ROLE OF FRA-2/AP-1 IN MYOGENIC PROGENITOR CELLS

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Abstract

Skeletal muscle constitutes ~40% of the body mass in metazoans and plays a crucial role throughout life. Skeletal muscle development prenatally and regeneration postnatally are critical for animal life. Unlike cardiac and smooth muscle, skeletal muscle maintains a robust regenerative capacity postnatally due to resident muscle stem cells, also known as satellite cells. Similar to skeletal muscle development, skeletal muscle regeneration is also a well orchestrated process. Many transcriptions factors and signaling pathways have been documented to be involved in this process, such as the myogenic regulatory factors (MRFs), the activator protein 1 (AP-1) family transcription factors and Notch signaling. Compared to MRFs, AP-1 is not a muscle-specific transcription factor, and its role in skeletal muscle regeneration and the mechanisms involved are still not well characterized. Similarly, the roles of signaling pathways during regeneration are much less defined in comparison to their role during development. Therefore, the purpose of this body of work was to investigate the role of AP-1 in myogenic progenitor cells.

Components of AP-1 have dynamic expression profiles during myogenesis in C2C12 cells, the expression profile of AP-1 is different among proliferative myoblasts, differentiating myoblasts, differentiated myotubes and reserve cells, indicating context-dependent spatial and temporal roles. We found that the expression profile of Fra-2/AP-1 is also dynamic in cultured single fibers, which harbor satellite cells. We observed Pax7 mRNA level went down accompanied with precocious differentiation upon konckdown of Fra-2, suggesting that Fra-2 may play a role in determining the fate of satellite cells by modifying the expression of the key specification factor, Pax7. In addition, inhibition of ERK 1/2 MAPK pathway, a known upstream kinase of Fra-2, led to the loss of Fra-2 and Pax7 expression at protein level.

Furthermore, loss of function using siRNA technology indicated that the effect of AP-1 on Notch signaling and its downstream target genes in satellite cells is Fra-2 specific, while c-Jun did not have the same effect. Fra-2 was also found to modulate the expression of Sbno1, a novel downstream effector of Notch signaling in myogenic cells.

In conclusion, this work provides some insight into the role of Fra-2/AP-1 in the regulation of satellite cells by looking into how Fra-2 might determine the fate of satellite cells by modulating the expression of Pax7.

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List of abbreviations

ANK	ankyrin
AP-1	activator protein 1
AT2R	angiotensin type 2 receptor
ATF	activating transcription factors
BMP	bone morphogenetic protein
BrdU	bromodeoxyuridine
BTD	β-trefoil domain
bZIP	basic region-leucine zipper
CBC	crypt base columnar
CDK	cyclin-dependent kinase
ChIP-seq	Chromatin immunoprecipitation sequencing
CNS	central nervous system
CoR	co-repressors
CRE	cAMP responsive elements
CSL	CBF1, Suppressor of Hairless, Lag1
DSL	Delta, Serrate, and Lag2
ECM	extracellular matrix
EGF	epidermal growth factor
ERK	extracellular-signal-regulated kinase
FGFs	fibroblast growth factors
GPI	glycosylphosphatidylinositol
GSK3β	glycogen synthase kinase 3β
Hes	hairy enhancer of split
Hey	hairy related
HGF/SF	hepatocyte growth factor/scatter
HSPGs	heparan sulfate proteoglycans
IGF	insulin-like growth factor
IGF1R	IGF-I receptor
IGFs	insulin-like growth factors
IL-6	interleukin-6
ISCs	intestinal stem cells
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JDP	Jun dimerization partners

JNK	Jun N-terminal kinase
LIF	leukemia inhibitor factor
LNRs	Lin–Notch repeats
Mam	Mastermind
MAPK	mitogen activated protein kinase
MCK	muscle creatine kinase
MDSCs	muscle-derived stem cells
MEF2C	myocyte specific enhancer binding factor 2c
MHC	myosin heavy chain
MRF	myogenic regulatory factor family
NA	numerical aperture
NECD	Notch extracellular domain
NICD	Notch intracellular domain
NLS	nuclear localization sequences
NRR	negative regulatory region
NSCs	neural stem cells
Pax7	paired domain transcription factor 7
PCP	planar cell polarization
PDGF	platelet-derived growth factor
PEST	Pro-Glu-Ser-Thr
PI3K	phosphoinositide 3'-kinase
PICs	PW1+ interstitial cells
RAM	RBPJ-associated molecule
RHR	Rel-homology regions
SMAD	small mother against decapentaplegic
S1P	sphingosine-1-phosphate
Shh	Sonic hedgehog
SOP	sensory organ precursors
TAD	transcriptional activation domain
TCFs	ternary complex factors
TGFβ	transforming growth factor-beta
Th	T helper
TNFα	tumor necrosis factor-α
TPA	phorbol 12-O-tetradecanoate-13- acetate
TRE	TPA response element

Review of literature

AP-1: Activator Protein-1 Transcription Factor

AP-1 is a ubiquitously expressed transcription factor, its expression and activity are induced by an array of physiological and environmental stimuli. In turn, AP-1 regulates a wide range of cellular processes, including cell proliferation, cell death and survival, differentiation and regeneration [1–5]. However, despite increasing insight into the physiological functions of AP-1, signalling pathways involved in regulating these functions and the target-genes mediating these functions are not well understood. As one of the first mammalian transcription factors identified [6], the complex physiological functions of AP-1 are still being unravelled.

1. 1 AP-1 components and structure

Activator protein 1 (AP-1) is a dimeric transcription factor consisting of basic region-leucine zipper (bZIP) proteins that belong to Jun and Fos, the closely related activating transcription factors (ATF) and the Maf subfamily [6–10].

Jun family proteins include c-Jun, JunB and JunD, which may form homodimers or heterodimers with members of the Fos family members. Members of the Fos family are c-Fos, FosB, Fra-1 and Fra-2, which can only form heterodimeric complexes with Jun members [11–13]. ATF proteins include CREB, ATF1, ATF2, LRF1/ATF3, B-ATF, which may form homodimers as well as heterodimers with Jun proteins [14]. There are two additional Jun dimerization partners (JDP), JDP1 and JDP2 [7]. Maf subfamily, which includes c-Maf, MafB, MafA, MafG/F/K and Nrl, is the least studied in terms of its activity [4,10].

AP-1 transcription factors harbour a basic DNA-binding domain and leucine zipper domain. The basic domain is responsible for DNA binding in a sequence specific manner. The leucine zipper domain mediates dimerization with other bZIP proteins. The transactivation domain regulates the transactivation activity (Figure 1). Dimerization between AP-1 components is a pre-requisite for DNA binding, occuring via hydrophobic interactions of leucine residues in the leucine zipper domain [6]. Although Jun proteins may form homodimers, Fos proteins do not form homodimers but can bind to DNA by forming Fos:Jun heterodimers that are more stable than Jun:Jun homodimers [11,15]. In mammalian cells, Jun:Jun and Fos:Jun dimers bind to a specific DNA sequence known as the phorbol 12-O-tetradecanoate-13- acetate (TPA) response element (TRE) (5'-TGAG/CTCA-3'), which can mediate transcriptional induction and tumor induction by TPA treatment [16]. In yeast, a Jun homologue, Gcn4p, recognizes the same DNA element indicating extensive evolutionary conservation of this *cis* element [2,17]. AP-1 complexes containing ATF, on the other hand, preferentially bind to the cAMP responsive elements (CRE, 5'-TGACGTCA- 3') [18]. JDP1 and JDP2 bind to both TRE and CRE [7].

Jun and Fos proteins differ significantly in terms of their DNA binding affinities and *trans*activation properties. c-Jun, c-Fos and FosB exhibit strong *trans*activation potential, while JunB, JunD, Fra-1 and Fra-2 are considered as weak *trans*activators. Each of these proteins is expressed and regulated differently. Different AP-1 dimer compositions exhibit differential promoter binding specificity and transactivation potential [8,14,19,20]. Hetero-dimerization with c-Fos enhances the transcriptional potential of c-Jun by forming a more stable dimer [11,15]. Hetero-dimerization with FosB, however, antagonizes c-Jun transcriptional potential [19,21]. Jun:ATF2 and Jun:Fos dimers promote tumor growth in melanoma, ATF2:ATF2 dimer, on the

other hand, inhibits that function [22,23]. Thus the relative expression of AP-1 subunits and their composition play critical roles in determining cell fate [20,21].

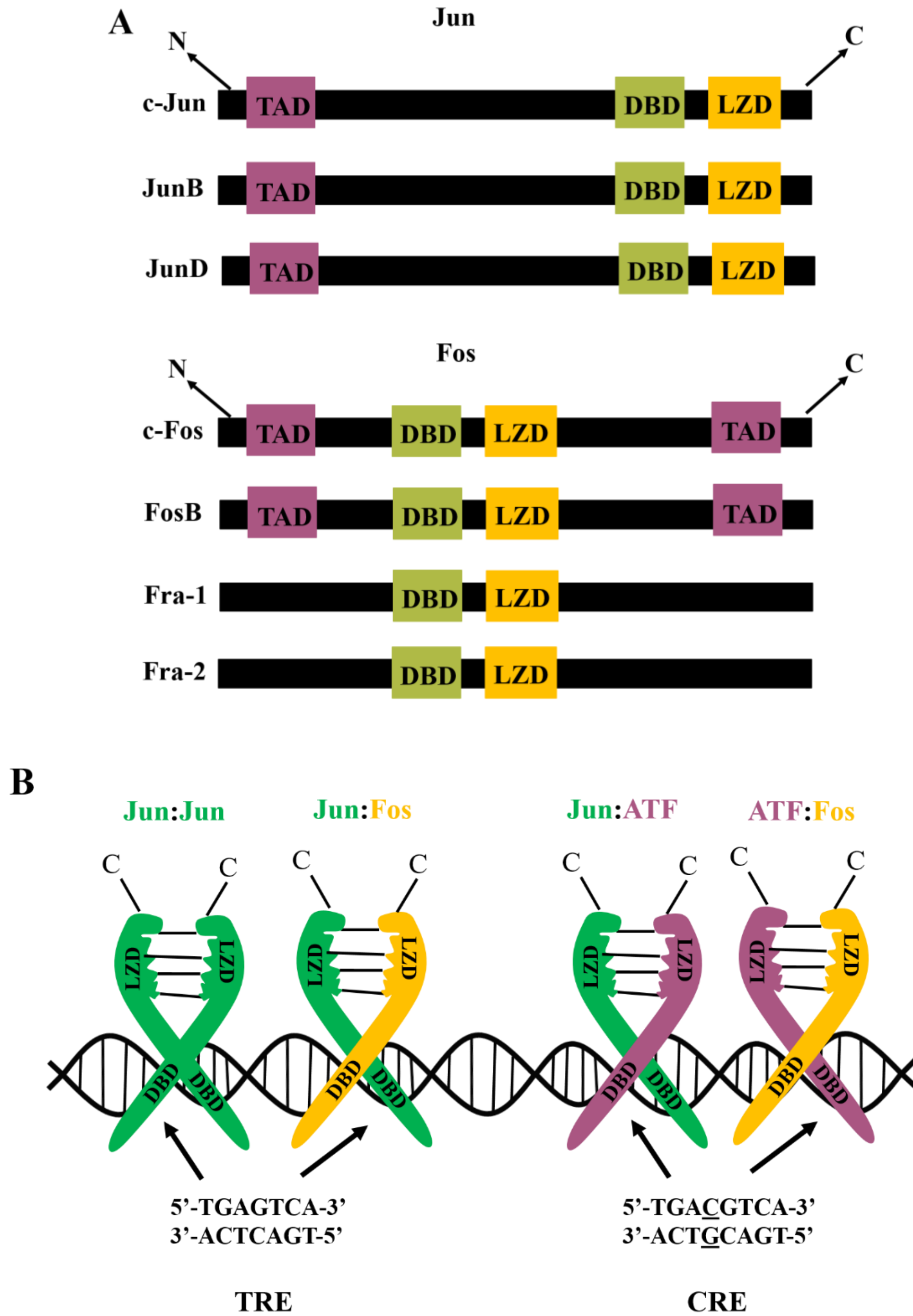


Figure 1: Schematic diagram showing the function domains (A) and dimerization and DNA binding properties (B) of AP-1. A: the locations of functional domains are indicated. N, amino terminus; TAD, transcription-activating

domain; DBD, DNA binding domain; LZD, leucine-zipper domain; C, carboxyl terminus. **B:** LZD mediates the dimerization between AP-1 components and is required for DNA binding. ATF, activation transcription factor; TRE, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element; CRE, cyclic AMP-responsive element. Note that CRE has an extra base (underlined) compared with TRE.

1.2 AP-1 function

AP-1 regulates both basal and inducible transcription of numerous genes containing AP-1 binding sites in their cis-regulatory regions. Each Jun protein exhibits unique roles, but Fos proteins show some functional redundancy [6]. AP-1 proteins are expressed in virtually all vertebrate cell types and some viruses, indicating that its activity is quite ubiquitous (Figure 2). AP-1 components are also found expressed and regulated differentially, which means that there are also elements of specificity wired into AP-1 complex function [24].

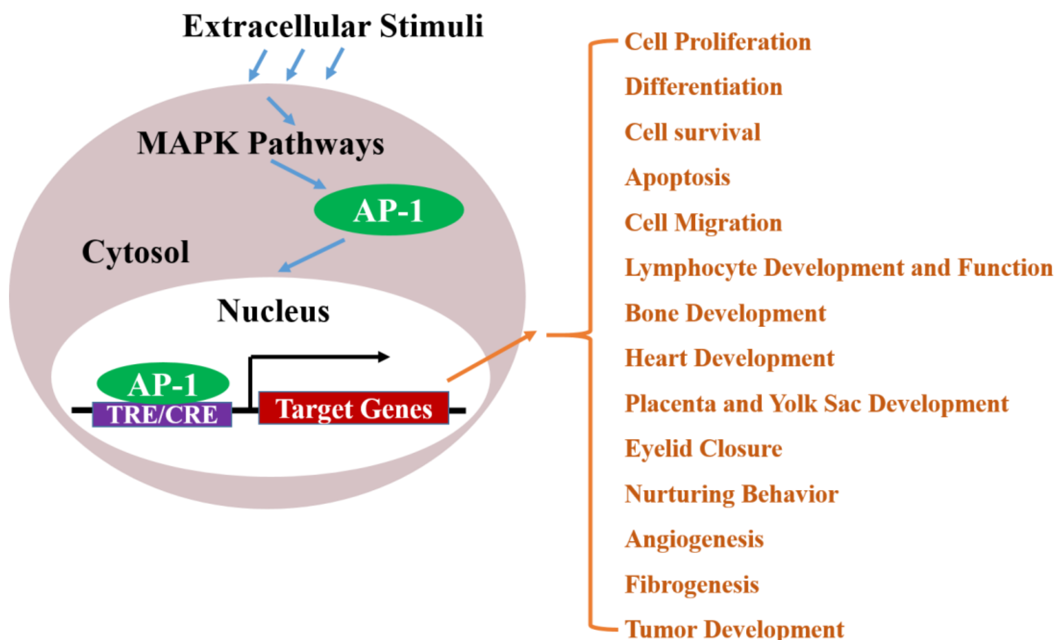


Figure 2: Schematic view of AP-1 functions. Activation of MAPK pathways by extracellular stimuli induces the post-translational modification of AP-1 subunits. Subsequently AP-1 translocates into the nucleus and drives target gene expression. AP-1 regulates a wide range of cellular processes as indicated above. The exact outcome is determined by the nature of the extracellular stimulus.

Loss-of-function experiments using ES cell technology and use of animal models has provided further understanding regarding the specific functions of different AP-1 components in mouse development. Absence of c-Jun results in embryonic lethality in mice due to defects in the heart, artery and liver [25–27]. Conversely, broad overexpression of c-Jun in transgenic mice does not affect morphological phenotype [28]. c-Jun regulates eyelid closure and skin tumor development through regulation of EGFR and HB-EGF [29]. Lack of JunB causes embryonic lethality between E8.5 and E10.0 due to vascular defects in the extra-embryonic tissues [30]. JunB is an important regulator in erythroid differentiation and a positive regulator in primarily controlling osteoblast as well as osteoclast activity [31,32]. JunB is also essential for endothelial cell morphogenesis by regulating core-binding factor β [33]. JunD deficient mice are viable. However, mutant males exhibit reduced postnatal growth, abnormalities in head hormone balance and defects in reproduction due to compromised spermatogenesis, while no defects in fertility were observed in female counterparts [34]. JunB and JunD can both regulate the differentiation and function of T helper (Th) cells by regulating cell-specific cytokines. JunB promotes Th2 lineage differentiation through regulating cyclinA, whereas JunD inhibits it by negatively affecting IL-4 expression [35,36]. Mice lacking c-Fos are viable and fertile but with an osteopetrotic phenotype due to lack of osteoclasts [37,38]. Prolonged expression of c-Fos is shown to stimulate osteoclast differentiation [39]. c-Fos expression is also induced during myelomonocytic differentiation and macrophage proliferation [40], while constitutive and inducible overexpression of c-Fos is reported to inhibit chondrocyte differentiation in vitro [41]. Mice lacking FosB develop normally although some adult mutant females display impaired nurturing behaviour [42,43]. Overexpression of DFosB, a dominant negative form of FosB [44–46], promotes differentiation in osteoblasts, while ectopic expression of DFosB in thymocytes disrupts normal T cell differentiation [47,48].

Induced FosB expression initiates osteogenic differentiation of mesenchymal cells through the upregulation of osteoblast genes [49]. Inactivation of Fra-1 leads to embryonic lethality due to abnormalities in the placenta and the yolk sac. Fra-1 deficient mice can be partly rescued and develop without obvious abnormalities. Ectopic Fra-1 expression is able to rescue Fra-1 knockout phenotype and promote osteoblast [50], indicating Fra-1 is dispensable for differentiation. However, embryo-specific Fra-1 knockout mice display osteopenia [32]. Fra-1 is also shown to inhibit follicular B cell differentiation into plasma cells by repressing Blimp1 expression [51]. Pups lacking Fra-2 die shortly after birth and exhibit defects in chondrocytes and osteoclasts [52,53]. Ectopic Fra-2 expression in transgenic mice perturbs normal eye development by contributing to TGF alpha signalling events [54]. Induced expression of Fra-2 is reported to cause pulmonary fibrosis in mice, possibly by bridging vascular remodeling and fibrogenesis [55]. Development of pulmonary fibrosis through a pathway involving the transcription factor Fra-2/AP-1. Fra-2 regulates B cell development by acting as an upstream regulator of IRF4 at multiple stages [56].

Table 1 Analysis of Jun and Fos knock-out mice

gene	Phenotype(s)	Affected organ(s)/cell type(s)
c-Jun	Embryonic lethality	Liver/hepatoblasts, Heart/outflow tract
JunB	Embryonic lethality	Extra-embryonic tissue/giant trophoblasts, yolk sac; Placenta/labyrinth layer
JunD	Male sterility	Testis/spermatogenesis
c-Fos	Osteopetrosis	Bone/osteoclasts
FosB	Nurturing defect	Brain/hypothalamus
Fra-1	Embryonic lethality	Extra-embryonic tissue/yolk sac; Placenta/labyrinth layer
Fra-2	Osteopetrosis	Bone/osteoclasts

AP-1 regulates cell proliferation, cell-cycle progression and differentiation mainly through its ability to modulate the expression of cell-cycle regulators such as cyclin D1, cyclin A, cyclin E, p53, p21Cip1, p16Ink4a and p19ARF [4,57]. Tissue-specific manipulation of c-Jun in mice has

documented that c-Jun is crucial for cell-cycle regulation in fibroblasts as well as in liver cells and keratinocytes [29,58,59]. Hepatocyte proliferation and liver regeneration are impaired in liver-specific deletion of c-Jun expression in mice [58]. Consistent with this finding, an early study showed that c-Jun expression was elevated during the expansion of liver cells [27]. c-Jun and JunB antagonistically regulate the proliferation and differentiation of fibroblasts during the formation of epithelia [60]. In agreement with this, c-Jun is found to promote epidermal neoplasia, whereas JunB inhibits it [61]. JunB is reported to inhibit proliferation in B-lymphoid cells, cell proliferation is enhanced in the absence of JunD in lymphocytes, indicating that JunB and JunD can also act as negative regulators of cell proliferation [36,62]. However, intriguingly JunB can substitute for c-Jun in mouse development and cell proliferation [63]. Thus, JunB plays a positive role in proliferation in the absence of c-Jun, and a negative one in the presence of c-Jun. JunD exhibits a similar role switch that depends on the presence of menin [64]. The fact that AP-1 can both promote and inhibit cell-cycle progression and proliferation are most likely due to the relative expression of distinct members and dimer compositions within the cell.

AP-1 was first identified as a transcription factor in HeLa cell extracts that binds to the promoter and enhancer region of the human metallothionein IIA gene and simian virus SV40 [65]. Since the Fos and Jun genes were initially found and studied in the genomes of oncogenic retroviruses, the connection of AP-1 to cancer has been immediately established. The first AP-1 component reported in mammalian tumors was c-Fos, c-Fos was reported sufficient to induce transformation of immortalized rat fibroblasts [66]. c-fos mRNAs were induced in small cell lung cancer cell upon bombesin treatment and is required for malignant progression of skin tumors [67,68]. Though c-Fos is conventionally considered to be oncogenic, c-Fos was recently reported to inhibit ovarian cancer progression by changing adhesion properties [69]. Constitutive expression of FosB and its

short form, FosB/SF, promotes proliferation and induces malignant cell transformation in rat-1A cells [70]. Surprisingly, FosB transgenic mice do not develop tumors [28]. Fra-1 and Fra-2 exhibit weak transforming potential comparing with the other Fos members. Fra-1 plays a critical role in the development of epithelial tumors through inducing morphological transformation and increasing *in vitro* invasiveness and motility of epithelial cells [71]. Fra-1 is suggested to promote breast cancer progression through regulating proliferation and invasiveness of breast cancer cells [72]. Though transgenic mice overexpressing Fra-2 do not develop tumors in a broad range of organs, Fra-2 overexpression is suggested to promote a more aggressive tumor phenotype and is probably involved in breast cancer progression by changing cellular adhesion properties [54,73,74]. *In vitro* studies show that c-Jun, the cellular homologue of v-Jun, causes oncogenic transformation in mammalian cells when co-expressed with an activated oncogene such as Ras or Src [19,75]. Whereas, c-Jun overexpression in transgenic mice does not promote tumor development [28]. c-Jun promotes cancer in mice by regulating the classical tumor initiation-tumor promotion process [76]. Breast cancer cell tumorigenesis is inhibited when c-Jun degradation is induced [77]. Neither JunB nor JunD exhibits transforming potential [78,79]. However, both JunB and JunD are involved in tumorigenesis suggesting that they are necessary but not sufficient for tumorigenesis. JunB functions as a gatekeeper for B-lymphoid leukemia through regulating the proliferative capacity of transformed leukemic cells [80]. In the absence of JunB, tumor angiogenesis is impaired in teratocarcinomas due to inability to efficiently recruit host-derived vessels [81]. While loss of stromal JunB does not affect tumor growth and angiogenesis [82]. JunD reduces tumor angiogenesis by protecting cells from oxidative stress [83]. Recent study also shows that the expression levels of JunB, JunD and phosphorylated c-Jun are positively correlated with tumor cell proliferation in diffuse large B-cell lymphomas [84].

AP-1 has a dual function in cell survival and apoptosis and the exact outcome is highly context dependent. AP-1 was first linked to apoptosis due to the observation of c-Fos and c-Jun induction preceding apoptosis [85–87]. Though such observations do not demonstrate Jun or Fos's functional involvement apoptosis, they opened the gate for more productive studies regarding the role of AP-1 in apoptosis. Light-induced apoptosis of retinal photoreceptors is impaired in c-Fos-deficient mice [88]. Activation of AP-1 is required for bufalin-induced apoptosis in human leukemia U937 cells [89]. In addition, AP-1 was reported to mediate NO-induced apoptosis in adult cardiomyocytes by acting downstream of JNK and ERK signalling pathway [90]. However, AP-1 is also involved in cell survival, which is anti-apoptotic. c-Jun was shown to protect cells against UV-induced cell death and promote cell survival via maintaining mitochondrial integrity and expression of the key regulators of ROS production [91–93]. Inhibition of AP-1 transcriptional activator function was shown to induce myc-dependent apoptosis in HL60 cells, indicating AP-1's anti-apoptotic role [94]. In addition, Fra-2 was recently reported to promote adipocyte survival by regulating PPARγ and hypoxia [95].

1.3 AP-1 regulation

Regulation of AP-1 activity can be achieved through changes of gene transcription and mRNA turnover, post-translational modifications and AP-1 subunit turnover, specific dimeric compositions of AP-1 subunits, as well as interaction with other transcription factors and cofactors [16,96,97] (Figure 3).

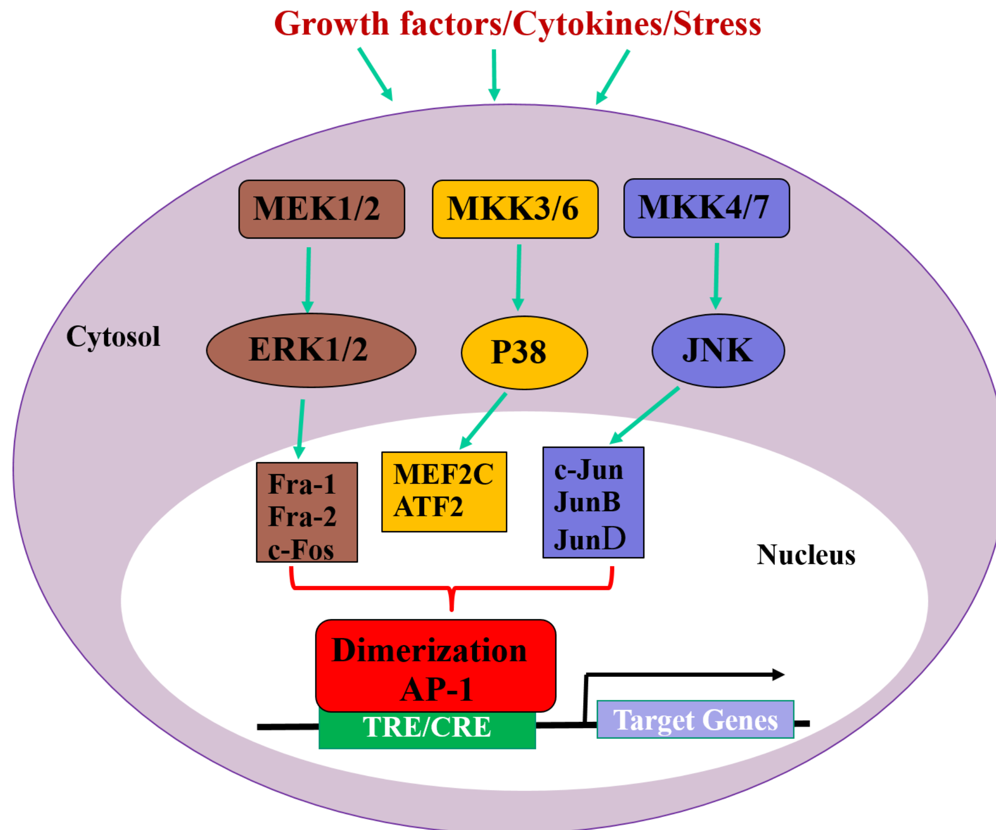


Figure 3: Schematic representation of MAPK signalling pathways regulating AP-1 proteins. A plethora of stimuli including cytokines, stress, and growth factors can activate signalling pathways that will subsequently modulate AP-1 and regulate its function.

AP-1 activity is regulated by a complex of physiological and pathological stimuli, including growth factors, cytokines, cell-matrix interactions, stress signals and oncogenic stimuli, as well as bacterial and viral infections. These stimuli induce the post-translational modification of AP-1 subunits by mitogen activated protein kinase (MAPK) cascades that lead to enhanced AP-1 activities [98,99].

Activation of Jun through the Jun N-terminal kinase (JNK) cascade is one of the most extensively documented mechanisms in the case of mitogen/stress-induced post-translational control [8,24]. JNKs belongs to the MAPK superfamily and target Jun proteins [100]. JNKs were

found to translocate to the nucleus upon activation and phosphorylate c-Jun within its N-terminal *trans*activation domain (residues Ser63 and Ser73), which potentiates the transactivation potential of c-Jun [101,102]. JNKs also phosphorylate and enhance the activity of ATF-2 and JunD [103,104]. In contrast, JunB phosphorylation by JNK is unclear though it has been proposed that Thr102 and Thr104 are target sites [105].

The extracellular-signal-regulated kinase (ERK) subgroup of MAPKs is another well studied pathway that regulates AP-1 activity. When activated by serum and growth factors, ERKs translocate to the nucleus, phosphorylate, and subsequently enhance the transcriptional potential of ternary complex factors (TCFs) that bind to Fos promoters [106]. Also, the duration of ERK1/2 activity was shown to determine the activation of c-Fos and Fra-1 and the composition and quantitative transcriptional output of AP-1 [107]. Furthermore, ERK1/2 directly phosphorylates Fra-1 and Fra-2, stabilizing them and possibly enhancing their DNA binding activity upon serum stimulation [5,108]. Though ERKs are mostly involved in the regulation of Fos proteins, ERK5 was shown to increase c-Jun expression by inducing phosphorylation and activation of the transcription factor myocyte specific enhancer binding factor 2C (MEF2C) [109].

In addition, MEKK1, a MAPK kinase kinase and an ubiquitin ligase that regulates both ERK1/2 and JNK, was reported to regulate the AP-1 dimer repertoire by controlling JunB transcription and Fra-2 protein stability [110]

Furthermore, p38, another subfamily of MAPKs, phosphorylates c-Fos and enhances AP1-driven gene expression in response to UV exposure [111]. Though p38 was conventionally linked to AP-1 regulation by its ability to directly phosphorylate ATF2, MEF2C and TCFs [112].

1.4 AP-1 in skeletal muscle

The role of AP-1 in skeletal muscle has been studied for more than two decades, yet the exact functions of this non-muscle-specific transcription factor are still being revealed. The proto-oncogene c-Jun was first documented to inhibit myogenesis by physically interacting with MyoD and suppressing its DNA binding activity [113]. Also c-Fos expression was found greatly decreased, which was a direct result of repression of the c-Fos promoter by MyoD upon muscle cell differentiation [114]. Interestingly, expression of c-Jun was maintained in mouse C2C12 and rat L6 myoblasts undergoing myogenic differentiation, although overexpression of c-Jun has been shown to block myogenic differentiation in C2C12 cells, suggesting that expression of c-Jun at physiological levels may not interfere with skeletal myogenesis whereas overexpression does [115]. This finding was confirmed by a later study, suggesting that the function of AP-1 in myogenesis is context and composition dependent [116]. AP-1 composition shifts from a predominant c-Fos:c-Jun dimer in myoblasts to a Fra-2:c-Jun/JunD complex in differentiating myoblasts [116].

Apart from its role in myogenesis, AP-1 was also reported to be involved in regulating the size of skeletal muscle, which is determined by the balance between overall rates of protein synthesis and degradation that are regulated by intracellular signaling networks. Increased AP-1 binding activity was observed in skeletal muscle during sepsis, which is associated with increased muscle proteolysis [117]. Blocking AP-1 via injection of virus containing the TAM67, a dominant negative form of c-Jun protein, into the gastrocnemius muscle of tumour-bearing rats resulted in a significant recovery of the muscle, indicating that AP-1 plays a negative role in protein accretion [118]. On the other hand, JunB was shown to maintain skeletal muscle mass and promotes hypertrophy through reducing protein breakdown [119]. Those contradictory findings can be partly

explained by the fact that in these studies, different AP-1 subunits were being studied and also different cellular systems were used. In addition, one study also reported that AP-1 plays a major role in skeletal muscle cellular plasticity by regulating the PGC-1α-controlled gene program of the hypoxia response [120].

Most of the studies mentioned above focused on the role of AP-1 in skeletal muscle proliferation and differentiation, AP-1 has also been implicated to be important in skeletal muscle regeneration. Transcripts for c-Fos and c-Jun genes are expressed in regenerating rat skeletal muscle at 3 h post-trauma, but the exact functions were not clear [121]. Another study showed that c-Jun transcriptional activity was induced upon exercise in human skeletal muscle[122]. In addition, c-Fos mRNA was induced upon repetitive stretch within several hours in skeletal muscle removed from rats [123]. AP-1 activity was higher in dystrophin-deficient diaphragm, which was further augmented by mechanical stretch. This mechanical stretch-induced activation of AP-1 was compromised by pre-treatment of muscle fibers with PD98059 (a MEK inhibitor) indicating that phosphorylation is involved [124]. A more recent study showed that Fra-2 is expressed in both reserve and satellite cell populations of skeletal muscle, implicating a possible role in skeletal muscle regeneration, though the mechanisms involved are not yet clear [5].

Furthermore, global analysis of MyoD and MEF2 target genes in skeletal muscle reported that AP-1 binding sites are enriched in neighbouring sequences, suggesting AP-1 may be intrinsically connected with the myogenic regulators and possibly more involved in skeletal muscle regulation than previously anticipated [125,126].

Skeletal Muscle regeneration

Skeletal muscle constitutes 40%-50% of the adult human body mass, allowing locomotion and providing form, support and stability to the body [127]. Under normal biological conditions, adult skeletal muscle mass is relatively stable due to its robust regeneration capacity allowing maintenance and repair following injury [128–130]. Skeletal muscle regeneration is a highly orchestrated process mediated mainly by satellite cells, although other cells lying outside the basal lamina may also contribute to the regeneration process [131,132]. Skeletal muscle regeneration is accompanied by the activation of various cellular responses, regulated by multiple signalling pathways.

2.1 Satellite cells

The satellite cell, since its identification by electron microscopy in 1961, has been the main focus of the skeletal muscle regeneration field. The presence of satellite cells was first detected in frog muscle, immediately followed by its confirmation in rat muscle [131,133]. Anatomically, those undifferentiated mononuclear cells located between the basal lamina and sarcolemma of skeletal muscle fibers [131] (Figure 4). Morphologically, satellite cells have a relatively high nuclear-to-cytoplasmic ratio, less organelle content, and a more compact nucleus compared with adjacent fiber myonuclei. These features are consistent with the finding that satellite cells are mitotically quiescent and transcriptionally less active than myonuclei under normal physiological conditions [134]. Satellite cells were later detected by phase-contrast microscopy on single myofiber explants base on their unique location [135]. Now, satellite cells can also be detected by fluorescence microscopy using specific biomarker. The most commonly used marker is the paired

domain transcription factor 7 (Pax7), which is specifically expressed in all quiescent and proliferating satellite cells of adult skeletal muscle [136,137]. Pax7 was shown to be critical for satellite cell specification and cell fate [138]. Other frequently used markers includes Pax3 [139], myogenic regulatory factor Myf5 [140], tyrosine receptor kinase c-Met [141], cell surface attachment receptor α7-intergin [142,143], transmembrane heparan sulfate proteoglycan syndecan-3 and syndecan-4 [144]. Practically, satellite cells are identified using combinations of the markers mentioned above, considering some of them are also expressed in other cell types within skeletal muscle.

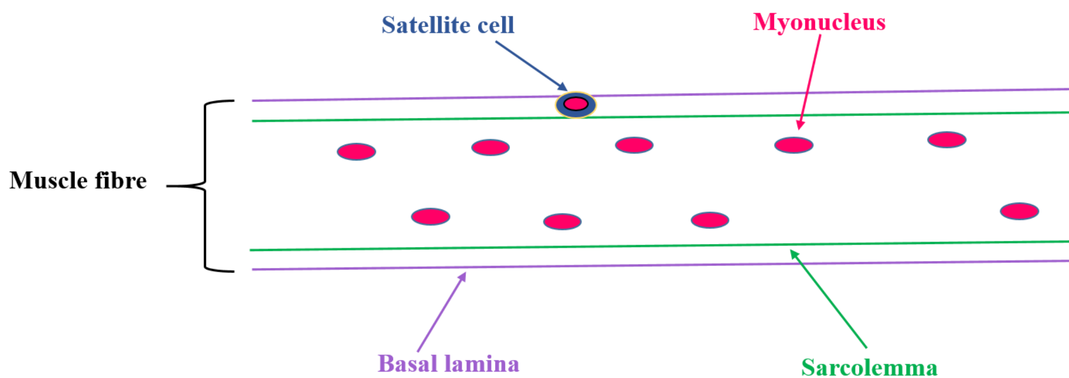


Figure 4: Anatomical location of satellite cells. Anatomically, satellite cells locate between the basal lamina and sarcolemma of skeletal muscle fibers.

Satellite cells together with all the other myogenic cell lineages share a common somitic origin, as suggested in early experiments using quail-chick chimeras [145]. Later evidences further suggested that adult satellite cells originate from the dermomyotome, an epithelial structure formed on the dorsal part of the somite [146–149]. Though another study suggested that the embryonic dorsal aorta can also give rise to adult satellite cells during fetal and postnatal muscle development [150]. The majority of skeletal muscle in vertebrates originates from somites with the exception of head muscles. Not surprisingly, different from satellite cells of trunk and limb muscles, adult

satellite cells of the various head muscles originate from their corresponding embryonic muscles [151].

Satellite cells are a heterogeneous population in terms of their gene expression profile and cell surface markers. All satellite cells express Pax7, but only a subset of satellite cells expresses Pax3, the paralog of Pax7 [152,153]. Though Pax3 and Pax7 have overlapping functions, it was shown that Pax3 cannot replace the anti-apoptotic function of Pax7 in adult muscle progenitor cells [153]. Immunofluorescence staining revealed that expression of CD34 and Myf5 are not present in a subpopulation of quiescent adult skeletal muscle satellite cells [154]. MyoD, Myogenin, and c-Met expression were also shown to be different among human skeletal muscle satellite cells, indicating their heterogeneity [155]. As mentioned previously, satellite cell origins of head muscle and body muscle are different. Consistent with this notion, two independent studies showed that satellite cells from head or body muscles express different molecular markers [151,156].

The heterogeneity of satellite cells is also reflected in their differentiation potential. Early studies using continuous infusion of bromodeoxyuridine (BrdU) labeling of satellite cells *in vivo* identified two distinct satellite cell populations, the responsive population and the reserve population [157]. It was found that the responsive population divides faster and acquires a proliferative fate, while the reserve population divides slower, suggesting a quiescent fate [157]. In agreement with this finding, a recent study showed that slow-dividing satellite cells retain long-term self-renewal capability in adult muscle [158].

Increasing evidences have revealed that satellite cells are heterogenic in terms of their stemness, indicating that only a small population of satellite cells are true stem cells [159–161]. It was reported that Numb, an inhibitor of Notch, was localized asymmetrically in actively dividing cells

and inhibition of Notch signaling by Numb results in the commitment of progenitor cells to the myoblast cell fate [162]. In agreement with this finding, a study using Myf5-Cre and ROSA26-YFP Cre-reporter alleles reported that $Pax7^{+}/Myf5^{+}$ cells are more prone to differentiation, while $Pax7^{+}/Myf5^{-}$ cells acquire a quiescent and undifferentiated state, suggesting satellite cells are a heterogeneous population composed of stem cells and committed progenitors [160]. Interestingly, Myf5 was recently found to contribute to Pax7-dependent long-term maintenance of adult muscle stem cells [163].

In addition, the heterogeneity of satellite cells can also be reflected in their cell fate potential. Satellite cells were found to acquire an adipocyte fate when exposed to a high oxygen environment [164]. Muscle satellite cells also exhibit multi-potentiality which is a common stem cell feature and are able to acquire osteocyte and adipocyte phenotypes, depending on the extracellular cues [165]. Thus, satellite cells have the potential to differentiate to other cell linages, and this is largely determined by the culture environment.

Satellite cells generally account for roughly 30% of the nuclei on myofibers in early postnatal muscle and this number is reduced to as low as 2% in adult muscles [166–168]. The distribution of satellite cells is denser at the ends of the myofibers, where the longitudinal growth of skeletal muscles takes place [169]. A few satellite cells can generate hundreds of satellite cells and restore regeneration capability, when transplanted into regeneration insufficient mice. Transplanted satellite cells can facilitate subsequent rounds of muscle regeneration, indicating that satellite cells are true muscle stem cells [170]. Stem cells are undifferentiated cells found in adult tissues that can give rise to functional progeny, while retaining the ability for self-renewal. Asymmetric division is a common function of stem cell self-renewal. Satellite cells, the muscle stem cells, are

undifferentiated cells in muscle tissue, they are mitotically quiescent under normal physiological conditions. Those cells may enter numerous rounds of proliferation in response to injury or stress to generate myoblasts that contribute to the repair of the existing damaged muscle fibres or generate new ones. Self-renewal through asymmetric division allows replenishment of the stem cell pool and generates more committed myogenic cells that contribute to skeletal muscle growth and regeneration. It has also been reported that satellite cells can undergo symmetric division, depending on the position of the daughter cells on the fiber [160](Figure 5). Non-canonical Wnt signaling is involved in the regulation of satellite cell self-renewal [171].

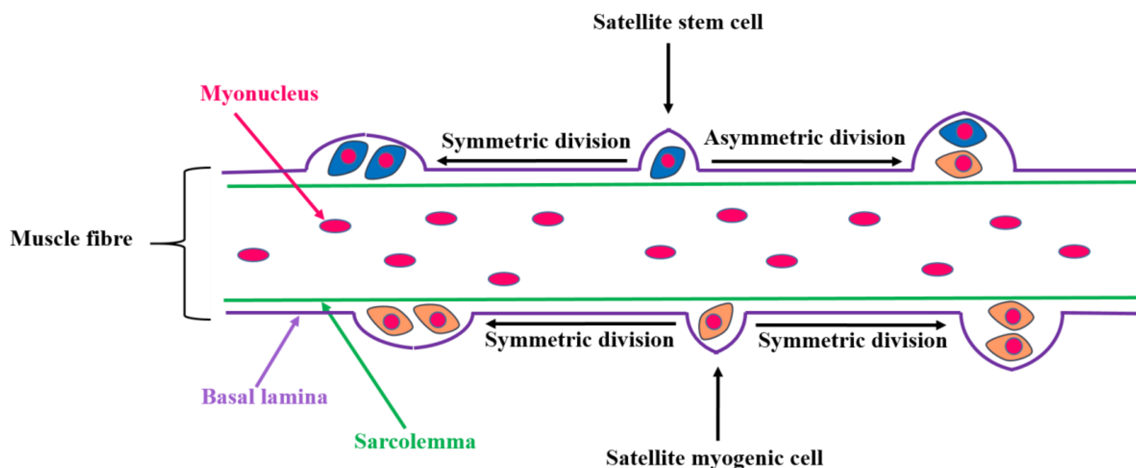


Figure 5: Symmetric and asymmetric division of satellite cells. Satellites cells are heterogeneous and can be divided into satellite stem cells and satellite myogenic cells. Satellite stem cells can divide symmetrically and asymmetrically. Satellite stem cells preferentially mediate satellite cell self-renewal and contribute to long-term muscle regeneration. While satellite myogenic cells preferentially differentiate following muscle injury.

2.2 Satellite cell mediated skeletal muscle regeneration

In mammalian species, adult skeletal muscle is a stable post-mitotic tissue with infrequent turnover of myonuclei [172]. Minor tears due to day-to-day wear can be repaired without causing cell death, inflammatory responses or involvement of satellite cells. However, when muscle is

subjected to severe injury, exposed to a toxic environment or pathological conditions, muscle repair is accompanied by the activation and proliferation of satellite cells, which are the main mediator of muscle regeneration in adulthood [173] (Figure 6).

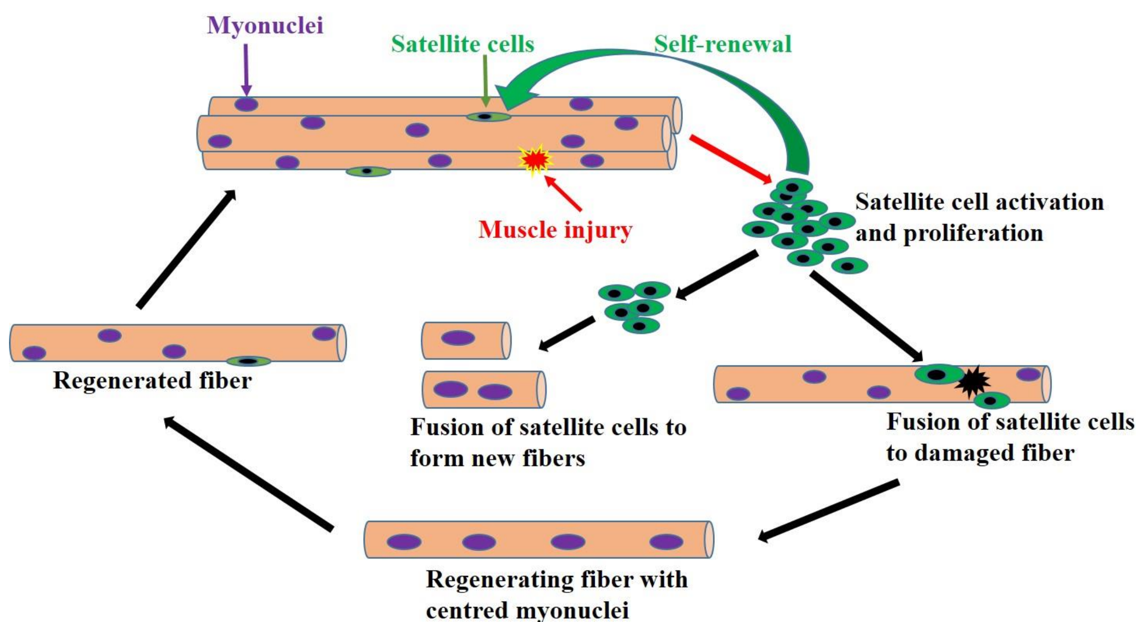


Figure 6: Satellite cell mediated muscle regeneration in response to muscle injury. In response to muscle injury, satellite cells become activated and proliferate. Some of the satellite cells will repopulate the satellite cell pool via self-renewal. Proliferating satellite cells will migrate to the damaged region and fuse with the existing myofiber to repair or fuse with each other to generate new myofibers. In the regenerating myofiber, the newly fused myonuclei are centred, but will later migrate and assume a more peripheral location.

Muscle regeneration shares similarities with muscle development during embryogenesis. In embryogenesis, all skeletal muscles are derived from mesodermal precursor cells and share somitic origin, except head muscles [139]. Study has shown that expression of Pax3 and Pax7 genes play an important role in specifying the satellite cell lineage of precursor cells [174]. Interestingly, Pax3 and Pax7 are co-expressed in most of the myotomal cells of the somite indicating their involvement during embryogenesis [175]. Pax3 is required for the migration of muscle precursor cells from the somite, while Pax7 is involved in the specification of muscle precursor cells [176]. During

development, up-regulation of MyoD and Myf5, the basic helix-loop-helix transcriptional activators of the myogenic regulatory factor family (MRF), is required for the specification of precursor cells to the myogenic lineage. MyoD:Myf5 double knockout mice displayed a total loss of skeletal muscle [177,178]. The myogenic cells expressing Myf5 and MyoD are termed myoblasts [175]. Following up-regulation of Myogenin and MRF4, the secondary MRFs, the terminal differentiation of those myoblasts into myocytes occurs, which leads to the expression of late differentiation markers, such as myosin heavy chain (MyHC) and muscle creatine kinase (MCK). Subsequently, those mono-nucleated myocytes fuse with each other to form multi-nucleated myotubes, which mature into contracting muscle fibers after innervation. Genome-wide loss of function study using CRISPER revealed that myomixer, a fusogenic micropeptide, is essential for fusion and muscle formation during embryogeniesis [179]. During the late stages of embryogenesis, a small population of those myogenic precursor cells maintain or resume the expression of Pax7 and escape from the differentiation pathway. Those cells are quiescent and mono-nucleated, anatomically associate closely with myofibers forming the satellite cell pool [180] (Figure 7).

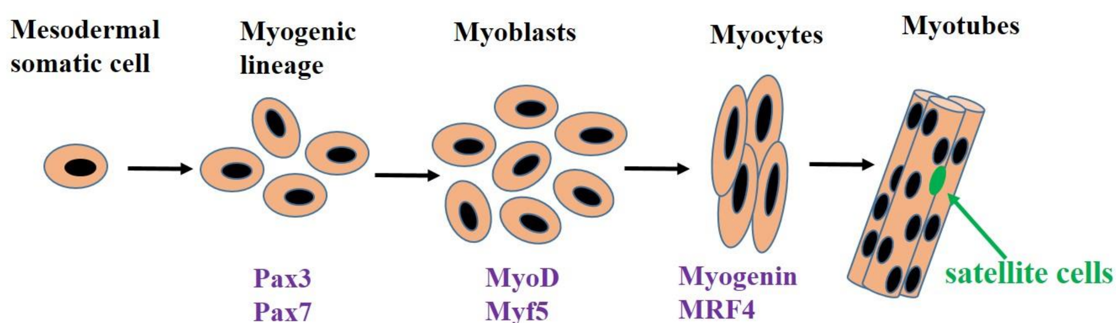


Figure 7: Model of embryonic skeletal muscle formation. Pax7 is required for the specification of myogenic lineage, while Pax3 contributes to myogenic cell expansion. MyoD and Myf5 induction by Pax7 commit cells into myogenic lineage and subsequently activate the expression of Myogenin and MRF4, directing progression of myogenesis and terminal differentiation. Finally, myocytes fusion generates multinucleated myotubes. Meantime, a distinct population, satellite cells, escape from fusion and remain in a quiescent undifferentiated state.

Skeletal muscle regeneration, like skeletal muscle development, is a highly orchestrated process although there are some contextual differences in the two processes. Muscle regeneration begins with necrosis in the damaged muscle fibers, myofiber necrosis subsequently induces inflammatory responses [181]. Following this, muscle regeneration involves activation, differentiation and self-renewal of satellite cells, as well as the maturation and remodeling of newly formed or repaired myofibers. As mention earlier, muscle regeneration shares similarities with embryonic myogenesis. This process recapitulates muscle development during embryogenesis in many but not all respects.

Under normal conditions, satellite cells are mitotically quiescent, those cells express Pax7 but not MyoD or Myogenin [140]. Interestingly, Myf5 expression was detected in the majority of quiescent satellite cells, indicating their commitment to the myogenic lineage [154]. Upon injury, satellite cells will be activated and start proliferating, and this is not restricted to the site of muscle injury. Studies have shown that localized damage at any site of a muscle fiber is able to activate all the satellite cells along the same myofiber, and migration of these satellite cells to the damaged site to facilitate regeneration [182]. In agreement with this finding, it was observed that activated satellites cells can migrate between myofibers and even across muscles during muscle growth and regeneration [183–185]. The c-Met receptor is essential for the migration of activated satellite cells into the limb bud [186]. By FACS analysis, muscle CD31(-) CD45(-) side population cells facilitate muscle regeneration by stimulating proliferation and migration of myoblasts [187]. CD34 can promote satellite cell motility and entry into proliferation to facilitate efficient skeletal muscle regeneration [188]. In addition, it was found that BRE facilitates skeletal muscle regeneration by promoting satellite cell motility [189].

Activated satellite cells start expressing muscle regulatory factors, MyoD and Myf5, which are followed by the expression of desmin and Myogenin [190,191]. However, MyoD expression does

not necessarily promote commitment to differentiation. Several studies has shown that some Pax7$^+$/MyoD$^+$ proliferating myoblasts can lose their MyoD expression and eventually go back to quiescence [192–194]. Pax7 overexpression has an inhibitory effect on MyoD in adult primary myoblasts. It has been proposed that that the ratio of Pax7 and MyoD activities is critical in determining satellite cell fate [195]. Satellite cells with a high ratio of Pax7 to MyoD remain in their quiescent state, whereas satellite cells with an intermediate ratio of Pax7 to MyoD are prone to proliferate, but not differentiate. Satellite cells with a low Pax7 to MyoD ratio initiate differentiation.

After sufficient rounds of proliferation, the majority of satellite cells start expressing Myogenin and Myf6 [196]. Interestingly, Myogenin was found to inhibit Pax7 expression in adult primary myoblast, which further down-regulate Pax7 expression and promote differentiation [195]. MyoD and Myogenin can also facilitate differentiation by up-regulating a wide range of muscle-related genes, such as MEF2 and Myosins [125]. These cells exit from the cell cycle and begin to fuse to damaged myofibers or each other to repair existing myofibers or form new myofibers de novo [197]. Some fibers express embryonic forms of MyHC, indicating that fibers are formed *de novo* [198]. *De novo* formation of myofibers during muscle regeneration takes place in two steps. At first, individual differentiated myoblasts fuse to one another forming nascent myotubes with few nuclei. This is followed by additional myoblasts being incorporated into the existing nascent myotubes, generating a mature myofiber with more nuclei and increased size [199]. β1 integrins regulate myoblast fusion via regulation of the formation of a protein complex important for fusion [200]. A recent study showed that Myomaker, the only muscle-specific protein known to be absolutely essential for fusion of embryonic and adult myoblasts, is also essential for muscle regeneration [201]. When the majority of the activated satellite cells start to differentiate, a small

population returns to repopulate the satellite pool, re-establish its anatomical location and maintains its ability for future regeneration.

2.3 Non-satellite cells with muscle regeneration potential

Satellite cells are the main mediator of skeletal muscle regeneration. However, increasing evidences revealed that non-satellite can also contribute to muscle regeneration.

The myogenic potential of bone marrow cells has been investigated for almost two decades. Early study found that bone marrow-derived cells can migrate into damaged muscle areas and undergo myogenic differentiation, suggesting the participation of bone marrow-derived cells in the regeneration of the damaged fibers [202]. Bone marrow was shown to assume a muscle satellite cell fate and participate in muscle regeneration after muscle damage [203]. This study also revealed that fetal liver cells have the same potential as bone marrow cell with respect to their involvement in muscle regeneration [203]. In agreement with those findings, a recent study also revealed that bone marrow-derived mesenchymal stem cells injection can improve muscle regeneration and increase muscle contractile function [204].

Side population cells are a subset of Sca-1(+)cells first found in bone marrow cells, they are characterized by their exclusion of dyes such as Hoechst33342 and Rhodamine 123 [205]. Those cells found in the skeletal muscle are termed muscle side population cells, they have been found to regenerate muscle fibers [206]. Muscle side population cells are heterogenic and can be differentiated by cell surface markers [149,207]. Muscle CD45(+) side population cells isolated from adult mouse can be induced to differentiate into myogenic cells with forced Pax7 expression or activation of Wnt signalling [208,209]. Muscle CD31(-) CD45(-)side population can contribute

to new fibres post injury [210]. In agreement with this finding, this subset has been shown to promote muscle regeneration by stimulating proliferation and migration of myoblasts [187]. Recent study found that the progeny of muscle side population cells expressing Abcg2 contributes to regenerated myofibers after injury [211].

PW1$^+$ interstitial cells (PICs), a subset of interstitial cells, can differentiate into skeletal muscle after isolation from both young and adult mice [212]. PICs are myogenic *in vitro* and can efficiently contribute to skeletal muscle regeneration *in vivo*. Those cells can also give rise to satellite cells and PICs [213]. Recent study also revealed that PICs can generate skeletal muscle and self-renewal both *in vivo* and in *vitro* [214].

Muscle-derived stem cells (MDSCs), mesoangioblasts and pericytes are all multipotent, they have been shown to display myogenic potential and facilitate regeneration following injury. MDSCs has been shown to contribute to regenerated myofibers and incorporate into the satellite cell niche in transplantation experiments [215]. Mesoangioblasts are highly proliferative when cultured *in vitro*, and can give rise to skeletal muscle following transplantation [216]. Pericytes of human skeletal muscle are found to be myogenic precursors distinct from satellite cells, and can generate numerous myofibers [217]. Recent study also found that pericytes contribute to successful muscle regeneration by balancing between myogenic and non-myogenic cells, that are activated by type-1 and type-2 pericytes respectively [218].

2.4 Regulation of skeletal muscle regeneration

Regulation of skeletal muscle regeneration following injury is fine-tuned process, which involves the coordination among a variety of secreted factors and multiple signalling pathways.

The microenvironment, including the extracellular matrix, also affects all aspects of regeneration (Figure 8).

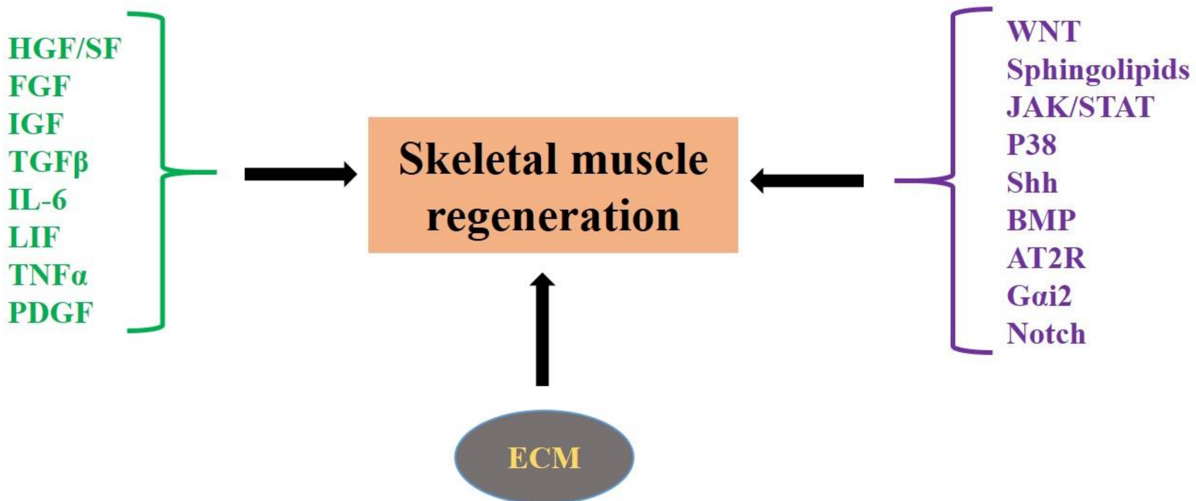


Figure 8: Schematic of skeletal muscle regeneration regulation. Skeletal muscle regeneration is under the regulation of various secreted factors, multiple signalling pathways and ECM.

Hepatocyte growth factor/scatter (HGF/SF) factor is one of the earliest secreted growth factors studied in muscle regeneration. Its cell surface receptor c-Met are found on both quiescent and activated satellite cells [219]. HGF/SF was found to activate quiescent skeletal muscle satellite cells via binding to c-Met in *vitro* [141]. HGF is present in muscle and can be released and bound to the extracellular matrix upon injury, and has the ability to activate quiescent satellite cells *in vivo* [220]. HGF/SF is the only known growth factor that activates quiescent satellite cells in skeletal muscle. It was found that HGF/SF can act directly on primary muscle cells to block differentiation, while promoting myoblast proliferation through synergy with factors in damaged muscle both *in vitro* and *in vivo,* indicating regeneration is not only regulated by myoblast number [221]. In this study, they also proposed that HGF inhibits the differentiation of myogenic precursor

cells probably by regulating Twist protein and p27 [221]. The dual function of HGF/SF in promoting proliferation and blocking differentiation involves activation of MAPK/ERK and phosphoinositide 3'-kinase (PI3K) pathways[222]. In addition, it was found that HGF/SF is expressed in cultured satellite cells, and that endogenous HGF/SF from satellite cells can act in an autocrine fashion. In this study, they proposed that direct administration of HGF/SF into damaged muscle may provide a useful approach for facilitating muscle repair, considering that HGF/SF plays a pivotal role in satellite cell activation [223].

Fibroblast growth factors (FGFs) are a large family of cytokines, involved in regulating many critical biological processes, including skeletal muscle development [224,225]. Accumulating evidences revealed that FGFs also play important roles in skeletal muscle regeneration. mRNA of FGF1, FGF2, FGF4, and FGF6 are detected in satellite cells, and has been shown to stimulate expansion of cultured satellite cells [226–228]. As a satellite cell mitogen, FGF2 was found to permit satellite cell proliferation by repressing myogenesis [229,230]. FGF-6(-/-) mutant mice show a severe regeneration defect following injury, possibly due to insufficient activation of satellite cells [231]. In contrast, it was reported that no skeletal muscle phenotypes were observed in FGF6-null mice [232]. However, injection of FGF6 was found to accelerate regeneration of the soleus muscle in adult mice [233]. A study using FGF6-null mice showed that FGF6 regulates muscle differentiation through a calcineurin-dependent pathway in regenerating soleus of adult mice [234]. Most recently, FGF was found to regulate the early steps of dedifferentiation during skeletal muscle regeneration in adult zebrafish [235]. In this study, they also showed that inhibition of MEK activity with U0126 mimicked the phenotype of the FGF receptor inhibition, also activated ERK (p-ERK) was detected in injured muscles, suggesting FGF signaling in regenerating myocytes involves the MAPK/ERK pathway [235].

Insulin-like growth factors (IGFs) are known to play important roles in controlling the growth and differentiation of skeletal muscle [236]. Recently, the importance of IGFs during muscle repair has become more apparent. IGFs can function in endocrine, autocrine, and paracrine fashions to promote satellite cell proliferation and differentiation [237]. Those process are all mediated by IGF-I binding to the IGF-I receptor (IGF1R), which is a ligand-activated receptor tyrosine kinase. IGF-1 and IGF-1 receptors are found to be induced by various types of exercise in skeletal muscles [238–240]. Targeted expression of IGF-1 transgene to skeletal muscle was shown to promote muscle regeneration, indicated by faster recovery of muscle mass [241]. Local expression of IGF-1 was found to accelerate muscle regeneration by rapidly modulating inflammatory response and reducing fibrosis and creating a qualitatively environment for more efficient muscle regeneration [242]. In agreement with those findings, overexpression of IGF-1 was reported to attenuate skeletal muscle damage and improve muscle regeneration and force recovery in soleus muscle [243]. In addition, conditional deletion of the IGF-1 gene specifically in mouse myeloid cells was shown to block the normal induction of local IGF-1 in damaged muscle, resulting in significantly compromised regeneration [244]. Activation of ERK signalling was implicated in the role of IGF-1 in skeletal muscle regeneration. A study found that IGF1R upon binding by IGF-1 can activate ERK pathway, which in turn activates other protein kinases and several transcription factors involved in proliferation [245,246]. Interestingly, the activation of the Ras/Raf/ERK pathway was shown to be required for satellite cell proliferation [245].

Transforming growth factor-beta (TGFβ) is a small family of multifunctional cytokines, consisting of TGFβ1, β2 and β3 and myostatin [247]. Accumulating evidence has indicated that they play important roles in skeletal muscle regeneration. TGFβ was revealed to inhibit skeletal muscle satellite cell differentiation in cultured rat satellite cells, and this inhibiting effect of TGFβ

on satellite cells is reversible [221]. Myostatin, a member of TGFβ family, was found to signal satellite cell quiescence and negatively regulates satellite cell self-renewal [248]. In this study, they showed that myostatin-deficient mice have more activated satellite cells compared with wild type. In contrast, addition of myostatin to myofiber explant cultures inhibits satellite cell activation [248]. Further investigation reported that myostatin signals through Pax7 to regulate satellite cell self-renewal [249]. The authors showed that myostatin can increase Pax7 expression level through ERK signaling pathway to help maintaining satellite cell quiescence [249]. In addition, blockage of TGFβ in old, injured muscle restores satellite cell mediated muscle regeneration *in vivo* [250]. Also pharmacological inhibition of myostatin/TGF-β receptor/pSmad3 signaling rescues muscle regenerative responses in mouse model of type 1 diabetes [251]. A gene profiling analysis of *in vivo* quiescent and activated muscle satellite cells revealed that TGFβ is highly expressed in quiescent satellite cells, suggesting it may be more involved in muscle regeneration than previously anticipated [252]. Another TGFβ family member GDF11, sharing 90% homology with myostatin, was recently shown to increase expression with age and inhibits skeletal muscle regeneration [253,254].

Other secreted factors that are also involved in skeletal muscle regeneration including Interleukin-6 (IL-6), Leukemia inhibitor factor (LIF), Tumor necrosis factor-α (TNFα) and Platelet-derived growth factor (PDGF). IL-6 was shown to be an essential regulator of satellite cell-mediated skeletal muscle hypertrophy [255]. The authors found that genetic loss of IL-6 blunts muscle hypertrophy *in vivo*. Also treatment with IL-6 promotes satellite cell proliferation via regulation of the cell-cycle-associated genes cyclin D1 and c-myc and STAT3 activation [255]. LIF was found to enhance regeneration in skeletal muscles after myoblast transplantation by increasing the number of dystrophin expressing fibers [256]. Study found that TNFα expression

was induced in cardiotoxin-injured soleus over the course of regeneration and it regulates muscle regeneration by activating p38 MAPK [257]. A most recent study revealed that PDGF-BB plays a protective role in muscular dystrophies by enhancing muscle regeneration through activation of satellite cell proliferation and migration [258].

Wnt signaling plays critical roles in many biological processes, including cell proliferation, cell fate determination, cell adhesion, cell polarity and morphology [259]. More and more studies have been revealing its implications in skeletal muscle regeneration. Canonical Wnt signaling pathway, also known as the Wnt/β-catenin pathway, was found to induce the myogenic specification of resident CD45(+) adult stem cells during muscle regeneration, suggesting a positive role in muscle regeneration [209]. In agreement with this finding, study also showed that canonical Wnt signalling induces satellite-cell proliferation during adult skeletal muscle regeneration [260]. Wnt/β-catenin pathway was recently revealed to regulate satellite cell myogenic potential through follistatin signalling [261]. In this study, the authors showed that the Wnt/β-catenin pathway induces follistatin expression in myoblasts and promotes myoblast fusion, and ectopic activation of the Wnt/β-catenin pathway *in vivo* promoted premature differentiation during muscle regeneration following injury [261]. In addition, a study using both *in vivo* and in *vitro* approaches found that the Wnt/β-catenin pathway in muscle progenitor cells regulates skeletal muscle regeneration [262]. In this study, they showed that β-catenin deficient myoblasts exhibit delayed differentiation, whereas myoblasts with constitutively active β-catenin undergo precocious growth arrest and differentiation [262]. In contrast, another study reported that increased Wnt signaling during aging converts satellite cells from a myogenic lineage to a fibrogenic one in aged mice, resulting in compromised muscle regeneration capacity. And this lineage conversion can be suppressed by inhibitors of canonical Wnt signalling [263]. Those conflicting findings are possibly

due to the different systems were being used to conduct experiments. Non-canonical Wnt signalling pathway, also known as the Wnt/PCP (planar cell polarization) pathway, is also involved in skeletal muscle regeneration. Activation of Wnt/PCP pathway by Wnt7a was found to drive the symmetric expansion of satellite cells, without any effect on the growth or differentiation of myoblasts, suggesting its role in regulating the muscle regenerative potential possibly via control of the homeostatic level of satellite stem cells [171].

Sphingolipids are a large group of naturally occurring glycolipids. They have important roles as signalling and regulatory molecules in cell proliferation, migration, death, and senescence [264,265]. Increasing evidences revealed that sphingolipids, particularly sphingosine-1-phosphate (S1P), also play important roles in skeletal muscle regeneration. A study has found that entry of muscle satellite cells into the cell cycle requires sphingolipid signaling, and inhibition of sphingolipid signaling reduces the number of satellite cells able to proliferate in response to mitogen stimulation both *in vitro* and *in vivo* [266]. Administration of S1P following myotoxic injury causes a significant increase of the regenerating fibers in both rat and mouse, and administration of anti-S1P antibody attenuates fiber growth [267]. S1P was also found to positively influence satellite cell proliferation and migration, facilitating the recruitment of satellite cells to the site of muscle damage [268]. In addition, S1P was reported to enhance satellite cell activation in dystrophic muscles by repression of cell cycle inhibitors via S1PR2/STAT3-dependent signaling [269]. S1P recently was shown to be involved in mediating epidermal growth factor-induced satellite cell activation, and this activation is S1P receptor dependant [270]. Interestingly, Ras/Raf/ERK pathway was found to be activated by S1P by transactivating epidermal growth factor receptor in rat-2 cells [271]. As mentioned earlier, the activation of the Ras/Raf/ERK pathway is required for satellite cell proliferation [245].

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway is also involved in skeletal muscle regeneration. Inhibition of JAK-STAT signaling was found to stimulate adult satellite cell function, indicated by pharmacological inhibition of Jak2 and Stat3 activity can promote symmetric expansion of satellite cells both *in vitro* and *in vivo* [272]. In agreement with this finding, transient Stat3 inhibition was found to promote satellite cell expansion and enhanced tissue repair in both aged and dystrophic muscle [273]. In contrast, the same study also found that IL-6-activated Stat3 signaling was shown to promote satellite myogenic lineage progression through myogenic differentiation 1 (Myod1) regulation. Conditional depletion of Stat3 in Pax7-expressing satellite cells stimulates satellite cell proliferation, but compromised myogenic differentiation due to inhibition of fusion to regenerating myofibers [273]. A recent study found that loss of Stat3 in MuSCs impairs their proliferation and self-renewal upon injury, and loss of Stat3 in MuSCs of dystrophic mice leads to severe fibrosis and inflammation, which are detrimental to muscle regeneration [274].

p38, a subgroup of the MAPKs, is involved in the regulation of multiple cellular processes, including senescence, apoptosis, cell-cycle arrest, RNA splicing and tumor formation [275,276]. Recent studies revealed that p38 is also involved in skeletal muscle regeneration. p38α/β MAPK was found to function as a molecular switch to activate the quiescent satellite cells [277]. The authors showed that p38α/β MAPK can reversibly regulate the quiescence of muscle satellite cells. Blockage of p38α/β MAPK promotes cell cycle withdrawal and prevents differentiation. Meantime insulating satellite cells from most external stimuli permits them to maintain a quiescent state [277]. Another study showed that genetic knockdown or pharmacological inhibition of p38α promotes Pax7 expression and expansion of satellite cells, and this effect is reversible [278]. Attenuation of p38-mediated miR-1/133 expression by FGF2 was found to facilitate myoblast

proliferation during the early stage of muscle regeneration [279]. Asymmetric activation of p38α/β MAPK by Par complex induces MyoD in only one daughter cell, which commits to myogenesis. While the other daughter cell adopts a quiescent satellite cell phenotype, preserving the stem cell population for future maintenance and repair of skeletal muscle tissue [280]. Polar distribution of the Par complex orients the plane of division and reinforces differential daughter cell fates through downstream signaling pathways [281,282]. Self-renewal and p38 MAPK signaling are found to be impaired in aged satellite cells, and the subcellular localization, duration of signaling, and timing of p38αβ MAPK activation are implicated to be highly involved in satellite asymmetric division self-renewal [283].

Sonic hedgehog (Shh) signalling was considered silent in post-natal life, though it plays crucial roles during embryonic development [284,285]. Increasing evidences revealed that Shh signalling is also involved in adult skeletal muscle regeneration. Shh pathway was found to regulate the cell fate of adult muscle satellite cells in mammals by acting as a proliferation and survival factor [286]. Shh signalling promotes proliferation and differentiation of adult muscle satellite cells, and Shh-induced Akt phosphorylation is required for its promotive effects on muscle satellite cells [287]. Inhibition of Shh signalling was found to reduce the number of myogenic satellite cells at injured site due to insufficient up-regulation of IGF-1 and impaired activation of MRFs, Myf-5 and MyoD [288]. The authors also observed Shh inhibition results in muscle fibrosis, increased inflammatory reaction and compromised motor functional recovery after injury [288]. Recent study also showed that intramuscular therapy with a plasmid encoding the human Shh gene increases the regenerative potential of the injured muscle by promoting satellite cell proliferation. At the molecular level, Shh treatment was shown to increase IGF-1, which will further facilitate muscle regeneration [289].

Other signalling pathways also involved in skeletal muscle regeneration includes the Angiotensin type 2 receptor (AT2R) signaling, Gαi2 signaling, bone morphogenetic protein (BMP) signalling and Notch signalling. AT2R signaling was shown to positively regulate myoblast differentiation and potentiates skeletal muscle regenerative potential via upregulation of phospho-ERK1/2 [290]. Gαi2 signaling was found to be required for skeletal muscle regeneration, satellite cell proliferation and differentiation [291]. Recent study revealed that BMP signalling permits population expansion by preventing premature myogenic differentiation in muscle satellite cells [292]. The involvement of Notch signalling in muscle regeneration will be discussed in detail later.

The extracellular matrix (ECM) is the non-cellular component consisting of collagens, elastin, fibronectin, laminin, and proteoglycans. The ECM provides essential physical scaffolding for the cellular components, also initiates crucial cues that are required for many cellular processes [293,294]. The ECM of skeletal muscle is composed of the interstitial matrix and the basement membrane. Upon injury, the damaged basement membrane hull is left behind where it can function as a scaffold to direct the fusion of newly generated myofiber [295]. ECM protein syndecan-3 and syndecan-4, members of cell-surface transmembrane heparan sulfate proteoglycans (HSPGs) family, specifically mark skeletal muscle satellite cells and are implicated in satellite cell maintenance and muscle regeneration. Inhibition of HSPGs sulfation with chlorate delays satellite cell proliferation due to altered MyoD expression, suggesting syndecan-3 and syndecan-4 sulfation is required for the progression of satellite cell myogenic program [144]. ECM was shown to provide instructive cues to satellite cells and instruct cell behavior during muscle regeneration [296]. Study also found that ECM protein collagen VI is a key component of the satellite cell niche and regulates satellite cell self-renewal and muscle regeneration [297]. This is demonstrated by lack of collagen causes impaired muscle regeneration and reduced satellite cell self-renewal after

injury. Also Collagen VI null muscles display significant decrease of stiffness, which results in compromised activity of wild-type satellite cells both *in vitro* and *in vivo* [297]. In agreement with this finding, it was observed that myotubes differentiate optimally on substrates with tissue-like stiffness, whereas either softer or stiffer gels greatly compromise their differentiation efficiency [298]. Loss of fibronectin in the ECM was found to affect the regenerative capacity of skeletal muscle in aged mice. The authors showed that fibronectin acts as a preferred adhesion substrate for satellite cells, and the loss of satellite cell adhesion to fibronectin in the ECM leads to compromised muscle regeneration [299]. Furthermore, ECM can act as reservoir for growth factors such as HGFs, FGFs, VEGFs, BMPs and TGF-β, which are inactive under normal condition due to binding by the ECM protein vitronectin, fibronectin, collagens and proteoglycans. On the other hand, proteins and proteoglycans of the ECM can also function as distributors of those growth factors during injury induced muscle regeneration. This is accompanied by remodeling of the ECM, which is mediated by metalloproteinases [300].

Notch signalling

Notch is a transmembrane receptor that is present in all animal species studied to date. It regulates local cell-cell communication and mediates cell fate determination [301]. The signalling cascade coordinated by Notch receptor, namely the Notch signaling pathway is highly conserved among organisms [302,303]. It was first discovered in 1917 by geneticist Thomas Hunt Morgan and his colleagues when they noticed notches in the wing blades of fruit flies [304]. Since its discovery, Notch signalling has been shown to play critical roles in many aspects of embryonic development, as well as differentiation, tissue homeostasis and regeneration [303,305].

3.1 Notch signalling ligands

Canonical Notch ligands are characterized by the presence of a DSL (Delta, Serrate, and Lag2) domain in their structure. And this DSL ligand family has been shown to induce the majority of Notch signaling [306,307]. In *Drosophila melanogaster*, there are two Notch DSL ligands, Delta and Serrate. In mammals, there are five Notch DSL ligands the Delta-like family (DLL1, DLL3 and DLL4) and the Jagged family (Jagged 1 and 2) based on homology to Delta and Serrate in *Drosophila melanogaster* [303]. Notch DSL ligands are transmembrane proteins sharing a common modular arrangement in their extracellular domains, which contains varying numbers of Epidermal Growth Factor (EGF)-like repeats and a cysteine-rich N-terminal DSL domain (Figure 9A). The DSL domain is present in all DSL ligands and evolutionarily conserved, and it is required for the interaction between Notch ligands and receptors. Serrate, Jagged 1 and Jagged 2 contain an additional cysteine-rich domain [308].

Unlike the activating canonical ligands that contain a DSL domain, non-canonical Notch ligands do not contain the DSL domain and comprise a group of structurally diverse proteins, including integral and glycosylphosphatidylinositol (GPI)-linked membrane proteins as well as some secreted proteins, which are implicated in the regulation of Notch receptor activity [309].

3.2 Notch signalling receptor

Notch receptors, like Notch ligands, are also transmembrane proteins. Notably, they are large single-pass type I transmembrane proteins. There are one in *Drosophila melanogaster* (Notch), two in *Caenorhabditis elegans* (LIN-12 and GLP-1) and four Notch receptors in mammals (Notch1–4), and they have been conserved from invertebrates to humans [301,310,311]. Notch

receptors are also multi-domain proteins (Figure 9B). The Notch extracellular domain (NECD) is relatively large and consists of 29 to 36 EGF-like repeats, which are subjected to post-translational modification by a variety of glycans and have been implicated in the regulation of Notch function [312]. Earlier study has shown that the EGF-like repeats 11–12 are required and sufficient to mediate the interaction between Notch ligands and receptors [313]. Following the NECD is the negative regulatory region (NRR), which consists of the three-cysteine rich Lin–Notch repeats (LNRs) and a region that links to the transmembrane and intracellular fragment. Study has found that the NRR can block the access of metalloproteinases to the S2 cleavage site of Notch to prevent premature activation of the receptor [302,303,314]. The Notch intracellular domain (NICD) is composed of four distinct regions, the RBPJ-associated molecule (RAM) domain, the ankyrin (ANK) repeats, a transcriptional activation domain (TAD) and a C-terminal Pro-Glu-Ser-Thr (PEST) sequence. Two nuclear localization sequences (NLS) are flanking the ANK repeats. The RAM and ANK domains are essential for the binding of NICD to CSL (CBF1, Suppressor of Hairless, Lag1) in the nucleus [315].

3.3 Nuclear effectors of Notch signalling

CSL (*CBF-1/RBP-Jκ, Su(H), Lag-1*), collectively named after the mammalian, *Drosophila melanogaster*, and *Caenorhabditis elegans* orthologous proteins, respectively, is a DNA binding transcription factor. As the key transducer of the Notch signalling, CSL is required for both activation and repression of Notch target genes. CSL shares similarity with the Rel transcription factor family. In comparison to Rel, CSL has an extra insertion of a central modified β-trefoil domain (BTD) between the two Rel-homology regions (RHR-N, RHR-C) [316] (Figure 9C). The RHR-N and BTD domains are the main domains mediating DNA and CSL interaction. There is

hydrophobic pocket within the BTD domain that is thought to mediate the interaction between CSL and NICD. In addition, beside CSL, the co-activator Mastermind (Mam) is also required to activate transcription. Unlike Notch ligands and receptors, Mam proteins from different species share little sequence homology except an extended α-helical domain formed in the N-terminal region. This extended α-helical domain contacts the RHR-N and RHR-C domains of CSL and the ANK domain of NICD in a trimeric complex [317,318].

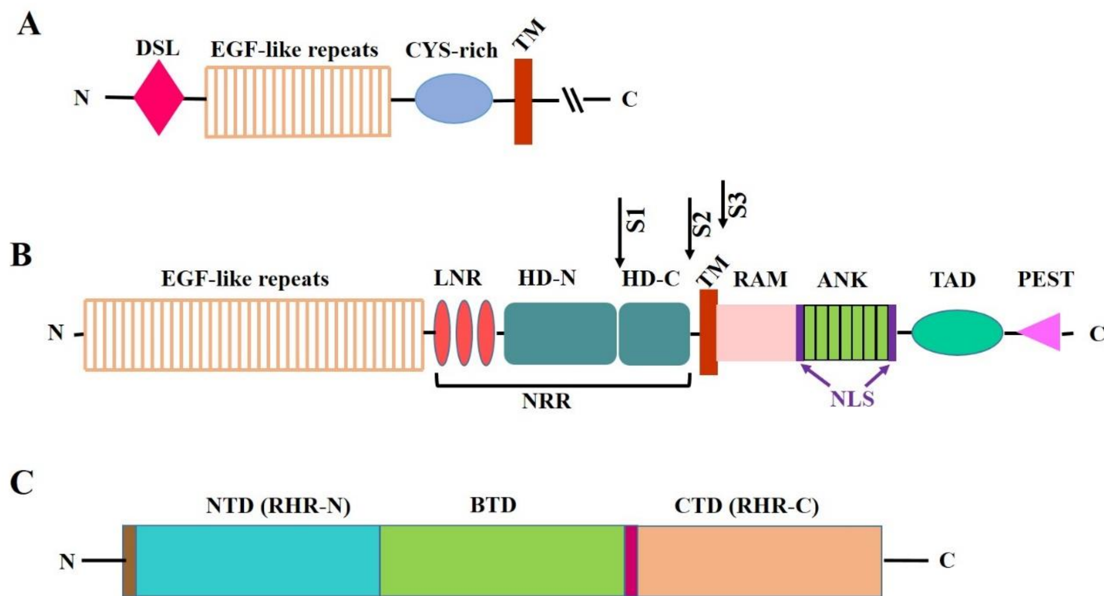


Figure 9: Schematics of Notch pathway components. A: Notch ligand contains DSL (Delta/Serrate/LAG-2), EGF-like repeats and a cysteine-rich (CYS-rich) domain, followed by single transmembrane-spanning segment (TM). **B:** Extracellularly, Notch receptor possesses multiple EGF-like repeats followed by the NRR, which consists of the LNR (Lin12-Notch Repeats) and the HD (Heterodimerization Domain). There is a single transmembrane-spanning segment (TM) following NRR. Intracellularly, Notch receptor contains RAM (RBP-J Associated Molecule) and ANK (ankyrin repeats) domains as well as TAD and PEST (proline, glutamate, serine, and threonine) domains. NLS (Nuclear localization signal) and S1, S2, and S3 cleavage sites are indicated. **C:** CSL (CBF1/RBP-J, Su(H), Lag1) consists of three domains: NTD (amino-terminal domains), BTD (β-trefoil domain), and CTD (carboxy-terminal domains). The NTD and CTD structurally resemble the RHR-N and RHR-C (Rel-homology region) domains, respectively.

3.4 The core of Notch signalling pathway

Notch signaling links the fate of one cell with its neighboring cell through physical interactions between the Notch receptor and the membrane-bound ligands that are differentially expressed in the apposing cell. And this is mediated by a successive proteolytic cleavage events which are described in detail in the following section (Figure 10).

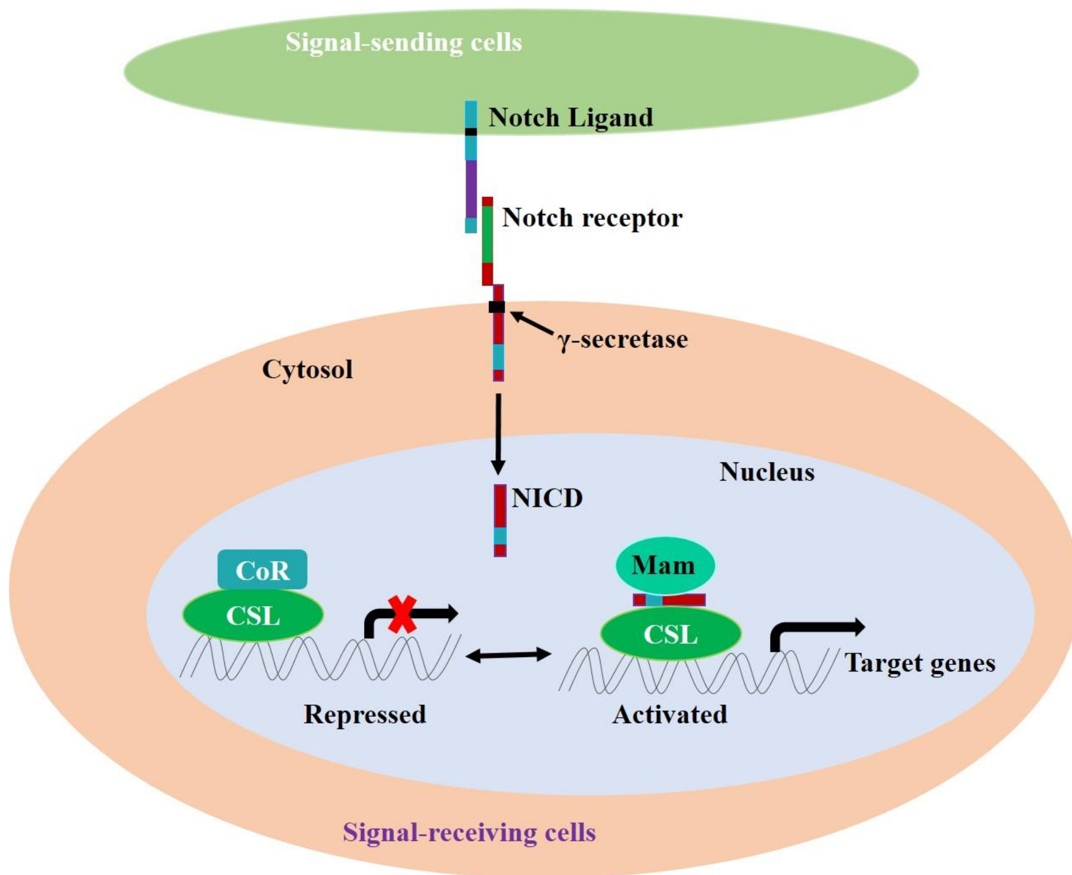


Figure 10: The core Notch signalling pathway. Binding of the Notch ligand on one cell to the Notch receptor on another cell results in two proteolytic cleavages of the receptor, generating a substrate for S3 cleavage by the γ-secretase complex. This proteolytic cleavage results in the release of the Notch intracellular domain (NICD), which translocates to the nucleus and interacts with the DNA-binding CSL (CBF1, Su(H) and LAG-1) protein. The co-activator Mastermind (Mam) is subsequently recruited to the CSL complex, replacing the co-repressors (CoR), and drive Notch target gene expression.

Newly synthesized Notch receptors are proteolytically cleaved in the NECD at the Golgi complex (at site S1) during their transport to the cell surface by a Furin-like protease, generating a functional heterodimeric Notch receptor located to the cell surface [319]. During Notch receptor synthesis and secretion, the NECD undergoes O-linked glycosylation which facilitates the proper folding of the Notch receptor and the interaction with its ligand DSL [312].

Notch signaling is initiated by ligand-receptor direct interaction between signal-sending cell and signal-receiving cell [302,303]. Ligand–receptor binding presumably induces a conformational change within the Notch receptors to expose the S2 cleavage site for proteolysis, which in turn facilitates the second NECD cleavage (at site 2) mediated by a metalloproteinase ADAM. This results in the shedding of most of the receptors external domain, which is trans-endocytosed into the signal-sending cell together with its ligand [320]. After shedding of the extracellular domain, Notch receptor becomes a γ-secretase substrate, which is further cleaved (at site 3) within the Notch receptor transmembrane domain by γ-secretase, a multiprotein complex containing Presenilin [321,322]. This then liberates the NICD from the membrane.

The liberated NICD translocates to the nucleus and heterodimerizes with the DNA binding transcription factor CSL, forming a short-lived nuclear transcription complex. Once bound to CSL, NICD recruits other transcriptional co-activators including Mam proteins (MAML1-3) to drive the expression of Notch target genes, such as members of the Hairy enhancer of split (Hes) or Hairy related (Hey or Hrt) genes [302,303]. In the absence of NICD, CSL forms complexes with a wide range of co-repressors to suppress Notch target genes transcription [302,303].

3.5 Function of Notch signalling

In general, trans-interactions between ligand and receptor that are expressed in apposing cells define activating events, while cis-interactions between ligand and receptor that are expressed in the same cell are inhibitory in nature [323,324]. Almost all tissues are affected by cell fate choices that are regulated by Notch signaling. The exact outcome of Notch signalling is highly context-dependent.

The most popular mode of Notch signalling is lateral inhibition, which describes the mechanisms cells use to adopt one particular cell fate while influencing the fate of its neighbouring cell [325]. Early evidence for this mechanism came from studies in the grasshopper embryo, where laser ablation of an emerging neuroblast caused a neighbouring cell, normally fated to remain epidermal, to instead adopt a neuroblast cell fate [326]. The classical demonstration of lateral inhibition is in the case of neuroblast differentiation within the neurogenic region of the Drosophila embryo. In this case, a cell that is fated to become a neuroblast inhibits its immediate neighbours from adopting the same fate [326,327]. During development, many other positional cues act in concert with lateral inhibition to mediate the specification of different cell types.

Another mode of Notch signaling in cell-fated decision has been documented during the differentiation of the *Drosophila melanogaster* sensory organ precursors (SOP) lineage, where the analysis of the SOP cell lineage gave new insight into the role of Notch signaling in binary cell-fate decisions. In this case Notch helps making lineage decisions by mediating the asymmetrical segregation of specific lineages regulators in the otherwise developmentally equivalent daughter cells, leading to asymmetric activation of Notch signaling, where one of the daughter cells becomes the signal-sending cell, whereas the other cell becomes the signal-receiving cell. Studies have shown that Numb and Neuralized (Neur) E3-ligase are asymmetrically inherited by the two daughter cells and subsequently involved in decision making of the cells, implicating the role of Notch in asymmetrical division [328,329]. The progeny again uses the Notch pathway in a second round of cell division to establish different cell fates [302].

In addition, Notch signaling is also crucial in the specification of borders between cellular fields by inducing intrinsic differences between two adjacent populations of cells, which can translate into distinct signalling populations. Two examples of the intrinsic differences induced by Notch

are restricted expression of ligands and Fringe glycosyl transferases, the later results in modifications to Notch and alters its capability to respond to ligands, together they will contribute to border specification [330–332].

Notch function has its longest history in the central nervous system (CNS) and is well known as a master regulator of neural stem cells (NSCs) and neural development. Early study showed that Notch1 is distributed asymmetrically in proliferating NSCs, with Notchl inherited selectively by one daughter cell that is fated to form new neuron, while the other daughter cell without Notch1 expression remains quiescent and maintains the neuron progenitor pool [333]. Differential expression of mammalian Numb, Numblike and Notch1 was also found in dividing neuron progenitor cells during mouse cortical neurogenesis, suggesting its role in mediating cell-fate decision [334]. Notch signaling is found to be involved in the generation of neurons/glia from NSCs in a stepwise process. At first Notch signalling inhibits the neuronal fate while promoting the glial cell fate of dividing NSCs. However, in the following step, Notch signalling inhibits the differentiation of both neurons and oligodendrocytes, while promotes the differentiation of astrocytes [335]. Recent study also showed that the NSCs that undergo asymmetric cell divisions express Notch1, whereas those that undergo symmetric cell divisions do not, indicating Notch1 expression is involved in initiating the switch between the symmetric to asymmetric cell divisions of NSCs [336]. Furthermore, Notch signalling is also required for neuronal migration in the Cerebral Cortex [337].

In addition, Notch signaling also plays important and complex roles in cell proliferation, cell differentiation and tumor formation, where it can play both positive and negative roles depending on the cellular context. Jagged1-induced Notch signaling was shown to promote the proliferation of multiple myeloma cells [338]. In consistent with this finding, Notch was found to maintain

blastema in a proliferative state and prevent differentiation during adult zebrafish fin regeneration [339]. Interestingly, Notch signalling was found to suppress the proliferation of early chondrogenic cells, surprisingly also inhibits the differentiation of those cells at the same time [340]. While study in keratinocyte found that Notch signaling is a direct determinant of growth arrest and promotes the differentiation keratinocyte [341]. Considering those studies are conducted using different cell types, recent study revealed that Notch signalling can also have different effects on the same cell type. Study in heart regeneration revealed that suppression of Notch signaling negatively affects cardiac regeneration due to insufficient cardiomyocyte proliferation, whereas, hyperactivation of Notch signaling also inhibits cardiomyocyte proliferation and heart regeneration, suggesting the dual role of Notch signalling in regulating of cell proliferation [342]. Notch signalling was first connected to cancer in 1991, TAN-1, the human homolog of the Drosophila notch gene, is found mutated due to chromosomal translocations in T lymphoblastic neoplasms [343]. The association of Notch with tumours is further studied and well characterised in human T cell acute lymphoblastic leukaemia using gain-of-function Notch1 mutations [344]. On the contrast, Notch1 was found to function as a tumor suppressor in mouse skin [345]. The developmental context of a cell appears to play major roles in dictating how activation of Notch signalling will affect the cell fate.

Furthermore, Notch signaling has been shown to be critical for the generation, specification, proliferation, differentiation and maintenance of stem cells in almost every system examined, including the CNS mentioned earlier. Notch signalling mediated by Notch1 but not Notch2 is found to be essential for the generation of hematopoietic stem cells from endothelial cells [346]. In agreement with this finding, study also showed that Notch signalling is crucial for the formation of intra-embryonic hematopoietic cells [347]. Activated Notch signaling in human embryonic stem

cells was found to be required for embryonic lineage commitment [348]. Notch signaling is involved in modulating the proliferation and differentiation of intestinal crypt base columnar (CBC) stem cells. The authors observed that Notch directly targets the CBC cell to maintain stem cell activity. Suppression of Notch impairs CBC stem cell proliferation, leading to rapid CBC cell loss and precocious differentiation of epithelial progenitors into secretory cell types [349]. Notch pathway molecules are revealed to be essential for the maintenance, but not the generation, of mammalian neural stem cells (NSCs). The authors showed NSCs are generated independent of RBP- Jκ, a key molecule in Notch signaling. Inhibition of Notch signaling enhances both neuronal and glial differentiation *in vitro*, and overexpression of Notch1 suppresses the differentiation of both lineages [350]. Notch signalling has been shown to mediate cell fate specification of multipotent intestinal stem cells (ISCs) in *Drosophila melanogaster*. The adult Drosophila midgut contains multipotent ISCs that can generate both enterocytes and enteroendocrine cells. ISCs with high level activated Notch signalling down-regulates the Notch signalling within their daughters and specify these daughters into enterocytes lineage. ISCs with low level or impaired Notch signaling specify their daughters to become enteroendocrine cells, suggesting that ISCs actively coordinate cell production with local tissue requirements to maintain homeostasis [351]. Recent study revealed that Notch1 and Notch2 together mediates intestinal stem cell homeostasis through regulating epithelial cell proliferation, cell fate determination during post-injury regeneration [352].

Notch signalling in skeletal muscle regeneration

As mentioned above, Notch signalling is involved in the regulation of stem cells in almost all systems studied. Stem cells in muscle are no exception. Accumulating evidences have revealed that Notch signaling plays several key roles in skeletal muscle regeneration [353]. Notch signaling

was reported to control satellite cell activation and cell fate determination during muscle regeneration upon injury. The authors found that Notch signalling is activated upon muscle injury, indicated by the upregulation of the Notch ligand Delta and NICD expression. Activation of Notch signalling also promotes the proliferation of satellite cells and the expansion of their progenies that contribute to regeneration. While inhibition of Notch signaling leads to the immediate commitment and differentiation of satellite cells and subsequently impairs muscle regeneration [162]. Interestingly, constitutive Notch activation was found to upregulate Pax7 expression and promotes the self-renewal of skeletal muscle satellite cells by inhibiting MyoD and myogenic differentiation, resulting in compromised muscle regeneration [354]. Earlier studies suggested that NICD can inhibit differentiation by directly interacting with MEF2C and blocking its transcriptional potential to activate myogenic genes [355]. Similarly, the Notch target Hey1 can also prevent differentiation of muscle progenitors by transcriptionally repressing key myogenic genes, including Myogenin and MEF2C [356]. In aged mice, skeletal muscle regeneration upon injury is compromised due to reduced satellite cells proliferation, resulting from insufficient activation of Notch signalling. Though the expression of Notch in satellite cells remains unchanged in aged mice compared with young mice. However, the induction of Notch ligand Delta in myofibers is no longer responsive to muscle injury, and thus diminished activation of Notch in aged muscle, suggesting that Notch signaling is a key determinant of muscle regenerative potential that declines with age [357].

From the findings mentioned above, it is obvious that activation of Notch signalling is crucial for the activation and proliferation of satellite cells. During muscle regeneration, inactivation of Notch signalling in proliferating precursor cells is required for their myogenic commitment and fusion [162]. Numb, a Notch signaling inhibitor that inhibits Notch signaling by inducing ubiquitination of the NICD, was shown to be involved in mediating this process [301]. Numb is

asymmetrically expressed in dividing progenitor cells, the daughter cell expresses Numb is fated to differentiate by activating the expression of Desmin, while the daughter without Numb expression remain proliferative [161,162].

Notch signaling is also involved in the maintenance of cell quiescence in resting muscle. In adult homeostatic muscle, it was found that Notch-1, -2, and -3 as well as high levels of the Notch/RBP-Jκ target genes Hes1 Hey1 and HeyL are expressed in quiescent satellite cells, indicating high level of Notch activity. Notch signaling was shown to be required to maintain the quiescent state of skeletal muscle stem cell. Conditional abrogation of Notch signalling by targeted deletion of RBP-Jκ leads to the spontaneous differentiation of muscle stem cells, thereby resulting in a severe depletion of the stem cell pool, indicating that satellite cells are sustained in a quiescent state by Notch activity [358,359]. In agreement with this notion, double knockout mice of Hey1 and HeyL, was found to have markedly reduced number of satellite cells, which is likely due to failure of satellite cells in remaining quiescent state [360]. Interestingly, in comparison to Notch1 mutants, Notch3-deficient mice have a seemingly opposite phenotype, with an abnormally increased number of quiescent satellite cells and hypertrophic regenerated muscle even after repetitive muscle injuries, indicating an antagonistic function with the other Notch receptors. Overexpression of Notch3 also activated the expression of Nrarp, a negative feedback regulator of Notch signaling, suggesting that Notch3 might act as a Notch1 repressor by activating Nrarp [361].

Cross-talk between Notch and other signalling pathways in skeletal muscle regeneration

Notch signaling also influences skeletal muscle regeneration through cooperation with other signaling pathways.

BMP signaling was shown to induce the proliferation of satellite cells and block their premature differentiation [292]. In this context, BMP signaling promotes Notch-dependent activation of Hes1 and Hey1 to inhibit differentiation of satellite cells, and the inhibitory effect can be restored by blocking Notch signaling [362].

TGF-β expresses at excessive level in old muscle, which leads to unusually high level of pSmad3 expression in satellite cells and subsequently affects their regenerative capacity. In this case, endogenous Notch and pSmad3 were found to antagonize each other in the control of satellite-cell proliferation. This is supported by the observation that activation of Notch blocks the TGF-β-dependent induction of the cyclin-dependent kinase (CDK) inhibitors p15, p16, p21 and p27, whereas inhibition of Notch induces them. In addition, Notch activity determines the binding of pSmad3 to the promoters of these negative regulators of cell-cycle progression in satellite cells [250].

Crosstalk between Notch and Wnt signaling in satellite cells seems to regulate the transition from an undifferentiated to a differentiated state during muscle regeneration. Study has revealed that a temporal switch from Notch to Wnt signaling in muscle stem cells is necessary for muscle regeneration. The authors observed that activation of Wnt signaling in differentiating myoblasts antagonizes Notch signaling and facilitates terminal differentiation during muscle regeneration. This switch is also mediated by glycogen synthase kinase 3β (GSK3β). In addition, inhibition of Notch signaling in regenerating muscle leads to elevated level of Wnt activity and premature differentiation, suggesting the temporal crosstalk between Notch and Wnt is critical for proper proliferative expansion followed by differentiation during muscle regeneration [363].

In summary, although much is known about the role of satellite cells in muscle maintenance and repair. Likewise, there is a huge amount of literature characterizing AP-1 function. However, to

date there are few studies addressing the role of AP-1 in satellite cells. Thus, in this dissertation, a number of studies were carried out to further delineate the role of AP-1 in muscle progenitor cells and satellite stem cells.

Statement of Purpose

Skeletal muscle, as the indispensable motor of body movement, possesses a robust regeneration capability, which is mainly mediated by satellite cells. The identification and use of satellite cells have attracted considerable interest from scientists in the muscle regeneration field. Satellite cells, also known as muscle progenitor cells, possess numerous advantages for adult muscle regeneration study, such as easy identification, self-renewal, and myogenic differentiation potential, yet, to date, the exact mechanisms controlling satellite cell biology during skeletal muscle regeneration are still incomplete. This is mostly due to the fact that each phase of skeletal muscle regeneration is highly regulated by complex signaling pathways. Despite progress made in the past decades, many questions remain unresolved. Further understanding of the signalling pathways and cellular regulators involved in skeletal muscle regeneration at the molecular level may therefore pave the way for therapeutic strategies and clinical applications in contexts in which muscle health is compromised, such as during aging (sarcopenia), myopathic diseases (cachexia, DMD), or inactivity (atrophy).

AP-1 has been documented as a versatile protein that is directly or indirectly involved in a variety of cellular processes. As a ubiquitously expressed transcription factor, it alters target gene expression in response to upstream signals upon its specific binding to its cognate DNA binding element, the TRE. Mutations to components of the AP-1 complex have profound effects on the cell, leading to a plethora of defects during vertebrate development. Previously, AP-1 has been implicated in playing a role in satellite cell biology by our group. These studies established a co-localization of Fra-2/AP-1 and the satellite cell marker Pax7. Also ERK MAPK was shown to phosphorylate Fra-2/AP-1 and stabilizes its activity [5]. However, the precise role of AP-1 in

muscle progenitor cells requires further characterization. ***The studies undertaken here were therefore aimed at further characterizing the role and mechanism of AP-1 function in skeletal muscle progenitor cells.***

In our studies, we used C2C12 mouse myoblast cell line, primary mouse myoblasts and single fiber culture as models to study myogenesis and skeletal muscle regeneration.

The C2C12 cell line is a subclone (produced by H. Blau, *et al*) of the mouse myoblast cell line established by D. Yaffe and O. Saxel from normal adult C3H mouse leg [364,365]. Mononucleated myoblasts when cultured in high serum conditions (10% FBS in DMEM) proliferate rapidly and then can be induced to differentiate when switched into low serum conditions (2% HS in DMEM). In low serum conditions, the majority of proliferating myoblasts withdraw from cell cycle and undergo steps in differentiation. Meantime, a small population escape from differentiation, remaining mononucleated and quiescent. Thus, the C2C12 cell culture effectively recapitulates the myogenic process *ex vivo* and serves as a great model to study myogenesis. These quiescent cells are termed reserve cells and can be identified by their lack of myogenic differentiation markers and enriched expression of cell cycle inhibitors. These characteristics of reserve cells are shared by resident satellite cells, rendering them a great analog to satellite cells and an useful substitute to study satellite cells [366,367].

Primary mouse myoblasts, compared with C2C12 cell line, though maybe more demanding to work with, are more relevant and reflective of the *in vivo* environment [368]. Same as C2C12 cell line, primary mouse myoblasts when cultured also recapitulate the myogenic process *ex vivo*. Primary mouse myoblasts can be purified and grown extensively *in vitro and* serve as a rather decent model to mimic the *in vivo* characteristics of muscle cells. The transplantation of these cells

can be potentially applied in myoblast-mediated gene therapy [368]. After we obtained preliminary data using C2C12 cell line, we validated the results in primary mouse myoblasts.

To better understand the characteristics of satellite cells, we employed a more relevant model system, single fiber culture, which allow for manipulation of satellite cells in a dish [369]. As we mentioned in literature review section, the complex behavior of satellite cells during skeletal muscle regeneration is tightly regulated by a variety of intrinsic factors, as well as extrinsic factors [370,371]. These extrinsic factors constitute satellite cell niche, thus when satellite cells are isolated along with single fibers, their niche remain relatively intact, providing a useful tool for investigating satellite cells.

Materials and Methods

C2C12 cell culture: C2C12 cell line was obtained from American Tissue Culture Collection (ATCC). Cells were maintained in growth media (GM) consisting of 10% foetal calf serum (FBS) in Dulbecco modified eagle's medium (DMEM) (HyClone), supplemented with 2mM L-glutamine (Invitrogen) and 100µg/ml penicillin/streptomycin (Invitrogen), according to ATCC recommendations. Cells were induced to differentiate around 80% confluency by switching into differentiation media (DM) consisting of 2% horse serum in DMEM supplemented with 2mM L-glutamine (Invitrogen) and 100µg/ml penicillin/streptomycin (Invitrogen). Cells were maintained in an incubator at 95% humidity, 5% CO2 and 37°C.

OP9/C2C12 co-culture: C2C12 cells were seeded onto a 10cm plate containing confluent OP9 stromal cells, which are derived from the calvaria of newborn M-CSF-deficient B6C3F1-*op*/*op* mice [372], and cultured in α-MEM medium (GIBCO/Invitrogen) supplemented with 20% FBS and 100µg/ml penicillin/streptomycin (Invitrogen). Co-culture cells were cultured in high serum DMEM medium and were induced to differentiate by switching into low serum medium for up to 4 days.

Primary cell culture: Primary myoblasts were isolated from 0 to 3-day-old C57BL/6 mice using a modified procedure described by Rando and Blau [368]. Briefly, cells were isolated from dissected muscle treated with collagenase/dispase (Sigma-Aldrich), and were pre-plated onto uncoated tissue culture plate for 30 min to remove fibroblasts. This 30min pre-plating was repeated during the following passages until obtaining a pure population of primary myoblast. Cells were maintained in F10 (Gibco) medium supplemented with human basic fibroblast growth factor (b-FGF, Sigma-Aldrich) and 20% FBS on collage-coated dishes (BD Biosciences). Cells were further

expanded and differentiated as described [368]. Myoblasts were never cultured for more than 10 passages after their date of isolation.

Fractionation of Myotubes and Reserve cells: C2C12 cells were induced to differentiate for 96 to 144 h in DM. Media was removed and cells were washed three times with cold 1X PBS (phosphate buffered saline) followed by addition of 1 ml of 0.125% trypsin in 1X PBS. MT contraction were monitored using an Axiovert 25 (Carl Ziess) light microscope. Trypsin was removed upon visual observation of MT contraction followed by addition of 1ml cold 1X PBS. Gentle swirling was applied to dislodge MTs, and they were transferred into a 1.5 ml tube. Residual MT were washed off by addition of cold 1X PBS. Reserve cells (which remained on the plate) were scraped off and transferred into a 1.5 ml tube.

Antibodies and inhibitors: The following antibodies were obtained from Santa Cruz: Fra-2 Q-20 (sc-604), c-Jun (H-79) (sc-1694), MyoD (M-318) (sc-760), ERK1 (C16) (sc- 93), Actin (I- 19) (sc-1616-R), dsRed (C-20) (sc-33354), donkey anti-goat IgG-HRP (sc-2020). The following antibodies were purchased from Cell Signalling: MEK 1/2 (#9122), phospho-MEK 1/2 (#9121), phospho-p44/p42 MAPK (Thr202/Tyr204) (#9106), phospho-Fra-1 (#3880), phospho-c-Jun (#9261). Myogenin (clone F5D) and MyHC (clone MF20) monoclonal antibodies were derived from hybridomas purchased from the Developmental Studies Hybridoma Bank (DSHB). Goat anti-rabbit IgG-HRP (170-6515) and goat anti-mouse IgG-HRP (170-6516) were purchased from BioRad Laboratories. PD98059 (#9900) and UO126 (#9903) was purchased from Cell Signalling.

Protein extraction and Western blot analysis: Cells were lysed and collected using NP-40 lysis buffer (0.5% Nonidet P-40, 50mM Tris-HCl pH 8, 150mM NaCl, 10mM sodium pyrophosphate, 1mM EDTA pH 8, and 0.1M NaF) containing 10μg/ml leupeptin and aprotinin, 5μg/ml pepstatin A, 0.2mM phenylmethylsulfonyl fluoride (PMSF), and 0.5mM sodium orthovanadate. Cytosolic

and nuclear extraction was performed using a NE-PER nuclear protein extraction kit (Thermoscientific, #78833). Protein concentrations were determined by Bradford protein assay (BioRad). Protein extracts were denatured using SDS loading buffer at 95°C for 5 min and then subjected to a 10% SDS-PAGE gel, transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), and blocked in 5% skim milk for 1 h prior to primary antibody incubation. Blots were incubated with the indicated primary antibody in 5% milk in PBS or Tris buffered saline (TBS)-T (10mM Tris-HCl pH 8.0, 150mM NaCl, 0.1% Tween-20) or 5% Bovine serum albumin (BSA) in TBS-T at 4 °C overnight with gentle agitation. Appropriate HRP-conjugated secondary antibody (1:2000) were added for 2 hours at room temperature. Protein was detected with Enhanced Chemiluminescence (ECL) western blotting substrate (Pierce).

Transfections: For siRNA experiments in C2C12, Lipofectamine (Invitrogen) was used according to the manufacturer's instructions. Cells were then harvested at indicated conditions. Primary myoblasts were transiently transfected with siRNA using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions.

Gene silencing: Three siRNAs (Mission® siRNA ID's: SASI_Mm01_00201000, SASI_Mm01_00201002, SASI_Mm01_00201004) targeting mouse Fra-2 were purchased from Sigma-Aldrich. They were reconstituted in nuclease free water (Ambion) and the concentration used was 50nM. Three siRNAs (Mission® siRNA ID's: SASI_Mm01_00046357, SASI_Mm02_00318895, SASI_Mm01_00046359 targeting mouse c-Jun were purchased from Sigma-Aldrich. They were reconstituted in nuclease free water (Ambion) and the concentration used was 100nM. a universal scrambled RNA was used at the equivalent concentration.

Chromatin Immunoprecipitation: Plates were washed briefly with 1X PBS (4°C) to remove media. Cells were incubated with 1% formaldehyde for 15 min at RT after which 0.125M of

glycine was added. Liquid was aspirated and cells were washed three times with 1X PBS (4°C). Cells were scraped in 1 ml of 1X PBS and protease and phosphatase inhibitors (4°C) and centrifuged for 5 min at 5000 rpm at 4°C to pellet cells. Cell pellet was resuspended with 1 ml of Wash buffer I (10mM HEPES pH 6.5, 0.5mM EGTA, 10mM EDTA, 0.25% Triton X-100, protease and phosphatase inhibitors) and incubated on ice for 5 min. Lysate was centrifuged for 5 min at 3000 rpm at 4°C, pelleted nuclei were resuspended in 1 ml of Wash buffer 2 (10mM, HEPES pH 6.5, 0.5mM EGTA, 1mM EDTA, 200mM NaCl, protease and phosphatase inhibitors) and incubated for 10 min on ice followed by centrifugation at 5000 rpm for 5 min at 4. To lyse nuclei, 250µl of SDS lysis buffer (50mM Tris-HCl pH 8.1, 1mM EDTA, 1% SDS, protease and phosphatase inhibitors, prepare fresh) was added. DNA was sonicated to approximately 250-500 bp fragments. Sonicated samples were centrifuged at max speed for 15 min at 4°C to remove insoluble materials. Sheared DNA resolved in supernatant, were collected to a clean tube. Sheared DNA was incubated with 1µg of Fra-2 primary antibody while protein G dynal magnetic beads were blocked using salmon sperm DNA overnight at 4°C. Pre-blocked protein G dynal magnetic beads were added to the IP reaction and incubated for 1h at 4°C. The magnetic beads were washed with low salt wash buffer (20mM Tris pH 8.1, 2mM EDTA, 150mM NaCl, 1% Triton-X 100, 0.1% SDS) followed by washing in high salt buffer (20mM Tris pH 8.1, 2mM EDTA, 500mM NaCl, 1% Triton-X 100, 0.1% SDS. Beads were then washed with a third wash buffer (20mM Tris pH 8.1, 250mM LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA). Protein-DNA complex were eluted from beads by incubating with elution buffer (0.1M $NaHCO_3$, 1% SDS) for 30min at room temperature. Supernatant was transferred to a clean tube and incubated with 5M NaCl overnight at 65°C. Isolated DNA was subjected to purification using the Qiagen PCR clean up kit, as per manufactures instructions.

RNA extraction: RNA extraction was carried out using the RNeasy Plus kit (Qiagen) and Qiashredder (Qiagen) according to manufacturer's instructions. cDNA conversion was carried out using Superscript III (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR: cDNA was diluted 1:10 prior to use. 2.5µl gDNA or cDNA was mixed with SYBR Green (BioRad) and 500nM primers in a final volume of 20µl. Each sample was prepared in triplicates and analyzed using Rotor-Gene Q (Qiagen). Parameters for qRT-PCR: 30s 95°C, [5s 95°C, 30s 60°C] x 40 cycles. Fold change (qRT-PCR) was quantified using the ΔΔCt method. Primers used in qRT-PCR are listed in appendix (Figure S6).

Immunofluorescence: Cells were washed three times with cold 1X PBS and fixed in 4% paraformaldehyde in PBS for 10 min and then permeabilized with 0.3% Triton X-100 in PBS for 5min at room temperature. Cells were blocked in 10% goat serum in 1X PBS for 1 h at room temperature and then incubated overnight with indicated primary antibody diluted (1:500) in 1.5% goat serum in 1X PBS at 4°C. Cells were washed three times with 1X PBS (10 min each) and then incubated with the specified tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) conjugated secondary antibody (Sigma-Aldrich) diluted (1:400) in 1.5% goat serum for 2 h in dark at room temperature, with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining (1:10,000; Sigma-Aldrich) in the last 10min. Cells were washed three times with 1X PBS (10 min each) and then left in 1X PBS at 4°C until imaging. Images were obtained using either a Carl Zeiss Axio Observer.Z1 (Photometrics Evolve 512 EMCCD camera) or LSM 700 AxioObserver microscope. Cells were imaged under 40× (EC Plan-Neofluar; 1.30 numerical aperture [NA] in oil), 63× (Plan-Apochromat; 1.40 NA in oil), and 100× (Alpha Plan-Apochromat; 1.46 NA in oil) objectives, using ZEN image acquisition software. Images were taken as a z-stack and projected orthogonally at maximum intensity.

Single fibre isolation, culture and immunofluorescence analysis: Myofibers were isolated from the extensor digitorum longus (EDL) muscle as described previously [369]. Briefly, the EDLs were removed from adult (6–8 weeks old) male mice with both tendons intact and digested with collagenase type I (2mg/ml in DMEM; Sigma-Aldrich). The muscles were then transferred to horse serum-coated plates, and myofibers were separated by trituration using heat-polished glass Pasteur pipettes. Fibers were incubated for up to 72 h in DMEM supplemented with 20% FBS and 1% chick embryo extract at 37 °C, 5% CO2. For immunocytochemistry, myofibers were fixed in 2% Paraformaldehyde in 1X PBS for 10 min at room temperature and washed three times with 1X PBS. Myofibers were permeabilized in 90% Methanol at -20°C for 6 min and incubated in blocking buffer (5% FBS in 1X PBS containing 0.01% triton X-100) for 30 min at room temperature prior to antibody incubation. Primary antibodies used were: Monoclonal mouse anti-Pax7 and anti-Myogenin (clone F5D) both from (DSHB) 1:1, Fra-2 (SantaCruz Q-20) 1:500 and phosphor-Fra-1 (Cell Signalling Technology #3880) 1:200. All primary antibodies were incubated with fibres overnight at 4°C. Secondary antibody incubation and image procedures are the same as for cell culture, mentioned above in Immunofluorescence.

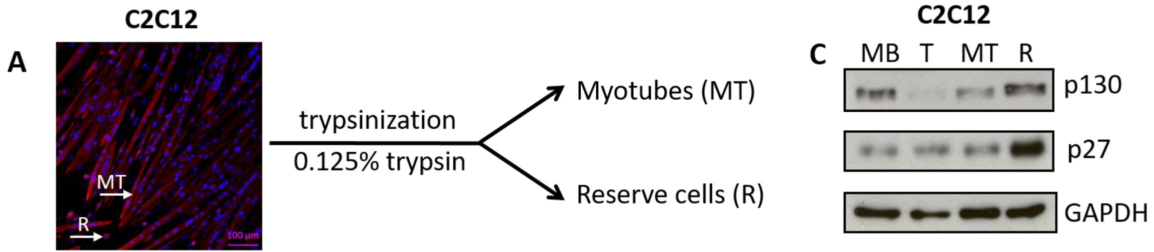
Statistical analysis: All experiments were performed at least three times. Data are presented as means ±SEM. Statistical analysis was done using one-tailed paired student t-test. Differences were considered statistically significant, if $p<0.05$. * is p-value ≤ 0.05, **is p-value ≤ 0.01, *** is p-value ≤ 0.001.

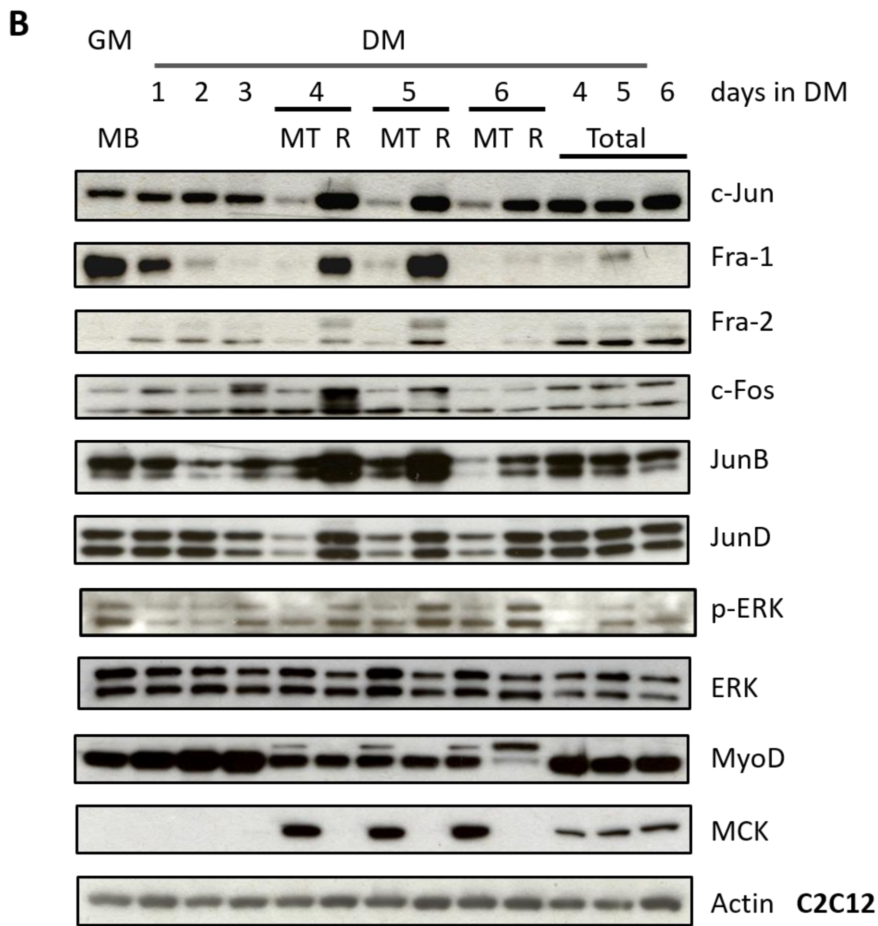
Results

AP-1 expression is maintained in and restricted to myogenic reserve cells during differentiation

Under differentiation conditions, C2C12 myoblasts have a binary fate, contributing to one of two distinct populations: multi-nucleated myotubes (MTs) or mono-nucleated quiescent "reserve" cells (R) that have some properties similar to adult muscle satellite cells (Figure 11A). Those two populations can be separated using mild trypsinization to assess their gene expression profile [373]. Previous studies in our laboratory have documented that AP-1 expression is maintained throughout the differentiation program. Fra-2 is the main Fos related AP-1 subunit expressed under these conditions and its stabilization upon phosphorylation by cytokine-mediated ERK1/2 signaling has been linked to the regulation of reserve cells and also satellite cells in adult muscle [5,116]. In order to better understand the role of AP-1 in muscle progenitor cells during skeletal muscle maintenance, we initially characterized the expression profile of AP-1 components during myogenesis in the reserve and differentiated populations. C2C12 cells were cultured in high serum condition and proliferating myoblasts were harvested when cells reached ~80% confluency. Differentiating C2C12 cells were cultured in low serum condition and harvested at the time points indicated. Western blotting analysis of this differentiation time course was carried out. MyoD and MCK expression were monitored in each immunoblot to assess differentiation. As previously reported, through the course of differentiation in the culture as a whole, the expression of several AP-1 components was maintained with the exception of Fra-1, whose expression diminished in differentiation medium (DM). However, when the reserve and MT populations were separated a different pattern of expression emerged in that we observed that the AP-1 factors were highly enriched in the undifferentiated reserve cell population and virtually absent in the differentiated

myotube population (Figure 11B), suggesting their possible involvement in maintaining the reserve cells in an undifferentiated state. Conversely their downregulation in the MT population correlates with the differentiated myotube phenotype. The undifferentiated state of the reserve cells was further reflected by their mono-nucleated phenotype and lack of differentiation markers such as MCK (Figure 11B) and their quiescent state was indicated by their enriched expression of p130 and p27, both of which are negative cell cycle progression regulators (Figure 11C) [373,374]. We also observed several immuno-reactive bands for Fra-2 in western blotting analysis consistent with our previous observations concerning ERK signaling to Fra-2 (Figure 11B). In addition, co-immunoprecipitation assays were performed at different time points to confirm the dimer composition of AP-1 in C2C12 cells (Figure 11D).





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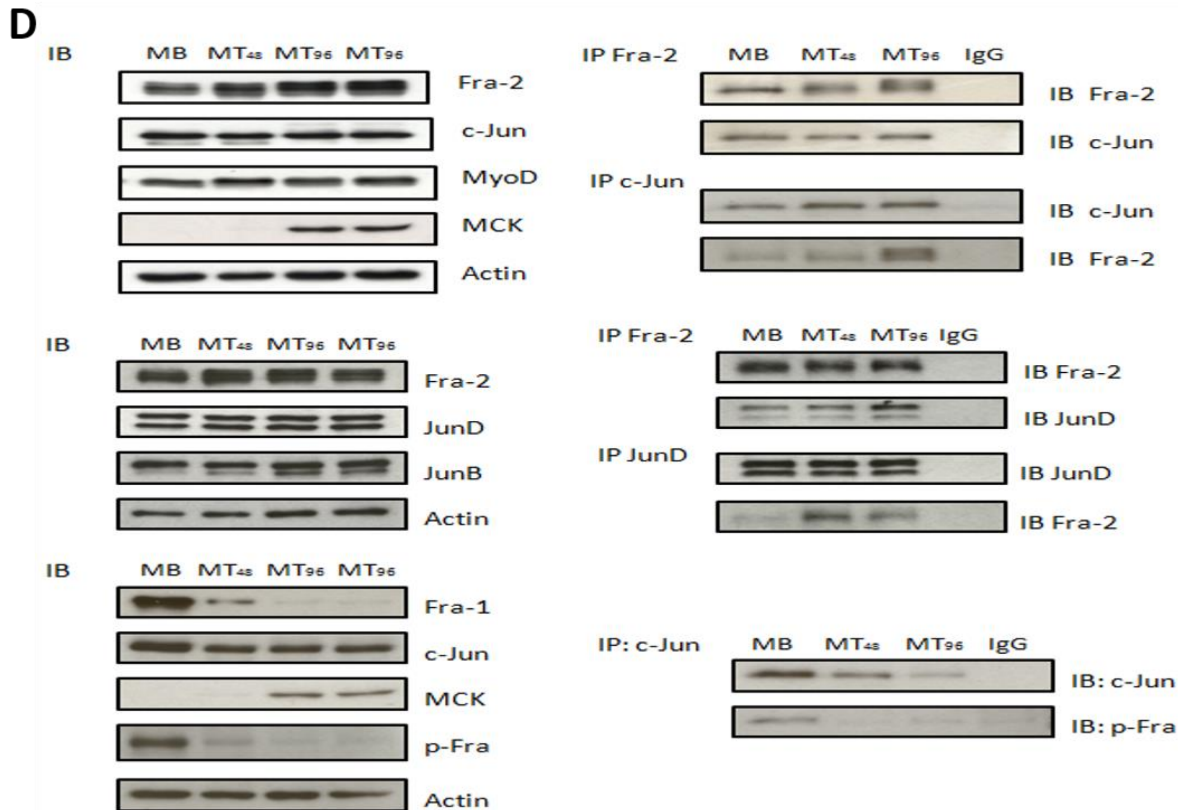


Figure 11. AP-1 expression is restricted to reserve cells. A: A 4-day culture of C2C12 cells were fixed and stained with DAPI (blue, nucleus) and MyHC (Red, MT), a marker of differentiation. Mono-nucleated reserved cells (R) and multi-nucleated myotubes (MT) are indicated by arrows. Cultures are separated into reserve cells (R) and myotubes (MT) using mild trypsinization or unseparated (Total) for subsequent analysis. **B:** Western blotting analysis was performed on the fractionated culture along with proliferating MB in GM and total differentiated culture (T) maintained in DM at the time point indicated to assess AP-1 subunit expression. MCK expression was used to indicate differentiation, Actin was used as loading control. **C:** Western blotting analysis was performed on a fractionated 4-day culture along with proliferating MBs to assess cell progression inhibitor expression. GAPDH was used as loading control. **D:** C2C12 cells were cultured and harvested at the time points indicated. Co-immunoprecipitation assays were performed using Fra-2, c- Jun and JunD antibodies as indicated (immunoprecipitation, IP) to show the interactions between endogenous AP-1 proteins. IB: immunoblot.

Depletion of Fra-2 expression in primary myoblast cultures invokes up-regulation of the cell cycle inhibitor p27

Using a loss of function approach, we next sought to investigate how Fra-2 impacts myogenesis and muscle progenitor cells. Previously we reported that loss of Fra-2 expression alters myogenic differentiation in C2C12 cells [5]. In an attempt to better understand how Fra-2/AP-1 regulates myogenesis, we isolated primary muscle cells from 1-3 days old neonatal mice (Figure 12A, B). We observed that Pax7 and Fra-2 are co-expressed in proliferating primary myoblasts. As cells progress to differentiation as indicated by Myogenin expression, the expression of Pax7, a satellite cell marker, diminished (Figure 12A, B). Depletion of Fra-2 expression was achieved using siRNA technology. In conditions where Fra-2 was depleted, we observed an increase in the cell cycle inhibitor p27 accompanied by an increase in the early differentiation marker Myogenin and late differentiation marker MCK compared to control conditions (Figure 12C). Thus, diminution of Fra-2 expression up-regulated both a cell cycle inhibitor (p27) and enhanced progression to differentiation, which was reflected by an observable increase in the expression of muscle specific differentiation markers (Myogenin and MCK). Interestingly, we observed in earlier experiments (Figure 11C), that p27 expression was enriched in reserve cells, where Fra-2 was also enriched. Collectively, based on these data, we hypothesized a possible role for Fra-2 in maintaining the undifferentiated state in muscle progenitor cells.

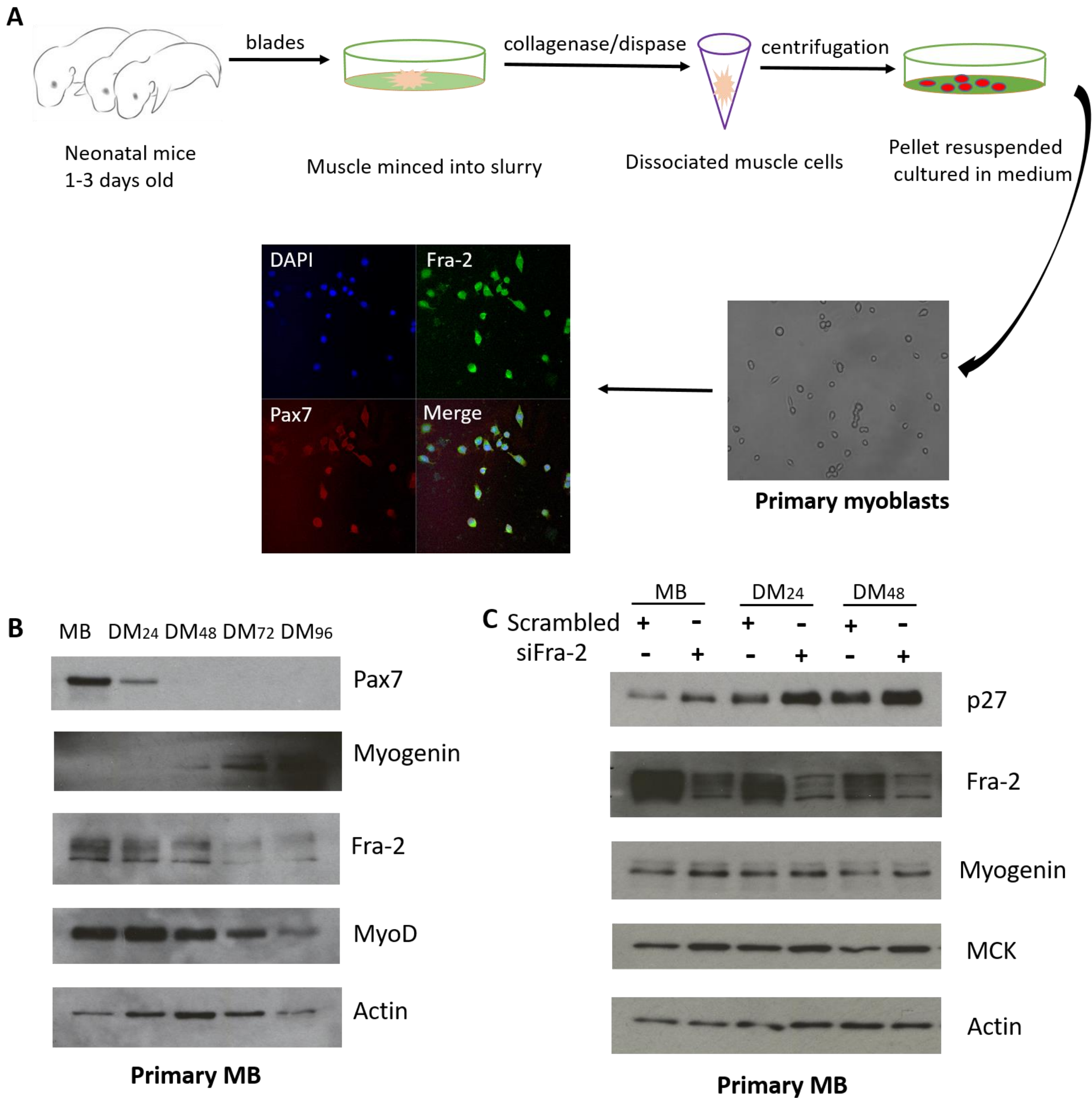


Figure 12. SiRNA depletion of Fra-2 enhances differentiation and up-regulation of cell cycle inhibitor p27. A: (Top panel) Schematic of primary muscle cell isolation and culture. (Bottom left panel) Cells were fixed and stained for Pax7 to indicate cells are primary muscle cells, DAPI indicates nucleus. (Bottom left panel) A 24 h culture of proliferating primary myoblasts under phase contrast microscopy. **B:** Primary muscle cells were harvested at the time indicated. Pax7, Fra-2, Myogenin and MyoD expression level were analyzed using western blotting. **C:** Primary myoblasts were transfected with siRNA targeting Fra-2. Cell cultures were harvested at proliferating MB, DM 24 h and DM 48 h stages respectively. Western blotting analysis was performed to determine Fra-2 levels and p27 expression. Myogenin and MCK were also analyzed to indicate differentiation, Actin was used as a loading control.

AP-1 expression in satellite cells and primary myoblasts

Reserve cells are a cell culture phenomenon and, in some respects are considered analogous to satellite cells due to their commitment to the myogenic lineage, shared mono-nucleated morphology and undifferentiated state. Satellite cells are the primary mediators of muscle regeneration. In view of the expression profile and loss of function data described above, we carried out further experiments to investigate the expression profile and role of Fra-2/AP-1 in satellite cells. To further characterize Fra-2's function in satellite cells, the well characterized single fiber culture model from mouse EDL (Extensor digitorum longus) was utilized. Cultures were made from 6-8 weeks old male C57BL/6 mice (Figure 13A). Immunofluorescence analysis of Fra-2 and Pax7 in single fiber cultures at 48h and 72h was performed (Figure 13B). Fra-2 was expressed in Pax7 positive satellites cells (48h) and not in the differentiated myofiber nuclei. In our earlier experiments we observed Fra-2 co-expressed with Pax7 in primary myoblasts (Figure 12A&B). Taken together, we propose that Fra-2 is a marker of myogenic progenitor cells. Pax7 expression was diminished as cells were differentiating. Expression of Pax7 was maintained in a small fraction of the cells at the 72h time point, indicating possible satellite cell pool renewal. Fra-2 expression was still observed, albeit at lower levels in the majority of satellite cell progeny. To verify this, we also performed immunofluorescence analysis of Fra-2 and Myogenin in single fiber cultures at 24h and 48h. We observed that Fra-2 was expressed in all of the Myogenin positive cells at 24h, while its expression was restricted to a much smaller population at 48h (Figure 13C), which indicates that Fra-2 expression is likely extinguished as the cells enter the differentiation program.

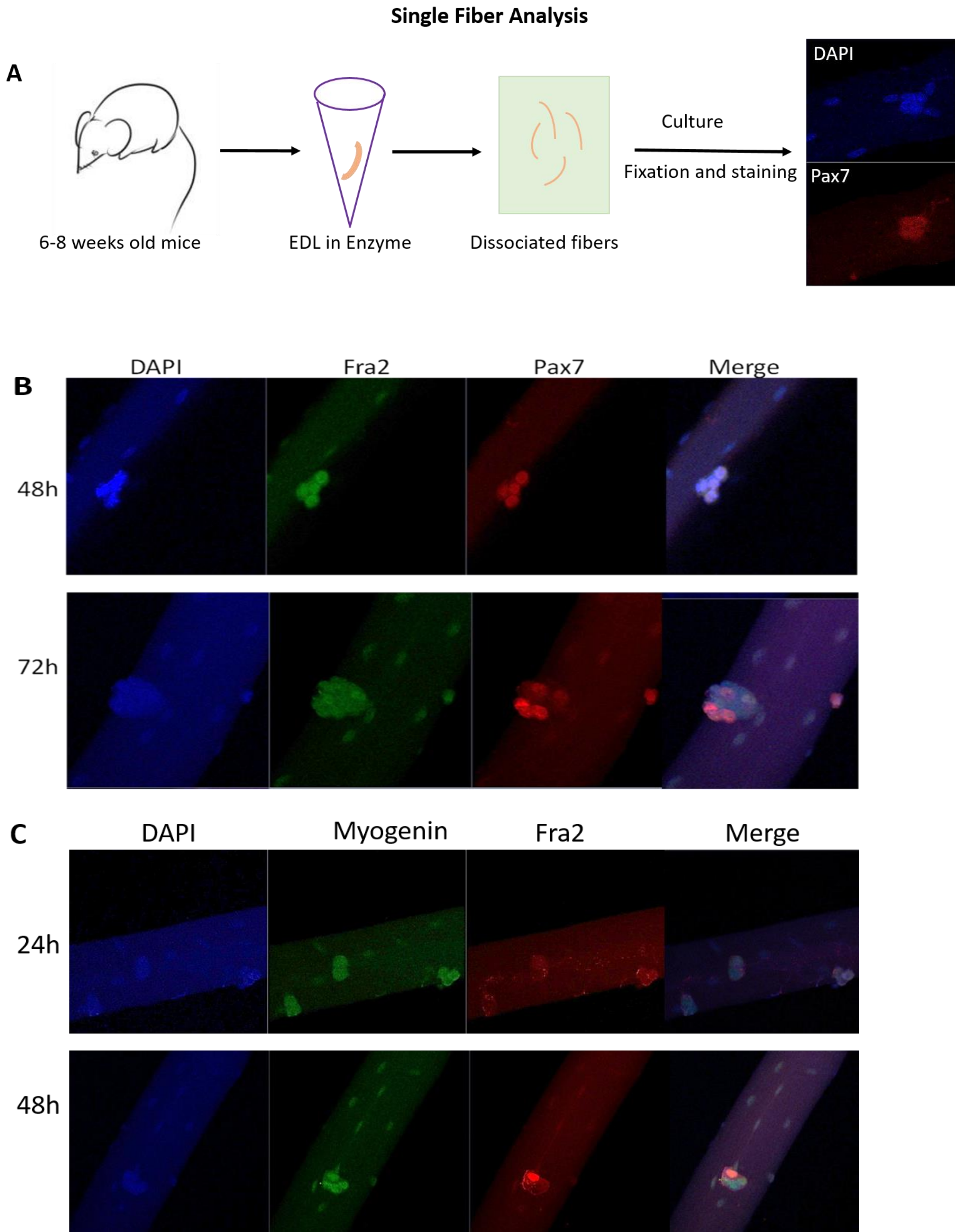
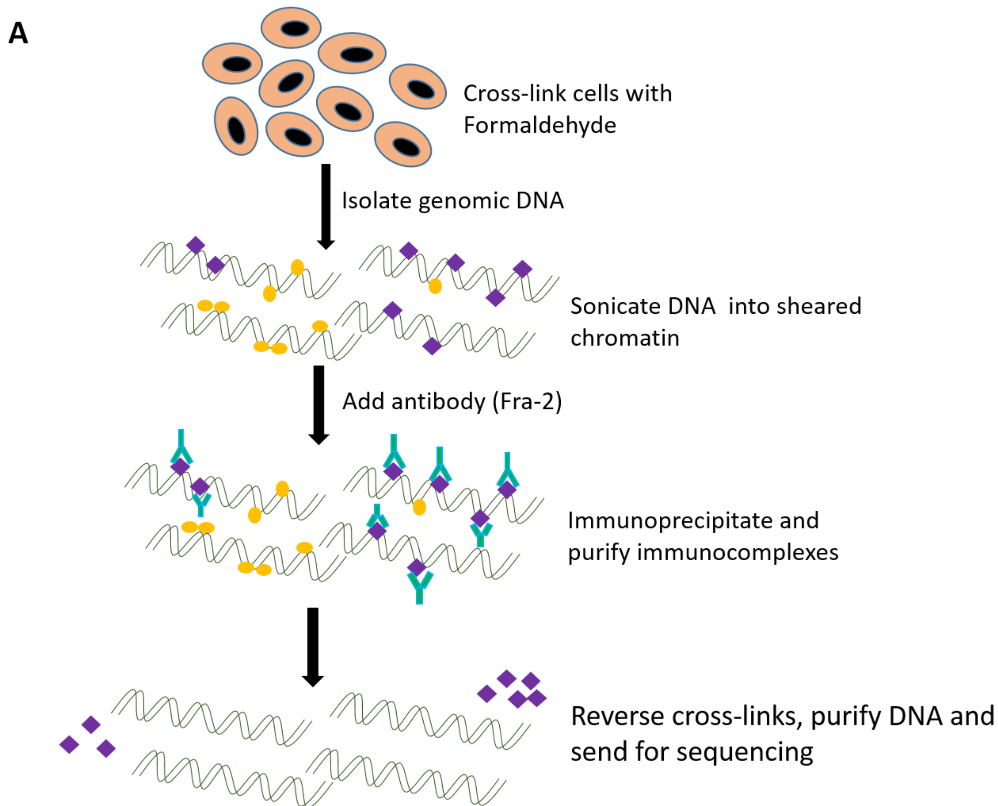


Figure 13. Fra-2 and Pax7 expression in activated satellite cells. A: Schematic of myofiber isolation, culture and staining. Myofibers was fixed at 24h after isolation and stained for Pax7 (red) to indicate satellite cells. DAPI (blue) indicates nucleus. **B:** Myofibers were dissected from EDL muscle of adult mice and cultured in a dish for the indicated

times (48h and 72h). Fibers were immune-stained for Fra-2 (green) and Pax7 (red) at 48h and 72h in culture. Nuclei were stained with DAPI (blue). **C:** Myofibers were dissected from EDL muscle of adult mice and cultured in a dish for the indicated times (24h and 48h). Fibers were immune-stained for Fra-2 (red) and Myogenin (green) at 24h and 48h in culture. Nuclei were stained with DAPI (blue).

ChIP-exo analysis of Fra-2 target genes in skeletal muscle

To address whether Fra-2 is in fact regulating differential sets of genes in proliferating and quiescent muscle progenitor cells, chromatin immunoprecipitation sequencing (ChIP-Seq) analysis was performed in proliferating cultured C2C12 myoblasts (GM, ~70% confluency) and quiescent reserve cells (DM 4d) using a Fra-2-specific antibody or a rabbit IgG control (Figure 14A). The efficiency of chromatin sonication was tested before sending to Peconic Genomics for sequencing (supplementary Figure 7). 716 gene associated binding events were identified in myoblasts, 2060 genes were observed in reserve cells. Nearby genes were identified using Genomic Regions Enrichment of Annotations Tool (GREAT) using the 5 + 1 kb basal promoter with 1 Mb extension rule [375]. Among the gene list, only 20 of them were common to both conditions (Figure 14B). Of interest to us was the identification of *Notch1* in reserve cells and the Notch ligand, *DLL1,* in myoblasts, Notch ligands and receptors have been well established to play a key role in satellite cells [162], suggesting that Fra-2 might play a role in regulating satellite cells by regulating the expression of Notch signaling components. Previous research showed that Notch regulates satellite cell self-renewal through direct transcription of Pax7 [354]. We therefore wanted to test whether Fra-2 is playing a role in myogenic progenitors by contributing to the regulation of competency for Notch signaling. Genes for HGF and wnt7a, two other known proteins which have been shown to play important roles in satellite cell division and activation respectively, were also identified [171,220].



B

	MB	R
Binding sites	716	2060
Gene associations	1164	2717
Within 10kb	136	281

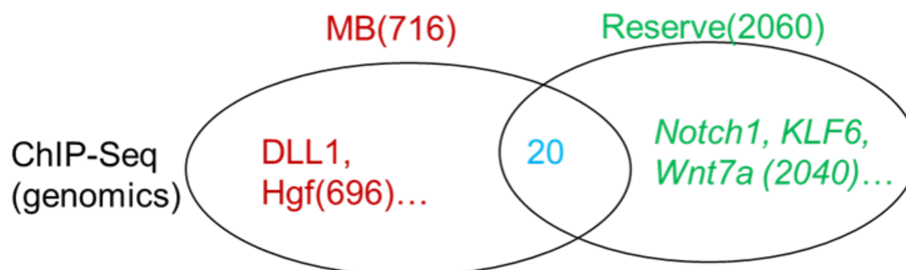


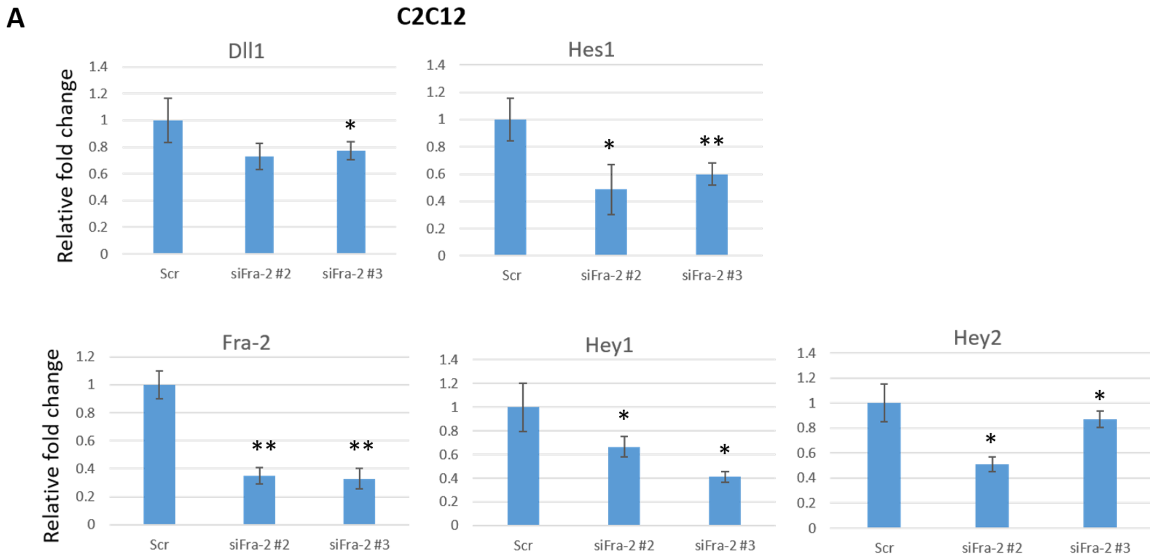
Figure 14. Identification of Fra-2 target genes in C2C12 myoblasts and reserve cells using ChIP-seq. A: Schematic of ChIP-Seq analysis. Proliferation C2C12 (GM) and reserve cells (4d in DM) were collected to identify Fra-2 target genes using ChIP-seq. A non-specific IgG antibody was used as a control. **B:** 716 genes was identified to

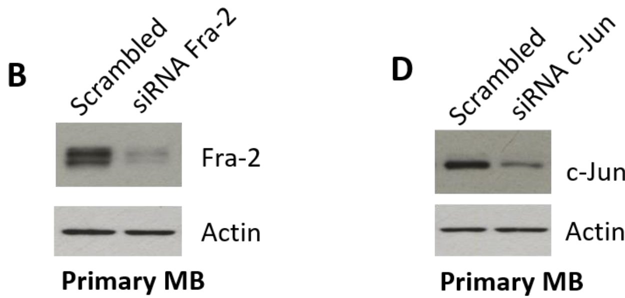
be associated with Fra-2 in myoblast, 2060 genes in reserve cells, among the genes list, with only 20 of them in common and are indicated in Venn diagram.

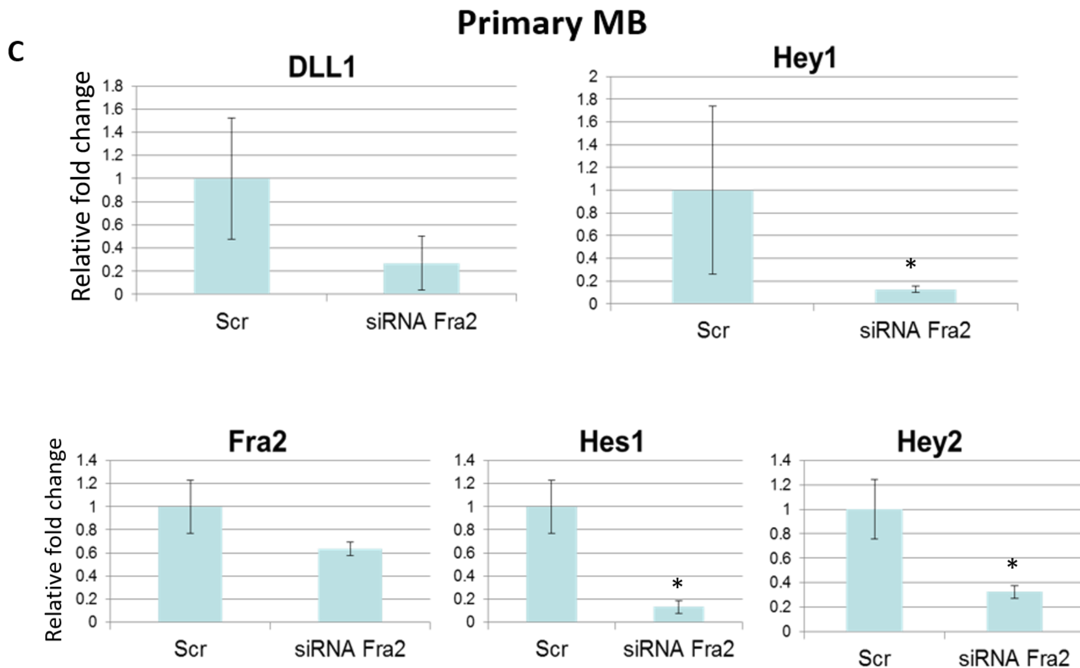
Loss of Fra-2 expression down-regulates Notch ligand and target gene expression

To test whether Notch ligand *DLL1* is regulated by Fra-2, we silenced Fra-2 expression using siRNA technology. Two independent siRNAs targeting Fra-2 were used. Cells were harvested 24h after transfection and followed by RNA isolation using RNeasy Plus Mini Kit. qRT-PCR was performed in triplicates. Notch ligand *DLL1* and Notch downstream target genes *Hes1*, *Hey1* and *Hey2* are down-regulated upon Fra-2 depletion (Figure 15A). To test whether this is also true in primary muscle cells, we transfected freshly isolated primary myoblast with siRNA targeting Fra-2 when cells. Efficiency of Fra-2 knockdown in primary myoblasts was assessed using western blotting analysis (Figure 15B). We observed the same effect on *DLL1* and Notch downstream target gene transcripts upon Fra-2 depletion (Figure 15C). Together, these data indicate that Fra-2 regulates Notch ligand expression and its downstream genes at the mRNA level.

Next, we tested whether Notch ligand and its downstream target genes are also regulated by c-Jun, a dimerization partner of Fra-2. Freshly isolated primary myoblasts were transfected with siRNA targeting c-Jun when cells reached ~50% confluency. Efficiency of c-Jun knockdown in primary myoblasts was assessed using western blotting analysis (Figure 15C). We observed no effect on Notch ligand and its downstream genes upon c-Jun depletion (Figure 15E). In combination with siFra-2 data described above, we propose that *DLL1* is indeed a Fra-2 target gene and losing Fra-2 but not c-Jun could not be compensated.







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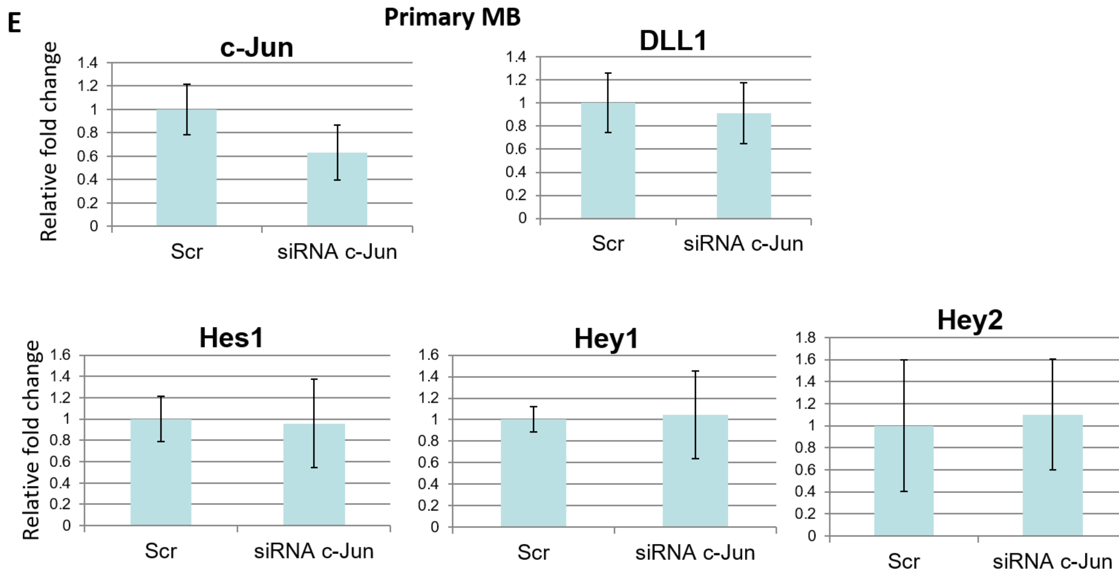


Figure 15. Notch ligand and its target genes are down-regulated at mRNA level upon Fra-2 knockdown, while mRNA levels of these genes are not affected upon c-Jun knockdown. A: C2C12 cells were transfected with a scrambled and two independent siRNAs targeted to Fra-2 for 24h. Total RNAs were isolated, followed by cDNA conversion and qRT-PCR. β-Actin was used as internal control. Results are means ± SEM of 3 independent experiments, performed in triplicates. The asterisk (*) indicates a statistically significant difference ($p<0.05$), when compared with the control cells. **B/D:** Primary myoblasts were transfected with scrambled siRNA, siRNA to Fra-2 or c-Jun and harvested 24h after transfection. Western blotting analysis was performed to indicate Fra-2 and c-Jun depletion efficiency at the protein level. **C&E:** Primary myoblasts were transfected with a scrambled siRNA, siRNA to Fra-2 or c-Jun for 24h. Total RNAs were isolated, followed by cDNA conversion and qRT-PCR. β-Actin was used as internal control. Results are means ± SEM of 3 independent experiments, performed in triplicates. The asterisk (*) indicates a statistically significant difference ($p<0.05$), when compared with the control cells.

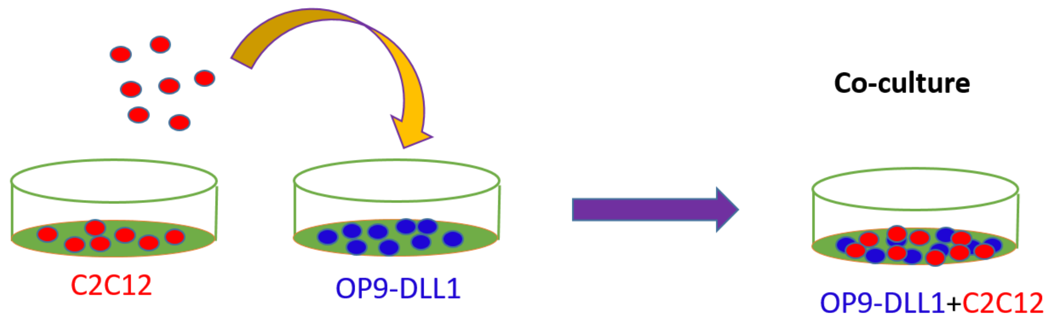
Fra-2 regulates Sbno1, a novel Notch downstream effector

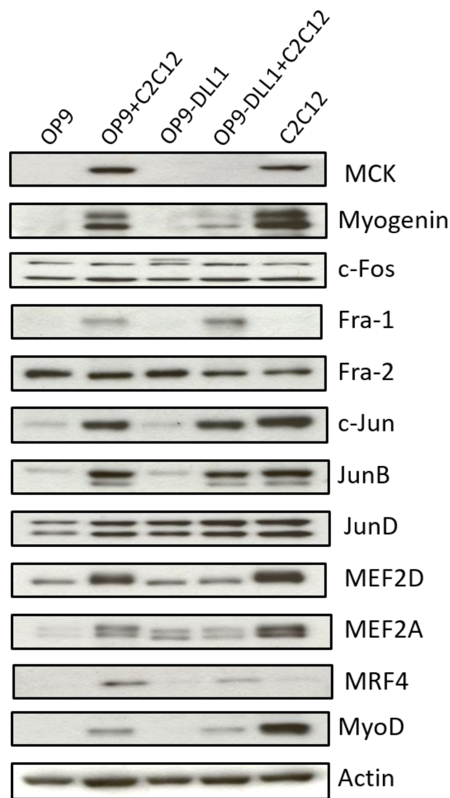
Notch signaling has been documented to inhibit myogenesis and plays a critical role in maintaining the quiescent state of muscle progenitor cells [358,376]. To better understand how Fra-2 regulates Notch signaling during myogenesis and regeneration, we activated Notch signaling using a well-characterized co-culture model, where Notch signaling pathway activation is achieved by co-culture of target cells (in our case muscle cells) with the OP9-Delta cells, a stable cell line expressing Notch ligands (Deltal) [377]. We observed inhibition of differentiation by Notch activation indicated by the absence of early differentiation marker Myogenin and late differentiation marker MCK (Figure 16A). To verify this inhibition was indeed caused by Notch activation, we treated C2C12 cells with a commercially available γ-secretase inhibitor L-685,458 (2μM), which inhibits the final cleavage of the Notch receptor, and thus inhibits Notch intracellular domain release and subsequent signaling. We observed enhancement of differentiation by the inhibitor as indicated by increased expression of MCK (Figure 16B).

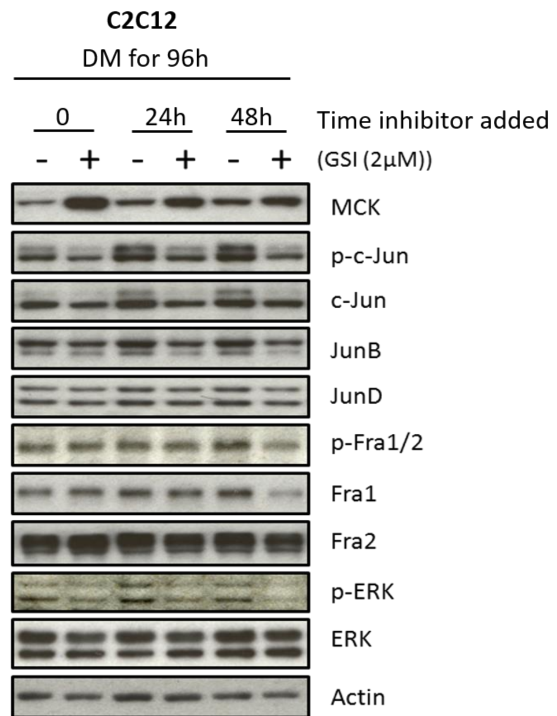
A previous study in our lab identified Sbno1 as a novel downstream effector of Notch signaling (unpublished, in preparation). To test whether exogenous expression of Sbno1 has the same inhibitory effect on differentiation of muscle cells as activated Notch signaling, primary myoblasts were transfected with Sbno1. We observed decreased expression of Myogenin and MCK upon exogenous expression of Sbno1 compared to control, indicating inhibition of differentiation (Figure 16C).

In our earlier experiments, we showed Fra-2 regulates Notch ligand and downstream target gene expression at the mRNA level (Figure 15A&C). To determine whether Sbno1, the novel Notch effector, is also regulated by Fra-2, we transfected freshly isolated primary myoblasts with two independent siRNAs targeting Fra-2. We indeed observed diminished expression of Sbno1,

suggesting that, like the other Notch target genes, Fra-2 expression is required for Notch mediated expression of Sbno1. Interestingly, we also observed increased expression of Numb when Fra2 is depleted, which acts as an antagonist for Notch by causing its selective endocytosis and degradation. Numb localizes asymmetrically and segregates into one daughter cell, where it influences cell fate by repressing signal transduction via the Notch receptor [378].







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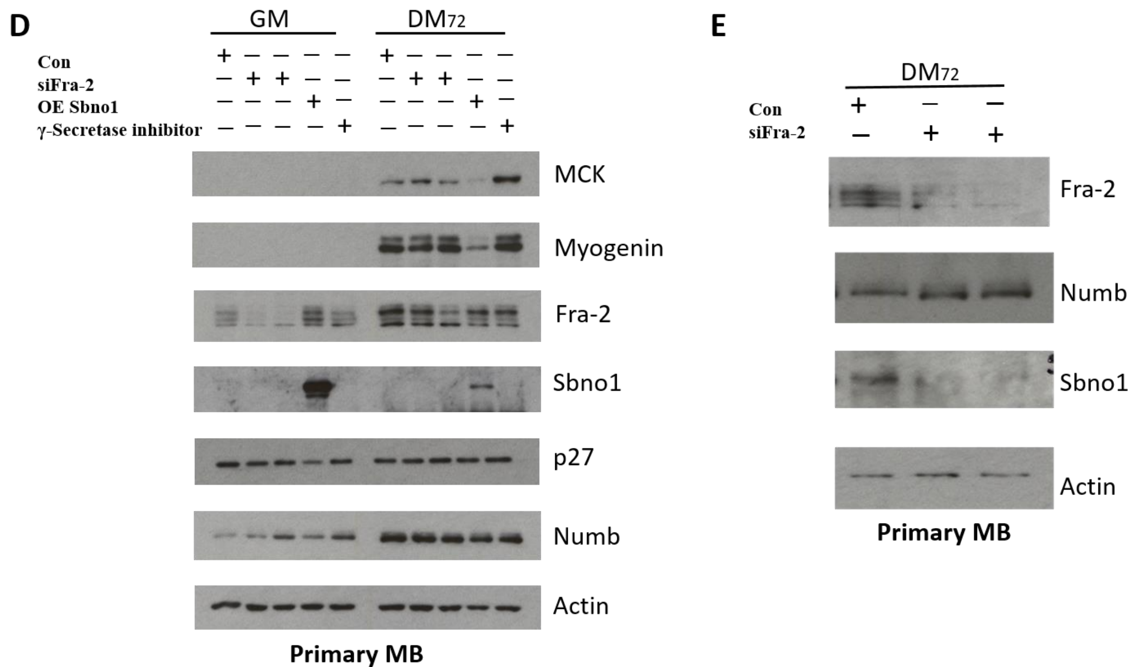


Figure 16. Fra-2 regulates the expression, a novel Notch downstream effector, Sbno1. A: Co-Culture Model System. **B**: C2C12 cells were co-cultured with the control OP9 cells and OP9-Deltal cells initially in GM and induced to differentiate for 96hrs in DM. Total cellular extracts were isolated and equal amount of cell extracts were subject to Western blotting analysis for detection of early and late differentiation markers. Actin was used as loading control. **C:** C2C12 cells were treated with γ-secretase inhibitor. Inhibitor was added at the time indicated. Cells were harvested after 96hrs in DM. Western blotting analysis was performed to analyze the effect. **D:** Primary myoblasts were transfected with Sbno1 or subject to γ-secretase inhibitor treatment. Cells were harvested at the time indicated and cell extracts were subject to Western blotting analysis to monitor differentiation. **E:** Two independent siRNA targeting Fra-2 were transfected in proliferating primary myoblasts. Cells were harvested at the time indicated. Western blot analysis was performed to determine level of Fra-2 knockdown and the effect on Sbno1. Actin was used as a loading control.

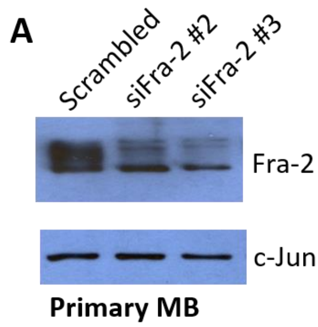
Fra-2, acting as downstream effector of ERK1/2 signaling, regulates Pax7 in muscle progenitor cells

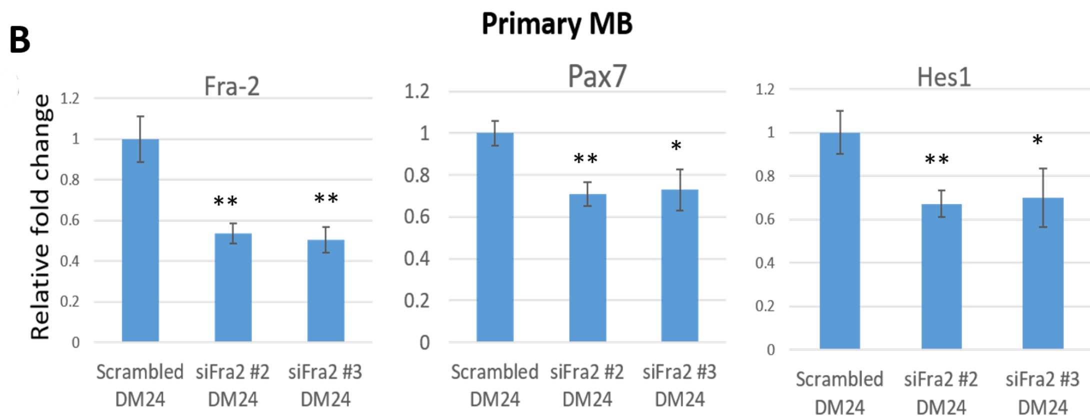
Notch signaling has been shown to actively promote the self-renewal of muscle progenitor cells through direct regulation of Pax7 [354]. Loss of Notch leads to precocious terminal differentiation of satellite cells and satellite cell pool exhaustion, reflected by diminished Pax7 positive cells. In our earlier experiments illustrated in Figure 15C& Figure 16D, we have observed that Fra-2 regulates Notch ligand and downstream target genes at the mRNA level, as well as its downstream effector Sbno1 at the protein level in primary myoblasts. We were interested to know if loss of Fra-2 impacts Pax7 expression in muscle progenitor cells. To address this question, we transfected freshly isolated primary muscle cells with two independent siRNAs targeting Fra-2. Efficiency of Fra-2 knockdown in primary myoblasts was assessed using western blotting analysis (Figure 17A). We observed that loss of Fra-2 results in decreased Pax7 expression at the mRNA level (Figure 17B).

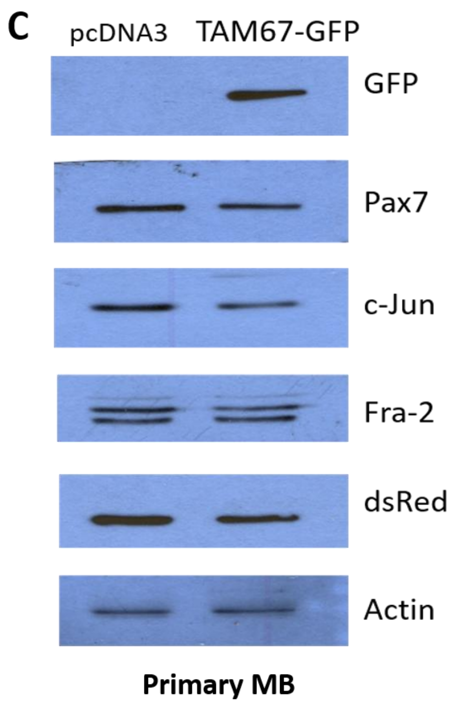
Next we tested whether suppression of Fra-2/AP-1 activity has the same effect as Fra-2 depletion using a dominant-negative c-Jun (TAM67), which is a previously well characterized general trans dominant inhibitor of AP-1 function [379]. Primary cells were transfected with TAM67-GFP and cell lysates were subjected to Western blotting and qRT-PCR analysis. We observed decreased Pax7 expression upon TAM67-GFP overexpression along with enhanced Myogenin expression (Figure 17C&D). This suggests that loss of Pax7 expression upon Fra-2/AP-1 inhibition leads to precocious differentiation of primary muscle cells.

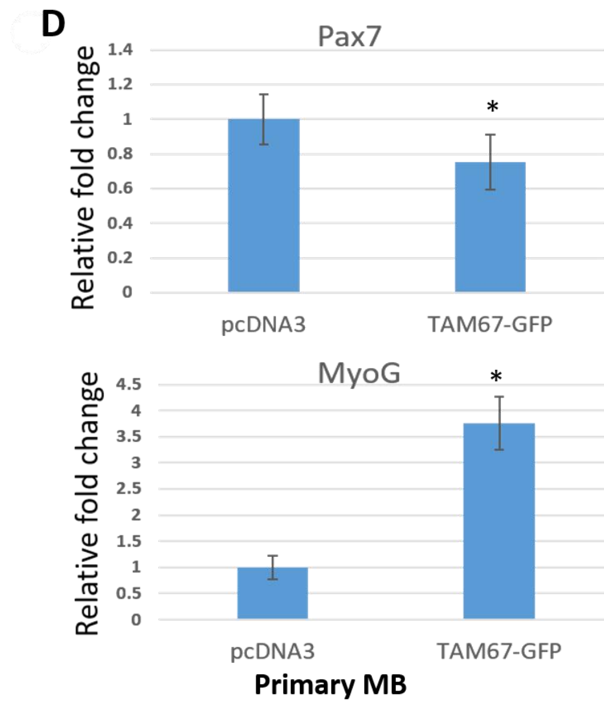
A previous study in our lab documented that Fra-2 is a direct phosphorylation target of ERK1/2, and phosphorylated Fra-2 at S320 and T322 stabilizes Fra-2 protein levels [5]. Also ERK1/2 has been reported to regulate the reversible quiescence of satellite cells by acting

downstream of the receptor for angiopoietin, Tyr kinase TIE2 [380]. As we have observed that Fra-2/AP-1 regulates Pax7 expression as illustrated in Figure 17B&D, we tested whether Fra-2 is one of the downstream effectors of ERK1/2 pathway in muscle progenitor cells. To test whether Fra-2 is regulated by ERK in satellite cells, EDL was isolated from 6-week-old male mice and single fibers were obtained by enzyme dissociation. Single fibers were cultured in growth medium for 24hr, and then subject to PD098059, UO126 (MEK inhibitors), or DMSO treatment. Cells were harvested 24hr after treatment, then analyzed by Western blotting analysis to determine expression levels of Fra-2 and Pax7. Upon ERK1/2 inhibition, both Fra-2 and Pax7 expression levels were decreased in comparison with controls (Figure 17E). This indicates that ERK signaling maintains both Fra-2 and Pax7 expression in muscle progenitor cells.









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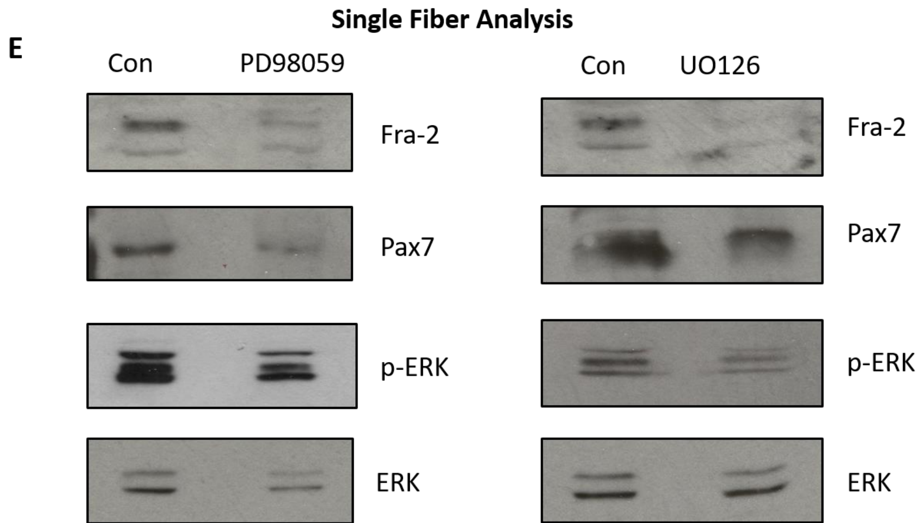

Figure 17. Pax7 is regulated by Fra-2 in muscle progenitor cell. A: Primary myoblasts were transfected with scrambled and siRNA to Fra-2. Cells were harvested 24h after transfection. Western blotting analysis was performed to indicate Fra-2 depletion efficiency at the protein level. **B:** Primary myoblasts were transfected with a scrambled and siRNA to Fra-2. Total RNA was isolated, followed by cDNA conversion and qRT-PCR. β-Actin was used as internal control. Results are means ± SEM of 3 independent experiments, performed in triplicates. The asterisk (*) indicates a statistically significant difference ($p<0.05$), when compared with the control cells. **C:** Primary myoblasts were transfected with pcDNA3 or TAM67-GFP for 24hr. Western blotting analysis was performed to verify exogenous expression at the protein level. **D:** Primary myoblasts were transfected with pcDNA3 or TAM67-GFP for 24hr. RNA was isolated and followed by cDNA conversion and qRT-PCR. β-Actin was used as internal control. Results are means ± SEM of 3 independent experiments, performed in triplicates. The asterisk (*) indicates a statistically significant difference ($p<0.05$), when compared with the control cells. **E:** Two independent inhibitors targeting MEK, PD98059 and UO126, were employed in single fiber culture. Western blotting analysis was performed to determine level of ERK1/2 inhibition and its effect.

Discussion

Extensive literature has documented that AP-1 plays critical roles during embryonic development as well as in tumorigenesis [2,3,381], while the role of AP-1 in muscle is still being unravelled. Previous studies identified that AP-1 also plays a role in skeletal muscle development and implicated its possible involvement in skeletal muscle regeneration [5,116]. In the current work, our data suggest that AP-1 might play a role in determining the fate of satellite cells by modulating the expression of Pax7 via regulation of Notch signalling in skeletal muscle. These data also indicate that the expression of AP-1 is enriched in mono-nucleated "reserve cells" in a differentiated culture indicating a possible role in the maintenance of the undifferentiated state. We also demonstrate that the expression of Fra-2/AP-1 in activated, proliferating and differentiating satellite cells suggests a possible involvement in skeletal muscle regeneration as well as in the re-establishment of the satellite cell population. Notch signalling has been documented as a crucial regulator of skeletal muscle regeneration [357,358,382][162,358,382,383]. Using ChIP-exo analysis, *Dll1*, *Hes1* and *Hey1* were identified as Fra-2/AP-1 downstream targets. Also, these studies document that Fra-2/AP-1 activity, which is regulated by ERK signalling, modulates the expression of Sbno1, a novel downstream effector of Notch signalling in myogenic cells.

AP-1 plays roles in proliferating primary myoblasts and reserve cells

The expression of AP-1 is maintained in cultured skeletal muscle cells with a relatively similar level under proliferating and differentiated conditions [5,116]. The expression of Fra-2 and c-Jun are however restricted to mono-nucleated "reserve cells" under differentiated condition [5].

Consistent with these data, here we show that the expression of AP-1 components are maintained throughout differentiation and are restricted to the minor undifferentiated population under differentiation conditions, indicating their possible role(s) in establishing or maintaining the undifferentiated state of "reserve cells" (Figure 11B). One possibility is that AP-1 plays differential roles in proliferating myoblasts and undifferentiated reserve cells, considering that they are completely different in terms of myogenic status, though both populations have some similarities in that they are mono-nucleated and lack differentiation markers. Compared to proliferating myoblasts, reserve cells possess enriched negative cell cycle progression regulators such as p130 and p27, indicating their quiescent state (Figure 11C). p130 is involved in the determination of reserve cells in differentiating myoblasts and can be used as a quiescent marker [384]. Forced expression of p130 in mouse C2C12 myoblasts blocks cell cycle progression and inhibits the differentiation program accompanied by reduced levels of the muscle-promoting factor MyoD [384]. Proliferating myoblasts receive signals from neighbouring cells which are also proliferating, while "reserve cells" receive signals from surrounding cells that are differentiated or committed to differentiation. Although AP-1 is expressed in both proliferating myoblasts and reserve cells, it is no surprise that AP-1 exerts differential sometimes opposite outcomes when affected by differing signals. Study has shown that AP-1 mediates hypertrophic growth in adult cardiomyocytes upon α-adrenergic stimulation, while the effect of AP-1 may shift from hypertrophy to apoptosis in response to additional activation of SMADs [385,386].

Depletion of Fra-2 in C212 cells enhances the expression of differentiation markers [5]. Our data in primary muscle cells agrees with this finding. Here, we show that p27, a cell cycle inhibitor, is up-regulated upon Fra-2 knockdown (Figure 12C). Together these data suggest that loss of Fra-2 and subsequent increased expression of cell cycle inhibitors such as p27 are critical for myoblasts

to withdraw from proliferation and commit to differentiation. Here we demonstrate that loss of Fra-2 enhances the commitment of myoblasts to the differentiation program and enrichment of Fra-2 contributes to the establishment and maintenance of the reserve cell population. Enhanced differentiation due to loss of Fra-2 expression is also observed in adipocytes [95], while Fra-2 deficient osteoblasts display a differentiation defect [53].

Our data also indicate that the establishment of reserve cells is more of an actively regulated process instead of a passive one. It is achieved partially via repression of proliferation through up-regulation of cell cycle progression regulators and inhibition of differentiation indicated by the absence of differentiation markers (Figure 11B&C). According to Fra-2/AP-1 ChIP-exo data, almost triple times of genes are modulated by Fa-2/AP-1 in reserve cells compared with in proliferating myoblasts (Figure 14B).

Fra-2/AP-1 plays a possible role in skeletal muscle regeneration

In our experiments employing the use of the single fiber culture model, we observed that Fra-2 expression weakens as activated satellite cells proliferate and differentiate, while Pax7 expression is maintained in a small fraction of the cells (Figure 13B). The expression of Pax7 in cells in direct contact with the fiber or in close proximity lose Pax7 expression, concomitant with reduced expression of Fra-2, while cells that are more distal from the fiber maintain their expression of Pax7 and Fra-2, thus indicating that signals from the myofiber may be involved in determining the fate of proliferating satellite cells. These observations are in agreement with the studies proposing asymmetric division of satellite cells [160].

Interestingly, we also observed that when all dividing activated satellite cells are in direct contact with the myofiber, which indicates symmetric division of satellite cells, Fra-2 expression is confined to a minority of proliferating satellite cells as some cells are committed to differentiation and start to express the differentiation marker Myogenin. Spatially, Fra-2^{+}MyoG^{+} satellite cells appear to separate from Fra-2^{-}MyoG^{+} cells as well as the myofiber (Figure 13C), supporting this idea that cell-cell communication among proliferating satellite cells also plays a critical role in cell fate decisions [371].

This expression pattern of Fra-2 in adult muscle progenitor cells suggests its possible involvement during skeletal muscle regeneration, especially in satellite cell fate decisions and possibly in the maintenance of the satellite cell pool.

Fra-2/AP-1 regulates cell fate in myogenic cells through Notch signalling

In cultured cells, how clonally equivalent cells assume different fates is still not very clear. Lateral inhibition imposed by Notch has been shown to be involved in this process in several systems but, so far, not in muscle [326,387,388]. Here we show that activated Notch signalling in cultured C2C12 cells inhibits differentiation indicated by decreased MCK expression, and this effect can be rescued by Notch inhibition (Figure16A&B). We also identify that the Notch ligand *Dll1* and receptor *Notch1* as Fra-2/AP-1 downstream target genes, which are selectively expressed on cells that will later assume different cell fates (Figure 14B). Here we show that depletion of Fra-2 down-regulates Hes1 and Pax7 gene expression in cultured primary myoblasts (Figure 17B). Inhibition of AP-1 using TAM67 also results in a decrease of Pax7 expression and an increase in Myogenin expression consistent with Fra-2 depletion data (Figure 17D). These data suggest that

loss of AP-1 leads to cellular differentiation by supressing Notch signalling and subsequently Pax7 expression. Collectively, these data implicate Fra-2 in the regulation of cell fate by Notch signalling in myogenic cells.

Notch signalling has been extensively investigated in skeletal muscle, revealing both direct and indirect effects, as well as contextual roles in regulating satellite cells and also niche establishment during skeletal muscle regeneration [350,354,389–391]. Most recently, Notch has been documented to activate distinct targets through the same Notch receptor by discriminating between ligands [392]. Ectopic expression of Dll1 was shown to promote myogenic differentiation, while ectopic expression of Dll4 inhibits it in chick neural crest [392] . Evidence reported here indicates that depletion of Fra-2 results in the down-regulation of Dll1 and its downstream genes both in cultured C2C12 cells and primary myoblasts (Figure 15A&C), while this effect is not observed upon c-Jun depletion (Figure 15E). Our previous studies documented how the variation in AP-1 complex composition can affect its function [116]. We conclude that Notch regulation by AP-1 during skeletal muscle regeneration depends primarily on Fra-2 function. Interestingly, c-Jun was reported to be critical for the exit of satellite cells from quiescence due to it being a key transcriptional target of the PI3K/mTORC1 signaling axis [393].

Besides regulating the Notch pathway by targeting its ligand/receptor and downstream genes, Fra-2/AP-1 can also affect Notch signalling by modulating its downstream effector, Sbno1 (unpublished data) at the protein level in primary culture (Figure 16D). We also report that loss of Fra-2 results in enhanced expression of Numb, a Notch inhibitor, which functions to segregate cells asymmetrically, dictate the outcomes of Notch and function as a cell fate determinant [394,395]. We conclude that Fra-2/AP-1 functions upstream of Notch signalling and determines

whether cells are quiescent, proliferative or assume a differentiated cell fate during muscle regeneration.

ERK 1/2 acts upstream of Fra-2/AP-1 in satellite cells

Previous work showed that Fra-2 is a primary ERK substrate in response to cytokine signalling in cultured C2C12 cells [5]. ERK1/2 signalling has also been reported to play a complex bi-phasic role in myogenesis [396]. Later study revealed that the localization of ERK1/2 dictates its function, in which case nuclear p-ERK promotes proliferation by preventing cell cycle exit while its cytoplasmic counterpart promotes differentiation [397]. In addition, ERK signalling is involved in maintaining the undifferentiated state in skeletal myoblasts by acting down stream of MEK1/2 activated by CT-1 [398]. Based on these reports, it is reasonable to propose that Fra-2/AP-1 may play a complex role in myogenic cells by acting as a direct substrate of ERK signalling. This bi-phasic role of ERK may explain what we mentioned earlier that Fra-2 plays differential roles in proliferating myoblasts and reserve cells.

To determine whether Fra-2 is regulated by ERK and its possible outcomes in satellite cells, we used two MAP kinase inhibitors to block ERK1/2 signalling in the cultured single fiber model. We document decreased levels of Fra-2 and Pax7 expression upon ERK inhibition (Figure 17E), indicating that Fra-2 is likely acting as a downstream effector of ERK1/2 in satellite cells since Fra2 is not expressed in the differentiated myofibers in these conditions, possibly influencing the fate of satellite cells via modulation of Pax7 expression. ERK signaling was shown to regulate reversible quiescence of the Pax7 satellite cell pool during muscle regeneration by acting downstream of Sprouty1 (*Spry1*), an inhibitor of receptor tyrosine kinase signalling [399,400].

Persistent activation of ERK signalling during adult muscle repair impairs the self-renewal of a subset of satellite cells [399]. ERK2 expression is specifically induced and activated by phosphorylation upon muscle injury, suggesting a key role in muscle regeneration [235]. This induced activation of ERK signaling upon injury is transient in mammals, while sustained ERK activation is found in salamander myotube regeneration [401]. Similarly, sustained ERK activation was also reported to impair nerve repair in adult Schwann cells [402]. The extent and pattern of ERK activation could therefore underlie the differences in regenerative competence between species and amongst tissues. In addition, ERK signalling is shown to be essential for zebrafish cardiac regeneration and induced expression of a dominant-negative form of MEK1 inhibits this process [403].

We therefore propose that ERK signalling might play a universal role in terms of regulating regeneration, and Fra-2/AP-1 as a ubiquitous downstream effector of this pathway maintains its expression in myogenic progenitor cells and may regulate the process of regeneration by modulation of Notch signalling, a known regulator a skeletal muscle development and regeneration.

Summary and future directions

AP-1 is ubiquitously expressed in many tissue types but also has been shown to exert tissue specific functions, which may be due to due to its complex and dynamic dimer composition. In skeletal muscle, dimer composition shifts from Fra-2:c-Jun to Fra-2:JunD when cells are starting to differentiate [116], indicating that each dimer combination may have a unique role in skeletal muscle.

In experiments described here, it is documented that all AP-1 compositions are enriched in reserve cells in a fully differentiated myogenic culture, implying that AP-1 may play a role in the establishment of the reserve cell pool during skeletal muscle differentiation. In these data, Fra-2/AP-1 is indicated to play a critical role in myogenic cells by modulating Pax7 expression through Notch activity. Notch function is, itself regulated by AP-1 modulation of its ligand (DLL1), receptor (Notch1) and subsequent downstream targets (Hes1/Hey1/Sbno1). Notch ligand and receptor are asymmetrically expressed during cell division, which is crucial for cell fate specification. Fra-2/AP-1 can further modulate Notch activity through inhibiting Numb, a Notch inhibitor, which is also asymmetrically expressed during cell division, allowing for differential cell fate. Loss of function using siRNA technology indicates that the effect of AP-1 on Notch signaling in myogenic progenitor cells is Fra-2 specific. Further investigation using pharmacological MEK/ERK pathway inhibitors UO126 and PD98059 indicate that ERK1/2 pathway is involved in AP-1 modulation in myogenic cells. We propose that Fra-2/AP-1 plays an important role in determining the fate of myogenic cells during proliferation, differentiation and satellite cell pool establishment and maintenance.

In summary, we provide novel insights into the role of Fra-2/AP-1 in myogenic progenitor cells, implicating Fra-2/AP-1 in the maintenance of the skeletal muscle stem cell quiescent phenotype and re-establishment of skeletal muscle stem cell pool. The data contained herein allow the following model to be proposed in which Pax7 expression in satellite cells is modulated by AP-1/Fra-2 through its effect on the competency for Notch signaling (Figure 18). Further experiments are needed to confirm this model.

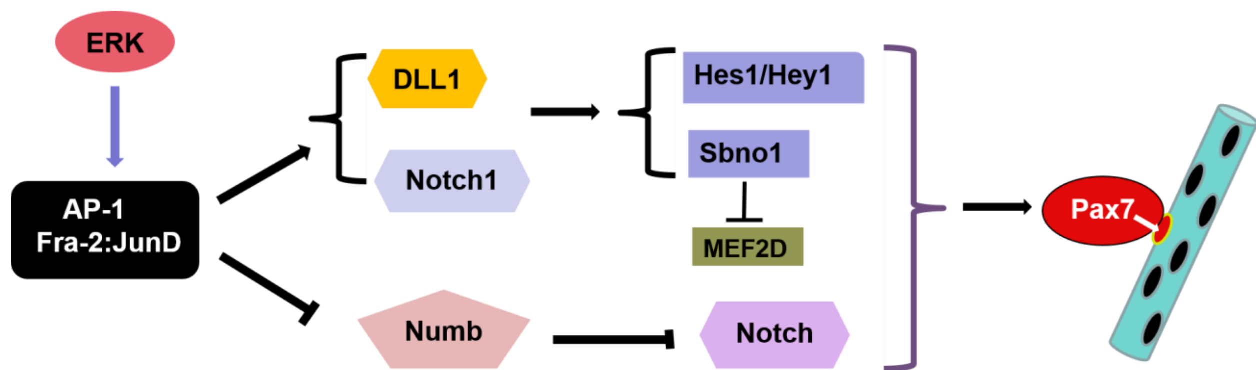

Figure 18. Proposed model for the regulation of myogenic progenitor cells by Fra-2. AP-1/Fra-2 regulates muscle progenitor cells by influencing competence for Notch signaling. This in turn influences the expression of Notch downstream effector genes and ultimately Pax7 expression.

These findings have important ramifications to the field of skeletal muscle regeneration and potentially in our understanding of the progression of skeletal muscle diseases, such as muscular dystrophy, muscle atrophy and muscle weakness. This knowledge may uncover potentially new therapeutic targets and alternatives for manipulation of skeletal muscle regeneration postnatally.

The data described here has been performed on C2C12 myoblasts, primary mouse myoblasts and single fiber cultures (*ex vivo*). Using cultured primary myoblasts and single fiber culture has several advantages over using immortalized cell lines (such as C2C12). However, removal of satellite cells from the *in vivo* environment may also alter phenotypic and differentiation properties

as a result of isolation from the surrounding cells and structures. Satellite cells have been documented to be greatly affected by their niche and surrounding ECM and this was why the single fiber analysis was carried out in which the niche and ultrastructure of the muscle fiber is maintained.

To further the physiological relevance of this work will require progression to an *in vivo* setting to further investigate the role of AP-1 in skeletal muscle regeneration. Blockade of Fra-2/AP-1 by delivery of upstream signaling pathway inhibitors *in vivo* could provide new evidence concerning whether Fra-2/AP-1are important in preventing muscle wasting diseases. Using geriatric mice to study the expression of AP-1 and its effect on muscle will also provide new insights into the role of AP-1 in myogenic progenitor cells and skeletal muscle regeneration. Gene (target genes of AP-1) and protein (co-factors of AP-1) profiling in young, adult and geriatric mice, or healthy and diseased mice will further our understanding the role of AP-1 in myogenic cells at the molecular and cellular levels, providing potentially new strategies to treat muscle diseases more effectively.

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Appendices

Appendix A: Supplementary figures

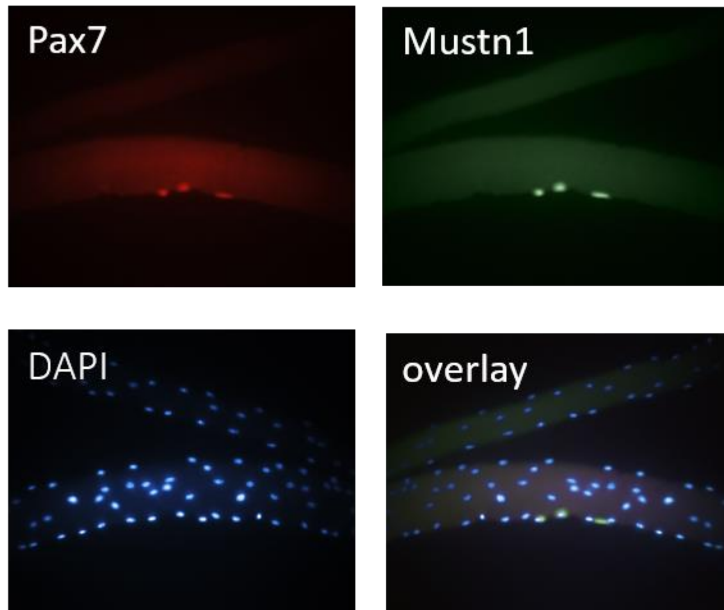

Figure S1: Mustn1 and Pax7 are co-expressed in satellite cells. Myofibers were dissected from EDL muscle of adult mice and followed by fixation. Fibers were immune-stained for Mustn1 (green) and Pax7 (red). Nuclei were stained with DAPI (blue). *Mustn1* gene contains MyoD binding sites and is enriched for AP-1 sites.

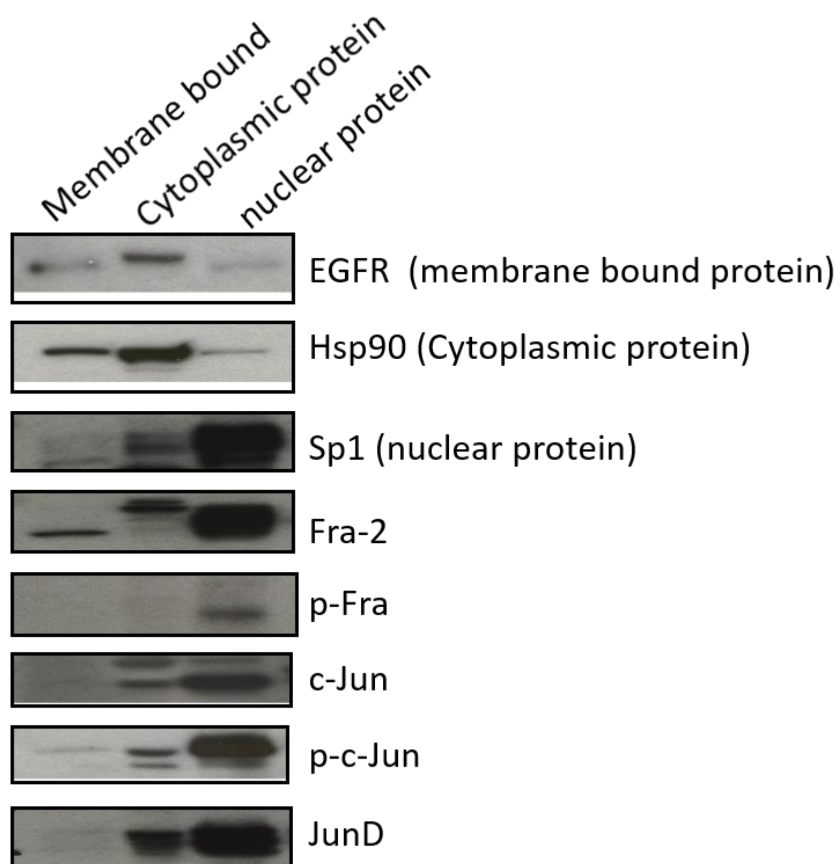

Figure S2: AP-1 subcellular location in C2C12 cells. C2C12 cells were cultured and harvested at proliferating condition. Subcellular fractionation was performed using subcellular protein fractionation kit (Thermo Fisher)**.** EGFR, Hsp90 and Sp1 were probed to indicate membrane bound protein, cytoplasmic protein and nuclear protein respectively.

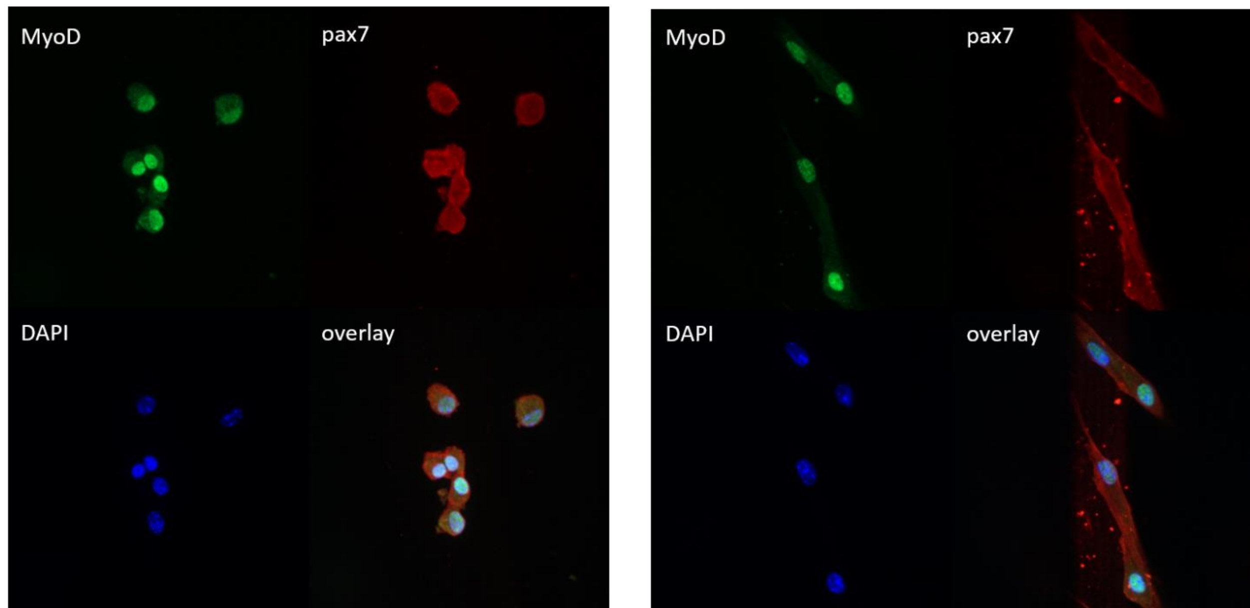

Figure S3: Pax7 expression in primary myoblasts diminishes as cells differentiate while MyoD expression persists. Primary myoblasts isolated from neonatal mice were cultured and fixed. Cells were immune-stained for MyoD (green) and Pax7 (red). Nuclei were stained with DAPI (blue).

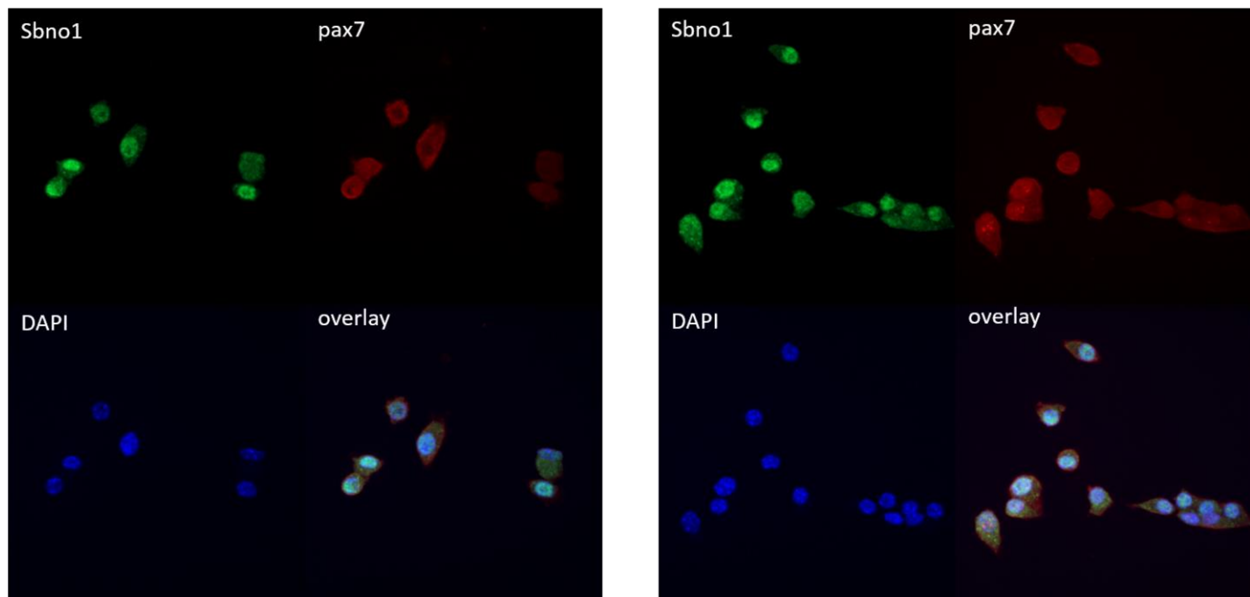

Figure S4: Sbno1 and Pax7 co-expresses in proliferating primary myoblasts. Primary myoblasts isolated from neonatal mice were cultured and fixed. Cells were immune-stained for Sbno1 (green) and Pax7 (red). Nuclei were stained with DAPI (blue).

Figure S5: Primers used in qRT-PCR

Fra-2	Fwd: 5'-AACTTTGACACCTCGTCCCG-3' Rev: 5'-CCAGGCATATCTACCCGGAAT-3'
c-Jun	Fwd: 5'AGATGAACTCTTTCTGGCCTGCCT-3' Rev: 5'-ACACTGGGCAGGATACCCAAACAA-3'
Pax7	Fwd: 5'-CTGGATGAGGGCTCAGATGT-3' Rev: 5'-GGTTAGCTCCTGCCTGCTTA-3'
DLL1	Fwd: 5'-TCAGATAACCCTGACGGAGGC-3' Rev: 5'-AGGTAAGAGTTGCCGAGGTCC-3'
Hes1	Fwd: 5'-TGAAGGATTCCAAAAATAAAATTCTCTGGG-3' Rev: 5'-CGCCTCTTCTCCATGATAGGCTTTGATGAC-3'
Hey1	Fwd: 5'-CGGACGAGAATGGAAACTTGA-3' Rev: 5'-CGGACGAGAATGGAAACTTGA-3'
Hey2	Fwd: 5'-AAGCGCCCTTGTGAGGAAA-3' Rev: 5'-TCGCTCCCCACGTCGAT-3'
Myogenin	Fed: 5'-CAGCTCCCTCAACCAGGAG-3' Rev: 5'-GACTGCAGGAGGCGCTGT-3'
β-Actin	Fwd: 5'-AAGTGTGACGTTGACATCCGTAA-3' Rev: 5'-TGCCTGGGTACATGGTGGTA-3'

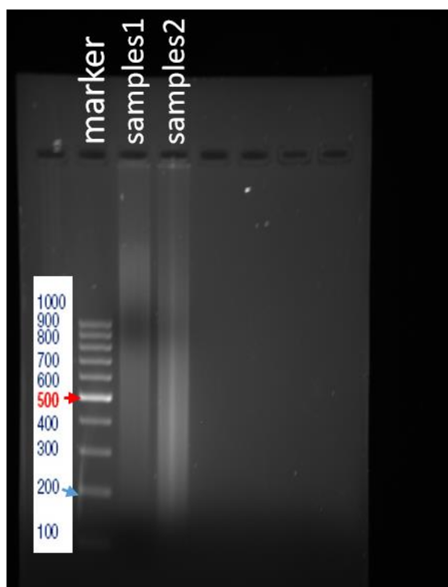

Figure S6: Testing the efficiency of chromatin sonication. Agarose gel (2%) electrophoresis of sonicated DNA to determine the fragment size of the crosslinked chromatin. Ideal DNA sonication is shown with fragment size ranging from 200~500 bp. High molecular weight unfragmented DNA can be seen on top of the gel.

Appendix B: Selected materials and methods

Cell Culture:

Reagents:

- DMEM (HyClone) supplemented with 100 µg/ml penicillin/streptomycin (Invitrogen) and 1% L-glutamine (Invitrogen) (added as required) for C2C12 cells
  - Growth Medium (GM): 10% fetal bovine serum (FBS)
  - Differentiation Medium (DM): 2% horse serum (HS)
- α-MEM (GIBCO) supplemented with 100 µg/ml penicillin/streptomycin (Invitrogen) and 1% L-glutamine (Invitrogen) for OP9 cells
  - GM: 20% FBS
- 1X Dulbecco's PBS
- Versene (VE) (0.2g of EDTA in 1L 1X PBS)
- 0.125% trypsin (HyClone) diluted in VE
- 1% gelatin for coating C2C12 culture plates
- Freezing Medium: Growth media supplemented with 5% DMSO

Cell Passaging:

- Aspirate media from cell culture
- Rinse cells briefly with 5 ml of VE, rinse cells with PBS only if they are primary myoblasts
- C2C12 and OP9: Remove VE or and add 1 ml of 0.125% trypsin solution to 100mm dish or 0.5ml to 60mm dish; primary myoblasts: Remove rinsing solution and add PBS instead
- Swirl the plate and pipette the trypsin solution or PBS
- Using a light microscope check if cells are completely detached
- Add 10 ml of GM and resuspend cells
- Count the cells in a haemocytometer (optional) and seed a dilution of cells that allows for sufficient cell (1:10 dilution is often used for C2C12 cells; 1:3 for OP9 cells; 1:3 for primary myoblasts)

- Plate cells accordingly: gelatin coated plates for C2C12 and OP9 cell; collagen coated plates for primary myoblast.

Inducing Muscle Cell Differentiation:

- Deplete cells at approximately 80% (60% for primary myoblasts) confluence of growth factors and wash cells gently twice with 1X PBS
- Re-feed cells with 10 ml DM
- Change media to fresh DM every 48h and monitor differentiation using a light microscope

Freezing Cells:

- Prepare a cell suspension of approximately 60% (40% for primary myoblasts) confluence and pellet the cells by centrifugation at 1500 g
- Resuspend the cells in freezing medium (1 plate 1 ml medium)
- Dispense cell suspension into freezing vials (1 plate of cells into 1 vial)
- Place vials into polystyrene a box in -80 °C freezer and freeze overnight
- Remove vials from polystyrene box. Place cells in -80 °C freezer for short-term storage, and for long term storage, place cells in liquid nitrogen freezer

Thawing Frozen Cells:

- Remove vial from the liquid-nitrogen freezer
- Thaw cells quickly in 37 °C water bath
- Invert vial a few times to dissociate cell clumps
- Transfer cells to a 15 ml conical tube containing 9 ml of media
- Centrifuge for 5 min at 1500 g, discard the supernatant and tap tube vigorously to remove cell clumps
- Suspend cells in 10 ml of GM
- Plate cells in 10 cm culture dish

Preparation of primary myoblasts culture:

Adaption of protocol by T. A. Rando, and H.M. Blau [368].

Materials:

- Sterile 1X PBS
- Ham's F-10 nutrient mixture (GIBCO) supplemented with 100 µg/ml penicillin/streptomycin (Invitrogen) (F-10$^+$)
- DMEM supplemented with 100 µg/ml penicillin/streptomycin (Invitrogen) (DMEM$^+$)
- Collagenase/Dispase/$CaCl_2$ solution
  - 1mg/ml Collagenase/Dispase (diluted in DMEM)
  - 2.5 mM $CaCl_2$, add fresh and use immediately
- F-10-based primary myoblast selection medium
  - F-10$^+$+20% FBS+2.5 ng/ml hFGF
- F-10/DMEM-based primary myoblast growth medium
  - F-10$^+$/DMEM$^+$ (half/half) +20% FBS+2.5 ng/ml hFGF
- Fusion medium
  - DMEM$^+$+5% HS

Methods:

- Sacrifice 5-10 neonatal mice by decapitation
- Rinse mice with 70% ethanol and remove the skin to expose hindlimb muscles
- Dissect the hindlimb muscles and place them in culture dish on ice in a drop of sterile PBS, keep the tissue moist and sterile
- Mince muscles to a slurry with razor blades in the culture dish in a sterile tissue culture hood
- Add collagenase/dispase/CaCl2 solution to tissue (~2 ml of per gram of tissue) and keep mincing for several min
- Transfer the minced tissue together with collagenase/dispase/CaCl2 solution to a sterile tube
- Incubate at 37°C for 25 min with occasional gentle trituration (make sure it's a fine slurry)
- Centrifuge the cells for 5 min at 350 X g
- Discard the supernatant and resuspend the pellet in 4-10 ml of F-10-based primary myoblast selection medium depending on the amount of tissue processed
- Transfer cells to a 60-100 mm collagen coated culture dish and incubate in a 37°C 5% CO_2 incubator

- Change to fresh F-10-based primary myoblast selection medium every 2 days
    - Transfer cells to a new plate if they have been on the same culture dish for more than 5 days, regardless of cell density
    - Do not grow cells at less than 10% confluence or over crowded
    - The F-10-based primary myoblast selection medium gives myoblasts a growth advantage over fibroblasts
- Pass cell when they are ready. Split them at no more than 1:5 dilution
    - Discard medium and wash the plate with 1X PBS
    - Leave a small amount of PBS in the dish, incubate at 37°C for 5 min
    - Dislodge cells from the plate by hitting the dish firmly in a sideways fashion against the edge of a table top (myoblasts come off more easily compare to fibroblasts)
- Pre-plate the cells for 15 min on a collagen coated dish
- Transfer cells in suspension to a new collagen coated dish (leave fibroblasts behind)
- Repeat pre-plating when passing cells until most of the fibroblasts are gone
- Switch cells into F-10/DMEM-based primary myoblast growth medium after the fibroblasts are no longer visible
    - Myoblasts are more compact and much smaller in diameter compared to fibroblasts when cultured on collagen
- Pure primary myoblasts can be used for experiments immediately, switch into differentiation medium around 40% confluence or frozen for storage

Transfection:

Lipofectamine transfections were performed in 60/100 mm cell culture dishes. The below protocols are for transfection of 100 mm plates, and reagents were scaled proportionally to surface area for transfection of 60 mm plates.

Lipofectamine (Invitrogen) transfection in C2C12 cells:

- Seed cells at 60% confluence in 10 cm plates 24 hours prior to transfection
- Prepare DNA-lipofectamine mixture
    - Dilute 5 µg of DNA or siRNA (50-100nM) in 500 µl of DMEM (no antibiotics or serum)
    - Mix 20 µl of Lipofectamine reagent with 500 µl of serum- and antibiotic-free media

- Incubate both mixtures for 5 min at RT
- Combine DNA-DMEM mixture and lipofectamine-DMEM mixture, mix well and incubate for 20-40 min at RT
- During incubation wash cells twice with 1X PBS and re-feed cells with 3 ml of serum- and antibiotic-free media
- Add DNA-lipofectamine mixture (1ml) drop wise to plates and gently rock
- Incubate cells with DNA-lipofectamine mixture for 5 h at 37°C and 5% CO2
- Supplement media with 20% FBS (1ml per plate) and mix well, incubate overnight
- Wash cells twice with 1X PBS and re-feed cells with 10 ml growth media, let cells recover and harvest at desired time points or switch into differentiation media

Lipofectamine 2000 (Invitrogen) transfection in primary myoblasts:

- Seed the cells at 60% confluence in 10 cm plates 24 hours prior to transfection
- Prepare DNA-lipofectamine mixture
    - Dilute a total of 10 µg of DNA in 500µl in Opti-MEM medium (Invitrogen)
    - Mix 15 µl of Lipofectamine 2000 reagent with 500 µl Opti-MEM medium
- Combine the DNA and Lipofectamine mixtures (1ml), mix well and incubate for a minimum of 5 min at RT
- During incubation wash cells twice with 1X PBS and re-feed cells with 4 ml of fresh growth medium
- Add the 1ml of DNA/Lipofectamine mixture drop wise to the plates and gently rock
- Incubate cells with DNA-lipofectamine mixture overnight at 37°C and 5% CO2
- Following the incubation, wash cells twice with 1X PBS re-feed cells with 10 ml fresh growth medium and allow the cells to recover for a minimum 16 hours prior to harvesting, pharmacological treatments or switch into differentiation media

Lipofectamine RNAiMAX (Invitrogen) transfection in primary myoblasts:

- Seed the cells at 70% confluence in 10 cm plates 24 hours prior to transfection
- Prepare DNA-lipofectamine mixture
    - Dilute siRNA (50-100nM) in 750 µl in Opti-MEM medium (Invitrogen)

- Mix 12.5 µl of RNAiMAX reagent with 750 µl Opti-MEM medium

- Combine the DNA and Lipofectamine RNAiMAX mixtures (1.5 ml), mix well and incubate for a minimum of 5 min at RT
- During incubation wash cells with 1X PBS and re-feed cells with 3.5 ml of fresh growth medium
- Add the 1ml of DNA/Lipofectamine RNAiMAX mixture drop wise to the plates and gently rock
- Incubate cells with DNA-lipofectamine RNAiMAX mixture overnight at 37°C and 5% CO2
- Following the incubation, wash cells twice with 1X PBS re-feed cells with 10 ml fresh growth medium and allow the cells to recover for a minimum 24 hours prior to harvesting or switch into differentiation media

Protein Extracts:

Keep protein samples on ice at all time (unless otherwise directed). Membrane, cytoplasmic and nuclear protein extracts were made using the Subcellular Protein Fractionation Kit for Cultured Cells (Pierce).

Reagents:

- 1xPBS (4°C)
- NP-40 Lysis buffer
  - 50 mM Tris, pH 8.0,
  - 150 mM NaCl,
  - 1 mM Sodium vanadate
  - 1 mM PMSF (add fresh)
  - protease inhibitor cocktail (add fresh, Sigma-Aldrich, P-8340)
- 2X SDS sample buffer (Biorad) (supplemented with 7.5% β-mercaptoethanol fresh as directed by manufacturer)

Whole Cell Extracts:

- Remove cells from the 37°C incubator and place on ice.

- Pour off media from cells
- Wash cells twice with cold 1xPBS
- Add 700 µl cold 1xPBS and gently scrape cells from the plates using a rubber scraper
- Collect cells (suspended in 1x PBS) into 1.5 ml eppendorph tubes.
- Spin cells at 1500xg for 5 min at 4°C
- Aspirate the supernatant and resuspend the pellet with five times (vol/vol) of that volume in NP-40 lysis buffer
- Vortex cells briefly and place on ice, repeat every 10 min for 40 min
- Centrifuge cell lysate at at 16.1 X 1000 G for 15 min at 4°C, and transfer supernatant to new tube
- Determine protein concentration by Bradford assay and store samples at - 80 °C

Membrane, Cytoplasmic and Nuclear Protein Extracts (Pierce):

- Wash cells twice with ice-cold 1X PBS
- Harvest cells with 1 ml trypsin-EDTA and then centrifuge at 500 × g for 5 min
- Wash cells by suspending the cell pellet with ice-cold 1X PBS
- Transfer 1-10 × 10^6 cells to a 1.5mL microcentrifuge tube and pellet cells by centrifugation at 500 × g for 2-3 min
- Use a pipette to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible
- Resuspend cell pellet with ten times (vol/vol) of that volume in ice-cold CEB containing protease inhibitors (add fresh)

  For following procedures, maintain the volume ratio of CEB:MEB:NEB:PEB reagents at 2:2:1:1, respectively.
- Incubate the tube at 4°C for 10 min with gentle mixing
- Centrifuge at 500 × g for 5 min. Immediately transfer the supernatant (cytoplasmic extract) to a clean pre-chilled tube on ice
- Resuspend cell pellet in ice-cold MEB containing protease inhibitors (add fresh)
- Vortex the tube at 16 000 × g for 5 sec and incubate tube at 4°C for 10 min with gentle mixing

- Centrifuge at 3000 × g for 5 minutes and transfer the supernatant (membrane extract) to a clean pre-chilled tube on ice
- Resuspend cell pellet in ice-cold NEB containing protease inhibitors (add fresh)
- Vortex on the highest setting for 15 secs and incubate tube at 4°C for 30 min with gentle mixing
- Spin the tube at 5000 × g for 5 min and transfer the supernatant (soluble nuclear extract) fraction to a clean pre-chilled tube on ice
- Prepare chromatin-bound extraction buffer by adding 5µL of 100mM $CaCl_2$ and 3µL of Micrococcal Nuclease (300 units) per 100µL of room temperature NEB
- Resuspend pellet in room temperature NEB containing protease inhibitors, $CaCl_2$ and Micrococcal Nuclease
- Vortex on the highest setting for 15 sec and incubate at RT for 15 min or in a 37°C water bath for 5 min
- Vortex on the highest setting for 15 sec and centrifuge the tube at at 16 000 × g for 5 min
- Transfer the supernatant (chromatin-bound nuclear extract) fraction to a clean pre-chilled tube on ice
- Determine protein concentration by Bradford assay and analyze samples by Western analysis

Bradford Assay:

Following collection of the cell lysate, the protein concentration can be determined by Bradford Assay.

- Thaw bovine serum album (BSA, 1µg/µl) and protein samples on ice
- Label 1.5 ml eppendorph tubes with increasing concentrations of BSA (0, 2, 4, 6, 8 µg/µl) and appropriate sample names, all conditions are in duplicates
- Add 800 µl, 798 µl, 786 µl, 794 µl, 792 µl of ddH_20 to the tubes with increasing concentrations of BSA, and add 799 µl ddH_20 to tubes with protein samples
- Add appropriate volume of BSA for the above labeled tubes accordingly and add 1 µl of cell lysate for each sample to the ddH_20
- Add 200 µl of Bradford reagent to each tube (BioRad)

- Mix well and incubate for 5 min at RT
- Determine optical density (OD) at 595nm for each sample using a spectrophotometer
- Generate a standard curve and use that to calculate protein sample concentrations

SDS-PAGE Analysis:

Reagents:

- 10% Resolving gel (15ml)
    - 5.9 ml dd$H_2$0
    - 3.8 ml 1.5M Tris pH 8.8
    - 5 ml 30% acrylamide;
    - 0.15 ml 10% SDS
    - 0.15 ml 10% APS
    - 0.006 ml TEMED
- Stacking gel (4ml)
    - 2. 7 ml dd$H_2$0
    - 0.5 ml 1.0 M Tris pH 6.8
    - 0.67 ml 30% acrylamide
    - 0.04 ml 10% SDS
    - 0.04 ml 10% APS
    - 0.004 ml TEMED
- 10 X Laemmli (1L)
    - dd$H_2$0 800 ml
    - Tris 30.3g
    - Glycine 144.2 g
    - SDS 10g
    - pH to 8.3
    - bring volume up to 1L with dd$H_2$0
- 1 X PBS

Methods:

- Prepare resolving gel and pour into Hoefer mini-gel apparatus, let the gel sit at RT for a minimum of 10 min to polymerize

- Pour stacking gel on top of resolving gel and insert appropriate combs, let the gel polmerize
- Immerse the bottom and centre well of mini-gel apparatus in 1X Laemmli buffer
- Load equal amounts of protein samples (prepare fresh) on gel
    - Calculate the appropriate volume for 15-20 µg of protein
    - Dilute protein samples with 3X SDS sample buffer
    - Boil samples at 100°C for 6 min, spin briefly, chill on ice for 5 min before loading
- Run a gel at 80 V through stacking gel and 120 V through resolving gel

Western Immunoblot:

Reagents:

- 1X Transfer buffer (100ml) (methanol, glycine, Tris Base)
- Methanol
- 1X PBS and/or 1X TBS
- Primary and Secondary antibody (as required)

Blocking buffer, washing solutions, ECL, and antibody diluent were prepared as per manufacturer's instruction.

Methods:

- Transfer protein from SDS-PAGE gel to Immobilon-P (Millipore) membrane by wet transfer at 20V for 16-20 h, or at 106V for 1h (use ice pack)
- Block membrane with 5 % (w/v) skim milk powder in PBS/TBS (depending on primary antibody manufacturers's requirements) for a minimum of 30 min at RT
- Incubate membrane with primary antibody diluted 1:100-1:10 000 in blocking solution overnight at 4 °C with gentle rocking
- Wash membrane with PBS/TBST (3 X 10 min each) with rocking
- Incubate membrane with secondary antibody diluted 1:2000-1:100 000 in blocking solution for 2 h at RT
- Wash membrane with PBS/TBST (3 X 10 min each)
- Prepare chemiluminescence reagents fresh and add to memebrane
- Incubate for 1-10 min (depending on the antibody) at RT

- Expose blot to film and develop using an automated developer in the dark room

Co-immunoprecipitation analysis:

- Mix 40 µl of ImmunoCruz beads (specie-specific to primary antibody) with 500 µl cold 1X PBS
- Add 1-5µg of primary antibody to PBS-ImmunoCruz beads mixture. Mix well and incubate O/N at 4°C with gentle rocking
- After overnight incubation, wash antibody-beads complex 3x in 1X PBS (cold) by centrifugation at max speed for 30 sec at 4°C
- Prepare protein lysates as described in protein extracts section
- Dilute 250-1000 µg total protein lysate in 1 ml of NP-40 lysis buffer and add to antibody-beads complex.
- Incubate O/N at 4°C with rocking
- After overnight incubation, wash immuno-complex 3x in 1X PBS (cold) by centrifugation at 5000 × g for 30 sec at 4°C
- Remove supernatant and wash pellet with 1 ml of 1X PBS (cold).
- Repeat washes twice more
- Resuspend pellet in 40 µl of 2 X SDS sample buffer and boil at 100°C for 5 min
- Load supernatant into SDS-PAGE gel (do not transfer beads) and followed by Western analysis

Immunofluorescence:

Reagents:

- Fixative: paraformaldehyde
- Permeabilize reagent: 90% ice-cold methanol
- Blocking reagent: 10% goat serum in 1X PBS

Methods:

- Wash cells several times with cold 1X PBS
- Fix cells with 4 % paraformaldehyde in 1X PBS for 10 min at RT

- Wash cells three times with cold 1X PBS
- Permeabilize cells with 90% methanol (cold) for 6 mins at -20°C
- Block cells with 10% goat serum in PBS at 37 °C for 30 min
- Incubate cells with primary antibody (1:50 –1:500 dilution) at 4°C for overnight
- Wash cells three times with 1X PBS
- Incubate cells with appropriate TRITC/FITC-conjugated secondary antibody (1 :200) in dark for 2 h at RT
- DAPI (4, 6-diamidino-2-phenylindole) staining in dark for 15 min at RT
- Wash cells several times with RT 1X PBS
- Add a drop of appropriate mounting media (DAKO) and cover slip
- Analyze using fluorescence microscopy

RNA Isolation:

As per Qiagen RNeasy Plus kit. See manufacture's protocol.

Chromatin Immunoprecipitation:

Day 1:

Preparation of cross-linked cells, isolation of nuclei and chromatin preparation:

- Wash cells with 1X PBS (4°C)
- Fix cell with 1% formaldehyde (270 µl of 37% formaldehyde diluted in 10 ml of 1X PBS) at RT for 10 min
- Quench cross-linking reaction by adding Glycine stock drop wise to plate at a final concentration of 0.125M, incubate for 5 min at RT
- Place dishes on ice, asperate liquid and dispose into formaldehyde waste
- Wash plates three times with 1X PBS (4°C), remove PBS and leave the plates as dry as possible
- Scrape cells in 1 ml PBS 4°C containing PMSF and protease inhibitor cocktail, followed by centrifugation for 5 min at 5000 rpm at 4°C
- Remove supernatants and resuspend cells with 1 ml of Wash buffer I
    - 10mM HEPES pH 6.5

- 0.5 mM EGTA
- 10mM EDTA
- 0.25% Triton X-100
- protease and phosphatase inhibitors

- Incubate on ice for 5 min followed by centrifugation for 5 min at 3000 rpm at 4°C
- Remove supernatant and resuspend nuclei in 1 ml of Wash buffer II
    - 10mM, HEPES pH 6.5
    - 0.5mM EGTA
    - 1mM EDTA
    - 200mM NaCl
    - protease and phosphatase inhibitors
- Incubate for 10 min on ice and centrifuge at 5000 rpm for 5 min at 4°
- Discard supernatant. Continue to day 2 immediately or nuclei samples can be frozen at -80°C

Day 2:

A. Nuclear lysis:

- Add 500 µl of nuclear lysis buffer (prepare fresh) to nuclei
    - 50mM Tris-HCl ph 8.1
    - 1mM EDTA
    - 1% SDS
    - protease and phosphatase inhibitors
- Shear DNA by sonication into approximately 250-500 bp fragments
- Centrifuge samples at max speed for 15 min at 4°C to remove insoluble residuals
- Transfer supernatant to clean new tubes
    - 2 X 200 µl aliquot for IP (Each 200 µl aliquot should have 1 x 10^6 cells)
    - 2 X 20 µl aliquots for agarose gel and input samples

B. Pre-Block Protein G Dynabeads:

- Block protein G Dynabeads with salmon sperm DNA in IP dilution buffer, for each IP (152 µl in total)

- 15 µl of beads
- 135 µl IP dilution buffer
  - 0.01% SDS
  - 1.1% Triton-X 100
  - 1.2 mM EDTA
  - 16.7 mM Tris-HCL pH 8.1
  - 167 mM NaCl
- 20 µg (2 µl of 10 mg/ml) salmon sperm

- Incubate overnight at 4°C with gentle rocking
- Make an extra IP

C. Lysate-antibody incubation:

- Determine the DNA sample concentration and use 25µg for each IP
- Dilute DNA sample 1:10 with IP dilution buffer
- Add 1-2µg of antibody and use equivalent amount of IgG for control
- Incubate overnight at 4°C with rotation

Day 3:

Incubation with Dynabeads and recovery of bound DNA:

- Mix 152 µl of pre-blocked beads to each IP reaction. Incubate at 4°C for 1 h with rotation
- Remove supernatant using magnet
- Wash dynabead-bound antibody:chromatin complex with 1 ml of cold IP Wash Buffer I
  - 20mM Tris pH 8.1
  - 2mM EDTA
  - 150mM NaCl
  - 1% Triton-X 100
  - 0.1% SDS
- Incubate at 4°C for 5-10 min with rotation and remove supernatant using magnet
- Wash dynabead-bound antibody:chromatin complexes with 1 ml of cold IP Wash Buffer II
  - 20mM Tris pH 8.1

- 2mM EDTA
- 500mM NaCl
- 1% Triton-X 100
- 0.1% SDS

- Incubate at 4°C for 5-10 min with rotation and remove supernatant using magnet
- Wash dynabead-bound antibody:chromatin complexes with 1 ml Wash Buffer III
    - 20mM Tris pH 8.1
    - 25 mM LiCl
    - 1% NP-40
    - 1% deoxycholate
    - 1mM EDTA
- Incubate at 4°C for 5-10 min with rotation and remove supernatant using magnet
- Wash dynabead-bound antibody:chromatin complexes twice in TE buffer at 4°C, each time 10 min and remove supernatant using magnet
- Free Protein-DNA complexes from Dynabeads by the addition of 300 µl freshly prepared Elution Buffer
    - 0.1M $NaCO_3$
    - 1% SDS

- Incubate with rotation for 30 min at RT
- Using magnet to attract Dynabeads, transfer the supernatant into clean tubes
- Treat recovered samples with 12 µl of 5M NaCl
- Incubate at 65°C overnight

Day 4:

Proteinase K Treatment:

- After overnight incubation, treat cell with freshly prepared Proteinase K treatment buffer
    - 6 µl 0.5M EDTA
    - 12 µl of 1M Tris-HCl pH 6.5
    - 1.2 µl of 10mg/ml proteinase K
- Incubate for 1 h at 45°C

DNA Purification (Using Qiagen PCR clean up kit):

- Purify DNA using Qiagen PCR clean up kit, as per manufactures directions
- The purified DNA can be analyzed immediately or stored at -20°C

Primary single fibre isolation culture and staining:

Regents:

- Fiber dissociation media (prepare fresh), heat to 37°C before use
    - 0.02g collagenase I (sigma) diluted in 10 ml DMEM supplemented with 100 µg/ml penicillin/streptomycin ($DMEM^+$)
    - 2 ml per mice (2 EDL)
- Fibre isolation media, keep at 37°C
    - $DMEM^+$ supplemented with 20% FBS
- Fibre growth media, heat to 37°C before use
    - $DMEM^+$ supplemented with 20% FBS and 1% Chick embryo extract (CEE)
- Fibre Differentiation media, heat to 37°C before use
    - $DMEM^+$ supplemented with 10% HS
- Fixation solution
    - 2% PFA in 1X PBS
- Blocking buffer
    - 1X PBS
    - 5% HS or Goat serum
    - 1% BSA
    - 0.5% Triton X-100
    - 0.01% NaAzide

Primary EDL muscle isolation and dissociation:

- Sacrifice 6-8 weeks old male mice (resting or injured) by cervical dislocation
- Spray the mice with 70% ethanol and remove the fascia with sterile scissors to expose muscles
- Identify TA muscle, EDL muscle and the tendons attaching to them

- Carefully dissect the TA muscle away from tendon (ankle) to tendon (knee) to expose the EDL muscle without damaging it
- Cut the EDL muscle from tendon (ankle) to tendon (knee) and lift it out of the leg, Avoid stretching of the EDL muscle during and after isolation
- Hold the EDL muscle by the tendon part and place it in collagenase I solution
- Incubate at 37°C for 1 h with occasional shaking (until fibres become loose), and check the digesting condition the EDL muscle under an inverted microscope
- Fill a 10cm plate (pre-coated with HS) with 5 ml of fiber isolation media
- Transfer the enzyme digested EDL muscle to the plate
- Wash the EDL muscle three time with fiber isolation media
- Using autoclaved-flame polished wide bore glass pipette, gently triturate the EDL muscle to dissociate fibers from tendons
- Remove fiber chunks from the plate
- Place the plate in an incubator and incubate at 37°C for 30 min
- Wash fibers with fiber isolation media. Only keep the straight and translucent ones and discard the hypercontracted ones

Primary single fiber culture:

- Using autoclaved-flame polished Pasteur pipet, transfer dissociated fibers to a new 6-well plate (pre-coated with HS) containing 2 ml fiber growth media
    - Fibers can be fixed immediately and proceed to staining to analyze satellite cells in the quiescent stage
- Culture fibers for 24 h to observe first division
- Culture fibers 48-72h to observe transition into myocytes (Myogenin activation)
    - Normally, large satellite cell clusters start to appear 72 h post isolation
- Transfer fibers into fiber differentiation media, and culture for 4-5 days to observe differentiation

Single fiber staining:

- Remove GM or DM and wash fibers three times with 1X PBS

- Fix fibers with fixation solution for 5 min at RT
- Wash fibers three times with 1X PBS
- Block fibers using blocking buffer O/N at 4°C or 1 h at RT, with shaking
- Incubate fibers with primary antibody diluted in blocking buffer for 2 h at RT or O/N at 4°C with shaking (300µl/well)
    - Pax7 1:2
    - Fra-2 1:500
    - p-Fra-2 1:200
    - c-Jun 1:500
    - Myogenin 1:5
- Wash fibers three times with 1X PBS
- Incubate fibers with appropriate TRITC/FITC-conjugated secondary antibody (1:1000) in dark for 2 h at RT with shaking
- DAPI (1:10 000) staining in dark for 10 min at RT
- Wash fibers several times with 1X PBS

Mounting stained fiber onto slides:

Materials:

- Superfrost Plus slides (fisher) microscope slides white
- Glass covers (22X50)
- Hydrophobic pen

Mehtods:

- Mark border of slides with Hydrophobic pen
- Transfer fibers on slide and remove excess liquid
- Add a drop of appropriate mounting media (DAKO), avoid bubbles
- Spread out fibres properly with tweezers, making sure they are aligned properly and not overlapping each other
- Remove excess mounting media
- Cover slip, avoid bubbles
- Dry the slides in the dark and the fluorescence images are captured using a confocal immunofluorescence microscopy