MOLECULAR MECHANISMS IN MYOGENESIS AND IN RHABDOMYOSARCOMA

by

DANQIONG SUN

B.S., China Agricultural University, 2004

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Biochemistry
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Abstract

Muscle satellite cells are the primary stem cells of postnatal skeletal muscle. Quiescent satellite cells become activated and proliferate during muscle regeneration after injury. They have the ability to adopt two divergent fates: differentiation or self-renewal. The Notch pathway is a critical regulator of satellite cell activation and differentiation. Notch signaling is activated upon the interaction of a Notch ligand present in a signal-sending cell with a Notch receptor present in a signal-receiving cell. Delta-like 1 (Dll1) is a mammalian ligand for Notch receptors. In this study, we found that Notch activity is essential for maintaining the expression of Pax7, a transcription factor associated with self-renewing satellite cells. We also demonstrated that Dll1 represents a substrate for several ADAM metalloproteases. Dll1 shedding takes place in a pool of Pax7-positive self-renewing cells, but Dll1 remains intact in differentiated myotubes. Inhibition of Dll1 shedding with a dominant-negative form of ADAM12 leads to elevated Notch signaling, inhibition of differentiation and expansion of the pool of self-renewing cells. We propose that ADAM-mediated shedding of Dll1 helps achieve an asymmetry in Notch signaling in initially equivalent myogenic cells and helps sustain the balance between differentiation and self-renewal.

Pax7 plays a key role in protecting satellite cells from apoptosis. The mechanism of Pax7 protecting muscle satellite cells from apoptosis is not well understood. In the second part of this study, we show that Pax7 up-regulates manganese superoxide dismutase (MnSOD) at the transcriptional level, suggesting the involvement of MnSOD in Pax7-mediated cell survival.

A specific chromosomal translocation involving the Pax7 gene and generation of a fusion protein Pax7-FKHR is found a childhood cancer, rhabdomyosarcoma. Furthermore, the level of the wild-type Pax7 is down-regulated in rhabdomyosarcomas. In the third part of this dissertation, we investigated the dominant-negative effect of Pax7-FKHR fusion protein on the wild-type Pax7, and found that the Pax7 protein level is down-regulated by Pax7-FKHR expression while the Pax7 mRNA level is not affected. We propose a specific microRNA-mediated inhibition of Pax7 mRNA translation by the oncogenic Pax7-FKHR fusion protein.

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Dedication

This dissertation is dedicated to my grandparents Qinghe Sun and Chongjie Fu and my parents Dr. Yi Sun and Suqin Cheng, for their love and support.

CHAPTER 1

INTRODUCTION

Muscle Satellite Cells

Adult skeletal muscle is composed of myofibers, which are highly specialized syncytia sustained by hundreds of post-mitotic myonuclei. Muscle satellite cells are mononucleated precursor cells that are defined by their position beneath the basal lamina of myofibers (Mauro, 1961; Yablonka-Reuveni, 1995). Satellite cells are the primary source for the postnatal muscle growth (Bischoff and Heintz, 1994). Upon injury, the activation and proliferation of satellite cells is responsible for the efficient repair and regeneration of damaged skeletal muscle (Whalen et al., 1990).

Quiescence, Activation and Self-renewal of Satellite cells

Satellite cells are quiescent under normal conditions in the adult muscle. Quiescent satellite cells are arrested at G0 stage of the cell cycle. They express a variety of signature proteins including CD34, M-cadherin, Pax7, syndecan-3, syndecan-4 and c-met (Beauchamp et al., 2000; Seale et al., 2000; Cornelison et al., 2000). FoxO and Rb families of transcriptional regulators, which promote quiescence in various cells, may also contribute to the reversible quiescent state of satellite cells (Yusuf and Fruman, 2003). A metalloprotease disintegrin, ADAM12 has been reported to up-regulate quiescence markers, retinoblastoma-related protein p130 and cell cycle inhibitor p27, and induce a quiescent-like phenotype in C2C12 myoblasts (Cao et al., 2003). The satellite cell niche, which is beneath the basal lamina of myofibers, is crucial for its maintenance of quiescence.

The activation of satellite cells from quiescent state and progression into proliferation and differentiation is controlled by various transcription factors. Myogenic regulatory factors (MRFs) play essential roles during satellite cell activation. This family includes myogenic determination factor 1 (MyoD), myogenic factor 5 (Myf5), myogenin and myogenic regulatory factor 4 (Mrf4) (Ludolph and Konieczny, 1995). Upon activation, satellite cells differentially express Myf5 (Beauchamp et al. 2000; Kuang et al., 2007). It has been further proposed that the Myf5 gene activity represents committed progenitors, whereas Myf5-absent cells contribute to the satellite cell reservoir (Kuang et al., 2007). MyoD expression appears during satellite cell activation and proliferation. The absence of MyoD impairs muscle regeneration (Megeney et al., 1996), delays the onset of myoblast differentiation and increases the propensity for self-renewal (Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). Myogenin is essential for muscle satellite cell

differentiation (Hasty et al., 1993). It has been reported that over-expression of myogenin results in down-regulation of the satellite cell marker, Pax7 (Olguin et al., 2007).

After satellite cell activation, the satellite cell pool is maintained *via* self-renewal, which involves withdrawal from the myogenic differentiation program. During muscle regeneration after injury, satellite cells are activated and proliferate. This is accompanied by co-expression of Pax7 and MyoD. Some of the cells then down-regulate Pax7, maintain MyoD and differentiate to produce myofibers (Halevy et al., 2004; Zammit et al., 2004), whereas others down-regulate MyoD expression while maintaining Pax7 and become quiescent to renew the satellite cell pool (Fig.1-1) (Zammit et al., 2004).

Two models have been proposed for satellite cell self-renewal, "asymmetric cell division model" and "stochastic cell fate model" (Dhawan and Rando, 2005). In "asymmetric cell division model", after satellite cell activation, an asymmetric division occurs during the first cell division, with one daughter cell returning to quiescence and forming a new satellite cell, while the other daughter cell undergoing proliferation and further generating fusion-competent myoblasts. In "stochastic cell fate model", the progeny of activated satellite cells are generated by symmetric cell divisions. At some point, one or more progeny adopts a different fate, returns to quiescence and forms a new satellite cell, while the remainders differentiate. The Notch inhibitor, Numb, is distributed asymmetrically during satellite cell division, which supports the asymmetric hypothesis in satellite cell self-renewal (Conboy and Rando, 2002).

Satellite cells are commonly identified by expression of the paired box transcription factor, Pax7. Pax7 plays an essential role in satellite cell maintenance and survival. Notch signaling is implicated in several steps of satellite cell proliferation, differentiation and self-renewal. The main focus of this dissertation is the modulation of Notch signaling in maintaining the balance between satellite cell differentiation and self-renewal, and the mechanism of Pax7 in protecting muscle satellite cells from apoptosis. In addition, a Pax7-related childhood cancer, rhabdomyosarcoma is also investigated.

Pax7 in Myogenesis and Rhabdomyosarcoma

Pax Genes

Pax genes encode a family of transcription factors that play key roles in tissue and organ formation during embryonic development. Pax proteins can be divided into four groups based on

their structures (Fig.1-2). All Pax proteins possess a paired domain, which is the defining characteristic of the family. The paired domain was originally identified in *Drosophila* Paired protein (Bopp et al., 1986; Treisman et al., 1991) and contains two sub-domains, PAI and RED. The N-terminal PAI domain binds to DNA directly. The consensus DNA binding site for paired domain is (G/T)T(T/C)(C/A)(C/T)(G/C)(G/C) with different nucleotide preferences of different members (Czerny et al., 1993; Epstein et al., 1994; Jun and Desplan, 1996). An eight-amino acid octapeptide is another conserved motif found in most of the Pax proteins with the exceptions of Pax4 and Pax6. The consensus sequences are HSIDGIL(G/S) for Pax3 and Pax7, YSI(N/S)G(I/L)LG for Pax2, Pax5 and Pax8 and HSV(S/T)(N/D)ILG for Pax1 and Pax9 (Noll, 1993). The octapeptide has transcriptional inhibitory function through interaction with other proteins to form repressor complexes (Smith, 1996). Several Pax proteins have a three helixed (Pax3, Pax4, Pax6 and Pax7), or a partial one helixed (Pax2, Pax5 and Pax8) homeodomain. The homeodomains share a conserved helix-turn-helix secondary structure. The homeodomains of Pax proteins contribute to DNA binding by recognizing the palindromic elements of TAAT(N)$_{2-3}$ATTA (Wilson et al., 1993).

Pax proteins play important roles during organogenesis, and their expression is tightly regulated spatially and temporally. Examples of Pax protein expression during tissue and organ development are Pax1 and Pax9 in skeleton (Wilm et al., 1998; Peters et al., 1999), Pax2, Pax3, Pax5, Pax6, Pax7 and Pax8 in central nervous system (Epstein et al., 1991; Stoykova et al., 1994), Pax2 and Pax8 in kidney (Dressler et al., 1993), Pax5 in B-cells (Nutt et al., 1999), Pax8 in thyroid (Mansouri et al., 1998), Pax4 and Pax6 in pancreas (Sosa-Pineda et al., 1997; St-Onge et al., 1997) and Pax3 and Pax7 in skeletal muscle (Tajbakhsh and Buckingham, 2000; Ben-Yair and Kalcheim, 2005; Gros et al., 2005).

Pax proteins are essential for growth and progression of cancers. The persistent expression of specific Pax proteins characterizes several tumors. Pax2 is frequently expressed in a number of primary human cancers, including brain, breast, colon, lung, lymphoma, melanoma, ovary and prostate cancers. Ovarian and bladder cancer cell lines depend on Pax2 for survival (Muratovska et al., 2003). It has been reported that melanoma cell lines depend on Pax3 for survival (Margue et al., 2000). Pax3 and Pax7 are commonly over-expressed in embryonal rhabdomyosarcoma (ERM) (Bernasconi et al., 1996; Barr, 1999; Tiffin et al., 2003). Pax5 is expressed in a variety of hematological malignancies, including follicular and non-Hodgkin's

lymphoma (Krenacs et al., 1998). Over-expression of Pax6 leads to cystic adenoma development and also occurs in the exocrine pancreas and intestine tumors (Salem et al., 2000). Pax8 is expressed in most thyroid cancers and expression of Pax8 correlates with a greater risk of tumor reoccurrence (Macchia et al., 1998; Castro et al., 2006). Furthermore, Pax genes are associated with specific chromosomal translocations in several tumors. In alveolar rhabdomyosarcoma (ARM), chromosomal translocations involving Pax3 or Pax7 genes, and fusion with the forkhead transcription factor (FKHR) gene, create oncogenic fusion proteins Pax3-FKHR or Pax7-FKHR (Barr et al., 1993; Galili et al., 1993; Shapiro et al., 1993; Davis et al., 1994). In a subset of lymphoplasmacytoid lymphoma, the Pax5 gene is involved in translocation and fusion with the immunoglobulin heavy chain (IGH) gene, which produces an oncogenic fusion protein Pax5-IGH (Busslinger et al., 1996; Iida et al., 1996). Pax8 is involved in chromosomal translocation and fusion with the peroxisome proliferator-activated receptor γ (PPARγ) gene to form an oncogenic fusion protein Pax8-PPARγ in thyroid tumors (Kroll et al., 2000; Cheung et al., 2003).

Pax7 and Pax3 in Myogenesis and Rhabodomysarcoma

Pax7 and Pax3 belong to the group III of Pax proteins, which contain a paired domain, an octapeptide, and a homeodomain with three α-helices. Binding to a target DNA occurs through the N-terminal paired domain and the homeodomain (Jun and Desplan, 1996). The transactivation domain includes a proline-, threonine- and serine-rich region and the C-terminus of Pax3/7 proteins (Jostes et al., 1990; Seo et al., 1998). Pax3 and Pax7 are partially overlapped in their expression during development. They are both expressed in dorsal neural tube and play essential roles in the development of neural crest cells (NCCs) (Kalcheim and Le Douarin, 1986). They are also expressed during skeletal muscle development and play critical roles in myogenesis.

A population of cells that express Pax3 and Pax7 but no other skeletal muscle specific markers was identified to constitute the resident muscle progenitor cells that subsequently become myogenic and form skeletal muscle in mouse embryos (Kassar-Duchossoy et al., 2005; Relaix et al. 2005). Pax3 and Pax7 are upstream regulators of the myogenic program (Buckingham, 2006). Pax3 and Pax7 regulate the expression of myogenic regulatory factors, MyoD and Myf5 (Relaix et al., 2006; Bajard et al., 2006). In the absence of both Pax7 and Pax3, skeletal muscle development is defective (Relaix et al. 2005). During embryonic muscle

development, both Pax7 and Pax3 function to ensure cell survival (Seale et al., 2000). The anti-apoptotic function of Pax7 is predominant in muscle satellite cells and cannot be replaced by its paralogue, Pax3 (Relaix et al., 2006).

Both Pax3 and Pax7 are involved in a childhood cancer, rhabdomyosarcoma. Rhabdomyosarcoma (RMS) is a highly malignant soft tissue tumor developed in children and young adults. Its two main subtypes are embryonal rhabdomyosarcoma (ERM) and alveolar rhabdomyosarcoma (ARM), with ARM being the more aggressive subtype (Tsokos et al., 1992). Approximately 70% of ARMs have one of the two specific chromosomal translocations occurring on chromosome 1 or 2 with chromosome 13 (Turc-Carel et al., 1986; Douglass et al., 1987; Wang-Wuu et al., 1988). The chromosomal translocation connects the 5'-end of the Pax3 or Pax7 gene to the 3'-end of the FKHR gene, and generates a fusion protein with the Pax3 or Pax7 DNA binding domain and the FKHR transcriptional activation domain (Fig.1-3) (Barr et al., 1993; Galili et al., 1993; Shapiro et al., 1993; Davis et al., 1994). The fusion proteins are more potent transcriptional activators than the wild-type Pax3 or Pax7 (Bennicelli et al., 1995; Scheidler et al., 1996). Studies of ARM tumors revealed that the wild-type Pax3 is up-regulated in ARM and its up-regulation is independent of fusion proteins, whereas the wild-type Pax7 is down-regulated in fusion positive ARM tumors (Tiffin et al., 2003; Tomescu et al., 2004). This indicates that fusion proteins may have a dominant negative effect on the wild-type Pax7.

Alternative Splicing of Pax7

The Pax7 gene consists of nine exons. Exons 2, 3 and 4 encode the paired domain. The homeodomain is encoded by exons 5 and 6. The transactivation domain is encoded by exons 6, 7 and 8 (Vorobyov et al., 1997; Barr et al., 1999). As a result of alternative splicing which occurs at the intron 2/exon 3 junction and the intron 3/exon 4 junction, alternate Pax7 transcripts include or exclude a tri-nucleotide, CAG, and a hexa-nucleotide, GTTTAG, respectively (Ziman and Kay, 1998). This produces four Pax7 isoforms a-d (Q+GL+, Q+GL-, Q-GL+, Q-GL-) with the inclusion or exclusion of a glutamine (Q) and a glycine-leucine dipeptide (GL), respectively. The various isoforms contain distinct tertiary structures and have different DNA binding affinities and specificities (Du et al., 2005). Another type of alternative splicing of Pax7 adds more complexity. Transcripts of Pax7 with alternative 3'-ends have been identified in both human and mice (Barr et al., 1999; Seale et al., 2000). Transcript Pax7A contains exons one through eight,

while transcript Pax7B contains exons one through nine. The alternative products encode proteins with distinct C-termini, which indicate different transactivation activity. Varied expression levels of alternative Pax7 transcripts have been reported in embryonic and adult muscles (Ziman et al., 1997; Lamey et al., 2004).

Pax7 in Satellite Cells

Pax7 has attracted particular attention in regulation of muscle satellite cells since it was reported that Pax7-deficient mice completely lack muscle satellite cells (Seale et al., 2000). However, it has been later shown that muscles of juvenile Pax7-null mice at P11 contain a reduced but substantial number of satellite cells. The number of satellite cells declined strongly during postnatal development. Muscle growth and regeneration was greatly compromised in Pax7-deficient mice (Oustanina et al., 2004). This implicates Pax7 in maintenance of muscle satellite cells and regeneration of skeletal muscle during postnatal growth (Oustanina et al., 2004; Kuang et al., 2006). Pax7 has a critical anti-apoptotic function in activated satellite cells, for which its paralogue, Pax3 cannot compensate. This explains the progressive satellite cell death in Pax7-deficient mice postnatally (Relaix et al., 2006). However, the mechanism of Pax7-mediated satellite cell survival is not clear. Pax7 can activate transcription in quiescent satellite cells and influence satellite cell fate by controlling the expression of myogenic regulatory factors (Zammit et al., 2006). Pax7 modulates the expression of Myf5 by interacting with Wdr5 and Ash2L, the core proteins of histone methyltransferase complex (McKinnell et al., 2008). Pax7 transcriptional activity is required for MyoD expression (Relaix et al., 2006). Constitutive expression of Pax7 delays the induction of myogenin expression in satellite cells (Zammit et al., 2006), whereas over-expression of myogenin results in down-regulation of Pax7 (Olguin et al., 2007).

Notch, Delta and ADAM12

Notch Signaling

Notch is the receptor of an evolutionarily conserved signaling pathway that regulates cell fate decision and differentiation. Notch was discovered first in *Drosophila*, where mutation in the Notch gene resulted in notches in their wingblades.

The Notch genes encode a family of type I single transmembrane proteins. In mammals, Notch is presented to a ligand as a heterodimer produced as a result of processing at S1 site by a furin-like convertase in the *trans*-Golgi, during the secretion process (Logeat et al., 1998; Nichols et al., 2007). The extracellular region of Notch includes 10 to 36 epidermal growth factor (EGF)-like repeats, three juxtamembrane repeats (also known as Lin-12 repeats) and the heterodimerization domains (Wharton et al., 1985; Yochem et al., 1988). Heterodimerization domains hold the Notch extracellular domain (NECD) with the Notch membrane-tethered intracellular domain non-covalently (Sanchez-Irizarry et al., 2004). The intracellular region of Notch is composed of the RBPjκ Associate Molecule (RAM) region, repeated structural motifs named Ankyrin repeats, a proline-, glutamine-, serine-, threonine-rich (PEST) domain, and a transactivation domain (TAD). Ankyrin repeats are flanked by nuclear localization signals (Fleming 1998; Lubman et al., 2004). They mediate the interaction between Notch and CSL DNA binding proteins. The PEST domain is involved in the degradation of Notch (Fig.1-4, right panel) (Fryer et al., 2004).

In the canonical Notch signaling pathway, activation of Notch requires the interaction between a DSL (Delta and Serrate in *Drosophila*, Lag2 in *C. elegans*) ligand expressed on the surface of the signal-sending cell and a Notch receptor expressed on the surface of the signal-receiving cell. There are five DSL ligands (Delta-like 1, 3 and 4, and Jagged 1 and 2) and four Notch receptors (Notch 1-4) in mammals. Ligand binding triggers sequential cleavages of Notch receptor, first by an ADAM protease at the S2 site in the juxtamembrane region of the extracellular domain, followed by γ-secretase at the S3 site in the transmembrane domain. The intracellular domain of Notch (NICD) is thus released from the membrane and translocates into the nucleus. Inside the nucleus, NICD forms a complex with CSL (CBF1, SuH, LAG1) DNA binding proteins and recruits the co-activator Mastermind to activate the target gene expression (Fig.1-5) (Bray, 2006; Fiuza and Arias, 2007).

The extensively studied Notch target genes include Hes and Hey genes in mammals. They are related to the Hairy and Enhancer of split [E(spl)] genes in *Drosophila* (Fischer and Gessler, 2003). In mouse and rat genomes, seven Hes (Hes 1-7) and three Hey (Hey 1, 2 and L) genes have been identified. All of these genes encode basic helix-loop-helix (bHLH) proteins. Hes1, Hes5, Hes7 and all members of the Hey gene family have been shown to be induced by Notch

(Fischer and Gessler, 2007). The Hes and Hey proteins are expressed in various tissues and have important functions during development and in adulthood.

In additional to its canonical signaling pathway, Notch can also regulate cellular processes in a CSL-independent manner. It has been reported that Notch signaling inhibits myogenic differentiation through a CBF1-independent pathway (Shawber et al., 1996; Nofziger et al., 1999).

Regulation of Notch Signaling

The regulation of Notch signaling is at multiple levels. Interaction of Notch with its ligands presented from the neighboring cells leads to *trans*-activation of Notch signaling. The extracellular cysteine-rich DSL region present in Delta and Jagged mediates the interaction with Notch EGF-like repeats 11 and 12 (Rebay et al., 1991; Fleming et al., 1998). The interaction leads to a conformational change that exposes the S2 site for cleavage by ADAM proteins. Notch receptor can also interact with its ligand within the same cell. This leads to *cis*-inhibition of Notch signaling by DSL ligands. It has been shown that in *Xenopus*, Delta-like 3 does not activate Notch signaling but exhibits only inhibitory effect in a cell-autonomous manner (Ladi et al., 2005). It has also been reported that during human keratinocyte differentiation, high level of Delta expression induces an inhibitory mechanism of Notch signaling (Lowell et al., 2000).

Proteolytic cleavage of Notch at the S2 site by ADAMs is a necessary step before the cleavage by γ-secretase and generation of NICD. Two ADAMs have been implicated in the S2 cleavage of Notch after ligand-receptor interaction. In *Drosophila*, Kuzbanian mediates the proteolytic processing of Notch (Rooke et al., 1996; Pan and Rubin, 1997; Sotillos et al., 1997; Lieber et al., 2002). In mouse cells *in vitro*, instead of Kuzbanian ortholog ADAM10, ADAM17 (also known as TACE) plays a prominent role in the cleavage and activation of Notch (Brou et al., 2000). However, ADAM10-deficient mice die at day 9.5 of embryogenesis (Hartmann et al., 2002), which resembles the effect of mutations in Notch. ADAM17-deficient mice die between embryonic day 17.5 and the first day after birth (Peschon et al., 1998), which suggests that other proteases can cleave Notch at the S2 site *in vivo*. Thus, ADAM10 may play a major role in the S2 cleavage of Notch *in vivo*, with other ADAMs making tissue-specific contributions, corresponding to their tissue specific expressions.

Notch and its ligands are glycoproteins and glycosylation plays an important role in regulating the ligand and receptor binding properties (Rampal et al., 2007; Stanley, 2007; Irivine, 2008). Notch receptors and DSL ligands have conserved sequences in the EGF-like repeats that can be modified by O- and N- linked glycans. Only O-glycan modifications have been reported to be indispensible for Notch signaling. In *Drosophila*, the glycosyltransferase O-fucosyltransferase-1 (OFUT1) is required for Notch activation. In addition to the O-fucose modification on Notch, OFUT1 is also required for the proper folding and trafficking of Notch to the cell surface (Okajima et al., 2008). The mammalian OFUT1, Pofut1, regulates Notch through its fucosyltransferase activity to achieve optimal ligand binding, but Pofut1 is not required for Notch cell surface expression (Stahl et al., 2008). The O-fucosylated sites can be further modified by Fringe, a β-1, 3-*N*-acetylglucosaminyltransferase. This modification is required for a subset of Notch-dependent events (Visan et al., 2006).

Recent studies have shown that Notch signaling is regulated by endocytosis. A number of proteins associated with endocytosis, including epsin, clathrin, dynamin and auxilin, have been implicated indispensible for DSL ligand activity in the signal-sending cells. Structural analysis of the Notch heterodimer has suggested that the ADAM cleavage site (S2 site) is contained in the negative regulatory region (NRR) composed of three Lin12/Notch repeats and the heterodimerization domain (Gordon et al., 2007). Considerable force is needed to expose the S2 cleavage site to ADAMs. Ligand endocytosis provides a good candidate to generate such force. Notch ectodomain undergoes *trans*-endocytosis and internalization by Delta-expressing cells. This process requires furin processing, but occurs independent of ADAM proteolysis (Nichols et al., 2007). Another possible mechanism explaining the requirement for ligand endocytosis is that trafficking into an endocytic compartment enables ligand post-translational modifications and makes DSL ligand competent to activate Notch after being recycled to the cell surface (Wang and Struhl, 2004; Le Borgne et al., 2005; Wilkin and Baron, 2005).

Functions of Notch

Notch signaling contributes to the decision between two alternative cell fates. In a population of cells, it is characterized as "lateral inhibition". In *Drosophila*, during neuroblast differentiation, all proneural clusters have ubiquitous but weak expression of both Delta and Notch. The cells that acquire a higher level of Delta differentiate into neuroblasts, while

activating Notch in their neighboring cells and directing them into alternative fate (Cabrera, 1990). The small difference within the roughly equivalent population of cells is thus amplified by Delta-Notch signaling. In mammals, it has been shown that lateral inhibition plays an important role during hair cell development in the inner ear (Kiernan et al., 2005). When it comes to the cell fate decision between two sister cells, Notch is associated with "asymmetric cell division". Asymmetric division of stem cells gives rise to one stem cell and one committed daughter cell, which is regulated by stem cell niche. It has been reported that upon apical-basal oriented cell divisions, a high level of Delta-like 1 expression is present in committed daughter cells that maintain contact with the plasmalemma, with a low level of Delta-like 1 expression in stem cells, suggesting the involvement of Notch signaling in oriented asymmetric cell division (Kuang et al., 2007). It has also been shown that Numb, a well-known Notch negative regulator, is distributed asymmetrically during satellite cell division (Conboy and Rando, 2002).

Defects in Notch signaling are involved in several human diseases. Mutations in Notch 1 and Notch 3 cause an autosomal vascular disorder resulting in the loss of the arteriolar vascular smooth muscle cells (Gridley, 2003; Harper et al., 2003). Mutations involving either the Notch heterodimerization domain or the PEST domain give rise to T-cell acute lymphoblastic leukemia (Weng et al., 2004). Mutations in Dll3 result in rib defects, causing abonormalities in vertebral segmentation and trunk size (Girdley, 2003). Mutations in Jagged 1 cause Alagille syndrome, characterized by kidney, eye, heart and skeleton developmental problems and defects in bile duct formation that leads to liver problems (Li et al., 1997; Oda et al., 1997).

Notch Signaling in Myogenesis

Notch signaling plays an important role in the development of myogenic lineage (Vasyutina et al., 2007a). Notch signaling is activated during satellite cell activation and proliferation (Conboy and Rando, 2002). Increased Notch signaling inhibits myogenic differentiation. Ectopic expression of NICD attenuates myogenic differentiation by down-regulating MyoD (Kopan et al. 1994; Conboy and Rando, 2002). It has also been reported that high levels of Dll1 suppress myogenic differentiation of cultured C2C12 cells by down-regulation of MyoD (Kuroda et al., 1999). A CBF1-independent inhibition of myogenic differentiation has been proposed in C2C12 myogenic cells, where the activity of MyoD is not affected but the expression of myogenin is down-regulated (Shawber et al., 1996; Nofziger et al.,

1999). Decreased Notch signaling promotes myogenic differentiation. Over-expression of Notch antagonist, Numb, up-regulates the expression of muscle differentiation genes (Conboy and Rando, 2002; Kitzmann et al., 2006). Inhibition of Notch signaling by using a pharmacological γ-secretase inhibitor (DAPT) enhances differentiation of murine and human myoblasts (Kitzmann et al., 2006). Studies utilizing conditional RBP-J mutant mice and hypomorph Dll1 mutant mice shed lights on the role of Notch signaling in maintaining the myogenic progenitor population in skeletal muscle (Schuster-Gossler et al., 2007; Vasyutina et al., 2007b). Both the conditional RBP-J and the hypomorph Dll1 mutations result in premature myogenic differentiation, leading to a depletion of the progenitor pool. This results in lack of muscle growth and severe muscle hypotrophy. Thus, Notch signaling is required for preventing uncontrolled differentiation and maintaining the progenitor population during muscle development. However, the mechanism responsible for regulation of Notch activity in myogenic cells is not fully understood.

Delta-like 1

Delta-like 1 (Dll1) is one of the canonical DSL ligands that interact with Notch receptors. Like Notch receptors, Dll1 is also a type I transmembrane protein. Mouse Dll1 contains a DSL domain and nine EGF-like repeats in its extracellular domain (Fig.1-5, left panel). The DSL domain together with the flanking N-terminal domain and the first two EGF-like repeats are responsible for the interaction with Notch receptors (Parks et al., 2006). After the transmembrane domain, the intracellular region of Dll1 contains multiple lysine residues and a C-terminal PDZ (PSD-95/Dlg/ZO-1) binding motif (Pintar et al., 2007).

Similar to the sequential proteolysis of Notch, Dll1 also undergoes regulated intramembrane proteolysis (RIP) in the juxtamembrane and transmembrane regions by ADAMs and γ-secretase, respectively. The cleavage by ADAMs generates a transmembrane and intracellular domain (TMIC) of Dll1 and an extracellular fragment (EC), which is released to the medium (Six et al., 2003; Dyczynska et al., 2007). ADAM10 was implicated in processing of mouse (Six et al., 2003) and rat Dll1 (LaVoie et al., 2003). The extent of Dll1 cleavage is reduced to ~50% in ADAM10-/- mouse embryonic fibroblasts (MEFs) compared to wild-type MEFs (Six et al., 2003), which indicates that other ADAMs may be capable to cleave Dll1 in ADAM10-/- cells.

Dll1 plays an essential role in regulating the canonical Notch signaling pathway. Dll1 can affect Notch signaling through cell-cell interaction in *trans*, and in a cell-autonomous manner in *cis*. Interaction between Dll1 and Notch receptor in the neighboring cell leads to sequential proteolytic cleavages and activation of Notch. Interaction between Dll1 and Notch within the same cell inhibits Notch signaling (Sakamoto et al., 2002; D'Souza et al., 2008). ADAM proteolysis of Dll1 has presumably dual effects on Notch signaling. ADAM proteolysis in the signal-sending cell would limit the ligand availability and down regulate Notch activity. RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a physiological inhibitor of ADAM10. In RECK-/- mice, Notch signaling is down-regulated because of excessive Dll1 shedding by ADAM10, which results in defective neurogenesis (Muraguchi et al., 2007). In cultured mammalian cells, soluble extracellular domain of Dll1 inhibits Notch activity (Trifonova et al., 2004). In addition to inhibition of Notch *trans*-activation, ADAM proteolysis of Dll1 may activate Notch signaling in a cell-autonomous manner by relief of *cis*-inhibition (Dyczynska et al., 2007; Zolkiewska, 2008).

After cleavage by ADAMs, the membrane-tethered Dll1 transmembrane and intracellular domain (TMIC) undergoes further proteolysis by γ-secretase (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six et al., 2003). The product of γ-secretase cleavage is the intracellular fragment (IC) of Dll1, which contains a typical RKRP sequence targeting it to the nucleus. Marked transcriptional stimulation of Gal4-luciferase reporter has been observed with co-transfection of Delta1-Gal4VP16 chimera. The transactivation is inhibited upon treatment with γ-secretase inhibitor (Ikeuchi and Sisodia, 2003). Dll1 IC has been reported to enhance transforming growth factor-β (TGF-β) induced transcriptional activity of Smad3 (Hiratochi et al., 2007).

Dll1 also mediates cellular processes through its C-terminal PDZ (PSD-95/Dlg/ZO-1) binding motif. Dll1 interacts with Activin receptor interacting protein 1 (Acvrinp1) (Pfister et al., 2003), disc large homolog 1 (Dlg1) (Six et al., 2004) and membrane-associated guanylate kinase with inverted domain arrangement 1 (MAGI1) (Mizuhara et al., 2005). It has been shown that PDZ interactions are dispensable for activation of Notch signaling. In fact, Delta with a mutated PDZ binding motif has enhanced signaling potential (Estrach et al., 2007).

ADAMs

ADAMs are type I transmembrane proteins that contain a metalloprotease and a disintegrin-like domain. ADAM stands for "A Disintegrin And Metalloprotease". Together with snake venom metalloproteases (SVMPs) and ADAMs containing thrombospondin repeats (ADAMTS proteins), ADAM proteins belong to the adamalysin family of the metzincin subgroup of zinc proteases. The matrix metalloproteases (MMPs) also belong to the metzincin subgroup. To date, 23 ADAMs have been identified in the human genome. Analysis of their structures shows that all ADAM proteins contain N-terminal signal peptide, pro-domain, metalloprotease, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic domains. Structural analysis revealed that the active site in the metalloprotease domain contains zinc and water atoms that are necessary for hydrolysis of ADAM substrates. The zinc binding consensus sequence is HEXXHXXGXXH. The zinc ion is coordinated by three conserved histidine residues and a downstream methionine (Stocker et al., 1995). ADAMs are involved in various biological events, including membrane protein shedding and proteolysis, cell adhesion and cell migration. They play important roles in development and diseases, such as cancer, asthma and Alzheimer's disease (Blobel, 2002; Seals and Courtneidge, 2003; Arribas et al., 2006).

ADAM proteins have important functions in regulation of Notch signaling because of their involvement in the proteolytic processing of both Notch receptors and DSL ligands. As previously described, two ADAMs, ADAM10 and ADAM17 have been implicated in the S2 cleavage of Notch. ADAM10 is also known to process Dll1, with potential contributions from other ADAMs. In addition to the cleavage of Dll1, mammalian Jagged 1 and Jagged 2 have been observed to be processed at the juxtamembrane region. In CHO cells, transfected Jagged 1 has been shown to be cleaved by ADAM17 (Ikeuchi et al., 2003; LaVoie et al., 2003).

ADAM12

ADAM12 is an active metalloprotease associated with development and regeneration of skeletal muscle (Yagami-Hiromasa et al., 1995; Gilpin et al., 1998; Engvall and Wewer, 2003). There are two splice variants existing in human, ADAM12-L and ADAM12-S. ADAM12-L is a transmembrane protein with a prototypical ADAM structure. ADAM12-S is a soluble protein where the transmembrane and cytoplasmic domains are replaced by 33 amino acids in the C-terminus (Fig.1-6). ADAM12 is synthesized in the rough endoplasmic reticulum and is modified

in the Golgi apparatus. Processing of the pro-domain takes place in *trans*-Golgi network by a furin-like pro-protein convertase. Cys179 in the pro-domain coordinates the active site zinc ion and keeps ADAM12 inactive by a cysteine-switch mechanism (Loechel et al., 1999; Cao et al., 2002). ADAM12 becomes an active metalloprotease after being delivered to the extracellular space (Loechel et al., 1998; Loechel et al., 1999).

Physiological ADAM12 substrates include insulin-like growth factor binding proteins (IGFBP)-3 and IGFBP-5 (Loechel et al., 2000; Shi et al., 2000), epidermal growth factor receptor (EGFR) ligands (Asakura et al., 2002; Kurisaki et al., 2003), placental leucine aminopeptidase (P-LAP) (Ito et al., 2004) and Delta-like 1 (Dyczynska et al., 2007). These proteolytic events regulate diverse cellular responses, including cell proliferation, differentiation, migration and invasion (Kveiborg et al., 2008).

Approximately 30% of ADAM12-deficient mice die before weaning (Kurisaki et al., 2003). Most muscles in the ADAM12-deficient mice appear normal, with some impaired formations of the neck and shoulder muscles. In some deficient mice, the interscapular brown adipose tissue is reduced. These findings suggest that ADAM12 may be involved in myogenesis and adipogenesis (Kurisaki et al., 2003; Masaki et al., 2005; Kveiborg et al., 2008).

During mouse embryogenesis, ADAM12 is expressed primarily in mesenchymal tissues that give rise to muscle and bone (Kurisaki et al., 1998). ADAM12 mRNA is not detected in adult muscle tissue in mice, but its transcription is up-regulated during muscle regeneration (Borneman et al., 2000; Galliano et al., 2000). During differentiation of C2C12 myogenic cells, ADAM12 expression is down-regulated in differentiated myotubes, whereas in a pool of quiescent reserve cells, ADAM12 expression is maintained, suggesting the role of ADAM12 in determination of reserve cells (Cao et al., 2003).

The expression of ADAM12 is markedly up-regulated in cancer of the breast, prostate, liver, lung, stomach, colon, bladder, brain and bone (Kveiborg et al., 2008). ADAM12 is also expressed by several tumor cell lines, including a rhabdomyosarcoma cell line (Gilpin et al., 1998). Recent studies using transgenic mouse models suggest that ADAM12 promotes tumor progression. In mice spontaneously developing breast cancer due to polyoma middle T antigen expression, forced expression of ADAM12 increased the tumor progression (Kveiborg et al., 2005), whereas in a mouse model for prostate cancer, knocking-out ADAM12 led to reduced tumor development (Peduto et al., 2006). ADAM12 has been identified as one of the candidate

cancer genes associated with human breast cancer in a large scale cancer mutation discovery and screen (Sjoblom et al., 2006). Three missense mutations in ADAM12 were identified, including a highly conserved aspartic acid in the metalloprotease domain changed to a histidine, a highly conserved glycine in the disintegrin domain changed to a glutamic acid, and a leucine in the cytoplasmic tail changed to a phenylalanine. The first two mutations of highly conserved residues have been shown to interfere with the intracellular trafficking of ADAM12 and result in loss of function ADAM12 at the cell surface (Dyczynska et al., 2008).

Goal of Study

Muscle satellite cells are commonly identified by the expression of Pax7. Pax7 is essential in protecting satellite cells from apoptosis (Relaix et al., 2006). However, the mechanism for Pax7-mediated satellite cell survival is not clear. When quiescent Pax7-expressing satellite cells are activated, they co-express Pax7 and MyoD. During proliferation, most of the cells down regulate Pax7 and differentiate, while others maintain Pax7 but lose MyoD and return to quiescence (Zammit et al., 2004). Notch signaling plays a critical role during this process in preventing myogenic differentiation and maintaining the satellite cell pool. The regulations of Notch activity in myogenic cells are not well understood. In alveolar rhabdomyosarcoma, Pax7 is involved in a specific chromosomal translocation. The generation of oncogenic fusion proteins Pax3-FKHR or Pax7-FKHR leads to decreased expression of Pax7 (Tiffin et al., 2003; Tomescu et al., 2004). The process of wild-type Pax7 protein down-regulation is yet to be fully investigated.

The goal of this dissertation was to gain a better understanding in these fundamental questions in myogenic differentiation and in rhabdomyosarcoma, with emphasis on the following aspects: the cleavage of Notch ligand, Dll1 by ADAM metalloproteases and the impact of Dll1 cleavage on Notch signaling and on muscle satellite cell self-renewal and differentiation; the involvement of other mediating factors in Pax7 anti-apoptotic function and in satellite cell survival; and the molecular mechanism of inhibition of Pax7 by the fusion protein generated in rhabdomyosarcoma.

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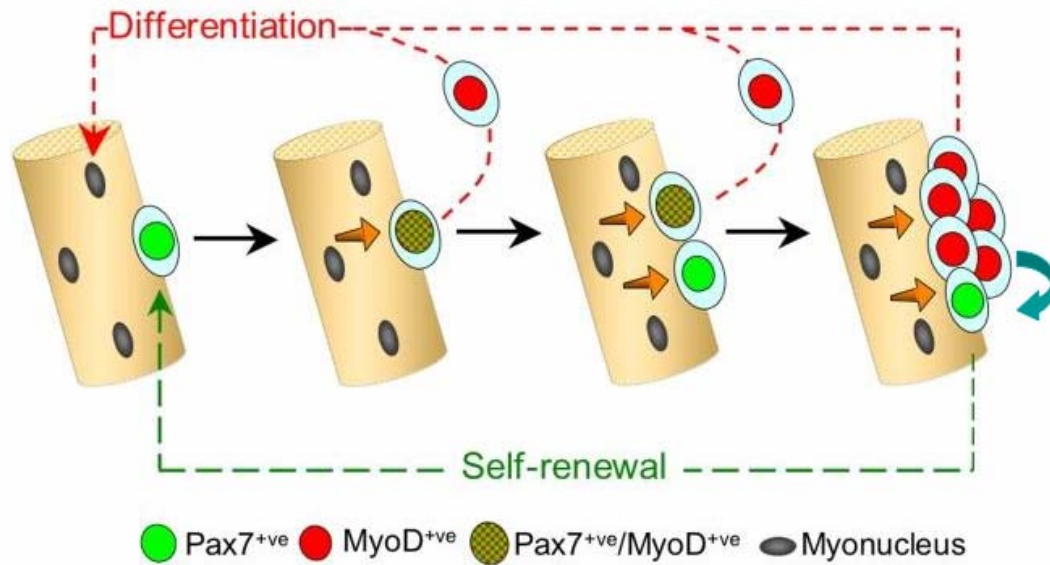
Figure 1-1. **Model of satellite cell self-renewal.** Quiescent satellite cells express Pax7 (Green). During muscle regeneration, quiescent satellite cells are activated and proliferate. Proliferating satellite cells co-express Pax7 and MyoD (green and red tartan). During satellite cell proliferation, most of cells down-regulate Pax7, maintain MyoD (red), and differentiate to produce myofibers (red pathway). Other cells down-regulate MyoD expression while maintaining Pax7 (green), and renew the satellite cell pool (green pathway). Signaling between the myofiber and the quiescent and proliferating satellite cells (orange arrows) and signaling between the cells within the clusters (blue arrow) are essential for divergent satellite cell fate determination.
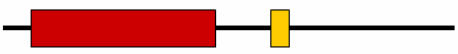
Figure 1-2. **Pax protein family.** Pax proteins are divided into four groups based on their structures. The paired domain (red) characterizes the family and is present in all Pax proteins. Members of group I, II and III have an octapeptide (yellow). Members of group II contain a partial one helixed homeodomain (blue), while members of group III and IV contain a three helixed homeodomain (blue). Pax3 and Pax7 belong to group III of the Pax protein family.
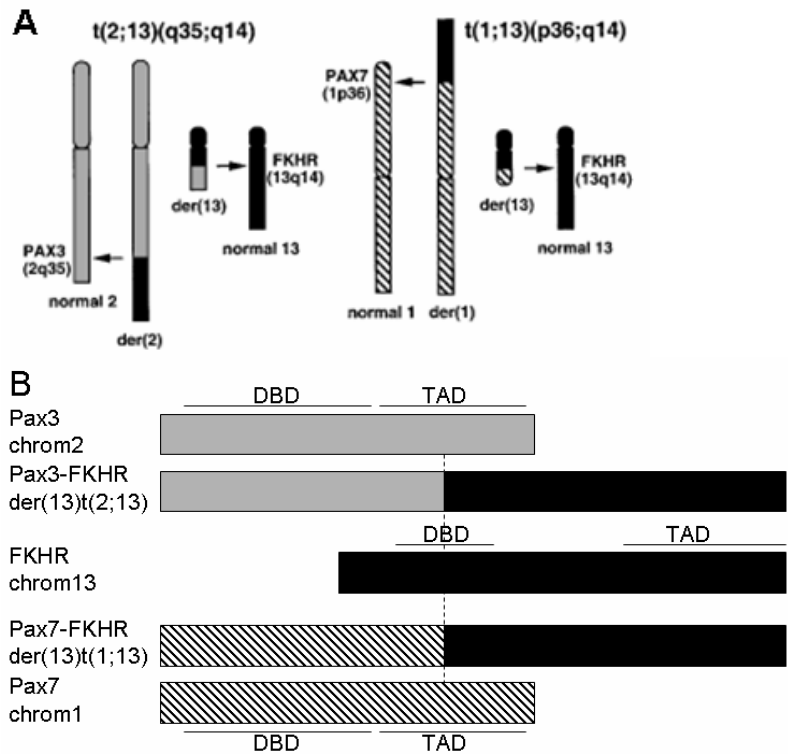
Figure 1-3. **Chromosomal translocations and generation of fusion proteins.** (A) Diagram of chromosomal translocations between chromosome 1 or 2 and chromosome 13 (Adapted with permission from © Barr, 2001. Originally published in *Oncogene.* 20: 5736-5746). (B) Comparison of the fusion products to the associated wild-type proteins. The DNA binding domains (DBD) of Pax proteins are connected with the transactivation domain (TAD) of the FKHR protein.
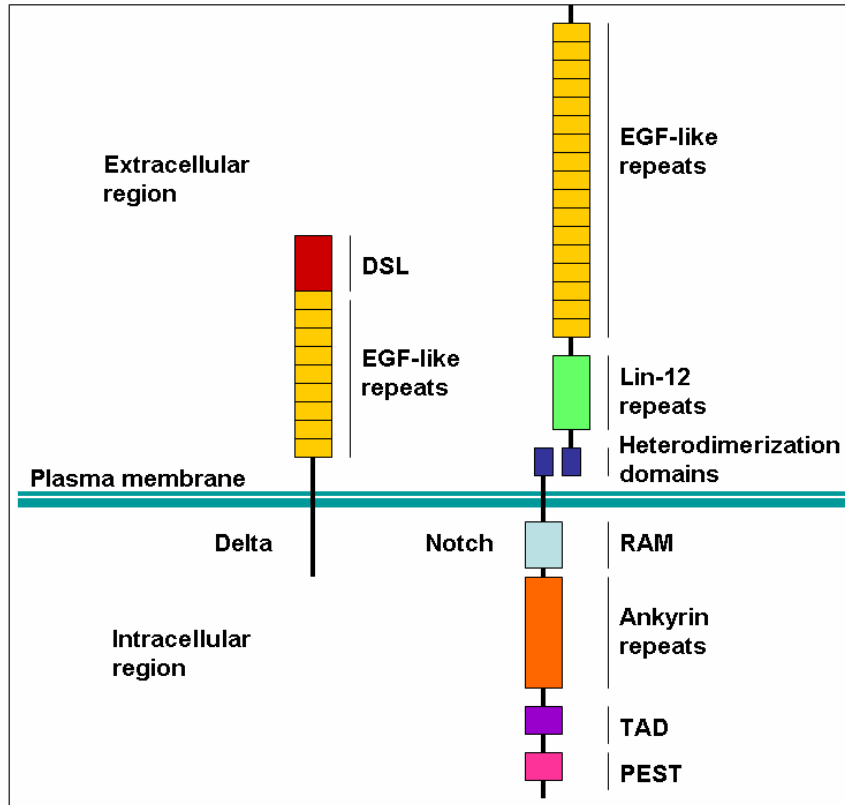
Figure 1-4. **Domains found in the Notch receptor and its ligand Delta.** The extracellular region of the Notch receptor includes EGF-like repeats (yellow), Lin-12 repeats (green) and heterodimerization domains (navy). The intracellular part of Notch contains RAM region (blue), Ankyrin repeats (orange), a transactivation domain (purple) and a PEST domain (pink) (*right panel*). Notch ligand Delta-like 1 contains a DSL domain (red) and nine EGF-like repeats (yellow) in its extracellular region (*left panel*).
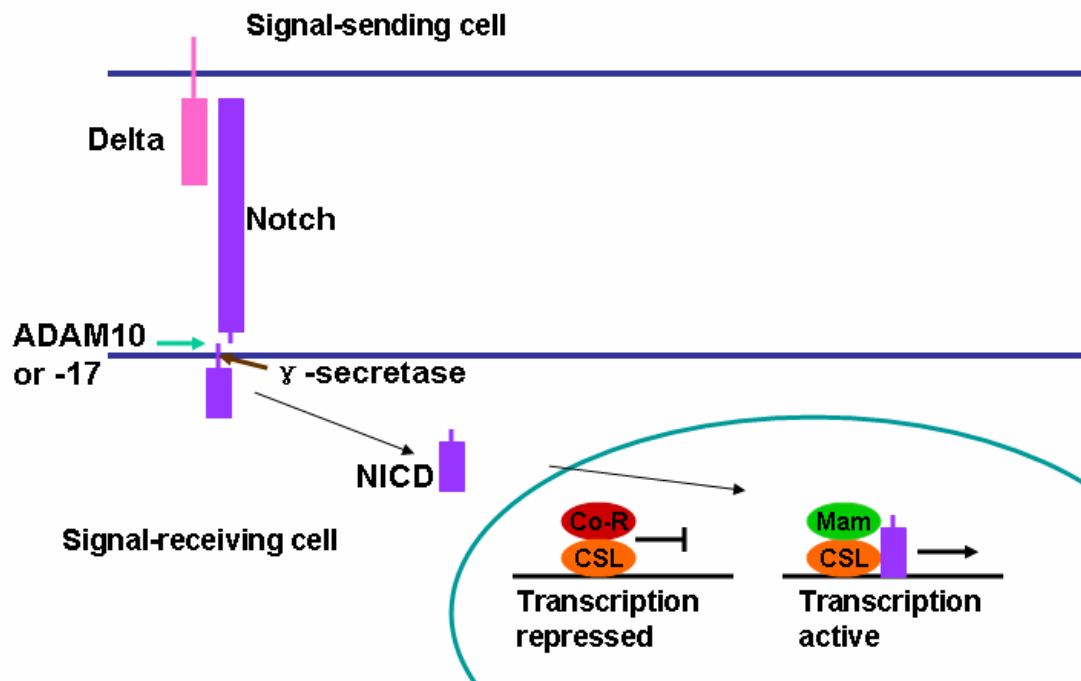
Figure 1-5. **Canonical Notch signaling pathway.** Binding of Delta ligand (pink) to the Notch receptor (purple) elicits sequential proteolytic cleavages of Notch receptor, first at the S2 site by ADAM metalloproteases (ADAM10 or ADAM17) (blue), followed with cleavage at S3 site by γ-secretase (brown). The Notch intracellular domain (NICD) is released and translocates into the nucleus. In the nucleus, NICD interacts with CSL DNA binding proteins (orange), recruits the co-activator Mastermind (green) and leads to the target gene expression.
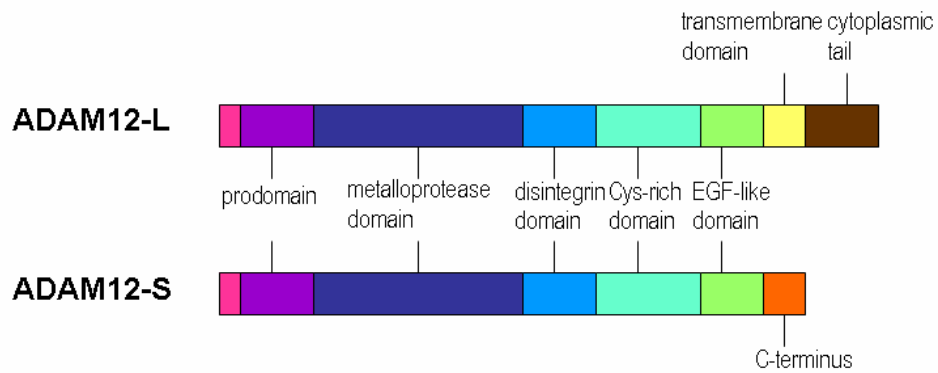
Figure 1-6. **Structures of human ADAM12-L and ADAM12-S.** Human ADAM12 exists in two forms as a result of alternative splicing. ADAM12-L is a transmembrane protein with a prototypical ADAM structure consisting of the N-terminal signal peptide (pink), pro-domain (purple), metalloprotease domain (navy), disintegrin domain (blue), cysteine-rich domain (light blue), EGF-like domain (green), transmembrane domain (yellow) and cytoplasmic tail (brown). ADAM12-S is a soluble protein with a short 33-amino acid C-terminus (orange).

CHAPTER 2

PROTEOLYTIC PROCESSING OF DELTA-LIKE 1 BY ADAM PROTEASES

Introduction

Notch signaling regulates cell fate decisions during development and in the adult (Lai, 2004; Kadesch, 2004; Louvi and Artavanis-Tsakonas, 2006). The signaling pathway is activated by direct interactions between Notch receptor, a transmembrane protein present at the surface of a signal-receiving cell, and a DSL (Delta/Serrate/Lag2) ligand, a transmembrane protein at the surface of a signal-sending cell. In mammals, there are four different Notch receptors (Notch 1–4), and five DSL ligands (Delta-like 1, 3, and 4 and Jagged 1 and 2). Ligand-bound Notch undergoes proteolytic cleavage at the S2 site in the extracellular domain, which is mediated by ADAM10 or ADAM17 (Brou et al., 2000; Mumm et al., 2000; Hartmann et al., 2002), members the ADAM family of metalloprotease-disintegrins (Blobel, 2005; Huovila et al., 2005). This is followed by the cleavage at the S3 site in the transmembrane domain of Notch by a γ-secretase complex (Mumm and Kopan, 2000; Selkoe and Kopan, 2003). The intracellular domain of Notch translocates to the nucleus, where it interacts with CSL (CBF1, Su(H), Lag-1) transcription factors and activates expression of target genes (Hayward, 2004).

Similar to their receptors, Notch ligands also undergo ADAM-mediated cleavage in their extracellular domains, which is then followed by processing by γ-secretase (Ikeuchi and Sisodia, 2003; Six et al., 2003; LaVoie and Selkoe, 2003; Bland et al., 2003). Two ADAMs were postulated to cleave Notch ligands in mammalian cells, ADAM10 and -17. ADAM10 was implicated in the processing of mouse (Six et al., 2003) and rat Delta-like 1 (Dll1) (LaVoie and Selkoe, 2003), whereas ADAM17 was suggested to cleave rat Jagged 1 (LaVoie and Selkoe, 2003). Proteolytic cleavage of Dll1 in ADAM10-/- mouse embryonic fibroblasts (MEFs) still amounts to 50% of the processing observed in ADAM10+/+ MEFs (Six et al., 2003), suggesting that ADAM10 is only partially responsible for Dll1 cleavage and other ADAMs may account for the remaining processing in ADAM10-/- cells. MEFs express several ADAM proteins with catalytically active metalloprotease domains, including ADAM9, -12, -15, and -17 (Sahin et al., 2004). However, to date, none of these ADAMs has been shown to be capable of cleaving Dll1.

Furthermore, although it is well established that proteolytic processing of Notch ligands down-regulates Notch signaling in neighboring cells (Qi et al., 1999; Mishra-Gorur et al., 2002), the effect of ligand cleavage on the Notch pathway within the same cell is less clear. Notch receptors can associate with their ligands in a cell-autonomous manner (Sakamoto et al., 2002; Katsube and Sakamoto, 2005; Ladi et al., 2005). Associations between receptors and ligands in

cis decrease Notch receptivity and attenuate the Notch pathway (Sakamoto et al., 2002; Katsube and Sakamoto, 2005; Ladi et al., 2005; Franklin et al., 1999). In *Drosophila*, proteolytic processing of Delta by ADAM10-like (Kuzbanian-like) alleviates the inhibitory effect of Delta on Notch in the same cell (Sapir et al., 2005). In contrast, processing of Jagged 1 in mammalian cells by ADAM17 was postulated to inhibit Notch signaling in *cis* due to competition of the C-terminal fragments of Jagged 1 and Notch for γ-secretase (LaVoie and Selkoe, 2003).

In this study, we have tested the ability of several ADAMs other than ADAM10 to cleave murine Dll1 and examined the effect of Dll1 cleavage on Notch signaling in the same cell. We show that ADAM12 can efficiently process Dll1 but not Notch1. Dll1 cleavage by ADAM12 activates Notch in a cell-autonomous manner. In addition, we show that two other ADAMs, ADAM9 and -17, but not ADAM15, are capable of Dll1 processing. ADAM9 and -17 are also capable to activate Notch in a cell-autonomous manner. The extent of processing of Dll1 transfected into ADAM9/12/15-/- MEFs is reduced when compared with the processing in wild type MEFs, suggesting that the endogenous ADAM9 and/or ADAM12 present in wild type MEFs contribute to Dll1 cleavage.

Results

We demonstrated that the full length Dll1 (FL Dll1, ~90kDa) was cleaved by ADAM12. The C-terminal Dll1 fragment (CTF Dll1, ~29kDa) was then cleaved by γ-secretase. The soluble N-terminal fragment of Dll1 (NTF Dll1, ~60kDa) was released to culture medium (Fig.2-1). The catalytically inactive mutant form of ADAM12, E349Q, did not process Dll1. ADAM12-catalyzed processing of Dll1 was more efficient at high cell density and occurred in *cis*. In addition, ADAM12 lacked α-secretase activity toward Notch1.

Having established that Dll1, a Notch ligand but not Notch itself, is cleaved by ADAM12, we examined the effect of Dll1 cleavage on Notch signaling. To monitor the activation status of the Notch pathway, we utilized CBF1-luc reporters containing four (Hsieh et al., 1996) or eight binding sites (Peng et al., 2000) for CBF1, a CSL transcription factor activated by Notch (Hayward, 2004). NIH3T3 cells were transiently co-transfected with mouse Notch1 and a Notch reporter and co-cultured with CHO cells stably transfected with Dll1 (CHO.Dll1) or with empty vector (CHO.Vec). Co-culture with CHO.Dll1 cells led to higher activity of the reporter than co-culture with CHO.Vec cells (Fig.2-2A), suggesting that the exogenous Dll1 expressed in

CHO.Dll1 cells activated Notch. The extent of this activation was more modest than reported previously in several other studies, in which quail QT6 or mouse L fibroblasts were employed to present Notch ligands to Notch-expressing cells (Ladi et al., 2005; Jarriault et al., 1998). One of the reasons of a modest increase in Notch activity in our system may be that co-culture with control CHO.Vec cells had already stimulated Notch 4-5-fold when compared with the conditions without CHO.Vec cells (results are not shown). This was most likely due to high expression levels of the endogenous Notch ligands in CHO cells that were capable of Notch activation. In such case, further increase of the amount of ligand by transfecting exogenous Dll1 might have had a limited effect and, understandably, might not have produced additional strong increase in Notch activity. Nonetheless, increased activity of the Notch reporter induced by co-culture with CHO.Dll1 *versus* CHO.Vec cells was observed for the reporter containing intact, but not mutated, CBF1 binding sites (Fig.2-2A), which validated our co-culture assay to measure Notch activation. When Dll1 was further co-expressed with Notch in NIH3T3 cells, the activation of Notch was abolished (Fig.2-2B) due to formation of Notch/Dll1 complexes in *cis* (Sakamoto et al., 2002; Katsube and Sakamoto, 2005; Ladi et al., 2005; Franklin et al., 1999). Most importantly, further co-transfection of Dll1-processing ADAM12 resulted in re-activation of the Notch reporter (Fig.2-2B). The catalytically inactive mutant of ADAM12, E349Q, which did not process Dll1, did not activate Notch either (Fig.2-2B). This result suggested that when Notch and Dll1 were expressed in the same cell, proteolytic processing of Dll1 increased the ability of Notch to receive signals and to activate its downstream signaling pathway.

We next examined the abilities of three other ADAMs, ADAM9, -15 and -17 to cleave Dll1. All ADAMs used in this study form stable complexes with Dll1. Co-transfection with Dll1 demonstrated that ADAM9 and -17, similarly to ADAM12, had catalytic activity toward Dll1, but ADAM15 was not able to process Dll1.

To test the effect of Dll1 cleavage by different ADAM proteins on Notch signaling, NIH3T3 cells were transiently co-transfected with mouse Notch1, CBF1-luc reporter containing eight CSL binding sites, and one of the ADAM constructs or empty vector. Notch signaling was similar in cells co-transfected with empty vector and with ADAM9, -12, -15 or -17 when NIH3T3 cells were co-cultured with CHO.Vec cells. Co-culture with CHO.Dll1 cells increased Notch reporter activity to a similar extent in all cases as a result of *trans*-activation. When NIH3T3 cells were further co-transfected with Dll1, Dll1 inhibited Notch signaling in *cis* in

vector-transfected cells. Most importantly, in cells co-transfected with ADAM9, -12 and -17, *cis*-inhibition of Notch activity by Dll1 in the same cell was relieved due to the efficient cleavage of Dll1 by these ADAM proteases. In ADAM15 co-transfected NIH3T3 cells, no relief of *cis*-inhibition was observed (Fig.2-3). This result indicated that proteolytic processing of Dll1 by ADAM9, -12 and -17 increased Notch signaling in a cell-autonomous manner.

To determine whether ADAM proteases studied in this work can process Dll1 when they are expressed at the endogenous levels, we compared the amount of Dll1 cleavage in wild type (wt) and ADAM9/12/15-/- triple knock-out (T) MEFs. Dll1 was transfected into wt-MEFs or T-MEFs, and the extent of Dll1 cleavage was examined by subjecting total cell lysates to immunoblotting with anti-Dll1 antibody (Fig.2-4A) or by immunoprecipitation of FL Dll1 and CTF Dll1 from ^{35}S-labeled cells (Fig.2-4B). Significant differences in the intensities of the bands corresponding to FL Dll1 and CTF Dll1 in Fig.2-4A precluded an accurate quantification of the extent of cleavage (the CTF Dll1/FL Dll1 ratio). These differences were even more pronounced in Fig.2-4B because the number of cysteine and methionine residues in FL Dll1 is ~10 higher than in CTF Dll1 (Six et al., 2003). Nevertheless, whereas the amounts of FL Dll1 in wt-MEFs and T-MEFs were similar, the level of CTF Dll1 in T-MEFs corresponded to ~60% of the level in wt-MEFs (Fig.2-4AB). Because ADAM15 is not capable of cleaving Dll1, this result suggested that ADAM9 and/or ADAM12 contributed to Dll1 processing in wt-MEFs.

At the end, we showed that the endogenous Dll1 was subject to proteolytic processing in confluent, differentiating myoblast cultures, giving rise to the 29-kDa fragment, and that the endogenous ADAM12 contributed to this processing.

Discussion

Mammalian Dll1 expressed in HEK293, COS, CHO, N2a, or NIH3T3 cells was cleaved by endogenous ADAMs present in these cells (Ikeuchi and Sisodia, 2003; Six et al., 2003; LaVoie and Selkoe, 2003). Transfection of Dll1 into ADAM10-/- or ADAM10+/+ MEFs demonstrated that the cleavage was reduced by ~50% in the absence of ADAM10 (Six et al., 2003). This result suggested that ADAM10 was only partially responsible for Dll1 cleavage and that other ADAMs catalyzed the remaining cleavage of Dll1 in ADAM10-/- cells. MEFs express several catalytically active ADAMs, including ADAM9, -12, -15, and -17 (Sahin et al., 2004), and we focused our study on these proteases. Here we show that ADAM9, -12, and -17, but not

ADAM15 are capable of Dll1 cleavage. Furthermore, processing of Dll1 transfected into ADAM9/12/15-/- MEFs was diminished when compared with processing in wild type MEFs (Fig.2-4). This result suggested that Dll1 was also subject to processing by the endogenous ADAM9 or ADAM12 or both (since ADAM15 was not capable of cleaving Dll1 even when over-expressed in COS-7 cells, most likely it did not contribute to Dll1 processing in MEFs at the endogenous level). Although the reduction of Dll1 cleavage in ADAM9/12/15-/- MEFs was rather modest (~40%), it was consistent with ~50% inhibition of Dll1 processing observed in ADAM10-/- MEFs (Six et al., 2003) and with the remaining activity of ADAM17, which might have also contributed to the processing.

The ability of ADAM9, -12 and -17 to cleave Dll1 allowed us to study the effect of ligand cleavage on Notch signaling. Although shedding of the extracellular domain of Notch ligand limits the ligand presentation in *trans* and terminates Notch signaling in a neighboring cell (Qi et al., 1999; Mishra-Gorur et al., 2002), cell-autonomous effects of ligand cleavage are less clear. Notch ligands form complexes in *cis* with Notch, which leads to sequestration of Notch receptors, reduction of Notch receptivity to signals from outside, and attenuation of Notch signaling (Sakamoto et al., 2002; Katsube and Sakamoto, 2005; Ladi et al., 2005; Franklin et al., 1999). Shedding of the extracellular domain of the ligand could relieve this inhibitory effect and activate Notch, a scenario that is supported by results of *in vivo* experiments in flies. Over-expression of Kuzbanian-like in juxta-marginal cells in *Drosophila* wing discs (which are characterized by high levels of Delta and low levels of Notch signaling) resulted in increased expression of Notch target genes (Sapir et al., 2005). Alternatively, the C-terminal fragment of a ligand could compete with Notch S2 cleavage product for γ-secretase and, thus, interfere with Notch signaling. Indeed, over-expression of an ectodomain-truncated Jagged 1 in COS-7 cells together with an ectomain-truncated Notch led to inhibition of the Notch function (LaVoie and Selkoe, 2003). However, expression of a truncated Jagged that mimicked an already cleaved Jagged, did not allow evaluation of the actual effect of the removal of the N-terminal portion of Jagged by ADAMs. In our studies, both Dll1 and Notch constructs represented the intact proteins, and the relative contribution of Dll1 ectodomain shedding (a stimulatory effect) and competition of Dll1 and Notch C-terminal fragments for γ-secretase (an inhibitory effect) were addressed. Our results demonstrate that ADAM12, capable of mediating a constitutive cleavage of Dll1 but not of Notch, activated Notch signaling in a cell autonomous manner, whereas the

catalytically inactive ADAM12 mutant did not process Dll1 and did not activate Notch (Fig.2-2B). Furthermore, Dll1-processing by ADAM9 and -17 increased the ability of Notch to activate its downstream signaling, while ADAM15, unable to cleavage Dll1, was unable to activate Notch (Fig.2-3). Thus, alleviation of the inhibition of Notch mediated by Dll1 appears to outweigh the generation of a Dll1 fragment that can compete for γ-secretase.

The essence of Notch signaling is the amplification of small differences in the levels of Notch receptors and their ligands between adjacent cells (Lai, 2004; Kadesch, 2004; Louvi and Artavanis-Tsakonas, 2006). Cells that are initially equivalent but at one point develop a bias toward receptors or ligands will eventually become signal-receiving and signal-sending cells, respectively. Amplification of the differences in receptor/ligand levels is mainly achieved by a transcriptional feedback mechanism (Lai, 2004; Kadesch, 2004; Louvi and Artavanis-Tsakonas, 2006). We propose that proteolytic cleavage of Dll1 may represent another mechanism for reinforcing small differences in the signaling capacities of different cells and for establishing the uni-directionality in Notch signaling.

Materials and Methods

Expression constructs

Mouse Dll1 cDNA was amplified by PCR using a full-length clone (ID 6402691; Invitrogen) as a template and cloned into pIRESpuro expression vector. c-Myc-Dll1 containing an internal c-Myc tag between amino acids 46 and 47, inserted into the SacII site in Dll1 cDNA, was cloned into pcDNA3.1 vector. Mouse full-length cDNAs of ADAM9, -12, -15, and -17 were cloned into pcDNA3.1 vector; these ADAMs contained c-Myc and His6 tags at their termini. The E349Q was generated by site-directed mutagenesis using QuikChange kit (Stratagene). Mouse Notch1 containing an intact extracellular domain and in which 348 C-terminal amino acids were replaced with 6 copies of c-Myc tag (pCS2+mN1FL6MT) was provided by R. Kopan (Washington University). CBF1 reporters containing four wild type or mutated CBF1 binding sites (pJH23A and pJH25A, respectively) were provided by D. Hayward (Johns Hopkins School of Medicine); Notch reporter containing eight CBF1 binding sites (pJT123A) was provided by P. D. Ling (Baylor College of Medicine).

Cells

NIH3T3 and CHO-K1 cells were obtained from American Tissue Culture Collection. NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, at 37 °C in the presence of 5% CO_2 under a humidified atmosphere. CHO-K1 cells were grown in F12K nutrient mixture, supplemented with 10% fetal bovine serum, at 37 °C in the presence of 5% CO_2 under a humidified atmosphere. MEFs isolated from ADAM9/12/15-/- (Sahin et al., 2004) or from wild type mice and immortalized with simian virus 40 large T antigen were grown on gelatin-coated dishes in DMEM containing 10% FCS and 100 μg/ml each penicillin and streptomycin.

Plasmid transfection

One day after plating, cells were transfected using Fugene6 (Roche Applied Science). In experiments with MEFs, the amount of total DNA was 1 μg/well in a 6-well plate; for CBF1 reporter assays, 2.05 μg of DNA/well was used (see below). For stable expression of c-Myc-Dll1 in CHO-K1 cells, transfected cells were grown for 2 weeks in the presence of Geneticin (800 μg/ml); a clone positive for c-Myc-Dll1 expression was isolated. Cells with the highest expression of cell-surface c-Myc were further selected by cell sorting using FACS Calibur (BD Biosciences).

Cell treatments

Before harvesting, MEFs were incubated with 1 μM L685,458 (a γ-secretase inhibitor) for 6 h.

Western blotting

Cellular proteins were extracted with extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (AEBSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 mM 1,10-phenanthroline; 0.5 ml extraction buffer/well in a 6-well plate). Cell extracts were centrifuged at 21,000 x g for 15 min, and supernatants were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 3% (w/v) dry milk and 0.3% (v/v) Tween 20 in DPBS, then incubated with primary antibody in blocking buffer, followed by incubation with horseradish peroxidase-labeled secondary antibody and

detection using the WestPico chemiluminescence kit (Pierce). Primary antibody rabbit anti-Dll1 (Santa Cruz Biotechnology, H-265; 0.2 μg/ml) was used. Secondary antibody was horseradish peroxidase (HRP)-conjugated anti-rabbit IgG. Intensities of the bands in Western blots were determined by densitometry and quantified using ScionImage software. Each experiment involving quantitative determination of Dll1 cleavage was repeated at least three times.

Metabolic labeling and immunoprecipitation

24 h after transfection with c-Myc-Dll1, MEFs were transferred to DMEM:methionine/cysteine-free DMEM (1:9) containing ^{35}S-labeled EasyTag Express protein labeling mix (267 μCi/ml, PerkinElmer Life Sciences). After 16 h, cell extract was collected, cell debris was removed by centrifugation, and supernatant was used for immunoprecipitation with anti-Dll1 antibody (H-265, 5 μg/ml). After pre-clearing, supernatants were incubated with antibodies and protein G-Sepharose 4 Fast Flow (Amersham Biosciences), the beads were washed three times with extraction buffer, and immuno-complexes were eluted with SDS-PAGE sample buffer and analyzed by electrophoresis and autoradiography.

CBF1 reporter assay

NIH3T3 cells in 6-well plates were transfected at 50% confluence with 0.5 μg of Notch1, 0.5 μg of CBF1 firefly luciferase reporter, 0.05 μg of *Renilla* luciferase (pRL-TK), 0.5 μg of ADAM12, ADAM12 E349Q, ADAM9, ADAM15, ADAM17 or empty pcDNA3.1 vector, and 0.5 μg of Dll1 or empty pIRES-puro vector. Twenty-four hours after transfection, CHO-K1 cells stably transfected with c-Myc-Dll1 or with empty vector were added (10^6 cells/well) and co-cultured for an additional 24 h. Firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase reporter assay system (Promega). The activity of *Renilla* luciferase was used as an internal control for transfection efficiency.

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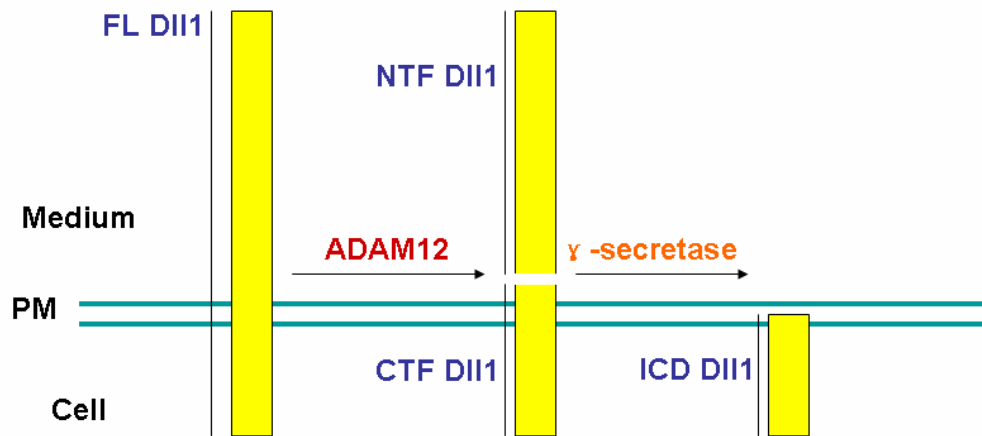
Figure 2-1. **Schematic diagram of the proteolytic processing of Dll1.** The full length Dll1 (FL Dll1, 90kDa, yellow) is cleaved by ADAM12 (red) to generate the C-terminal Dll1 fragment (CTF Dll1, 29kDa, yellow) and the N-terminal fragment of Dll1 (NTF Dll1, 60kDa, yellow). The CTF Dll1 containing the transmembrane and intracellular domain is then cleaved by γ-secretase (orange) to produce the intracellular domain of Dll1 (ICD Dll1, 26kDa, yellow). The soluble NTF Dll1 is released to medium.

Figure 2-2. **Proteolytic processing of Dll1 by ADAM12 increases Notch signaling in a cell-autonomous manner.** A, NIH3T3 cells were co-transfected with mouse Notch1 and either a CBF1 reporter (CBF1-Luc; pJH23A) or the same reporter in which CBF1 binding sites were mutated (Mut CBF1-Luc; pJH25A). After 24 h, cells were co-cultured with CHO cells stably transfected with vector only (CHO.Vec; white bars) or with CHO-cells stably transfected with Dll1 (CHO.Dll1; black bars). The activities of firefly luciferase, normalized to *Renilla* luciferase as internal control, were assayed 24 h later. B, NIH3T3 cells were transiently co-transfected with Notch1, a CBF1 reporter (pJT123A) and Dll1, wild type ADAM12 (A12), or catalytically inactive ADAM12 (E349Q A12) as indicated. After 24 h, cells were co-cultured for an additional 24 h with CHO.Vec (white bars) or CHO.Dll1 cells (black bars) followed by measurement of luciferase activity. Notice that co-expression of Dll1 with Notch in the same cell inhibits Notch signaling induced by CHO.Dll1 (black bars) in the absence of A12 or in the presence of the E349Q mutant (*, $p<0.05$). In the presence of catalytically active A12 the inhibition was diminished and was not statistically significant. In A and B, error bars represent S.E. of the mean ($n = 3$).
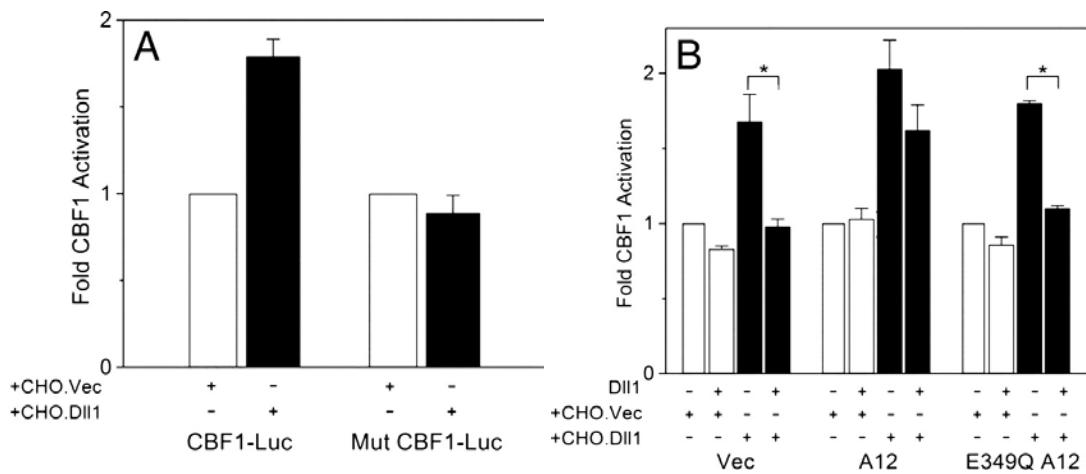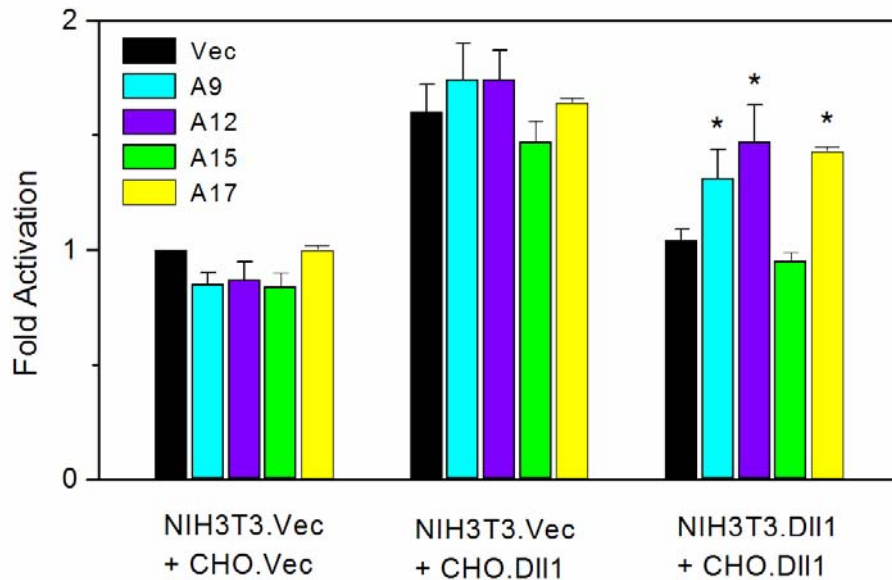
Figure 2-3. **Proteolytic processing of Dll1 by ADAM9, -12 and -17 increases Notch signaling in a cell-autonomous manner.** NIH3T3 cells were transiently co-transfected with Notch1, a CBF1 reporter (pJT123A) and the empty pIRES-puro vector or Dll1, the empty pCDNA3.1 vector (Vec, black), ADAM9 (A9, blue), ADAM12 (A12, purple), ADAM15 (A15, green) or ADAM17 (A17, yellow) as indicated. After 24 h, cells were co-cultured for an additional 24 h with CHO.Vec or CHO.Dll1 cells followed by measurement of luciferase activity. Notice that co-expression of Dll1 with Notch in the same cell inhibits Notch signaling induced by co-culture with CHO.Dll1 cells in the empty pCDNA3.1 vector-transfected NIH3T3 cells. In the presence of A9, A12 and A17 the inhibition was diminished (*, $p<0.05$), while in presence of A15, the inhibition remained. Error bars represent S.E. of the mean (n = 3).
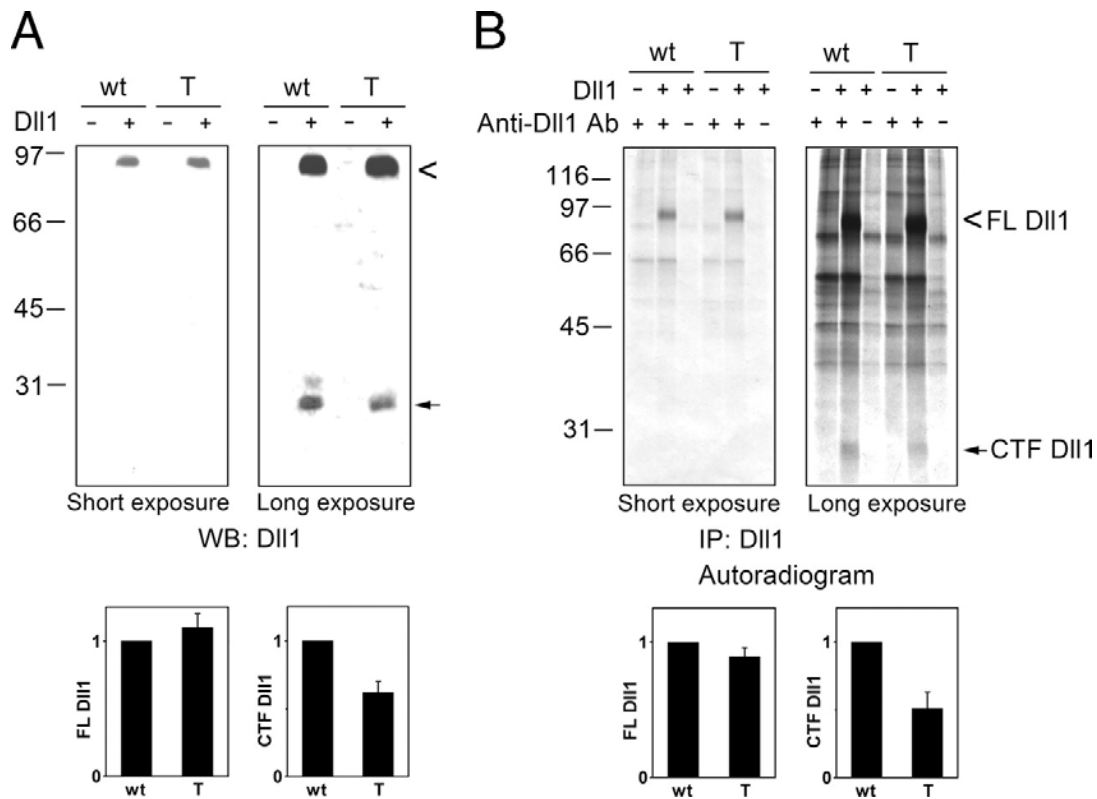
Figure 2-4. **Endogenous ADAM9 and/or ADAM12 contribute to Dll1 cleavage in MEFs.** SV40-immortalized MEFs isolated from wild type mice (wt) or from ADAM9/12/15-/- triple knock-out mice (T) were transiently transfected to express Dll1 as indicated. A, after 48 h, Dll1 processing was analyzed by Western blotting with anti-Dll1 antibody. The intensities of the bands corresponding to FL Dll1 were determined after short exposure times (left), and the intensities of the bands representing CTF Dll1 were measured after long exposure times (right). The amounts of FL Dll1 and CTF Dll1 in T-MEFs were normalized to the amounts in wt-MEFs; the results (mean from three different experiments, ± S.E.) are shown below each Western blot. B, 24 h after transfection cells were metabolically labeled with [^{35}S]methionine + cysteine, and 16 h later cell lysates were subjected to immunoprecipitation using anti-Dll1 antibody, as indicated. The immunoprecipitates were resolved by SDS-PAGE and analyzed by autoradiography. The intensities of the bands corresponding to FL Dll1 and CTF Dll1 were determined after short exposure times (left) and long exposure times (right), respectively, and the results were plotted as in A (mean ± S.E., n = 2). In A and B, cells were incubated for 6 h in the presence of 1 μM γ-secretase inhibitor L685,458 before harvesting.

CHAPTER 3

THE ROLE OF DELTA-LIKE 1 SHEDDING IN MUSCLE CELL SELF-RENEWAL AND DIFFERENTIATION

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The role of Delta-like 1 shedding in muscle cell self-renewal and differentiation.

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Introduction

Skeletal muscle development and regeneration in vertebrates requires a careful balance between myogenic differentiation and the maintenance of progenitor cells (Buckingham, 2006). During embryonic development, myogenic progenitor cells give rise to myoblasts that further undergo skeletal muscle differentiation. A population of progenitor cells is set aside and later these progenitors generate satellite cells. Satellite cells are the primary stem cells of post-natal skeletal muscle (Dhawan and Rando, 2005; Collins, 2006; Shi and Garry, 2006; Zammit et al., 2006; Le Grand and Rudnicki, 2007). During muscle growth or regeneration after injury, quiescent satellite cells become activated, proliferate, and then either differentiate or return to the satellite quiescent state. The ability to adopt two divergent fates, differentiation or entry into an un-differentiated quiescent state, is maintained by myogenic cells *in vitro*. Studies utilizing isolated myofibers or myogenic cell cultures show that activated, proliferating satellite cells express both Pax7, a paired-box transcription factor, and MyoD, a basic helix-loop-helix myogenic determination factor. Some cells then down-regulate Pax7, maintain MyoD, and differentiate, and some cells down-regulate MyoD, maintain the expression of Pax7, and remain un-differentiated (Halevy et al., 2004; Zammit et al., 2004). Since quiescent Pax7$^+$/MyoD$^-$ cells generated *in vitro* resemble quiescent satellite cells, the mechanisms regulating the generation of the pool of Pax7$^+$/MyoD$^-$ cells may be similar to the mechanisms involved in satellite cell self-renewal (Zammit et al., 2004). Consistently, in the absence of MyoD, satellite cells show an increased propensity for self-renewal rather than differentiation, which results in a deficit in muscle regeneration (Megeney et al., 1996; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999).

The Notch pathway is an evolutionary conserved signaling mechanism that plays critical roles in cell fate decisions during embryonic development and in the adult. The pathway is activated when one of the Notch ligands, a transmembrane protein present at the surface of a signal-sending cell, binds to a Notch receptor present in a signal-receiving cell. In mammals, there are five Notch ligands, (Delta-like 1, 3, and 4, and Jagged 1 and 2) and four Notch receptors (Notch 1-4). The ligand-receptor interaction is followed by the sequential cleavage of the receptor by an ADAM protease and by γ-secretase, leading to the release of the intracellular domain of Notch, NICD, from the plasma membrane and its translocation to the nucleus. Inside the nucleus, NICD forms a complex with the transcription factor CBF1 (also known as RBP-J)

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and the coactivator Mastermind, and it activates target gene expression (Kadesch, 2004; Bray, 2006; Hurlbut et al., 2007).

The Notch pathway is a critical regulator of myogenesis in cultured myogenic cells, in vertebrate embryos, and in post-natal regenerating muscle (Luo et al., 2005; Vasyutina et al., 2007a). Early muscle development, as well as activation of satellite cells upon muscle injury, are accompanied by activation of Notch 1 signaling (Conboy and Rando, 2002; Brack et al., 2008). Notch signals inhibit myogenesis by blocking the expression and activity of the myogenic determination factor MyoD (Kopan et al., 1994; Shawber et al., 1996; Kuroda et al., 1999; Wilson-Rawls et al., 1999). Consequently, manipulations that activate the Notch pathway inhibit myogenic differentiation and manipulations that decrease the level of Notch signaling promote differentiation. Ectopic expression of a constitutively active form of Notch 1 in myogenic cells cultured *in vitro* (Kopan et al., 1994; Shawber et al., 1996; Conboy and Rando, 2002) or co-culture of myogenic cells with cells overexpressing Notch ligands (Lindsell et al., 1995; Shawber et al., 1996; Jarriault et al., 1998; Kuroda et al., 1999) blocks myogenic differentiation. Overexpression of Numb, a negative regulator of Notch (Conboy and Rando, 2002; Kitzmann et al., 2006), or inhibition of γ-secretase activity (Kitzmann et al., 2006) promotes cell differentiation. Constitutive activation of Notch signaling in muscle cells during chick limb development by overexpression of Delta1 prevents MyoD expression and leads to inhibition of myogenesis *in vivo* (Delfini et al., 2000; Hirsinger et al., 2001). Furthermore, myoblasts lacking Stra13, a basic helix-loop-helix transcription factor that modulates Notch signaling, exhibit increased proliferation and defective differentiation (Sun et al., 2007). Megf10, a novel multiple epidermal growth factor repeat transmembrane protein that impinges on Notch signaling stimulates myoblast proliferation and inhibits differentiation (Holterman et al., 2007).

Two recent studies have directly established that Notch signaling is critical for muscle development in the mouse. These studies also revealed that in addition to being a negative regulator of myogenic differentiation, Notch is a positive and essential regulator of muscle progenitor cells. First, Dll1 hypomorph mutant mice show premature myoblast differentiation in the embryo, depletion of progenitor cells, and severe muscle hypotrophy (Schuster-Gossler et al., 2007). Second, conditional mutagenesis of RBP-J in mice results in a similar premature differentiation, depletion of progenitor cells, and lack of muscle growth (Vasyutina et al., 2007b). Both studies indicate that Notch signaling initiated by Dll1 ligand and mediated by RBP-

J is essential for maintaining a resident pool of myogenic progenitor cells and preventing their differentiation during muscle development. In the adult, Notch plays an important role in satellite cell expansion during muscle regeneration, and inadequate Notch signaling caused by reduced expression of Dll1 in aging muscle contributes to the loss of its regenerative potential (Conboy et al., 2003).

In summary, it appears that the two pools of cells that are generated simultaneously during myogenesis *in vivo* or in tissue culture, i.e., terminally differentiated cells and undifferentiated cells with progenitor-like properties, have opposing requirements for Notch signaling. While Notch activation must be relieved in cells progressing into differentiation, Notch signaling must be sustained (or elevated) in progenitors/self-renewing satellite cells/reserve cells to prevent their differentiation. The mechanisms responsible for the regulation of Notch activity in a population of myogenic cells are not well understood.

We and others have shown that, in certain cell systems, the extracellular domains of several Notch ligands, including Dll1, are shed from the cell surface by ADAM proteases (Ikeuchi and Sisodia, 2003; Six et al., 2003; Dyczynska et al., 2007). Ligand shedding down-regulates Notch signaling in neighboring cells (Mishra-Gorur et al., 2002) and may stimulate Notch signaling in a cell-autonomous manner (Dyczynska et al., 2007). Modulation of the Notch pathway by ligand shedding plays an important role in the developing wing in *Drosophila* (Sapir et al., 2005) and in cortical neurogenesis in mice (Muraguchi et al., 2007). The extent of ligand shedding and its potential role in modulating Notch signaling during myogenic differentiation has not been examined.

In this study, we show that Notch signaling is required for maintaining Pax7 expression in cultures of differentiating mouse primary myoblasts and C2C12 cells. Furthermore, stimulation of Notch activity increases expression of Pax7 and, consistent with previous reports, inhibits myogenic differentiation. Dll1 is proteolytically processed in a pool of C2C12 reserve cells that are Pax7-positive, quiescent and un-differentiated, but Dll1 remains intact in differentiated myotubes. Incubation of primary myoblasts or C2C12 cells with a soluble, dominant-negative mutant form of ADAM12 leads to inhibition of Dll1 cleavage, elevation of Notch signaling, expansion of the pool of Pax7^{+}/MyoD^{-} cells, and reduction of the number of Pax7^{+}/MyoD^{+} cells. We propose that the proteolytic processing of Dll1, a stochastic event, helps achieve an

asymmetry in Notch signaling in a pool of initially equivalent myogenic cells and helps sustain the balance between differentiation and maintenance of undifferentiated cells.

Results

It has been shown previously that Notch is activated during satellite cell activation *in vivo*, and that active Notch is present in proliferating primary myoblasts *in vitro*, where it enhances myoblast proliferation and inhibits differentiation (Conboy and Rando, 2002). We examined the amount of active Notch in cultures of myogenic cells at the stage when most of the cells exit the cell cycle and undergo differentiation. Primary mouse myoblasts or C2C12 mouse myogenic cells were incubated in growth medium (GM) containing 10% FBS for 24-48 hours until they were 90-100% confluent, then they were transferred to differentiation medium (DM) containing 2% HS and were incubated for additional 3 days. Early differentiation markers myogenin and cell cycle inhibitor p21 increased during incubation of cells in DM, indicating that some cells progressed into differentiation (Fig.3-1A). MyoD and Pax7, a marker of non-differentiating cells, were expressed in cells in GM and remained high in cells incubated in DM (Fig.3-1A). The number of proliferating primary cells that incorporated BrdU after the 3-hour pulse labeling decreased from ~55% at day 0 to ~10% at day 3 in DM, and the number of BrdU-labeled C2C12 cells decreased from ~70% at day 0 to ~5% at day 3 (Fig.3-1B). Importantly, the level of active Notch 1, determined by Western blotting using epitope-specific anti-Notch 1 antibody that recognizes NICD only after Notch 1 is cleaved by γ-secretase, declined much less dramatically (by ~15% in primary cells and by ~40% in C2C12 cells) between day 0 and day 3 in DM (Fig.3-1AB). After separation of Pax7$^-$ differentiated C2C12 myotubes from Pax7$^+$ undifferentiated reserve cells at day 3, NICD was detected exclusively in the reserve cells fraction (Fig.3-1C). These results suggest that there is no direct correlation between the number of proliferating cells and the total Notch activity in cultures of differentiating myogenic cells. Instead, Notch 1 remains active in a population of Pax7$^+$ cells that have stopped proliferation and remain undifferentiated.

To examine the role of Notch in maintaining the pool of Pax7$^+$ cells, primary myoblasts were incubated for 1 day in DM in the presence of GM6001, a broad-spectrum metalloproteinase inhibitor (Grobelny et al., 1992), or DAPT, a potent and selective inhibitor of γ-secretase activity (Dovey et al., 2001). GM6001, by inhibiting ADAM-mediated cleavage of Notch at the S2 site,

prevents the subsequent cleavage at the S3 site by γ-secretase, whereas DAPT directly blocks cleavage and activation of Notch by γ-secretase. Indeed, as shown in Fig.3-2A, both GM6001 and DAPT treatment effectively eliminated the active Notch 1, NICD. After treatment with DAPT, the levels of MyoD, myogenin, and p21 were slightly increased, consistent with stimulation of myogenic differentiation upon inhibition of Notch activity (Conboy and Rando, 2002; Kitzmann et al., 2006). Notably, both GM6001 and DAPT dramatically decreased the expression level of Pax7 (Fig.3-2AB), indicating an absolute requirement for the active Notch in the maintenance of Pax7-positive cells. Interestingly, the level of Pax3, the paralogue of Pax7 with partially overlapping functions in myogenic cells (Relaix et al., 2006; Buckingham and Relaix, 2007), was not strongly affected by DAPT treatment (Fig.3-2A). Inhibition of Notch by DAPT in C2C12 cells produced similar effects, although inhibition of Pax7 expression was not as potent as in primary myoblasts (Fig.3-2C).

To explore whether increasing Notch activity has any effect on Pax7, we first infected primary myoblasts with retroviruses encoding a constitutively active Notch 1, caNotch. caNotch lacks a major portion of the extracellular domain and is processed to NICD in a ligand-independent manner (Ohtsuka et al., 1999). In contrast to the endogenous NICD which is very hard to visualize by immunofluorescence microscopy (Schroeter et al., 1998), the exogenous NICD can be easily detected in the nuclei of infected cells. We observed that the number of Pax7^{+} cells was ~2-fold higher among NICD-positive than NICD-negative cells on the same microscopic slide (Fig.3-3A). Pax7 expression was not changed in cells infected with control virus (bearing a truncated, inactive Notch 1, detected with anti-FLAG antibody; Fig.3-3A). In an alternative approach to stimulate the Notch pathway, primary myoblasts or C2C12 cells were co-cultured with CHO cells stably transfected with mouse Dll1 or with empty vector (Dyczynska et al., 2007). As reported previously (Lindsell et al., 1995; Shawber et al., 1996; Jarriault et al., 1998; Kuroda et al., 1999), co-culture with Dll1-transfected cells inhibited myogenic differentiation, as judged by decreased expression of MyoD and myogenin (Fig.3-3B). Importantly, the level of Pax7 both in primary myoblasts and in C2C12 cells co-cultured with Dll1-transfected CHO cells was dramatically increased (Fig.3-3B). Collectively, these results indicate that Notch is a critical regulator of the balance between Pax7^{+} and Pax7^{-} cells.

The requirement for the active Notch in Pax7-positive cells and decline of Notch activity in differentiating cells suggest that there must be a heterogeneity in Notch signaling among post-

mitotic myogenic cells cultured *in vitro*. To get insight into possible mechanisms responsible for the heterogenic levels of Notch signaling, we examined the expression, distribution, and proteolytic processing of Dll1, a Notch ligand that plays critical roles in muscle development *in vivo* (Schuster-Gossler et al., 2007). Similar to the proteolytic processing of Notch, mammalian Dll1 undergoes a sequential cleavage by ADAM proteases and then by γ-secretase (Ikeuchi and Sisodia, 2003; Six et al., 2003; a diagram is depicted in Fig.3-4A). The immediate consequence of ADAM-mediated shedding of the extracellular domain of Dll1 is down-regulation of Notch signaling in neighboring cells (Mishra-Gorur et al., 2002; Muraguchi et al., 2007; Sapir et al., 2005) and, possibly, activation of Notch signaling in the same cell (Dyczynska et al., 2007). We have previously observed a cleaved form of the endogenous Dll1 in cultures of primary myogenic cells (Dyczynska et al., 2007). Here, we have used cultures of C2C12 cells incubated for 3 days in DM to separate well-differentiated myotubes (Pax7-negative, low Notch activity) from reserve cells (Pax7-positive, high Notch activity; see Fig.3-1C). When total cell extract was subjected to Western blotting using antibody specific for the C-terminus of Dll1, three Dll1 bands were detected: the ~90-kDa full-length form (Dll1^{FL}), the 29-kDa ADAM-cleavage product spanning the transmembrane and the intracellular domains of Dll1 (Dll1^{TMIC}), and the 26-kDa γ-secretase cleavage product comprising the intracellular domain and a short C-terminal segment of the transmembrane domain (Dll1^{IC}). Remarkably, after separation into the myotube and reserve cell fractions, Dll1^{TMIC} and Dll1^{IC} were present only in reserve cells, and no Dll1^{TMIC} or Dll1^{IC} were observed in myotubes (Fig.3-4B). In contrast, Dll1^{FL} was more abundant in myotubes than in reserve cells (Fig.3-4B). This indicates that the proteolytic processing of Dll1 in differentiating C2C12 cells is asymmetrical, with significantly more cleavage detected in Pax7-positive reserve cells than in Pax7-negative myotubes.

To determine whether the asymmetrical cleavage of Dll1 in Pax7-positive *vs* Pax7-negative cells is a mere consequence of different proteolytic activities in these two populations of cells or whether it plays a more direct, causal role in establishing an imbalance in Notch signaling and generating two pools of myotubes and reserve cells, we intended to inhibit ADAM-mediated cleavage of Dll1, the first and obligatory step in Dll1 processing. Since several different ADAM proteases expressed in myogenic cells are capable of cleaving Dll1, including ADAM9, 10, 12, and 17 (Dyczynska et al., 2007), and since ADAM10 and ADAM17 also cleave Notch (Brou et al., 2000; Hartmann et al., 2002; Mumm et al., 2000), we adopted a dominant-negative approach

rather than knocking down expression of individual ADAMs or using pharmacological inhibitors of ADAM activities. We showed previously that ADAM12 cleaves Dll1 but it does not process Notch, and that ADAM12 forms complexes with Dll1 (Dyczynska et al., 2007). Here, we used the soluble extracellular domain of the catalytically inactive mutant form of ADAM12, expressed and purified from *Drosophila* S2 cells (recombinant protein X, Fig.3-5A), to block the processing of Dll1 by endogenous ADAM proteases. When COS-7 cells were transfected to express murine Dll1, the Dll1^{FL} and Dll1^{TMIC} forms were observed in Western blots (Fig.3-5B; Dll1^{TMIC} is the predominant cleaved form and Dll1^{IC} is poorly detected when Dll1 is overexpressed (Ikeuchi and Sisodia, 2003; Six et al., 2003; Dyczynska et al., 2007)). In the presence of exogenously added purified protein X, the extent of Dll1 cleavage was reduced by ~50% (Fig.3-5B), which validated the use of protein X as a dominant-negative modulator of Dll1 cleavage. In primary myoblasts incubated for 1 day in differentiation medium containing protein X, the level of Notch signaling was increased, as demonstrated by the elevated amount of NICD (Fig.3-5C.a). Furthermore, a higher transcriptional activity of the CBF1-luciferase reporter gene was observed in primary myoblasts or C2C12 cells incubated in the presence of protein X (Fig.3-5C.b). Our interpretation of these results is that protein X, by binding to Dll1, prevents its cleavage by ADAMs but it does not interfere with Dll1 binding and activation of Notch in *trans*. Thus, higher level of Notch activity in the presence of protein X suggests that ADAM-mediated shedding of Dll1 contributes to down-regulation of Notch signaling in a pool of cells during myogenic differentiation *in vitro*.

Down-regulation of Dll1 cleavage by protein X had also a negative effect on the progression through a myogenic lineage, as the total levels of MyoD, myogenin, and p21 were decreased by ~50%, 25%, and 25%, respectively (Fig.3-6A). In contrast, expression of Pax7 was slightly increased after 1 day incubation of cells in differentiation medium containing protein X (Fig.3-6A). The effect of protein X on the level of MyoD, myogenin, p21, and Pax7 were abolished in the presence of γ-secretase inhibitor DAPT (Fig.3-6A), suggesting that the effect of protein X was mediated through the activation of Notch signaling, as shown in Fig.3-5C. Similar inhibition of MyoD, myogenin, and p21 expression and elevation of Pax7 expression was observed in C2C12 cells upon incubation of cells in DM supplemented with protein X (Fig.3-6B). Furthermore, immunofluorescence analysis of cells using anti-MyoD and anti-Pax7 antibody demonstrated that protein X specifically decreased the pool of Pax7$^+$/MyoD$^+$ myoblasts

and increased the pool of Pax7$^+$/MyoD$^-$ myoblasts, whereas the pool of Pax7$^-$/MyoD$^+$ myoblasts did not seem to be affected (Fig.3-6C). These results suggest that the shedding of Dll1, which is partially blocked in the presence of protein X, is important in maintaining the balance between Pax7$^+$/MyoD$^+$ and Pax7$^+$/MyoD$^-$ cells.

Discussion

This study provides an insight into the role of Notch signaling in sustaining the balance between myogenic differentiation and the maintenance of undifferentiated cells *in vitro*. Our studies suggest that the proteolytic processing of Dll1, a Notch ligand, plays an important role in modulation of Notch signaling and myogenic cell fate determination.

It has been previously shown that the Notch pathway is critical for satellite cell activation and myogenic precursor cell expansion in postnatal myogenesis (Conboy and Rando, 2002). New genetic evidence indicates that Notch signaling initiated by Dll1 and mediated by RBP-J is essential for maintaining a pool of myogenic progenitor cells and for preventing their differentiation during muscle development in mice (Schuster-Gossler et al., 2007; Vasyutina et al., 2007b). In accordance with these studies, we find that Notch signaling is critical in maintaining expression of Pax7, a marker of the undifferentiated state, in quiescent myoblasts *in vitro*. Inhibition of the Notch pathway using pharmacological inhibitors of either ADAM proteases (GM6001) or γ-secretase (DAPT) abolishes Pax7 expression, and stimulation of Notch signaling expands the pool of Pax7$^+$ cells.

Our results differ from those obtained by Conboy and Rando, where down-regulation of Notch signaling in differentiating myoblast by retrovirally delivered Notch antagonist Numb did not have an effect on the level of Pax7 (Conboy and Rando, 2002). It is possible that application of pharmacological inhibitors of the Notch pathway in our studies might have resulted in a more complete and uniform inhibition of Notch signaling than expression of Numb. Our results are more in line with the study by Kuang et al., in which treatment of freshly isolated proliferating satellite cells with DAPT for 3 days of culture significantly reduced the total number of cells due to a decrease of the number of Pax7$^+$/MyoD$^-$ cells (Kuang et al., 2007). While the results of Kuang et al. further support the notion that Notch signaling is vital for satellite cell expansion, our results indicate that Notch is also required to maintain Pax7 expression in a pool of quiescent myoblasts, after they exit the cell cycle. Interestingly, forced expression of Delta1 and activation

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of the Notch pathway during early avian myogenesis *in vivo* resulted in down-regulation of MyoD and complete lack of differentiated muscles, but the exit from the cell cycle was not blocked, suggesting that Notch signaling acts in post-mitotic myogenic cells to control a critical step of muscle differentiation (Delfini et al., 2000; Hirsinger et al., 2001). Thus, Notch signaling acts at multiple steps of the muscle development and regeneration processes.

We propose a model in which the level of Notch activity plays a crucial role in cell fate determination after myoblasts exit the cell cycle (Fig.3-7). According to this model, the Notch pathway is turned on in activated, proliferating satellite cells that are $Pax7^+/MyoD^+$. Upon cell cycle exit, Notch signaling is down-regulated in a subset of $Pax7^+/MyoD^+$ cells and it is maintained (or further up-regulated) in self-renewing $Pax7^+/MyoD^-$ cells that replenish the pool of satellite cells. As $Pax7^+/MyoD^+$ cells progress into differentiation, they express myogenin, a negative regulator of Pax7 expression (Olguin et al., 2007), and become Pax7-negative. The loss of Pax7-positive cells in DAPT-treated cultures may thus be related to the effect of Notch on MyoD: low levels of Notch signaling promote high MyoD, induction of myogenin and, in consequence, loss of Pax7. In contrast, expansion of Pax7-positive cells observed after stimulation of the Notch pathway may be a consequence of decreased MyoD and myogenin expression. If this is the case, modulation of Pax7 expression by Notch signaling should be blunted in MyoD-/- myoblasts, a prediction that remains to be tested. To our knowledge, Pax7 is not directly regulated by any of the known Notch target genes.

If the level of Notch signaling is set at different levels in $Pax7^-/MyoD^+$ and $Pax7^+/MyoD^-$ cells, the question remains: How are these different levels of Notch signaling simultaneously and spontaneously achieved in two pools of initially equivalent myogenic cells? One mechanism could involve an asymmetric cell division that generates two daughter cells: one with a high Notch activity and one with a low Notch activity (Kuang et al., 2008). Numb is distributed asymmetrically during satellite cell division and it has been postulated that the cell inheriting Numb is the one that acquires low Notch activity and proceeds into differentiation (Conboy and Rando, 2002). However, Numb has been also shown to be asymmetrically segregated to cells that inherit all the older template DNA strands (Shinin et al., 2006), suggesting that Numb-receiving cells are self-renewing (according to the immortal DNA strand hypothesis; Cairns, 1975), rather than differentiating ones. Furthermore, the level of Numb increases significantly after the onset of differentiation (Conboy and Rando, 2002) and, in chick embryo, it is promoted

by MyoD expression (Holowacz et al., 2006). This pattern of Numb expression suggests that Numb may reinforce, rather than initiate, the low Notch activity in differentiating cells. In addition, the results presented here and in several other reports (Lindsell et al., 1995; Shawber et al., 1996; Jarriault et al., 1998; Kuroda et al., 1999) have demonstrated that co-culture of myoblasts with Dll1- or Jagged1-overexpressing cells inhibits myogenic differentiation, suggesting that limited ligand availability rather than Numb, may be responsible for the decline of Notch activity in differentiating myogenic cells.

Recent studies indicate that satellite cells are a mixture of stem cells and committed myogenic progenitors (Collins, 2006; Kuang et al., 2007; Zammit et al., 2006) and that asymmetric division of stem cells *in vivo* yields one stem cell and one committed daughter cell (Kuang et al., 2007). The two daughter cells show asymmetric expression of Dll1, with higher Dll1 level (and most likely lower Notch activity) in the committed cell. This asymmetric cell division is favored by a specific stem cell niche and it occurs perpendicular to the muscle fiber, with Dll1 being expressed in the cell that maintains contact with the plasmalemma (Kuang et al., 2007). Whether such oriented cell division with asymmetric expression of Dll1 takes place in satellite cells cultured *in vitro* and deprived of the niche regulation is not clear. It appears that the modulation of Notch signaling among cells cultured *in vitro* may be achieved in large part by stochastic mechanisms (Losick and Desplan, 2008). We propose that one of these mechanisms involves the proteolytic processing of Dll1 by ADAM proteases (Fig.3-7), a hypothesis supported by two observations. First, in C2C12 cells, we detect the cleaved Dll1 in undifferentiated reserve cells but not in differentiated myotubes. Second, inhibition of Dll1 processing by soluble, catalytically inactive extracellular domain of ADAM12, protein X, elevates the global Notch signaling and increases the pool of Pax7$^+$/MyoD$^-$ cells, with the concomitant decrease of the pool of Pax7$^+$/MyoD$^+$ cells (Figs.3-5 and 3-6). These studies confirm and extend our previous observations obtained for C2C12 cells, where the soluble protein X inhibited myogenic differentiation (Yi et al., 2005) and overexpression of the wild-type ADAM12 decreased MyoD expression (Cao et al., 2003).

According to the model in Fig.3-7, Dll1 shedding helps establish a balance between Pax7$^+$/MyoD$^+$ and Pax7$^+$/MyoD$^-$ cells after the exit from the cell cycle. Proteolytic processing of Dll1 by ADAMs in some cells leads to ligand depletion and down-regulation of Notch signaling in neighboring cells. Cells in which Dll1 cleavage takes place would acquire higher level of

Notch activity than their neighbors, leading to down-regulation of MyoD. Cells in which the cleavage of Dll1 does not occur or occurs less efficiently would attain lower level of Notch signaling and maintain MyoD expression. Inhibition of Dll1 processing by soluble protein X did not seem to have a direct effect on the number of Pax7$^-$/MyoD$^+$ cells (Fig.3-6C), and thus the balance between Pax7$^+$/MyoD$^+$ and Pax7$^-$/MyoD$^+$ cells may not be controlled by the cleavage of Dll1. Furthermore, since MyoD is a positive regulator of Delta-1 in *Xenopus* (Wittenberger et al., 1999), it is possible that MyoD stimulates Dll1 expression and further up-regulates Notch signaling in neighboring Pax7$^+$/MyoD$^-$ cells. This would provide another means to increase Dll1 expression in cells with declining Notch activity, in addition to the relief of the transcriptional repression mediated by Notch (Greenwald, 1998; Wilkinson et al., 1994). Stimulation of Numb expression by MyoD (Holowacz et al., 2006), on the other hand, should down-regulate Notch and further consolidate the differences in Notch signaling among MyoD$^+$ and MyoD$^-$ cells.

While other stochastic models of myogenic cell fate determination invoke random changes in the level of expression of the Notch pathway components, myogenic factors, or Pax7, we place the main emphasis on the proteolytic processing of Dll1 as the initial trigger of the asymmetry in Notch signaling between seemingly equivalent cells. Since Dll1 cleavage occurs at the cell surface, is cell-density dependent, and may be influenced by intracellular events (Dyczynska et al., 2007; Zolkiewska, 2008), it combines features of a stochastic, as well as regulated mechanism of Notch modulation. Finally, our model is not mutually exclusive with oriented cell division, which may be most relevant *in vivo* where it becomes subject to niche regulation. The extent to which Dll1 shedding contributes to the regulation of Notch signaling and myogenic cell fate determination during muscle development, growth, and regeneration *in vivo* remains to be determined.

Materials and Methods

Expression constructs

Dll1-pcDNA3.1 expression vector has been described previously (Dyczynska et al., 2007; 2008). Notch reporter vector containing eight CBF-1 binding sites (pJT123A) was provided by P. D. Ling (Baylor College of Medicine). caNotch-AP retroviral vector directed expression of the constitutively active mouse Notch 1 spanning the transmembrane region, the RAM23 domain, the cdc10/ankyrin repeats, and the nuclear localization signal (aa 1704-2192), and c-AP vector

lacking the RAM23 and cdc10/ankyrin repeats sequences, was a negative control (Ohtsuka et al., 1999). caNotch-AP and c-AP vectors were obtained from R. Kageyama and C. Takahashi (Kyoto University).

Cells

C2C12 and COS-7 cells were obtained from American Tissue Culture Collection; *Drosophila* S2 cells were from Invitrogen; the retroviral packaging cell line Phoenix Eco was provided by Dr. Garry P. Nolan (Stanford University). C2C12, COS-7, and Phoenix Eco cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37˚C in the presence of 5% CO_2 under a humidified atmosphere. CHO cells stably transfected with mouse Dll1 or with empty vector were grown in F12K nutrient mixture supplemented with 10% FBS and 800 μg/ml G418, as described (Dyczynska et al., 2007). Drosophila S2 cells were cultured at 27˚C in Schneider's Drosophila medium containing 10% heat inactivated FBS. Primary myoblasts were isolated from hindlimbs and forelimbs of neonatal C57BL/6 mice (2-5 days old) as described (Rando and Blau 1994). The muscle tissue was incubated in Dulbecco's phosphate-buffered saline (DPBS) with 1% collagenase II (Invitrogen), 2.4 U/ml dispase II (Roche) and 2.5 mM $CaCl_2$ for 45 minutes at 37˚C, and then passed through 100-μm nylon mesh filter (BD Biosciences). The filtrate was centrifuged, the cell pellet was suspended in Ham' F-10 medium (Cambrex) containing 20% FBS and 1% penicillin/streptomycin, pre-plated for 30 minutes on collagen I-coated plates, and then plated on tissue culture-treated plastic plates. To stimulate differentiation, 90-100% confluent primary myoblasts or C2C12 cells were transferred to DMEM containing 2% horse serum.

Plasmid transfection and retroviral infection

Transient transfections were performed using Fugene 6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol, one day after plating cells. For generation of retroviruses, virus packaging Phoenix Eco cells were transfected with a retroviral expression vector (15 μg plasmid DNA per 100-mm plate) using calcium phosphate precipitation method, viral supernatants were harvested 48 hours later, supplemented with 5 μg/ml polybrene, and used to infect primary myoblasts.

Protein expression and purification

The cDNA fragment encoding the extracellular domain of ADAM12 (protein X, amino acids 32–707, containing the E349Q mutation) was cloned into the pMT/BiP/V5-HisA vector (Yi et al. 2005). *Drosophila* S2 cells stably transfected with protein X (Yi et al. 2005) were incubated for 5 days in the presence of 0.5 mM $CuSO_4$. Culture medium was collected 5 days later, and protein X was purified by sequential chromatography on a chelating Sepharose column (Amersham Biosciences) and a nickel-nitrilotriacetic acid-agarose column (Qiagen) (Yi et al., 2005). The final column eluate was dialyzed against DMEM, supplemented with 2% HS or 10% FBS, and added to cells.

Cell treatments

Primary myoblasts were cultured in growth medium until 90%-100% confluency and then they were incubated for 24 hours in differentiation medium (DMEM plus 2% horse serum) with 1 μM DAPT (Calbiochem), 5 μM GM6001 (Chemicon; both dissolved in DMSO), or DMSO alone. Confluent C2C12 cells were incubated for 1 day in DM with 5 μM DAPT or DMSO. In the experiments analyzing the effect of protein X, medium was prepared by adding 2% HS (for primary myoblasts and C2C12 cells) or 10% FBS (for COS-7 cells) directly to the solution of protein X (final concentration: 2 μM) dialyzed against DMEM or to DMEM that was retrieved as the external dialysis solution. For analysis of cell proliferation, primary myoblasts or C2C12 cells were incubated with 10 μM 5-Bromo-2'-deoxyuridine (BrdU) for 3 hours prior to fixation and staining with anti-BrdU antibody. For ^{35}S -labeling, C2C12 cells were incubated for 3 days in DM and then for 16 h in methionine/cysteine-free DM containing EasyTag 200 μCi/ml Expre[^{35}S][^{35}S]-Protein Labeling Mix (PerkinElmer).

Separation of myotubes and reserve cells

Differentiating cultures of C2C12 cells were separated into myotubes and reserve cells essentially as described earlier (Kitzmann et al., 1998; Cao et al., 2003). C2C12 cells were incubated for 3 days in DM and subjected to mild trypsinization (0.05% trypsin and 1 mM EDTA in DPBS, 1 minute treatment). Detached myotubes were collected first and the remaining undifferentiated reserve cells were detached by 5 min incubation with 0.25% trypsin and 1 mM

EDTA in DPBS. The purity of the myotube and reserve cell fractions was assessed by Western blotting with anti-integrin α7A and anti-Pax7 antibodies, respectively.

Cell co-culture experiments

Primary myoblasts or C2C12 cells were plated in 6-well plates. One day later, when cells were ~70% confluent, CHO cells stably transfected with mouse Dll1 or empty vector were added (5 x 10^5 cells/well) and incubated in DM without G418. Twenty four hours later, cells were washed and 300 μl of extraction buffer was added to wells.

Western blotting

Cells were incubated in extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (AEBSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 mM 1,10-phenanthroline) for 15 minutes at 4˚C. Cell extracts were centrifuged at 21,000xg for 15 min, supernatants were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked in DPBS containing 3% (w/v) dry milk and 0.3% (v/v) Tween 20, then incubated with primary antibodies in blocking buffer, followed by incubation with horseradish peroxidase-labeled secondary antibodies and detection using the WestPico chemiluminescence kit (Pierce). The following primary antibodies were used: rabbit anti-Dll1 (H-265, Santa Cruz Biotechnology, 1:200), mouse anti-p21 (F-5, Santa Cruz Biotechnology, 1:1000), mouse anti-myogenin (F5D, Santa Cruz Biotechnology, 1:200), rabbit anti-MyoD (C-20, Santa Cruz Biotechnology, 1:500), mouse anti-MyoD (5.8A, Lab Vision, 1:500), mouse anti-Pax7 (ascites, Developmental Studies Hybridoma Bank, 1:250), mouse anti-Pax3 (ascites, Developmental Studies Hybridoma Bank, 1:500), rabbit anti-cleaved Notch 1 (Val1744, Cell Signaling, 1:500), mouse anti-α-tubulin (Sigma, 1:100,000), rabbit anti-integrin α7A (a gift from Stephen J. Kaufman, 1:2000). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies.

Immunofluorescence

Cells grown on glass coverslips or in plastic chamber wells were fixed with 3.7% paraformaldehyde in DPBS for 20 minutes and permeabilized with 0.1% Triton X-100 in DPBS

for 5 min. Cells were blocked in DPBS containing 5% donkey serum (v/v) and 1% BSA (w/v), then incubated with primary antibodies diluted in 1% BSA, followed by incubation with fluorophore-conjugated secondary antibodies. The primary antibodies used were: mouse anti-Pax7 (supernatant, Developmental Studies Hybridoma Bank, 1:5), rabbit anti-MyoD (C-20, 1:50), goat anti-desmin (Santa Cruz Biotechnology, 1:50), rabbit anti-cleaved Notch 1 (Val1744, 1:50); rabbit anti-FLAG (Affinity BioReagents, 1:1000). For detection of BrdU-stained nuclei, fixed cells were treated with 70% ethanol and 50 mM glycine, pH 2.0, then with 4N HCl for 15 minutes at room temperature to denature DNA, and then cells were stained with rat anti-BrdU antibody (Abcam, 1:100). Secondary antibodies were coupled Alexa488, rhodamine Red-X or AMCA. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Coverslips were mounted on slides and examined by Axiovert 200 inverted fluorescent microscope (Zeiss).

Luciferase reporter assays

Primary myoblasts or C2C12 cells grown in 96-well plates were transfected at 60% confluency with 0.05 μg CBF1 firefly luciferase gene reporter vector and 0.005 μg *Renilla* luciferase (pRL-TK) vector as an internal control for transfection efficiency. Twenty four hours after transfection, cells were transferred to DM. Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega) at day 0 and day 1 in DM.

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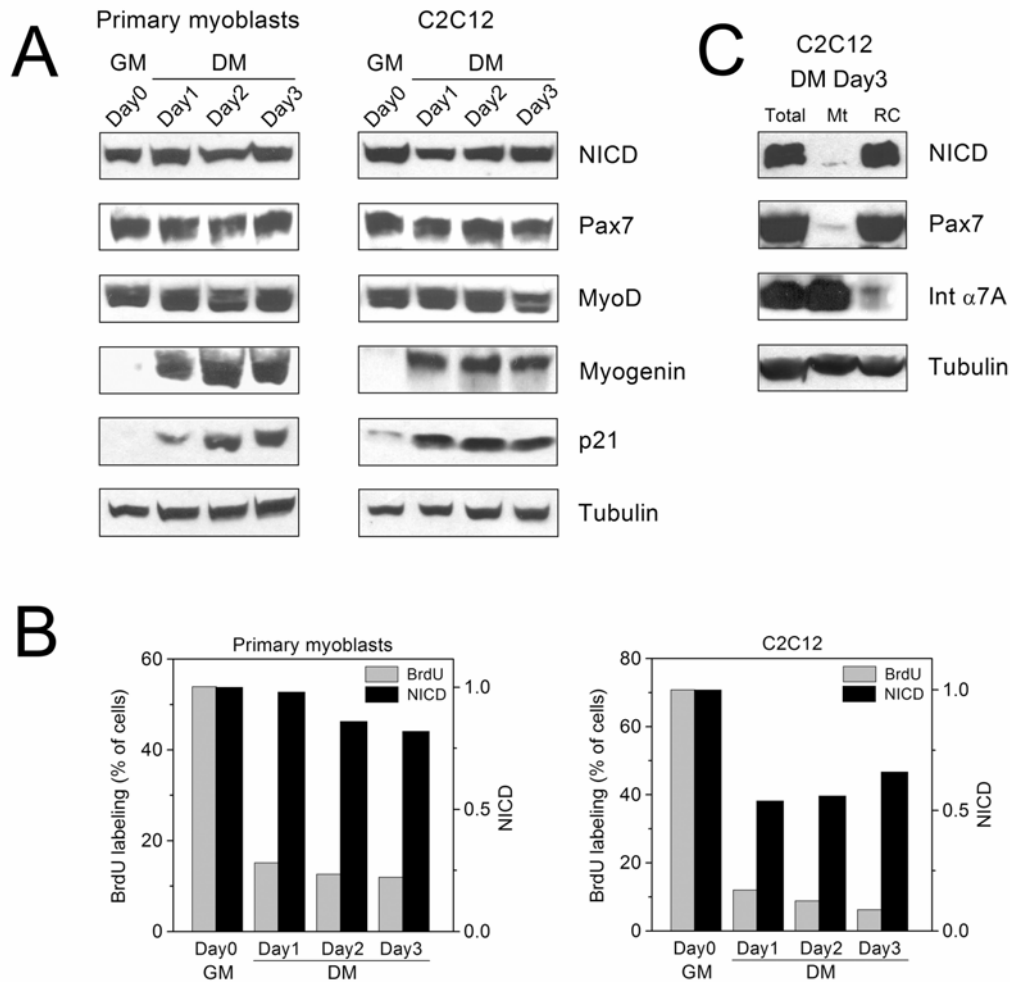
Figure 3-1. **Notch activity in myogenic cells during differentiation *in vitro*.** Primary mouse myoblasts or C2C12 cells were incubated in growth medium until they were 90-100% confluent (GM, Day0) and then they were transferred to differentiation medium (DM, Day1- Day3). (A) The levels of active Notch 1 (NICD), Pax7, MyoD, myogenin, p21, and tubulin were determined by Western blotting. NICD was detected using epitope-specific antibody against γ-secretase-cleaved Notch 1. (B) The amount of NICD in panel A was quantified by gel densitometry and normalized to the amount of tubulin (black bars); the amount of NICD at Day0 is set as 1. Percent of BrdU-positive cells was determined after 3-hour pulse BrdU labeling (gray bars). Experiments in A and B were repeated three times with similar results, representative experiments for primary myoblasts and C2C12 are shown. (C) C2C12 cells incubated for 3 days in DM were subjected to partial trypsinization to separate myotubes (Mt, integrin α7A-positive) from reserve cells (RC, Pax7-positive). The amount of NICD in Mt and RC fractions and in total cell lysate was determined by Western blotting.
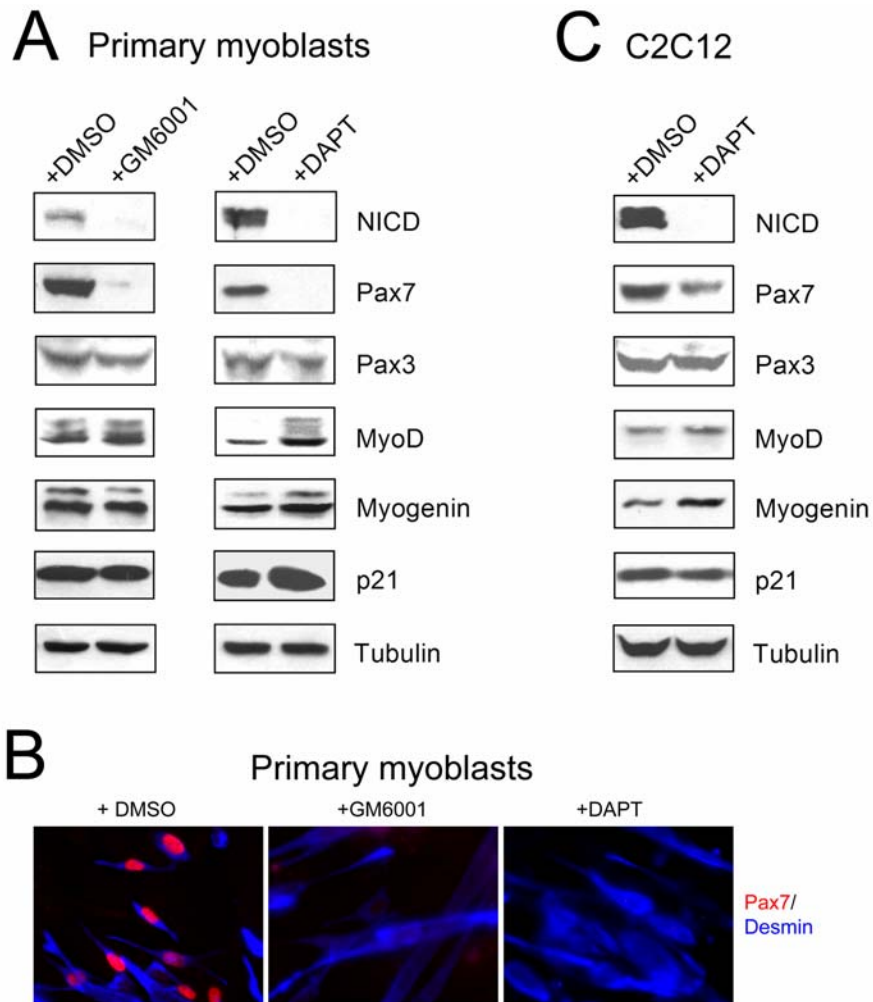
Figure 3-2. **Notch activity is essential for the maintenance of Pax7-positive cells during myogenic differentiation *in vitro***. (A) Primary myoblasts, incubated in GM until 90-100% confluency, were transferred to DM and were incubated for 1 day in the presence of DMSO or 5 μM GM6001, a metalloproteinase inhibitor (left), or in the presence of DMSO or 1 μM DAPT, a γ-secretase inhibitor (right). The levels of NICD, Pax7, Pax3, MyoD, myogenin, and p21 were determined by Western blotting, tubulin is a gel-loading control. A representative experiment out of three is shown. (B) Primary myoblasts incubated for 1 day in DM in the presence of DMSO, 5 μM GM6001, or 1 μM DAPT were stained with mouse anti-Pax7 and goat anti-desmin antibodies, and then with Rhodamine Red-X conjugated anti-mouse IgG and AMCA-conjugated anti-goat IgG antibodies. (C) Confluent C2C12 cells were incubated for 1 day in DM in the presence of DMSO or 5 μM DAPT and the levels of NICD, Pax7, Pax3, MyoD, myogenin, p21, and tubulin were analyzed by Western blotting.
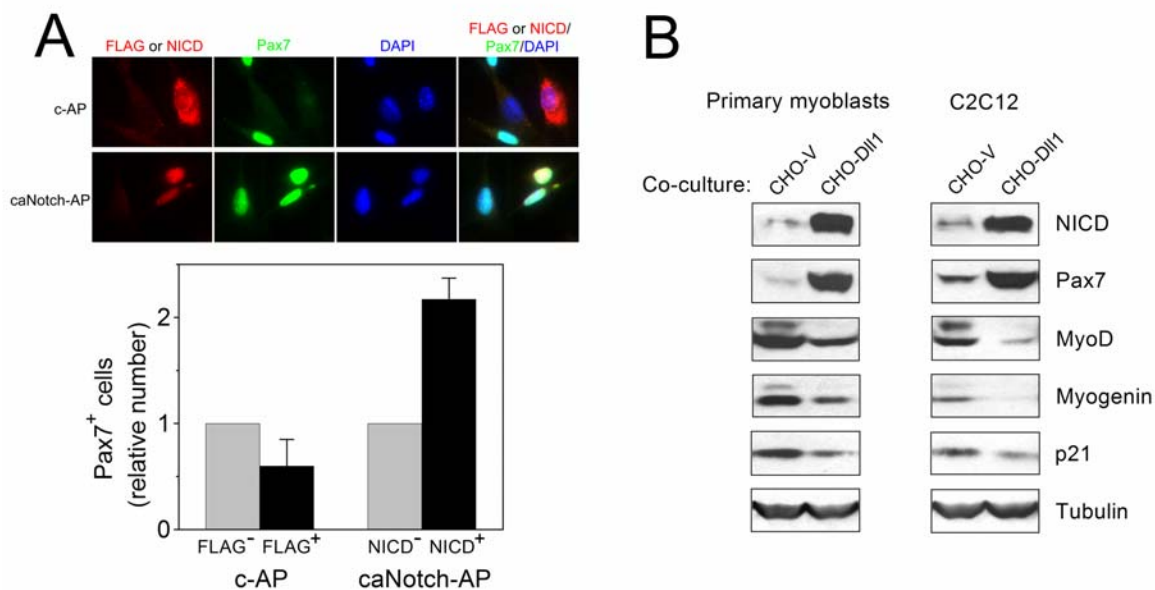
Figure 3-3. **Notch stimulation expands Pax7-positive cells during myogenic differentiation**
in vitro. (A) Primary myoblasts were infected with retroviruses containing constitutively active
mouse Notch 1 (caNotch-AP) or with control retroviruses (c-AP). One day after infection, cells
were transferred to DM and, one day later, cells were fixed, co-stained with mouse anti-Pax7 and
rabbit anti-cleaved Notch 1 (caNotch-AP-infected cells) or anti-FLAG antibodies (c-AP-infected
cells), and analyzed by immunofluorescence microscopy. The relative number of Pax7-positive
cells among NICD-negative and NICD-positive cells (or FLAG-negative and FLAG-positive
cells) on the same slide was calculated (mean ± s.e.m., n=3; at least 200 Pax7-positive cells were
counted in each determination). (B) Primary myoblasts or C2C12 cells (~70% confluent) were
co-cultured for 1 day with CHO cells stably transfected with mouse Dll1 (CHO-Dll1) or with
empty vector (CHO-V). The levels of NICD, Pax7, MyoD, myogenin, p21, and tubulin were
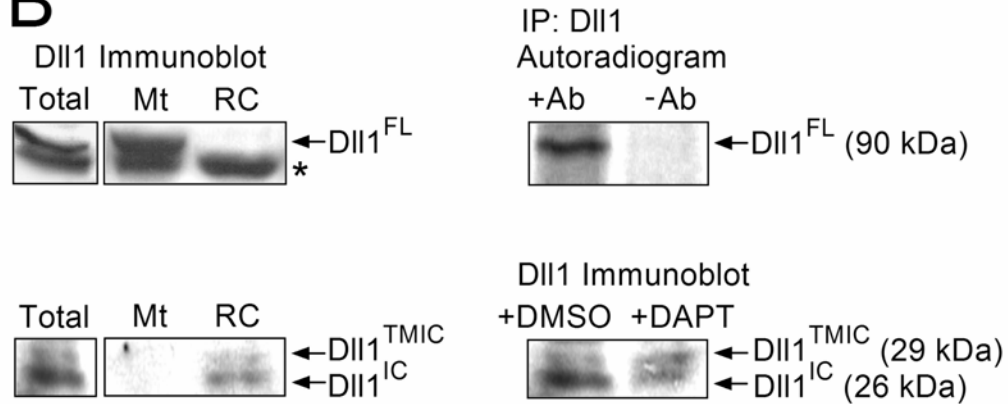analyzed by Western blotting.
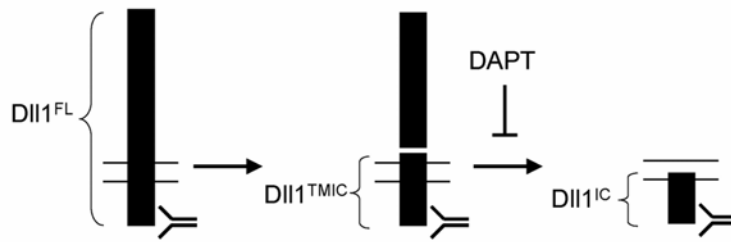
Figure 3-4. **Proteolytic processing of Dll1 in reserve cells**. (A) Schematic diagram of the sequential cleavage of Dll1. The full-length Dll1 ($Dll1^{FL}$, 90 kDa) is cleaved by an ADAM. The transmembrane and intracellular domain fragment ($Dll1^{TMIC}$, 29 kDa) is then cleaved by γ-secretase and the intracellular domain ($Dll1^{IC}$, 26 kDa) is released. The γ-secretase-mediated cleavage is inhibited by DAPT, the antibody recognition site is located in the intracellular domain of Dll1. (B) *Left*, C2C12 cells incubated in DM for 3 days were separated into myotubes and reserve cells, as in Fig.3-1, lyzed, and immunoblotted with anti-Dll1 antibody. Arrows indicate $Dll1^{FL}$, $Dll1^{TMIC}$, and $Dll1^{IC}$, respectively; asterisk marks a non-specific band (left panels). The position of $Dll1^{FL}$ corresponds to the position of the radioactive 90-kDa $Dll1^{FL}$ band detected in the immunoprecipitate from [^{35}S]-labeled C2C12 cells (*right, top*). $Dll1^{TMIC}$ and $Dll1^{IC}$ contain ~10 times less cysteine and methionine residues than the full-length Dll1 and give weak signals in autoradiograms. The identities of $Dll1^{TMIC}$ and $Dll1^{IC}$ are confirmed by the relative increase in the abundance of $Dll1^{TMIC}$ and decrease in the abundance of $Dll1^{IC}$ after treatment of C2C12 cells with DAPT (*right, bottom*).
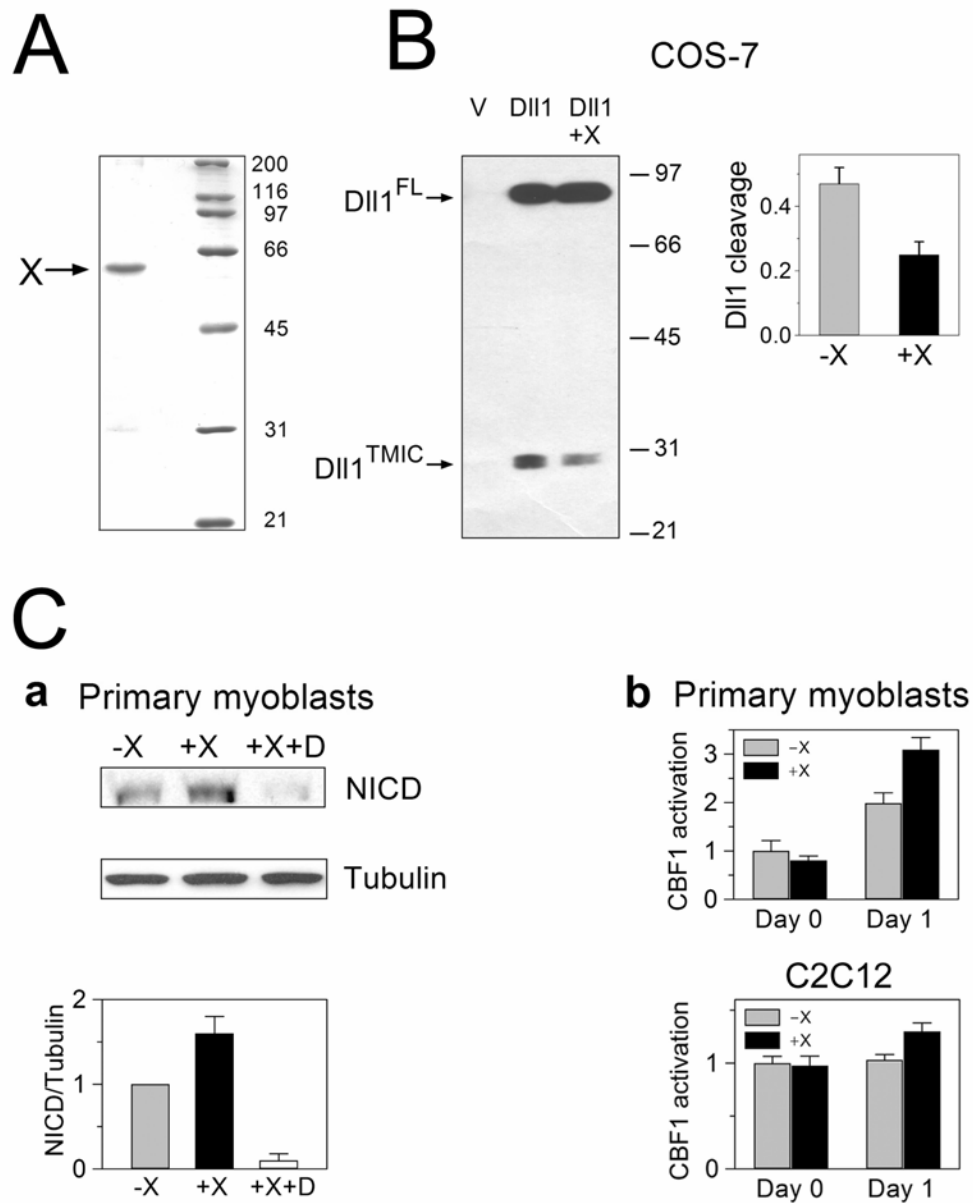
Figure 3-5. **Soluble, catalytically-inactive extracellular domain of ADAM12 inhibits Dll1 processing and stimulates Notch signaling in myoblasts**. (A) Coomassie blue-stained SDS-PAGE gel showing the recombinant, soluble, extracellular domain of mouse ADAM12 containing the E349Q mutation in the catalytic site (protein X), expressed in *Drosophila* S2 cells and purified from culture medium. The molecular weight markers (kDa) are shown on the right. (B) Inhibition of Dll1 cleavage by protein X. *Left*, COS-7 cells transfected to express Dll1 were incubated for 24 hours in the absence or presence of 2 μM protein X. *Right*, Cell extracts from Dll1-transfected and empty vector (V)-transfected cells were analyzed by Western blotting using

antibody against the cytoplasmic domain of Dll1. $Dll1^{FL}$ and $Dll1^{TMIC}$ are indicated with the arrows, positions of the molecular weight markers are on the right. *Left*, The extent of Dll1 cleavage was calculated as the ratio of band intensities of $Dll1^{TMIC}$ and $Dll1^{FL}$ (mean ± s.e.m., n=3). (C) The effect of protein X on Notch signaling. (a) *Top*, Primary myoblasts were incubated for 1 day in DM without protein X or with 2 μM protein X, in the absence or presence of 1 μM DAPT (D). The level of active Notch, NICD, was analyzed by Western blotting using an epitope-specific antibody, tubulin is a gel-loading control. *Bottom*, The amount of NICD was quantified by densitometry and normalized to the amount of tubulin (mean ± s.e.m., n=3). (b) Primary myoblasts or C2C12 cells were transfected with a CBF1-luciferase reporter and pRL-TK vector, 24h after transfection cells were transferred to DM (Day 0) and incubated for additional 24h (Day 1), in the absence (gray bars) or presence (black bars) of 2 μM protein X. The relative firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay. Fold of CBF1 activation over the level at Day 0, in the absence of protein X, was calculated. The data represent the means ± s.e.m. from three measurements, the experiment was repeated twice with similar results.
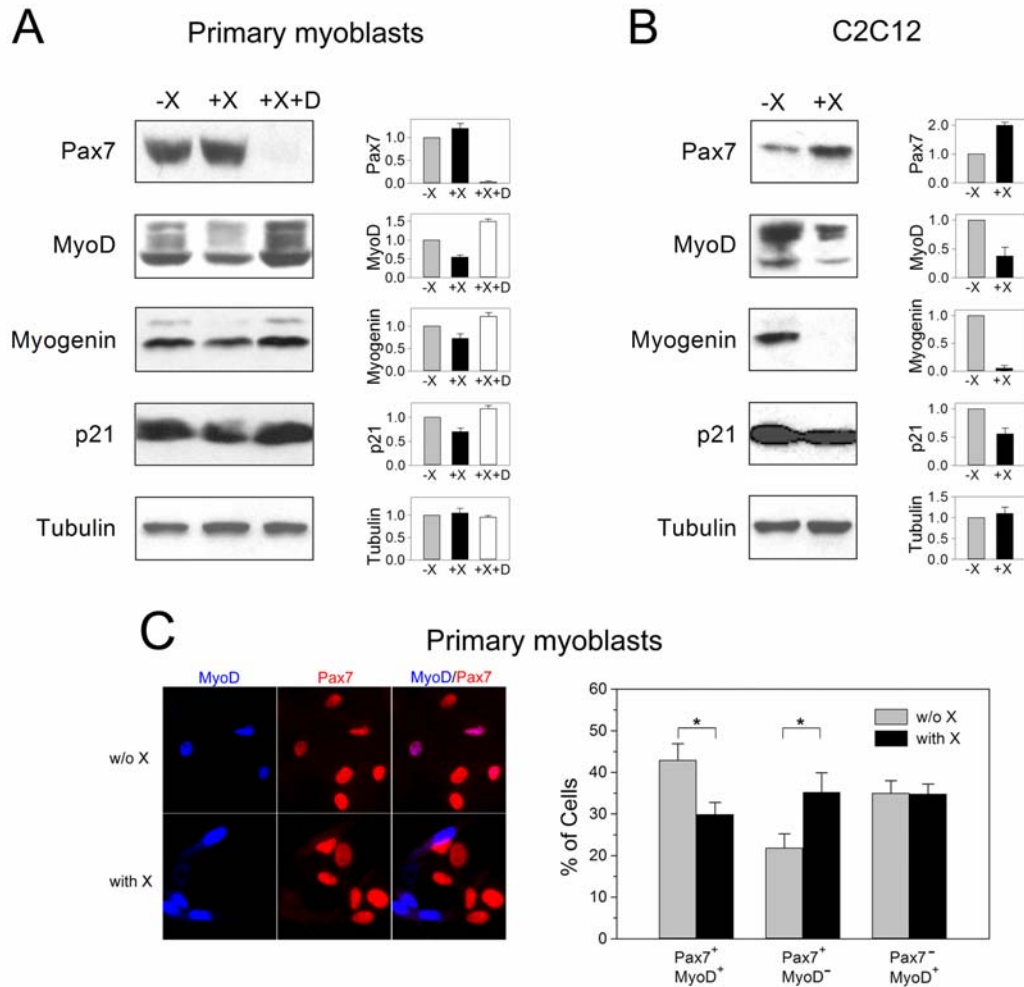
Figure 3-6. **The effect of protein X on the myogenic progression and Pax7 expression**. (A) Primary myoblasts were incubated for 1 day in DM without protein X or with 2 μM protein X, in the absence or presence of 1 μM DAPT (D). Levels of Pax7, MyoD, myogenin, p21, and tubulin expression were examined by Western blotting, band intensities were quantified by densitometry and are plotted on the right. Data represent mean values ± s.e.m. from three different experiments. (B) C2C12 cells were incubated for 1 day in DM without or with 2 μM protein X, and the levels of Pax7, MyoD, myogenin, p21, and tubulin expression were examined as in panel A. (C) *Left*, Primary myoblasts incubated for 1 day in DM without or with 2 μM protein X were co-stained with rabbit anti-MyoD and mouse anti-Pax7 antibodies and with AMCA-conjugated anti-rabbit IgG and rhodamine Red-X-conjugated anti-mouse IgG antibodies. Two representative images are shown. *Right*, The numbers of Pax7^{+}/MyoD[+], Pax7^{+}/MyoD[-], and Pax7^{-}/MyoD^{+} cells were counted in 15 different microscopic fields, 300-400 cells were analyzed for each

experimental condition (without and with protein X). The data show mean values of cells counted on 3 different slides, error bars represent standard error of the mean (*, p<0.05). The experiment was repeated twice with similar results.
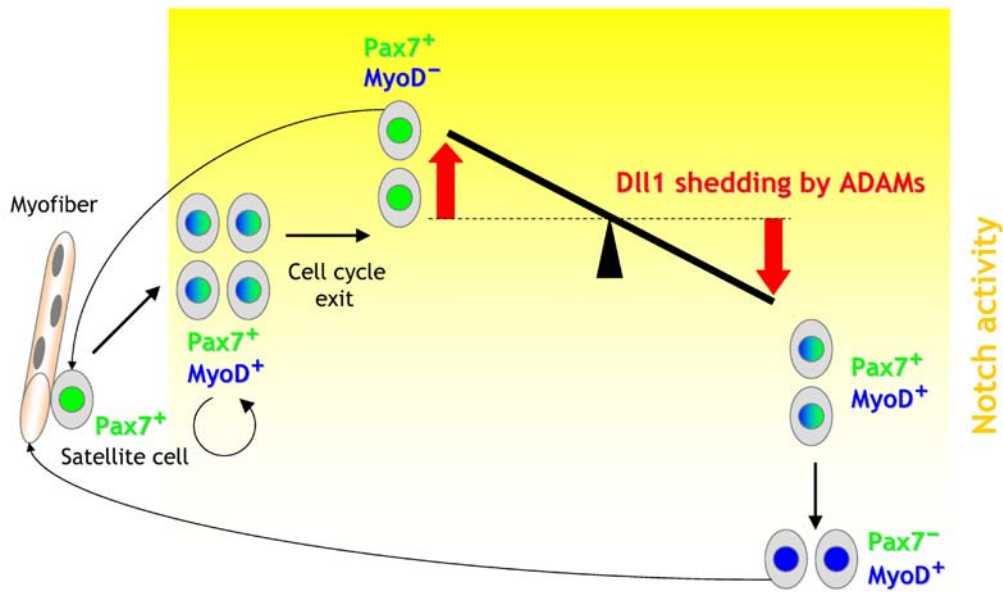
Figure 3-7. **Proposed model of modulation of Notch activity by Dll1 shedding during myogenic differentiation**. The Notch pathway is active in proliferating Pax7$^+$/MyoD$^+$ cells derived from Pax7$^+$ quiescent satellite cells. Upon exit from the cell cycle, Notch signaling is down-regulated in Pax7$^+$/MyoD$^+$ cells that later become Pax7$^-$/MyoD$^+$, progress into differentiation, and eventually fuse to give rise to myofibers. The level of Notch activity (shown as the yellow gradient) is maintained (or up-regulated) in Pax7$^+$/MyoD$^-$ cells that replenish the pool of satellite cells. The balance between Pax7$^+$/MyoD$^-$ and Pax7$^+$/MyoD$^+$ cells is maintained by Dll1 shedding by ADAM proteases in a stochastic and cell density-dependent manner. Dll1 shedding in a pool of cells leads to ligand depletion and down-regulation of Notch signaling in neighboring cells, maintenance of MyoD expression, and eventually loss of Pax7 expression. Cells in which Dll1 cleavage takes place acquire higher level of Notch activity than their neighbors, leading to down-regulation of MyoD and sustained Pax7 expression.

CHAPTER 4

TRANSCRIPTIONAL UP-REGULATION OF MnSOD BY PAX7

Introduction

Reactive oxygen species (ROS) are highly reactive oxygen ions, free radicals and peroxides which damage cellular constituents, including proteins, lipids, DNA and RNA. Cells have developed defense system against ROS damage through enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Superoxide dismutase catalyzes the dismutation of superoxide. Three mammalian superoxide dismutases have been discovered. CuZn-SOD (SOD1) is a homodimer that bears copper and zinc, and is found in the cytoplasm. EC-SOD (SOD3), a copper and zinc-containing tetramer, functions in the extracellular space (Zelco et al., 2002). Manganese superoxide dismutase (MnSOD), also known as superoxide dismutase-2 (SOD2), is a mitochondrial enzyme that represents a major cellular defense system against superoxide free radicals (Faraci et al., 2004). MnSOD forms homotetramers and binds one manganese ion per subunit.

Certain properties of skeletal muscle, including high metabolic activity and high level of heme-containing proteins, render it particularly susceptible to injury from reactive oxygen species (Chan et al., 1994). Together with other antioxidant enzymes, including CuZn-SOD, glutathione peroxidase and catalase, mRNA level and enzyme activity of MnSOD decrease as muscle differentiation occurs. This correlates with the fact that differentiated myotubes are more susceptible to oxidative injury compared with undifferentiated myoblasts (Alexa et al., 1999).

The Human MnSOD gene has been shown to be induced by TPA (a phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate) *via* activation of a CREB-1/ATF-1-like factor (Kim et al., 1999). Cytokines also induce MnSOD expression in insulin-producing cells. Two interleukin-1β-responsive elements have been identified, with one located in the promoter region and the other one located in the second intron of the MnSOD gene. NF-κB is required for this process, as it cooperates with CCAAT/enhancer-binding proteins in the promoter region and with octamer and Ets factors in the intronic region (Darville et al., 2000). Vascular Endothelial Growth Factor (VEGF) also activates MnSOD transcription through NF-κB, and the activation is dependent on a second intronic NF-κB consensus motif (Abid et al., 2004).

MnSOD is a transcriptional target of the FOXO family of forkhead transcription factors (Kops et al., 2002; Essers et al., 2004; Adachi et al., 2007). Forkhead transcription factors are characterized by the presence of a highly conserved DNA binding domain, with a structure of three α-helices and two large loops, often referred to as winged-helix motif (Weigel et al., 1990).

FOXO (Forkhead box, class O) factors are the only known forkhead proteins regulated by the PKB/Akt pathway. Protein kinase B (PKB/Akt) is a negative regulator of FOXOs. In response to insulin, FOXO proteins are phosphorylated by PKB/Akt. This results in their nuclear exclusion (Brunet et al., 1999; Kops et al., 1999) and subsequent proteasomal degradation (Matsuzaki et al., 2003). In proliferating cells, active PKB/Akt drives cellular proliferation and protects cells from apoptosis (Coffer et al., 1998; Datta et al., 1999). In quiescent cells, when PKB/Akt is silent, FOXO family members play an important role in controling quiescence, oxidative stress and apoptosis (Burgering et al., 2003). The mammalian FOXO family of forkhead transcription factors includes FOXO1 (FKHR), FOXO3a (FKHRL1) and FOXO4 (AFX).

In human colon carcinoma cells, when the protective mechanism of PKB-mediated signaling is absent, active FOXO3a protects quiescent cells from oxidative stress by transcriptional up-regulation of MnSOD. This results in protection of cells from reactive oxygen species and antagonizes apoptosis caused by glucose deprivation (Cops et al., 2002). In *C. elegans*, the absence of PKB signaling activates the FOXO homologue DAF-16 and generates a phenotype characterized by increased resistance to oxidative stress (Ogg et al., 1997; Paradis et al., 1998; Honda et al., 1999). DAF16, AFX, FKHR and FKHRL1 share a common consensus binding sequence of 8bp (5`-TTGTTTAC-3`) called DBE. The transcriptional activation is controlled in a dose-dependent manner by FOXO expression and is sensitive to the number of DBE elements (Furuyama et al., 2000). Two FOXO-binding elements (DBE) have been identified in the human MnSOD promoter region: one inverse DBE at position -1249 to -1241 (GTAAACAA; DBE1) and one suboptimal DBE at position -997 to -989 (TTGTTTAA; DBE2). It has been shown that only DBE1 is required for activation of MnSOD through FOXO3a (Cops et al., 2002).

Pax proteins are a family of transcription factors characterized by a paired domain. Pax proteins play critical roles in formation of tissues and organs during embryogenesis (Tremblay and Gruss, 1994). Pax7 and Pax3 are two closely related family members that mark myogenic progenitor cells and regulate their entry into myogenic differentiation (Relaix et al., 2005). Pax7 and Pax3 share a common structure containing a paired domain, an octapeptide and a homeodomain. Pax7 is required for maintenance of the satellite cell pool in skeletal muscle and to protect satellite cells from apoptosis. The anti-apoptotic function of Pax7 cannot be replaced by Pax3 (Relaix et al., 2006).

It has been shown that Pax7 interacts with the histone methyltransferase (HMT) complex Wdr5-Ash2L-MLL2 and directs the methylation of histone H3 lysine4 (H3K4). Methylation of H3K4 causes chromatin to become more permissive for transcription. As a result, Pax7 stimulates transcriptional activation of target genes and regulates the myogenic development program. The expression of Myf5, a myogenic regulatory factor, is increased by this mechanism (McKinnell et al., 2008). By analyzing target genes identified using Pax7-chromatin immunoprecipitation (ChIP), a core motif GTCAC was recognized as the Pax7 consensus binding site. Pax7 directly occupies this site within target genes. The target genes with one or more such sites include the transcription factors Gbx1 and Eya4, the neurogenic cytokine receptor CntfR, the neuronal potassium channel Kcnk2, and the signal transduction kinase Camk1d *in vivo* and in cultured cells (White et al., 2008).

Pax7 is essential for maintenance of muscle satellite cells. In skeletal muscle of Pax7 knockout mice, satellite cells are present, but exist in reduced numbers as the mice mature (Oustanina et al., 2004). This is due to satellite cell death. While MnSOD knockout mice exhibit neonatal lethality, heterozygous MnSOD knockouts have normal life-span, but show increased oxidative damage and incidence of apoptosis (Van et al., 2001).

Pax7 is essential for protecting muscle satellite cells from apoptosis, but the mechanism of its action is not clear. As both Pax7 and MnSOD protect quiescent cells from apoptosis, we asked whether Pax7 exerts its anti-apoptotic function through up-regulation of MnSOD. In this study, we show that in C2C12 cells, exogenous expression of Pax7 up-regulates MnSOD promoter in a dose-dependent manner. In COS7 cells, transfection of Pax7 increases MnSOD protein expression. Up-regulation of MnSOD promoter by Pax7 is not mediated by FOXO family members. Two Pax7 consensus binding sites are found in the MnSOD promoter sequence. However, activation of the MnSOD promoter by Pax7 does not occur through direct binding to these sites.

Results

It has been shown that Pax7 is essential for protecting muscle satellite cells from apoptosis (Relaix et al., 2006). MnSOD knockout mice show increased oxidative damage and incidence of apoptosis (Van et al., 2001). We examined the effect of exogenous Pax7 expression on the MnSOD promoter. C2C12 myogenic cells were co-transfected with luciferase reporter under the

control of MnSOD promoter, pRL-TK vector and mouse Pax7 expression vector or empty vector. pRL-TK vector, which expresses *Renilla* luciferase, was used as an internal control for transfection efficiency. 24 hours after transfection, MnSOD promoter activity was measured as a ratio of firefly luciferase activity to *Renilla* luciferase activity. With the increased expression of Pax7 protein, MnSOD promoter activity was induced in a dose-dependent manner (Fig.4-1AB). The increased expression of Pax7 was confirmed by Western blotting using the same cell lysates as used for measurement of luciferase activity (Fig.4-1C). These results show that Pax7 activates MnSOD at the transcriptional level in a dose-dependent manner.

To examine the up-regulation of MnSOD protein by Pax7, we used COS7 cells, which are characterized by higher transfection efficiency than C2C12 cells. It has been shown that MnSOD is a transcriptional target of forkhead FOXO proteins (Kops et al., 2002; Essers et al., 2004; Adachi et al., 2007). FOXO proteins are phosphorylated and excluded from nucleus in response to insulin. Upon serum withdrawal, FOXO proteins are de-phosphorylated, translocated into nucleus and become active transcription factors (Brunet et al., 1999; Kops et al., 1999). We reasoned that serum free condition would help activate MnSOD if FOXO proteins are involved. On the other hand, if FOXO proteins are not mediating Pax7 up-regulation of MnSOD, insulin should decrease the background MnSOD protein level and make the specific up-regulation by Pax7 more prominent. Here, we incubated COS7 cells under three different conditions for 24 hours after transfection with Pax7 expression vector or empty vector. Cells were incubated in normal growth medium, serum free medium and serum free medium with 100 nM insulin for an additional 24 hours. Under all conditions, the endogenous MnSOD proteins were increased after exogenous expression of Pax7, with about 10% increase in growth medium, about 30% increase in serum free medium and about 50% increase in serum free medium with insulin, respectively (Fig.4-2AB). Together, these results show that over-expression of Pax7 up-regulates MnSOD protein expression.

It has been shown that FOXO family members share a common 8bp consensus binding sequence, called DBE (Furuyama et al., 2000). There are two DBE elements in human MnSOD promoter, one inverse DBE at position -1249 to -1241 (DBE1), and one suboptimal DBE at position -997 to -989 (DBE2) (Fig.4-3A). A single C to G mutation in the AAACAA sequence of DBE1 completely abolished FOXO3a-mediated expression of MnSOD, while a single G to C substitution in the TTGTTT sequence of DBE2 does not affect FOXO3a-mediated activation in

human colon carcinoma cells (Cops et al., 2002). Here, we generated the C to G point mutation in DBE1 and the G to C point mutation in DBE2 sequences of MnSOD promoter respectively and generated the double DBE mutant with both point mutations (Fig.4-3A). After co-transfection of these mutants with Pax7 expression vector into C2C12 or COS7 cells, Pax7-mediated up-regulation of MnSOD promoter was not affected by the point mutations (Fig.4-3BC). These results suggest that the transcriptional up-regulation of MnSOD by Pax7 does not involve FOXO proteins.

A Pax7 consensus binding site, GTCAC, has been recently identified by analysis of target genes recognized by Pax7-chromatin immunoprecipitation (White et al., 2008). Our 3340-base pair MnSOD promoter luciferase construct contains the MnSOD promoter fragment and part of intron 1. After analysis of the sequence, we identified one Pax7 consensus binding site at position +118 to +123 within intron 1 and one inverse Pax7 consensus binding site at position -1854 to -1849 within the promoter region (Fig.4-4A). As there is no literature report indicating which nucleotide is essential for Pax7 binding, we decided to delete all five nucleotides within each site. We generated a single deletion mutant with the Pax7 binding site in intron 1 deleted and a double deletion mutant with the inverse Pax7 binding site in promoter region deleted in addition to the first deletion (Fig.4-4A). After co-transfection of deletion mutants with Pax7 expression vector, luciferase reporter assay suggested that the mutant promoter constructs could be activated by Pax7 expression to the same extent as the wild type promoter construct in both C2C12 and COS7 cells (Fig.4-4BC). Together, these results show that the transcriptional up-regulation of MnSOD by Pax7 does not occur through direct binding of Pax7 to the MnSOD promoter.

Discussion

In muscle satellite cells, Pax7 plays a critical role in cell survival, an essential function that cannot be replaced by Pax3 (Relaix et al., 2006). As a result, in Pax7 knockout mice, muscle satellite cells are rapidly lost due to apoptosis as the mice mature (Oustanina et al., 2004). So far, the mechanism of how Pax7 performs its anti-apoptotic function is not known. MnSOD heterozygous knockout mice have increased oxidative damage and incidence of apoptosis (Van et al., 2001). Here, our study suggests that Pax7 increases MnSOD expression at the

transcriptional level. This provides an insight into the mechanism of Pax7-mediated muscle satellite cell survival.

MnSOD is a mitochondrial enzyme that represents a major cellular defense system against superoxide free radicals (Faraci et al., 2004). Both C2C12 and COS7 cell lines that we tested have high levels of the endogenous MnSOD protein. NF-κB and FOXO proteins are well-known positive regulators of MnSOD transcription (Darville et al., 2000; Abid et al., 2004; Kops et al., 2002; Essers et al., 2004; Adachi et al., 2007). Our study shows that exogenous Pax7 activates MnSOD at the transcriptional level and leads to increased MnSOD protein expression (Fig.4-1 and Fig.4-2). To determine whether Pax7 is indispensable for MnSOD expression, comparing the MnSOD protein levels in myoblasts of Pax7-/- mice with their wild-type control, or decreasing the endogenous Pax7 expression by siRNA would provide more accurate answer. Since Pax7-deficient mice do not exhibit neonatal lethality as MnSOD homozygous knockouts do, Pax7 may not be essential for the maintenance of MnSOD expression. However, this does not exclude the possibility that MnSOD is necessary for mediating the anti-apoptotic function of Pax7 in quiescent muscle satellite cells. One approach to determine the necessity of MnSOD should be to transfect MnSOD-/- myoblasts with Pax7. If Pax7 loses its anti-apoptotic function in the absence of MnSOD, then one can conclude that MnSOD is a critical mediator of cell survival for Pax7.

FOXO family members play an important role in control of quiescence, oxidative stress and apoptosis (Burgering et al., 2003). MnSOD is a transcription target of FOXO proteins (Kops et al., 2002; Essers et al., 2004; Adachi et al., 2007). FOXO proteins, MnSOD and Pax7 all have essential functions in protecting quiescent cells from apoptosis. We examined the involvement of FOXO proteins in Pax7-mediated up-regulation of MnSOD transcription. Although DBE1 has been shown to be important for FOXO3a mediated activation of MnSOD expression (Kops et al., 2002), both DBEs proved to be non-essential for increased MnSOD promoter activity by Pax7 (Fig.4-3). Since DBE is a common consensus binding sequence shared by DAF16, AFX, FKHR and FKHRL1 (Furuyama et al., 2000), our findings suggest that the activation of MnSOD transcription by Pax7 is independent of the activation of MnSOD transcription by FOXO proteins. The increased MnSOD protein after Pax7 transfection upon addition of insulin, which favors the nuclear exclusion and inactivation of FOXO proteins, also suggests that FOXO proteins are not involed (Fig.4-2).

Recent studies shed insight into the mechanism of Pax7 function as a transcriptional activator. It has been shown that Pax7 interacts with histone methyltransferase (HMT) complex Wdr5-Ash2L-MLL2 and directs the methylation of histone H3 lysine4. Binding of HMT-Pax7 to Myf5, which is a myogenic regulatory factor, resulted in its increased expression (Mckinnell et al., 2008). This model involves chromatin modification, and appears to be a rather broad spectrum activation. It has been shown that by this mechanism Pax7 increases expression of a number of genes. However, this mechanism is less important in our experiments, as the transfected MnSOD promoter construct we used is independent of chromatin regulation. By analysis of target genes identified using Pax7-chromatin immunoprecipitation (ChIP), a core motif GTCAC is recognized as the Pax7 consensus binding site. Pax7 directly occupies this site within target genes (White et al., 2008). We identified two Pax7 consensus binding sites in the MnSOD promoter and have those sites deleted in our mutant constructs. Our results show that the identified Pax7 binding sites are not involved in MnSOD promoter activation (Fig.4-4). This indicates that activation of MnSOD by Pax7 may be indirect and may require other transcription factors.

Pax7 activates MnSOD at the transcriptional level. Our study provides a possible answer to the question how Pax7 mediates cell survival in muscle satellite cells. We have excluded the involvement of FOXO proteins and the direct binding to Pax7 consensus binding sites in the MnSOD promoter. The mechanism by which Pax7 activates MnSOD promoter remains to be examined. The co-factors involved in the activation and the precise sites in the MnSOD promoter await determination.

Materials and Methods

Expression constructs

pMnSOD3340 luciferase reporter construct was a gift of Dr. Boon Chock, NHLBI. pMnSOD3340 DBE1 mutant, DBE2 mutant and double mutant were generated using QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) according to the manufacture's protocol with primers 5'-CTGACGTCTGTAAAGAAGCCCAGCCCTTC-3' and 5'-CACACCATTCAGGATTGTTCTTTAACTGTTGAGAGAGCA-3', respectively. pMnSOD3340 Pax7 binding site deletion mutant and Pax7 binding site together with inverse Pax7 binding site double deletion mutant were created with primers 5'-

GAAAGGGGACCCGGGCCCCAAGGGGC-3' and 5'-TCTCATAGCTAGTGCCCTTAAAACTGCAGTACCTCC-3', respectively.

Mouse Pax7-pcDNA3 expression vector was provided by Dr. Micheal Rudnicki, University of Ottawa. pcDNA3 empty vector was generated by deletion of the Pax7 cDNA between two HindIII sites.

Cells

C2C12 and COS7 cells were obtained from American Tissue Culture Collection. C2C12 and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in presence of 5% CO_2 under a humidified atmosphere.

Plasmid transfection

Transient transfections were performed using Fugene 6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol, one day after plating cells.

Cell treatments

24 hours after transfection, COS7 cells were incubated in normal growth medium (GM), serum free medium (SFM) or SFM with 100 nM insulin for additional 24 hours before samples were collected for Western blotting.

Western blotting

Cells were incubated in extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (AEBSF), 5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin A, 10 mM 1,10-phenanthroline) for 15 min at 4°C. Cell extracts were centrifuged at 21,000xg for 15 min. Supernatants were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked in DPBS containing 3% (w/v) dry milk and 0.3% (v/v) Tween 20, then incubated with primary antibodies in blocking buffer, followed by incubation with horseradish peroxidase-labeled secondary antibodies and detection using the WestPico chemiluminescence kit (Pierce). The following primary antibodies were used: mouse anti-Pax7

(ascites, Developmental Studies Hybridoma Bank, 1:4000), rabbit anti-MnSOD (Stressgen, 1:10,000), mouse anti-α-tubulin (Sigma, 1:100,000). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG.

Luciferase reporter assay

Cells in 6-well plates were transfected at 60% confluency with 0.5 μg pMnSOD3340 firefly luciferase reporter or firefly luciferase reporter with MnSOD promoter containing mutations, 0.05 μg *Renilla* luciferase (pRL-TK) vector, together with Pax7 expression vector or control empty vector. 24 hours after transfection, firefly and *Renilla* luciferase activities were measured using Dual-Luciferase reporter assay system (Promega). The activity of *Renilla* luciferase was used as an internal control for transfection efficiency.

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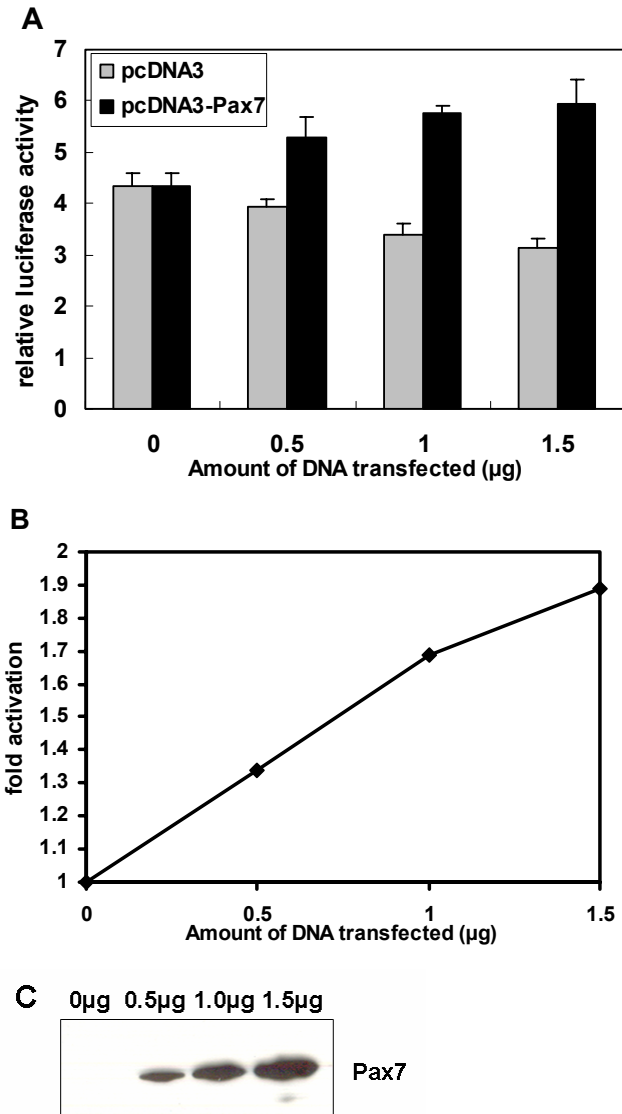
Figure 4-1. **MnSOD promoter activity is increased by Pax7 transfection in a dose-dependent manner.** C2C12 cells were co-transfected with MnSOD firefly luciferase reporter, pRL-TK vector and mouse Pax7 expression vector or empty vector. (A) 24 h after transfection, the relative firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay. The data represent the mean ± s.e.m. from two measurements. The experiment was repeated twice with similar results. (B) The ratios of the relative luciferase activities from Pax7-transfected cells to the empty vector-transfected cells were calculated. (C) Western blotting with anti-Pax7 monoclonal antibody was performed to show the increased expression of Pax7 protein in C2C12 cells.
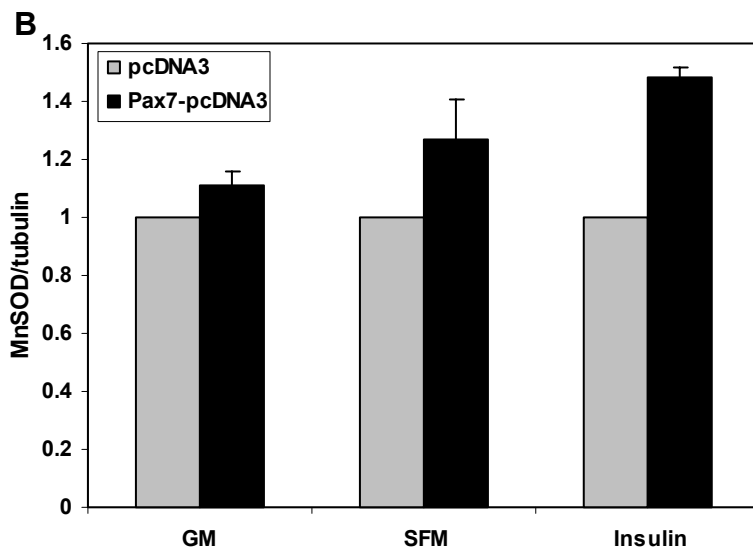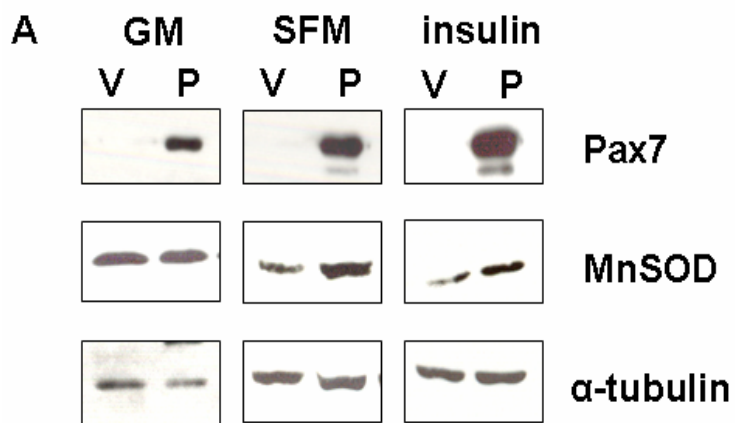
Figure 4-2. **MnSOD protein expression is up-regulated by Pax7 transfection.** (A) COS7 cells were transfected with either Pax7 expression vector or empty vector as control. 24 h after transfection, cells were incubated in growth medium (GM), in serum free medium (SFM) or in SFM with 100 nM insulin for additional 24 h. The expression levels of Pax7 and MnSOD proteins were examined by Western blotting, and α-tubulin was used as loading control. (B) Band intensities of MnSOD protein were quantified by densitometry, and normalized to the amount of tubulin. Data represent the mean values ± s.e.m. from three different experiments.
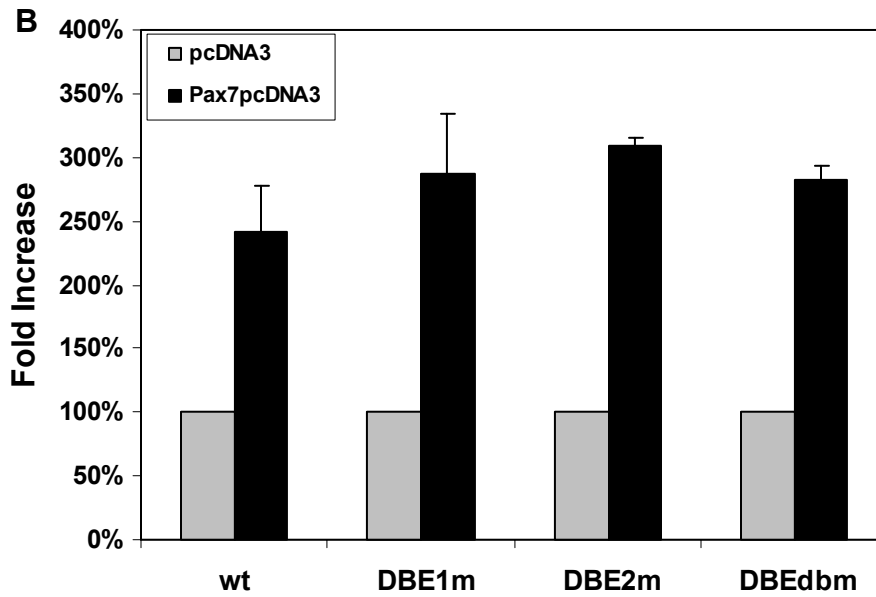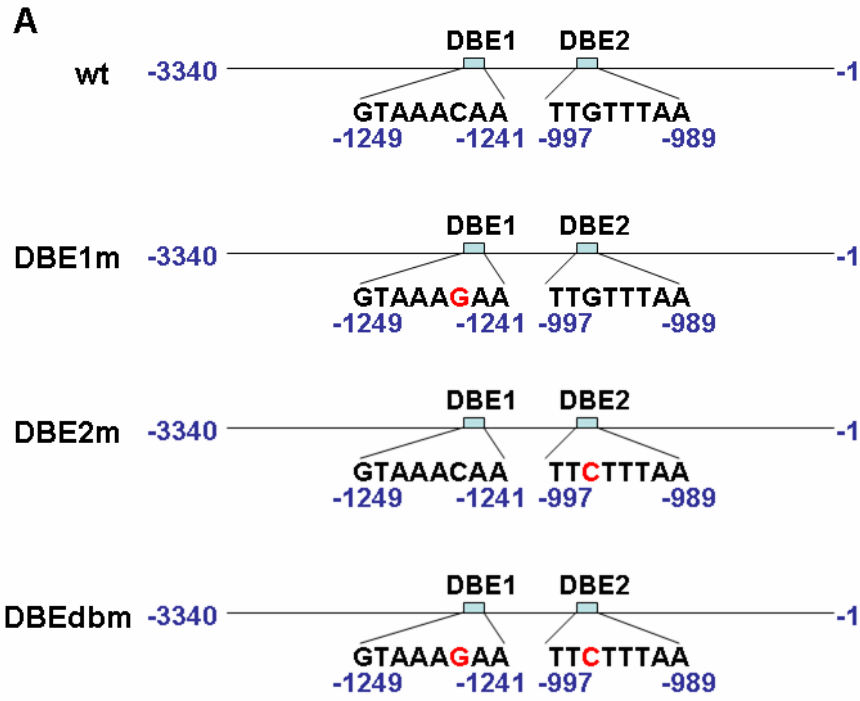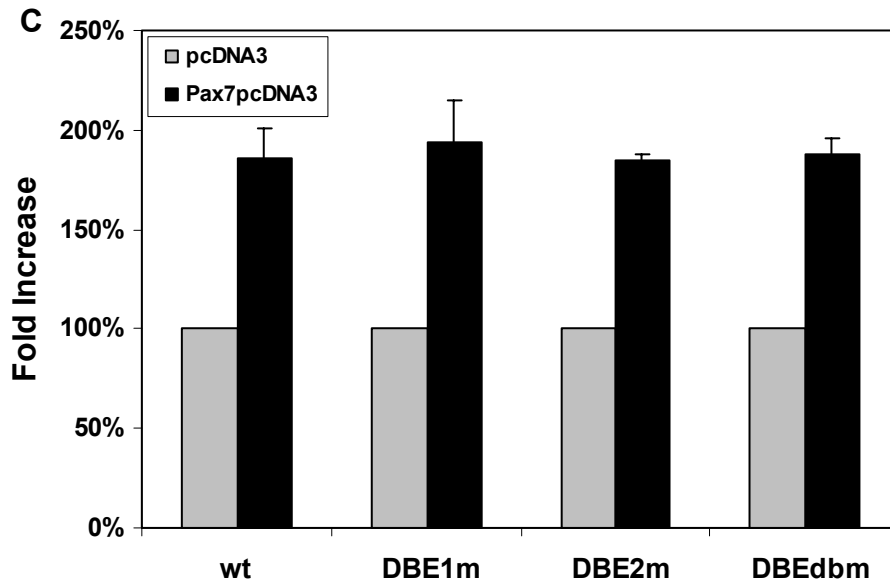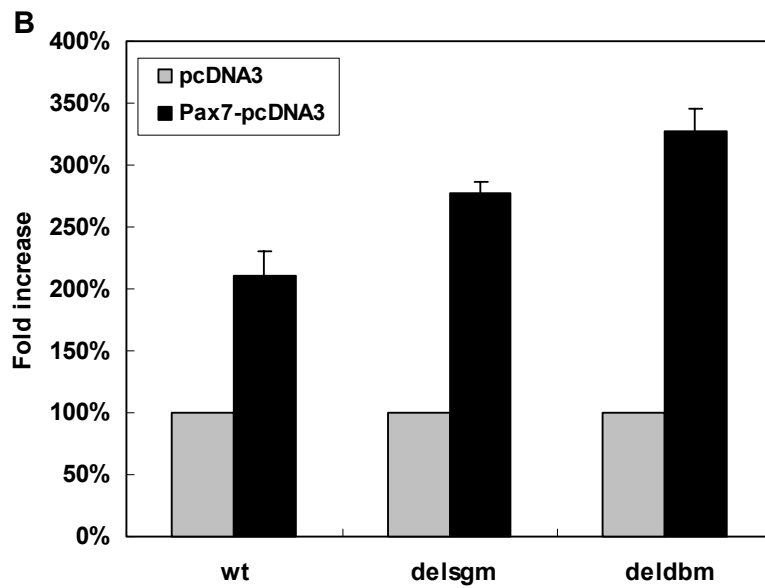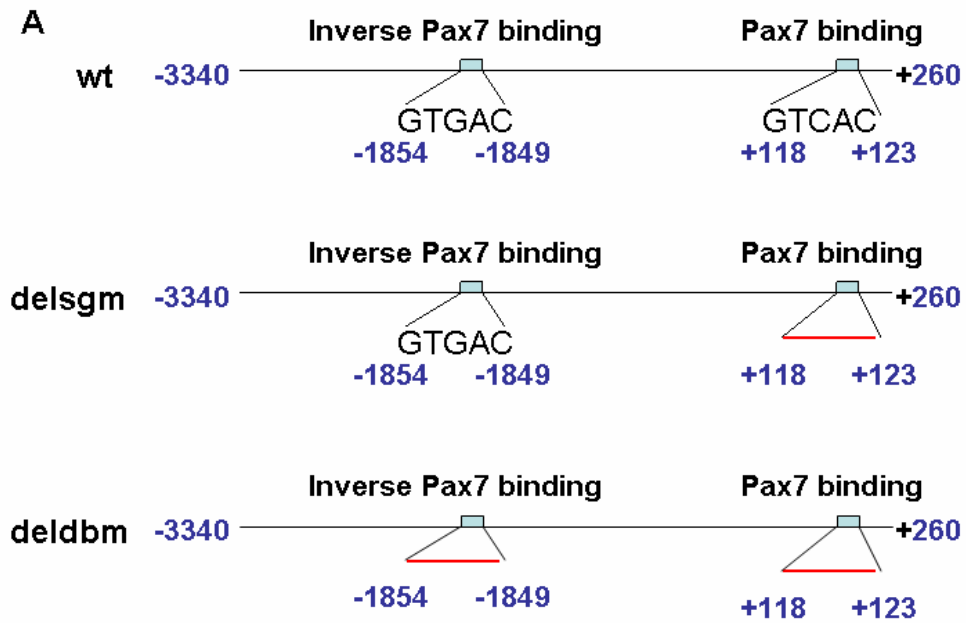
Figure 4-3. **Up-regulation of the MnSOD promoter by Pax7 does not involve FOXO proteins.** (A) The linearized MnSOD promoter containing two consensus FOXO proteins binding (DBE) sites (wt), carrying a point mutation in DBE1 site (DBE1m), carrying a point mutation in DBE2 site (DBE2m) and carrying both point mutations in DBE1 and DBE2 sites (DBEdbm). C2C12 cells (B) or COS7 cells (C) were co-transfected with luciferase reporter under the control of MnSOD promoter or MnSOD promoter mutants, pRL-TK vector and mouse Pax7 expression vector or empty vector. 24 h after transfection, the relative firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay. The data represent the means ± s.e.m. from two measurements. The experiment was repeated twice with similar results.

A

wt   -3340   Inverse Pax7 binding   Pax7 binding   +260
GTGAC   GTCAC
-1854   -1849   +118   +123

delsgm   -3340   Inverse Pax7 binding   Pax7 binding   +260
GTGAC
-1854   -1849   +118   +123

deldbm   -3340   Inverse Pax7 binding   Pax7 binding   +260
-1854   -1849   +118   +123

B

Fold increase

- □ pcDNA3
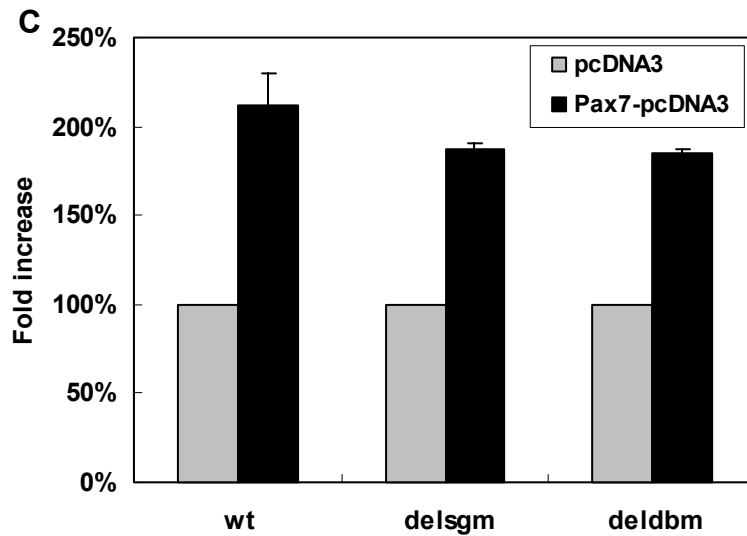- ■ Pax7-pcDNA3

wt   delsgm   deldbm

Figure 4-4. **Up-regulation of MnSOD promoter by Pax7 is not through consensus Pax7 binding sites.** (A) The linearized MnSOD promoter fragment containing two consensus Pax7 binding sites (wt), carrying a single Pax7 binding site deletion mutation (delsgm), and carrying double Pax7 binding sites deletion mutations (deldbm). C2C12 cells (B) or COS7 cells (C) were co-transfected with luciferase reporter under the control of MnSOD promoter or MnSOD promoter mutants, pRL-TK vector and mouse Pax7 expression vector or empty vector. 24 h after transfection, the relative firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay. The data represent the means ± s.e.m. from two measurements. The experiment was repeated twice with similar results.

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CHAPTER 5

DECREASED EXPRESSION OF PAX7 PROTEIN BY RHABDOMYOSARCOMA INITIATOR PAX7-FKHR FUSION PROTEIN

Introduction

Rhabdomyosarcoma (RMS) is a highly malignant soft tissue tumor found in children and young adults. Its two main subtypes are embryonal rhabdomyosarcoma (ERM) and alveolar rhabdomyosarcoma (ARM). ARM is the more aggressive subtype (Tsokos et al., 1992). RMS cells express a number of skeletal muscle markers, such as desmin, sarcomeric actin, sarcomeric myosin heavy chain and MyoD, which suggests its myogenic origin (Merlino and Helman, 1999). Embryonal RMS is characterized by loss of heterozygosity on the short arm of chromosome 11 (Loh et al., 1992). Approximately 70% of alveolar RMS has one of two specific chromosomal translocations, which fuse the Pax3 or Pax7 gene on chromosome 1 or 2 to the FKHR gene on chromosome 13 (Turc-Carel et al., 1986; Douglass et al., 1987; Wang-Wuu et al., 1988). Among patients with the chromosomal translocation-positive ARMs, the Pax7-FKHR fusion protein is present in ~20% cases.

The chromosomal translocations break within intron 7 of paired box transcription factor Pax3 or Pax7 and within intron 1 of the forkhead transcription factor FKHR. The product connects 5'-end of Pax3 or Pax7 to the 3'-end of FKHR, and generates a fusion protein with Pax3 or Pax7 DNA binding domain and the FKHR transcription activation domain (Fig.1-3) (Barr et al., 1993; Galili et al., 1993; Shapiro et al., 1993; Davis et al., 1994). The fusion proteins are more potent transcriptional activators than wild-type Pax3/7 or FKHR due to several reasons. First, the FKHR transactivation domain is insensitive to the inhibitory effects of the N-terminal Pax3 or Pax7 domains (Bennicelli et al., 1995). Second, unlike the wild-type FKHR, the transcriptional activity and the nuclear localization of fusion proteins are not regulated by Akt phosphorylation (Peso et al., 1999). Third, the fusion genes are over-expressed in ARM. The Pax7-FKHR over-expression is associated with gene amplification. The Pax3-FKHR over-expression results from copy number-independent fusion gene transcription (Davis and Barr, 1997). Thus, Pax3-FKHR and Pax7-FKHR use "gain of function" mechanism relative to the wild-type Pax3 and Pax7 proteins in ARM.

Pax3 and Pax7 are closely related Pax family members that play essential roles in neural and muscle development. They are characterized by an N-terminal DNA binding paired box domain, a short conserved octapeptide motif and a complete homeobox. They also have a proline-, serine- and threonine-rich C-terminal transactivation domain (Jun and Desplan, 1996; Seo et al., 1998). Pax3 and Pax7 partially overlap in their expression during development. They

both have essential functions in the development of embryonic neural cells (Kalcheim and Le Douarin, 1986). Pax3 and Pax7 are also crucial for embryonic development of skeletal muscle (Kassar-Duchossoy et al., 2005; Relaix et al. 2005). Pax7 is indispensible in the maintenance of muscle satellite cells (Seale et al., 2000; Oustanina et al., 2004; Kuang et al., 2006). The number of satellite cells declines strongly during postnatal development in Pax7-null mice. This is a result of the anti-apoptotic role played by Pax7, which cannot be compensated by Pax3. Pax7 also helps to maintain the quiescent state of satellite cells by delaying the onset of myogenic differentiation (Zammit et al., 2006).

Ubiquitination is a post-translational modification that controls protein stability. It has been revealed that Pax3 is regulated by mono-ubiquitination and proteasomal degradation. The amino acid lysine 475 at the C-terminal region of Pax3 is responsible for the susceptibility to mono-ubiquitination. The corresponding residue in Pax7 is an alanine (Ala486), which is not a target for ubiquitination. As a result, Pax7 is not susceptible to proteasomal degradation (Boutet et al., 2007).

The Pax7 gene consists of nine exons. Exons 2, 3 and 4 encode the paired domain. The homeodomain is encoded by exons 5 and 6. The transactivation domain is encoded by exons 6, 7 and 8 (Vorobyov et al., 1997; Barr et al., 1999). As a result of alternative splicing which occurs at the intron 2/exon 3 junction and the intron 3/exon 4 junction, there are four alternative Pax7 transcripts with the inclusion or exclusion of a tri-nucleotide, CAG, and a hexa-nucleotide, GTTTAG, respectively. This results in the inclusion or exclusion of a glutamine (Q) and a glycine-leucine dipeptide (GL) in four Pax7 isoforms (Q+GL+, Q+GL-, Q-GL+, Q-GL-)(Ziman and Kay, 1998). The isoforms contain distinct tertiary structures and have different DNA binding affinities and specificities. Corresponding to the wild-type Pax7 isoforms, there is a consistent pattern of co-expression of Pax7-FKHR isoforms in ARM tumors. The DNA binding ability and transcriptional activity of these Pax7-FKHR isoforms also vary (Du et al., 2005).

The chromosomal translocation occurs in one set of the chromosomes, with the other set expressing the wild-type Pax3 or Pax7 and FKHR proteins. The wild-type Pax3 and Pax7 proteins share the same DNA binding domains with the oncogenic Pax3-FKHR and Pax7-FKHR fusion proteins, and may compete with each other for DNA binding sites and protein interactions. Thus, the oncoproteins may need to down-regulate the wild-type proteins in order to circumvent the interferences. Studies of RMS tumor samples and RMS cell lines reveal that

wild-type Pax3 is up-regulated in both ERM and ARM. Its up-regulation is independent of fusion genes (Barr et al., 1999; Tiffin et al., 2003). While elevated wild-type Pax7 expression is also observed in ERM (Bernasconi et al., 1996; Barr et al., 1999) and in fusion negative ARM (Tiffin et al., 2003), Pax7 expression is reduced in primary ARM tumors with Pax-FKHR fusion genes (Tiffin et al., 2003), and in Pax3-FKHR or Pax7-FKHR expressing ERM cell lines (Tomescu et al., 2004). However, the molecular mechanism of how fusion proteins down-regulate the wild-type Pax7 is not clear.

In this study, we show that co-transfection with Pax7-FKHR fusion protein decreases the level of wild-type Pax7 protein in the presence or absence of a proteasomal inhibitor. In a human HEK293 cell line stably expressing exogenous Pax7 protein under the control of SV40 promoter, although Pax7 protein was decreased in a dose-dependent manner, Pax7 mRNA level was not affected by Pax7-FKHR transfection. MicroRNA microarray analysis revealed that more than twenty miRNAs were significantly up-regulated upon expression of Pax7-FKHR fusion protein. However, none of these up-regulated miRNAs targeted the short fragment of human Pax7 3'-UTR present in our vector.

Results

It has been shown that Pax7 expression is reduced in Pax-FKHR fusion positive primary ARM tumors (Tiffin et al., 2003), and in Pax3-FKHR or Pax7-FKHR expressing ERM cell lines (Tomescu et al., 2004). To examine the direct effect of Pax7-FKHR fusion protein on Pax7 expression, we co-transfected C2C12 myogenic cells with human Pax7 expression vector and different doses of human Pax7-FKHR expression vector. On Western blots, both Pax7 (57kDa) and Pax7-FKHR (94kDa) can be detected by an antibody recognizing the N-terminal consensus sequence of Pax proteins. The exogenous Pax7 protein was decreased upon co-expression of the fusion protein (Fig.5-1A). Pax7-FKHR may form heterodimers with the wild-type Pax7 and affect Pax7 protein stability. To determine whether the decreased Pax7 was due to protein degradation, we treated C2C12 cells with a proteasomal inhibitor, MG132, 24 hours after transfection. In the presence of a proteasomal inhibitor, Pax7-FKHR fusion protein was stabilized and a more pronounced decrease of wild-type Pax7 was observed with the fusion protein co-transfection (Fig.5-1B). This result is consistent with the observations from a study on the regulation of Pax3 proteasomal degradation (Boutet et al., 2007). The amino acid lysine 475

at the C-terminal region of Pax3 is responsible for its susceptibility to mono-ubiquitination and proteasomal degradation. The corresponding residue in Pax7 is an alanine (Ala486), which is not a target for ubiquitination. As a result, Pax7 is not susceptible to proteasomal degradation. Furthermore, within the Pax7-FKHR fusion protein, the C-terminal part containing the Ala486 is replaced by the C-terminal domain of FKHR protein, which may contain an ubiquitination and proteasomal degradation target site. To obtain a higher expression level of Pax7 and Pax7-FKHR proteins, we transfected COS7 cells with a fixed dose of human Pax7 expression vector and increased doses of human Pax7-FKHR expression vector. COS7 cells were treated with MG132, 24 hours after transfection. A dose-dependent decrease of the wild-type Pax7 protein was observed with the increasing levels of the fusion protein (Fig.5-1C). Together, these co-transfection results show that the wild-type Pax7 protein is decreased by co-expression of Pax7-FKHR fusion protein. The decreased Pax7 protein level is independent of proteasomal degradation.

Transient transfection may cause the initial unevenness in Pax7 expression level, which interferes with the effect induced by Pax7-FKHR. We next generated HEK293 cells stably expressing exogenous human Pax7. HEK293 cell line is a human cell line which does not have endogenous Pax7 expression. After transfection with human Pax7 expression vector or empty vector, HEK293 cells were selected for two weeks in the presence of G418. As determined by immunofluorescence microscopy, approximately 70% of HEK293 cells were Pax7-positive after selection (Fig.5-2A). Pax7-expressing HEK293 cells were transfected with Pax7-FKHR fusion protein, and treated with MG132, 24 hours after transfection. With the increasing amounts of the fusion protein, Pax7 protein level was reduced to ~70%, ~40% and ~30%, respectively, as compared to the empty vector transfected HEK293-Pax7 cells (Fig.5-2B). These results indicate that Pax7 protein is decreased by Pax7-FKHR fusion protein expression in a dose-dependent manner.

The exogenous Pax7 introduced to HEK293 cells is under the control of a constitutively active SV-40 promoter, which makes the expression of Pax7 independent of its natural promoter regulation. After excluding the involvement of proteasomal degradation, we next asked whether the mRNA level of Pax7 is affected by Pax7-FKHR fusion protein expression. Similar to the previous experiment, HEK293 stably transfected cells were treated with MG132, 24 hours after transfection of the fusion protein. After total RNA extraction and reverse transcription, we used a

PCR primer set detecting only the wild-type Pax7, not Pax7-FKHR fusion protein. PCR amplification of Pax7 occurred only in Pax7-expressing HEK293 cells, not in empty vector transfected cells without or with Pax7-FKHR transfection (Fig.5-3A). Semi-quantitative PCR results suggested that in HEK293-Pax7 cells, the mRNA level of Pax7 was not changed by transfection and increasing expression of Pax7-FKHR (Fig.5-3A). β-actin was used as an internal control for total RNA amount. The unaltered Pax7 mRNA level upon the fusion protein transfection was confirmed by real time PCR experiment using the same cDNA preparations (Fig.5-3B). Taken together, these results suggest that although the protein level of Pax7 was decreased markedly, the mRNA level of Pax7 is not changed by Pax7-FKHR fusion protein expression.

To further compare Pax7 mRNA stability with and without fusion protein transfection, we treated HEK293-Pax7 cells with actinomycin D to inhibit transcription and prevent the synthesis of new mRNA. HEK293 cells stably expressing Pax7 were transfected with either empty vector or Pax7-FKHR expression vector. 24 hours after transfection, cells were treated with MG132 for 10 hours, followed by treatment with actinomycin D in the presence of MG132. Total mRNA from vector transfected and Pax7-FKHR transfected cells was collected 0, 1, 2, 4 and 6 hours after adding actinomycin D. Pax7 mRNA levels were determined by semi-quantitative PCR. As expected, Pax7 mRNA level was decreased in both vector- and fusion protein-transfected cells after addition of actinomycin D. Importantly, the decay rate of Pax7 mRNA in Pax7-FKHR fusion protein-transfected cells was similar to the decay rate in empty vector-transfected cells, with a rapid reduction to ~60% after 1 hour treatment and then more modest reduction to 40-50% during the 6 hour-incubation (Fig.5-4). This result indicates that Pax7 mRNA stability is not affected by the fusion protein transfection, and confirms that the decreased Pax7 protein is not due to the decrease in mRNA level.

The deceased protein expression without changes in mRNA level suggests a specific Pax7-FKHR-induced inhibition of Pax7 mRNA translation. MicroRNAs (miRNAs) can block the translational machinery and prevent protein translation without causing mRNA degradation. Mammalian microRNAs frequently target the 3'-UTR and function as negative regulators of gene expression. The human Pax7 expression construct, which we introduced to HEK293 cells, includes a short fragment of 3'-UTR containing several potential miRNA target sites. These potential miRNAs include hsa-miR-19b-2*, hsa-miR-19b-2* and hsa-miR-665 from the Sanger

Institue Database (microrna.sanger.ac.uk), and hsa-miR-575 from the TargetScan Database (www.targetscan.org). HEK293 cells were transiently transfected with empty vector, Pax7 expression vector, or Pax7-FKHR expression vector. 24 hours after transfection, cells were treated with MG132 for 10 hours and total RNA samples were collected. MicroRNA microarray analysis was performed by LC Sciences (Houston, TX). After in-depth data analysis, the relative expressions for the microRNAs that changed significantly (ANOVA, p-value < 0.01) were sorted with a hierarchical clustering analysis (Fig.5-5). Although more than twenty miRNAs were significantly up-regulated in fusion-protein transfected cells, compared to empty vector- or Pax7-transfected cells, none of predicted miRNAs for Pax7 3'-UTR were included in the list.

Discussion

Approximately 70% of alveolar RMS has one of the two specific chromosomal translocations on chromosome 1 or 2 with chromosome 13, which generates oncogenic fusion proteins Pax3-FKHR or Pax7-FKHR, respectively (Barr et al., 1993; Galili et al., 1993; Shapiro et al., 1993; Davis et al., 1994). The chromosomal translocation occurs in one set of the chromosomes, with the other set expressing wild-type Pax3 or Pax7 and FKHR proteins. The wild-type proteins are presumably able to perform their normal functions. The wild-type Pax3 and Pax7 can potentially compete for binding sites with the fusion proteins as they share the same DNA binding domains. In fusion-positive ARM tumors, Pax3-FKHR and Pax7-FKHR are expressed at higher levels than the wild-type Pax3 and Pax7, respectively. Over-expression of Pax3-FKHR and Pax7-FKHR fusion proteins ensures a critical level of gene products for the oncogenic effects of these fusions (Davis and Barr, 1997). Fusion proteins could also down-regulate the wild-type Pax3 and Pax7 to circumvent the interfering effect. Our finding of the down-regulation of the wild-type Pax7 protein with the Pax7-FKHR fusion protein transfection is consistent with previous studies, where Pax7 expression was reduced in primary ARM tumors with Pax-FKHR fusion genes (Tiffin et al., 2003), and in Pax3-FKHR or Pax7-FKHR expressing ERM cell lines (Tomescu et al., 2004). This study provides an insight into the dominant-negative effect of Pax7-FKHR fusion protein on the wild-type Pax7.

It has been shown that wild-type Pax3 is up-regulated in both ERM and ARM, in the absence or in the presence of the fusion genes (Barr et al., 1999; Tiffin et al., 2003). Thus, unlike

down-regulation of the wild-type Pax7, the wild-type Pax3 protein would either be up-regulated or unaffected by the expression of fusion proteins. This remains to be examined.

In this study, we generated a HEK293 cell line with stable expression of exogenous human Pax7 protein. In our system, while the Pax7 expression is decreased by Pax7-FKHR transfection in a dose-dependent manner (Fig.5-2B), the mRNA level of Pax7 is not affected (Fig.5-3). It has been reported that the endogenous Pax7 mRNA level is decreased markedly in long-term stable Pax3-FKHR- or Pax7-FKHR-expressing ERM cells (Tomescu et al., 2004). The discrepancy can be explained by the differences of the experimental systems. The exogenous Pax7 protein tested in our system is under the control of SV40 promoter, while the expression of the endogenous Pax7 in ERM cells is regulated by its natural promoter and is influenced by the tumor cell environment. Furthermore, in our system, the introduction of the fusion protein Pax7-FKHR was through transient transfection and we examined its short-term effect, while the down-regulation of endogenous Pax7 mRNA is more sensitive to the long-term effects of Pax3-FKHR and Pax7-FKHR proteins. Taken these two factors into consideration, the down-regulation of the wild-type Pax7 protein expression by fusion proteins could be at multiple levels, with an mRNA quantity-dependent mechanism, possibly through the promoter regulation, and an mRNA quantity-independent inhibition of protein translation.

It has been suggested that miRNAs can function as either tumor suppressors or oncogenes (Esquela-Kerscher and Slack, 2006). A recent comprehensive analysis of miRNA expression profiles of 27 sarcomas, including rhobdomyosarcoma, has demonstrated that different histological types of sarcoma have distinct miRNA expression patterns (Subramanian et al., 2008). MicroRNA microarray analysis was performed to determine the effect of oncogenic fusion protein Pax7-FKHR on miRNA expression profile (Fig.5-5). To our disappointment, none of the predicted miRNAs targeting the short fragment of human Pax7 3'-UTR present in our vector was significantly up-regulated by fusion protein expression. However, expression of more than twenty miRNAs was significantly increased in fusion protein-transfected cells compared with vector- and Pax7-transfected cells. Although 3'-UTR of mRNA is the predominant target for miRNAs in mammals, miRNAs can also down-regulate protein expression through interaction with the coding region of mRNA. During miRNA recognition, it is essential that the ~7nt sites in mammalian mRNA match the seed region of miRNA. The possible recognition of

the coding region of Pax7 mRNA by the up-regulated miRNA candidates is to be further explored.

The oncogenic fusion protein Pax7-FKHR decreases the wild-type Pax7 protein expression without altering the Pax7 mRNA stability. This study provides an insight into the mechanism of Pax7-FKHR dominant-negative effect on the wild-type Pax7. As our Pax7 expression system is free of its natural promoter regulation and is irresponsive to proteasomal degradation, we propose a specific inhibition of Pax7 mRNA translation involving miRNAs. Although the expression of the predicted miRNAs targeting the short fragment Pax7 3'-UTR is not increased by Pax7-FKHR transfection, whether the significantly up-regulated miRNA candidates target the coding region of Pax7 mRNA needs to be determined.

Materials and Methods

Expression constructs

Human Pax7(Q+GL-)-pcDNA3 and Pax7-FKHR(Q+GL-)-pcDNA3 expression vectors were provided by Dr. Frederic G. Barr (University of Pennsylvania School of Medicine).

Cells

C2C12 and COS7 cells were obtained from American Tissue Culture Collection; HEK293 cells were purchased from Clontech. C2C12, COS-7 and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37˚C in presence of 5% CO_2 under a humidified atmosphere.

Plasmid transfection and selection of stably transfected cells

Transient transfections were performed using Fugene 6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol, one day after plating cells. Stably transfected HEK293 cells were selected for two weeks with 1 mg/ml G418.

Cell treatments

24 hours after transfection, when cells were 90%-100% confluent, they were treated with 10 µM MG132 (EMD Biosciences, dissolved in DMSO) or DMSO alone for 10 hours. For the mRNA stability experiment, actinomycin D (Sigma, dissolved in DMSO) was added to the

culture medium at a concentration of 10 μg/mL to inhibit RNA transcription. Cells were harvested at indicated times after addition of actinomycin D and used for total RNA isolations.

Western blotting

Cells were incubated in extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (AEBSF), 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 mM 1,10-phenanthroline) for 15 min at 4°C. Cell extracts were centrifuged at 21,000xg for 15 min, and supernatants were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked in DPBS containing 3% (w/v) dry milk and 0.3% (v/v) Tween 20, then incubated with primary antibodies in blocking buffer, followed by incubation with horseradish peroxidase-labeled secondary antibodies and detection using the WestPico chemiluminescence kit (Pierce). The following primary antibodies were used: mouse anti-Pax7 (ascites, Developmental Studies Hybridoma Bank, 1:500), rabbit anti-Pax (H-150, Santa Cruz Biotechnology, 1:5000), mouse anti-α-tubulin (Sigma, 1:100,000). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG.

Immunofluorescence

Cells grown on coverslips were fixed with 3.7% paraformaldehyde in DPBS for 20 min and permeabilized with 0.1% Triton X-100 in DPBS for 5 min. Cells were blocked in DPBS containing 5% donkey serum (v/v) and 1% BSA (w/v), then incubated with primary antibody mouse anti-Pax7 (supernatant, Developmental Studies Hybridoma Bank, 1:5) diluted in DPBS containing 1% BSA, followed by incubation with Rhodamine-RedX-conjugated anti-mouse secondary antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Coverslips were mounted on slides and examined by Axiovert 200 inverted fluorescent microscope (Zeiss).

Reverse transcription PCR

Total RNA was extracted using TRIzol Plus RNA purification kit (Invitrogen) according to the manufacturer's protocol. RNA quantity and quality were determined by spectrophotometry. For each reaction, 1 μg RNA was treated with DNase I (Invitrogen) at room temperature for 15 min. cDNA samples were generated with Oligo(dT) using SuperScript III First-Strand Synthesis

for RT-PCR kit (Invitrogen). PCR reactions were performed using BIO-X-ACT Short DNA Polymerase kit (Bioline). The primer pairs for human Pax7 were 5'-GCAAGCAGCGACGCAGTCG-3' (forward) and 5'-GAGAAGTCAGCCTGTGGCT-3' (reverse). The primer pairs for human β-actin were 5'-GCTCGCGTCGACAACGGCTC-3' (forward) and 5'-CAAACATGATCTGGGTCATCTTCT-3' (reverse).

Real time PCR

Real time reactions were performed in 25 μL mixture containing 12.5 μL of iQ SYBR Green Supermix (BIO-RAD), 1 μL of each primers (5 μM) and 10.5 μL of 1250 times dilution of cDNA preparations from reverse transcription. The primer pairs for human Pax7 were 5'-CCACAGCTTCTCCAGCTACTCTG-3' (forward) and 5'-GGGTTGCCCAAGATGCTC-3' (reverse). The primer pairs for human β-actin were 5'- TTGCCGACAGGATGCAGAA-3' (forward) and 5'-GCCGATCCACACGGAGTACT-3' (reverse). Real time quantifications were performed using the BIO-RAD iCycler iQ system. The fluorescence threshold value was calculated using iCycler iQ system software. The relative change was calculated using $2^{-\Delta\Delta C_T}$ method.

MicroRNA microarray

Total RNA extraction was performed using miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. miRNA expression profiling was determined by microRNA microarray analysis using 856 human miRNA array probes (version 12.0; LC Sciences, Houston TX). ANOVA statistic test was performed to determine the significant changes (p-value < 0.01) of different samples.

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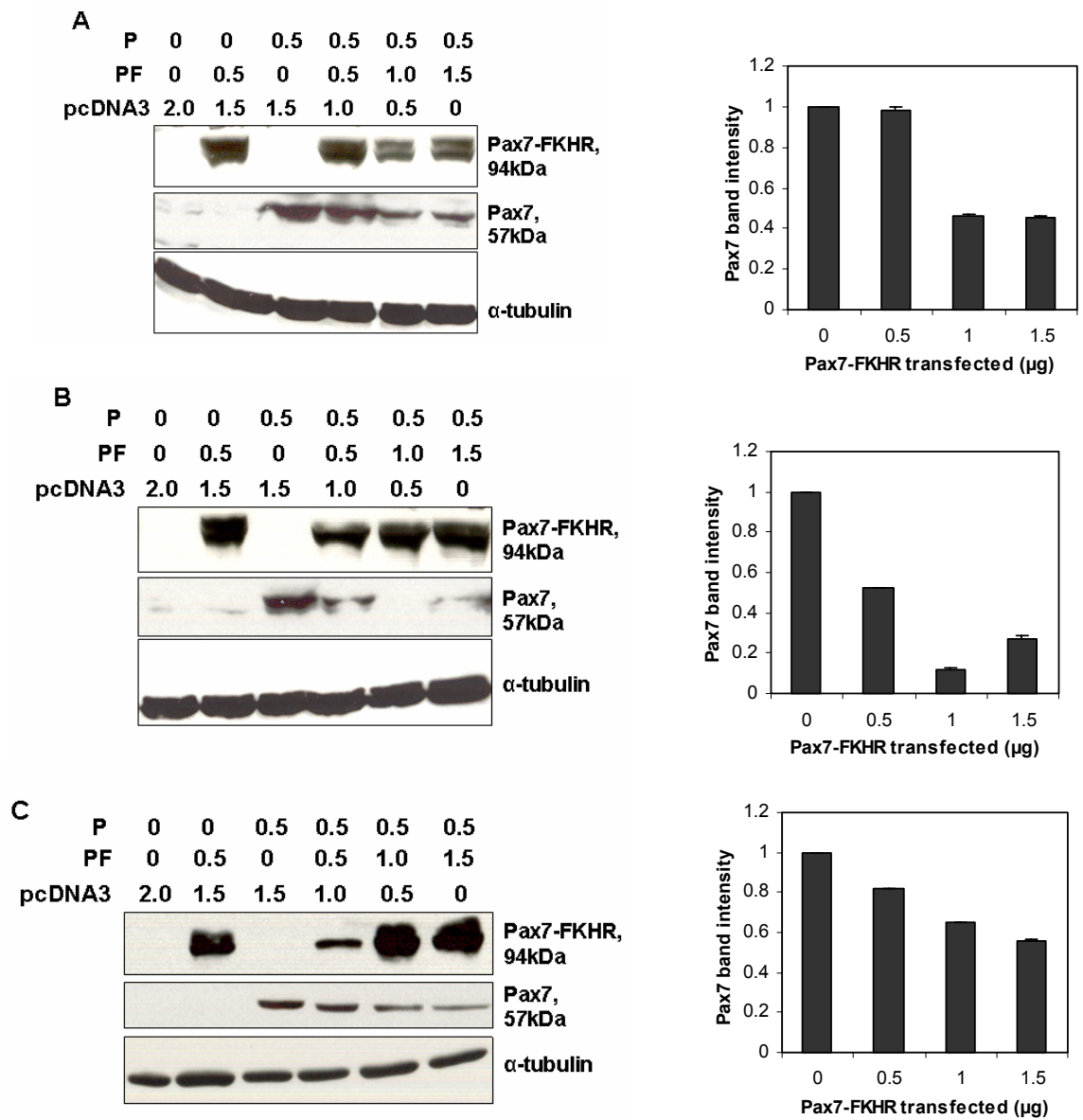
Figure 5-1. **Pax7 protein is decreased by co-transfection of Pax7-FKHR in the absence and in the presence of a proteasomal inhibitor.** C2C12 cells (A and B) or COS7 cells (C) were co-transfected with human Pax7 expression vector (P) and human Pax7-FKHR expression vector (PF), or empty pcDNA3 vector at the indicated amounts (μg). 24 hours after transfection, cells were treated with DMSO (A) or 10 μM MG132 (B and C) for 10 hours. Western blotting with anti-Pax antibody was performed to show the expression of Pax7-FKHR and Pax7. α-tubulin was used as a loading control. Band intensities of Pax7 protein were quantified by densitometry. Data represent the mean values $\pm$ s.e.m. from three measurements.
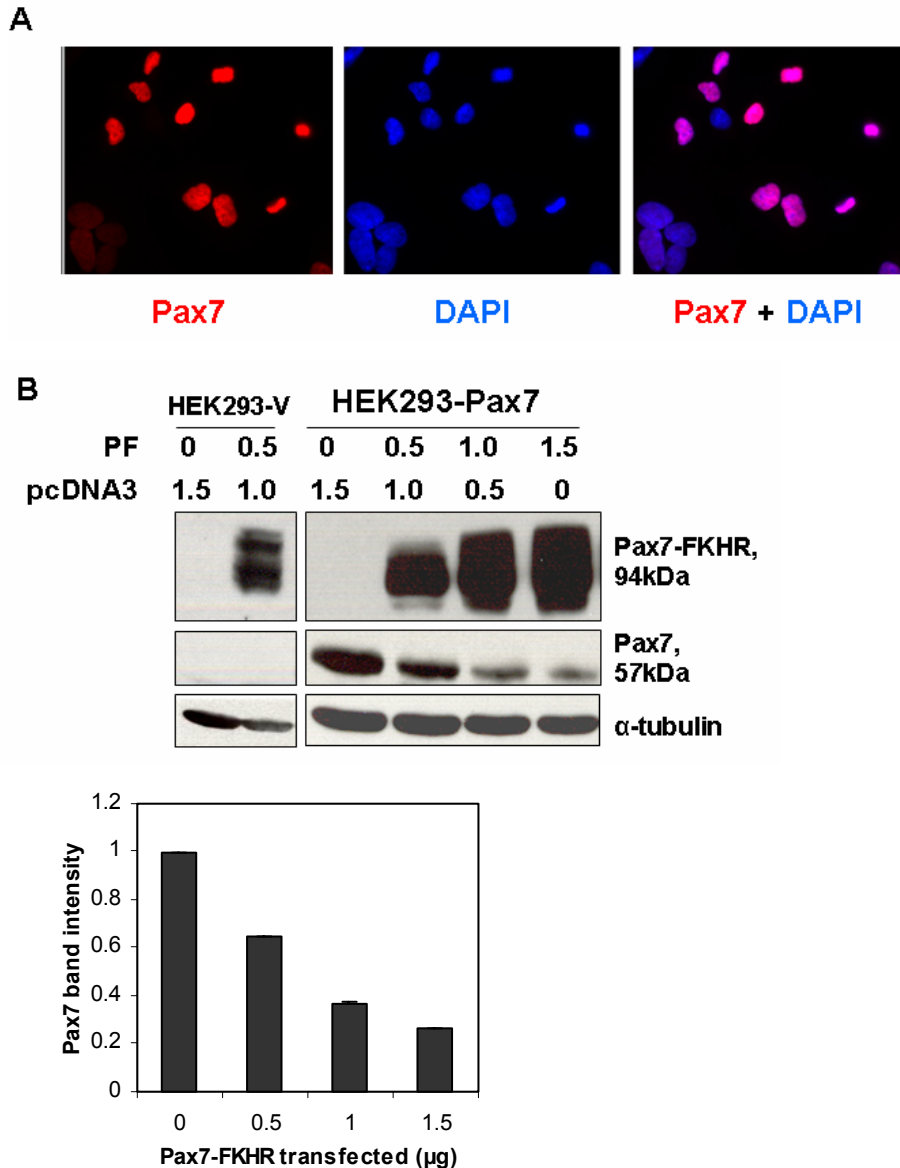
Figure 5-2. **Pax7 protein in stably transfected-HEK293 cells is decreased by Pax7-FKHR transfection in a dose-dependent manner.** (A) HEK293 cells were transfected with human Pax7 expression vector and selected for two weeks in the presence of 1 mg/ml G418. Stably transfected HEK293 cells (red) were stained with anti-Pax7 monoclonal antibody. Nuclei (blue) were stained with DAPI. (B) HEK293 cells stably transfected with empty vector (HEK293-V), or stably expressing Pax7 (HEK293-Pax7) were transiently transfected with Pax7-FKHR expression vector (PF), or empty pcDNA3 vector at the indicated amounts (μg). 24 hours after transfection, cells were treated with 10 μM MG132 for 10 hours. Western blotting with anti-Pax antibody and anti-Pax7 monoclonal antibodies was performed to show the expression of Pax7-

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FKHR and Pax7, respectively. α-tubulin was used as loading control. Band intensities of Pax7 protein were quantified by densitometry. Data represent the mean values ± s.e.m. from three measurements. The experiment was repeated twice with similar results.
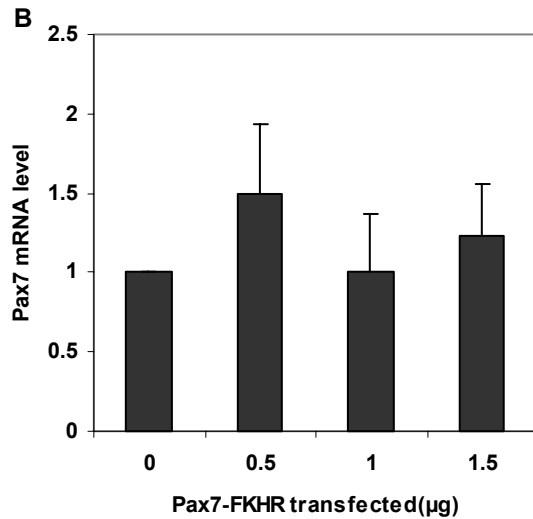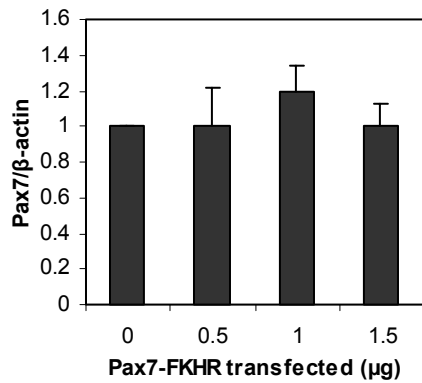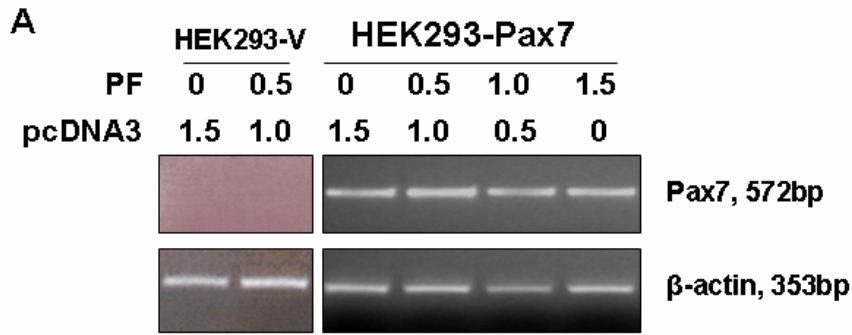
Figure 5-3. **Pax7 mRNA in stably transfected-HEK293 cells is not affected by Pax7-FKHR transfection.** HEK293 cells stably transfected with empty vector (HEK293-V), or stably expressing Pax7 (HEK293-Pax7) were transiently transfected with Pax7-FKHR expression vector (PF), or empty vector at the indicated amounts (μg). 24 hours after transfection, cells were treated with 10 μM MG132 for 10 hours. Total RNA samples were extracted. (A) After reverse transcription, PCR reactions were performed using primer sets for Pax7 and β-actin, respectively. Band intensities of Pax7 and β-actin were quantified by densitometry. Data represent the mean

values ± s.e.m. from three different experiments. (B) Real time PCR experiments were performed using the same cDNA samples as in A. Relative changes were calculated using $2^{-\Delta\Delta C_T}$ method. Data represent the mean values ± s.e.m. from three different experiments.
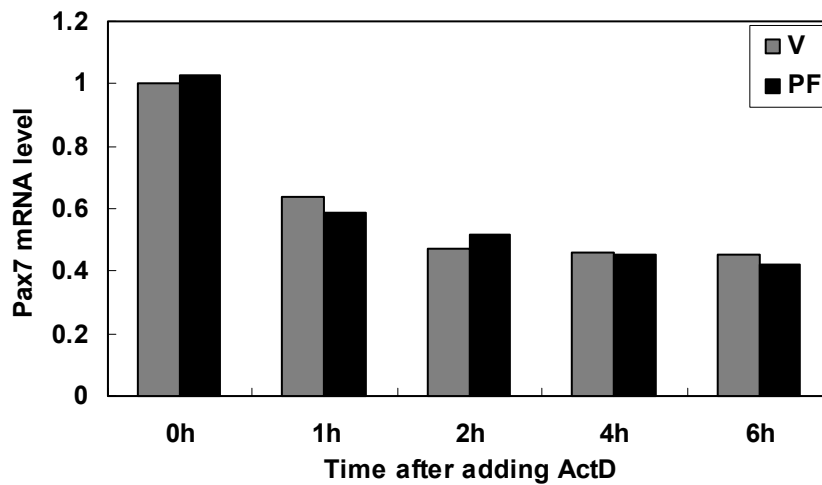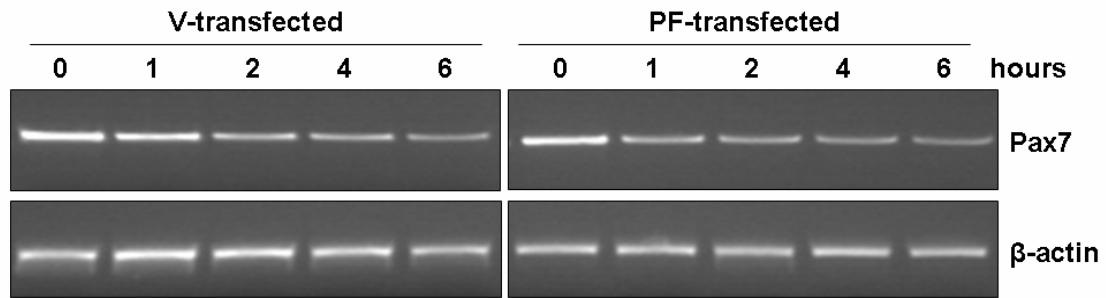
Figure 5-4. **Pax7 mRNA stability is not affected by transfection of Pax7-FKHR.** HEK293 cells stably expressing Pax7 were transiently transfected with empty vector (V-transfected) or Pax7-FKHR expression vector (PF-transfected). 24 hours after transfection, cells were treated with 10 μM MG132 for 10 hours. Cells were then incubated with actinomycin D (ActD), and total RNA samples were collected at 0h, 1h, 2h, 4h and 6h for both vector and fusion protein transfected cells. Reverse transcription and PCR reactions were performed. Band intensities of Pax7 and β-actin were quantified by densitometry. The amount of Pax7 mRNA was normalized to the amount of β-actin. The experiment was repeated twice with similar results.
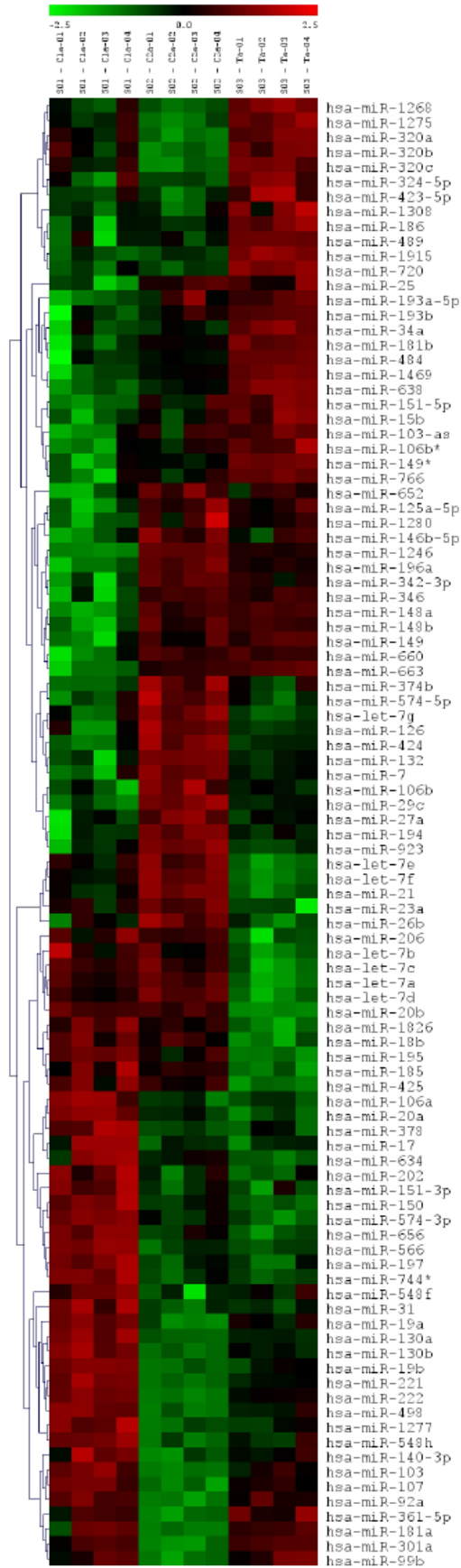
Figure 5-5. **Profile of microRNA expression in vector-transfected, Pax7-transfected and Pax7-FKHR-transfected HEK293 cells.** HEK293 cells were transiently transfected with empty vector (C1a), Pax7 expression vector (C2a) or Pax7-FKHR expression vector (Ta). 24 hours after transfection, cells were treated with 10 µM MG132 for 10 hours. Total RNA samples were extracted and microRNA microarray analysis was carried out by LC Sciences (Houston, TX). Each row represents the expression level for a single miRNA and each column represents a single repeat of each sample. Each sample has four repeats for a given miRNA. The red or green color indicates relatively high or low expression level, respectively. The median of each group for a given microRNA is set as 0. The range of relative expression values is from -2.5-fold to 0 to +2.5-fold. The relative expressions for the miRNAs that changed significantly (ANOVA, p-value < 0.01) are sorted with a hierarchical clustering analysis. Notice that the miRNAs which are red only in Ta samples are the miRNAs up-regulated by transfection of the fusion protein.

CHAPTER 6

CONCLUSIONS

The first half of this dissertation is about the cleavage of Notch ligand Dll1 by ADAM metalloproteases (Chapter 2) and the effect of Dll1 shedding on muscle cell self-renewal and differentiation (Chapter 3).

The Notch ligand Dll1 undergoes proteolytic cleavage by ADAMs. ADAM10 has been implicated in the processing of mouse Dll1. However, proteolytic cleavage of Dll1 in ADAM10-/- mouse embryonic fibroblasts (MEFs) still amounts to 50% of the processing observed in ADAM10+/+ MEFs (Six et al., 2003), suggesting the involvement of other ADAMs in the remaining Dll1 processing in ADAM10-/- cells. In our study, the reduction of Dll1 cleavage in ADAM9/12/15-/- MEFs indicates that the endogenous ADAM9 or ADAM12 or both are also capable of cleaving Dll1 (ADAM15 is not capable of Dll1 processing even when over-expressed). This study expands the role of ADAMs in processing Notch ligand and suggests their function in modulating Notch signaling.

The Notch pathway is activated upon interaction of a DSL ligand in a signal-sending cell with a Notch receptor in a signal-receiving cell, which is known as *trans* activation. A high level of DSL ligand expression in the Notch-expressing cell can inhibit Notch signaling in a cell-autonomous manner, which is known as *cis* inhibition. Proteolytic processing of Notch ligand Dll1 by ADAM metalloproteases can presumably have dual effects on Notch signaling: down-regulation of Notch signaling by reducing the ligand availability and up-regulation of Notch signaling by relieving the *cis* inhibition (Fig.6-1). Both effects of ADAM-mediated Dll1 shedding on Notch signaling have been observed in our studies.

Ligand shedding can affect Notch signaling in a cell-autonomous manner. It has been reported for mammalian Jagged 1 that the C-terminal fragment of the ligand could compete with Notch for γ-secretase and inhibit Notch signaling (LaVoie and Selkoe, 2003). Our results demonstrate that the relief of the *cis* inhibition of Notch mediated by Dll1 outweighs the generation of a Dll1 fragment that can compete for γ-secretase cleavage. While co-transfection of Dll1 and Notch into the signal receiving cells decreased the Notch signaling due to *cis* inhibition, further co-transfection of Dll1-processing ADAM12 into the signal-receiving cells led to activation of Notch. The effect of Notch activation was not observed with a catalytically inactive mutant of ADAM12. Furthermore, other ADAMs, including ADAM9 and ADAM17, which were capable of Dll1 cleavage, were also capable of relieving Dll1-mediated *cis* inhibition. In contrast, ADAM15, an ADAM that was not involved in Dll1-processing, did not activate Notch

signaling. These results show a consistent correlation of Dll1-processing by ADAMs with the relief of Dll1-mediated *cis* inhibition and activation of Notch signaling. Whether Dll1-mediated *cis* inhibition of Notch signaling can be relieved by endogenous ADAM cleavage and whether this effect occurs *in vivo* remains to be determined.

Ligand shedding can affect Notch signaling in neighboring cells. RECK (reversion-inducing cysteine-rich protein with Kazal motifs), a physiological inhibitor of ADAM10, is specifically expressed in nestin-positive neural precursor cells (NPCs). RECK decreases the ectodomain shedding of Notch ligand by directly inhibiting the proteolytic activity of ADAM10. RECK-deficient NPCs undergo precocious differentiation, which is associated with increased ADAM10 activity and consequently excessive Dll1 shedding that leads to impaired Notch signaling in neighboring cells (Muraguchi et al., 2007). In our study, the soluble catalytically inactive extracellular domain of ADAM12, protein X is capable of binding to Dll1 and preventing its cleavage by ADAMs, without interfering with Dll1 and Notch interaction in *trans*. Thus, protein X is a dominant-negative modulator reducing Dll1 cleavage by ADAMs. Global Notch signaling is elevated in primary myoblast or C2C12 myogenic cells incubated with protein X, as indicated by the increased amount of NICD and increased activity of Notch reporter.

Notch signaling acts at multiple steps of muscle development and regeneration process. Notch pathway is activated during muscle satellite cell activation and myogenic precursor cell expansion in postnatal myogenesis (Conboy and Rando, 2002). Recent genetic evidences suggest that Notch signaling initiated by Dll1 and mediated by RBP-J is essential for maintaining a pool of myogenic progenitor cells and preventing their differentiation during muscle development (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). This is consistent with our findings that inhibition of Notch signaling decreases the expression of muscle satellite cell marker, Pax7, while stimulation of Notch signaling expends the pool of Pax7$^+$ cells.

Notch has been implicated in asymmetric cell division. It has been reported that Pax7$^+$Myf5$^-$ satellite cell undergoes asymmetric cell division perpendicular to the muscle fiber, and generates a basal Pax7$^+$Myf5$^-$ cell contributing to the satellite cell reservoir and an apical Pax7$^+$Myf5$^+$ cell exhibiting precocious differentiation. The two daughter cells show asymmetric expression of Dll1, with higher Dll1 level and low Notch activity in the committed Pax7$^+$Myf5$^+$ cell (Kuang et al., 2007). In our study, we have utilized cultured satellite cells *in vitro* and they are deprived of niche regulation. The modulation of Notch signaling among cultured cells *in*

vitro may be achieved by stochastic mechanisms (Losick and Desplan, 2008). We propose that one of the mechanisms involves the proteolytic processing of Dll1 by ADAM proteases. Dll1 shedding helps establish a balance between $Pax7^+MyoD^+$ and $Pax7^+MyoD^-$ cells after exit from the cell cycle. Proteolytic processing of Dll1 by ADAM proteins in some cells leads to ligand depletion and down-regulation of Notch signaling in neighboring cells. Cells in which Dll1 cleavage takes place would acquire higher level of Notch activity than their neighbors, leading to down-regulation of MyoD. Cells in which the cleavage of Dll1 does not occur efficiently would attain lower level of Notch signaling and maintain MyoD expression.

The second half of this dissertation is about the mechanism of Pax7 functioning as a key regulator of muscle satellite cell survival (Chapter 4) and the decreased Pax7 protein with expression of Pax7-FKHR fusion protein generated by a specific chromosomal translocation in rhabdomyosarcoma (Chapter 5).

Pax7 is essential for maintenance of muscle satellite cells. In skeletal muscle of Pax7 knockout mice, satellite cells are present, but exist in reduced numbers during postnatal development (Oustanina et al., 2004). This is due to the anti-apoptotic function of Pax7, which cannot be compensated by its paralogue, Pax3 (Relaix et al., 2006). The heterozygous MnSOD knockout mice exhibit increased oxidative damage and incidence of apoptosis (Van et al., 2001), suggesting a critical role of MnSOD in cell survival. Our goal was to explore the potential and the mechanism of MnSOD-mediating Pax7 anti-apoptotic function. The up-regulation of MnSOD by Pax7 at the transcriptional level provides an insight into the capacity of MnSOD in mediating Pax7 function. However, whether MnSOD is indispensible for Pax7-regulated satellite cell survival needs to be further addressed. The up-regulation of MnSOD by Pax7 is not dependent on FOXO proteins, which are well-known positive regulators of MnSOD promoter. A recent study identified a consensus DNA sequence, GTCAC, which is recognized by Pax7 and mediates the Pax7 binding directly (White et al., 2008). We have analyzed the Pax7 binding sites in MnSOD promoter, but they are not required for Pax7-mediated activation. The question how Pax7 up-regulates MnSOD promoter remains open and possible co-factors and key elements await for identification.

Pax7 is involved in a chromosomal translocation that leads to alveolar rhabdomyosarcoma (ARM). The chromosomal translocation fuses the Pax7 gene on chromosome 1 to the FKHR gene on chromosome 13 and generates an oncogenic fusion protein Pax7-FKHR. It has been

observed that the wild-type Pax7 is down-regulated in fusion positive ARM tumors (Tiffin et al., 2003), and in Pax-FKHR expressing ERM cell lines (Tomescu et al. 2004), implicating that fusion proteins have a dominant-negative effect on the wild-type Pax7 in order to circumvent the possible competition and to impair the normal Pax7 protein function. Consistent with previous findings, our results show that expression of the wild-type Pax7 protein is down-regulated upon transfection of Pax7-FKHR fusion protein in HEK293 cells stably expressing exogenous Pax7. This down-regulation is independent of Pax7 promoter regulation and cannot be diminished by application of proteasomal inhibitor. As the Pax7 mRNA level is not affected by fusion protein transfection, we propose a specific inhibition of Pax7 mRNA translation involving microRNAs. A recent comprehensive analysis of miRNA expression profiles of 27 sarcomas, including rhobdomyosarcoma, has demonstrated that different histological types of sarcoma have distinct miRNA expression patterns (Subramanian et al., 2008). Although in fusion protein-transfected cells, the predicted miRNAs targeting the 3'-UTR of Pax7 do not appear to be significantly up-regulated, as revealed by microRNA expression profiling, the potential of the up-regulated miRNA candidates in blocking the translation of Pax7 mRNA needs to be examined.

In conclusion, this dissertation provides insights into the proteolytic processing of Notch ligand by ADAM metalloproteases and the effect of ligand shedding on Notch signaling and on muscle cell self-renewal and differentiation. This dissertation also sheds light on the mechanism of Pax7-mediated muscle satellite cell survival and the dominant-negative effect on the wild-type Pax7 by oncogenic fusion protein Pax7-FKHR in rhabdomyosarcoma.

For future studies, a mouse model with a mutated Dll1, which cannot be cleaved by ADAM metalloproteases should be generated. The effect of Dll1 shedding on Notch signaling and on myogenic differentiation *in vivo* could be assessed more accurately with this non-cleavable Dll1 knock-in mutant. Other mediators of the up-regulation of MnSOD by Pax7 should be identified and the requirement of MnSOD for Pax7-mediated cell survival should be determined. The involvement of the miRNAs up-regulated by fusion protein in decreasing the wild-type Pax7 expression should be examined and the potential of the miRNAs as a target for rhabdomyosarcoma treatment should be evaluated.

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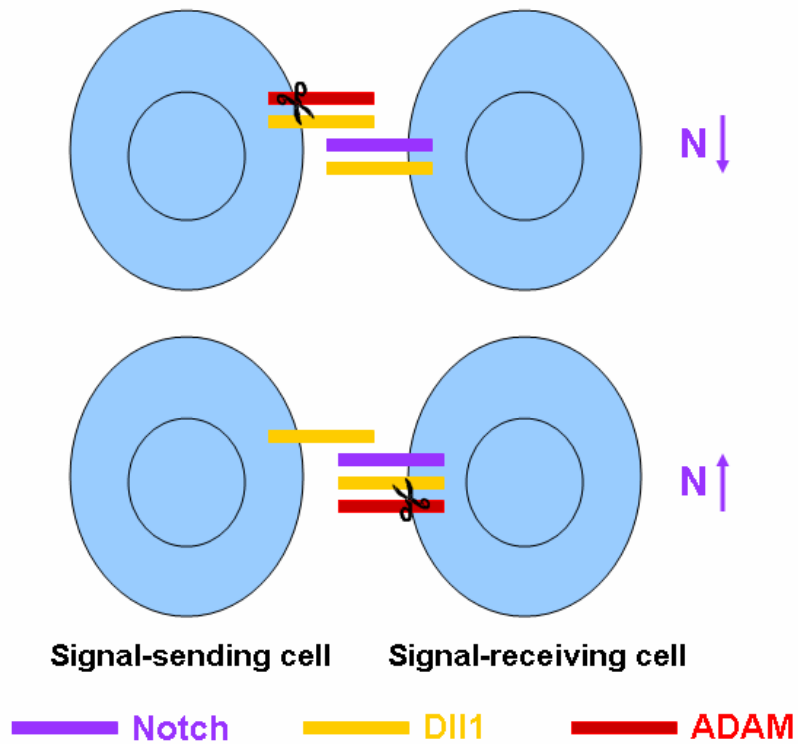
Figure 6-1. **Dll1 shedding by ADAM metalloproteases has dual effects on Notch signaling.** Proteolytic cleavage of Dll1 by ADAM metalloproteases in the signal-sending cell leads to reduced ligand availability and down-regulation of Notch signaling in the neighboring cell (upper panel). Proteolytic cleavage of Dll1 by ADAM metalloproteases in the signal-receiving cell leads to relief of *cis* inhibition and up-regulation of Notch signaling in a cell-autonomous manner (lower panel).

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