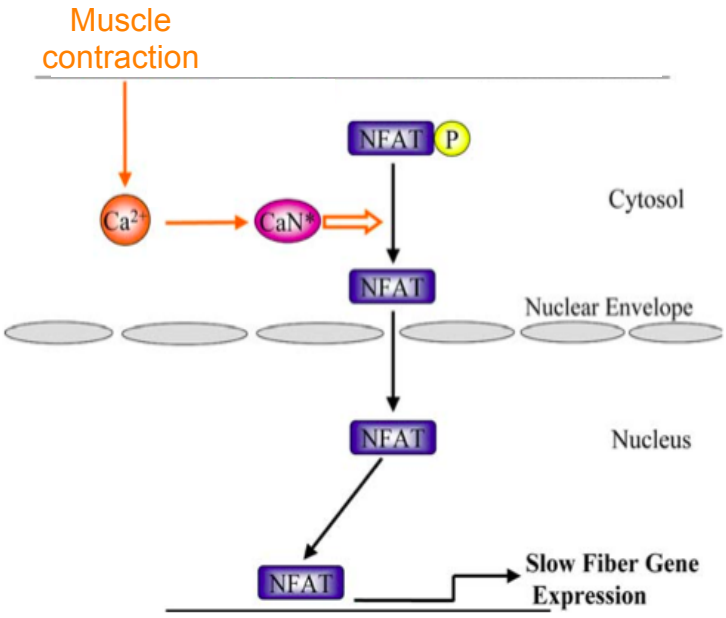

Figure 19. A diagram for Ca2+/Calcineurin/NFAT pathway for activation of slow skeletal muscle fibre gene expression. Elevated cytosolic Ca2+, produced in a muscle fibre during muscle contraction activates the cytoplasmic Ca2+ dependent phosphatase Calcineurin (CaN). CaN dephosphorylates cytoplasmic NFAT-P and NFAT then translocates to the nucleus where it activates genes implicated in slow muscle phenotype.
(Modified from Liu et al, 2005)

Several studies have implicated this signalling pathway in muscle plasticity. Activation of Calcineurin in skeletal myocytes selectively up-regulates slow-fibre-specific gene promoters while inhibition of Calcineurin activity by administration of Cyclosporin A to intact animals promotes slow-to-fast fibre transformation (Chin et al., 1998). Similarly, transgenic mice that express activated Calcineurin under the control of the muscle creatine kinase (MCK) enhancer exhibited an increase in slow muscle fibres with an increased expression of proteins implicated in oxidative metabolism like Myoglobin (Naya et al., 2000). Finally, mice lacking isofrms alpha and beta of Calcineurin A showed a dramatic down-regulation in the oxidative/slow fibre type programme in multiple muscles (Parsons et al., 2003).

III.2. Calmodulin Kinase/MEF2/HDAC.

The MEF2 transcription factor has been implicated in fast-to-slow fibre type transformation. Both NFAT and MEF2 consensus binding sequences are present within the transcriptional control regions of multiple slow fibre type-specific genes (Chin et al., 1998). The transcriptional activity of MEF2 is suppressed by members of the family of class II histone deacetylase proteins (HDAC; HDAC4, 5, 7, and 9), which form complexes with MEF2 within the nucleus. The repression of nuclear MEF2 by class II HDACs is regulated by

the phosphorylation status of HDACs, which in turn is regulated by nuclear Calmodulin dependent kinase (CaMK). CaMK is activated upon nuclear calcium increase during muscle activity. This leads to phosphorylation of HDACs in the nucleus, allowing phosphorylated HDAC to bind to the 14-3-3 protein and move out of the nucleus. The translocation of HDAC from nucleus to cytoplasm displaces HDAC from MEF2, thereby relieving the inhibition on MEF2 transcriptional activity and activating the expression of slow fibre genes. [Figure 20] (Liu et al., 2005b).

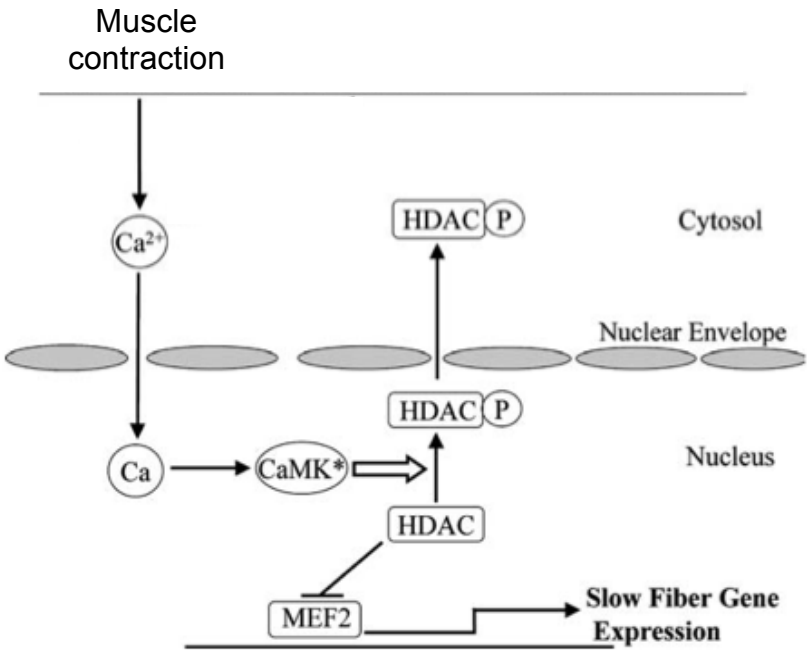

Figure 20. A diagram of the Ca2+/CaMK/HDAC pathway for activation of slow skeletal muscle fibre gene expression. Elevated nuclear calcium leads to the activation of intranuclear CaM kinase. Activated nuclear CaMK phosphorylates HDAC in the nucleus, allowing HDAC to exit from the nucleus via the nuclear export system and thereby removing the HDAC repression of MEF2 activation of slow fibre type gene expression.

Several studies have implicated CaMK in fibre type transition. Liu et al have shown that HDAC4 translocation from nucleus to cytoplasm upon repetitive slow fibre type electrical stimulation was blocked by CaMK inhibitor KN-62 in cultured adult skeletal muscle fibres (Liu et al., 2005a). Furthermore, transgenic mice that selectively express in skeletal muscle a constitutively active form of calcium/Calmodulin-dependent protein kinase IV (CaMKIV) showed increase in type I fibre in skeletal muscle (Wu et al., 2002). CaMKII, a member of the Calmodulin kinase family may play a role in muscle fibre type transition. It has been shown that CaMK II is sensitive to Ca2+ oscillations (De Koninck and Schulman, 1998) and is activated during hypertrophic growth and endurance adaptations (Chin, 2004).

III.3. The PGC-1α coactivator.

PGC-1α (Peroxysome proliferator-activated receptor-γ coactivator-1α) is a Transcriptional coactivator whose activity is controlled by calcium oscillations. The expression of PGC-1α is increased by exercise training in skeletal muscle (Baar et al., 2002). The subsequent activation of calcium signaling activates calcineurin and Calmodulin kinase whose activity leads to the activation of several transcription factors, such as CREB and MEF2 (Wu et al., 2002). PGC-1α regulates its expression by a positive autoregulatory loop by coactivating MEF2 on its own promoter (Handschin et al., 2003). Moreover Zong et al. have shown that AMPK (AMP activated protein kinase K) is required for exercise-induced PGC-1α expression (Zong et al., 2002). PGC-1α induces the expression of ERRα (estrogen-related receptor α), which activates the expression of NRF-1, NRF-2, and ERRα itself. Finally, PGC-1α activates the expression of slow-twitch fibre genes by coactivating MEF2 and simulates mitochondrial biogenesis and oxidative metabolisms by coactivating NRF-1 and NRF-2 [Figure 21] (Lin et al., 2005).

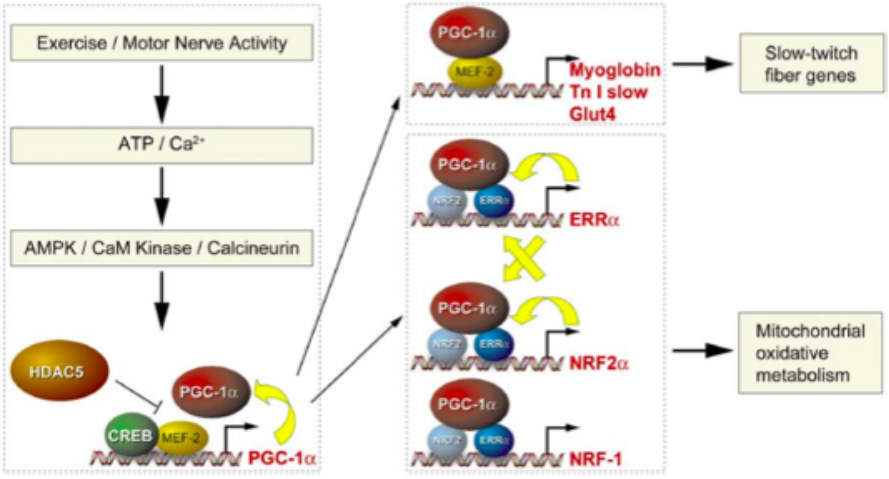

Figure 21. Regulation of PGC-1α expression in skeletal muscle and mechanisms by which PGC-1α stimulates mitochondrial gene expression. Coactivation of MEF2 by PGC-1α provides a positive feed-forward signal to rapidly induce PGC-1α expression following muscle contraction. PGC-1α induces the expression of ERRα, which activates the expression of NRF-1, NRF-2, and ERRα itself. These molecular events lead to the stimulation of nuclear-encoded mitochondrial genes. PGC-1α also simultaneously regulates the expression of slow-twitch muscle fibre genes through coactivation of MEF2.

PGC-1α is a powerful regulator of gene expression that stimulates mitochondrial biogenesis and fibre type switching in skeletal muscle. Gain and loss of function studies in mice have elucidated some aspects of its functions. PGC-1α specific over-expression in skeletal muscle under the control of a muscle creatine kinase (MCK) promoter leads to fast-to-slow fibre type conversion. Muscles normally rich in type II fibres activate genes of

mitochondrial oxidative metabolism, express proteins characteristic of type I fibres, such as Troponin I (slow) and Myoglobin, and show a much greater resistance to electrically stimulated fatigue (Lin et al., 2002). PGC-1α null mice exhibit reduced mitochondrial number and respiratory capacity in slow-twitch skeletal muscle. Furthermore, PGC-1α deficiency caused metabolic dysfunctions with abnormal increase body fat, cardiac dysfunction, and problems with temperature homeostasis in cold conditions (Leone et al., 2005). Similarly, specific inactivation of PGC-1α in skeletal muscle leads to a shift from oxidative type I and IIa toward type IId and IIb muscle fibres with reduced endurance capacity and increased muscle damage after endurance exercises (Handschin et al., 2007).

IV. MicroRNA control of skeletal muscle development and physiopathology.

MicroRNAs (miRNAs) constitute a class of 21-25 nucleotide (nt) noncoding RNAs many of which are evolutionarily conserved in metazoans. MiRNAs regulate gene expression post-transcriptionally, primarily by associating with the 3' untranslated region (UTR) of their target mRNAs. MiRNA are transcribed by RNA polymerase II from intergenic, intronic, and exonic regions of the genome. Intergenic miRNAs are transcribed independently under the control of their own transcriptional regulatory elements. Intronic and exonic miRNAs are located within the introns and the exons of host genes and are usually, but not always co-transcribed and coexpressed with their host genes. A subset of intronic miRNAs are transcribed in the opposite orientation of their host genes and have their own cis-regulatory elements (Bartel, 2004).

IV.1. MiRNA biogenesis and function.

MiRNAs are transcribed as a precursor molecule called pri-miRNAs, which can encode single or multiple miRNAs. Pri-miRNAs fold into a hairpin structure containing an imperfectly base-paired stem and are processed by the endonuclease Drosha into 60-100 nt hairpins known as pre-miRNAs. The pre-miRNAs are exported from the nucleus to the cytoplasm where they are cleaved by the endonuclease Dicer to yield imperfect miRNA-miRNA* duplexes. The miRNA strand is selected to become mature miRNA, while most often the miRNA* strand is degraded. Occasionally both strands give rise to functional miRNAs. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which recognizes specific targets and induces posttranscriptional genes silencing [Figure 22] (Liu and Olson)

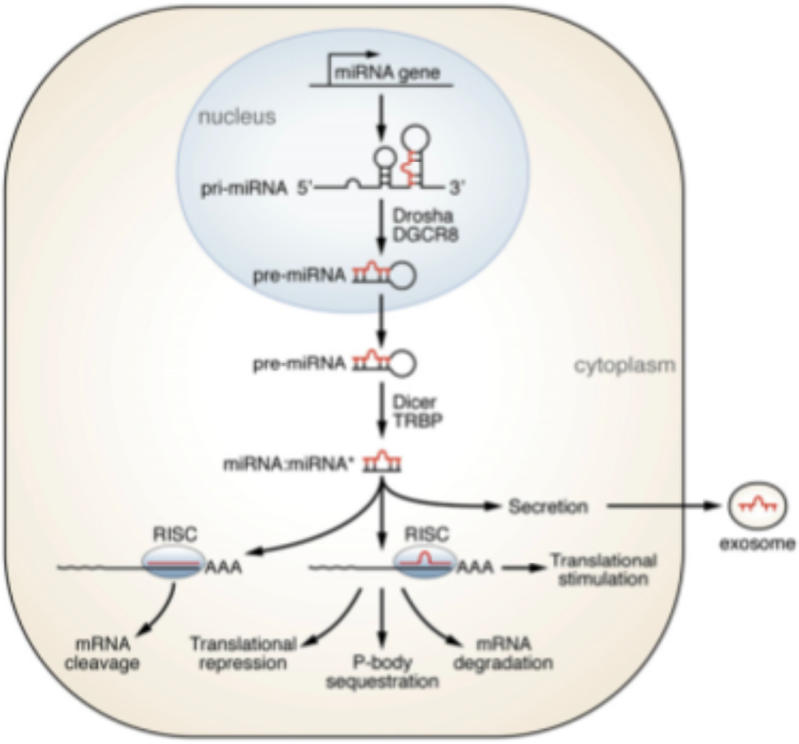

Figure 22. MiRNA biogenesis
(From Liu et al. 2008)

Several mechanisms have been proposed for post-transcriptional gene regulation by miRNA. MiRNAs pair imperfectly to target mRNA and inhibit initiation of translation, mark target mRNAs for degradation by deadenylation, or sequesters target mRNA into cytoplasmic P bodies (Filipowicz et al., 2008). In rarer cases, when there is a perfect match between miRNA and target mRNA this will lead to mRNA cleavage. While miRNAs commonly act to repress their mRNA targets, in rare cases they have also been reported to promote translation their target mRNA. For example, it has been reported that the let-7 miRNA which normally represses translation of tumour necrosis factor-α (TNFα) in proliferating cells is able to up-regulate its translation during cell cycle arrest (Vasudevan et al., 2007). Recently, miRNAs have been detected in circulating plasma microvesicles called exosomes, indicating that miRNAs may be secreted and may mediate intercellular communications (Gibbings et al., 2009; Hunter et al., 2008).

IV.2. Muscle specific miRNAs.

MyomiRs (myo = muscle + miR = miRNA) are miRNAs that are highly enriched in cardiac and skeletal muscle (McCarthy, 2008). The canonical myomiRs identified so far are miR-1, miR-133, and miR-206 and belong to the so-called miR-1 family. The first description of canonical myomiRs was provided by the work of Sempere et al (2004) who showed that their expression is highly enriched in both human and mouse heart and skeletal muscle (Sempere et al., 2004). Subsequent microarray studies confirmed their muscle specificity and that miR-206 is primarily restricted to skeletal muscle (Baskerville and Bartel, 2005 ; Beuvink et al., 2007 ; Liang et al., 2007).

The miR-1 family consists of six members clustered into three bicistronic pairs [Figure 23]. MiR-1-1 and miR-1-2 are identical and differ from miR-206 by 4 nucleotides. MiR-133a-1 and miR-133a-2 are identical and differ from miR-133b by 2 nucleotides. Phylogenetically, all myomiRs derive from the ancient miR-1. The original paralogous gene cluster (miR-1 and miR-133) comes from an initial local gene duplication of miR-1. The other gene clusters come from two "non-local" genomic duplications resulting in the new clusters being locate

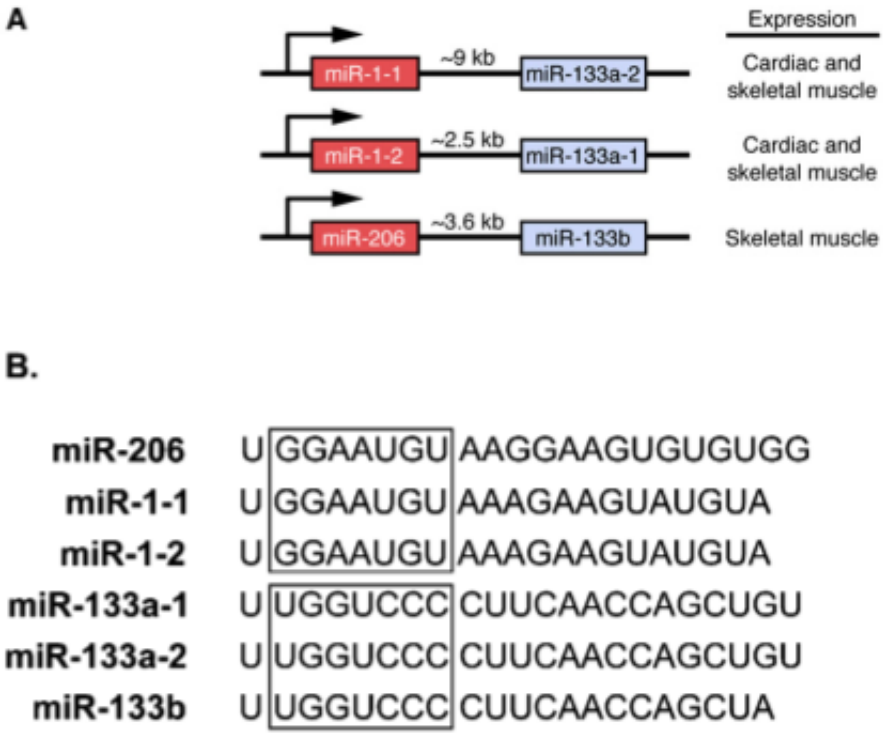

Figure 23. Muscle specific miRNA

A. Bicistronic pairs of the miR-1 family and the muscle tissues in which they are expressed are shown. (from Liu et al. 2010)

B. Sequence alignment of each muscle-specific miR. The "seed" region is boxed for the miR-1/206 and the miR-133a/b groups to emphasize their respective conservation. (from McCarthy. 2008)

IV.3. MyomiRs and skeletal muscle development.

IV.3.1. The requirement of the miRNA biogenesis pathway for skeletal muscle development.

Loss of function experiments in the miRNA generating enzyme Dicer have shown that miRNAs play essential roles in mouse development. Loss of Dicer1 leads to lethality at embryonic day (E) 7.5 (Bernstein et al., 2003). In order to circumvent the early embryonic lethality associated with the deletion of Dicer, tissue specific and conditional null alleles of Dicer have been generated. Deletion of Dicer in skeletal muscle progenitors using Cre recombinase under the control of *Myod1* regulatory elements, which direct gene expression in skeletal muscle as early as E9.5 caused skeletal muscle hypoplasia and perinatal death of mutant mice (O'Rourke et al., 2007).

IV.3.2. Regulation of myomiR expression in muscle.

The muscle specific miRNAs control the expression of several genes in cardiac and skeletal muscle (for review see (Williams et al., 2009)). Cardiac and skeletal muscle specific transcription of myomiRs is tightly regulated. In vertebrates, muscle specific expression of miR-1-1/133a-2 and miR-1-2/133a-1 clusters appears to be controlled by two separate enhancers, one upstream of each gene cluster and the other intronic (Liu et al., 2007). The myogenic transcription factors SRF, MEF2, and MYOD1 control the expression of miR-1 and miR-133a in cardiac and skeletal muscle through these enhancers. MEF2 directly activates transcription of a bicistronic primary transcript encoding miR-1-2 and miR-133a-1 via the intragenic enhancer while SRF directs their cardiac specific expression through the upstream enhancer (Liu et al., 2007 ; Zhao et al., 2005). In addition, in SRF and MEF2 deficient mice, miR1 and miR133a are strongly downregulated (Liu et al., 2007 ; Niu et al., 2008). Skeletal muscle specific expression of the miR-206/133b primary transcripts is thought to be controlled by an upstream regulatory region bound by MYOD1 and MYOG (Rao et al., 2006). Also, in fibroblasts converted to myogenic lineage by MYOD1 over-expression, MYOD1 directly activates the expression of miR-206 (Rosenberg et al., 2006).

IV.3.3. MyomiRs in skeletal muscle proliferation and differentiation.

The proliferation and differentiation of skeletal muscle cells is mutually exclusive. The proliferating muscle cells, myoblasts, actively expand under growth conditions, whereas they quickly exit from the cell cycle in response to growth factor depletion, under differentiation conditions, and fuse to form terminally differentiated multinucleated myotubes (Parker et al., 2003 ; Pownall et al., 2002). In C2C12 myoblasts, miR-1 promotes myogenesis by repressing

the expression of histone deacetylase 4 (HDAC4), a transcriptional repressor of the MEF2 transcription factor (Chen et al., 2006). Indeed, histone acetyltransferases and deacetylases have been implicated in the control of cell proliferation and differentiation (Lehrmann et al., 2002; Magnaghi-Jaulin et al., 2000). Thus, the repression of HDAC4 by miR-1 establishes regulatory loop in which the up-regulation of miR-1 by MEF2 causes further repression of HDAC4 and increased activity of MEF2, which drives C2C12 differentiation [Figure 24].

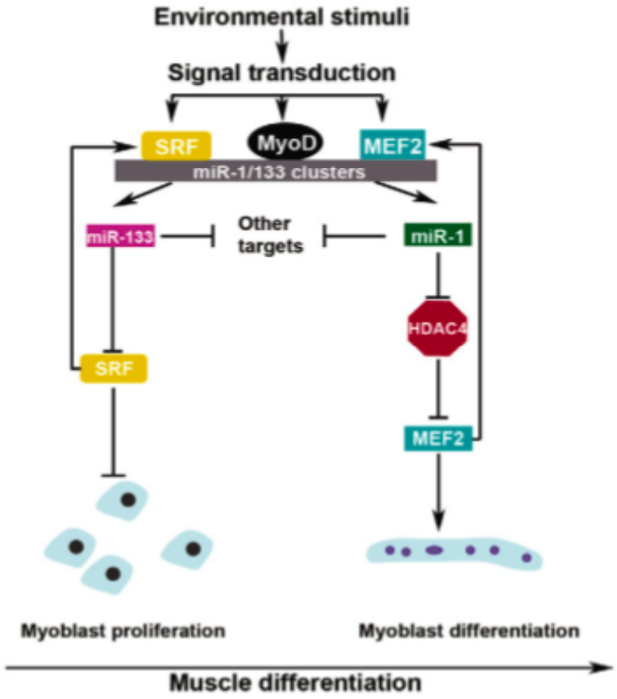

Figure 24. Model of miR-1- and miR-133-mediated gene regulation during muscle proliferation and differentiation. Tissue-specific expression of miR-1 and miR-133 clusters is controlled by the transcription factors SRF, MEF2 and MYOD1. MiR-1 promotes muscle differentiation by repressing the expression of HDAC4 (histone deacetylase 4), a signal-dependent inhibitor of muscle differentiation that represses MEF2 activity. MEF2, in turn, potently activates the expression of myoblast-differentiation genes and miR-1. MiR-133, however, reduces protein levels of SRF, a crucial regulator of muscle differentiation, thereby enhancing the proliferation of myoblasts and inhibiting their differentiation.

By contrast, miR-133 promotes the proliferation of myoblasts and inhibits their differentiation at least in part by repressing SRF(Chen et al., 2006). miR-133 also inhibits the translation of polypyrimidine tract-binding protein (nPTB), which controls differential transcript splicing during skeletal muscle cell differentiation (Boutz et al., 2007). Paradoxically, miR-1 and miR-133 exert opposing effects on skeletal muscle development despite originating from the same miRNA polycistronic transcript. Interestingly, embryonic stem cell (ESC) differentiation towards cardiomyocytes is promoted by miR-1 and inhibited by miR-133 (Ivey et al., 2008). These studies support the idea that miR-1 and miR-133

regulate myogenesis by regulating the balance between key regulatory pathways for muscle cell proliferation and differentiation [Figure 24].

Similar to miR-1, miR-206 promotes myoblast differentiation. The developmental regulation of miR-206 is recapitulated *in vitro* using C2C12 myoblasts. Upon initiation of differentiation, there is a steady induction of miR-206 as well as miR-1 and miR-133a (Kim et al., 2006). MiR-206 has been shown to inhibit the expression of the gap junction protein connexin 43 also called gap junction protein, alpha 1 (GJA1) during fusion of C2C12 myoblasts into myotubes (Anderson et al., 2006). *GJA1* is known to be a major component of gap-junctions and has been shown to be important for muscle regeneration and *in vitro* differentiation (Araya et al., 2005). It is thought that the down-regulation of GJA1 during perinatal muscle development is necessary for the proper formation of the mature neuromuscular junction. MiR-206 also represses the translation of p180 subunit of DNA polymerase-α (*polA1*), thereby promoting myogenesis by inducing the transition from cell proliferation to cell quiescence (Kim et al., 2006). In addition it has been shown that miR-206 mediates the repressing effects of MYOD1 on folistatin-like 1 (*Ftl1*) and Utrophin (*Utrn*) during C2C12 myoblast differentiation (Rosenberg et al., 2006).

IV.3.4. MyomiRs in skeletal muscle disorders.

Primary skeletal muscle disorders involve different groups of diseases, including muscular dystrophies, inflammatory myopathies and congenital myopathies. Although there is increasing clarification of the primary aberrant cellular processes responsible for these conditions, the secondary pathogenic cascades are still mainly obscure. Recent studies have begun to link miRNAs to certain muscle-related diseases (Eisenberg et al., 2007 ; McCarthy and Esser, 2007 ; McCarthy et al., 2007).

The expression of miR-206 was increased in the diaphragm, but not in the hind limb of the dystrophin-deficient *mdx* mice, an animal model for muscular dystrophy (McCarthy et al., 2007). The fact that diaphragm is the most affected muscle in the *mdx* mouse suggests that the elevated level of miR-206 may contribute to the severity of the disease by repressing the expression of genes whose function is necessary to compensate the loss of *Dystrophin* function. This proposition is supported by the finding that miR-206 expression was only modestly changed in *mdx* hindlimb muscles, which do not display phenotype as severe as the *mdx* diaphragm (Stedman et al., 1991).

The expression of miR-1 and miR-133a was decreased during overload-induced muscle hypertrophy (McCarthy and Esser, 2007). Down-regulation of miR-1 and miR-133a expression may serve to promote adaptation to overload by removing post-transcriptional

repression of necessary target genes. Paradoxically, the pri-miRNA expression level of miR-1 and miR-133a was not down-regulated, but rather increased upon functional overload which may indicate the requirement of additional regulation of miRNA biogenesis which does not appear to involve the canonical pathway (McCarthy and Esser, 2007). Nevertheless, further studies are needed to elucidate this apparently paradoxical miRNA biogenesis pathway.

In addition to those studies of miRNA expression in muscle disorders, a direct genetic link has connected miRNA function to muscular hypertrophy (Clop et al., 2006). A single nucleotide polymorphism (SNP) within the 3' UTR of Myostatin gene, responsible for the exceptional muscularity of Texel sheep, results in the formation of a functional miR-1/miR-206 target site. Myostatin is a member of the transforming growth factor-β family and functions to repress muscle growth. This mutation leads to the translational repression of Myostatin which phenocopies the 'muscle doubling' that results from the loss in Myostatin in mice, cattle and humans (Lee, 2004 ; Tobin and Celeste, 2005).

V. TEAD/TEF family of transcription factors.

V.1. Identification and characterisation of the TEAD/TEF family.

In addition to the well defined MRFs of the bHLH family whose functions have been discussed above, my thesis work concerns the distinct TEAD/TEF family of transcription factors, that have long been thought to be involved in muscle differentiation and homeostasis, but whose precise roles have remained elusive. The TEAD family of transcription factors was first identified through the purification and cloning of the first mammalian TEF factor, TEF1 (TEAD1), as a factor binding to the GT-IIC and Sph enhansons of the SV40 enhancer where it regulates transcription from early and late promoters (Davidson et al., 1988 ; Xiao et al., 1991). This family shares a highly conserved DNA binding domain (DBD) called the TEA domain (Andrianopoulos and Timberlake, 1991), and consists of four members including TEAD1 (TEF-1, NTEF-1), TEAD2 (ETF, ETEF-1, TEF-4), TEAD3 (DTEF-1, TEF5, ETFR-1), and TEAD4 (RTEF1, TEF-3, ETFR-2, FR-19) [Table 1].

Name	Alternative Name	Percent Identity to TEF-1	Percent Identity to TEF-1 within the TEA Domain
TEF-1	NTEF-1, Tead1,TCF13	...	...
ETF	ETEF-1, EtdF,TEF-4, Tead2	64%	100%
RTEF-1	TEF-3,Tcf13r1, ETFR-2,FR-19, Tead4	74%	100%
DTEF-1	TEF-5,Tcf13r2, ETFR-1, Tead3	70%	99%
Amino acid identity was determined among human TEF-1 family members.			

Table 1. The TEAD/TEF Family Members. (modified from Yoshida, 2008).

Each TEAD family member has multiple names as shown in parenthesis above and in Table 1. The TEA domain is also referred to as the ATTS domain because it appears in yeast, vertebrate, and fly transcription factors AbaA, TEC1, TEF1, and Scalloped (Campbell et al., 1992). AbaA regulates development of the asexual spores in *Aspergilus nidulans* and terminates vegetative growth, (Andrianopoulos and Timberlake, 1994), TEC1 is involved in the activation of Ty1 retrotransposon in yeast *Saccharomyces cerevisiae,* (Laloux et al., 1990) and the *Drosophila* gene scalloped plays important roles during wing development (Bray, 1999). Conservation of the TEA domain in multiple organisms indicates its critical role in regulation of gene transcription. Comparison of the TEA domain from yeast to human indicates a remarkable degree of conservation [Figure 25].

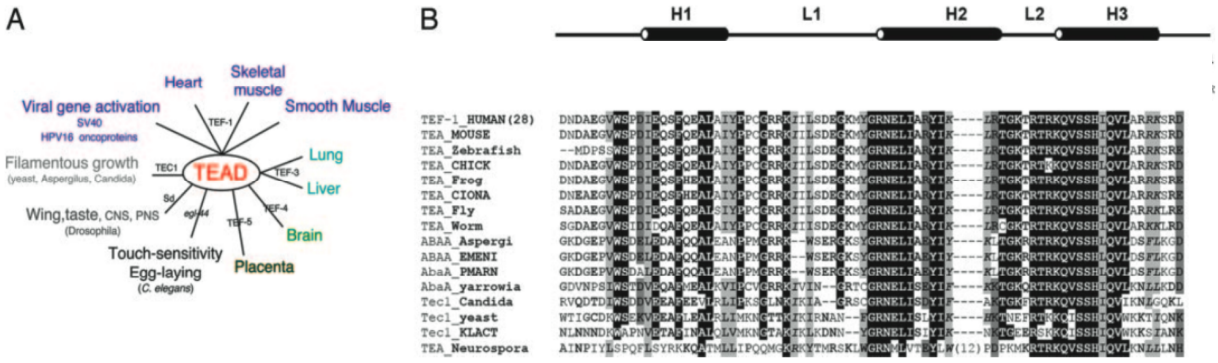

Figure 25. (A) The TEAD factors are widely expressed and have different functions. (B) The TEA domain is evolutionarily conserved.
(Modified from Anbanandam et al, 2006)

The TEA domain was formally identified as a DBD by functional dissection of the human TEAD1 factor (Hwang et al., 1993). The consensus DNA binding site of the TEAD family is 5'-CATTCCA/T-3' and is called the MCAT element (Cooper and Ordahl, 1985; Farrance et al., 1992). TEAD family members bind to double-stranded form of the MCAT element, but not to the single-stranded MCAT element (Carlini et al., 2002). In contrast to many transcription factors that bind cooperatively to palindromic sites, several members of the TEAD family have been shown to bind cooperatively to tandem repeats, but non-cooperatively to spaced or inverted repeats (Davidson et al., 1988 ; Jacquemin et al., 1996). The ability to bind cooperatively to tandem, but not spaced, repeats is an intrinsic property of the TEA domain. This cooperativity is required for efficient binding to regulatory elements, such as the SphI+II enhansons of the simian virus 40 (SV40) enhancer or the DF-3 element enhansons in the human Chorionic Somatomammotropin-B (hCS-B) gene enhancer, which are composed of tandemly repeated low-affinity binding sites (Davidson et al., 1988 ; Jacquemin et al., 1996).

In addition to DNA binding, the TEA domain is also a target of the SV40 large T antigen (TAg) oncoprotein, which interacts with this domain and may modulate the DNA-binding and/or transcriptional properties of TEAD1 (Berger et al., 1996). Interestingly, a single amino acid change in TAg (S189N), which disrupts interaction with the TEA domain also results in defective transformation function.

Recently, the three dimensional (3D) structure of the TEA domain has been solved and shown to comprise a three-helix bundle with a homeodomain fold (Anbanandam et al., 2006) (see also below). Structure-function correlations have shown that the L1 loop is essential for cooperative binding of TEAD molecules to tandemly duplicated MCAT sites. This suggests that the interactions between TAg and TEA domain factors are involved in cellular transformation and gives interesting cues regarding the regulation of TEAD factors activity.

The translation of several TEAD family members may be subject to control as the translation of TEAD1, TEAD3, and TEAD4 are initiated at isoleucine (AUU), leucine (UUG), and isoleucine (AUA) codons, respectively, that lie upstream of the first methionine codon (Jiang et al., 1999 ; Stewart et al., 1996; Xiao et al., 1991). In TEAD2, the methionine (AUG) codon is used for the initiation of translation (Jiang et al., 1999; Yasunami et al., 1995).

The tissue distribution of TEAD family members has been examined by a number of studies (Azakie et al., 2005; Azakie et al., 1996 ; Jacquemin et al., 1996 ; Stewart et al., 1996 ; Xiao et al., 1991 ; Yasunami et al., 1995 ; Yasunami et al., 1996 ; Yockey et al., 1996). Indeed, TEAD1, TEAD3, and TEAD4 are widely expressed in multiple tissues including the skeletal muscle, pancreas, placenta, lung, and heart. In contrast to these three factors, TEAD2 is selectively expressed in a subset of embryonic tissues including the cerebellum, testis, and distal portions of the forelimb and hindlimb buds as well as the tail bud, but it is essentially absent from adult tissues (Yasunami et al., 1995). TEAD2 has also been shown to be expressed from the 2-cell stage during development (Kaneko et al., 1997).

V.2. The MCAT element.

In vertebrates, The MCAT element has been found in number of cardiac, smooth, and skeletal muscle-specific genes, including cardiac Troponin T (Mar and Ordahl, 1988), β-myosin heavy chain (β-MHC) (Rindt et al., 1993), smooth muscle α-actin (SM α-actin) (Swartz et al., 1998), and skeletal α-actin (Karns et al., 1995). It has been shown to play a key role in the transcriptional regulation of these genes, although it is also present in the promoter/enhancer regions of nonmuscle genes, including the forkhead box A2 gene (*Foxa2)* (Sawada et al., 2005), paired box gene 3 (*Pax3)* (Milewski et al., 2004), and hCS-B (Jiang et al., 1999). Some muscle-specific genes contain two MCAT elements in their promoter and

enhancer regions, whereas others contain only a single MCAT element [Table 2]. Mutational analysis of the cardiac Troponin T promoter that contains two MCAT elements has shown that both elements are required for the full muscle-specific transcriptional activity in cultured muscle cells (Mar and Ordahl, 1988).

Gene	Species	Sequence and Position	Conserved in Human?	Reference
Cardiac troponin T	chicken	MCAT1: CATTCCT (-95/-89 bp) MCAT2: CATTCCT (-72/-66 bp)	Yes	(Mar and Ordahl, 1988)
β-MHC	mouse	distal: CATTCCA (-275/-281 bp)* proximal: CATGCCA (-205/-211 bp)*	Yes	(Rindt et al., 1993)
SM α-actin	rat	MCAT2: CATTCCT (-314/-320 bp)* MCAT1: CATTCCT (-178/-184 bp)*	No	(Swartz et al., 1998)
Skeletal α-actin	mouse	CATTCCT (-69/-63 bp)	Yes§	(Karns et al., 1995)
α-MHC	rat	CATTCCA (-42/-48 bp)*	Yes	(Gupta et al., 1994)
β-acetylcholine receptor	rat	CATTCCT (-49/-43 bp)	No	(Berberich et al., 1993)
Myocardin	mouse	CATTCCA (-30 kb)	Yes§	(Creemers et al., 2006)
α1c-adrenergic receptor	mouse	CATGCCA (-916/-910 bp)	Yes	(O'Connell et al., 2001)
α-tropomyocin	flog	CATTCCT (-59/-65 bp)*	No	(Pasquet et al., 2006)

Table 2. MCAT elements in the muscle specific genes.

**MCAT elements are located in reverse orientation.*

§MCAT1 in human cardiac troponin T gene is CATCCCC, and human β-acetylcholine receptor MCAT sequence is CATTCCC.

(from Yoshida, 2007)

One of major question is how the MCAT motif can direct muscle-specific gene expression on one hand and non-muscle specific gene expression on the other. Larkin et al., (Larkin et al., 1996) proposed a model to answer this question. They proposed that the sequences flanking the MCAT elements may modulate cell specific transcriptional activity of target genes by recruiting specific factors that may regulate positively or negatively the activity of the TEAD factors on target promoters.

V.3. The structure of TEAD/TEF family.

V.3.1 Functional domains of TEAD/TEF Factors.

The TEAD proteins are closely related not only in the TEA domain but also in the C-terminal domain called the YAP1 (Yes/src associated protein 1) binding domain (YBD), whereas the N-terminal region preceding the TEA domain and the proline-rich region following the TEA domain are more variable. The most conserved region is the TEA domain [Figure 26]. The human TEAD factors are more than 99% identical in the DBD (Yoshida,

2008). The C-terminal domain, that contains the YAP1 binding domain (YBD) is also highly conserved (Chen et al.).

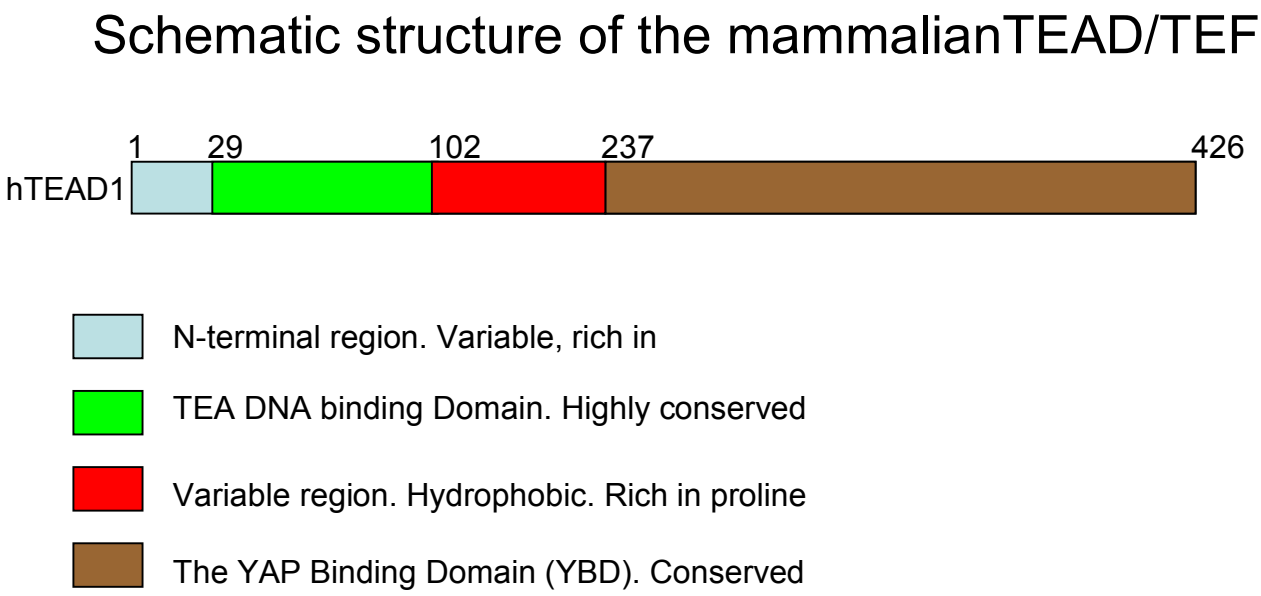

Figure 26. The overall structure of the mammalian TEAD factors is schematized. The domains are defined both by their conservation among the family members and by their amino acid compositions. The numbers represent the amino acid coordinates of each domain in the prototype member TEAD1. The corresponding amino acid coordinates in the TEAD2, TEAD3, TEAD4 factors are similar.
(Modified from Jacquemin et al, 1997)

V.3.2. The TEA/ATTS DNA binding domain.

The first 3D structure of TEA/ATTS of human TEAD1 domain was identified in 2006 and comprises a three helix bundle with a homeodomain fold (Anbanandam et al., 2006) [Figure 27].

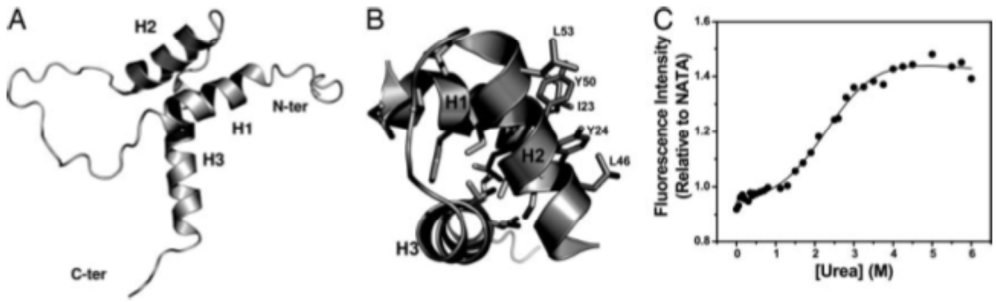

Figure 27. The 3D structure of the TEA domain. (A) Front view. (B) Down view, the hydrophobic residues (sticks) contribute to the core packing and hydrophobic surface patch.(C) The TEA domain unfolds with a midpoint of a 2.5 urea. Fluorescence intensity is relative to that of an equimolar solution of N-acetyl tryptophanamide (NATA).
(from Anbanandam et al, 2008)

The TEA domain has a folded globular structure made of three α-helices, H1, H2, and H3. H1 and H3 are nearly anti-parallel and pack on either side of the H3. The TEA domain consists of 28 hydrophobic residues and the core contains only 12 residues with relatively low hydrophobic contacts. Subsequently, the TEA domain was predicted to have a low thermodynamic stability. Indeed, the TEA domain unfolds irreversibly with a mid point of urea denaturation of 2.5M (Anbanandam et al., 2006). The H1-H3 contact creates a hydrophobic patch that consists of 5 amino acids, I23, Y24, L46, Y50, L53 figure. This surface is likely to be crucial in protein-protein interactions.

The TEA domain binds DNA with nanomolar affinity. The consensus DNA sequence bound by the isolated TEA domain is N[A/T/G]G[AT/C]ATNT and differs from the MCAT sequence. This suggests that other domains of the full-length TEAD proteins participate in binding specificity perhaps by inducing conformational changes in the TEA domain. Indeed, Jiang et al. have shown that alternative splicing of TEAD1 mRNA in regions immediately after the TEA domain altered its DNA binding properties (Jiang et al., 2000).

The DNA recognition surface is located in the H3 helix and contains three serines (Anbanandam et al., 2006). This is in agreement with biochemical data showing that the phosphorylation of ser-102 by protein kinase A (Gupta et al., 2000) or of ser-91 by protein kinase C diminishes DNA binding activities (Jiang et al., 2001).

V.3.3. The YAP1 dinding domain (YBD).

The C-terminal region of the TEAD family has recently been shown to mediate interaction with the transcriptional coactivator YAP1 (Yes Associated Protein 1). The YBD 3D structure from human TEAD1 and 2 and mouse TEAD4 has been described (Anderson et al.; Li et al. ; Tian et al.,). The three structures are strikingly similar and reflect the conservation of the YBD through evolution. The YBD adopts an immunoglobulin-like structure and is composed of 12 β strands and four α helices [Figure 28].

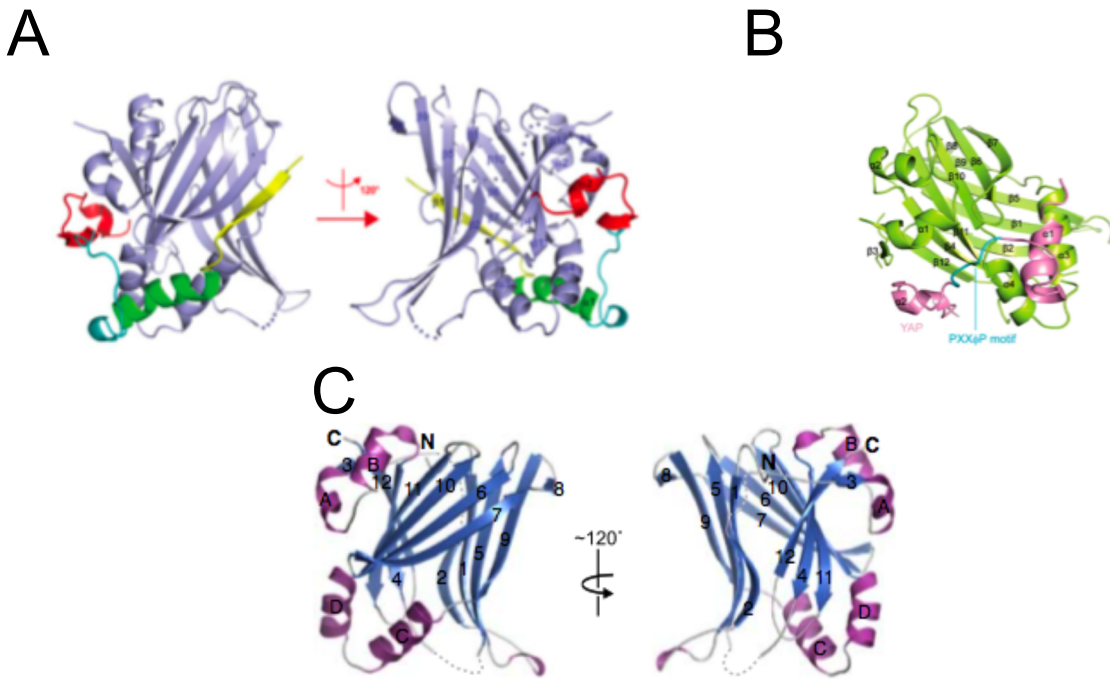

Figure 28. The 3D structure of YBD from (A) Li et al, 2010. (B) Chen et al, 2010. The YBD was crystallised together with the TEAD binding domain of YAP1 (Yes/src associated protein kinase). (C) Tian et al, 2010.

The β strands form two β sheets that pack against each other to form a β sandwich with one β sheet composed of strands β1, β2, β5,β8, β9 and the other consisting of β3, β4, β6, β7, β10, β11, β12. The α helices form two helix-turn-helix motifs where each one connects two β strands.

V.3.4. The Proline-rich region.

Although the proline-rich region is not conserved at the primary sequence level, all TEAD family members are proline-rich (16-25%) in this region. The proline-rich region of rat TEAD1 is only 20% identical with rat TEAD4 (Mahoney et al., 2005). The proline-rich region is also required for full interaction with the YAP1 and related TAZ transcriptional coactivators (Vassilev et al., 2001). Therefore, the proline-rich region probably accounts for the differential interaction of the TEAD proteins with YAP/TAZ.

V.3.5. The N-terminal region.

The N-terminal region of TEAD proteins is amongst the less conserved in the TEAD family. The TEAD1 N-terminal region has a net negative charge and contains a high concentration of serines which are potential sites of phosphorylation. The N-terminal region of TEAD1 is required for its interaction with the transcription factor MAX (Gupta et al., 2000).

Furthermore, the full transcriptional activation by TEAD1 requires its N-terminal region to synergise with the proline rich-region and the YBD probably by forming a functional transactivation surface (Hwang et al., 1993).

V.4. Regulation of the transcriptional activity of the TEAD family.

V.4.1. Post-transcriptional modifications: Phosphorylation.

To date, the unique post-transcriptional modification described for the TEAD family members is phosphorylation. Ueyama et al. have shown that phosphorylation of TEAD4 on serine 322 is required for alpha1-adrenergic mediated cardiac myocyte hypertrophy. Mutation of this residue reduced the alpha1 adrenergic mediated activation of a reporter gene by 70% compared to wild type TEAD4 (Ueyama et al., 2000).

On the other hand, activation of protein kinase A induces phosphorylation of TEAD-1 at Serine 102 and activates the transcription of the α-MHC gene in cardiomyocytes (Gupta et al., 2000). In fact, phosphorylation of TEAD1 at serine 102 reduces its binding activity on the MCAT element within the α-MHC gene and did not affect its binding to the MAX transcription factor. The inhibition of DNA binding and the stimulation of transcriptional activity seem to be contradictory. Furthermore, Jiang et al., have shown, however, that TEAD1 has two isoforms, TEAD1 β and γ, which are expressed in the heart and have increased DNA binding affinity for the MCAT element (Jiang et al., 2000). Interestingly, serine 102 is converted to Valine in these isoforms, which prevents their phosphorylation by protein kinase A and may allow them to mediate muscle specific gene expression instead of full length TEAD1.

V.4.2. Interaction with cofactors.

Like most transcription factors, the transcriptional activity of the TEAD factors is dependent on interaction with cofactors. Multiple cofactors for the TEAD family members have been described amongst which are; the p160 family of nuclear receptor coactivators (SRC1, TIF2, RAC3) (Belandia and Parker, 2000), a Src/Yes associated protein YAP1 (Vassilev et al., 2001), the related TAZ (WWTR1) (Mahoney et al., 2005), and vestigial-like (VGLL) family of cofactors: VGLL-1 (TONDU) (Vaudin et al., 1999), VGLL-2 (VITO-1) (Maeda et al., 2002a), VGLL-3 (Mielcarek et al., 2009), and VGLL-4 (Chen et al., 2004c).

V.4.2.1. The Vestigial-like family of cofactors.

Vestigial-like proteins are the vertebrate orthologues of the *Drosophila*, Vestigial, a transcriptional coactivator for Scalloped and required for wing formation (Paumard-Rigal et al., 1998). Mammalian vestigial-like factors have broad patterns of expressions, but VGLL-2 is restricted to skeletal muscle. VGLL-2 is expressed in the differentiating somites and branchial arches during embryogenesis and is exclusively expressed in skeletal muscle in the adult (Maeda et al., 2002a). VGLL-4 is highly enriched in the heart, but its expression is detected in brain, kidney, small intestine, lung and placenta (Chen et al., 2004c). VGLL-3, is mainly expressed in the myogenic lineage during embryonic development and at later developmental stages is predominantly found in the nervous system. In adult mice, VGLL-3 was detected in different tissues, including skeletal muscle, heart, kidney, liver and brain. VGLL-1, is expressed in lung, kidney and placenta, and in foetal heart (Vaudin et al., 1999).

Vestigial-like proteins may regulate positively or negatively TEAD dependent transcriptional activity. During differentiation of C2C12 skeletal muscle cells, expression of VGLL-2 is increased and VGLL-2 protein is translocated from the cytoplasm to the nucleus. VGLL-2 interacts with TEAD1 and TEAD4 (Maeda et al., 2002a), and differentially regulates the binding activity of TEF-1 family members to MCAT elements. VGLL-2 decreased TEAD1 binding to MCAT elements while it increased TEAD4 binding. (Gunther et al., 2004) (Chen et al., 2004b). Cotransfection assays in differentiated C2C12 cell have shown that VGLL-2 increased TEAD4 dependent transcription, while repressing TEAD1 activity (Maeda et al., 2002a).

V.4.2.2. The YAP/TAZ transcriptional coactivators.

YAP1 (Yes/Src associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif also known as WWTR1, for WW-domain containing transcription regulator 1) are related transcriptional coactivators linking extracellular signalling events to transcriptional regulation in the nucleus (Vassilev et al., 2001 ; Wang et al., 2009). Human YAP1 and TAZ share several domains and overall display 46% identity and about 60% similarity [Figure 29] (Wang et al., 2009).

Initially identified as a 65 kDa binding partner of c-YES, chicken YAP1 was named YAP65 (Sudol, 1994). Subsequently human and mouse orthologues of c-YAP65 were cloned (Sudol et al., 1995). Interestingly, mouse YAP1, but not the original human YAP1, possess a tandem WW domain raising the question as to whether there are different YAP isoforms in mammals. Komuro et al. identified a human YAP1 isoform with tandem WW domains similar

to the mouse YAP1 (Komuro et al., 2003) [Figure 23]. This isoform was named YAP2 and YAP65 was named YAP1.

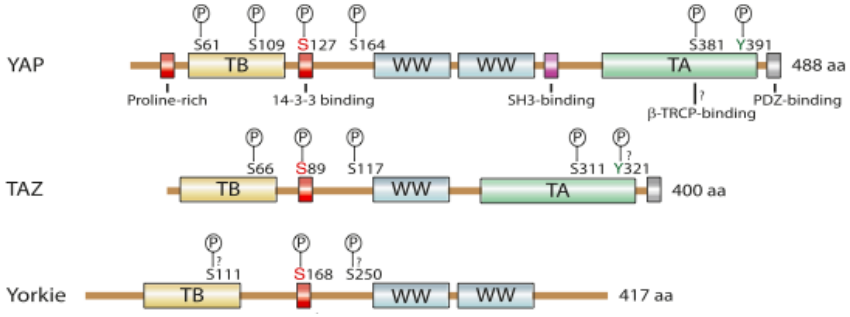

Figure 29. Organisation of YAP, TAZ and Yorkie (the Drosophila homologue of YAP)
Illustrated are the proline rich-motif, TEAD-binding (TB) domain, 14-3-3 binding site, WW domain, SH3-binding motif, transcriptional activation (TA) domain, and PDZ-binding motif. Ovals with the P letter inside denote phosphorylation. The 14-3-3 binding residue is highlighted in red. (from Wang et al. 2009)

YAP1 is part of a stable multiprotein complex containing TEAD2, and interacts with essentially the same affinity with all TEAD proteins (Vassilev et al., 2001). TAZ on the other hand differentially interacts with the TEAD family members and TEAD1 appears to display the highest affinity (Mahoney et al., 2005).

YAP1 and TAZ are expressed in a wide range of tissues including skeletal muscle and heart (Komuro et al., 2003 ; Kanai et al., 2000), and therefore are able to regulate TEAD transcriptional activity in the majority of tissues where TEAD proteins are expressed.

YAP/TAZ are the major downstream effectors of the Hippo tumour supressor pathway. Components of the Hippo pathway are highly conserved from *Drosophila* to mammals (Zhao et al.). The *Drosophila* protein Hippo is a protein kinase that is activated by upstream signals (Harvey et al., 2003). The mammalian orthologue of Hippo is the MST (mammalian ste20 lilke) kinase (Harvey and Tapon, 2007). In mammals, under certain conditions (e.g. low cell density), YAP1 and TAZ localize in the nucleus where they act as coactivators for several transcription factors including TEAD factors, and promote cell proliferation, cell survival and inhibit apoptosis (Huang et al., 2005 ; Lei et al., 2008). Upon cell contact inhibition, the Hippo pathway is activated and the MST kinase activates the protein kinase LATS that phosphorylates YAP1 and TAZ to promote their binding to 14-3-3 proteins and their translocation to the cytoplasm, thus inhibiting their transcriptional coactivation potential[Figure 30] (Zhao et al., 2007 ; Lei et al., 2008 ; Zhang et al., 2008a ; Oka et al., 2008).

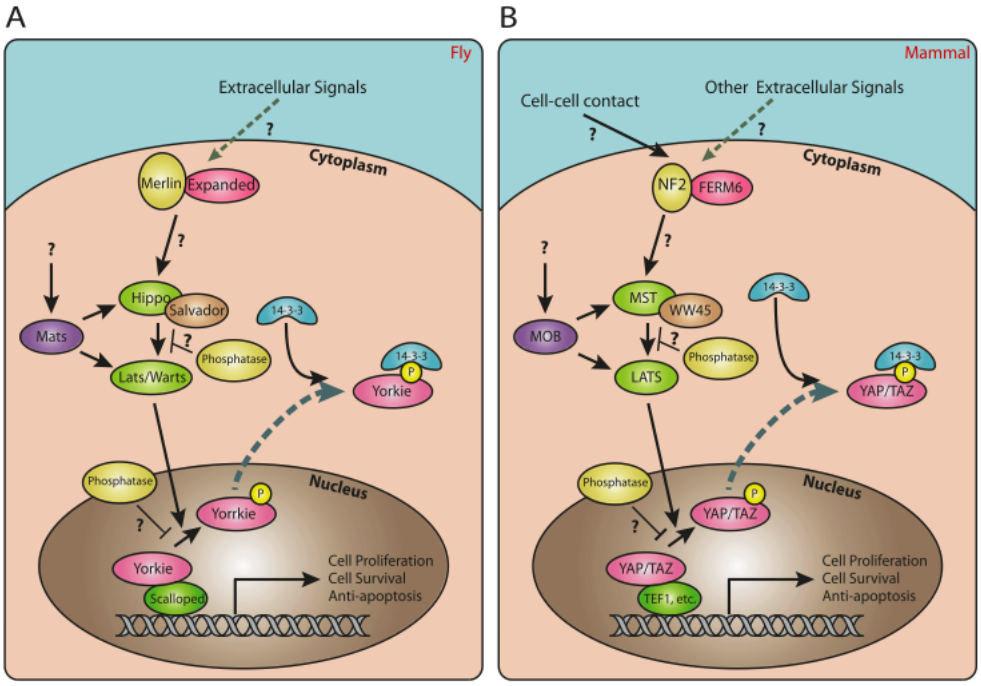

Figure 30. Comparaison of the Drosophila Hippo and mammalian Hippo-like signalling pathways. A. Model for Yorkie regulation by the hippo pathway. In the nucleus, Yorkie interacts with Scalloped and functions as transcriptional coactivator to promote cell proliferation, stimulate cell survival, and inhibit apoptosis. Upon activation of the Hippo pathway, Yorkie is phosphorylated by Large Tumor Suppressor (Lats), and binds to 14-3-3 proteins. As a results, Yorkie localizes to the cytoplasm, and its transacting function is inhibited. The phosphorylation may occur in the cytoplasm, and 14-3-3 binding may just sequester Yorkie to this compartment. B. Model for YAP1 and TAZ regulation by the Hippo-like pathway. Under certain conditions (e.g., low cell density), YAP1 and TAZ localize to the nucleus, where they interact with transcription factors (e.g. TEAD factors) and function as transcriptional coactivators to promote cell proliferation, stimulate cell survival, and inhibit apoptosis. Other conditions, such as cell-cell contact inhibition, stimulate the Hippo-like pathway in mammals. LATS kinase phosphorylates YAP1 and TAZ and promote binding to 14-3-3 proteins. As a result, YAP1 and TAZ translocate to the cytoplasm, leading to inhibition of their transcriptional activation potential. (From Wang et al. 2009)

The MST kinase is regulated upstream by the membrane-associated proteins NF2 and FERM6 and also by MOB1 and WW45. The extracellular signals that activate the pathway have yet to be fully defined.

Much interest for this pathway has arisen from the characterisation of its role in organ growth in Drosophila and tumour suppression in mammals. Several lines of evidence highlight the importance of the Hippo pathway in human cancer. Mutation of the Hippo pathway components, such as the NF2 tumor suppressor, is known to contribute to human tumorigenesis (McClatchey and Giovannini, 2005). More importantly, YAP1 is the candidate oncogene in the human chromosome 11q22 amplicon, which is evident in several human

cancers (Overholtzer et al., 2006 ; Zender et al., 2006). Furthermore, YAP and TAZ are highly expressed in a wide spectrum of human cancer cell lines and various primary tumors (Chan et al., 2008 ; Dong et al., 2007). YAP1 and TAZ over-expression stimulates proliferation of cultured cells and allow them to overcome cell contact inhibition (Zhao et al., 2007 ; Lei et al., 2008). In addition, YAP1 and TAZ over-expression in MCF10A cells induces epithelial-mesenchymal transition (EMT), which is a hallmark of tumorigenic transformation (Chan et al., 2008 ; Overholtzer et al., 2006). Moreover, elevated YAP1 protein levels and increased nuclear localization have been observed in multiple human cancer tissues (Zhao et al., 2007). Interestingly, YAP1 over-expression causes a dramatic increase in liver size and eventually leads to tumor growth (Camargo et al., 2007 ; Dong et al., 2007).

The interaction of TEAD factors with their transcriptional coactivators YAP1/TAZ is important in mediating their oncogenic potential. It has been shown that the TEAD family members are required for YAP1-induced cell growth, oncogenic transformation, and epithelial mesenchymal-transtion (Zhao et al., 2008).

The Hippo pathway is also important in the control of organ size in Drosophila. Scalloped and Yorkie are the *Drosophila* orthologues of TEAD and YAP1 respectively. Similarly to what occurs in mammals, transcription factor Scalloped acts together with the coactivator Yorkie to regulate Hippo pathway-responsive genes in *Drosophila* (Zhang et al., 2008b ; Wu et al., 2008 ; Goulev et al., 2008). Scalloped over-expression enhances, whereas its inactivation suppresses, tissue overgrowth caused by Yorkie over-expression (Zhang et al., 2008b).

V.4.3. Interaction of TEAD factors with other transcription factors.

The widely expressed TEAD proteins interact with several transcription factors to control muscle-specific gene expression. It has been proposed that expression of muscle-specific genes is controlled by unique combinations of transcription factors that are expressed ubiquitously or cell-selectively. TEAD proteins interact with SRF, MEF2, and MAX transcription factors and these interactions are thought to participate in the control of their muscle specific target genes (Azakie et al., 2005 ; Gupta et al., 2001 ; Gupta et al., 1997 ; Maeda et al., 2002b).

SRF is a MADS box transcription factor that binds to consensus sequence CC(A/T)(6)GG found in the promoter region of several serum-inducible and muscle-specific genes. It has been shown that SRF and TEAD1 interact physically via the MADS and TEA

domains, respectively. In transient transfection assays, a positive cooperative effect of SRF and TEF-1 was observed when DNA-binding sites for both factors were intact, while mutation of either site abolished their synergistic effect. This interaction was required for the activation of the skeletal α-actin promoter (Gupta et al., 2001). Furthermore, TEAD1 has also been shown to interact with MAX to positively regulate α-MHC expression in primary cultures of cardiomyocytes (Gupta et al., 1997). On other hand, TEAD3 has been reported to interact with MEF2 and this interaction cooperatively increases the activity of the cardiac Troponin T promoter in cardiomyocytes (Azakie et al., 2005).

V.4.4. Importance of the MCAT element flanking sequence.

The sequences flanking the MCAT element and their binding factors are capable of modulating the transcriptional activity of MCAT element-containing promoters (Larkin et al., 1996). Switching the flanking sequence of the MCAT1 element of the cardiac Troponin T (cTNT) promoter for that of the SV40 GTIIC element abolished muscle-specific expression of a reporter gene. These results indicate that MCAT flanking regions are required for repressing expression in non-muscle cells. Butler et al have shown that the chromatin-modifying enzyme PARP binds specifically to both TEAD1 and the sequences flanking the MCAT1 element of the cTNT promoter. PARP binding to the MCAT element was significantly reduced when this was replaced by those of SV40 GTIIC enhanson. PARP can poly-ADP-ribosylate TEAD1 *in vitro* and inhibition of the PARP enzymatic activity repressed expression of an MCAT1-dependent reporter in transfected primary muscle cells (Butler and Ordahl, 1999). Together, these data implicate PARP as an auxiliary protein that interacts with and modifies TEAD1 on the flanking region of the MCAT1 element to control muscle-specific transcription of the cTNT gene.

Another example of the role of MCAT element flanking regions in the control of muscle-specific gene expression is given by single stranded DNA binding proteins (SSBP). Carlini et al., have shown that although mutation of the MCAT1 element of the mouse smooth muscle (SM) α-actin promoter decreased its transcriptional activity, mutation of the flanking regions increased its activity in fibroblast and smooth muscle cells. They found that the flanking sequences surrounding the MCAT1 element constitute the binding site for three single-stranded DNA-binding proteins, Purine-rich binding protein-α and β (Purα, Purβ respectively), and MSY1(called also Y box protein 1 (YBP1)) (Carlini et al., 2002). This binding site shows a high degree of polypurine/polypyrimidine (Pu/Py) asymmetry. Purα□, Purβ interact with the purine-rich strand and MSY1 with the complementary pyrimidine-rich strand. Loss of function studies using small interfering RNA (siRNA) have shown that

knockdown of Purβ, but not Purα, increased the transcriptional activity of the SM α-actin gene and concordantly, over-expression of Purβ, but not Purα, decreased its activity (Knapp et al., 2006). Taken together, these results suggest that the sequence flanking the MCAT elements contribute to the regulation of MCAT element-dependent genes.

V.4.5. Alternative splicing.

Several isoforms for the TEAD family members have been described (Stewart et al., 1994 ; Zuzarte et al., 2000 ; Jiang et al., 2000). Some TEAD1 isoforms lack a putative nuclear localisation signal (NLS) and are localized in the cytoplasm, whereas most isoforms are located in the nucleus (Zuzarte et al., 2000). SV40 large T-Ag transformed fibroblasts express several alternate spliced forms of TEAD1. The alternative splicing concerned the third α-helix of the TEA domain and the immediate downstream region (Zuzarte et al., 2000), which modulates the DNA binding and functional properties of these isoforms (Jiang et al., 2000). Some TEAD1 isoforms contained an additional VTSM (for Valine, Threonine, Serine, Methionine amino) motif, which is a potential phosphorylation site for Protein Kinase C and casein Kinase II allowing them to be regulated differentially from the canonical TEAD1 protein (Jiang et al., 2000).

V.5. TEAD4/TEF3 transcription factor.

V.5.1. TEAD4/TEF3 cloning.

Human TEAD4 was cloned in my host laboratory from cDNA libraries of Hela cells while mouse TEAD4 was cloned from cDNA libraries of retinoic acid-differentiated embryonic stem cells and a 10.5-day mouse embryo (Jacquemin et al., 1996).

Two degenerate oligonucleotides deduced from the sequence of the TEA domain were used in polymerase chain reaction (PCR) amplification experiments with cDNA libraries from either human or mouse cells. Amplification products of the expected size were cloned and their DNA sequence determined. The cDNA libraries were rescreened with the novel partial cDNAs, and full-length clones encoding hTEAD4, mTEAD4, were isolated. Alignment of the amino acid sequences of hTEAD1, hTEAD3, showed that overall hTEAD4 is 76% identical to hTEAD1.

V.5.2. TEAD4/TEF3 in early development.

TEAD4, together with TEAD1 and TEAD2, is among the first transcription factors to be expressed during zygotic gene activation in mouse 2-cell embryos. The role of TEAD4 in

early development have been subsequently been studied in knockout mice by Yagi et al., and Nishioka et al. (Yagi et al., 2007 ; Nishioka et al., 2008).

TEAD4 knockout mice die early in embryogenesis because of a defect in embryonic implantation. *Tead4*$^{-/-}$ morulae do not produce trophoblast stem cells, trophectoderm or blastocoel cavities, and therefore do not implant into the uterine endometrium (Yagi et al., 2007). *Tead4*$^{-/-}$ embryos do not express *Cdx2* (caudal type homeobox 2) that is required for the establishment of trophoblast stem (TS) cells nor eomesodermin (*Eomes*), that acts downstream of *Cdx2*, and is required for post-implantation extraembryonic tissue development (Russ et al., 2000 ; Strumpf et al., 2005). Another transcription factor whose expression is regulated by TEAD4 during trophectoderm formation is GATA3. GATA3 is expressed in the trophoblast lineage *in vivo* and its ectopic expression is sufficient to induce trophoblast genes in ES cells (Ralston et al., ; Nishioka et al., 2009). GATA3 expression in the trophectoderm was greatly reduced upon TEAD4 inactivtion (Nishioka et al., 2009). Furthermore it has been shown that GATA3 regulates trophoblast development downstream of TEAD4 independently of CDX2 (Ralston et al.).

Nishioka et al. elegantly showed that the Hippo signaling pathway controls TEAD4 activity to distinguish mouse trophectoderm from the inner cell mass (Nishioka et al., 2009). In the outside cells of the early embryo, YAP1 localises to the nucleus and anticipates CDX2 expression. Modulation of TEAD4 or YAP1 activity by mRNA injection into blastocysts leads to changes in CDX2 expression. In contrast, in the inner cell mass, YAP1 is phosphorylated and is cytoplasmic. This is in agreement with the model of Hippo signalling described above in cultured cells, where LATS phosphorylation of YAP1 leads to its cytoplasmic localisation (Zhang et al., 2008a ; Zhao et al., 2007). Indeed, LATS2 over-expression in embryos greatly reduced YAP1 accumulation in the nucleus of injected cell (Nishioka et al., 2009).

From these observations, Nishioka et al. propose a model in which TEAD4 promotes trophectoderm development in the outer cells, whereas the activation of Hippo pathway through in the inner cells mass induces cytoplasmic localisation of YAP1 and therefore inactivation of TEAD4 [Figure 31].

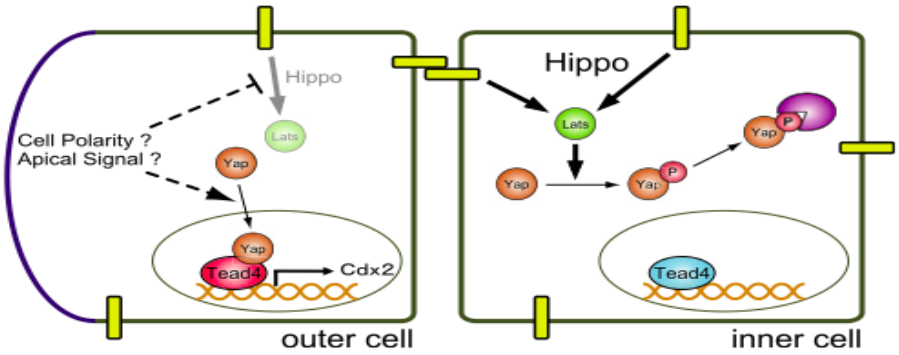

Figure 31. A model of cell position-dependent fate specification in pre-implantation embryos. (from Nishioka et al. 2009).

V.5.3. TEAD4 in muscle development and physiopathology.

V.5.3.1. TEAD4 in skeletal muscle development.

At mid-gestational stages in the developing mouse embryo, TEAD4 expression becomes restricted to the developing skeletal muscle, while TEAD1 is expressed in the developing myocardium and in skeletal muscle precursors (Jacquemin et al., 1996). TEAD4 and TEAD1 are expressed in the developing skeletal muscles derived from epaxial and hypaxial lineages as well as the head muscles derived from the unsegmented paraxial mesoderm. The expression of TEAD1 and TEAD4 in muscle is maintained at late stages of embryogenesis where a heterogeneity of TEAD4, but not TEAD1, expression is seen, beginning around 15.5 dpc. Interestingly, this corresponds to the time at which muscle innervation and fibre type differentiation begin suggesting that TEAD4 may be differentially expressed in different fibre types (Jacquemin et al., 1996).

Nevertheless despite the above observations, conditional knockout of TEAD4 in post-implantation embryos had no obvious morphological phenotype and muscle development appeared normal (Yagi et al., 2007). This may be explained by compensatory effects of the others TEAD family members. Such redundancy has been previously noted for other myogenic factors, such as MYF5 and MYOD1 (Rudnicki et al., 1993).

Further evidence for redundancy amongst the TEAD factors in muscle and in other tissues comes from other knockout mice studies. TEAD2 inactivation gives rise to viable adult animals and therefore has no evident non-redundant function. Inactivation of TEAD1 by gene-trap leads to embryonic lethality due to abberant cardiac development (Chen et al., 1994), although expression of many putative cardiac muscle TEAD target genes appeared normal. The cardiac defects may therefore also have arisen from defects in placenta development. In contrast, a double knockout of TEAD1 and TEAD2 has a more dramatic phenotype with multiple growth and morphological abnormalities (Sawada et al., 2008). The genetic studies of mouse TEAD factors therefore indicate that different members of the

family can have both specific and redundant functions. This functional redundancy may account for the fact that knockouts of a single family member do not show an obvious skeletal muscle phentotype.

V.5.3.2. TEAD4 in cardiac arrythmias.

Transgenesis has also been used to study TEAD4 function in heart. Transgenic mice with cardiac-specific expression of human TEAD4 under the control of rat α-MHC promoter developed cardiac arrythmias (Chen et al., 2004a). These arrythmias was due to cardiac conduction defects correlated with dephosphorylation of Connexin 40 and Connexin 43 (also known respectively as gap junction protein, alpha 5 and alpha 1 (Gja5) and (Gja1) and up-regulation of protein phosphatase 1 (PP1). Indeed, the authors of this work found that over-expression of PP1 in HeLa cells dephosphorylated these cardiac connexins. Confocal microscopy revealed increased levels of dephosphorylated connexin 43 at the cardiac gap junctions in TEAD4-transgenic mice, suggesting that defective conduction is a result of impaired gap-junction conductance rather than assembly. Because chronic dephosphorylation of connexins impairs the gap-junction conductance, this is likely to be the cause of arrhythmias in TEAD4 transgenic mice.

V.5.3.3. TEAD4 in cardiac hypertrophy.

Cardiac hypertrophy is a thickening of the heart muscle (myocardium) which results in a decrease in size of the chamber of the heart, including the left and right ventricles. Healthy cardiac hypertrophy (physiological hypertrophy) is the normal response to healthy exercise or pregnancy, which results in an increase in the heart muscle mass and pumping ability. Pathological hypertrophy occurs in a number of pathological conditions including hypertension, valvular disease, myocardial infraction, and cardiomoyopathy (Yoshida, 2008). At the cellular level, cardiac hypertrophy is characterized by an increase in cell size and protein synthesis and by reactivation of the fetal cardiac genes including β-MHC and skeletal α-Actin (Simpson et al., 1991). In cultured neonatal rat cardiomyocytes, stimulation of a1-adrenergic signalling has been shown to induce cardiac hypertrophy and activate transcription of the β-MHC gene and the skeletal a-actin gene in a MCAT and TEAD4-dependent manner (Stewart et al., 1998 ; Karns et al., 1995 ; Kariya et al., 1994).

Thesis project.

TAF4b.

The original subject of my thesis project was the study of the TAF4b subunit of the general transcription factor TFIID in mouse embryonic stem cells. TAF4 and TAF4b are two parologous subunits of the TFIID complex (Dikstein et al., 1996). While TAF4 is almost ubiquitously expressed, TAF4b expression is may be more restricted and in strongly over-expressed in the male germ cells in the testis and in the granulosa cells of the ovary. TAF4b inactivation in mice leads to both male and female sterility (Freiman et al., 2001 Falender et al., 2005). However, experiments performed in my host lab, showed that inactivation of TAF4, unlike several other TAFs, did not lead to cell cycle arrest and apoptosis. In *Taf4*$^{-/-}$ MEFs, increased association of TAF4b with TFIID could be observed together with changes in gene expression (Mengus et al., 2005). The *Taf4*$^{-/-}$ MEFs are characterised by activation of the TGFb signalling pathway leading to serum free autocrtine growth. These observations suggested that TAF4 and TAF4b containing TFIID complexes may have different target genes and differential abilities to respond to signalling pathways and transcriptional activators.

In a first set of experiments, I examined TAF4b expression in various mouse tissues and cell lines by immunoblot and noted that TAF4b was well expressed in mouse ES cells. To identify TAF4b associated proteins and potential target genes and I used bacterial artificial chromosome (BAC) recombineering to generate a targeting vector for modification of the *Taf4b* gene to express a C-terminally tandem-affinity 3XFLAG-SBP (streptavidine binding peptide) tagged TAF4b. The modified BAC was electroporated in mouse ES cells and clones expressing TAP-tagged TAF4b from a homologously recombined allele were identified. Extracts from the undifferentiated ES cells were prepared and subjected to tandem affinity purification. While immunoprecipitation with FLAG M2 resin resulted in an efficient purification of the tagged TAF4 protein, the second SBP purification step was extremely inefficient and despite several changes to the protocols could not be improved.

While a single purification step did not yield a highly purified complex, I nevertheless analysed the anti-FLAG immunoprecipitates by mass spectrometry. TAF4b and the other TBP and TAF-subunits of the TFIID complex could be identified in the precipitated fraction from the TAF4b-tagged cells, but not from control untagged cells.

I also attempted to perform chromatin immunoprecipitation (ChIP) experiments from the cells expressing tagged TAF4b. The experiments that I performed as well as those performed by other members of the groups at this time indicated that FLAG-ChIP was

characterised by a high background. I made several ChIP experiments followed by qPCR to detect TAF4b occupancy at several target genes, however no enrichment could be seen at house keeping promoters such as those of the ribosomal protein RPLP0 or previously described TAF4b target genes such as c-Jun. Given the difficulties in performing tandem affinity purification (TAP) and ChIP with the 3XFLAG-SBP combination, we decided to terminate this aspect of my work.

Role of TEAD4 in muscle development.

During this time, I worked together with Dr Aurore Morlon in the laboratory who used BAC recombineering and homologous recombination to introduce the 3XFLAG-SBP TAP-tag on the C-terminus of the *Tead4* gene in ES cells. Clones expressing TAP-tagged TEAD4 were isolated, however the same technical difficulties observed with TAF4b also arose during the purification of tagged TEAD4. The first choice of the 3XFLAG-SBP as a tag combination therefore proved disappointing. It should however be stressed that the tagging of TEAD4 in ES cells was performed before the publication of the results showing its critical role in trophoblast specification (Yagi et al., 2007). The idea of identifying target genes and protein partners of TEAD4 at this early stage therefore remains pertinent.

In parrallel with the tagging of TEAD4 in ES cells, we also generated C2C12 cells expressing ectopic C-terminal 3XFLAG-HA-tagged TEAD4 with aim of identifying its target genes by ChIP and its protein partners by mass spectrometry. Despite the lack of a clear demonstration of a role for TEAD factors in skeletal muscle, we were prompted to perform these experiments by the observation that ChIP-chip performed by Blais et al. (Blais et al., 2005) showed that TEAD4 is a direct target of the MYOD1 and MYOG transcription factors in C2C12 cell differentiation. Up-regulation of TEAD4 by MYOD1 and MYOG during differentiation was proposed then to activate transcription of a downstream gene expression programme of muscle-specific structural genes. The observation that TEAD4 was a target of MYOD1 and MYOG provided further evidence that it would play an important role in differentiation, however only a small number of muscle target genes of the TEAD family have been identified, and even less are known in other tissues.

The more recent spate of results concerning the role of the TEAD/YAP complex in promoting cell proliferation via the Hippo pathway raised the question as to how the TEAD factors can play such contrasting roles in, on the one hand, promoting proliferation and on the other, activating muscle-specific genes during the cell cycle arrest that accompanies muscle differentiation.

The aim of this project was therefore to determine whether the TEAD factors had indeed a role in muscle differentiation, and to identify TEAD4 target genes in differentiating C2C12 cells to understand its specific role in this process. Our original hypothesis was that cell-type differences in the repertoire of TEAD target genes between muscle and non-muscle cells may help to explain how the TEAD factors can promote both differentiation or proliferation in a cell specific manner. The results of these experiments are described in the accompanying manuscript

RESULTS

1) Cooperation of TEAD transcription factors with MYOD1 and Myogenin is essential for C2C12 cell differentiation.

Manuscript in preparation

Cooperation of TEAD transcription factors with MYOD1 and Myogenin is essential for C2C12 cell differentiation.

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Running Title : TEAD regulates myoblast differentiation.

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Abstract.

The TEAD family of transcription factors (TEAD1-4) comprise the conserved TEA/ATTS DNA binding domain that recognises the MCAT element present in the promoter of several muscle specific genes. Despite extensive genetic analysis, the precise function of the TEAD factors in muscle specification and differentiation has proved elusive due to redundancy amongst the family members. Here we show that expression of the isolated TEA/ATTS DNA binding domain, that acts as a dominant negative repressor of all TEAD factors, in C2C12 myoblasts completely inhibits their differentiation into myotubes. The TEAD4 factor is potently induced during C2C12 differentiation under the control of MYOD1 and Myogenin. ShRNA silencing of TEAD4 expression results in abnormal C2C12 cell differentiation characterised by the formation of shortened myotubes. Chromatin immunoprecipitation coupled to array hybridisation (ChIP-chip) shows that TEAD4 occupies 864 promoters including those of MYOD1 and Myogenin, multiple muscle structural genes and miRNAs required for differentiation. Almost 40% of TEAD4 target genes are also occupied by MYOD1 suggesting an extensive cooperation between these two factors during differentiation. Immunoblot and RNA expression analyses indicate that TEAD factors cooperate with MYOD1 to induce Myogenin and CDKN1A expression and regulate Caveolin 3 and Dysferlin essential for myoblast fusion. In contrast, TEAD4 represses expression of the growth factor CTGF and the transcriptional cofactor YAP1 to promote differentiation Together these results show that TEAD factor activity is essential for C2C12 cell differentiation and that TEAD4 plays a critical role in regulation the genes required for myoblast fusion.

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 rather degenerate motifs of the simian virus 40 (SV40) enhancer (Davidson et al., 1988; Xiao et al., 1991). Subsequent studies have shown that the TEAD factors make a highly conserved family of eukaryotic DNA-binding proteins [(Jacquemin et al., 1996) (for review see (Jacquemin and Davidson, 1997; Yoshida, 2008)]. 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) (Andrianopoulos and Timberlake, 1991; Burglin, 1991). 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 enhancer (Anbanandam et al., 2006). Most members of the family also bind cooperatively to tandemly repeated binding sites such as those found in the SV40 enhancer, the somatomammotropin (hCS)-B gene enhancer and the connective tissue growth factor (CTGF) proximal promoter (Davidson et al., 1988; Jacquemin et al., 1997; Leask et al., 2003). Cooperative binding requires the L1 loop of the homeodomain fold. DNA binding of TEAD1 has also been shown to be modulated by phosphorylation.

Members of the mammalian TEAD family are expressed in a variety of tissues, with particularly prominent expression in the nervous system and muscle, where their function has been well studied. 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 genes (Chen et al., 2004a; Chen et al., 1994; Mar and Ordahl, 1988; Mar and Ordahl, 1990). A number of these genes, for example cardiac troponin T, β-MHC and Myocardin, have been shown to have functional MCAT motifs in their regulatory regions (Yoshida, 2008). TEAD4 may play a role in cardiac hypertrophy, characterized by increased cell size and reactivation

of fetal cardiac genes (Karns et al., 1995). Stimulation of α1-adrenergic signalling has been shown to induce cardiac hypertrophy and activate transcription of the β-MHC gene and the skeletal α-actin gene in a MCAT and TEAD-dependent manner in cultured neonatal rat cardiomyocytes (Maeda et al., 2002b; Ueyama et al., 2000). Similarly, cardiac muscle-specific overexpression of TEAD4 in transgenic mice has been shown to induce arhythmias *in vivo* (Chen et al., 2004a).

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 genes (Creemers et al., 2006). In the case of the smooth muscle α-actin gene, it appears that it is regulated by TEAD4 in myofibroblasts, but by TEAD1 in differentiated smooth muscle cells (Gan et al., 2007). Similarly, the conserved tandem MCAT binding sites in the CTGF proximal promoter seem to be involved in TGFβ-mediated activation of this gene (Leask et al., 2003). CTGF is a critical factor inducing myofibroblast proliferation and matrix deposition.

Additional evidence for a role of TEAD4 in muscle differentiation comes from the observation that it is specifically expressed in developping skeletal muscle in mouse embryos (Jacquemin et al., 1996). Furthermore, chromatin immunoprecipitation (ChIP)-chip shows that TEAD4 is a direct target of the MYOD1 and MYOG transcription factors in C2C12 cell differentiation (Blais et al., 2005). 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 leathality due to lack of trophectoderm specification (Yagi et al., 2007). Conditional TEAD4 inactivation subsequent to the specification of the trophectoderm 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 (Wu et al., 2008; Zhang et al., 2008a; Zhao et al., 2008) (Fernandez and Kenney, ; Grusche et al.,). TEAD factors interact with the YAP1 and TAZ/WWTR1 coactivators that are phosphorylated and inhibited by the Hippo tumor suppressor pathway (Mahoney et al., 2005 ; Vassilev et al., 2001). TEAD factors are also required for YAP-induced cell growth, oncogenic transformation, and epithelial-mesenchymal transition (Zhao et al., ; Zhao et al., 2008). 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 TEAD DBD in C2C12 cells. We show that general 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 864 genes including *Myod1* and *Myog* and is required for activation of *Myog* expression during differentiation. In addition, TEAD4 directly activates expression of the Dysferlin (*Dysf*) and Caveolin 3 (*Cav3*) genes to regulate myoblast fusion. Together these results show that TEAD factors are required for C2C12 differentiation and TEAD4 plays a specific role in myoblast fusion via regulation of the *Dysf* and *Cav3* genes and acts in a positive feedback loop with *Myog*. In contrast, TEAD4 knockdown leads to up-

regulation of CTGF 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 Affymetrix array data shows that undifferentiated C2C12 cells express all members of the TEAD family, with lowest expression of TEAD1 and higher expression of TEAD2 and TEAD4. (Fig. 1A and Supplemental Fig. 1). The expression of TEAD1 and TEAD4 is strongly upregulated during differentiation, while that of TEAD2 and TEAD3 is unaffected (Fig. 1A and Supplemental Fig. 1). To address the function of these factors in C2C12 cells, we had to consider the potential redundancy between the closely related TEAD proteins. We chose to generate C2C12 cells stably expressing a Flag-tagged-TEAD1 DBD fused to a nuclear localisation signal. The DBD is essentially identical in all members of the family and can be used as a dominant negative inhibitor of TEAD function (Hwang et al., 1993). Expression of the isolated DBD (Fig. 1B lane 2) leads to diminished upregulation of TEAD4 during differentiation, as well as a loss of MYOG, CDKN1A and βMHC expression (Fig. 1C) and almost completely inhibits differentiation, where only a few short fused myotubes are observed (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 whose expression is strongly induced during C2C12 cell differentiation (Fig. 1A, and 1C and D, lanes 1-4). To address the role of this protein, we used lentiviral vectors expressing shRNA to make a stable knock down of TEAD4. Compared to cells 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. Loss of TEAD4 expression induced 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 βMHC (Fig. 2B). In the ShA and ShB knockdown cells, the vast majority of myotubes are significantly shorter than in the controls, with only 2-3 nuclei per myotube. However, the majority of cells in the culture showed βMHC expression indicating that they had begun to differentiate, 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.

Dexamethasone (Dex) treatment has been shown to augment myoblast fusion (Montano and Lim, 1997; Yoshiko et al., 1998). Treament of control ShC cells with Dex led to the formation of thickened myotubes that fused to form complex syncytial like structures (Fig. 3A). TEAD4 expression was enhanced in the presence of Dex showing a more rapid induction (Fig. 3B, 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. Even in the presence of Dex, however, 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 myoblast differentiation and that TEAD4 plays an essential role in 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 to not allow ChIP of TEAD4, we established C2C12 cells that stably express Flag-HA tagged (F-H-)TEAD4 (Fig. 1C lanes, 3 and 4). The exogenous tagged protein is expressed to a level comparable to that of the endogenous TEAD4 in undifferentiated cells, but as it is not subject to the regulation of the endogenous protein and is not up-regulated it makes up less of the TEAD4 population in differentiated cells (data not shown). Expression of the endogenous protein did not affect myoblast proliferation, nor 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. 4A).

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 (Delacroix et al., 2010; Kobi et al.,2010) (Materials and methods and see Supplemental text). This analysis identified 864 promoters with at least one TEAD4 occupied site (Supplemental Table 1). Taking into account the divergent promoters a total of 926 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)(also called inside region) (Fig 4B 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*) and *Myod1* genes (Figs 4A, 5A, C-E and Supplemental Table 1).

In addition, TEAD4 occupies sites at 17 miRNA genes including the muscle-enriched Mmu-mir-206 (Figs. 4A and 5B and Supplemental Table 1). Furthermore, 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 (Anderson et al., 2006; Kim et al., 2006; Townley-Tilson et al.). Likewise, TEAD4 occupies a site at the locus encoding mmu-mir-214 that promotes skeletal muscle differentiation (Flynt et al., 2007; Juan et al., 2009; Liu et al.). Occupancy of a subset of these sites was confirmed by ChIP-qPCR experiments (Fig. 4A).

Ontology analysis of the occupied genes revealed several potential functions for TEAD4. In addition to expected classes such as sarcomere, contractile fibre and cytoskeleton most of which are 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 (Fig. 4D and Supplemental Table 2) as well as potential oncogenes and anti-oncogenes. 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 cell proliferation (see discussion).

Analysis of the DNA sequences at the TEAD4 occupied peaks using the MEME programme (http://meme.nbcr.net/meme4_1/cgi-bin/meme.cgi) (Bailey, 1994)) identified two closely related motifs (MCAT-A and MCAT-B) that were highly represented in the TEAD4 occupied sites (Fig. 6A). Each motif contains the highly conserved 5'-ATTCC-3' core of the previously defined MCAT motif, but the MCAT-A motif shows extended conservation with a highly conserved position. MEME analysis identified 103 MCAT-A motifs and 198 MCAT-B

motifs (Supplemental Table 3). The TEAD4 occupied motifs at other sites are likely degenerate versions of these motifs. We also 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 that of the ChIP-chip data on MYOG of Blais et al., (Blais et al., 2005). In the ChIP-chip experiments of Blais et al, 137 MYOG-occupied promoters were identified. Of these, 21 are also occupied by TEAD4 (Fig. 6B and Supplemental Table 4). We also compared the TEAD4 ChIP-chip data with the MYOD1 and MEF2 ChIP-chip data from the same study. MYOD1 and MEF2 were found to occupy 126 and 28 promoters respectively. Comparison with the TEAD4 data set shows that 13 promoters were commonly occupied by MYOD1 and TEAD4 and 12 by TEAD4 and MEF2 (Fig. 6B and Supplemental Table 4). Several promoters like those of *Acta1*, *Ing3* and *Myog* are occupied by all three factors.

These comparisons did not reveal a large overlap of TEAD4 and MYOD1 target genes, however, a more extensive ChIP-seq data set for MYOD1 has been reported (Cao et al.). The raw data set was down-loaded and re-analysed by our MACS and GPAT pipeline (see Material and methods and Supplemental text). The GPAT analysis was performed using a 5 kb window around the gene coordinates allowing annotation of sites that were located 5 kb upstream of the TSS, within the gene and 5 kb downstream of the polyadenylation site. This allowed us to identify a set of MYOD1 occupied genes and compare them with the TEAD4 data set. Two comparisons were made. We first compared the genes that were occupied by TEAD4 at the proximal promoter region (-5kb to +2kb relative to the TSS) with MYOD1 occupancy as described above. In this case, 351 genes were occupied by both MYOD1 and TEAD4 (Fig. 6C and Supplemental Table 4). We also identified 248 genes where the MYOD1 sites are located ≤ 5kb form the TEAD4 site. Thus 40% of TEAD4 occupied genes are also MYOD1 target genes and 28% of the TEAD4 occupied sites are

located ≤ 5kb from a MYOD-occupied site. These data support the idea that MYOD1 and TEAD4 cooperate to activate a large series of muscle target genes including key regulatory molecules *Myog*, and *Cdkn1a*, structural components *Jup*, *Myl6b*, and *Ttn*, and components of the neuromuscular junction, *Musk* and *Chrna1*.

Zhao et al., have reported ChIP-chip results for TEAD1 in human MCF7 breast cancer cells (Zhao et al., 2008). We compared these TEAD1 occupied genes with those occupied by TEAD4 in C2C12 cells. From the data provided by Zhao et al, we identified mouse orthologues of 1831 of the 2323 genes occupied by TEAD1 in MCF7 cells. Of these only 122 were occupied by TEAD4 in C2C12 cells (Fig. 6D and Supplemental Table 4). Nevertheless, amongst the genes that are occupied in each cell type are genes important for proliferation such as *Cdkn1a*, and *Ctgf* and *Myod1*. Thus TEAD factors occupy a rather distinct set of genes in C2C12 and MCF7 cells.

Direct regulation of C2C12 cell differentiation genes by TEAD4

An important observation from the ChIP-chip results is that TEAD4 binds to the TEAD1 promoter (Fig. 5F). As shown above, TEAD1 expression is strongly induced upon differentiation (Fig. 1A). In differentiating cells expressing the ShB RNA, *Tead1* activation is strongly diminished (Fig. 7A). These results show that TEAD4 directly regulates TEAD1 expression during myogenic differentiation. As TEAD1 and TEAD4 bind essentially the same recognition sequence, the changes in expression of TEAD4 target genes in the ShB cells may be due to loss of TEAD4, TEAD1 or both proteins.

The ChIP analysis indicates that TEAD4 occupies a site in the *Myog* promoter (Fig. 5) 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. 7B). In contrast, TEAD4 knock-down does not affect MYOD1 expression that appears even mildly increased in the ShB-expressing cells (Supplemental Figure 3A). Together, these results show that TEAD4 is essential for *Myog* activation in C2C12 cell differentiation either due to its presence at the promoter, or due to its ability to induce *Tead1* expression which could then also occupy and regulate the *Myog* promoter, but not because of loss of MYOD1 expression.

It has previously been shown that activation of *Cdkn1a* ($p21^{cip1}$) expression by MYOD1 is involved in cell cycle arrest during myogenic differentiation (Guo et al., 1995; Halevy et al., 1995). ChIP-chip and ChIP-qPCR analysis shows that TEAD4 occupies a site in the first intron of the *Cdkn1a* gene (Supplemental Fig. 4A and Supplemental Table 1). CDKN1A is strongly expressed in differentiating control cells with strongest expression observed at days 1 and 3 (Fig. 1C and D, lanes 1-4), but its expression is strongly diminished in the ShB or DBD-expressing cells. These observations indicate that TEAD and MYOD1 cooperate to activate CDKN1A expression during 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. 4 and Supplemental Fig. 2). RT-qPCR experiments show that expression of Mmu-mir-206, -1-2 and -133a-1 are all significantly down-regulated in the ShB cells (Fig. 7C-E). TEAD4/TEAD1 is therefore required for the normal activation of these miRNAs during C2C12 cell differentiation.

In addition to the above, TEAD4 is also found associated with the promoters of many muscle structural genes such as *Acta2*, Titin (*Ttn*) or *Anrkd2*, but also other genes that are involved in differentiation like Calsequestrin 1 (*Casq1*) or Myocardin-like 2 (*Mkl2)* that acts as a transcriptional coactivator for serum response factor (Cen et al., 2004; Parmacek, 2007) (Supplemental Fig. 4B and C and data not shown). The expression of these genes is down-

regulated in the ShB cells (Fig. 7F-H and data not shown). Interestingly, TEAD4 also occupies the promoters of the *Ankrd1* (CARP) and *Ankrd23* (DARP) genes (Supplemental Fig. 4D and E) that together with *Ankrd2* constitute the MARPs (muscle ankyrin repeat proteins) and interact with Titin to form a signalling complex (Kojic et al., 2004; Miller et al., 2003). The MARPs also appear to contribute to the coordination of proliferation and apoptosis during myogenic differentiation through the TRP53 network (Bean et al., 2008). Thus, the TEAD factors may regulate all of the key structural components of the complex, but does not seem to occupy the promoter of the Calpain 3 component. Unfortunately, as there are no oligonucleotide probes on the Agilent array for the Myopalladin component of the complex, direct TEAD4 regulation of this gene cannot be assessed. These data highlight the diverse roles of genes regulated by the TEAD factors, in transcription, signalling and structural aspects of muscle differentiation.

TEAD4 directly regulates genes required for myoblast fusion.

The results presented above show that shRNA-mediated TEAD4 knockdown leads to defective myoblast fusion. 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. 5E and F, and Supplemental Fig. 4F and data not shown) all of which have been shown to be involved in myoblast fusion (Bansal and Campbell, 2004; Conti et al., 2009; Galbiati et al., 1999; Han and Campbell, 2007; Liu et al.). RT-qPCR experiments in cells expressing the ShSC and ShB RNAs show that the expression of each of these genes is normally up-regulated during differentiation, while their activation is strongly reduced in the TEAD4 knockdown cells (Fig. 8). We also observed a reduced expression of Myoferlin (*Myof*), a protein related to Dysferlin, in the ShB cells, although as for Myopalladin, there are no oligonucleotide probes on the

Agilent array at this promoter so direct TEAD4 regulation cannot be assessed. Reduced Dysferlin and Caveolin 3 protein expression can also be observed in the ShB knockdown cells in both the presence and absence of Dex treatement compared to the ShSC cells and in the cells expressing the DBD (Fig. 3B and see also Supplemental Fig. 3A and B). Together these results show that TEAD4/TEAD1 directly regulates expression of several genes required for normal myoblast fusion accounting for the shortened myotubes seen upon its knockdown.

TEAD4 represses CTGF expression to promote myogenic differentiation.

As described in the introduction, TEAD factors interact with the YAP transcriptional activator to promote proliferation. In breast cancer cells for example, proliferation is mainly due to direct activation of the *Ctgf* promoter by the TEAD-YAP1 complex (Zhao et al., 2008). ChIP-chip shows that TEAD4 occupies the *Cgtf* promoter and a second internal binding site in differentiated C2C12 cells (Fig. 9A). To ask whether TEAD4 regulates CTGF expression during differentiation, we performed RT-qPCR experiments in the ShSC and ShB cells. In the control cells, *Ctgf* expression is only mildy increased during differentiation (Fig. 9D). However, contrary to the genes shown above that require TEAD4 for activation, *Ctgf* expression is up-regulated by TEAD4 knockdown. Thus while TEAD factors activate *Ctgf* expression in breast cancer cells, they repress its expression in differentiating C2C12 cells.

We also noted that TEAD4 occupies the promoter of the genes encoding the YAP1 and related WWTR1 coactivators (Fig. 9B and C). Similar to what was observed for *Ctgf, Yap1* expression is also mildly up-regulated by TEAD4 knockdown, while expression of Wwtr1 is unaffected (Fig. 9E-F). These results show that TEAD factors repress CTGF and YAP1 coactivator expression in C2C12 cells to promote their differentiation.

Discussion.

TEAD factor cooperation with MYOD1 is essential for 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 factors such as MYOD1 that activates the *Myog* and *Mef2* genes which then together act to regulate downstream genes involved in the above processes. 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 multiple downstream targets, but they are also essential for the initial events of the differentiation process. Hence, the TEAD factors join the previously described myogenic proteins as essential components in the transcription regulatory networks of myoblast differentiation.

The TEAD proteins directly regulate two genes that are essential for the initial steps of the differentiation process. We show that TEAD4 occupies the *Myog* promoter and expression of the TEAD-DBD or TEAD4 knockdown both lead to a loss of its activation. Since loss of TEAD function does not affect MYOD1 expression, the TEAD factors must therefore normally cooperate with MYOD1 to activate *Myog* expression. Blais et al have shown that MYOD1 and MYOG bind to the TEAD4 promoter (Blais et al., 2005), 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 and MYOG (Fig. 10).

A second gene that is activated in the initial stage of differentiation is *Cdkn1a* encoding the CDK-inhibitor p21. Like *Myog*, *Cdkn1a* expression is activated by MYOD1 during differentiation (Halevy et al., 1995; Lassar et al., 1994), and we show that this also requires cooperation with the TEAD factors as CDKN1A expression is not properly induced when TEAD function is inhibited. We propose therefore that cooperative action of TEAD and MYOD1 factors is required to active *Myog* and *Cdkn1a* expression and initiate myogenic

differentiation. This cooperativity is likely not limited to these two promoters as we identified more than 300 promoters that are occupied by both TEAD4 and MYOD1 suggesting that the cooperativity between these two factors plays a major role in differentiation. It is probable that TEAD4 ChIP-seq would reveal a much larger set of occupied sites and further increase the number of genes commonly occupied with MYOD1.

ShRNA-mediated down-regulation of TEAD4 leads to the appearance of shortened myotubes. This phenotype is less marked than the almost complete inhibition of differentiation seen upon expression of the TEAD4 DBD. This may be explained by a low residual TEAD4 expression that may be sufficient to initiate the differentiation process, or indicate a redundant contribution of the other members of the family. Indeed, silencing of TEAD4 expression inhibits TEAD1 activation, but does not affect the expression of the other members of the family. Expression of many of the muscle structural genes or fusion genes is not completely abolished upon TEAD4 silencing, but show only diminished expression, again suggesting that other TEAD factors can contribute to their expression. However, in the absence of TEAD4 and TEAD1, the other TEAD factors cannot assure the normal activation of genes required for fusion leading to the appearance of shortened myotubes. In particular, the strong down-regulation of CAV3 expression may account for the diminished myoblast fusion.

Gene ontology analysis of the ChIP-chip results not only reveals TEAD4 occupancy of the promoters of an extensive set of muscle structural genes, but also many other cellular processess and many genes involved in transcriptional regulation. While not all of these genes are not de-regulated upon TEAD4 silencing in differentiating C2C12 cells, these observations highlight the potential of the TEAD family to regulate these genes in other cell types.

Our observations demonstrate an important role for the TEAD factors in myoblast differentiation *in vitro*, yet this function has not been clearly demonstrated *in vivo*. TEAD4 inactivation leads to preimplantation lethality due to is requirement for trophectoderm

formation (Nishioka et al., 2009; Nishioka et al., 2008; Yagi et al., 2007) and post-implantation inactivation gives rise to viable adult animals with no obvious phenotype. In contrast, TEAD2 inactivation gives rise to viable adult animals, while inactivation of TEAD1 by gene-trap leads to embryonic lethality due to abberant cardiac development (Chen et al., 1994). A double knockout of TEAD1 and TEAD2 however has a more dramatic phenotype with several growth and morphological abnormalities (Sawada et al., 2008).

Together these results show that TEAD factors can have both specific and redundant functions accounting for the fact that single knockouts do not show an obvious skeletal muscle phentotype. This is in agreement with our *in vitro* studies showing that loss of all TEAD function through dominant negative inhibition is required to see a complete inhibition of differentiation. However, although TEAD4 silencing *in vitro* leads to shortened myotubes, the TEAD4-null animals appear normal. This may be explained by compensation by other TEAD factors during development in the animal model, that does not occur *in vitro* when an accute loss of TEAD4 expression is induced by shRNA silencing. Further studies will be required to determine the function of each TEAD factor in C2C12 differentiation.

Cell-specific regulation of proliferation genes by TEAD factors.

The results presented here indicate that 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* (Lian et al., ; Zhao et al., ; Zhao et al., 2008). It should be noted however that stable expression of the TEAD1-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 *Ctgf* promoter and the promoters for other growth promoting genes in C2C12

cells. While the repertoire of TEAD-occupied genes is quite different in C2C12 and MCF7 cells, we clearly see TEAD4 occupancy of the *Ctgf* locus in differentiated C2C12 cells.

Our results rather show that TEAD factors repress *Ctgf* expression during differentiation. This repression may be critical for the differentiation process as it has been shown that CTGF can inhibit C2C12 differentiation and even induce de-differentiation (Vial et al., 2008). 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. It is tempting to speculate that that opposite may be true for CDKN1A expression that may be activated by TEAD factors in C2C12 cells, but repressed in proliferating cells

What are the mecansims that account for this differential regulation?. It has been established that TEAD factors interact with YAP1 to activate CTGF and other genes of the proliferative programme. However, in C2C12 cells, it has been shown that YAP1 is phosphorylated and exported from the nucleus during differentiation (Watt et al.). Moreover, expression of a non-phosphorable YAP1 mutant that remains nuclear inhibits differentiation. Based on these observations, we propose that the export of YAP1 from the nucleus results in loss of TEAD activation of CTGF. In absence of YAP1, TEAD remains associated with and represses the *Ctgf* promoter. Nevertheless, TEAD factors activate other genes in differentiated cells showing that they act via other cofactors in the absence of YAP1, but that these cofactors must show promoter specificity and do not allow activation of CTGF. Thus, cofactor exchange during differentiation may account for the altered promoter specificity of TEAD factors. It has previously been proposed that the Vestigial (VGLL) family of proteins may act as cofactors for the TEAD factors in muscle cells (Chen et al., 2004b; Gunther et al., 2004; Maeda et al., 2002a; Mielcarek et al., 2002). Exchange of YAP1 for VGLL cofactors may therefore be an essential event in muscle differentiation. Initial immunopurification of TEAD4 from proliferating C2C12 cells followed by mass-spectrometry clearly reveals its

association with YAP1 and the related WWTR1 cofactors (our unpublished data). Analogous experiments in differentiated C2C12 cells will reveal which coactivators associate with TEAD4 in absence of YAP1.

As mentioned above, the identification of TEAD4 occupied genes in C2C12 cells reavels an extensive set of genes involved in cell cycle including the key cyclins D1 and D2 and the TRP53 signalling pathway as well as oncogenes such as MYC and ETS1. It has previously been shown that TEAD activation of Cyclin D1 drives the proliferation of neuronal precursors during development (Cao et al., 2008). Further studies will reveal when and where TEAD factor regulation of its other target cell cycle and proliferation genes is relevant for normal and pathological processes.

Materials and Methods.

C2C12 cell culture and differentiation.

C2C12 cells were cultured and differentiated under standard conditions as previously described (Perletti et al., 2001). C2C12 cell lines expressing Flag-HA tagged TEAD4 and the Flag tagged TEAD1-DBD were generated by infection with the the corresponding pBABE retroviruses and puromycin selection as described (Delacroix et al., 2010). Cells expressing the ShRNAs were generated by infection with the appropriate pLKO.1 lentiviral vectors and puromycin selection. 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.

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.

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 (Delacroix et al., 2010; Kobi et al.). 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 3.

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) (Bailey, 1994)). Comparisons with the previously described ChIP-chips from Blais et al., (Blais et al., 2005) were performed with Excel. For comparison with the MYOD1, the ChIP-seq data sets were down-loaded from the Sequence Read Archive public data base and the data re-analysed for peak detection using MACS (http://liulab.dfci.harvard.edu/MACS/) (Zhang et al., 2008b). Peaks were then annotated using GPAT [(Krebs et al., 2008), http://bips.u-strasbg.fr/GPAT/Gpat_home.html] using a window

of +/- 5kb with respect to the coordinates of the beginning and end of RefSeq transcripts. The list of MYOD1 occupied genes was then compared to the TEAD4 list using Excel.

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 δCt method was used to calculate relative fold changes normalized against beta-actin expression.

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 δCt 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'-CGCAAGGATGACACG CAAATTCGT-3').

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Legends to Figures.

Figure 1. Expression TEAD factors in C2C12 differentiation. **A**. (Left panel) RT-qPCR of TEAD1, TEAD4 expression during days 1-7 of C2C12 cell differentiation. (Right panel) RT-qPCR of TEAD4 in ShA and ShB expressing C2C12 cells compared to control ShSC expressing C2C12 cells during differentiation **B**. Immunoblot analysis of undifferentiated C2C12 cells stably expressing the Flag-tagged TEAD1-DBD or Flag-HA tagged full length TEAD4 using anti-TEAD4 and anti-Flag antibodies. **C**. Immunoblot analysis of the expression of the indicated proteins during differentiation of control C2C12 cells and cells expressing the TEAD1-DBD. **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 TEAD1-DBD or the TEAD4 shRNAs. **A.** Phase contrast images of the indicated C2C12 cells at day 7 of differentiation. Magnification 20X. **B.** Macro-immunofluorescence of the indicated C2C12 cells at day 7 of

differentiation using anti-bMHC 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.

Figure 3. Effect of dexamethasone on C2C12 differentiation. **A**. Immunofluorescence of the indicated C2C12 cells at day 7 of differentiation in presence or absence of Dex using anti-bMHC antibody. **B.** Immunoblot analysis of expression of the indicated proteins in the cells differentiated in presence or absence of Dex.

Figure 4. 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.

Figure 5. 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 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.

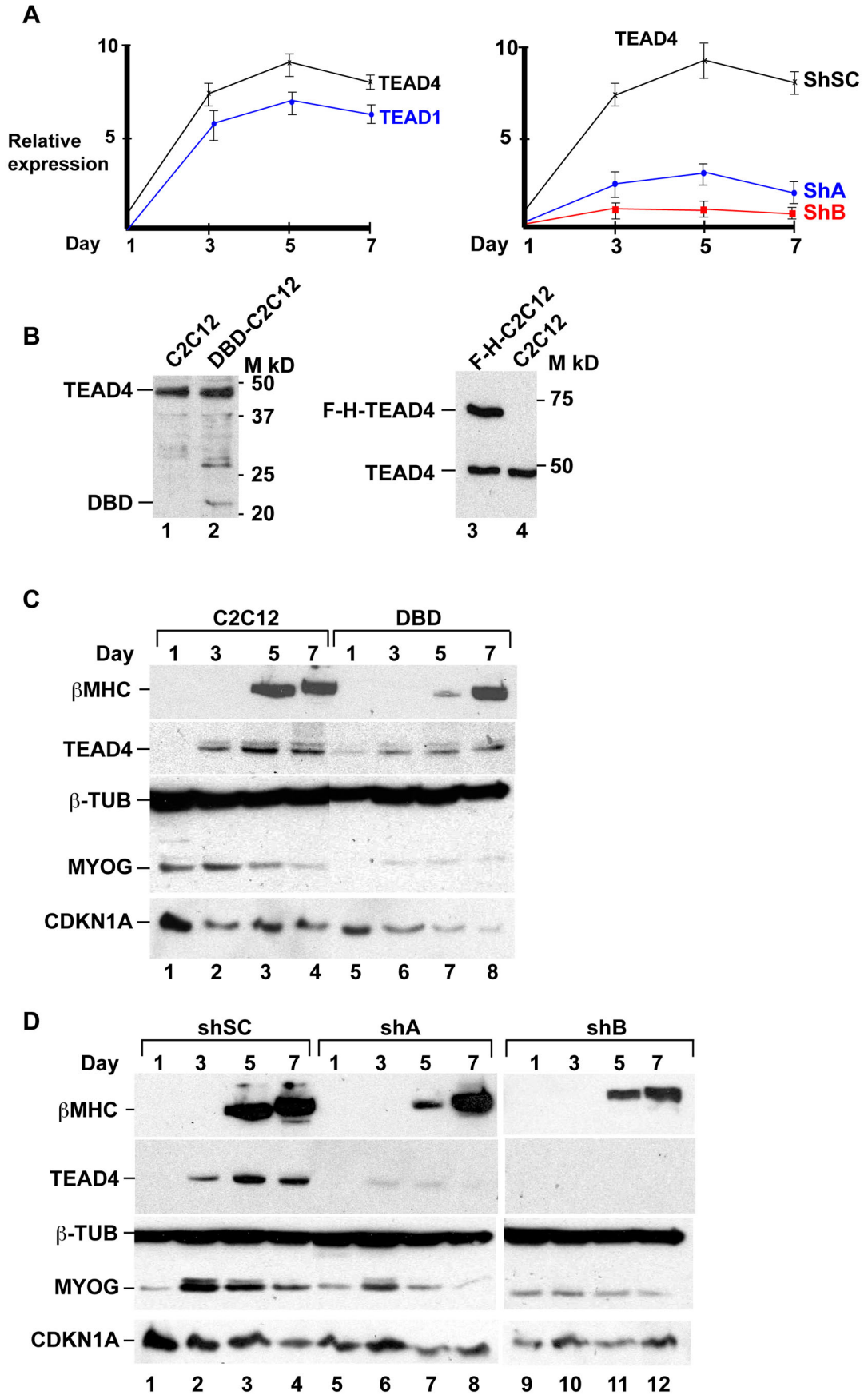
Figure 6. Characterisation of TEAD4 occupied genes. **A**. The 500 nucleotides surrounding the highest scoring oligonucleotide at each TEAD4 peak was analysed by the MEME programme. The consensus Logos for the two identified MCAT-related motifs are shown. **B-D**. Venn diagrammes indicating the overlap between the TEAD4 C2C12 and the MYOG C2C12, MYOD1 C2C12 and MEF2 C2C12 ChIP-chip data sets, the TEAD4 ChIP-chip data set and the MYOD1 ChIP-seq data set, the TEAD4 C2C12 and TEAD1 MCF7 ChIP-chip data sets, respectively.

Figure 7. Changes in gene expression upon TEAD4 knock-down. **A-H**. 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.

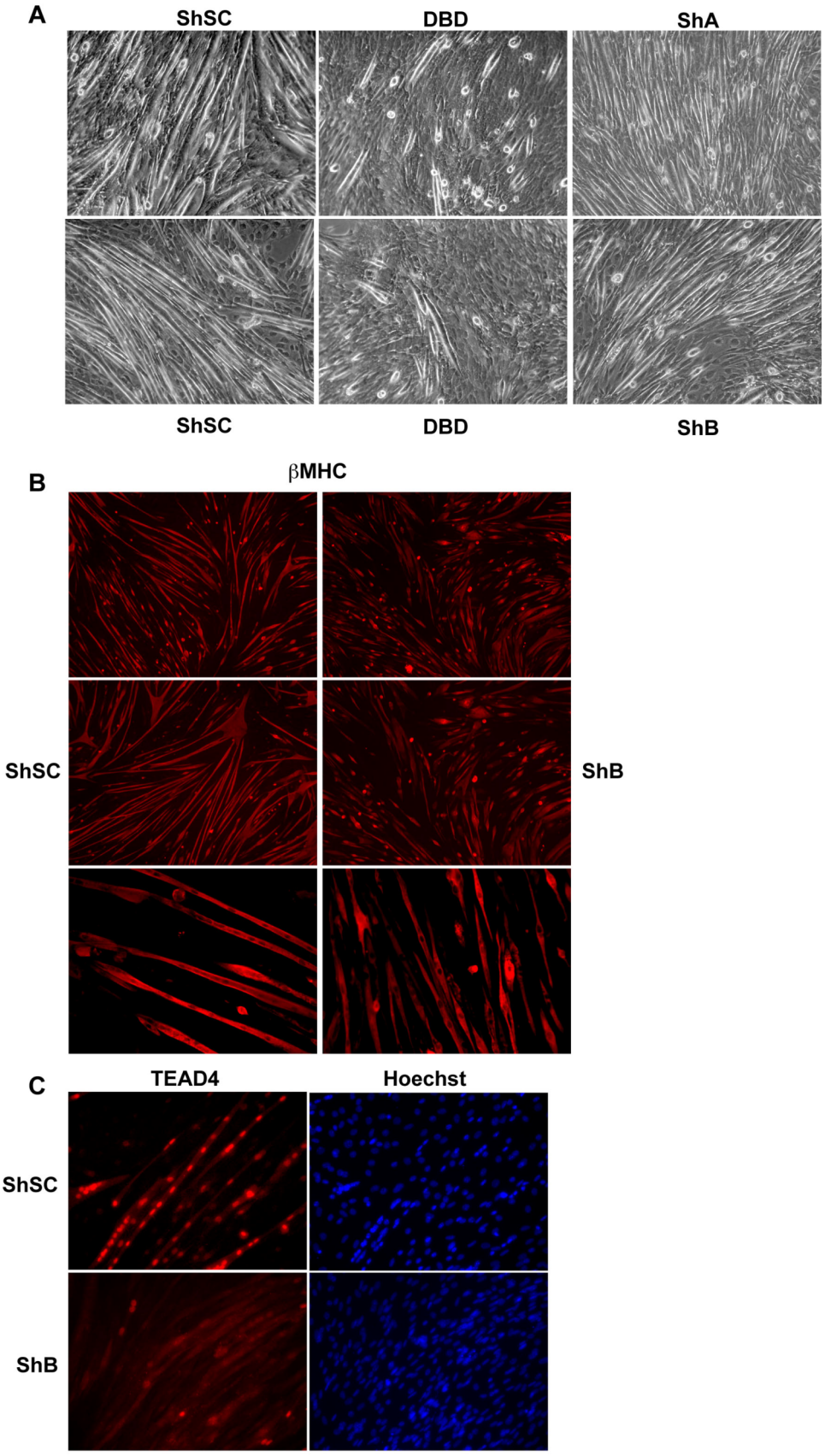
Figure 8. TEAD4 regulation genes required for Myoblast fusion. **A-E**. RT-qPCR quantification of the expression of the indicated genes in control ShSC-expressing C2C12 cells and cells expressing ShB from day 1 to day 7 of differentiation.

Figure 9. TEAD4 represses CTGF expression. A-C. TEAD4 occupancy of the *Ctgf*, *Yap1* and *Wwtr1* promoters. UCSC screenshots of the anti-Flag ChIP-chips on the cells expressing the tagged TEAD4 (F-TEAD4) and the un-tagged control cells at the indicated promoters. The TEAD4 binding sites are indicated by the arrows. **B**. 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.

Figure 10. Model for TEAD factors in C2C12 cell differentiation. Schematic network of interplay between the TEAD factors, MYOD1 and MYOG that controls expression of downstream genes required for differentiation. Arrowheads show positive regulation. The figure illustrates the mutual regulation of TEAD4 and MYOG, activation of TEAD1 expression by TEAD4, activation of downstream structural genes, cell cycle regulation, and miRNAs involved in differentiation. TEAD4 also represses CTGF and YAP1 expression.



Benhaddou et al., Fig. 1



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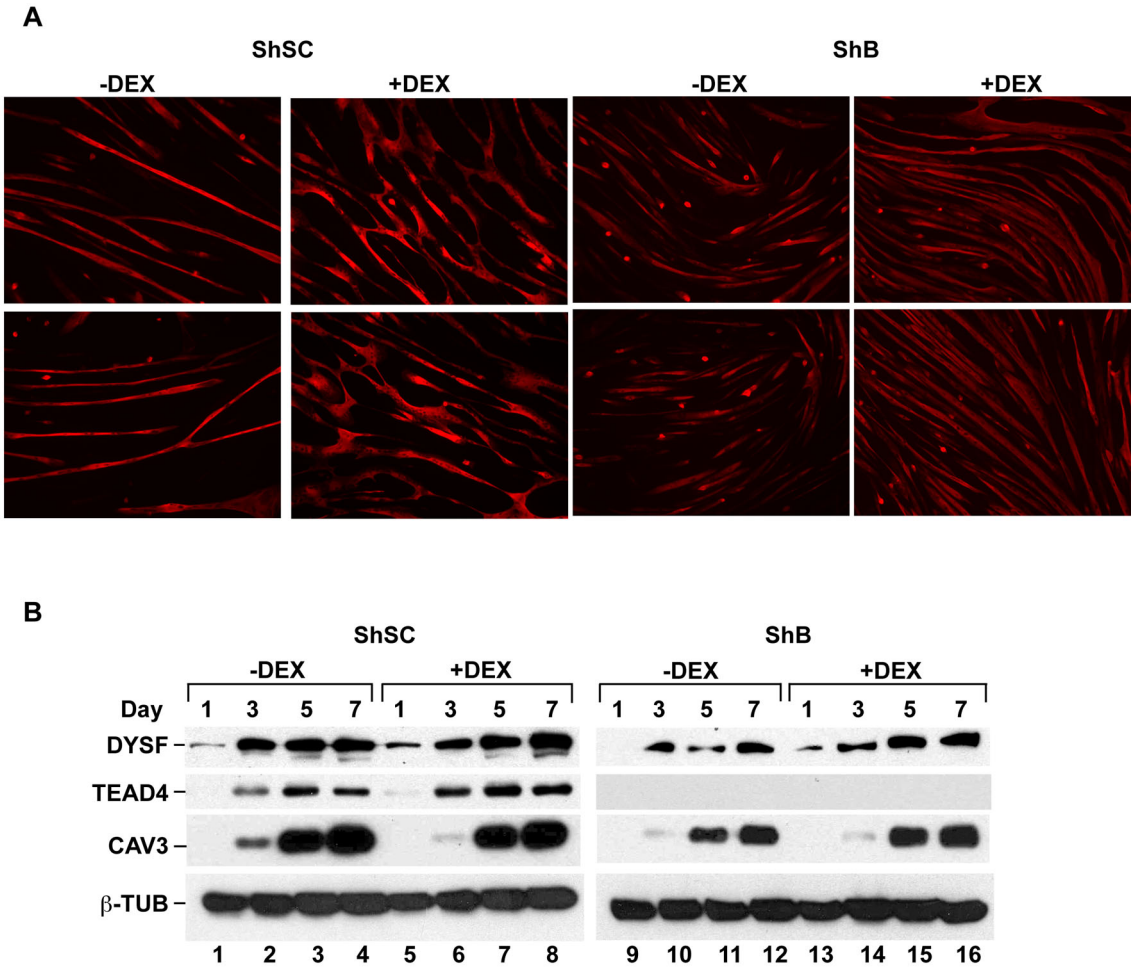

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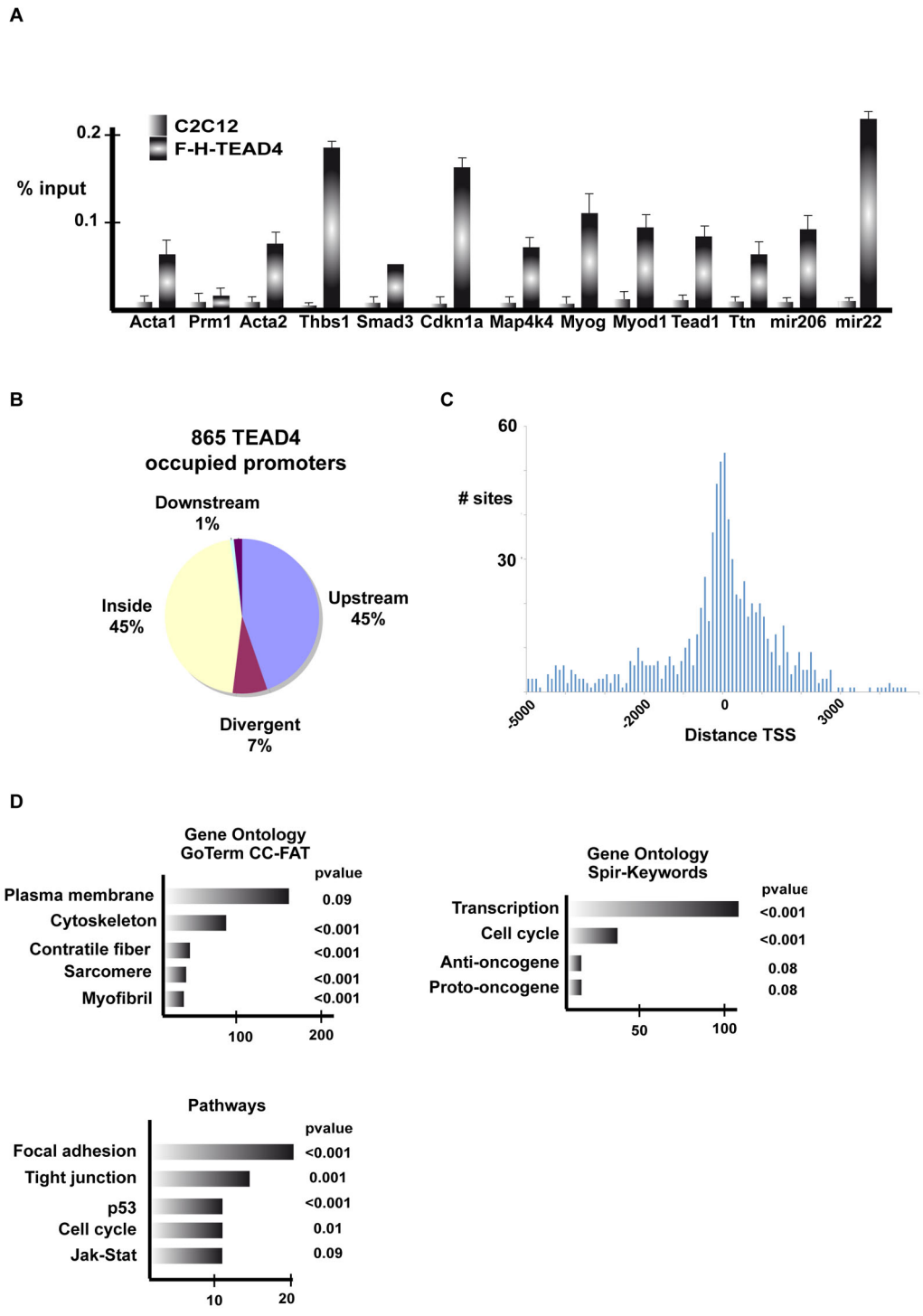

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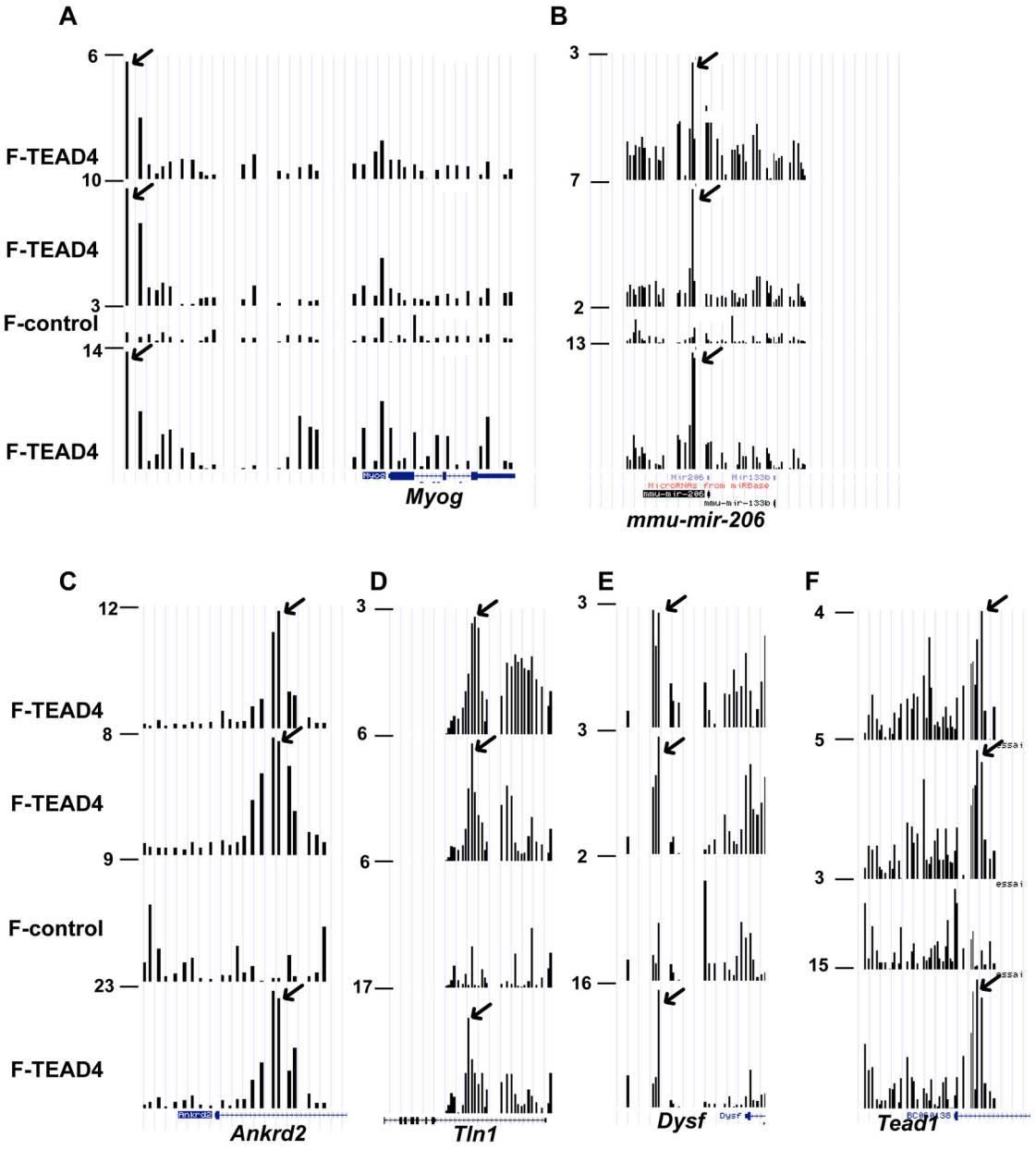

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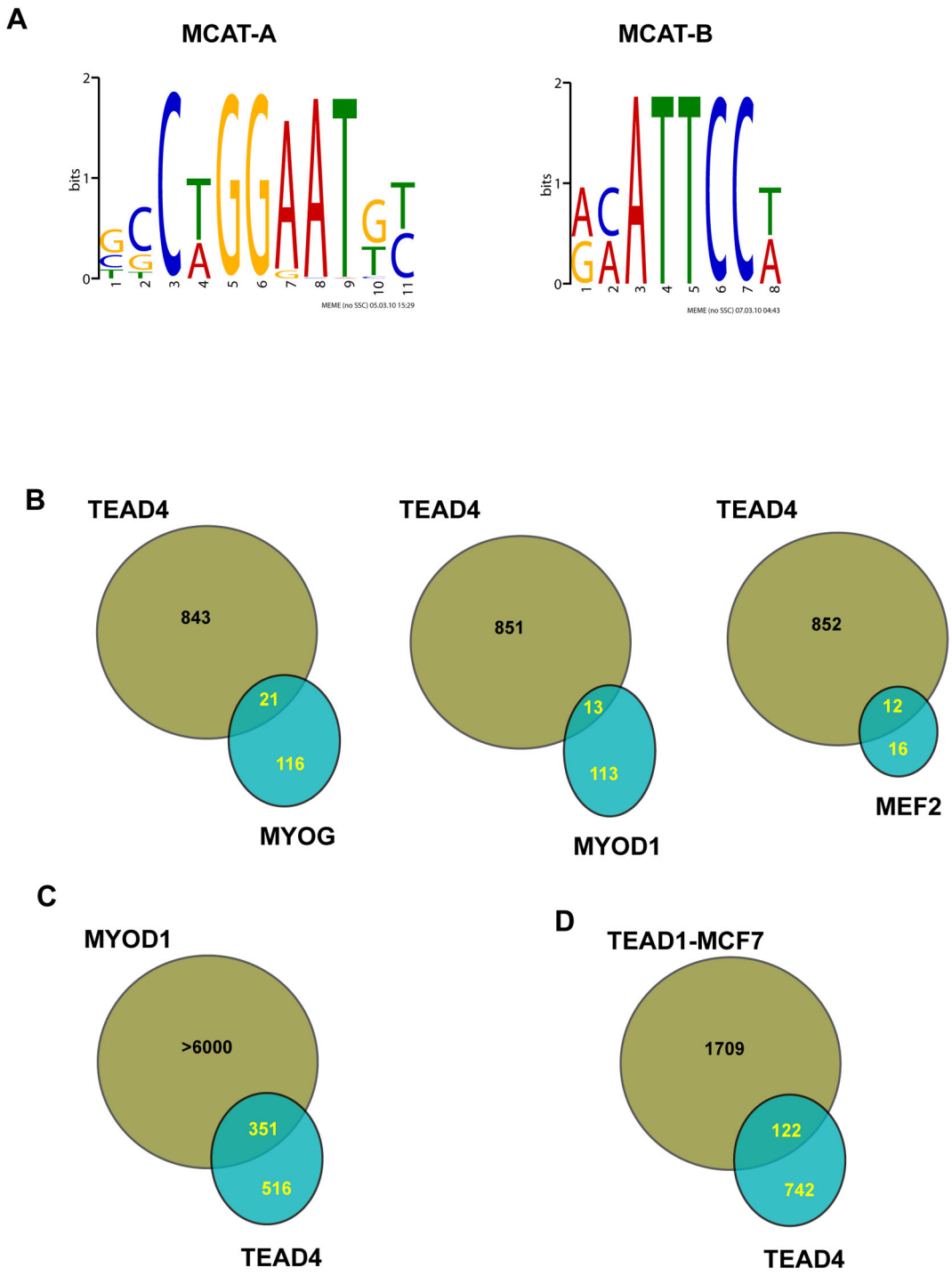

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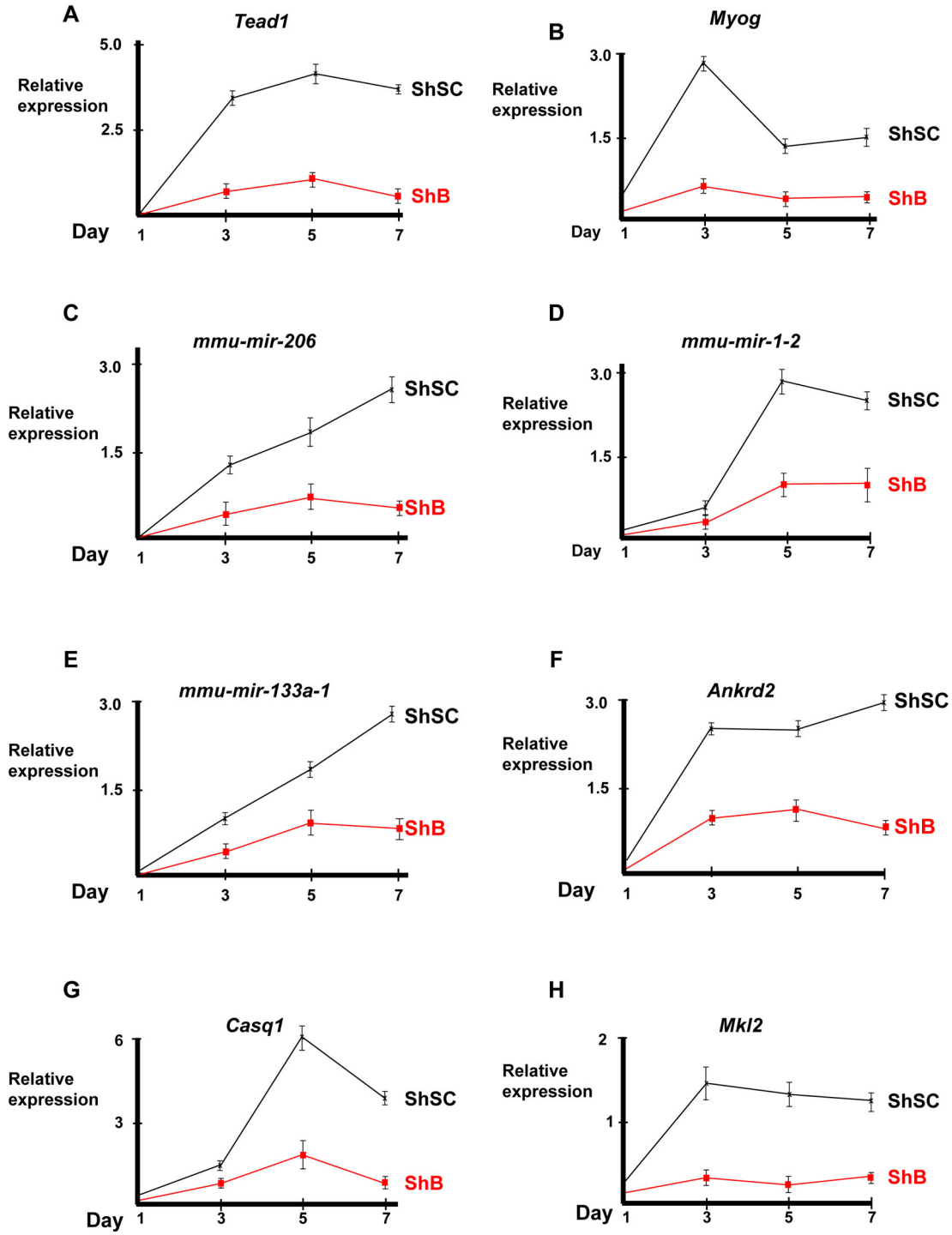

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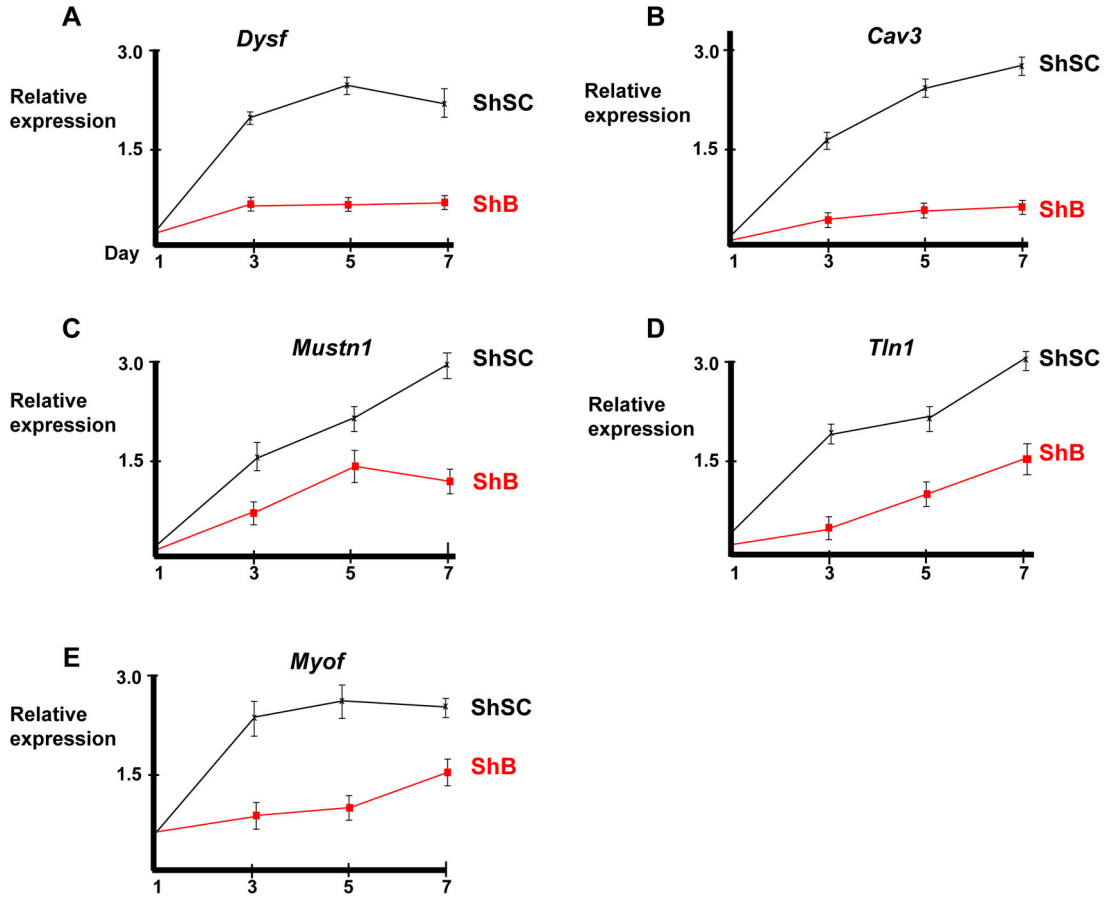

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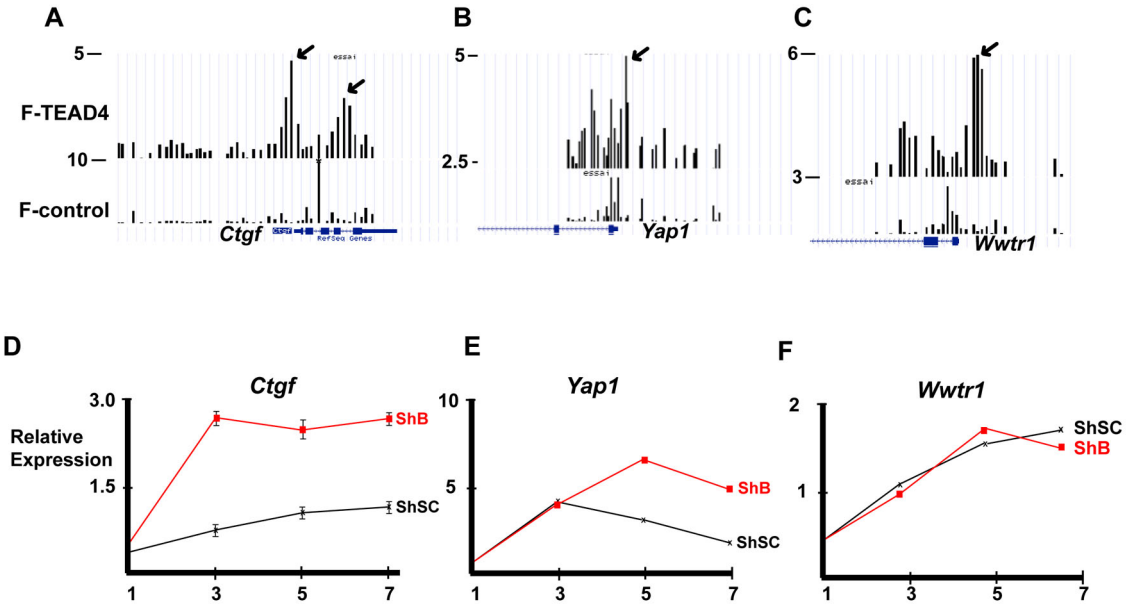

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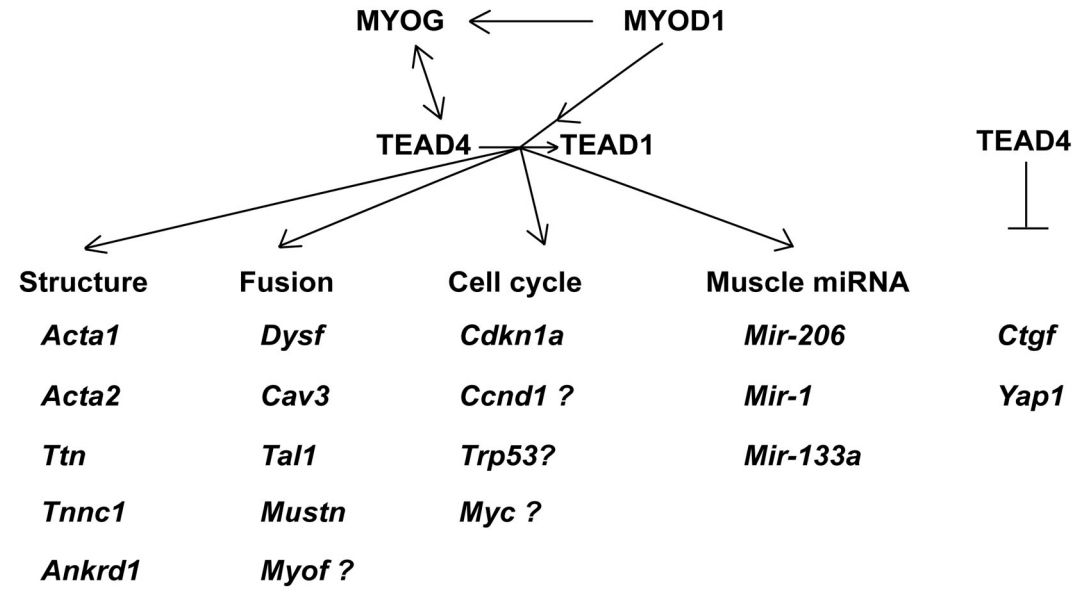


Benhaddou et al., Fig. 10.

Supplemental Material

Material and Methods.

ChIP.

Cells were crosslinked with formaldehyde 0.4% for 10 min at room temperature and the reaction was stopped by adding glycine to final concentration 0.2M for 10 minutes at room temperature. Fixed cells were rinsed twice with PBS and resuspended in 100 ul of lysis buffer (10 mM EDTA pH 8, 50 mM Tris-HCl pH 8, SDS 1 %). Lysate was sonicated 30 min (30 sec on / 30 sec off) in Diagenode water bath-sonicator and centrifuged at 14000 rpm for 10 min. The cleared supernatant was used immediately in ChIP experiments or stored at -80°C.

50-150 ug of sonicated chromatin was diluted 10 times in ChIP Dilution Buffer (SDS 0.01%, Triton X-100 1.1%, 1.2 mM EDTA pH 8, 16.7 mM Tris-HCl pH 8 and 167 mM NaCl) and pre-cleared for 1 hour, rotating at 4°C, with 50 ul blocked beads (Protein G Sepharose (PGS) 50% slurry incubated for 4 hours minimum with BSA 0.5 mg/mL and yeast tRNA 0.2 mg/mL) before the overnight incubation with 2-5ug of the M2 resin. The bound material was recovered after a 2 hours incubation, rotating at 4°C, with 30 ul blocked PGS. The beads were washed, for 5 minutes, once in Low Salt Buffer (SDS 0.1%, Triton X-100 1%, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8 and 150 mM NaCl), twice in High Salt Buffer (SDS 0.1%, Triton X-100 1%, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8 and 500 mM NaCl), twice in LiCl Buffer (0.25 M LiCl, NP-40 1%, Na Deoxycholate 1%, 1 mM EDTA pH 8 and 10 mM Tris-HCl pH 8) and twice in TE. ChIPed material was eluted by two 15 minute incubations at room temperature with 250 ul Elution Buffer (SDS 1%, 0.1 M NaHCO3). Chromatin was reverse-crosslinked by adding 20 ul of NaCl 5M and incubated at 65°C for 4 hours minimum and DNA was submitted to RNase and proteinase K digestion and extracted by phenol-chloroform.

ChIP-chip

For ChIP-chip, input chromatin and ChIPed material were amplified using the GenomePlex Complete Whole Genome Amplification Kit (WGA2 087K044, Sigma) and labelled. Samples were hybridised to the extended promoter array from Agilent covering -5kb to +2kb regions of 17 000 promoters.

Data were extracted with Agilent *Feature extraction* and analysed by *ChIP Analytics*. Data were subjected to Blank substraction, Inter-array median, Intra-array (dye-bias) median and Intra-array Lowness (intensity-dependent) normalisations. The Whitehead error model was used and the peak detection was performed with Pre-defined Peak Shape detection v2.0 with a p-value < 0.005 for non-parametric test and a peak-score > 3 for EVD-based score (based on T. Kaplan & N. Friedman "Model-Based Analysis of High resolution Chromatin Immunoprecipitation" Technical Report 2006-11, School of Computer Science & Engineering, Hebrew University 2006).

The output .tsv tables were further analysed using a script to detect individual oligonuleotides whose IP/Input ratio in the ChIP from the tagged cells was more than two-fold higher than in the ChIP from control cells. The selected probes were then screened to determine whether their upstream and downstream neighbours also had similar characteristics. The probes of this list were then sorted for those with a normalised log ratio greater than 1.5.

Legends to supplemental figures and tables.

Supplemental Figure 1.

A. Expression of TEAD factors in C2C12 cells. Affymetrix values of expression of each TEAD factor in myoblasts and day 7 differentiated C2C12 cells.

Supplemental Figure 2.

TEAD4 occupancy mmu-mir1-1 and 133 loci. 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 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.

Supplemental Figure 3.

Expression of MYOD1 and CAV3 in differentiating C2C12 cells. **A**. Immunoblot analysis of the expression of the indicated proteins during differentiation of control C2C12 cells and cells expressing the ShB RNA. **B**. Immunoblot analysis of CAV3 expression during differentiation of control C2C12 cells and those expressing the TEAD1-DBD.

Supplemental Figure 4.

TEAD4 promoter occupancy. **A-F.** UCSC screenshots at the indicated promoters. For simplicity only an anti-Flag ChIP-chip on the tagged cells is shown. The TEAD4 binding sites are indicated by the arrows. The values on the Y axis show the normalised IP/Input ratio.

Supplemental Table1.

TEAD4 bound genes in differentatiated C2C12 cells. Excel table of annotated loci bound by TEAD4 indicating the sequence, genomic localisation and identity of the peak oligonucleotide together with normalised ratio, p-value, peak score (from the second Flag-ChIP-chip replicate), primary (MGI gene symbol) and secondary gene annotations, transcript accession number, and location of binding sites with respect to TSS.

Supplemental Table 2.

Gene ontology of TEAD4-occupied genes. Pages 1 and 2 show the results of the Go-term CC-FAT and SP_PIR_KEYWORDS analysis (http://david.abcc.ncifcrf.gov/) and page 3 the results of the KEGG pathway analysis.

Supplemental Table 3.

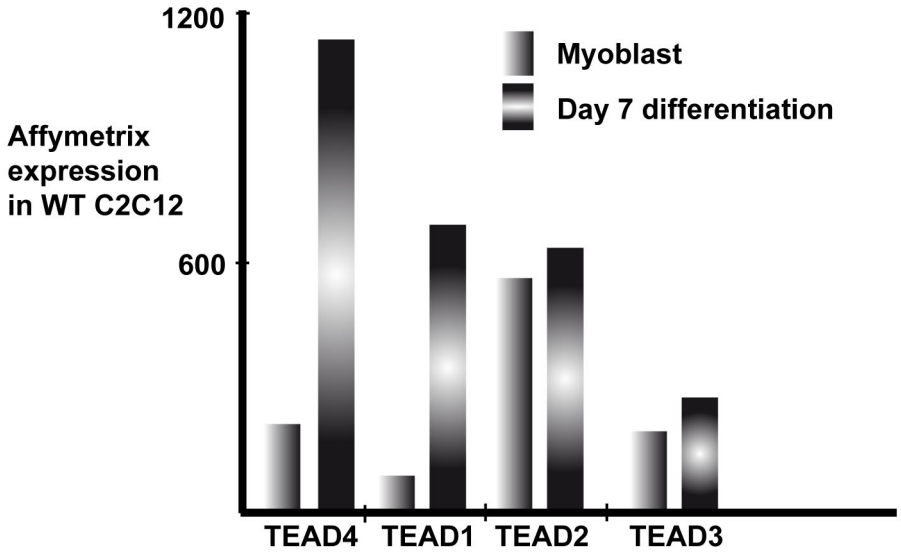
Presence of the MCAT-A and MCAT-B motifs in TEAD4 occupied genes.

Supplemental Table 4.

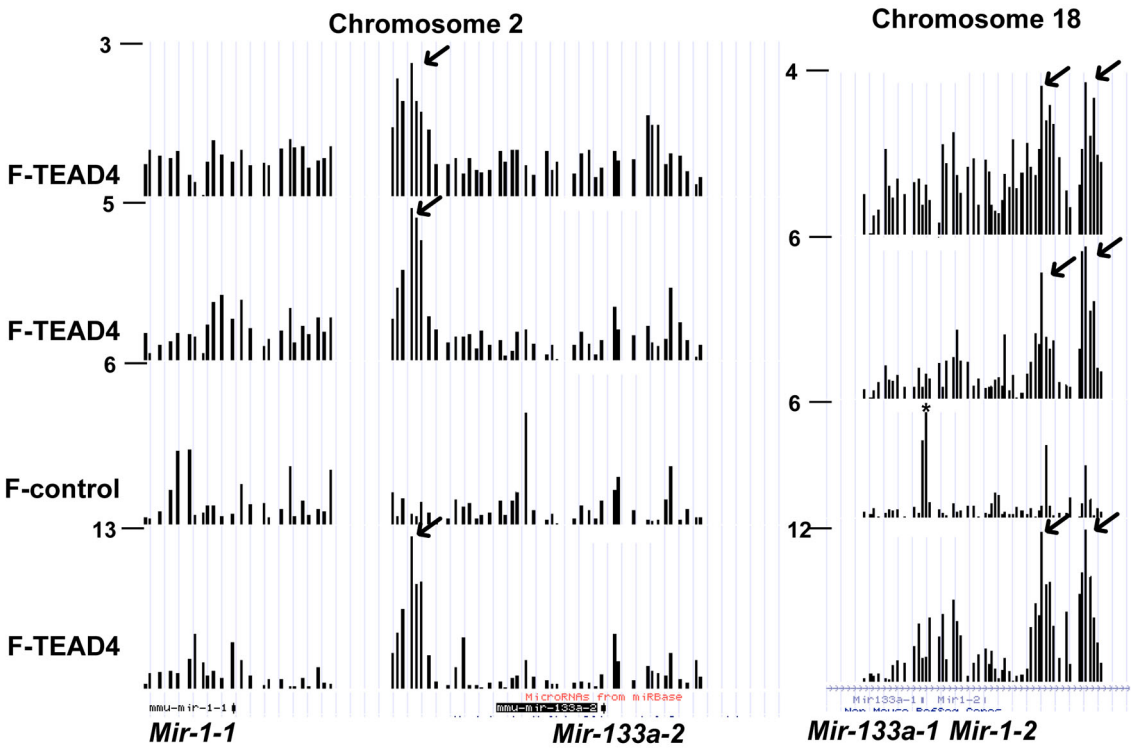
Comparison of the TEAD4 occupied genes with those occupied by MYOG, MYOD1 and MEF2 (ChIP-chip data sets, pages 1-3), with MYOD1 ChIP-seq data set (page 4) and the TEAD1 MCF7 ChIP-chip (data set page 5).

Supplemental Table 5.

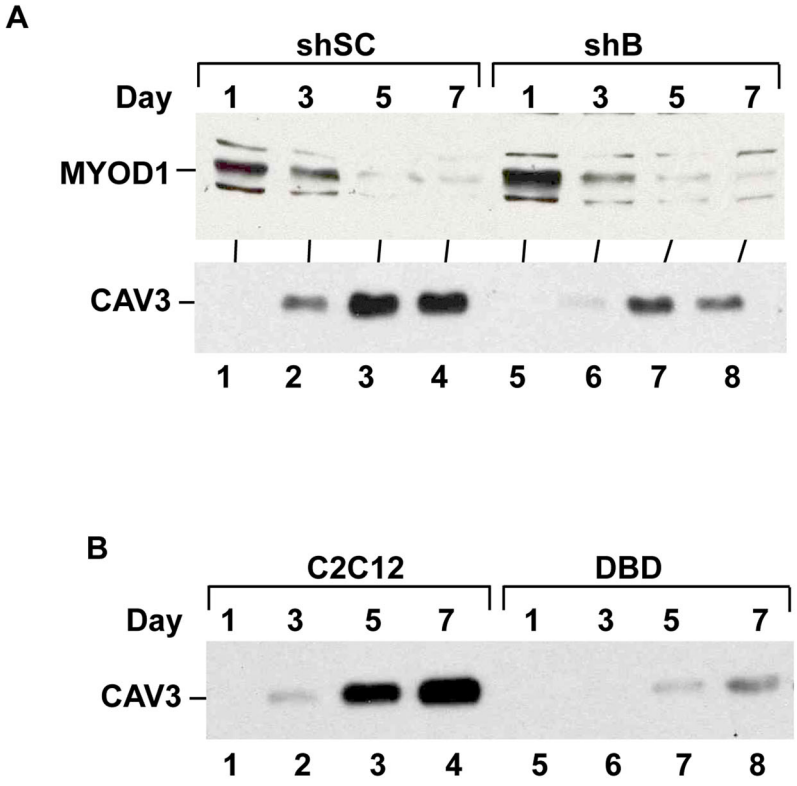
Sequences of oligonucleotides used for ChIP-qPCR and RT-qPCR.



Benhaddou et al., Supplemental Figure 1.



Benhaddou et al., Supplemental Fig. 2



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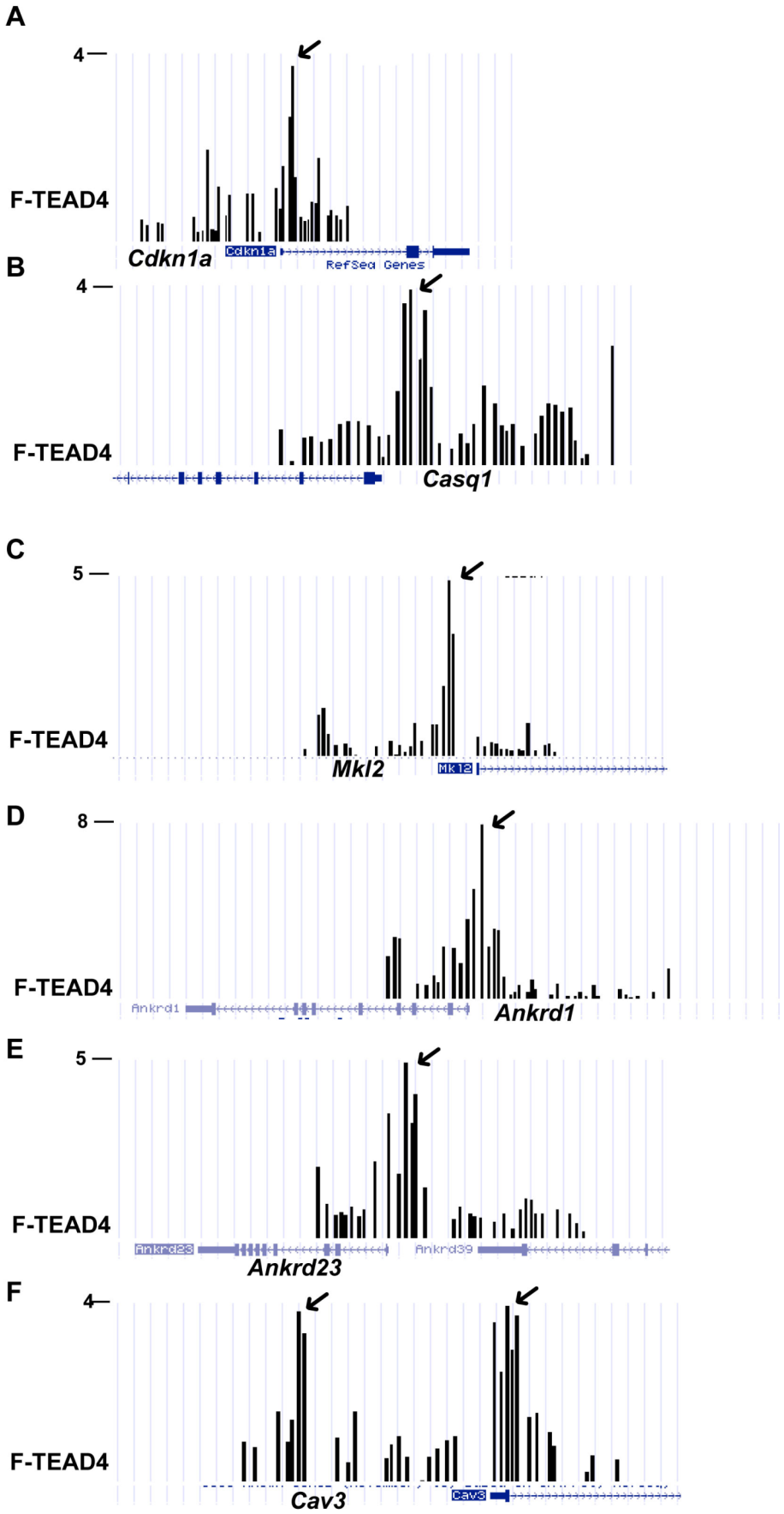

Benhaddou et al., Supplemental Fig. 4

2) Purification of TEAD4 and proteomic studies.

Material and methods

Flag Immunoprecipitation,

Extracts were prepared using a modification of the Dignam protocol (Dignam 1990). Briefly, cells were lysed in hypotonic buffer (10 mm Tris-HCl at pH 7.65, 1.5 mm $MgCl_2$, 10 mm KCl) and disrupted by Dounce homogenizer. The cytosolic fraction was separated from the pellet by centrifugation at 4°C. The nuclear-soluble fraction was obtained by incubation of the pellet in high-salt buffer (to get a final NaCl concentration of 300 mM). The nuclear-insoluble fraction was obtained by further incubating the pellet with spermidine and spermine containing sucrose buffer (20 mM Tris at pH 7.65, 15 mM NaCl, 60 mM NaCl, 0.34 mM Spermine(Sigma), 0.5 mM Spermidine (Sigma)). Tagged proteins were immunoprecipitated with anti-Flag M2-agarose (Sigma), eluted with Flag peptide (0.5 mg/mL), Flag peptides was first buffered with 50 mM Tris-Cl (pH 8.5), then diluted to 4 mg/mL in TGEN 150 buffer (20 mM Tris at pH 7.65, 150 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0 0.01% NP40), and stored at −20°C until use. Between each step, beads were washed in TGEN 150 buffer. Complexes were resolved by SDS-PAGE and stained using the Silver Quest kit (Invitrogen).

Identification of proteins was carried out using an ion-trap mass spectrometer (ThermoFinnigan LTQ-XL)

As described above, our results suggested that exchange the YAP1 and VGL family cofactors may in part account for the observation that TEAD factors activate CTGF expression in proliferating and cancer cells, while they repress its expression in differentiated C2C12 cells. To begin to address this possibility, we took advantage of the cells expressing Flag-HA tagged TEAD4 to perform immunopurification followed by mass spectrometry. Protein extracts were prepared proliferating C2C12 myoblasts expressing Flag-HA-TEAD4 (F-H-TEAD4) as well as control cells infected with the empty vector. We also generated NIH3T3 fibroblast cells expressing F-H-TEAD4 in order to compare the protein partners associated with TEAD4 in myoblasts. In each cell line, two types of extract were prepared. A standard soluble nuclear fraction was first obtained through 0.3M KCL extraction of the lysed nuclei (SNI). The insoluble chromatin associated proteins were then extracted by DNaseI digestion of the nuclear pellet (INE). The presence of tagged TEAD4 in each fraction was verified by Western blot (Figure 26 A, lanes 4 and 8).

The extracts were then immunoprecipitated with Flag antibody and TEAD4 and its associated proteins eluted by competition with Flag peptide.

Western blot with Flag antibody shows efficient precipitation of TEAD4 in the extracts from the C2C12 cells expressing tagged TEAD4, with little or no tagged TEAD4 remaining in the imunoprecipitate supernatant fraction, while no TEAD4 was precipitated from the control cells (Figure 26A). Following SDS PAGE and silver nitrate staining (Figure 26B), F-H-TEAD4 was clearly visible and as expected present only in the immunoprecipitate from the extracts of tagged cells (Figure 26B, lanes 2 and 4). In addition to actin as a major contaminant in each lane, several other proteins could be detected specifically in the extracts from the tagged cells that stained much weaker than the TEAD4.

These immunoprecipitated protein fractions (Figure 26B) were concentrated using Milipore centrifugal filters and were sent for analysis by mass-spectrometry. Several independent immunopurifications were performed and analysed by mass spectrometry on the platforms of different laboratories. We prepared also extracts from the NIH3T3 cells in an analogous manner that were analysed by mass-spectrometry. A summary of the obtained results is given in Table 3.

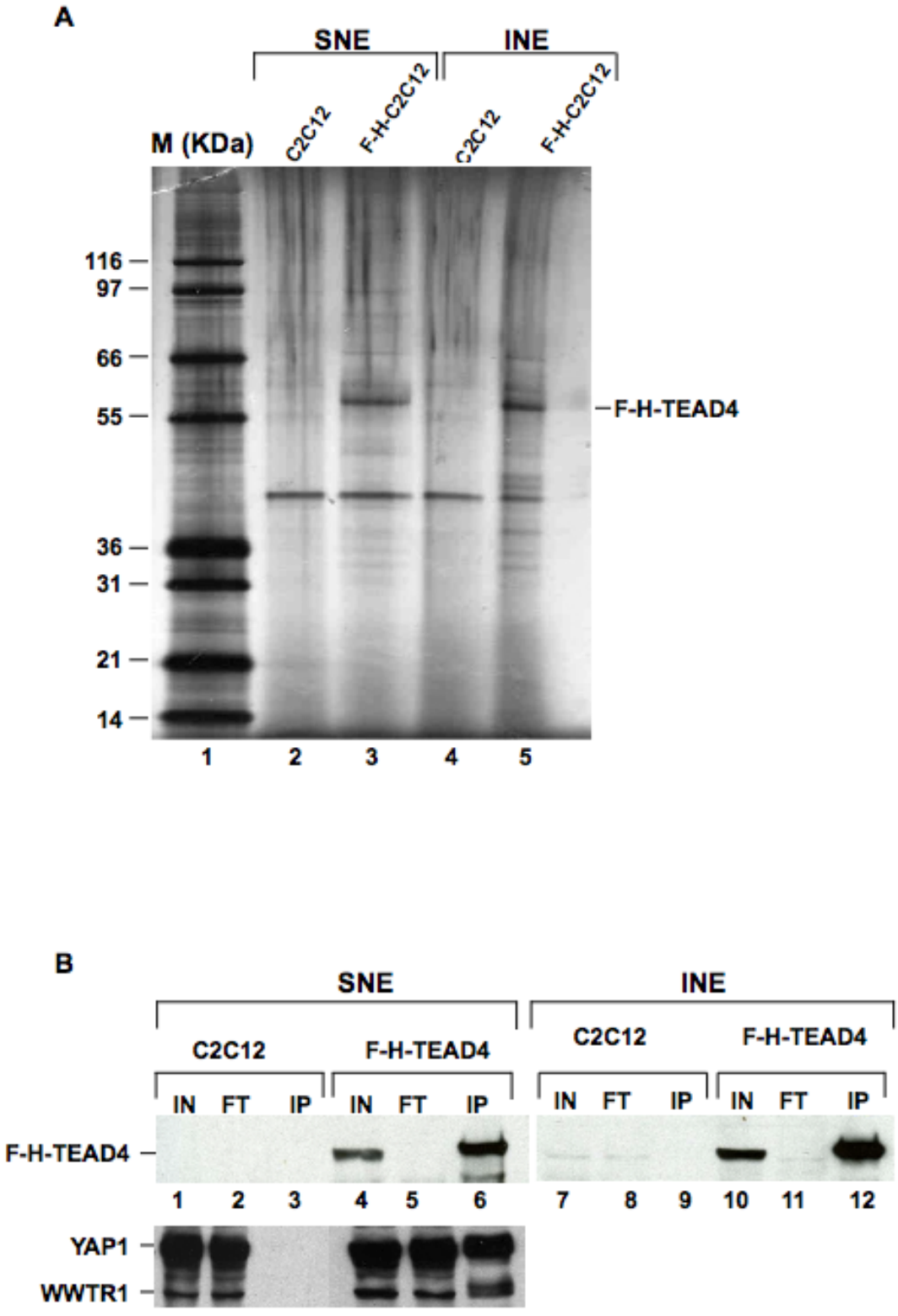

Figure 26. Purification of F-H-TEAD4 and identification of its protein partners. A. Silver staining of the soluble (SNE) and the insoluble (INE) nuclear extracts from F-H-C2C12 cells and control C2C12 cells. B. Western blot analysis of the immunopurification of TEAD4 protein. IN : input, SN : Supernatant, IP : immunopurified fraction.

In a first experiment, all fractions were analysed on the Orbi-trap FTMS instruments at the Sanger Centre UK. It is noteworthy that although we could observe several specific bands in the F-H-C2C12 fraction that were not present in the untagged control, only a small number of protein partners were identified by mass spectrometry analysis probably due to material loss during concentration. Two other immunopurifications were made from tagged and untagged NIH3T3 and C2C12 cells. Soluble and chromatin associated fractions were prepared from NIH3T3 cells were analysed by Dr Gonzales on the Orbi-trap platform at the IPBS in Toulouse. The soluble nuclear extract from the C2C12 cells was analysed by Linear Ion Trap (LIT) spectrometer that was recently acquired at the IGBMC. Table 3 summarizes the results obtained by the 3 experiments.

	IPBS		WTSI		IGBMC
	3T3SNE	3T3INE	C2C12SNE	C2C12INE	C2C12SNE
TEAD4	18/33,3%	9/19,4%	11/*	13/*	14/15,93%
YAP1	8/21,5%		1/*	2/*	6/15,25%
WWTR1	3/11,1%		2/*		
RBBP4		2/3,5%		1/*	2/4,94%
THRAP3		5/9,9%			
VGLL3	1/4,9%				
YWHAQ(14-3-3 protein theta)	2/9,8%				4/13,88%
YWHAB(14-3-3 protein beta)	2/9,8%	4/14,2%			
YWHAZ(14-3-3 protein zeta)	4/17,6%				
BIN1					2/3,23%
HDAC2					3/6,35%

*Table 3. Summary of results obtained from 3 independent mass spectrometry analyses. The number of peptides identified per protein and percentage of coverage are indicated between brackets. SNE, Soluble Nuclear Extract. INE, Insoluble Nuclear Extract. IPBS, Institut de Pharmacologie et de Biologie Structurale. WTSI, Wellcome Trust Sanger Institute. IGBMC, Institut de Génétique et de Biologie Moléculaire et Cellulaire.*No percentage of coverage communicated by the platform of Mass spectrometry of WTSI. TEAD4 (TEA domain family member 4), YAP1 (Ses/Src binding protein1), WWTR1 (WW domain containing transcription regulator 1), RBBP4 (retinoblastoma binding protein 4), THRAP3 (thyroid hormone receptor associated protein 3), VGLL3 (Vestigial like protein 3), BIN1(bridging integrator 1), HDAC2(Histone deacetylase2)*

This analysis allowed us to identify YAP1 and WWTR1 in the soluble nuclear TEAD4 extract in both differentiated C2C12 and NIH3T3 cells. Additionally, YAP1 was also identified in the chromatin associated form of TEAD4 in C2C12 cells. The presence of YAP1 and WWTR1 in the TEAD4 immunoprecipitated fraction from the soluble nuclear extract of tagged, but not untagged, cells was confirmed by Western blot analysis (Figure 26A lower panel).

RBBP4 is specifically associated with the chromatin bound TEAD4 in NIH3T3 cells, but is also found in the soluble fraction in C2C12 cells. RBBP4 has been identified in several chromatin interacting complexes and thus may be involved in association of the TEAD4-coactivator complex with chromatin. While YAP1 was a previously identified TEAD cofactor, RBBP4 in a novel TEAD cofactor.

We further identified the mediator complex subunit THRAP3 in the same fraction as RBBP4. HDAC2 is present in the soluble fraction of TEAD4 suggesting that it may function as a transcriptional repressor. Other interesting protein partners of TEAD4 are the 14-3-3 proteins YWHAB, YWHAZ and YWHAQ. These proteins are known to interact with phosphorylated YAP1/WWTR1 and translocate them from nucleus to cytoplasm.

BIN1, also called amphiphysin 2, was found to specifically associate with the soluble protein complex of TEAD4. *Bin1* gene mutation causes centronuclear moypathy (Nicot et al., 2007) and has been shown to repress Myc-dependent transcription (Chang et al., 2007).

The above results clearly show that TEAD4 can be found associated with YAP1 and WWTR1 in extracts from C2C12 cells. In addition, we did not readily observe peptides from members of the VGLL family, only in one experiment did we detect a VGLL3 peptide. While the mass-spectrometry results are not quantitative, they do not argue for abundant TEAD/YAP1 complexes and rarer TEAD4/VGLL (or WWTR1) complexes. We conclude therefore that in proliferating C2C12 cells TEAD4 preferentially associates with YAP1/WWTR1 rather than the VGLL-family members. Nevertheless, we note that with the exception of TEAD4, the other proteins, although reproducibly detected, are identified with only very low peptide coverage. Obviously additional experiments with higher amounts of starting material will be required to confirm the above conclusions. It will also be important to perform analogous experiments in differentiated C2C12 cells to determine the TEAD4 partners as YAP1 is exported from the nucleus during differentiation.

3) A role for TEAD4 in muscle regeneration ?.

As our results show a role for TEAD4 in myoblast fusion and myotube formation, we then asked whether TEAD4 may play a role in physiopathological conditions in mouse skeletal muscle. This chapter will describe preliminary results we obtained in Notexin (NTX) induced muscle degeneration-regeneration.

NTX induced degeneration-regeneration.

Muscle regeneration is an important feature of skeletal muscle in response to injury or exercise overload. Muscle regeneration is characterized by two phases: a degenerative phase and a regenerative phase (Charge and Rudnicki, 2004). In the degenerative phase, damaged myofibres undergo necrosis and inflammatory macrophages invade the muscle where they phagocytose cellular debris and may activate myogenic cells (Almekinders and Gilbert, 1986 ; Lescaudron et al., 1999). In the regenerative phase, the satellite stem cells are activated to proliferate and undergo an asymeteric division to give rise to a population of committed myogenic cells that differentiate and fuse to existing damaged fibres for repair or to one another for a new myofibre (Darr and Schultz, 1987 ; Kuang et al., 2007; Snow, 1977). NTX is a phospholipase A_2 neurotoxin peptide extracted from snake venom that blocks neuromuscular transmission by inhibition of acetylcholine release and induces degeneration.

To artificially induce a cycle of degeneration-regeneration in mouse skeletal mouse we injected NTX in the right tibialis anterior and as a control we injected PBS in left tibialis anterior. As seen in Figure 27, NTX injected muscle was severely injured and inflammatory infiltrates and mononuclear cell proliferation are already seen (arrows).

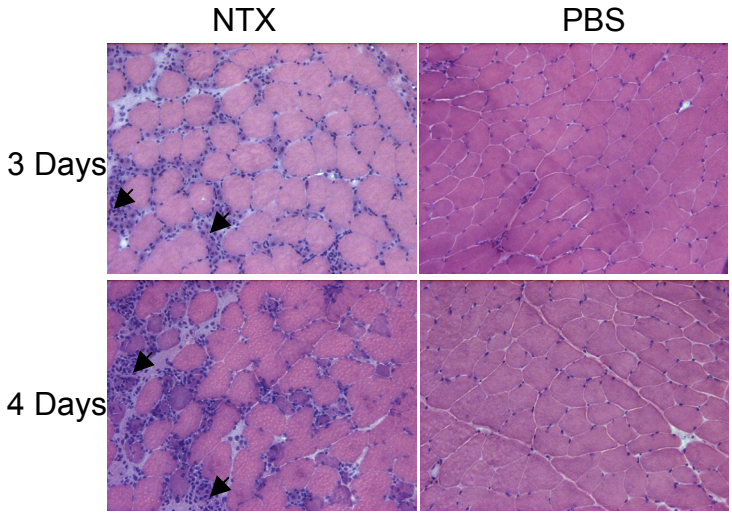

Figure 27. Hematoxylin and eosin staining of transversal sections of tibialis anterior 3 and 4 days after injection of PBS or Notexin (NTX).

Western blot analysis of TEAD4 expression showed that it was strongly induced in NTX injected muscle compared to PBS injected muscle (Figure 28A). The expression of its target gene Myogenin was also increased, confirming that the muscle was regenerating and new muscle fibres were forming. Desmin, a cytoplasmic intermediate filament, implicated in myoblast fusion during muscle regeneration (Smythe et al., 2001), was also induced after NTX injection (Figure 28A). Utrophin is important for neuromuscular junction formation and is expressed in newly formed muscle fibres and then replaced by dystrophin in mature fibres (Rosenberg et al., 2006 ; Ohlendieck et al., 1991). NTX injection induces *de novo* Utrophin expression in regenerating muscle (Figure 28A). Mir-206 plays an important role in muscle regeneration (Yuasa et al., 2008) and is a target gene for TEAD4 (see above). qRT-PCR showed that mir-206 expression is upregulated in NTX injected mice compared to control and its expression increases with time (Figure 28 B).

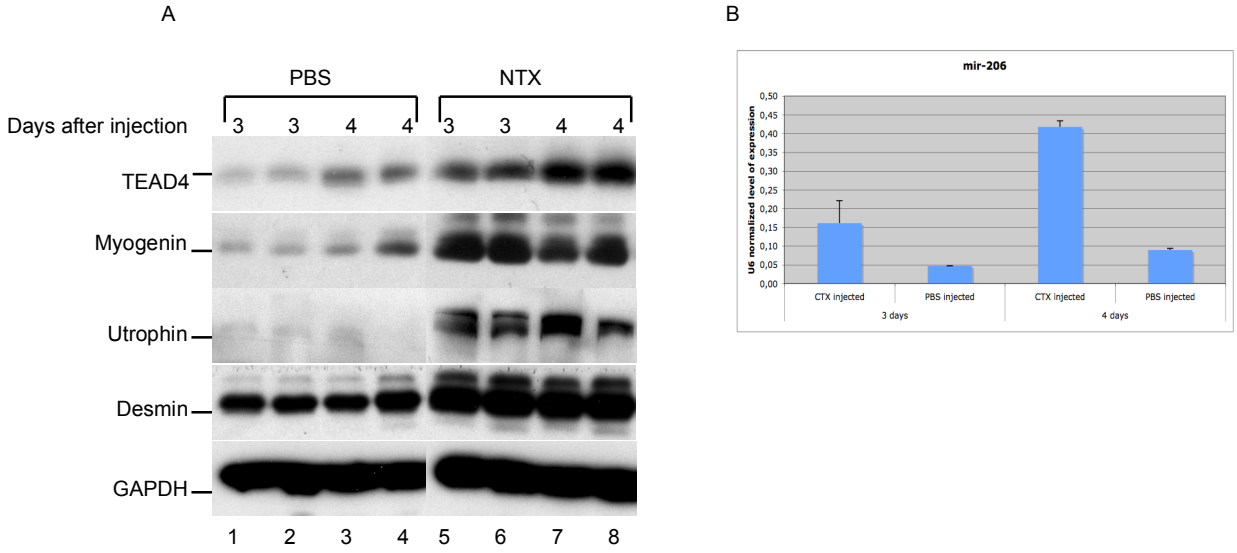

Figure 28. Notexin (NTX) induced muscle regeneration. (A) Mice were injected with 200µl of NTX (1µg/ml) in the right Tibialis Anterior and with PBS in the left one. Mice were sacrificed 3 and 4 days after injection and the injected regions of the muscle were dissected and protein extracts were prepared and analysed by western blot analysis with the indicated antibodies. GAPDH was used as a loading control. B. RNA was extracted from the same muscle as in A and subjected to reverse transcription followed by quantitative PCR to assess the expression of mir-206. The expression level was normalized to U6 RNA.

These results confirmed that we induced a cycle of degeneration and regeneration after NTX injection and that TEAD4 is induced during the regeneration phase. This up-regulation of TEAD4 is accompanied by that of its target genes mir-206 and MYOG. As we have shown that TEAD factors are essential for activation of these genes in differentiating C2C12 cells, their coordinate activation during regeneration suggest that TEAD4 or any combination of the TEAD factors may be required to activate their expression also during regeneration. A similar

proposition was formulated by Zhao et al. who showed that TEAD2 was also induced during muscle regeneration (Zhao et al., 2006). It is probable therefore that TEAD4 and TEAD2 may act together to promote expression of the differentiation programme of regenerating muscle.

DISCUSSION
and
PERSPECTIVES

1) TEAD4 propagates and potentiates the transcriptional regulatory network initiated by MYOD1.

In the limb bud, proliferating myoblasts express MYOD1 which mark their engagement in the myogenic programme. Subsequently, expression of Myogenin and MRF4 is up-regulated and cells begin their terminal differentiation programme. The differentiation programme progresses with activation of muscle-specific genes, such as MHC, and fusion of differentiated myocytes into myotubes (Francis-West et al., 2003). The maturation process ends when the myofibre becomes innervated by a motor neuron and electrically insulated from other myofibres by the intramysium connective tissue (Anderson et al., 2006).

This myogenic differentiation programme is at least in part recapitulated during *in vitro* differentiation of C2C12 cells (Ferri et al., 2009). We show that all four TEAD family proteins are expressed at least at the RNA level in differentiating C2C12 cells. The TEAD4 promoter is bound by MYOD1 and MYOG in C2C12 myotubes (Blais et al., 2005) and TEAD4 expression is induced during C2C12 differentiation (Hsu et al., 1996). We also show that TEAD1 is strongly induced probably under the control of TEAD4, but that TEAD2 in particular is expressed at high levels in both myoblasts and myotubes. The expression of multiple members of the TEAD family and the potential for redundancy is a major obstacle in deciphering TEAD function during differentiation. Nevertheless, by blocking TEAD activity with a dominant negative or the selective knockdown of TEAD4 coupled with ChIP-chip analysis we have gained new insight into TEAD function.

Comparison of our TEAD4 ChIP-chip analysis and those of (Blais et al., 2005) and (Cao et al.) revealed that a significant number of genes bound by MYOD1 are also bound by TEAD4 (Figure 6B and 6C of the manuscript and supplemental table 4). This constitutes a feed-forward regulatory loop in which the transcriptional programme initiated by MYOD1 is potentiated by TEAD4. TEAD4 is also present on promoters of genes that are bound by MYOG showing that TEAD factors cooperate with the MRFs to activate many critical genes in the differentiation programme.

MYOD1 expression is down-regulated after the onset of C2C12 differentiation (Singh et al.). This raises the question of how the differentiation signal initiated by MYOD1 is relayed until terminal differentiation and maturation of myotubes. The MYOG promoter has also been shown to be bound and regulated by MYOD1 (Blais et al., 2005). Upon *in vitro* differentiation of C2C12 cells, MYOG protein levels reach a peak around day 3 of differentiation and are subsequently down-regulated, whereas TEAD4 protein reached a peak later around day 5 of differentiation. These observations suggest that MYOD1 initiates

myogenic differentiation, while TEAD4 and MYOG sustain the differentiation process until the maturation and innervation of myotubes (discussed below).

The above considerations are in agreement with previous reports that TEAD factors are downstream effectors of the differentiation programme and this may indeed be the case for TEAD4 and TEAD1 that are induced during differentiation. Nevertheless, it is important to note that loss of all TEAD function in cells expressing the DBD blocks differentiation at a very early stage, as no induction of MYOG or CDKN1A is observed. This shows that other TEAD factors must cooperate with MYOD1 to initiate the programme by activation of these critical genes. TEAD2 is strongly expressed in myotubes and is a good candidate to fulfil this role. Together, our results show that cooperation between different TEAD factors MYOD1 and MYOG is required to both initiate and propagate the differentiation signal.

2) TEAD factors and cell cycle control.

Ontology analysis of TEAD4 target genes revealed a large number of genes involved in cell cycle control (figure 4D of the manuscript and supplemebtal table 2-KEGG pathways). Amongst them, we identified genes from the TRP53 signalling pathway including TRP53 itself as well as several cyclins. We have shown, that during C2C12 differentiation, TEAD4 is required for the normal expression of the p21 protein. Reduced levels of p21 leads to less efficient cell cycle exit and reduced differentiation in C2C12 cells lacking TEAD4. Interestingly however, TEAD4 also occupies the cyclin D1 and cyclin D2 promoters whose expression drives cell proliferation. This observation highlights the capacity of TEAD factors to promote or repress proliferation depending on the cellular context. The factors that influence the outcome of this choice remain to be determined.

In oncogenic transformation (for example in MCF7 breast cancer cells) TEAD factors bind to the CTGF promoter and activate its expression to stimulate proliferation(Zhao et al., 2008). In differentiating C2C12 cells, CTGF expression shows little change. However, in C2C12 cells where TEAD4 is down-regulated CTGF is significantly induced. These results show that in MCF7 cells, TEADs act to directly stimulate CTGF expression to promote cell cycle progression, while in C2C12 cells, TEAD4 directly represses its expression to allow cell cycle exit and differentiation.

The proliferative effects of TEAD factors in MCF7 cells are due to their interaction with the oncogene protein YAP1 that mediates transcriptional activation of target genes (Zhao et al., 2008). Interestingly, YAP1 is expressed in proliferating C2C12 myoblasts and as we have shown, interacts with nuclear TEAD4 while during differentiation it is phosphorylated and translocated to the cytoplasm (Watt et al.). It is possible that in the absence of active

YAP1 in the nucleus of differentiating C2C12 cells, TEAD4 may interact with repressor complexes to mediate CTGF inhibition. Indeed in our proteomic analysis, we found that TEAD4 potentially forms a complex with RBBP4 and HDAC2. RBBP4 is part of the Mi-2 complex which has been implicated in chromatin remodeling and transcriptional repression associated with histone deacetylation. RBBP4 is also part of co-repressor complexes, which are integral components of transcriptional silencing (Wolffe et al., 2000). While this complex did not seem so abundant in myoblasts, it may be favoured in differentiated cells where YAP1 is absent from the nucleus.

Several studies have shown that TEAD factors act together with cofactors of the VGLL family, in particular VGLL2, to activate muscle specific genes. It is thus possible that while the TEAD-VGLL complexes activate these genes, they are not active at the CTGF promoter. In the future, it will be important to perform immunopurification and proteomics on TEAD factors from differentiated cells. It will also be informative to perform ChIP-seq to determine co-occupancy of regulatory elements at activated and repressed genes by TEAD factors and co-activators such as VGLL2 or co-repressors such as the HDACs and RBBP4. Such analysis will help to understand how different TEAD-cofactor combinations regulate gene expression in differentiated C2C12 cells.

3) TEAD4 and the combinatorial control of gene expression during C2C12 myoblast differentiation.

Sequence analysis of the 500 bp flanking region of TEAD4 binding sites revealed that a consenus MCAT motif is present in 45% of TEAD4 target genes (data not shown). Up to 92% of MCAT element containing peaks also show an E-box and thus are potential targets for MYOD1 and MYOG. However, the E-box sequence is short and degenerate and clearly not all of these sites are occupied. This is shown by comparison with the MYOD1 ChIP-seq results where only a small number of these sites are actually occupied.

A motif search for MEF2 binding sites using the consensus 5'- YTAWWWWTAR-3' and Serum Response Element (SRE) 5'- CCWWWWWWGG-3', the binding site for Serum Response Factor (SRF), where W= A or T, Y= C or T, and R= A or G, shows that 10% of TEAD4 target genes contain at least one or the other motif in the binding site peak (data not shown). These combinations of transcription factor binding sites (TFBS) may be critical for the control of gene expression. For example, promoter analysis of the Desmin gene revealed the presence of a MEF2 binding site and two E-boxes (Li and Capetanaki, 1994). Li et al. showed that Desmin gene transcription depends on cooperative interactions between these sites. MEF2 and MYOG bHLH proteins have also been shown to synergistically activate the

muscle creatine kinase (MCK), myosin light chain1/3 (Myl1), and Myog genes (Kaushal et al., 1994).

Several studies showed that SREs of skeletal α-actin and α-MHC cooperate positively with the MCAT element to control their expression (MacLellan et al., 1994) ; Karns, 1995 #618}. A combinatorial interaction between these two elements is found to be necessary for α1-adrenergic, transforming growth factor-β, and stretch-induced activation of skeletal α-actin gene expression in cardiac myocytes and slow twitch skeletal muscle fibres (MacLellan et al., 1994 ; Karns et al., 1995 ; Carson et al., 1996). Indeed, SRF and TEAD1 directly interact both *in vivo* and *in vitro* (Gupta et al., 2001). On the other hand, MEF2 and TEAD1 were shown to directly interact to regulate muscle specific promoters (Maeda et al., 2002b).

Our analysis of the TEAD4 binding site sequences shows however that aside these well studies examples, TEAD-SRF and TEAD-MEF2 cooperativity is not a general phenomenon, much less so than cooperation with MYOD1. However, it will be important in the future to obtain ChIP-seq data for these factors and to compare them with the TEAD4 data rather than to base the conclusion only on bioinformatic analysis.

4) TEAD4 is implicated in the control of neuromuscular junction formation.

The neuromuscular junction (NMJ) forms following a series of complex interactions between motor neurons and muscle fibres that express a large number of genes that are required for these interactions (Burden, 2002). Our ChIP-chip analysis suggests that TEAD4 governs the expression of genes involved in different aspects of synapse formation and function like MuSK, Acetylcholine receptor γ (Chrng), Acetylcholine receptor α1 (Chna1), (supplemental table 2-GO term CC) that has been shown to be important component of NMJ (Liyanage et al., 2002). It has already been shown that MYOD1, MYOG and MEF2 target important components of the NMJ in C2C12 myotubes (Blais et al., 2005). Taken together, our results combined with previous published data strongly suggest that muscle cells have their own intrinsic transcriptional program for establishing synapses and that these networks are controlled, at least in part, by MYOD1, MYOG, MEF2 and TEAD4. Interestingly TEAD4, but not TEAD1, is differentially expressed in skeletal muscle myofibres at E15.5 which is the time at which muscle innervation and fibre type differentiation begin. This suggests that TEAD4 plays a role in skeletal muscle terminal differentiation and maturation *in vivo* and may be implicated in NMJ formation.

5) TEAD4 and skeletal muscle maturation.

Inactivation of TEAD4 in post-implantation embryos showed no obvious muscle defects (Yagi et al., 2007 ; Nishioka et al., 2008). Nevertheless, these authors did not investigate the metabolic and regenerative capacities of *Tead4*$^{-/-}$ muscle. As we have shown that TEAD4 is required for C2C12 myoblast fusion and differentiation, we hypothesised that it may play a role in skeletal muscle regeneration. We have shown that TEAD4 is up-regulated during NTX-induced muscle regeneration together with miR-206. MiR-206 is a target of TEAD4 and also of MYOD1 and MYOG (Rao et al., 2006) that are induced during skeletal muscle regeneration (Charge and Rudnicki, 2004). MiR-206 regulates connexin 43 (also known as GJA1, gap junction protein, alpha 1) expression during skeletal muscle development (Anderson et al., 2006). GJA1 is an important component of gap junctions. It has been shown that *in vitro* fusion and differentiation of myoblasts require functional gap junctions (Mege et al., 1994; Proulx et al., 1997). Furthermore, GJA1 is up-regulated during skeletal muscle regeneration and is required for normal myogenesis *in vitro* and adult muscle regeneration *in vivo* (Araya et al., 2005). MiR-206 down-regulates GJA1 expression after myoblast fusion by inhibiting translation of its mRNA (Anderson et al., 2006). Despite the requirement for GJA1 during the initial phase of myogenic differentiation, its subsequent down-regulation and gap junction communication is important in generating insulated muscle fibres that are singly innervated for fine motor control. Thus, TEAD4 makes a feed-forward loop with MYOG and MYOD1 during muscle regeneration to maintain miR-206 expression until late stages of terminal maturation of muscle fibre and their subsequent innervation.

6) TEAD4 and skeletal muscle physiopathology.

Does TEAD4 or other TEAD factors play a role in centronuclear myopathies (CNM), a group of congenital myopathies classically defined by the presence of an abnormally high number of muscle fibres with nuclei present in the central part of the fibre?. The centrally localized nuclei may reflect a default in myofibre maturation. X linked myotubular myopathy XLMTM1 is one example of CNM caused by mutations in the *Mtm1* gene. So far, few studies have investigated the role of transcription factors in the pathogenesis of CNM. Given the role of the TEAD factors in myofibre structure and maturation, this now becomes a pertinent question.

To gain insights in the role of TEAD4 in XLMTM1, we checked its expression in *Mtm1*$^{-/-}$ muscle compared to wild type muscle and found that it is up-regulated (our unpublished data). This up-regulation may reflect a “poised” maturation process as previously suggested by centrally localized nuclei. Transcriptome analysis of *Mtm1*$^{-/-}$ muscle (Laporte, J.

unpublished data) revealed that the majority of deregulated genes were up-regulated which may reflect direct and indirect consequences of increased TEAD4 expression. A comparison of TEAD4 target genes with the transcriptome data revealed a subset of common genes. Together, these data suggest that the deregulation of TEAD4 expression may contribute to the physiopathological phenotype observed in *Mtm1*$^{-/-}$ mice. An important question in the field is how mutations in the *Mtm1* gene that codes a ubiquitously expressed lipid phosphatase that acts on phosphatidylinositol 3-monophosphate [PI(3)P], a lipid implicated in intracellular vesicle trafficking and autophagy, can lead to transcriptional deregulation.

Another gene mutated in CNM is *Bin1* (Amphyphysin 2). *Bin1* is mutated in autosomal recessive CNM. Our proteomic analysis showed that the BIN1 protein is associated with TEAD4 protein in the nuclear soluble fraction of C2C12 myoblasts. *Bin1* encodes several isoforms of a nucleo-cytoplasmic adaptor protein, one of which was initially identified as a MYC-interacting protein which represses MYC dependent transcription (Sakamuro et al., 1996; Wechsler-Reya et al., 1998). *Bin1* is induced during C2C12 differentiation with generation of several isoforms by alternate splicing. Interestingly in C2C12 myoblasts BIN1 is exclusively nuclear and its over-expression reduced C2C12 cell proliferation and increased their differentiation (Wechsler-Reya et al., 1998), while BIN1 knockdown repressed differentiation and prevented induction of p21, a phenotype similar to TEAD4 knockdown. Taken together these results suggest that BIN1 may act as a transcription co-factor for TEAD4 or act on signalling pathways that converge on the TEAD proteins. Further experiments will be required to address these possibilities.

7) TEAD4 and fast-slow muscle fibre type transition.

Our ChIP-chip experiments identified miR-206 as a gene bound and regulated by TEAD4 during C2C12 differentiation. In addition to GJA1, MiR-206 is predicted to regulate the expression of MEF2A and HDAC4, downstream effectors of the Calmodulin kinase pathway as well as RCAN1 (regulator of Calcineurin 1) a regulator of the calcineurin/NFAT pathway (McCarthy, 2008). Additionally, miR-206 is predicted to regulate the expression of SOX6, *Pur*β and SP3, all known as transcriptional repressors of the slow myosin heavy chain gene (Ji et al., 2007 ; Hagiwara, 2007). The idea that miRNAs may have a role in regulating skeletal muscle fibre type is supported by the finding that loss of miR-214 expression leads to a reduction in slow fibre type (Flynt et al., 2007). Interestingly miR-214 is also a target gene of TEAD4. Furthermore TEAD4 occupies the promoter of the slow-twitch isoform TroponinC1 (Tnnc1), but not the fast twitch isoform promoter. Perhaps TEAD4 regulation of miR-206 contributes to fibre type specification.

Other TEAD family members have already been implicated in the control of slow fibre type phenotype. It has been shown that TEAD1 binds multiple muscle MEF2 and A/T-rich elements during fast-to-slow skeletal muscle fibre type transition and its over-expression in transgenic mouse striated muscles produces a slower skeletal muscle contractile phenotype (Karasseva et al., 2003; Tsika et al., 2008). Furthermore, expression and DNA binding activity of TEAD1 was decreased in denervated rat soleus, which is associated with a decreased expression of slow type I MHC and an increased expression of the faster MHC isoforms (Huey et al., 2003).

8) A strategy to test TEAD function *in vivo*.

Despite all of the data which we and others have accumulated concerning TEAD function in muscle, their role *in vivo* in muscle specification and development has remained elusive due to redundancy amongst the different members. Our experiments suggest an alternative strategy to address this question by generating transgenic mice in which the TEA/ATTS DBD could be expressed in developing muscle under the control of a skeletal muscle promoter, or more specifically in satellite cells under the control of the Pax7 promoter. This strategy would bypass the problem of redundancy amongst the family members. In addition, there are also Cre recombinase dependent strategies to induce transgene expression allowing inhibition of TEAD activity in adult muscle or under regeneration conditions. We would predict that this approch should provide many novel insights into the role of TEAD factors in muscle development and physiopathology.

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