REGULATION AND PROTEOLYTIC ACTIVITY OF ADAM12 METALLOPROTEASE

by

EMILIA A. SOLOMON

M.S., University of Gdansk, Poland 2003

AN ABSTRACT OF A DISSERTATION

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Abstract

ADAMs (a disintegrin and metalloprotease) can influence multiple cellular processes involved in normal development and pathogenesis. ADAM12 expression levels are elevated in many pathological conditions including cancer, cardiovascular disease, and muscle regeneration. Recently, ADAM12 has emerged as a candidate cancer gene in a comprehensive genetic analysis of human breast cancers. The regulation of ADAM12 expression is poorly understood. Identification of new substrates for ADAM12 metalloprotease can expand our knowledge of processes in which ADAM12 is involved.

Here, we show that ADAM12 expression is upregulated by transforming growth factor β (TGFβ), an essential signaling pathway for many cellular processes. This upregulation requires proteosomal degradation of a transcriptional repressor SnoN. Furthermore, breast cancer cell lines expressing high levels of SnoN have significantly impaired induction of ADAM12 by TGFβ, suggesting an inverse correlation between SnoN and the extent of regulation of ADAM12 by TGFβ.

Additionally, we demonstrate that ADAM12 is one of the metalloproteases involved in shedding a Notch ligand, Delta like 1 (Dll1). The Notch signaling pathway plays a crucial role in cell fate decision during development and in adults. Cleavage of Dll1 by ADAMs occurs in *cis* and results in activation of Notch signaling in a cell-autonomous manner. Furthermore, the intracellular domain of Dll1 created after cleavage further enhances TGFβ signaling in response to TGFβ.

Our analysis of breast cancer-associated mutations in the ADAM12 gene showed a lack of proper proteolytic processing of the ADAM12 protein and its mislocalization to the endoplasmic reticulum . Additionally, ADAM12 mutants show a dominant-negative effect on the processing of the wild-type ADAM12 and result in loss of the functional ADAM12 at the cell surface.

Collectively, our results indicate a new mechanism of regulation of ADAM12 expression, expand the role of ADAM12 in the regulation of Notch signaling, and characterize cancer-associated mutations in the ADAM12 gene.

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Dedication

I dedicate this work to my parents Maria and Czesław, and to my husband CJ. Their love, understanding, and support have been a solid foundation for this study.

Chapter 1

Introduction

1.1 Family of ADAM metalloproteinases

The ADAM (a disintegrin and metalloprotease) family, together with astacins and matrix metalloproteases (MMPs), form the metzincin family of metalloproteinases. ADAMs, snake venom metalloproteases (SVMPs), and ADAMTS (ADAMs containing thrombospondin motifs) belong to the adamalysins subfamily. ADAMs are expressed in all animals ranging from worms to humans. As of today, 40 ADAM genes are found in various species, and 23 known ADAMs are present in humans (http://people.virginia.edu/∼jw7g/Table_of_the_ADAMs.html).

The typical domain layout of ADAMs is shown in Fig. 1.1. They possess signal sequences at their N-termini that direct the protein to the secretory pathway. This is followed by the prodomain which assures proper protein folding and maintains enzyme latency. The metalloprotease domain of many, but not all, ADAMs contains a zinc-binding consensus sequence (HEXGHXXGXXHD) and a downstream methionine. ADAMs lacking one or more critical features of the consensus sequence do not show catalytic activity. The disintegrin domain is immediately C-terminal to the metalloprotease, and is responsible for interactions between ADAMs and integrins (White, 2003). This is followed by a cysteine-rich domain which is involved in substrate interactions (Iba *et al.*, 2000; Gaultier *et al.*, 2002). Most ADAMs have an epidermal growth factor (EGF)-like domain after the cysteine-rich domain, followed by a transmembrane domain and a cytoplasmic tail. The

cytoplasmic domain varies widely in length and sequence. Several ADAMs contain motifs in their cytoplasmic domain that can act as binding sites for proteins containing the SH3 (Src homology region-3) domain, and serine, threonine, and tyrosine residues that can be phosphorylated by various kinases (Seals & Courtneidge, 2003).

ADAMs are capable of proteolytically processing multiple proteins, and therefore they are involved in many biological processes, such as adhesion, migration, and intracellular signaling. ADAMs have been implicated in the development and in pathological processes including cancer (Mochizuki & Okada, 2007), inflammation (Charrier-Hisamuddin *et al.*, 2008), neurodegeneration, and fibrosis. Most studies on ADAM12 focused on the role of ADAM12 in cancer (Iba *et al.*, 1999; Kveiborg *et al.*, 2005; Peduto *et al.*, 2006; Kodama *et al.*, 2004), but ADAM12 is also involved in muscular dystrophies (Borneman *et al.*, 2000; Galliano *et al.*, 2000), cardiac hypertrophy (Fedak *et al.*, 2006) and neurological disorders such as multiple sclerosis (Toft-Hansen *et al.*, 2004). Genetic analysis has shown polymorphism of the ADAM12 gene in patients with osteoarthritis (Valdes *et al.*, 2004, 2006) and Alzheimer's disease (Harold *et al.*, 2007). The clinical importance of ADAMs requires an understanding how their activity is regulated. This work will focus on the current knowledge of how ADAMs are regulated, with an emphasis on ADAM12.

1.1.1 Regulation of ADAMs activity

The activity of ADAM metalloproteases can be regulated by several mechanisms including gene expression, post-transcriptional control through trafficking, intracellular localization, and zymogen activation or inhibition.

Gene expression

ADAMs have different expression patterns. ADAM2, 7, 18, 20, 21, 29, and 30 expression is restricted to testis, where they are involved in spermatogenesis and sperm function. ADAM8 is present in hematopoetic cell types. Other members of the family have broader spectrum of expression in somatic tissues. Some ADAMs are also upregulated during pathological events

such as cancer (ADAM8, 9, 12, 15, 17, 19, 28) (Mochizuki & Okada, 2007) or asthma (ADAM8, 9, 12, 33) (Paulissen *et al.*, 2006). In Silico Transcriptomic Datamining studies have shown the presence of ADAM12 in placenta, mesenchymal, and adult stem cell populations (Edwards *et al.*, 2008)

Several members of the ADAM family are regulated at the transcriptional level. Foxm1 (transcriptional factor) is responsible for the regulation of ADAM17 expression (Kim *et al.*, 2005). Tumor Necrosis Factor-α induces ADAM8 expression through interferon-regulating factor 1 (Schlomann *et al.*, 2000), and ADAM9 expression is induced by oxygen species generated in response to cell stress (Sung *et al.*, 2006). TGFβ is involved in upregulation of ADAM12 in hepatic stellate cells (Le Pabic *et al.*, 2003) and ADAM19 in alveolar epithelial cells (Keating *et al.*, 2006).

Some of the ADAMs are regulated by alternative splicing of their mRNAs and generate various transmembrane or soluble protein forms. Human ADAM12 encodes both a long transmembrane form (ADAM12L) and a short form, lacking the transmembrane and cytoplasmic tail (ADAM12S) (Gilpin *et al.*, 1998). Similar alternative splicing occurs for ADAM9 (Mazzocca *et al.*, 2005), ADAM11 (Katagiri *et al.*, 1995), and ADAM28 (Roberts *et al.*, 1999). The ADAM15 gene can give rise to as many as 13 different splice variants with different cytoplasmic tails (Kleino *et al.*, 2007). Change in the cytoplasmic domain can influence interaction with cytosolic proteins. Interestingly, recent data suggest that different variants of human ADAM15 are differently expressed in human mammary carcinoma tissue and show differential association with prognosis in breast cancer (Zhong *et al.*, 2008).

Post-transcriptional control and trafficking

ADAM proteins are glycoproteins. Glycosylation is required for the full enzyme activity. Mutations in glycosylation sites of ADAM10 lead to its accumulation in the endoplasmic reticulum, decreased enzymatic activity, or an increased susceptibility for proteolysis (Escrevente *et al.*, 2008).

ADAMs are first transported to the endoplasmic reticulum (ER) and are trafficked through the Golgi apparatus to the cell membrane (Fig. 1.2). Maturation of ADAMs occurs in the Golgi where

the prodomain is cleaved at the consensus motif RX(R/K)R by pro-protein convertases (PCs) such as furin. PCs are also regulated by proteolysis, which adds an additional level to the regulation of metalloprotease activation (Seidah *et al.*, 2008). The prodomain functions through a cysteine switch mechanism blocking activation of the metalloproteases. ADAM8 (Schlomann *et al.*, 2002) and ADAM28 (Howard *et al.*, 2000) are able to autocatalytically remove the prodomain. The prodomains also functions as a chaperone, helping in proper protein folding (Cao *et al.*, 2002; Leonard *et al.*, 2005). Sometimes, the prodomain can stay non-covalently associated with the mature enzyme (Gonzales *et al.*, 2004; Wewer *et al.*, 2006).

ADAM proteins are regulated by trafficking and localization of the enzyme to the particular sites within the cell, for example ADAM19 and ADAM17 are present and function in lipid rafs (cholesterol-rich membrane microdomains) in the membrane. The cytoplasmic domain plays an important role in the proper localization of ADAMs. Replacement of the cytoplasmic domain of ADAM12 with the cytoplasmic tail of ADAM9 leads to a significant increase in the transport of the protein to the cell surface, suggesting that the cytoplasmic domain plays a role in the exit of ADAM12 from the ER (Cao *et al.*, 2002). Proline-rich motifs in the cytoplasmic domain are also important for proper basolateral localization of ADAM10 in polarized epithelial cells (Wild-Bode *et al.*, 2006).

Additional control of ADAMs' activity occurs through protein internalization. ADAM17 is rapidly removed from the cell surface by endocytosis (Doedens & Black, 2000). Epidermal growth factor (EGF) can upregulate the mature form of ADAM17 present on the membrane, possibly by enhancing stabilization of the protein (Santiago-Josefat *et al.*, 2007).

Intracellular signaling

The cellular localization of ADAMs and their substrates is important in the regulation of shedding activity. Specificity of ADAMs' substrate cleavage and what triggers the activation is not fully understood. The presence of proline-rich SH3-binding sites in the cytoplasmic domain, as well as phosphorylation sites, suggests that their function may be regulated through different binding

partners and phosphorylation.

Mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways have been implicated in the regulation of ADAMs activity. As was mentioned before, the subcellular localization of ADAMs plays a key role in their regulation. Furthermore, intracellular signaling is involved in the trafficking of ADAMs. For example, ADAM17 is localized mainly to the Golgi apparatus, and Erk-MAPK mediated phosphorylation of a tyrosine in the ADAM17 cytoplasmic tail translocates it to cell surface (Soond *et al.*, 2005). ADAM12 interacts with the p85α domain of phosphatidyl inositol 3-kinase (PI3K) and facilitates its recruitment to the membrane (Kang *et al.*, 2001). Tks5/Fish, a Src substrate, interacts with ADAM12, 15, and 19, and ADAM12 was shown to co-localize with Tks5 (Abram *et al.*, 2003). ADAM12 binds and activates Src kinase, potentially activating Tks5/Fish (Kang *et al.*, 2000).

Another possible regulation mechanism of ADAMs' activity is by direct or indirect binding of adapter proteins. Several SH3-domain containing proteins, such as PACSIN3 and Eve-1, can interact with the cytoplasmic tail of ADAM12 and are required for the activation of its proteolytic activity (Mori *et al.*, 2003; Tanaka *et al.*, 2004). PKCδ phosphorylates the cytoplasmic domain of ADAM9, stimulating shedding of heparin-binding EGF-like growth factor (HB-EGF) (Izumi *et al.*, 1998).

Phorbol myristate acetate (PMA) has been shown to transiently activate ADAM17 through the PKC pathway, but longer treatments led to decreasing amount of ADAM17 at cell surface, probably due to endocytosis (Doedens & Black, 2000). PMA has been also shown to induce ADAM12 translocation through PKCε. This translocation requires catalytic activity of PKCε (Sundberg *et al.*, 2004). Ionomycin stimulated Ca^{2+} influx upregulates ADAM10 and enhances shedding of chemokines.

G-protein-coupled receptors (GPCR) can activate ADAMs, which leads to activation of EGFR. Exactly how GPCR activates ADAMs is not fully understood, but current results suggest involvement of Src. Src is required for the GPCR-mediated activation of phosphatidyl inositol-3 kinase (PI3K), subsequently necessary for the activation of phosphatidyl inositol-dependent kinase-1

(PDK-1). PDK-1 activates ADAM17 through phosphorylation and shedding of EGFR (Zhang *et al.*, 2006).

The modification of ADAM cytoplasmic tails by phosphorylation may lead to conformational changes, and consequently to activation of ADAMs. Interaction with SH3-containing proteins can result in an accumulation of ADAMs and their substrates in specific subcellular localizations, thus allowing substrate processing. Involvement of different signaling pathways can explain the cell and/or stimuli-specific manner of ADAMs regulation.

Inhibitors

The tissue inhibitors of metalloproteases (TIMPs) are major natural inhibitors of ADAMs. TIMPs are also able to inhibit matrix metalloproteases, but they generally display much greater selectivity towards ADAMs. Each of the four TIMPs inhibits one or more ADAMs at physiologically relevant concentrations. TIMP-1 inhibits ADAM10, and TIMP-2 inhibits ADAM12. TIMP-3 has a broader inhibition range (ADAM10, 12, 17, 28, 33), and TIMP-4 inhibits ADAM28 and ADAM33 (Huovila *et al.*, 2005). ADAM10 proteolytic activity in the embryonic brain is also directly inhibited by the reversion-inducing cysteine-rich protein with Kazal motif (RECK) (Muraguchi *et al.*, 2007), indicating that additional natural inhibitors of ADAM activity may exist.

1.1.2 ADAMs sheddase activity

The major function of ADAMs is the release of the extracellular domains of transmembrane proteins by cleaving them in their juxtamembrane region, known as shedding. ADAMs are capable of shedding type-I and type-II transmembrane protein, as well as GPI-anchored molecules. ADAMs activity leads to cell-surface remodeling, regulation of growth factors, and the cell ability to respond to extracellular stimuli. Functionally, ADAM mediated proteolysis has three major roles (Fig. 1.3). First, shedding of the membrane-bound factors leads to the release of active peptides. Then, soluble factors activate downstream signaling (Fig. 1.3a) in an autocrine manner (binding of active ligands to their receptors on the same cells) or a paracrine manner (ligands bind to the

receptors on opposite cells). Epidermal growth factor (EGF) ligands, such as amphiregulin, betacellulin, EGF, epigen, epiregulin, heparin-binding EGF-like growth factor (HB-EGF), neuregulin 1-4, and TGF-α, are synthesized in the inactive, membrane-bound form. These EGF ligands must be cleaved by members of the ADAM family in order to be activated. Different ADAMs shed different EGF ligands. ADAM17 mainly cleaves TGF-α, HB-EGF, epiregulin, and amphiregulin (Sahin *et al.*, 2004; Sahin & Blobel, 2007). ADAM10 is involved in the shedding of EGF and betacellulin (Sahin *et al.*, 2004). ADAM12 cleaves HB-EGF, and this function of ADAM12 is linked to cardiac pathophysiology (Asakura *et al.*, 2002).

The second functional role of ADAMs' sheddase activity is abrogation of protein function (Fig. 1.3b). ADAMs are able to selectively decrease the amount of specific proteins in the membrane or inactivate receptors and ligands. They can shed proteins in *cis* (in the same cell) or in *trans* (on the surface of an opposing cell). Trans-shedding induces cellular repulsion of two cells. The released ectodomains also act as soluble antagonists. A perfect example of abrogation of protein function by ADAMs is proteolysis of cell adhesion molecules (CAMs), such as cadherins or selectins. Cadherins are important for cell-cell adhesion, and cleavage by ADAMs plays a role in tissue morphogenesis, wound healing, and cell migration. Releasing of intracellular domain after cleavage, in some cases, can lead to intracellular signaling. ADAM12 is capable of cleaving several extracellular matrix proteins such as collagen IV, fibronectin, and gelatin (Roy *et al.*, 2004).

Additionally, ADAMs' shedding can be a prerequisite for regulated intramembrane proteolysis (RIP; Fig. 1.3c). Transmembrane proteins cleaved in the extracellular domain are followed by further cleavage in the intramembrane region. This intramembrane cleavage leads to the release of the intracellular domain (ICD), which may participate in signal transduction. A good example of ADAM function in RIP is the Notch signaling pathway. ADAMs' cleavage of the Notch receptor is necessary for subsequent cleavage by γ-secretase and the release of the intracellular domain, which further transduces the signal. This pathway is described in more detail in Section 1.2. A second example is the shedding of the amyloid precursor protein (APP) in the central nervous

system (CNS). ADAM9, 10, and 17 are capable of cleaving APP. It is hypothesized that increasing these ADAMs' activity could help patients with Alzheimer's disease (Deuss *et al.*, 2008).

1.2 Notch signaling

The Notch signaling pathway is evolutionarily conserved between species from *C. elegants* to humans. Notch plays critical roles in apoptosis, cell proliferation, differentiation, and lineage decision during embryonic development as well as during self-renewing processes in adulthood (Bray, 2006). Aberrant gain or loss of Notch signaling is implicated in many human diseases, including developmental syndromes (Hansson *et al.*, 2004), adult-onset diseases (Louvi *et al.*, 2006), and cancer (Miele *et al.*, 2006).

The primary proteins involved in the Notch signaling pathway are Notch receptors, DSL (Delta /Serrate/Lag2) ligands, and nuclear effectors. In mammals, there are four Notch receptors (Notch 1-4), and five ligands (Delta-like (Dll) 1, 3, 4, Jagged 1, 2).

Notch is a transmembrane protein containing several EGF-like repeats and a conserved negative regulatory region (NRR) composed of three Lin12/Notch repeats (LNR) and a heterodimerization domain (HD). EGF-like repeats 11 and 12 are essential for ligand binding. NRR helps to keep Notch in a metalloprotease resistant conformation before ligand-induced activation (Gordon *et al.*, 2007). The intracellular part of Notch contains the RAM (RBP-Jκ association module) domain, six ankyrin repeats, and a C-terminal PEST (proline/glutamic acid/serine/threonine-rich motifs) domain. The PEST domain contains degradation signals. All Notch ligands at the N-terminus contain a cysteine-rich DSL domain, which is essential for its interaction with receptors. Similar to Notch receptors, ligands contain in their extracellular domains various number of EGF-like repeats. Jagged (and Serrate in *Drosophila*) ligands also contain a cysteine-rich domain between the EGF-like repeats and the transmembrane domain. This domain is not present in the Delta class of ligands (Kopan & Ilagan, 2009).

Notch is glycosylated and cleaved by a furin-like convertase at site 1 (S1) within the secretory pathway (Logeat *et al.*, 1998). This cleavage leads to formation of a heterodimer, held to-

gether by non-covalent interactions, that is present in cell membrane. Notch signaling requires interactions between receptors present on the surface of the signal-receiving cell and a ligand located on a signal-sending cell. This interaction triggers the cleavage of Notch by ADAM metalloproteases in site 2 (S2) and generates Notch extracellular truncation (NEXT). Two ADAMs, ADAM17 and ADAM10, are capable of Notch cleavage at the S2 site (Brou *et al.*, 2000; Pan & Rubin, 1997). Generation of NEXT is a prerequisite for intramembrane cleavage of Notch by γ-secretase complex at site 3 (S3) (De Strooper *et al.*, 1999; Wolfe & Kopan, 2004). Cleavage at S3 frees the Notch intracellular domain (NICD) to enter the nucleus. NICD cannot bind directly to DNA. Rather, NICD forms a heterodimer with CSL (CBF1 in mammals, also known as RBP-Jκ in mouse, Su(H) in *Drosophila* and Lag1 in *C. elegans*) transcription factors through the RAM domain. Ankyrin repeats of NICD associate with CSL and help recruit the coactivator Mastermind/Lag3. This Mastermind-CSL-NICD interaction leads to transcriptional activation of the responsive genes. An overview of Notch signaling pathway is shown in Fig. 1.4.

Notch ligands also undergo ADAM-mediated cleavage in the juxtamembrane region of the extracellular domain (Six *et al.*, 2003; Dyczynska *et al.*, 2007). This cleavage is followed by γ-secretase cleavage in the intramembrane region (Ikeuchi & Sisodia, 2003). Until recently only, ADAM17 have been implicated in cleavage of Dll1 and Jagged1 (LaVoie & Selkoe, 2003), and ADAM10 was known to be involved in the cleavage of Dll1 (Six *et al.*, 2003; Qi *et al.*, 1999). In Chapter 3, our results show that ADAM12 and ADAM9 are also capable of cleaving Dll1 (Dyczynska *et al.*, 2007). The intracellular domain (ICD) of Notch ligands is released after cleavage by γ-secretase, and is translocated to the nucleus (Bland *et al.*, 2003). The Notch ligand ICD does not contain any recognizable DNA binding motifs, but the ICD of Dll1 can interact with transcriptional factors such as Smad2/3/4 and enhance Smad dependent transcription (Hiratochi *et al.*, 2007). Also, the soluble ICD of Jagged1 can activate gene expression through the AP1 transcription factor (LaVoie & Selkoe, 2003). This suggests a bi-directional function of the Notch signaling pathway where, not only the cell with the Notch receptor can receive signal, but also the cell presenting the ligand.

Only the membrane-bound ligand is able to activate Notch signaling. Therefore, ADAM-mediated processing of ligands results in a lack of functional ligands on the cell surface and down-regulation of Notch signaling in a neighboring cell. This has been demonstrated during cortical development in the mouse embryo. Inhibition of ADAM10, which mediates shedding of Notch ligands, leads to impaired Notch signaling (Muraguchi *et al.*, 2007).

Interestingly, interactions between Notch ligands and Notch in *trans* activate the Notch pathway, whereas ligand binding to Notch in *cis* inhibits Notch signaling (Katsube & Sakamoto, 2005). Interaction of Notch with its ligand on the same cell requires different EGF-like repeats of Notch (24-29) than interaction in *trans*. Notch ligands presented in the same cell as Notch can interact with each other, and this interaction leads to decreased receptivity to Notch signals (Sakamoto *et al.*, 2002). A possible function for shedding of the ligand by ADAM proteases is relieving cis-inhibition. This hypothesis is discussed in Chapter 3.

1.3 Transforming growth factor β (TGFβ) signaling pathway

The TGFβ signaling pathway is important during development and in adulthood. This conserved mechanism of signaling is involved in regulation of proliferation, differentiation, apoptosis, and extracellular matrix remodeling. It is not surprising that dysregulation of this pathway leads to many disorders such as cancer, fibrosis, and vascular disease, as well as, hereditary conditions including familial primary pulmonary hypertension and hereditary hemorrhagic telangiatacsia (HHT) (Gordon & Blobe, 2008).

The primary components in TGFβ signaling are ligands, receptors, and intracellular effectors–Smads. More then 60 members of the TGFβ family have been identified. Among these, ligands consist of three TGFβ isoforms, four activins, ten bone morphogenic proteins (BMPs), and eleven growth and differentiation factors (GDFs) (Feng & Derynck, 2005). The characteristic feature of the TGFβ family of ligands is the "cysteine knot" formed by three intramolecular disulfide bonds between six cysteine residues, which are highly conserved between ligands (Feng & Derynck, 2005). All ligands are synthesized as dimeric pre-proteins and are secreted. All three mammalian

TGFβ isoforms, TGFβ1, 2 and 3, are secreted in the latent form, where the latency associated peptide (LAP) is non-covalently attached to the active peptide. LAP must be removed in order to release the active ligand. This removal occurs by proteolytic cleavage or physical interactions of LAP with other proteins, such as integrins (Annes *et al.*, 2003).

Two classes of TGFβ receptors exist: type I (TβRI) and II (TβRII). In vertebrates there are five type-II receptors and seven type-I receptors, called activin receptor-like kinases (ALK). They are Serine/threonine kinase receptors. On the membrane, receptors form homodimers. Type-II receptors are constitutively active. Type-I receptor is inactive, due to presence of Glycine/Serine-rich (GS) region in the kinase domain (Huse *et al.*, 1999). Upon ligand binding receptors are brought together and type II receptor phosphorylates the GS region of type I receptor, allowing its activation.

Smads are the only TGFβ receptor substrates with a demonstrated ability to propagate signals (Ross & Hill, 2008). Receptor-activated Smads (R-Smads; Smad1, Smad2, Smad3, Smad5, Smad8) are activated by TβRI. They contain conserved two Mad-homology (MH) domains: N-terminal MH1 and C-terminal MH2, connected by a linker. The MH1 domain is responsible for DNA binding (except in Smad2) and the MH2 domain is responsible for interaction with receptors, Smad-Smad interaction, and binding of the transcription factors, co-activators, and co-repressors. Linkers can be phosphorylated by MAPKs, glycogen synthase kinase-3β, and cyclin dependent kinases. Common-Smads (co-Smad; Smad4) form a heteromeric complex with activated R-Smads (Moustakas *et al.*, 2001). The primary difference between co-Smad and R-Smad is that co-Smad does not contain a phosphorylation site in its C-terminus. Additionally, two inhibitory Smads (I-Smads) are present, Smad6 and Smad7. These I-Smads can interact with TβRI, ubiquitin ligases, and protein phosphatase 1 (PP1) to inhibit signaling by enabling degradation or dephosphorylation of active receptors. I-Smads are also upregulated in response to TGFβ, leading to a negative-feedback loop mechanism.

TGFβ signaling is surprisingly straightforward. Upon binding of active TGFβ to TβRII, TβRII forms a complex with TβRI. Formation of this complex leads to the phosphorylation of

TβRI. This signal is further transduced by R-Smads proteins. Smad2 and Smad3 transduce the signal activated by TGFβ or activin, and Smad1, Smad5, and Smad8 are activated in response to BMP. R-Smads are phosphorylated by TβRI. Upon phosphorylation, R-Smads form a complex with Smad4 and translocate to the nucleus where they collaborate with distinct transcription co-regulators to induce or block TGFβ responsive genes. A simplified overview of the TGFβ pathway is presented in Fig. 1.5. The Smad binding element (SBE) is a short sequence 5'-AGAC-3' or complement 5'-GTCT-3' that binds to the MH1 domains of Smad3/4 complexes. This binding is very weak and requires a series of repeated SBE sequences and additional transcription factors interacting with Smad complexes. Additionally, non-Smad pathways are involved in TGFβ signaling. One can distinguish three signaling mechanisms in which non-Smad pathways affect the Smad-dependent pathway. Non-Smad pathways can directly modify Smad function, and Smads can modify the function of non-Smad proteins and transmit signals to other pathways. TGFβ receptors can directly phosphorylate non-Smad proteins (Moustakas & Heldin, 2005). The non-Smad pathways involved in TGFβ signaling are p38 MAPK, c-Jun MAPK, PI3K-AKT, mTOR and PP2A. Interplay between Smad-dependent and non-Smad pathways allow the cell-type and context-dependent activation of responsive genes.

ADAM12 has been shown to be upregulated by TGFβ in human activated hepatic stellate cells. mRNA of both long and short human ADAM12 isoforms is upregulated after three days of treatment (Le Pabic *et al.*, 2003), and this upregulation is decreased by PI3K and MAPK inhibitors (Le Pabic *et al.*, 2003, 2005). Additionally, ADAM12 has been shown to interact with TGFβ receptor type II. This interaction modulated receptor trafficking and positively regulated the TGFβ signaling (Atfi *et al.*, 2007).

1.3.1 SnoN as transcriptional repressor

SnoN and closely related Ski were discovered as oncogenes, able to transform fibroblasts when over-expressed (Boyer *et al.*, 1993). However, more recent studies of heterozygous sno (Shinagawa *et al.*, 2000) and ski (Shinagawa *et al.*, 2001) mice have shown that they can function as

tumor suppressors. SnoN is ubiquitously expressed at low levels, and has an altered expression in cancer (Zhang *et al.*, 2003; Fukuchi *et al.*, 2004; Poser *et al.*, 2005; Akagi *et al.*, 2008), liver regeneration (Macias-Silva *et al.*, 2002), and obstructive neurophathy (Tan *et al.*, 2006). The fact that SnoN and Ski can interact with Smad2/3 and Smad4 opens new possibilities for regulation of TGFβ signaling (Stroschein *et al.*, 1999; Sun *et al.*, 1999; Luo *et al.*, 1999; Xu *et al.*, 2000).

In humans, the Sno gene is alternatively spliced forming SnoN, SnoN2, SnoI, and SnoA (Nomura *et al.*, 1989; Pearson-White, 1993; Pearson-White & Crittenden, 1997). All four isoforms share 366- amino acids from exon 1. SnoN is the largest of the Sno isoforms, 684 amino acid long, in human cells. SnoN2 has 46 amino acid deletion in exon 3 (Pearson-White & Crittenden, 1997). SnoI contains the first 399 residues of SnoN, and SnoA has only first exon common with other forms. Rodents only express SnoN and SnoN2 with a different tissue distribution (Pelzer *et al.*, 1996; Pearson-White & Crittenden, 1997). The functional implications of various Sno splice forms are currently not known. SnoN in the N-terminus contains a DS domain which has extended homology between Ski and Sno proteins. This is followed by a SAND-like domain (Sp100, AIRE-1, NucP41/75 and DEAF-1 protein) found in chromatin remodeling nuclear proteins. The SAND-like domain does not directly bind DNA but is responsible for Smad4 binding. The C-terminal part contains α-helical dimerization domains. A schematic representation of human Sno isoforms is shown in Fig. 1.6a. Mouse SnoN/SnoN2 are shorter and do not contain dimerization domain.

Overexpression of SnoN inhibits the ability of TGFβ to induce transcription (Stroschein *et al.*, 1999; Sun *et al.*, 1999). Crystal structures reveal that Ski and SnoN interact with the R-Smads through their N-terminal region and with co-Smad through SAND-like domain, suggesting that Ski/SnoN disrupt the formation of functional complex between Co- and R-Smads. Ski and SnoN also prevent binding of R-Smads to transcriptional co-activators p300/CBP (Wu *et al.*, 2002). Additionally, SnoN and Ski bind directly to the nuclear hormone receptor co-repressor (N-coR) and mSin3A, components of the histone deacetylase (HDAC) complex, and repress TGFβ responsive genes (Luo *et al.*, 1999; Akiyoshi *et al.*, 1999; Nomura *et al.*, 1999). Other cellular partners for

SnoN and Ski include different transcriptional factors pRb, GATA1, Gli3, RAPα (retinoic acid receptor α) (Deheuninck & Luo, 2009). SnoN is mostly localized to the nucleus, but some reports suggest that SnoN can work in the cytoplasm by sequestering Smad proteins (Krakowski *et al.*, 2005).

TGFβ induction leads to degradation of SnoN and, to a lesser extent, Ski. Several E3 ubiquitin ligases have been shown to be directed to SnoN in response to TGFβ. Smad ubiquitin regulatory factor (Smurf)-2 interacts with Smad2 in a TGFβ-dependent manner and targets SnoN for ubiquitin mediated degradation by proteosome (Bonni *et al.*, 2001). Smad3 recruits the anaphase-promoting complex (APC) and causes ubiquitination and degradation of SnoN through the interaction of Destruction-box (D box) in SnoN with the CDH1 subunit of APC (Stroschein *et al.*, 2001). Arkadia, an E3 ubiquitin ligase, has also been shown to form a complex with Smad2/3 and SnoN, and lead to degradation of SnoN only in the presence of phosphorylated Smad2 or Smad3 (Levy *et al.*, 2007; Nagano *et al.*, 2007). The rapid degradation of SnoN in response to TGFβ releases the repression of responsive genes, allowing activation by binding of R-Smads/co-Smad complexes to SBE. Schematic activation of TGFβ responsive genes by degradation of SnoN is shown in Fig. 1.6b.

SnoN is also upregulated in response to TGFβ signaling through direct binding of the Smad2/Smad4 complexes to SBE in the SnoN promoter. This upregulation occurs within short times of TGFβ treatment (Sun *et al.*, 1999; Stroschein *et al.*, 1999), suggesting a negative feedback mechanism between TGFβ signaling and SnoN.

1.4 Goal of the study

To better understand how ADAM12 is regulated and how it functions, we focused on three goals.

- Previous studies show that ADAM12 can be upregulated by TGFβ in hepatic stellate cells (Le Pabic *et al.*, 2003), but the general mechanism of how ADAM12 is regulated by TGFβ is not fully understood. My goal was to investigate the TGFβ-Smad pathway and its involvement in the regulation of ADAM12 in different cell lines.

- ADAMs are important in the Notch signaling pathway, but little is known about ligand cleavage by ADAMs. My goal was to test several ADAMs for their ability to proteolytically cleave Dll1. Main focus has been put on what effect this will have on Notch signaling in the cell in which cleavage takes place and on Notch signaling in the neighboring cell.

- Recently, ADAM12 emerged as a Candidate Cancer Gene in a comprehensive genetic analysis of human breast cancers and three somatic mutations were observed at significant frequencies in breast cancers (Sjöblom *et al.*, 2006). The effect of these mutations on ADAM12 function have been tested.

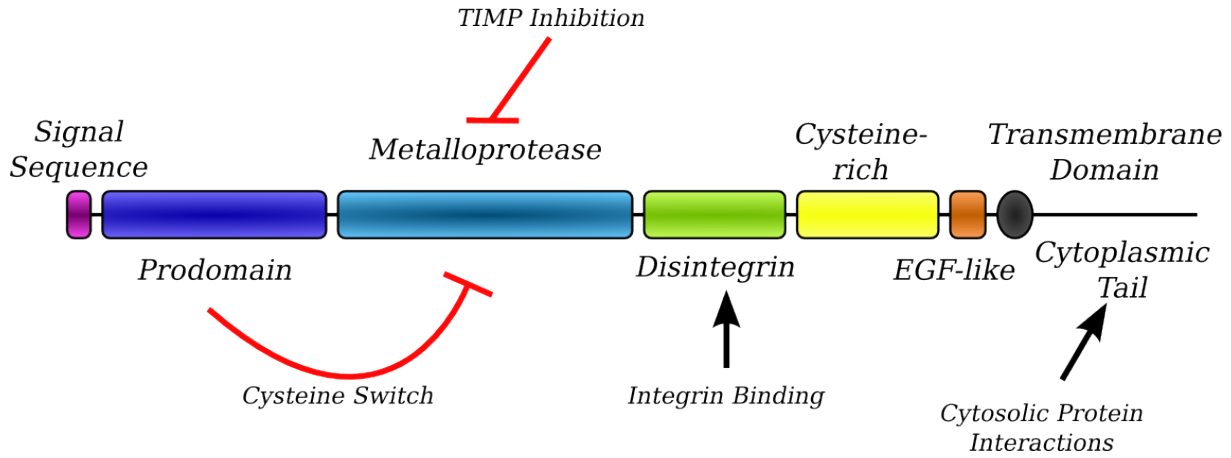

Figure 1.1: **Domain structure of ADAM metalloproteases.** Typical ADAMs contain a signal sequence at the N-terminus, followed by prodomain, metalloprotease, disintegrin, cysteine-rich EGF-like, transmembrane domain, and cytoplasmic tail. Prodomain blocks the active site of metalloprotease by the "cysteine switch" mechanism, where a cysteine residue in the prodomain forms a bond with zinc in the catalytic site. Tissue inhibitors of metalloprotease (TIMP) are natural inhibitors of metalloprotease activity. Cytoplasmic tail contains several putative binding motifs and kinase target sites. Based on Huovila *et al.* (2005).

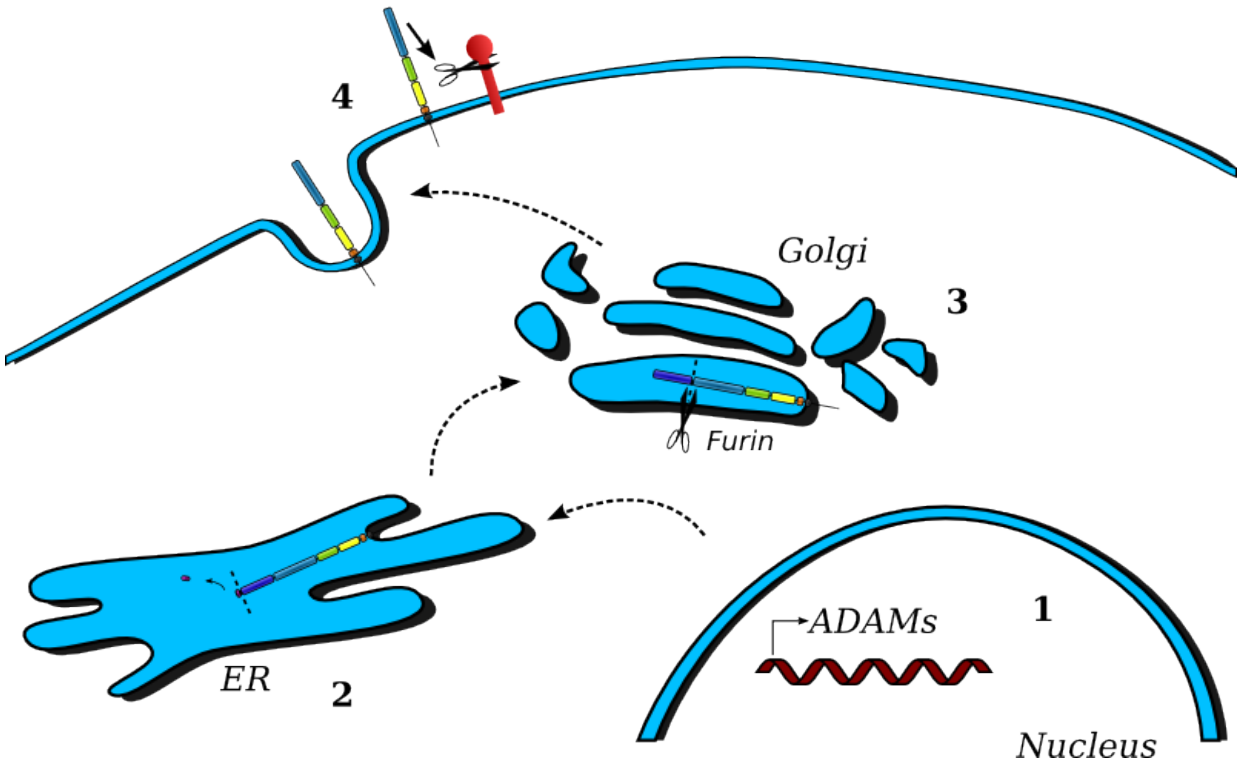

Figure 1.2: **An overview of ADAMs synthesis and processing.** ADAMs can be regulated at the transcriptional level (1). They are synthesized in the endoplasmic reticulum (ER), where the signal peptide is removed (2). Maturation of ADAMs occurs in the Golgi apparatus, where furin cleaves the prodomain (3) and an active form is transported to the membrane. ADAMs are also regulated by interactions of cytosolic proteins with the cytoplasmic tail. ADAMs presented at the cell surface are able to shed the ectodomains of their substrates (4).

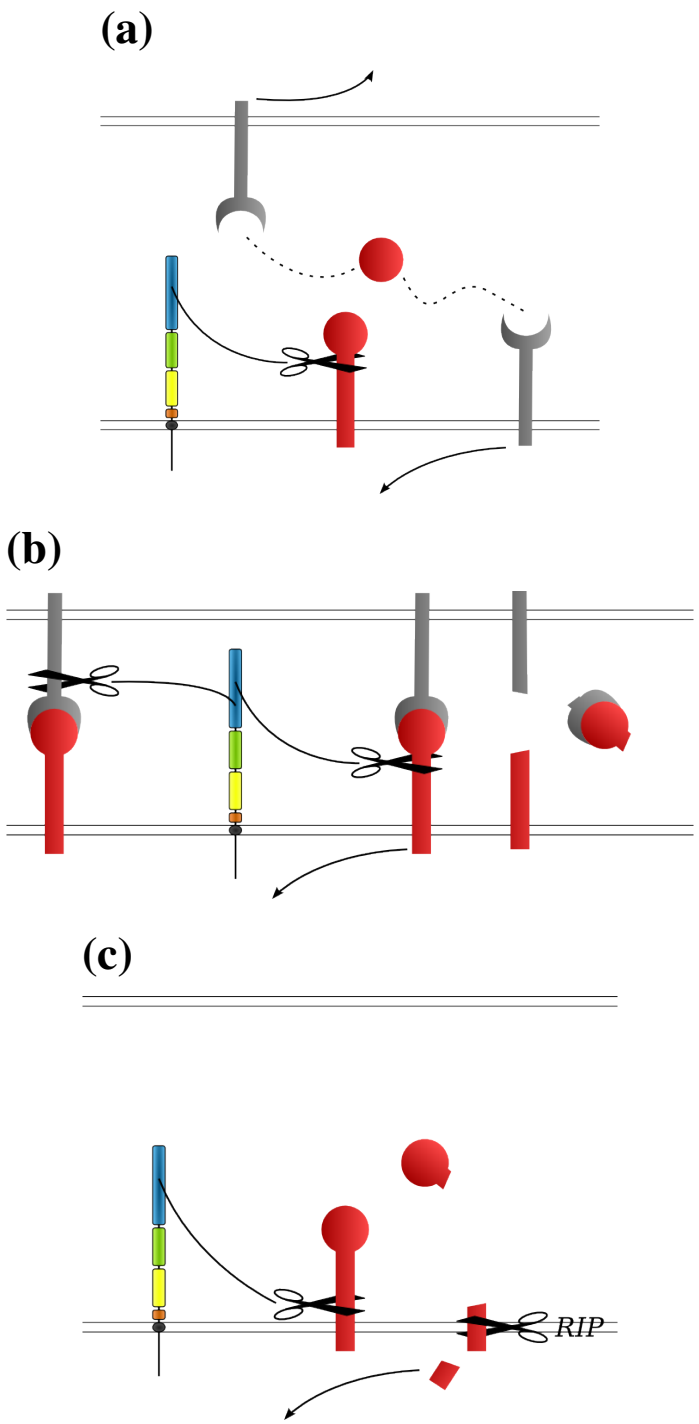

Figure 1.3: **Functional roles of ADAMs sheddase activity.** (a) Shedding releases active peptide from pro-proteins which leads to autocrine and paracrine signaling. (b) Abrogation of protein function, repulsion by trans-shedding. The released ectodomains can be sequestrated by soluble factors. (c) Shedding is a prerequisite for regulated intramembrane proteolysis (RIP). Released intracellular fragments can act as transcription factors. Based on Reiss & Saftig (2009).

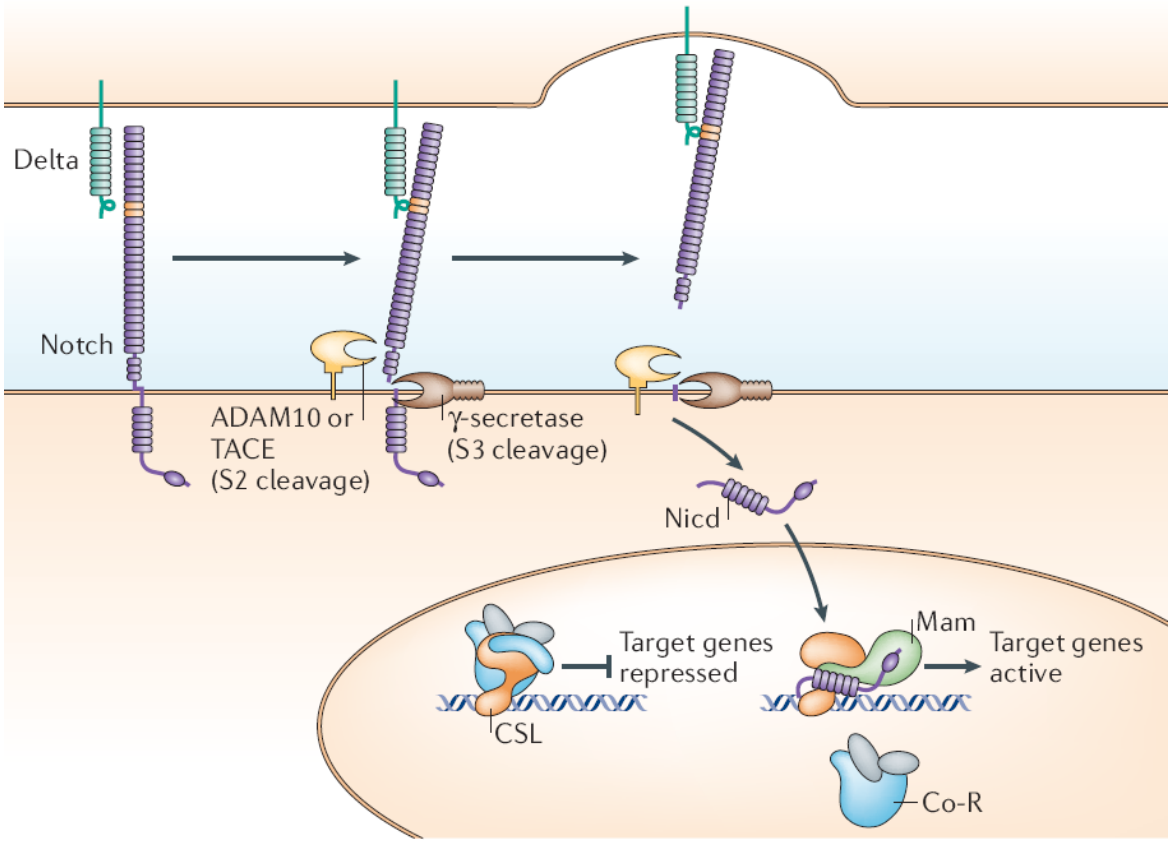

Figure 1.4: **Notch signaling pathway.** Delta or Jagged (Notch ligands) are present on a signal-sending cell whereas Notch receptor is present on an opposite, signal-receiving cell. After Notch binds to its ligands, two proteolytic cleavages occur, the first cleavage at the S2 site by ADAM10 or TACE (ADAM17) is followed by a cleavage by the γ-secretase complex at the S3 site. Nicd (Notch intracellular fragment) is released from the membrane and transported to the nucleus, where it interacts with CSL (CBF1, Su(H) and Lag1). The coactivator Mastermind (Mam) is also required for releasing the repression and transcriptional activation. Adapted by permission from Macmillan Publishers Ltd: Nature Rev Mol Cell Biol (Bray, 2006), copyright (2006).

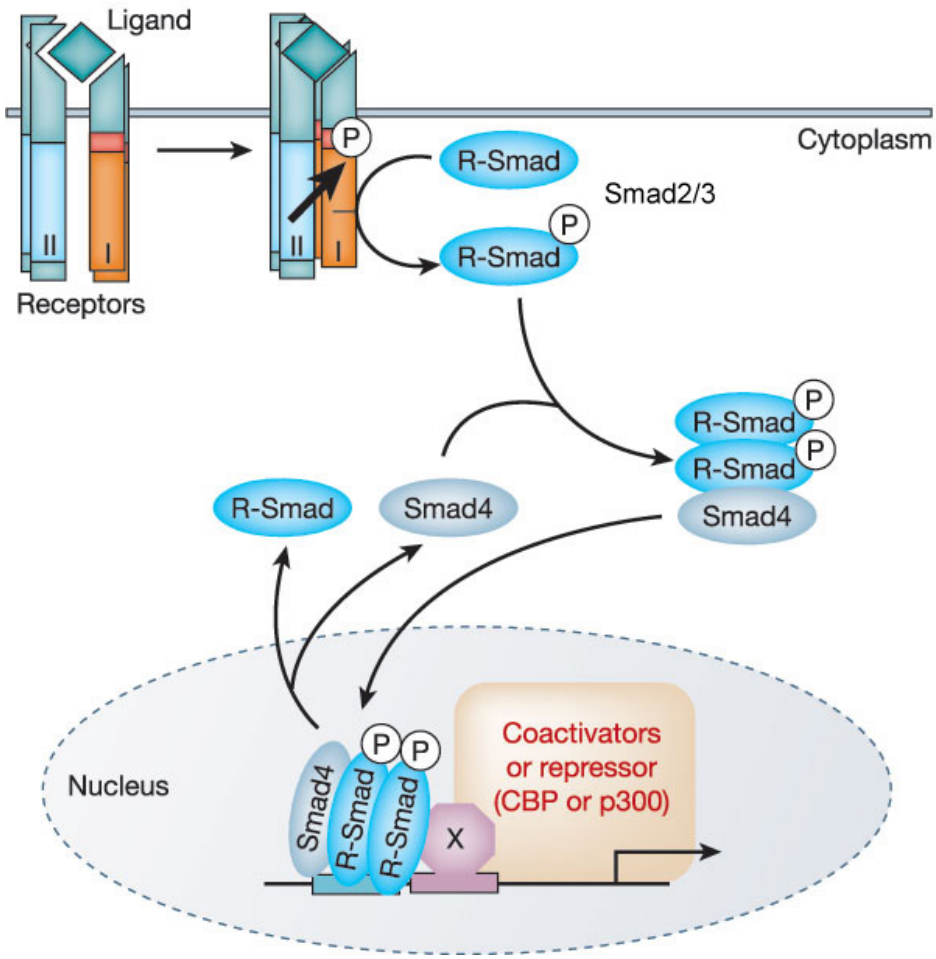

Figure 1.5: **TGFβ signaling pathway** Ligand binds to TGFβ receptors, and brings them together. TGFβ receptor type II (TβRII) phosphorylates TGFβ receptor I (TβRI). Active TβRI phosphorylates R-Smads, which leads to complex formation with Smad4. R-Smad/Smad4 complex translocates to the nucleus where it interacts with co-repressors or co-activators and regulates TGFβ responsive genes. Adapted by permission from Macmillan Publishers Ltd: Nature (Derynck & Zhang, 2003), copyright (2003).

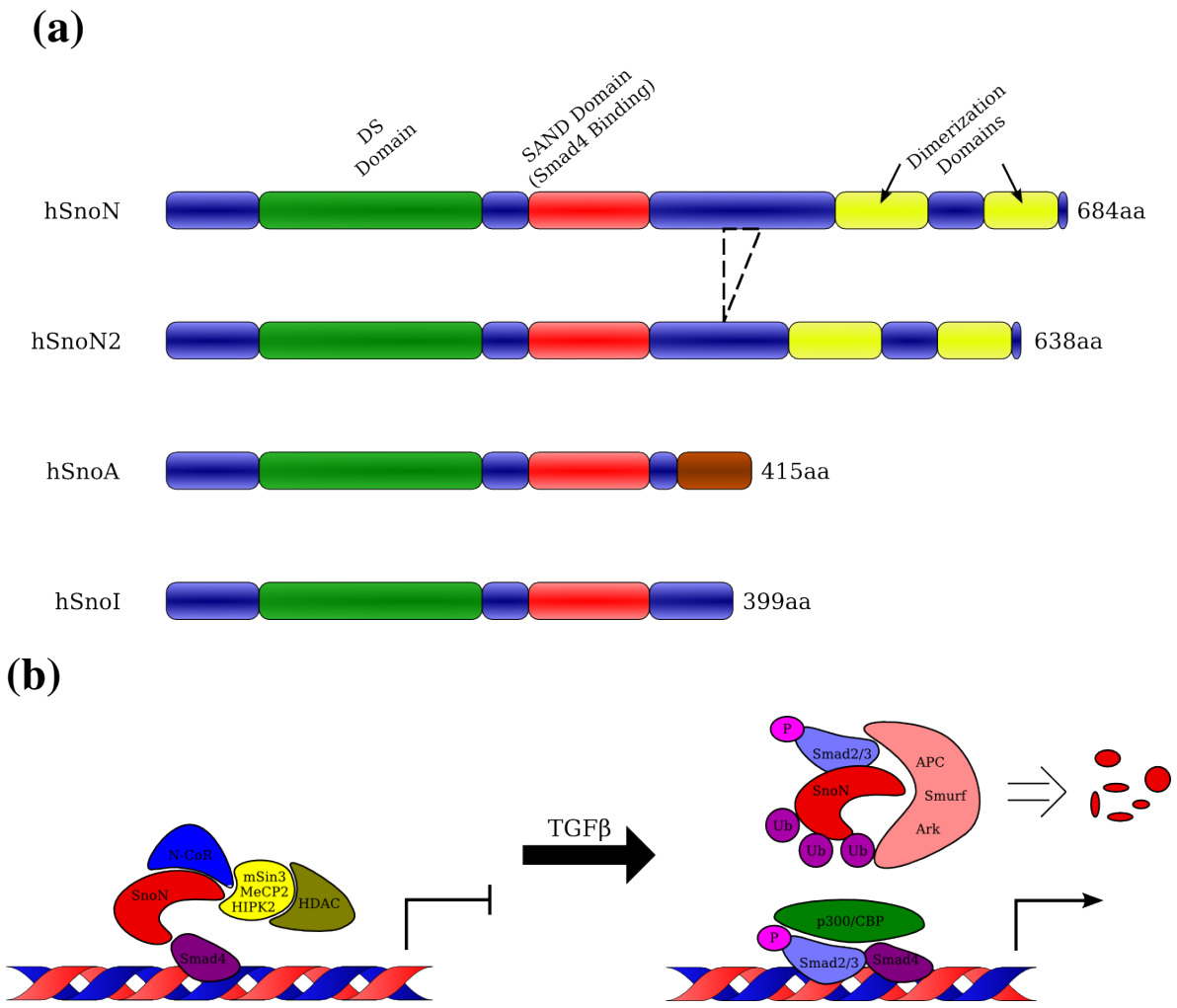

Figure 1.6: **Domain structure of SnoN family of proteins (a) and mechanism of regulation by SnoN (b).** (a) Schematic representation of human Sno isoforms with structural domains. SnoN is the longest isoform containing 684 amino acid residues. SnoN2 has a deletion of amino acids 431-476 (denoted with dashed lines) due to alternative splicing of exon 3. SnoA has first 366 amino acids identical to the other isoforms, and contains 49 residues (shown in brown) which are specific for this isoform. SnoI is a truncated form of SnoN. DS domain (green) is a Ski/Sno conserved homologous region. SAND-like domain (red) and two α-helical dimerization domains are shown in yellow. Figure based on Pot & Bonni (2008) and Deheuninck & Luo (2009). (b) SnoN, Smad4 and various transcriptional co-repressors and histone deacetylase complex (HDAC) maintain repressed state of TGFβ responsive genes. After TGFβ stimulation, receptor phosphorylated Smad2/3 bring E3 ubiquitin ligases (APC/Smurf/Arkadia) leading to degradation of SnoN and activation of transcription. Figure based on Luo (2004).

Chapter 2

The role of SnoN in transforming growth factor β1-induced expression of ADAM12

The data presented in this chapter have been submitted as a journal article:

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The role of SnoN in transforming growth factor β1-induced expression of ADAM12

2.1 Abstract

Increased expression of metalloprotease-disintegrin ADAM12 is a hallmark of several pathological conditions, including cancer, cardiovascular disease, and certain inflammatory diseases of the central nervous system or the muscoskeletal system. We show that transforming growth factor β1 (TGFβ1) is a potent inducer of ADAM12 mRNA and protein in mouse fibroblasts and in mouse and human mammary epithelial cells. Induction of ADAM12 mRNA is detected within 2 h of treatment with TGFβ1, is Smad2/Smad3-dependent, and is a result of derepression of the ADAM12 gene. SnoN, a negative regulator of the TGFβ signaling pathway, is a master regulator of ADAM12 expression in response to TGFβ1 stimulation. Overexpression of SnoN in NIH3T3 cells reduces the magnitude of ADAM12 induction by TGFβ1 treatment. Downregulation of SnoN expression by shRNA leads to elevation of basal levels of ADAM12 expression and enhances TGFβ1-induced expression of ADAM12. In a panel of TGFβ1-responsive cancer cell

lines with high expression of SnoN, induction of ADAM12 by TGFβ1 is significantly impaired, suggesting that the endogenous SnoN plays a role in regulating ADAM12 responses to TGFβ1. Identification of SnoN as a repressor of the ADAM12 gene should contribute to advances in the studies on the role of ADAM12 in tumor progression and in the development of other pathologies.

2.2 Introduction

ADAM12, a member of the metalloprotease-disintegrin family of proteins, has been implicated in the progression of cancer, cardiovascular disease, osteoarthritis, and neurological disorders (Kveiborg *et al.*, 2008). ADAM12 gene is frequently mutated in human breast cancers (Sjöblom *et al.*, 2006; Wood *et al.*, 2007), and cancer-associated mutations cause mislocalization of ADAM12 protein in cells and alter its function (Dyczynska *et al.*, 2008). Missense single nuclear polymorphism in the ADAM12 gene shows strong association with osteoarthritis (Valdes *et al.*, 2004, 2006). In addition to changes in its amino acid sequence, expression levels of ADAM12 are significantly increased in many pathological states. For example, ADAM12 expression levels are 20-30-fold higher in human breast tumors than in normal mammary epithelium (Iba *et al.*, 1999; Kveiborg *et al.*, 2005; Lendeckel *et al.*, 2005; Roy *et al.*, 2004; Mitsui *et al.*, 2006; Turashvili *et al.*, 2007). ADAM12 expression is also markedly upregulated in cancers of the liver, lung, stomach, colon, prostate, bladder, and in glioblastoma (Le Pabic *et al.*, 2003; Mino *et al.*, 2009; Carl-McGrath *et al.*, 2005; Peduto *et al.*, 2006; Fröhlich *et al.*, 2006; Kodama *et al.*, 2004). Increased ADAM12 expression levels are found in the cardiac tissue of patients with hypertrophic obstructive cardiomyopathy (Fedak *et al.*, 2006) and in mice with angiotensin II-induced hypertension and cardiac hypertrophy (Wang *et al.*, 2009a,b). During inflammatory responses and aseptic osteolysis associated with loosened hip replacement implants, ADAM12 is upregulated in the interface tissue around loosening implants (Ma *et al.*, 2005). In the experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, ADAM12 level is markedly increased in T cells that infiltrate spinal cords (Toft-Hansen *et al.*, 2004).

The mechanisms regulating ADAM12 expression, in particular those that may be responsible

for altered levels of ADAM12 in various pathological states, are poorly understood. Previous studies employing hepatic stellate cells, a mesenchymal cell type in hepatic parenchyma, have indicated that ADAM12 expression is induced by transforming growth factor β (TGFβ) (Le Pabic *et al.*, 2003, 2005). The TGFβ signaling pathway is initiated when one of the family members, e.g. TGFβ1, β2, or β3, binds to a complex of TGFβ type I and type II serine/threonine kinase receptors (TβRI and TβRII, respectively) and induces phosphorylation and activation of TβRI by TβRII. TβRI then phosphorylates receptor Smads (R-Smads), Smad2 and Smad3. Phosphorylated Smad2/3 associate with the common partner Smad4 and translocate to the nucleus, where they regulate transcription of target genes (Massagué *et al.*, 2005; Feng & Derynck, 2005). In addition, receptor activation in certain cell types leads to Smad-independent responses via the activation of mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), and Rho family members (Moustakas & Heldin, 2005; Derynck & Zhang, 2003).

SnoN and the related Ski protein are negative regulators of TGFβ signaling. They bind to nuclear Smad complexes and repress their transcriptional activities (Deheuninck & Luo, 2009; Lönn *et al.*, 2009; Pot & Bonni, 2008). In response to TGFβ stimulation, SnoN (and to a lesser extent Ski) undergoes ubiquitination and rapid proteasomal degradation (Stroschein *et al.*, 1999; Sun *et al.*, 1999). The ubiquitin ligases implicated in ubiquitination of SnoN, the anaphase promoting complex (APC), Smurf2, and Arkadia, are recruited to SnoN via the phosphorylated R-Smads (Bonni *et al.*, 2001; Stroschein *et al.*, 2001; Wan *et al.*, 2001; Nagano *et al.*, 2007; Levy *et al.*, 2007).

Previous study on the regulation of ADAM12 expression by TGFβ in hepatic stellate cells used rather long (24 - 72 h) stimulation times and showed that ADAM12 induction was partially blocked by inhibitors of MAPKs, PI3K, or p70S6 kinase (Le Pabic *et al.*, 2003, 2005). Based on these results, it was postulated that induction of ADAM12 expression by TGFβ might be Smad-independent, but a direct role of R-Smads in the regulation of ADAM12 expression has not been tested. In this report, we investigate short term (0 - 24 h) effects of TGFβ on ADAM12 mRNA and protein levels in mouse fibroblasts. We find that TGFβ causes derepression of the ADAM12 gene

in a Smad2/3-dependent manner, and that the repressor responsible for the negative regulation of ADAM12 expression is SnoN. Our studies uncover a new mechanism of ADAM12 regulation by TGFβ that may contribute to aberrant expression of ADAM12 in various diseases.

2.3 Materials and Methods

Cell culture

NIH3T3 fibroblasts, HT1080 fibrosarcoma cell line, DU145 prostate cancer cell line (American Type Culture Collection), and retroviral packaging cell line Phoenix Eco (G. P. Nolan, Stanford University) were grown in DMEM supplemented with 10% FBS. SMAD2$^{-/-}$ MEFs (E. Bottinger, Mount Sinai School of Medicine), SMAD3$^{-/-}$ MEFs (K. Flanders, NCI), ADAM9/12/15$^{-/-}$ (C. P. Blobel, Hospital for Special Surgery), and wild-type MEFs were grown in DMEM containing 10% FCS and 1% penicillin/streptomycin. Normal mouse mammary gland epithelial cell line NMuMG and human MCF7 breast cancer cells were grown in DMEM with 10% FBS and 10 μg/ml insulin. Normal human mammary epithelial cells MCF-10A were cultured in DMEM-F12 supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 20 ng/ml hEGF, 10 μg/ml bovine insulin, 100 ng/ml cholera toxin and 1% penicillin/streptomycin. MDA-MB-468 breast cancer cells were cultured in Liebovitz's L-15 medium supplemented with 10% FBS. MDA-MB-435S melanoma cells were maintained in Liebovitz's L-15 medium supplemented with 10% FBS and 10 μg/ml bovine insulin. MDA-MB-231 breast cancer cells were cultured in DMEM-F12 medium supplemented with 10% FBS. T47D breast cancer cells were cultured in RPMI-1640 medium supplemented with 10% FBS. Cells were plated 24 h before experiment and allowed to reach $\sim$70% confluency. The medium was changed at the time of TGFβ1 treatement. Cells were treated with 2 ng/ml of TGFβ1 (R&D Systems) for 24 h (unless indicated otherwise), 10 μM MG132 (EMD Biosciences) for 16 h, 5 μg/ml actinomycin D (Sigma-Aldrich) for 15 min prior to and during TGFβ1 treatment, 5 μg/ml cycloheximide (Sigma-Aldrich) for 2 h prior and during TGFβ1 treatment; or 10 μM SB-431542 (Sigma-Aldrich) 30 min prior and during TGFβ1

treatment; control incubations with vehicle alone were included in each experiment.

Viral transduction

Human SnoN cDNA was transferred from pCI-Neo HA-hSnoN plasmid vector into the retroviral pBMN-I-GFP vector (both from Addgene). Phoenix Eco cells were transfected with SnoN retroviral vector (15 μg plasmid DNA/100-mm plate) using calcium phosphate precipitation method. Viral supernatants were harvested 48 hours later, supplemented with 5 μg/ml polybrene, and used without further dilution for infection of NIH3T3 cells. For SnoN knockdown, NIH3T3 cells were incubated with MISSION^{TM} Lentiviral shSnoN Transduction Particles (Sigma, clone ID TRCN0000088306) or with MISSION^{TM} Non-Target shRNA Control Transduction Particles (Sigma, SHC002V), according to the manufacturer's instructions. After one day, media containing retroviral particles were replaced with fresh media, and after additional 24 h, stably transduced cells were selected with 2 μg/ml of puromycin for 7 days.

Immunoblotting

Immunoblotting was performed as described (Dyczynska *et al.*, 2007). For ADAM12 detection, cell extracts were enriched for glycoproteins using concanavalin A agarose prior to SDS-PAGE and Western blotting (Cao *et al.*, 2002). The following primary antibodies were used: rabbit anti-ADAM12 cytoplasmic peptide antibody (Cao *et al.*, 2002, 1:3000), rabbit anti-ADAM9 (Cao *et al.*, 2002, 1:400), goat anti-ADAM15 (R&D Systems, 1:100), rabbit anti-SnoN (H-317, Santa Cruz Biotechnology, 1:1,000), mouse anti-α-tubulin (Sigma, 1:100,000). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG antibodies.

RNA analysis

Total RNA was extracted using PureLink Micro-to-Midi Total RNA Purification System containing TRIzol (Invitrogen). Northern blot analysis was performed using NorthernMax kit (Ambion). Membranes were hybridized with ADAM12 cDNA probe (nt 161-2202) or β-actin probe provided

with the kit. Probes were labeled using DECAprime II Random Primed DNA Labeling kit (Ambion) and [α-^{32}P] dATP. For RT-PCR analysis, RNA (1 μg) was treated with deoxyribonuclease I (Invitrogen), followed by reverse transcription using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo(dT) primers. Semi-quantitative PCR was performed in 50 μl reaction volumes using 1 μl cDNA, 0.2 mM dNTPs, 2 units of BIO-X-ACT Short DNA Polymerase (Bioline), and 1 μM primers (Table 2.1). PCR reaction conditions were: 94°C, 30s; 55°C, 30s; 72°C, 45s; 29-32 cycles for mADAM12, and 24-26 cycles for mGAPDH. PCR products were resolved in 2% agarose/TAE gels, visualized after ethidium bromide staining and UV illumination, and quantified by densitometry. Quantitative RT-PCR was performed in a total volume of 25 μl in a 96-well spectrofluorometric thermal cycler (iCycler, Bio-Rad). The final reaction mix contained 10.5 μl of diluted cDNA (1:5 for human cDNA, 1:50 for mouse cDNA), 12.5 μl iQSYBR Green Supermix, and 0.4 μM primers (Table 2.1). PCR conditions were: 95°C, 30s; 55°C, 30s; 72°C, 40s. The relative expression of ADAM12 mRNA, normalized to mouse GAPDH or human β-actin, was calculated using the $2^{(-\Delta\Delta Ct)}$ method.

Statistical analysis

Paired t test was used to compare values of two groups. When fold change in ADAM12 expression was calculated (stimulus- or inhibitor-treated cells versus vehicle control), data were analyzed by one sample t test (GraphPad Prism Software, San Diego, CA).

2.4 Results

Previous reports have shown that treatment of human hepatic stellate cells with TGFβ1 induces expression of ADAM12 (Le Pabic *et al.*, 2003, 2005). Here, we extended the analysis of TGFβ1 effects on ADAM12 expression to other cell types. We observed that treatment of mouse NIH3T3 fibroblasts or normal mouse mammary epithelial cell line NMuMG for 24 h with TGFβ1 led to dramatic increase in ADAM12 protein levels (Fig. 2.1a, 2.1b). The levels of two other ADAMs, ADAM9 and ADAM15, were not changed after similar TGFβ1 treatment (Fig. 2.1c), demonstrat-

Table 2.1: Primer sequences

Primer	Sequence	Product size
Primers used for semi-quantitative RT-PCR		
mADAM12 (F)	TAA AAC GTA CAG CTT AGA GC	300 bp
mADAM12 (R)	CTT GTC AAC GTG ATT GGC GAT CTC	
mGAPDH (F)	TCG GTG TGA ACG GAT TTG	228 bp
mGAPDH (R)	GCC GAT CCA CAC GGA GTA CT	
Primers used for real-time qRT-PCR		
mADAM12 (F)	GGA TGT GCC TCT TCA ACC TAC	134 bp
mADAM12 (R)	AGC GTT ACA GCA GCG ATT C	
hADAM12L (F)	AGC CAC ACC AGG ATA GAG AC	106 bp
hADAM12L (R)	CGC CTT GAG TGA CAC TAC AG	
mGAPDH (F)	GCC TTC CGT GTT CCT ACC	101 bp
mGAPDH (R)	GCC TGC TTC ACC ACC TTC	
hβ-actin (F)	TTG CCG ACA GGA TGC AGA A	101 bp
hβ-actin (R)	GCC GAT CCA CAC GGA GTA CT	

ing that among three ADAMs tested, the effect of TGFβ1 was specific for ADAM12.

Induction of ADAM12 protein in cells that were starved in 0.5% serum for 24 h prior to adding TGFβ1 was comparable to the induction observed in the presence of 10% serum (Fig. 2.2a). Therefore, all subsequent experiments were performed using media supplemented with 10% FBS, without starvation. It has to be stressed that when cells were incubated for prolonged times ($\sim$48 h) without adding fresh media, the basal level of ADAM12 expression was significantly elevated, and it was efficiently reduced by adding SB-431542, an inhibitor of TβRI (Fig. 2.2a). This result suggests that the increase in the basal level of ADAM12 expression was most likely due to the autocrine/paracrine effect of the endogenous TGFβ1 produced in NIH3T3 cells that was accumulating over time in cell medium. The induction of ADAM12 protein in NIH3T3 cells by exogenously added TGFβ1 was dose-dependent and reached a maximum at 2 ng/ml of TGFβ1 (Fig. 2.2b, 2.2c), the concentration used in the remaining part of this study. The upregulation of ADAM12 protein was evident after 8 h of stimulation of cells with TGFβ1 (Fig. 2.2d, 2.2f). Semi-quantitative reverse-transcription PCR (RT-PCR) analysis further demonstrated that TGFβ1 treatment increased the level of ADAM12 mRNA, and the changes in ADAM12 mRNA preceded the changes in ADAM12 protein levels (Fig. 2.2e, 2.2f).

Pre-treatment of cells with actinomycin D, an inhibitor of transcription, completely blocked the upregulation of ADAM12 protein (Fig. 2.3a), indicating that induction of ADAM12 expression by TGFβ1 occurred at the transcriptional level. Consistently, real-time quantitative RT-PCR (qRT-PCR) (Fig. 2.3b) or Northern blot analysis of NIH3T3 cells treated with TGFβ1 (Fig. 2.3c) showed that the induction of ADAM12 mRNA by TGFβ1 was also blocked by pre-treatment of cells with actinomycin D.

Smad2 and Smad3 are the main mediators of the transcriptional responses to TGFβ1. To determine whether Smad2 and/or Smad3 are involved in TGFβ1-induced upregulation of ADAM12 expression, we examined the effect of TGFβ1 on ADAM12 levels in Smad2- or Smad3-deficient cells. As shown in Fig. 2.4, the induction of ADAM12 by TGFβ1 in Smad2$^{-/-}$ or Smad3$^{-/-}$ mouse embryonic fibroblasts (MEFs) was significantly impaired when compared to wild-type

MEFs, indicating that Smad2/3 did play a role in upregulation of ADAM12.

Two lines of evidence further suggested that induction of ADAM12 expression by TGFβ1 involves derepression of the ADAM12 gene. First, pretreatment of cells with cycloheximide, an inhibitor of translation, did not abolish the induction of ADAM12 mRNA by TGFβ1 (Fig. 2.5a). In fact, cycloheximide alone increased the level of ADAM12 mRNA (Fig. 2.5b). These results suggest that de novo protein synthesis is not required for the upregulation of ADAM12 by TGFβ1 and that cycloheximide might block synthesis of a transcriptional repressor acting on the ADAM12 promoter/gene. Second, pre-treatment of cells with MG132, a proteasomal inhibitor, efficiently blocked the induction of ADAM12 protein and mRNA by TGFβ1 (Fig. 2.5c, 2.5d). This result, together with the effect mediated by cycloheximide, indicates that TGFβ1 signaling leads to proteasomal degradation of a repressor of the ADAM12 gene. SnoN, a negative regulator of TGFβ1 signaling, is known to be rapidly degraded in response to TGFβ1 stimulation, and thus may be a good candidate for a repressor of the ADAM12 gene. Indeed, analysis of SnoN protein levels in cell treated with TGFβ1 confirmed that SnoN, a 75-kDa protein, was degraded within 30 min of TGFβ1 treatment and remained at a low level for up to 4 h after adding TGFβ1 (Fig. 2.5e). As SnoN expression is upregulated by TGFβ1 signaling as a part of the negative feedback loop mechanism that limits the duration and strength of the TGFβ1 signals (Stroschein *et al.*, 1999), the level of SnoN began to rise and eventually returned to its initial level after 8-16 h of TGFβ1 treatment (Fig. 2.5e).

To determine whether SnoN is directly involved in TGFβ1-induced upregulation of ADAM12, we first studied the effect of SnoN overexpression on the level of ADAM12 in TGFβ1-treated cells. NIH3T3 cells were transduced with SnoN or control retroviruses, and 24 h later they were incubated for 16 h with or without TGFβ1. As shown in Fig. 2.6a, 2.6b, overexpression of SnoN caused $\sim$50% reduction in ADAM12 expression in response to TGFβ1, and this effect was statistically significant. The lack of a stronger inhibition of ADAM12 expression by SnoN may be caused by the fact that the efficiency of viral transduction was only $\sim$50-60% (as determined by GFP fluorescence, result not shown).

Next, we examined the effect of knocking down the expression of the endogenous SnoN in NIH3T3 cells on the expression levels of ADAM12. NIH3T3 cells were infected with lentiviral SnoN shRNA or control shRNA particles, and cells with stable incorporation of shRNA vectors were selected in the presence of puromycin. The level of the endogenous SnoN protein in shSnoN cells was reduced to an undetectable level (Fig. 2.7a). Induction of ADAM12 mRNA by TGFβ1 was more potent and occurred with faster kinetics in shSnoN cells than in shRNAControl, as revealed by qRT-PCR (Fig. 2.7b). Consistently, after 16 h of TGFβ1 treatment, the level of ADAM12 protein was $\sim$2.5-fold higher in SnoN-deficient cells than in control cells (Fig. 2.7c, 2.7d). Furthermore, the level of basal ADAM12 protein expression, which was most likely due to the autocrine/paracrine effect of the endogenous TGFβ1 produced in NIH3T3 cells (as shown in Fig. 2.2a) was also $\sim$2-fold higher in SnoN-deficient than in control cells (Fig. 2.7c, 2.7d).

As the induction of ADAM12 by TGFβ1 was very efficiently blocked by MG132 (Fig. 2.5c, 2.5d), suggesting that degradation of a transcriptional repressor is required for ADAM12 expression, we asked whether MG132 would be equally potent in blocking the induction of ADAM12 in SnoN-deficient cells. If SnoN is the repressor of the ADAM12 gene, then in the absence of SnoN, MG132 should have little effect on ADAM12 expression. As shown in Fig. 2.7c, right panel, incubation of shSnoN cells with TGFβ1 in the presence of MG132 led to the induction of ADAM12 expression, whereas no induction was observed in control cells under the same conditions. The induction of ADAM12 in shSnoN cells in the presence of MG132 was more modest than in the absence of the inhibitor, but this can be explained by the effective levels of SnoN in these cells. While no SnoN was detected in shSnoN cells in the absence of the inhibitor, MG132 treatment resulted in a significant accumulation of SnoN (Fig. 2.7c). This most likely was the result of incomplete double stranded mRNA degradation, active synthesis of SnoN protein, and very potent inhibition of SnoN degradation. The accumulation of SnoN in MG132-treated shSnoN cells might be the reason why SnoN knockdown by shRNA did not fully bypass the inhibitory effect of MG132 on the induction of ADAM12 by TGFβ1. Collectively, reduced induction of ADAM12 in cells overexpressing SnoN, increased basal and TGFβ1-induced levels of ADAM12 in shSnoN

cells in the absence of MG132, and a partial induction of ADAM12 by TGFβ1 in shSnoN cells treated with MG132 suggest that SnoN is a repressor of the ADAM gene in NIH3T3 cells and that degradation of SnoN after TGFβ1 treatment is responsible for derepression of the ADAM12 gene.

To determine whether the role of SnoN in TGFβ1-induced expression of ADAM12 observed in NIH3T3 cells can be extended to other cell types, we examined the effects of TGFβ1 on ADAM12 expression in human cancer cell lines, which typically express higher levels of SnoN than untransformed cells. For our analysis, we selected several cell lines in which the major components of the canonical TGFβ1 signaling pathway remain intact and which are responsive to TGFβ1 signals: MDA-MB-231, MDA-MB-435S, DU145, and HT1080 (Zhu *et al.*, 2007; Kim *et al.*, 1996; Pouliot & Labrie, 1999). In agreement with previous reports, the level of SnoN in cancer cells was significantly higher than in normal mammary epithelial cell line MCF10A (Fig. 2.8a). The basal level of ADAM12 mRNA in all four cancer cell lines was also higher than in MCF10A cells, but it was not inhibited by SB-431542 (results not shown), indicating that this was not a result of an elevated autocrine/paracrine TGFβ1 signaling. Upon TGFβ1 treatment, there was a $\sim$7-fold increase in ADAM12 expression in MCF10A cells and a much lower ($<$2-fold) increase in MDA-MB-231, MDA-MB-435S, DU145, and HT1080 cells. As expected, no induction of ADAM12 was detected in Smad4-deficient MDA-MB-468 cells, or in T47D and MCF7 cells, which express very low levels of TβRII and do not respond to TGFβ1 signals (Lynch *et al.*, 2001) (Fig. 2.8b). These results suggest that, among TGFβ1-responsive cells, there is an inverse correlation between SnoN levels and the extent of ADAM12 induction by TGFβ1.

2.5 Discussion

The induction of ADAM12 expression by TGFβ1 was first reported in hepatic stellate cells (Le Pabic *et al.*, 2003, 2005). Our studies demonstrate that induction of ADAM12 by TGFβ1 is a more general phenomenon and it takes place in fibroblasts and in epithelial cells. TGFβ1 does not appear to cause general upregulation of ADAM proteins, as at least two other ADAMs, ADAM9 and ADAM15, are not affected by the cytokine treatment (Le Pabic *et al.*, 2003, and this report).

Up to our knowledge, the only other ADAM reported to be upregulated by TGFβ1 is ADAM19, whose mRNA levels increase in alveolar epithelial cells treated with TGFβ1 (Keating *et al.*, 2006). The same study showed that TGFβ1 treatment leads to downregulation of ADAM28 mRNA level, further highlighting the specificity of TGFβ1 in regulating the expression of individual ADAMs.

According to previous reports, the induction of ADAM12 by TGFβ1 in hepatic stellate cells occurred with slow kinetics, as the level of ADAM12 mRNA was not significantly changed before 24 h of TGFβ1 treatment (Le Pabic *et al.*, 2003). Furthermore, the effect of TGFβ1 on ADAM12 expression was partially blocked by inhibitors of MAPKs, PI3K, or p70S6 kinase (Le Pabic *et al.*, 2005), indicating that TGFβ1 might upregulate ADAM12 via SMAD-independent pathways. In this report, we show that the induction of ADAM12 by TGFβ1 in mouse fibroblasts is more rapid, and increased levels of ADAM12 mRNA are detected within $\sim$2 h after TGFβ1 treatment. ADAM12 induction is completely blocked upon inhibition of transcription or proteasomal degradation, but is not blocked by cycloheximide, an inhibitor of translation. Based on these observations, we conclude that TGFβ1 induces ADAM12 expression by relieving a transcriptional repression of the ADAM12 gene. We show that SnoN, a transcriptional repressor that is efficiently degraded after TGFβ1 stimulation, is involved in regulation of ADAM12 expression. Overexpression of SnoN reduces ADAM12 induction, and downregulation of SnoN expression by shRNA leads to increased levels of both basal and induced ADAM12 expression. At this point, we are not able to conclude whether SnoN is the sole repressor controlling ADAM12 expression. Our attempts to test whether MG132 still blocks ADAM12 induction by TGFβ1 in shSnoN cells were compromised by the fact that MG132 treatment leads to build up of SnoN protein in these cells. Nevertheless, a modest induction of ADAM12 by TGFβ1 in shSnoN cells in the presence of MG132, compared to a complete block of induction in control cells where SnoN levels are much higher, clearly indicates that SnoN is a repressor that needs to be degraded in order to derepress the ADAM12 gene. Another possible candidate for repression of ADAM12 is a SnoN-related protein Ski, but its degradation in response to TGFβ1 appears to be less efficient than that of SnoN (Stroschein *et al.*, 1999).

Although SnoN was first described as a negative regulator of TGFβ1 signaling, it is not a universal repressor of TGFβ1 responsive genes. Rather, SnoN acts in a gene-specific manner, and inhibition of the proteasome leads to abrogation of certain TGFβ1 target gene regulation, without any effect on other TGFβ1 target genes (Zhang *et al.*, 2002). Furthermore, recent reports indicate that under certain circumstances SnoN can act as a positive mediator of transcription (Sarker *et al.*, 2005). Indeed, a microarray analysis of human lung cancer A549 cells demonstrated that a large set of genes is downregulated in cells lacking SnoN, suggesting that SnoN may function as a transcriptional activator, in addition to acting as a transcriptional repressor of the Smad proteins (Zhu *et al.*, 2007). Thus, the effect of SnoN on a particular target gene is not easily predictable, and a negative or positive regulation is possible. As shown in this study, in the case of ADAM12, SnoN is a negative regulator of its expression.

ADAM12 expression is dysregulated in many cancers, cardiac hypertrophy, during aseptic loosening of hip replacement implants, and in the EAE (Kveiborg *et al.*, 2008; Fedak *et al.*, 2006; Wang *et al.*, 2009a,b; Ma *et al.*, 2005; Toft-Hansen *et al.*, 2004). Each of these pathological conditions is accompanied by increased levels of TGFβ1 and/or abnormal expression of SnoN (Pot & Bonni, 2008; Massagué, 2008; Leask, 2007; Holt *et al.*, 2007; Swanborg & Stepaniak, 2001). It is tempting to speculate that upregulation of ADAM12 expression in cardiac hypertrophy, in inflammatory responses related to osteolysis, or in the EAE is directly linked to activation of TGFβ1 signaling. Furthermore, during fibrotic kidney disease after obstructive injury, SnoN is downregulated due to enhanced ubiquitin-mediated degradation, which in turn is a result of TGFβ1-induced expression of Smurf2 ubiquitin ligase (Yang *et al.*, 2003; Tan *et al.*, 2006). It is possible that a similar chain of events leads to degradation of SnoN during fibrosis associated with cardiac hypertrophy, which would explain high levels of ADAM12 expression in hypertrophic myocardium. In cancer, the situation is more complex, as both TGFβ1, a positive ADAM12 regulator, and SnoN, a negative ADAM12 regulator, are elevated. Our studies in cancer cells show that increased basal expression of ADAM12 is not due to the autocrine/paracrine effects of TGFβ1 produced by some of these cell lines. Rather, it is an inherent feature of cancer cells and may be a result of genetic

or epigenetic changes associated with the oncogenic transformation. Increased basal levels of ADAM12 expression also do not correlate with increased SnoN levels in cancer cells. However, cancer cells have many other components of the TGFβ1 pathway aberrantly expressed, including TGFβ receptors and Smads, and direct correlations between absolute expression levels of ADAM12 and SnoN in different cell types may be difficult to establish. Importantly, our results indicate that there is an inverse correlation between the level of SnoN in cancer cells and the ability of TGFβ1 to induce ADAM12 expression.

TGFβ signaling plays dual roles during tumor development. During early phases of tumorigenesis, TGFβ acts as a tumor suppressor by limiting cancer cell proliferation and enhancing differentiation. In later stages, TGFβ promotes tumor growth by stimulating cell migration, invasion, and metastasis, by modifying tumor microenvironment, and by modulating host immune responses (Massagué, 2008; Bierie & Moses, 2006). High expression of SnoN makes cancer cells resistant to the anti-proliferative effects of TGFβ (a pro-oncogenic role of SnoN) and limits their metastatic potential (an anti-tumorigenic activity of SnoN) (Zhu *et al.*, 2007). Interestingly, both tumor-promoting and tumor-suppressing roles have been postulated for ADAM12. Transgenic expression of ADAM12Δcyt (lacking the cytoplasmic domain) in mammary tumors of MMTV-PyMT mice accelerates tumor growth, which is consistent with tumor-promoting function of ADAM12 (Kveiborg *et al.*, 2005). On the other hand, ADAM12 is frequently mutated in breast cancer (Sjöblom *et al.*, 2006; Wood *et al.*, 2007), and cancer-associated mutations cause mislocalization of ADAM12 protein in cells and interfere with its function at the cell surface, suggesting that the wild-type ADAM12 may also play an anti-tumor role (Dyczynska *et al.*, 2008). We believe that identification of SnoN as a repressor of the ADAM12 gene will contribute to advances in studies on the role of ADAM12 in tumor progression and in the development of other pathological conditions.

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Footnotes

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Abbreviation list

TβRI and TβRII, TGFβ type I and type II serine/threonine kinase receptors, respectively; DMEM; Dulbecco's modified Eagle medium; FBS, fetal bovine serum; EAE, experimental autoimmune encephalomyelitis; qRT-PCR, real-time quantitative reverse transcription PCR

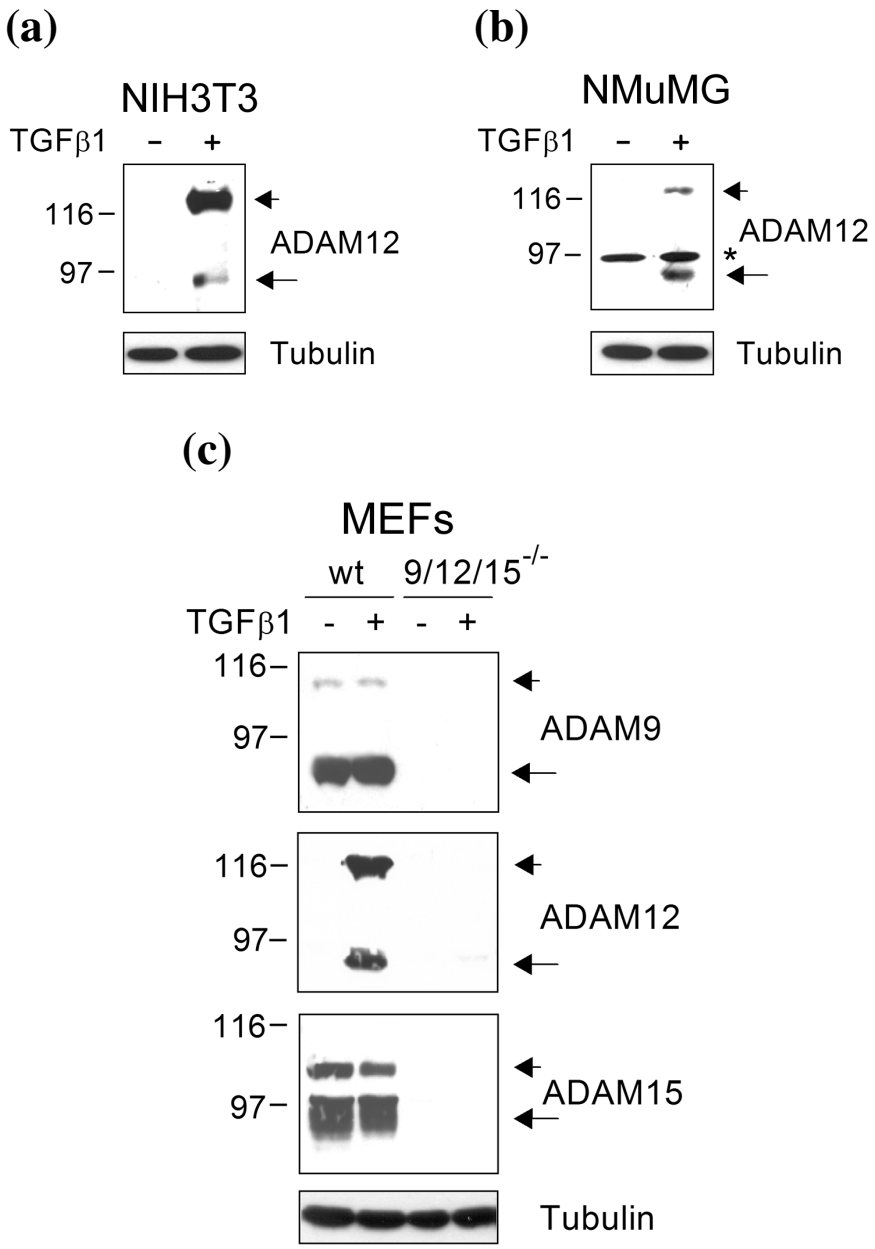

Figure 2.1: **Induction of ADAM12 expression by TGFβ1.** (a) NIH3T3 fibroblasts, (b) normal mouse mammary gland epithelial cells NMuMG, and (c) mouse embryonic fibroblasts (MEFs) (wt, wild-type; 9/12/15$^{-/-}$, isolated from triple knockout mice lacking ADAM9,12, and 15) were incubated for 24 h in the presence of TGFβ1 (2 ng/ml), as indicated. Cells were lyzed and glycoprotein-enriched fractions were analyzed by Western blotting using anti-ADAM antibodies; tubulin is a loading control. The full-length ADAM proteins are indicated by short arrows, the mature forms lacking the pro-domains are indicated by long arrows, asterisk denotes a non-specific band. Experiments in Fig. 2.1 were performed by Dr. Emilia Syta.

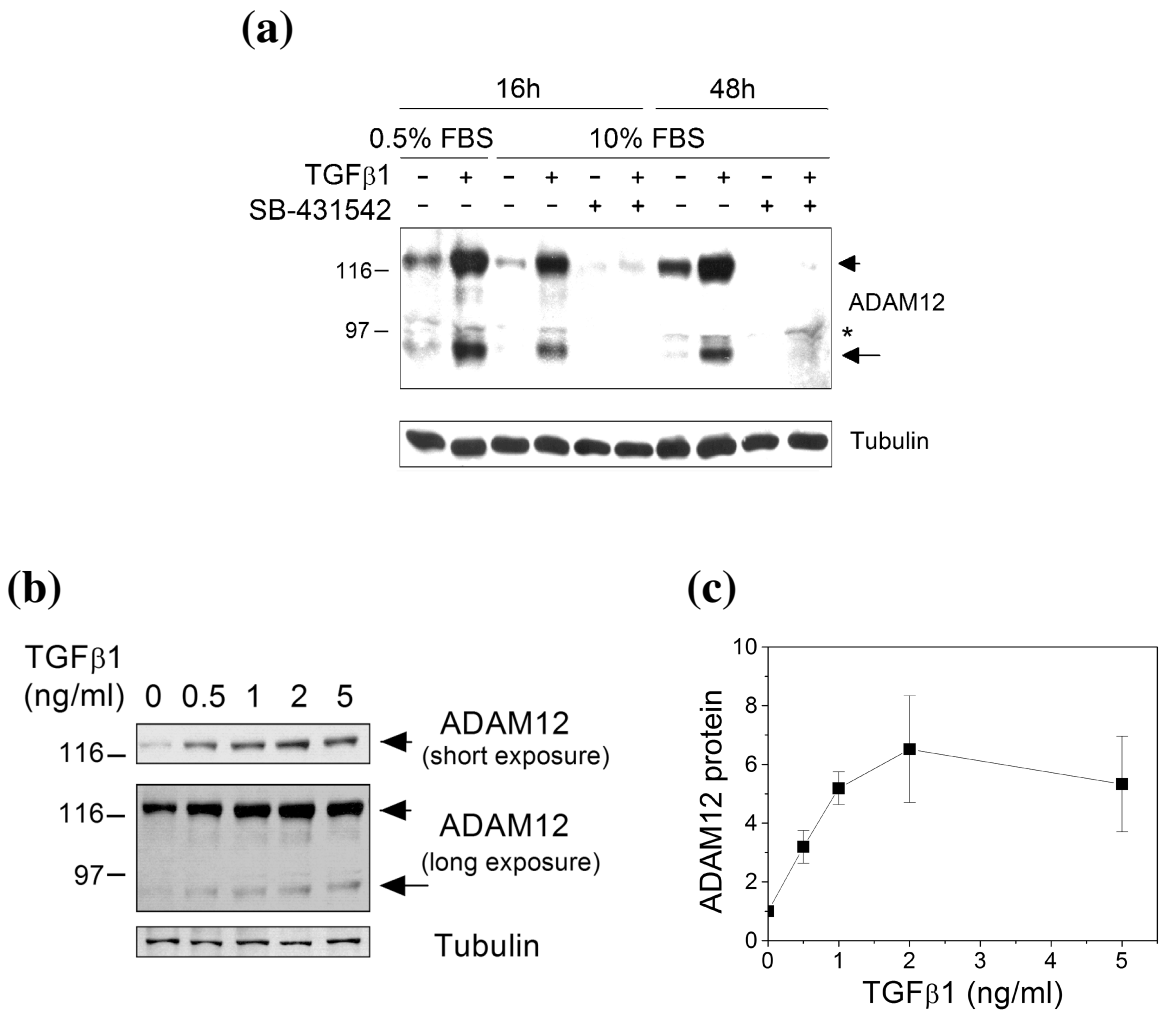

Figure 2.2: **Characterization of TGFβ1-induced expression of ADAM12.** (a) The effect of serum on the induction of ADAM12 by TGFβ1. NIH3T3 cells were pre-incubated for 24 h in the presence of 0.5% or 10% FBS, as indicated, followed by treatment with 2 ng/ml of TGFβ1 for additional 16 h or 48 h. In some cases, 10 μM SB-431543, an inhibitor of TβRI, was added 30 min prior to TGFβ1 treatment. (b), (c) TGFβ1 dose-response of the induction of ADAM12. (b) NIH3T3 cells were incubated for 24 h with the indicated doses of TGFβ1, and the levels of ADAM12 protein were analyzed as in Fig. 2.1. Quantitative differences in the abundance of the full-length ADAM12 (short arrow) are best shown after short exposure of the immunoblot, the mature ADAM12 (long arrow) is visualized after long exposure. (c) The intensities of the bands corresponding to the full-length ADAM12 protein in panel (b) were quantified using densitometry and ScionImage software. The data represent the means +/- SEM from 3 independent determinations.

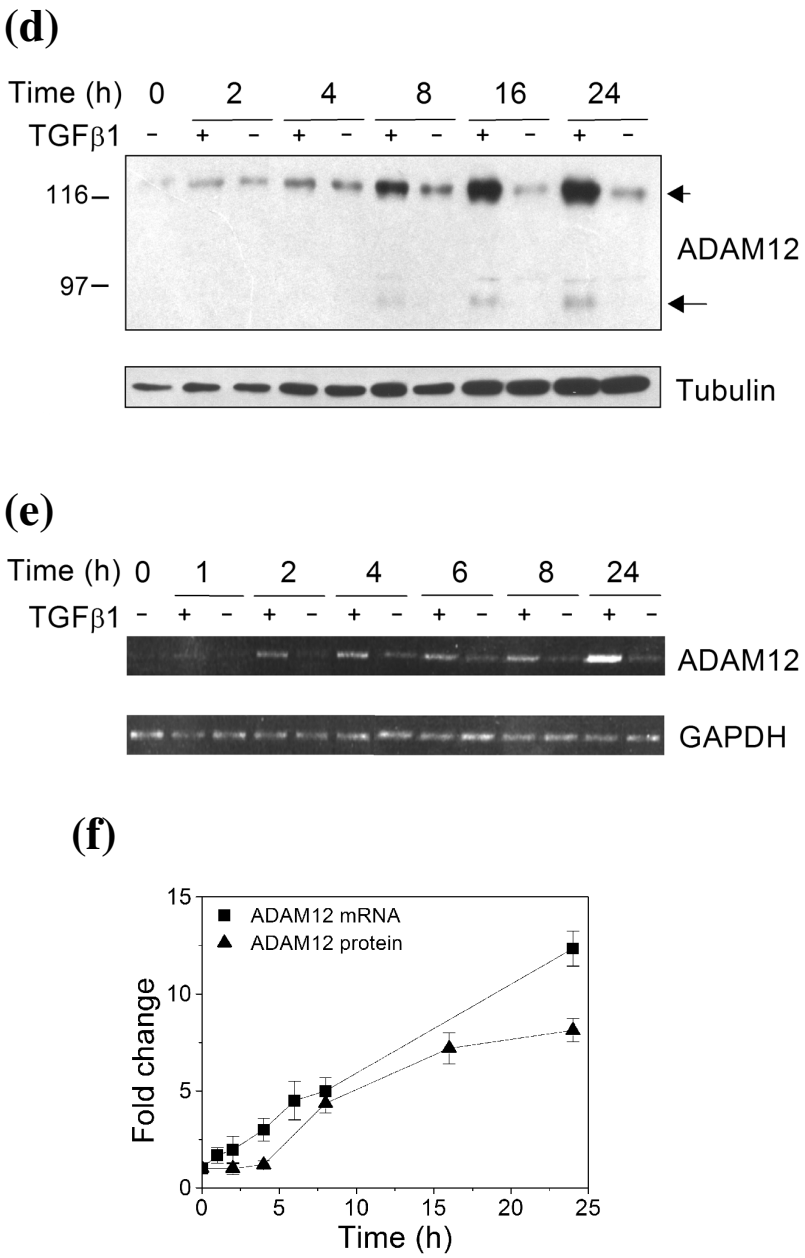

Figure 2.2: **Characterization of TGFβ1-induced expression of ADAM12 (continued).** (d) - (f) Time course of ADAM12 induction by TGFβ1. NIH3T3 cells were incubated without or with 2 ng/ml of TGFβ1 for the indicated times. (d) The levels of ADAM12 protein were analyzed by Western blotting, as in Fig. 2.1. (e) The levels of ADAM12 mRNA were analyzed by semi-quantitative RT-PCR, GAPDH is a gel-loading control. (f) The intensities of the bands corresponding to the full-length ADAM12 protein in (d) and ADAM12 mRNA in (e) were quantified using densitometry and ScionImage software. The data represent the means +/- SEM from 3 independent determinations. Experiment in (e) was performed by Dr. Hui Li.

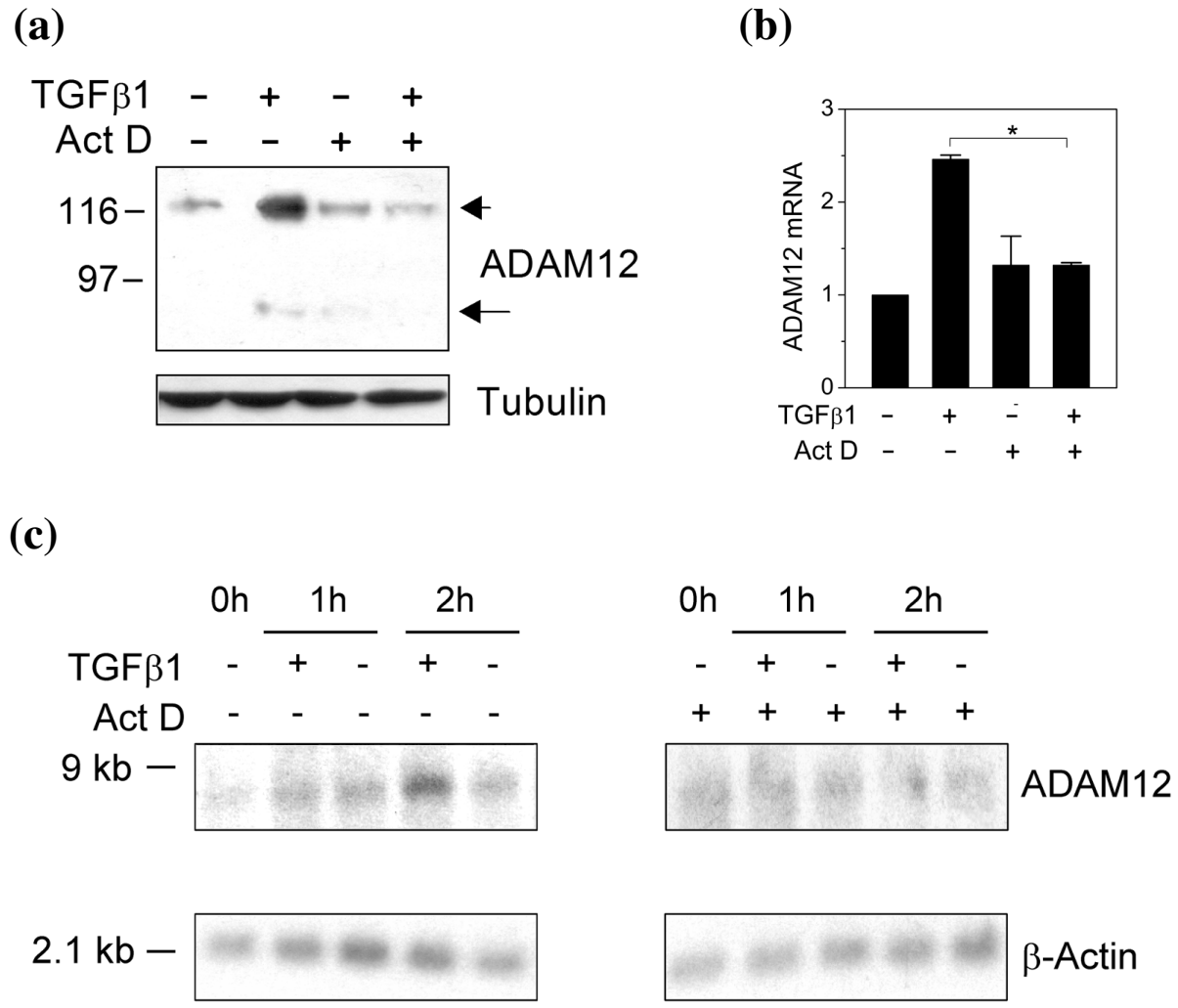

Figure 2.3: **Induction of ADAM12 expression by TGFβ1 occurs at the transcriptional level.** (a) NIH3T3 cells were pretreated for 15 min with 5 μg/ml actinomycin D or with DMSO, then incubated for 8 h with or without TGFβ1, followed by Western blotting with anti-ADAM12 antibody, as in Fig. 2.1. Tubulin is a gel loading control. The experiment was repeated 3 times with similar results. (b) Quantitative real-time RT-PCR analysis of ADAM12 expression. NIH3T3 cells were pretreated for 15 min with 5 μg/ml actinomycin D or with DMSO, and then treated without or with 2 ng/ml of TGFβ1 for 4 h. The level of ADAM12 mRNA was normalized to GAPDH. The data represent the means +/- SEM from 3 independent experiments. Asterisk indicate statistically significant effect ($p < 0.05$) of inhibitor treatment. (c) Northern blot analysis of ADAM12 expression. NIH3T3 cells were pretreated for 15 min with 5 μg/ml actinomycin D or with DMSO, and then treated without or with 2 ng/ml of TGFβ1 for indicated times. Total mRNA was extracted and hybridized with ADAM12 and β-actin probes. The analysis was repeated 3 times with similar results.

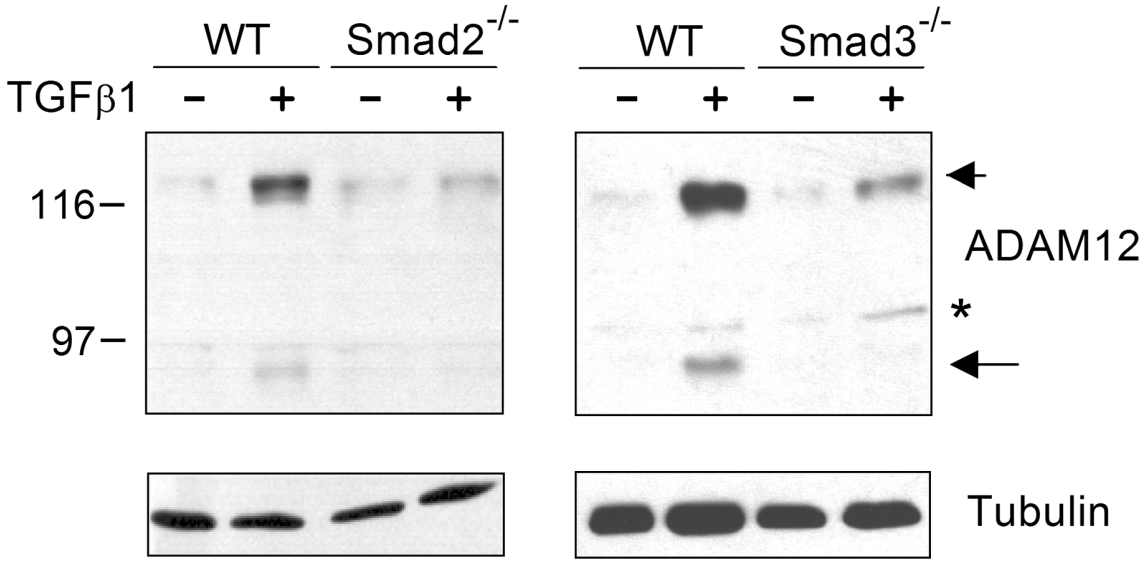

Figure 2.4: **Induction of ADAM12 by TGFβ1 is reduced in Smad2- and Smad3-deficient cells.** Smad2$^{-/-}$, Smad3$^{-/-}$, or the corresponding wild-type (WT) MEFs were incubated for 24 h with or without 2 ng/ml TGFβ1, followed by analysis of ADAM12 expression by Western blotting, as in Fig. 2.1; tubulin is a loading control. The full-length ADAM proteins are indicated by short arrow, the mature forms lacking the pro-domains are indicated by long arrow, asterisk denotes a non-specific band.

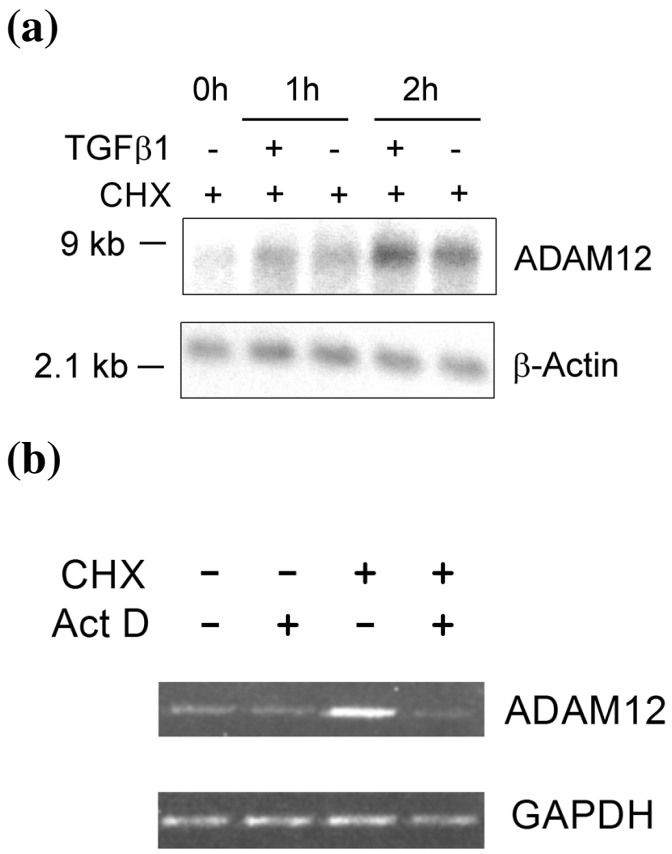

Figure 2.5: **Induction of ADAM12 expression by TGFβ1 involves derepression of the ADAM12 gene.** (a), (b) ADAM12 expression is induced in the absence of protein synthesis. In (a), NIH3T3 cells were pretreated for 2 h with 5 μg/ml cycloheximide (CHX), an inhibitor of translation, and then treated with or without 2 ng/ml of TGFβ1 for indicated amounts of time, with the continuous presence of CHX. Total RNA was extracted and hybridized with ADAM12 or β-actin probes. In (b), NIH3T3 cells were pre-treated for 15 min with 5 μg/ml actinomycin D or DMSO, and then treated for 4 h with or without 5 μg/ml CHX, as indicated. The level of ADAM12 mRNA was evaluated by semi-quantitative RT-PCR, GAPDH is an internal control. The experiment shown in (a) and (b) were repeated 3 times with similar results. Experiment in (b) was performed by Dr. Hui Li.

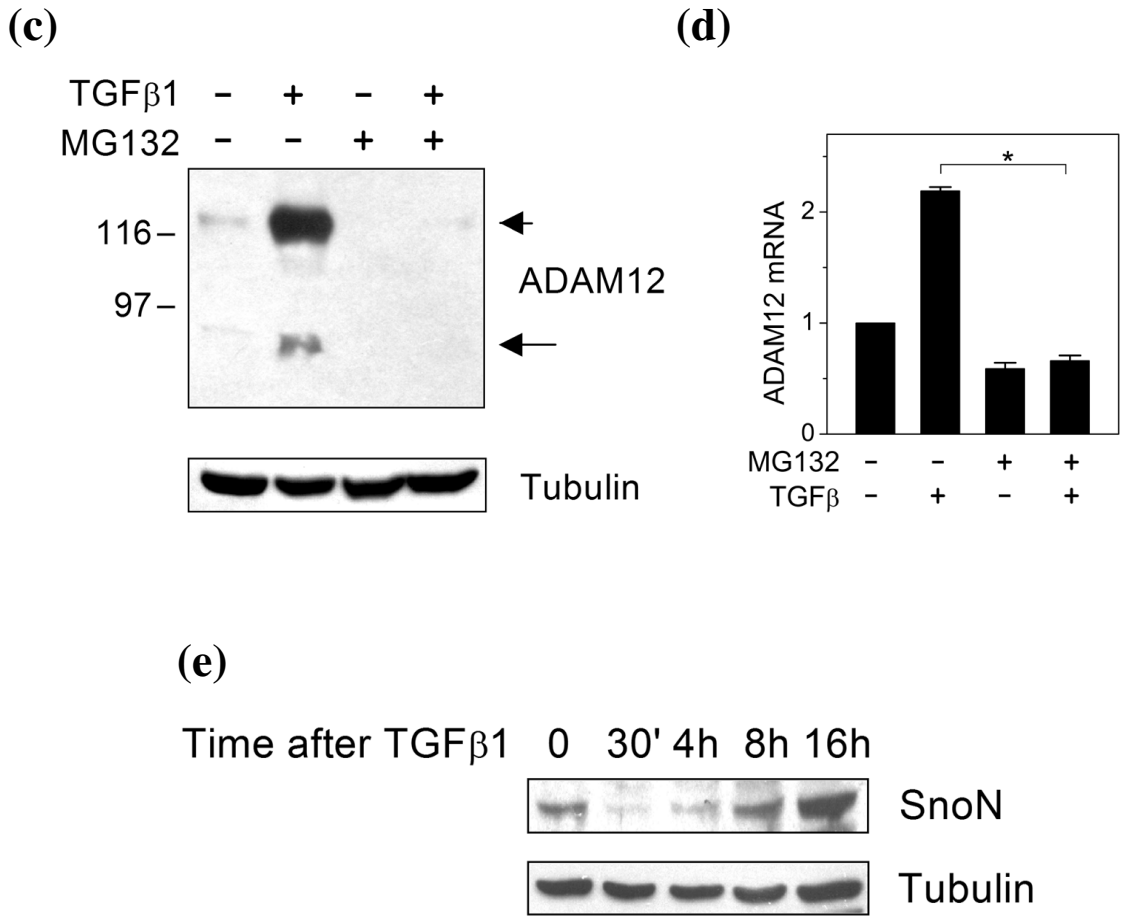

Figure 2.5: **Induction of ADAM12 expression by TGFβ1 involves derepression of the ADAM12 gene (continued).** (c), (d) Induction of ADAM12 by TGFβ1 is blocked by a proteasomal inhibitor. NIH3T3 cells were pretreated for 1 h with or without 10 μM MG132, and then treated for 16 h (c) or 4 h (d) with or without 2 ng/ml TGFβ1, in the presence or absence of MG132, as indicated. The level of ADAM12 protein (c) was analyzed by Western blotting, as in Fig. 2.1; the level of ADAM12 mRNA was quantified by real time qRT-PCR (d). In (d), ADAM12 mRNA was normalized to GAPDH mRNA; the data represent the means +/- SEM from 2 independent experiments. Asterisk indicate statistically significant effect ($p < 0.05$) of inhibitor treatment. (e) The level of SnoN repressor at the indicated times after adding TGFβ1 was evaluated by Western blotting.

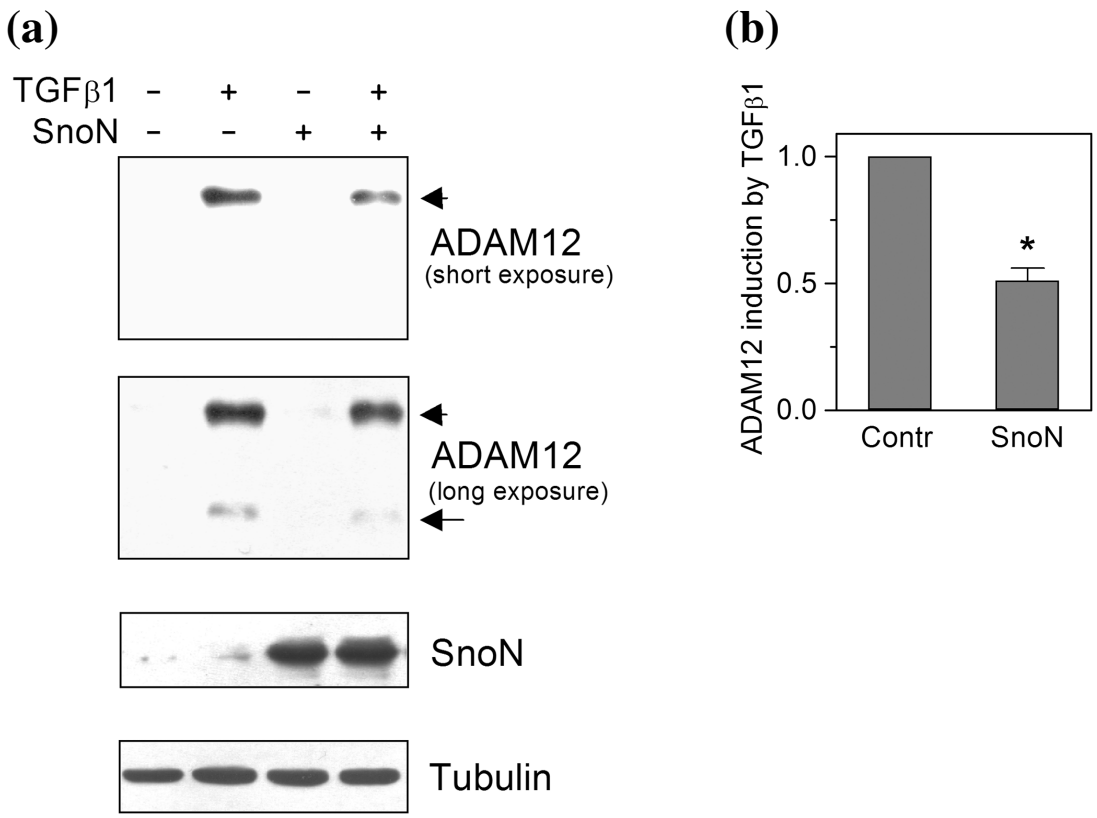

Figure 2.6: **Overexpression of SnoN partially inhibits the induction of ADAM12 by TGFβ.** (a) NIH3T3 cells were transduced with SnoN or control retroviruses, and 24 h later they were treated for additional 16 h with or without 2 ng/ml of TGFβ1. ADAM12 and SnoN levels were examined by Western blotting. The full length ADAM12 (short arrow) and the mature ADAM12 (long arrow) are best visualized at short and long film exposures, respectively. (b) The experiment shown in panel (a) was repeated three times, and the relative changes in the level of ADAM12 protein were quantified by densitometry and Scion Image; the data represent the means +/- SEM; asterisk indicates statistically significant effect ($p < 0.05$) of SnoN.

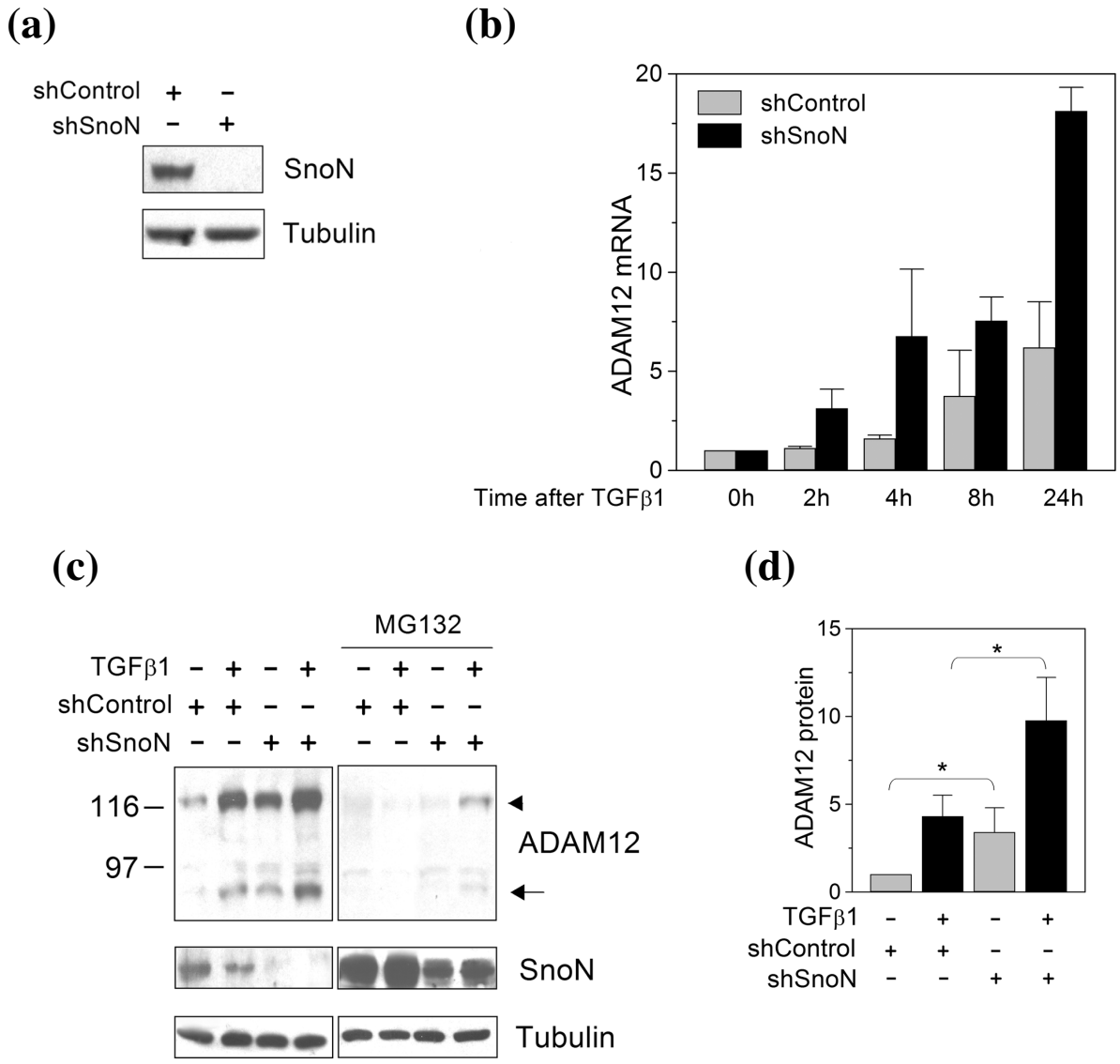

Figure 2.7: **Knockdown of SnoN expression increases basal and TGFβ1-induced ADAM12 expression.** NIH3T3 cells were stably transduced with SnoN shRNA or control shRNA lentiviruses. (a) The level of SnoN protein in shSnoN or shControl cells was evaluated by Western blotting. (b) shSnoN and shControl cells were incubated with 2 ng/ml of TGFβ1 for indicated times. ADAM12 mRNA levels, normalized to GAPDH mRNA, were measured by qRT-PCR; fold changes over the levels at time 0 are shown. (c) shSnoN and shControl cells were incubated for 16 h with 2 ng/ml TGFβ1, in the absence or presence of 10 μM MG132, followed by evaluation of ADAM12 and SnoN protein levels. (d) The intensities of the bands corresponding to the full-length ADAM12 protein in panel (c) were quantified using densitometry and ScionImage software. The data represent the means +/- SEM from 3 independent experiments. Asterisks indicate statistically significant differences ($p < 0.05$) compared to shControl cells.

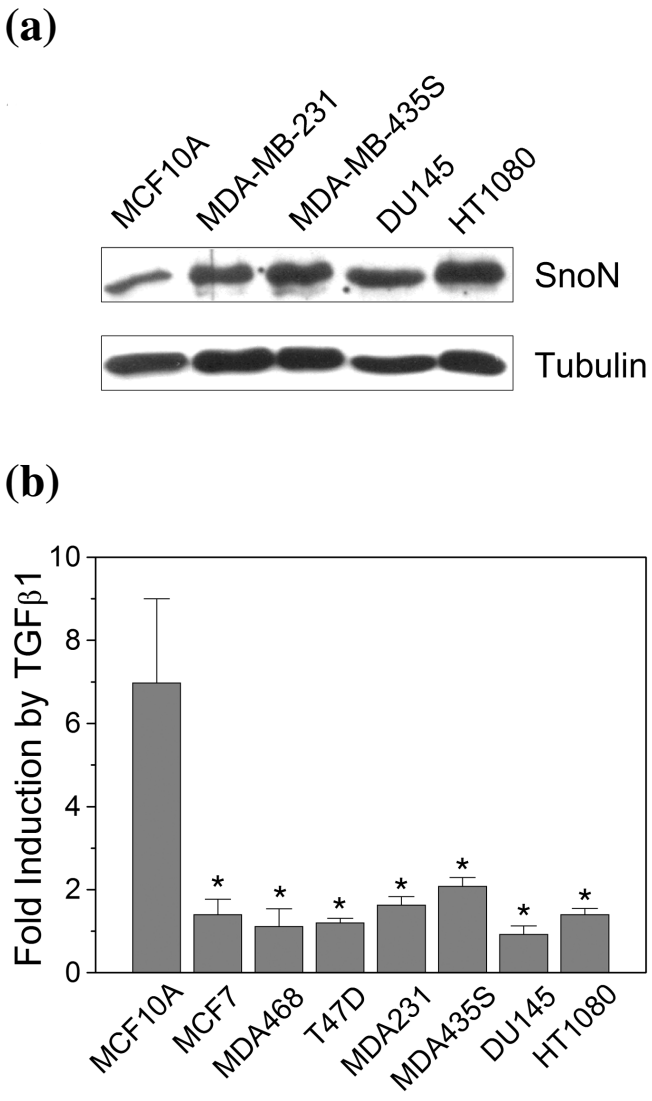

Figure 2.8: **Diminished induction of ADAM12 by TGFβ1 in cancer cell lines.** (a) The level of SnoN expression in different cell lines. (b) Fold induction of ADAM12 mRNA, normalized to β-actin mRNA, after 24 h treatment with 2 ng/ml TGFβ1 was evaluated by qRT-PCR. The data represent the means +/- SEM from 2 independent experiments. Asterisk indicate statistically significant differences ($p < 0.05$) compared to MCF10A cells. MCF10A are untransformed human mammary epithelial cells, MDA-MB-231, MCF7, MDA-MB-468 and T47D are breast cancer cell lines, MDA-MB-435S are melanoma cells, DU145 is a prostate cancer line, and HT1080 is a fibrosarcoma cell line.

Chapter 3

Proteolytic processing of Delta-like 1 by ADAM proteases

The data presented in this chapter have been published in the following journal article:

Dyczynska E., Sun D., Yi H., Zolkiewska A.

Proteolytic processing of Delta-like 1 by ADAM proteases

J Biol. Chem. 282:436-444 (2007)

3.1 Abstract

Delta-like 1 (Dll1) is a mammalian ligand for Notch receptors. Interactions between Dll1 and Notch in trans activate the Notch pathway, whereas Dll1 binding to Notch in *cis* inhibits Notch signaling. Dll1 undergoes proteolytic processing in its extracellular domain by ADAM10. In this work we demonstrate that Dll1 represents a substrate for several other members of the ADAM family. In co-transfected cells, Dll1 is constitutively cleaved by ADAM12, and the N-terminal fragment of Dll1 is released to medium. ADAM12-mediated cleavage of Dll1 is cell density-dependent, takes place in *cis* orientation, and does not require the presence of the cytoplasmic domain of ADAM12. Full-length Dll1, but not its N- or C-terminal proteolytic fragment, co-immunoprecipitates with ADAM12. By using a Notch reporter construct, we show that Dll1 processing by ADAM12 increases Notch signaling in a cell-autonomous manner. Furthermore,

ADAM9 and ADAM17 have the ability to process Dll1. In contrast, ADAM15 does not cleave Dll1, although the two proteins still co-immunoprecipitate with each other. Asn-353 present in the catalytic motif of ADAM12 and other Dll1-processing ADAMs, but absent in ADAM15, is necessary for Dll1 cleavage. Dll1 cleavage is reduced in ADAM9/12/15$^{-/-}$ mouse embryonic fibroblasts (MEFs), suggesting that the endogenous ADAM9 and/or ADAM12 present in wild type MEFs contribute to Dll1 processing. Finally, the endogenous Dll1 present in primary mouse myoblasts undergoes cleavage in confluent, differentiating myoblast cultures, and this cleavage is decreased by ADAM12 small interfering RNAs. Our findings expand the role of ADAM proteins in the regulation of Notch signaling.

3.2 Introduction

Notch signaling regulates cell fate decisions during development and in the adult (Lai, 2004; Kadesch, 2004; Louvi & 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, 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 & Kopan, 2000; Selkoe & Kopan, 2003). The intracellular domain of Notch translocates to the nucleus, where it interacts with CSL 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 & Sisodia, 2003; Six *et al.*, 2003; LaVoie & Selkoe, 2003; Bland *et al.*, 2003). Two ADAMs were postulated to

cleave Notch ligands in mammalian cells, ADAM10 and -17. ADAM10 has been implicated in the processing of mouse (Six *et al.*, 2003) and rat Delta-like 1 (Dll1) (LaVoie & Selkoe, 2003), whereas ADAM17 was suggested to cleave rat Jagged 1 (LaVoie & 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 & Sakamoto, 2005; Ladi *et al.*, 2005). Associations between receptors and ligands in *cis* decrease Notch receptivity and attenuate the Notch pathway (Ladi *et al.*, 2005; Sakamoto *et al.*, 2002; Katsube & Sakamoto, 2005; Franklin *et al.*, 1999). In Drosophila, proteolytic processing of Delta by ADAM10-like (Kuzbanian-like; Kul) 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 & Selkoe, 2003).

In this work, 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, which has not been previously implicated in Notch signaling, can efficiently process Dll1, but not Notch1. We demonstrate that Dll1 cleavage by ADAM12 activates Notch in a cell-autonomous manner. In addition, we show that two other ADAMs, ADAM9 and 17, are capable of Dll1 processing, and we identify a residue within the ADAM catalytic motif that appears necessary for the cleavage of Dll1. 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. Finally, we show that the endogenous Dll1 present in primary mouse myoblasts undergoes cleavage in confluent, differentiating myoblast cultures, and this cleavage is decreased by ADAM12 small interfering RNAs (siRNAs).

3.3 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. An untagged ADAM12 was generated by introducing a stop codon after the C-terminal Lys residue. The E349Q and N353S ADAM12 mutants and the S354N ADAM15 mutant were 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). p3PTlux vector was obtain from Addgene (plasmid 11767) (Wrana *et al.*, 1992).

Cell culture and plasmid transfection

COS-7, NIH3T3, and CHO-K1 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM; COS-7 and NIH3T3) or F12K nutrient mixture (CHO-

K1), supplemented with 10% fetal bovine serum, at 37°C in the presence of 5% CO_2 under a humidified atmosphere. Primary myoblasts were isolated from 2-3-day-old C57BL/6 mice according to Rando & Blau (1994) and grown on collagen I-coated plates in DMEM supplemented with 20% fetal bovine serum and 100 μg/ml each penicillin and streptomycin. 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.

One day after plating, cells were transfected using Fugene6 (Roche Applied Science). In most experiments 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 FACSCalibur (BD Biosciences).

RNA interference

Three different Silencer siRNA duplexes specific for mouse ADAM12 and a negative control siRNA (control #1) were purchased from Ambion. The sequences of sense RNA strands were: CCAGAGAGGAGCUUACGAAtt (siRNA1); GCCAAUGAAAAACACCACUtt (siRNA2); GCAAAUUACCACAUUGUCUtt (siRNA3). Mouse primary myoblasts were trypsinized and transfected with individual siRNA duplexes (30 nM) using siPORT *NeoFX* transfection reagent (Ambion) as cells attached to plates. One day after transfection, confluent cells were transferred to differentiation medium (DMEM with 2% horse serum). The level of expression of ADAM12 and the amount of Dll1 cleavage were analyzed 40 h later.

Cell treatments

In some experiments, before harvesting, cells were incubated for 3 h with 5 μM lactacystin (a proteasomal inhibitor), for 6 h with 1 μM L685,458 (a γ-secretase inhibitor), for 1 h with 1 μM

ionomycin, or for 1 h with 25 ng/ml phorbol myristate acetate (PMA). Cells were serum-starved for 30 min before and during the PMA treatment.

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 antibodies in blocking buffer, followed by incubation with horseradish peroxidase-labeled secondary antibodies and detection using the West-Pico chemiluminescence kit (Pierce). To detect the endogenous ADAM12 in primary myoblasts, cell extracts were enriched for glycoproteins using concanavalin A-agarose beads (20-μl bed volume/ml cell extract) before SDS-PAGE and Western blotting (Cao *et al.*, 2002). The following primary antibodies were used: rabbit anti-ADAM12 cytoplasmic peptide antibody (1:3000), rabbit anti-ADAM12 disintegrin antibody (1:3000), rabbit anti-Dll1 (Santa Cruz Biotechnology, H-265; 0.2 μg/ml), mouse anti-c-Myc (clone 9E10, Upstate Biotechnology; 1 μg/ml). Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG (heavy and light chain-specific) or HRP-conjugated anti-rabbit IgG (Fc fragment-specific, Jackson ImmunoResearch; 0.8μg/ml; used in the experiment shown in Fig. 3.4c). 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

To immunoprecipitate the N-terminal fragment of Dll1 from culture media, 24 h after co- transfection with c-Myc-Dll1 and ADAM12, COS-7 cells were transfered 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 medium was collected, cell debris was removed by centrifugation, and supernatant was used for immunoprecipitation with 9E10 antibody (5 μg/mL). To immunoprecipitate c-Myc-tagged ADAMs or untagged ADAM12, 36 h after transfection cells were lysed with extraction buffer, cell extracts were centrifuged at 21,000 x g for 15 min, and supernatants were used for immunoprecipitation with 9E10 antibody (5 μg/ml) or with anti-ADAM12 cytoplasmic peptide antibody (1:250), respectively. To immunoprecipitate Dll1 from transfected MEFs or the endogenous Dll1 from primary myoblasts, anti-Dll1 antibody (H-265, 5 μg/ml) was used. After preclearing, supernatants were incubated with antibodies and protein G-Sepharose 4 Fast Flow (Amersham Biosciences), the beads were washed three times with extraction buffer, and immunocomplexes were eluted with SDS-PAGE sample buffer and analyzed by electrophoresis and autoradiography or Western blotting.

Luciferase 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 ADAM12 or empty pcDNA3.1 vector, and 0.5 μg of Dll1 or empty pIRES-puro vector. A9/12/15$^{-/-}$ MEFs cells in 6-well plates were with 0.5 μg ADAM12, ADAM12 E349Q or empty pcDNA3.1 vector, 0.5 μg p3TPlux firefly luciferase reporter, 0.05 μg pRL-TK and increasing amounts of c-Myc-Dll1 or empty pcDNA3.1 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) to NIH3T3 cells and co-cultured for an additional 24 h. MEFs treated with 2 ng/ml TGFβ1 or vehicle (BSA/HCl) for 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.

3.4 Results

First, we asked whether ADAM12 is capable of processing Dll1. When untagged Dll1 was transfected into COS-7 cells, antibody specific for the C terminus of Dll1 detected the full-length protein of ∼90 kDa and a low level of the C-terminal Dll1 fragment (CTF) of ∼29 kDa in cell lysates (Fig. 3.1a). Co-transfection of wild type mouse ADAM12, but not the catalytically inactive E349Q mutant, strongly increased the abundance of CTF (Fig. 3.1a), suggesting that ADAM12 cleaved Dll1. To follow the fade of the N-terminal portion of Dll1, we introduced c-Myc tag between amino acids 46 and 47 of Dll1 (Fig. 3.1b). After metabolic labeling of transfected cells with [^{32}S]methionine+cysteine, we immunoprecipitated the ∼60kDa soluble N-terminal fragment (NTF) of Dll1 from cultured medium (Fig. 3.1b, middle panel). The amount of detected NTF was much higher when cells were co-transfected with wild type ADAM12, indicating that Dll1 cleavage took place in intact cells and that NTF was released to medium.

ADAM12-catalyzed processing of Dll1 (as well as the processing mediated by endogenous ADAMs present in COS-7 cells) was more efficient at high cell density (∼90% confluence at the time of assaying) than at low cell density (∼50% confluence; Fig. 3.2a). This result suggested that the cleavage might have taken place in *trans* orientation. However, co-transfection of ADAM12 and Dll1 yielded significantly more CTF than co-culture of cells that were singly transfected with Dll1 or with ADAM12 (Fig. 3.2b), indicating that the cleavage occurred in *cis*. Thus, cell density dependence of Dll1 cleavage suggests that interactions of Dll1 or ADAM12 with proteins present on the surface of neighboring cells may be required for the efficient processing of Dll1.

It was reported that removal of the extracellular domain of Dll1 was followed by cleavage of CTF by γ-secretase (Ikeuchi & Sisodia, 2003; Six *et al.*, 2003; LaVoie & Selkoe, 2003). In our experiments the product of γ-secretase cleavage (ICD, intracellular domain of Dll1, ∼3 kDa smaller than CTF) was poorly detectable in immunoblots, but it was augmented after cells were treated with lactacystin, a proteasomal inhibitor (Fig. 3.3a). Incubation of cells with L685,458, a γ-secretase inhibitor, resulted in increased accumulation of CTF (Fig. 3.3a). These results suggest that at least part of ADAM12-generated CTF Dll1 is further processed by γ-secretase to produce

ICD Dll1, and the ICD fragment is then subject to proteasomal degradation.

ADAM10- or ADAM17-mediated cleavage of several substrate proteins is increased by ionomycin or phorbol esters, respectively (Sahin *et al.*, 2004; Reiss *et al.*, 2005; Maretzky *et al.*, 2005; Nagano *et al.*, 2004). Here, ADAM12-mediated processing of Dll1 was not increased by ionomycin and was only weakly stimulated by PMA (Fig. 3.3b). This result indicates that ADAM12 is capable of constitutive rather than stimulated cleavage of Dll1.

To test whether ADAM12 can also cleave Notch, a receptor for Dll1, we co-transfected COS-7 cells with ADAM12 and Notch1 and cultured cells at high density. As a positive control, we used ADAM17, which acts as α-secretase for Notch and, upon ligand binding, cleaves Notch at the S2 site (Brou *et al.*, 2000). After stimulation of cells with PMA, the S2 cleavage product NEXT, was clearly detected in ADAM17-cotransfected cells (Fig. 3.3c). In contrast, the S2 product was not detected in ADAM12-transfected cells, suggesting that ADAM12 lacks α-secretase activity toward Notch1.

ADAM12-mediated Dll1 processing did not require an intact cytoplasmic tail of ADAM12, as the full-length ADAM12 and the XT fragment containing a 169-amino acid deletion at the C terminus (Yi *et al.*, 2005) processed Dll1 equally well (Fig. 3.4a). Dll1 and ADAM12 co-immunoprecipitated together by an antibody specific to the C-terminus of ADAM12 (Fig. 3.4b). Interestingly, the immunocomplexes contained the full-length Dll1, but not the 29-kDa CTF (Fig. 3.4c). The N-terminal extracellular fragment of Dll1 generated after ADAM12 cleavage was, however, also excluded from the ADAM12-Dll1 immunocomplexes (Fig. 3.4d), suggesting that only the full length Dll1 was capable of interacting with ADAM12. The lack of 60-kDa NTF Dll1 in ADAM12 immunoprecipitates is consistent with the result shown in Fig. 3.1b, in which the N-terminal fragment of Dll1 was released to medium and was not found in association with cells.

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. 3.5a), 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. 3.5a), 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. 3.5b) due to formation of Notch/Dll1 complexes in *cis* (Sakamoto *et al.*, 2002; Katsube & 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. 3.5b). The catalytically inactive mutant of ADAM12, E349Q, which did not process Dll1 (Fig. 3.1), did not activate Notch either (Fig. 3.5b). 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 tested whether Dll1 can serve as a substrate for three other ADAMs that have not been previously implicated in Dll1 cleavage, ADAM9, -15, and -17. All these ADAMs as well as ADAM12 were engineered to contain a C-terminal c-Myc tag for a common detection with

anti-c-Myc antibody (Fig. 3.6). 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 (Fig. 3.6a). After comparing the amounts of the 29-kDa fragment of Dll1 and the amounts of mature, catalytically active forms of each ADAM, we concluded that the cleavage of Dll1 occurred with the following potency order: ADAM17 >ADAM12 >ADAM9. Interestingly, all ADAM proteins used in this study formed stable complexes with Dll1, as assessed by co-immunoprecipitation with anti-c-Myc antibody (Fig. 3.6b). Thus, ADAM15 interacted with Dll1 but was unable to cleave it. Importantly, ADAM15 contains a consensus sequence of the catalytic site of zinc-dependent metalloproteases and appears catalytically active (Martin *et al.*, 2002).

To better understand molecular features that are required for Dll1 cleavage, we compared the sequences of the catalytic site of Dll1-processing ADAMs and of ADAM15. In addition to the newly identified Dll1-processing enzymes ADAM9, -12, and -17, we included the sequence of the catalytic site of ADAM10, a well established Dll1 cutter (Six *et al.*, 2003; LaVoie & Selkoe, 2003). The consensus motif for ADAM proteases is HEXXHXXGXXH (Kopan *et al.*, 1994), with three His residues binding a zinc ion at the active site and Glu being the catalytic residue (Fig. 3.7a). We observed that all Dll1-cleaving ADAMs contained an Asn after the second His. In contrast, ADAM15 contained a Ser (Ser-354) at this position. We thus asked whether the identity of the amino acid following the second His was important for Dll1 cleavage. We generated two mutants, N353S ADAM12 and S354N ADAM15. When expressed in COS-7 cells, both mutants were processed correctly, and both their nascent and mature forms lacking the pro-domains were detected (Fig. 3.7b). Although the N353S ADAM12 mutant completely lost its ability to process Dll1, the S354N ADAM15 mutant did not gain catalytic activity towards Dll1 (Fig. 3.7b). The lack of Dll1 processing by N353S ADAM12 was not due to its lost capacity to interact with Dll1, as demonstrated in the co-immunoprecipitation experiment (Fig. 3.7c). We thus conclude that Asn-353 in ADAM12 is necessary for Dll1 cleavage, but the presence of Asn at the corresponding position in ADAM15 is not sufficient for the cleavage. Fig. 3.7d shows a predicted structure of the metalloprotease domain of ADAM12, obtained by homology modeling using the structure of

ADAM33 metalloprotease as a template. It is apparent that the side chain of Asn-353 faces the catalytic cleft, where it might participate in the interactions with Dll1.

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. 3.8a) or by immunoprecipitation of FL Dll1 and CTF Dll1 from ^{35}S-labeled cells (Fig. 3.8b). The level of Dll1 cleavage mediated by endogenous proteases in MEFs was significantly lower than the cleavage observed in COS-7 overexpressing ADAM9, -12, or- 17 (compare Fig. 3.8a and Fig. 3.6a). Significant differences in the intensities of the bands corresponding to FL Dll1 and CTF Dll1 in Fig. 3.8a precluded an accurate quantification of the extent of cleavage (the CTF Dll1/FL Dll1 ratio). These differences were even more pronounced in Fig. 3.8b 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. 3.8a and 3.8b). Because ADAM15 is not capable of cleaving Dll1 (Fig. 3.6a), this result suggested that ADAM9 and/or ADAM12 contributed to Dll1 processing in wt-MEFs.

Activation of the Notch pathway inhibits myogenic differentiation *in vitro* and *in vivo* (Kopan *et al.*, 1994; Shawber *et al.*, 1996; Nofziger *et al.*, 1999; Kuroda *et al.*, 1999; Delfini *et al.*, 2000), suggesting that this pathway may act to maintain the myogenic cells in an undifferentiated proliferating state (Conboy & Rando, 2002; Luo *et al.*, 2005; Kitzmann *et al.*, 2006; Shinin *et al.*, 2006). Here, we asked whether the endogenous Dll1 present in myogenic cells interacts with ADAM12, whether it undergoes proteolytic processing, and what role ADAM12 might have in this processing. To address these questions, we used mouse primary myoblasts, which express the endogenous Dll1 and ADAM12 and in which the expression of both proteins increases during the first few days after transfer to differentiation medium (Kitzmann *et al.*, 2006; Cao *et al.*, 2003; Dahlqvist *et al.*,

2003). As shown in Fig. 3.9a, the endogenous ADAM12 co-immunoprecipitated with the endogenous Dll1 from primary myoblasts. Metabolic labeling of cells with [^{35}S]methionine+cysteine followed by immunoprecipitation with anti-Dll1 antibody revealed the presence of antibody-specific bands of 90 and 29 kDa, corresponding to the FL Dll1 and CTF Dll1, respectively. Transfection of cells with three different siRNAs targeting ADAM12, but not with a control siRNA, decreased ADAM12 protein levels by ∼50-60% and reduced the amount of CTF Dll1 by more than 50% (Fig. 3.9b). Thus, we conclude that the endogenous Dll1 is subject to proteolytic processing in confluent, differentiating myoblast cultures, giving rise to the 29-kDa fragment, and that the endogenous ADAM12 contributes to this processing.

3.5 Discussion

Notch ligands undergo proteolytic processing by ADAMs, but the identities of ADAMs that can mediate the cleavage, as well as the consequences of such processing are not clear. In *Drosophila*, the best characterized Notch ligand is Delta. Co-transfection experiments in *Drosophila* S2 cells demonstrated that three ADAMs could cleave Delta: Kuzbanian (a *Drosophila* homolog of ADAM 10), Kuzbanian-like (Kul), and DTACE (a homolog of ADAM17) (Qi *et al.*, 1999; Sapir *et al.*, 2005). Interestingly, DMeltrin, a *Drosophila* homolog of ADAM12, clearly lacked the ability to process Delta (Sapir *et al.*, 2005).

Mammalian Dll1 expressed in HEK293, COS, CHO, N2a, or NIH3T3 cells was cleaved by endogenous ADAMs present in these cells (Ikeuchi & Sisodia, 2003; Six *et al.*, 2003; LaVoie & 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 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. ADAM17 is the closest relative of ADAM10, and its activity towards Dll1 has been somewhat expected (Six *et al.*, 2003). Dll1 processing in CHO cells was not affected, how-

ever, by ADAM17 inhibitors batimistat or TAPI-1, raising questions about the ability of ADAM17 to cleave Dll1. Processing of Dll1 by ADAM9, -12 or -15 has not been tested previously.

Here we show that ADAM 9, -12, and -17, but not ADAM15, when co-transfected with Dll1 into COS-7 cells, are capable of Dll1 cleavage. Although we have not determined the exact cleavage sites in Dll1, each of these ADAMs generated a CTF of the same size, suggesting that the cleavage sites were either identical or located very close to each other. In the case of ADAM12, we provided evidence that Dll1 processing was accompanied by the release of the N-terminal fragment of Dll1 to medium, it was cell density-dependent and occurred in *cis*, and it was weakly stimulated by PMA. Furthermore, processing of Dll1 transfected into ADAM9/12/15$^{-/-}$ MEFs was diminished when compared with processing in wild type MEFs. 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 overexpressed 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 ($\sim$40%), it was consistent with $\sim$50% inhibition of Dll1 processing observed in ADAM10$^{-/-}$ MEFs (Six *et al.*, 2003) and with the remaining activity of ADAM17, which might have also contributed to the processing.

Our studies show that Asn-353 in ADAM12 is required for Dll1 cleavage. Replacement of Asn-353 with a Ser residue found in ADAM15 completely abolished ADAM12 activity toward Dll1. Consistently, other ADAMs capable of cleaving Dll1, namely ADAM9 and -17 (this study) and ADAM10 (Six *et al.*, 2003), also contain an Asn residue at the corresponding position, suggesting that this may be a general feature of Dll1-processing ADAMs. Currently, high resolution structures of metalloprotease domains are available only for ADAM17 and ADAM33. Analysis of the architecture of the catalytic site in ADAM17 and in the predicted structure of ADAM12 modeled onto the closely related ADAM33 (this work) indicates that the side chain of Asn-410 in ADAM17 and of Asn-353 in ADAM12 face the catalytic cleft and, thus, might be involved in the interaction with Dll1. Replacement of Ser-354 in ADAM15 with Asn, however, did not increase Dll1 cleavage by ADAM15, indicating that the presence of Asn at that position is not sufficient

for Dll1 processing and that other molecular events in ADAMs are required for efficient cleavage of the Dll1 substrate.

The ability of ADAM12 to cleave Dll1 and the apparent lack of activity toward Notch 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 & 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. Overexpression 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, overexpression of an ectodomain-truncated Jagged 1 in COS-7 cells together with an ectodomain-truncated Notch led to inhibition of the Notch function (LaVoie & 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. Thus, alleviation of the inhibition of Notch mediated by Dll1 appears to out-weigh 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 & Artavanis-Tsakonas, 2006). Cells that are initially equivalent but at one point develop a bias towards 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 & 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.

During myogenic differentiation *in vitro*, a pool of seemingly equivalent cells assume different fates that coincide with different levels of Notch signaling (Kitzmann *et al.*, 2006). In proliferating cells and then in cell cycle-arrested but undifferentiated "reserve cells," the Notch activity is high, whereas in differentiated myotubes the level of Notch signaling is low (Kitzmann *et al.*, 2006). Although Notch signaling is generally regulated at multiple levels, one of the most direct mechanisms involves regulation of expression and membrane localization of Notch ligands. In *Xenopus*, expression of XDelta-1 is positively regulated at the transcriptional level by MyoD (Wittenberger *et al.*, 1999). In *Drosophila*, expression of Delta has been recently shown to be negatively regulated by dmiR-1 (Kwon *et al.*, 2005), a muscle-specific microRNA (Rao *et al.*, 2006). Our results suggest that in mouse myogenic cells, the Dll1 protein may be additionally regulated at the post-translational level by cell surface proteolysis and that ADAM12 is one of the ADAM proteases mediating this processing (Fig. 3.9). Interestingly, gene profiling experiments suggest that several ADAMs capable of cleaving Dll1, including ADAM9, -10, and -12, are strongly up-regulated during muscle regeneration *in vivo* ((Zhao & Hoffman, 2004); data are available at the Children's National Medical Center web site), and several reports suggested a role for ADAM12 in myogenesis (Yagami-Hiromasa *et al.*, 1995; Kurisaki *et al.*, 2003). Whether Dll1 processing by ADAMs indeed represents a mechanism of regulation of Notch signaling during myogenic differentiation associated with muscle regeneration remains to be determined.

Acknowledgements

We thank Drs. Raphael Kopan for the Notch1 plasmid and Diane Hayward and Paul D. Ling for the CBF1 reporters. We also thank Dr. Elena Tasheva for help in studying cleavage of Notch1.

Footnotes

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Abbreviation list

Dll1, Delta-like 1; ICD, intracellular domain of Dll1; PMA, phorbol myristate acetate; IP, immunoprecipitate; siRNA, small interfering RNA; MEF, mouse embryonic fibroblast; DMEM, Dulbeccos modified Eagles medium; CTF, C-terminal fragment; NTF, N-terminal fragment; CHO, Chinese hamster ovary; wt, wild type; WB, Western blot.

ADAM12 enhances TGFβ signaling in Dll1-dependent manner (Not included in the *J Biol. Chem.* paper)

Our previous study showed that ADAM12 is strongly up-regulated by TGFβ1 at protein and mRNA level in mouse embryonic fibroblasts (MEFs), NIH3T3 fibroblasts, and NMuMG epithelial cells (Chapter 2). ADAM12 cleaves Dll1 which is followed by cleavage of Dll1 by γ-secretase and release of the ICD of Dll1 from the membrane. ICD of Dll1 can interact with Smad2, Smad3, and Smad4 and enhance Smad-dependent transcription in response to stimulation of cells with TGFβ1 (Hiratochi *et al.*, 2007) (Fig. 3.10a). To test whether ADAM12 is able, through cleavage of Dll1, to enhance TGFβ signaling we co-transfected ADAM9,12,15$^{-/-}$ MEFs with Dll1 and ADAM12. We monitored the response to TGFβ1 stimulation with p3TP-Lux reporter, before and after TGFβ1 treatment (Fig. 3.10b). We noticed that Dll1 did not increase cellular responses to TGFβ1 in the absence of ADAM12. In contrast, when cells where co-transfected with wild type ADAM12 and Dll1, Dll1 stimulated TGFβ1 responses in a dose-dependent manner. This effect was not observed when cells were transfected with catalitycally inactive ADAM12. Thus, ADAM12-mediated cleavage of Dll1 increases cell responsiveness to TGFβ1.

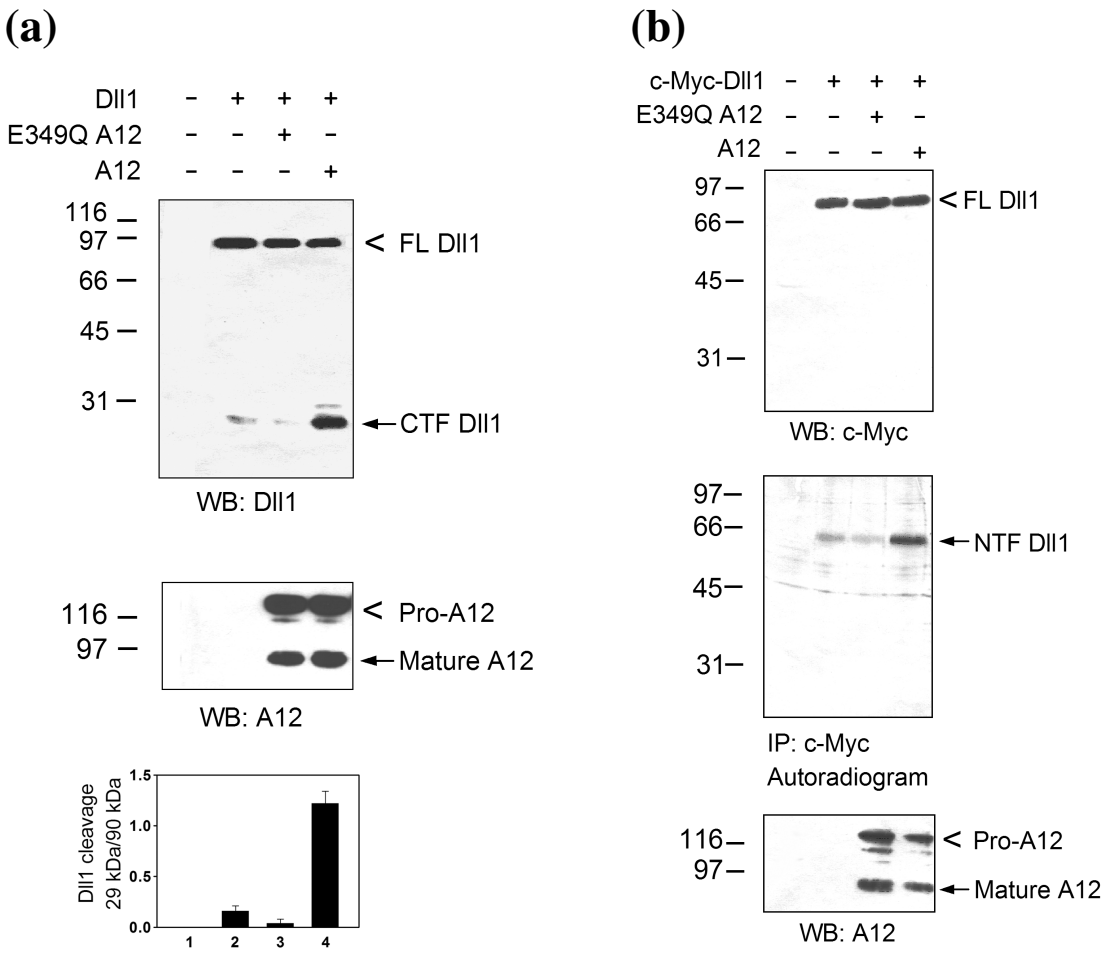

Figure 3.1: **ADAM12 cleaves Dll1 in intact cells.** (a), COS-7 cells were transiently transfected with two empty vectors (lane 1), with mouse Delta-like 1 and empty ADAM12 expression vector (Dll1; lane 2), co-transfected with Dll1 and catalytically inactive mutant form of ADAM12 (E349Q A12; lane 3), or cotransfected with Dll1 and wild type ADAM12 (A12; lane 4). Total cell lysates were analyzed by Western blotting using antibody specific for the C terminus of Dll1 (top panel) or for the C-terminal domain of ADAM12 (middle panel). Full-length (FL, 90-kDa band) and the C-terminal fragment (CTF, 29-kDa band) of Dll1 are indicated; the nascent pro-form of ADAM12 and the mature form of ADAM12 lacking the pro-domain are shown. The extent of Dll1 cleavage was determined as the ratio of intensities of the 29- and 90-kDa Dll1 bands (mean +/- S.E., n = 5, bottom panel). (b) cells were transfected as in (a); Dll1 construct contained c-Myc tag in the N-terminal domain. Cells were metabolically labeled with [^{32}S]methionine+cysteine, cell lysates were analyzed by Western Blotting using anti-c-Myc antibody (top) or anti-ADAM12 antibody (bottom), and the NTF of Dll1 was immunoprecipitated from medium using anti-c-Myc antibody and visualized by SDS-PAGE and autoradiography (middle). Experiment in (a) was performed by Dr. Haiqing Yi.

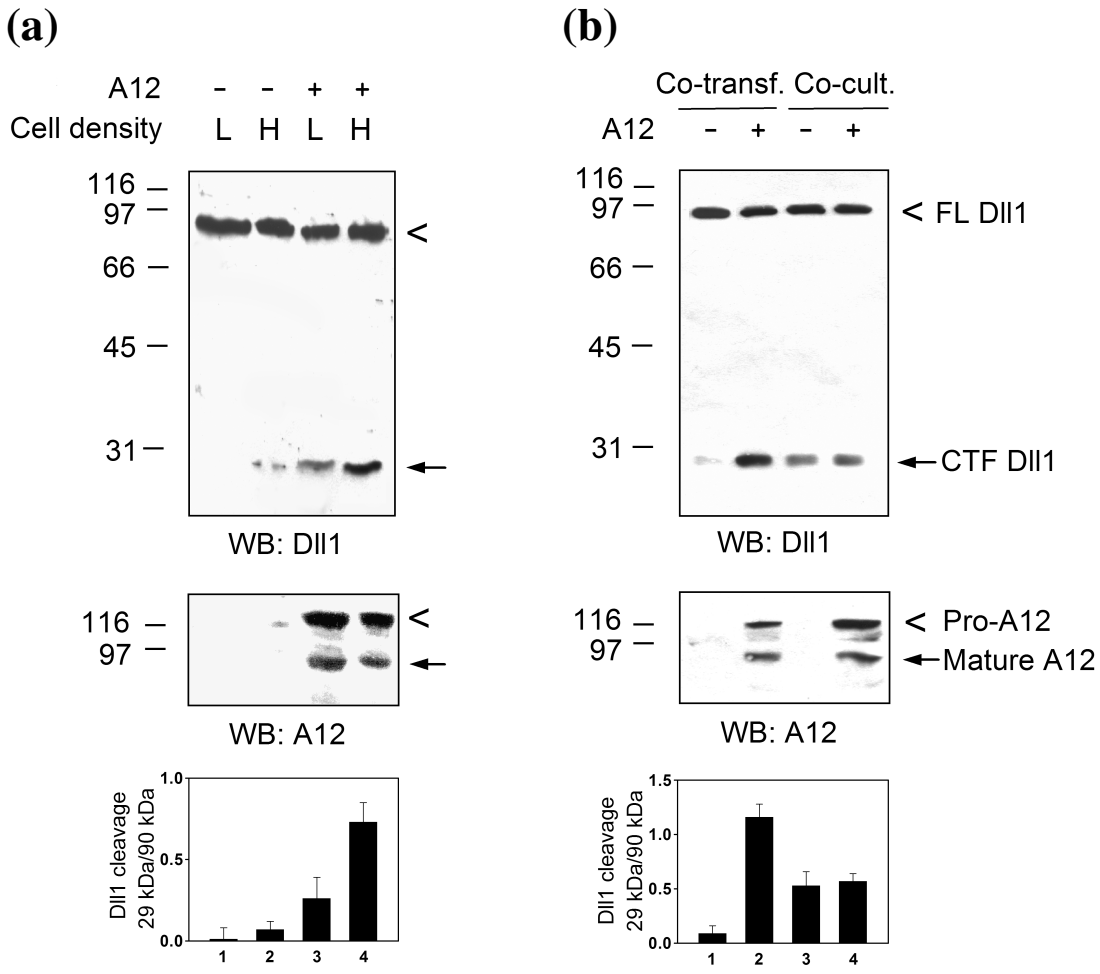

Figure 3.2: **Dll1 cleavage by ADAM12 is cell density-dependent and occurs in *cis*.** (a) COS-7 cells were co-transfected with Dll1 and either empty ADAM12 expression vector (lanes 1 and 2) or with vector containing ADAM12 cDNA (lanes 3 and 4). Cells were ∼50% confluent (low density (L)) or ∼90% confluent (high density (H)) at the time of harvesting. (b) COS-7 cells (∼90% confluent) were co-transfected with Dll1 and empty vector (lane 1) or Dll1 and ADAM12 (lane 2). Alternatively, cells (∼50% confluent) were transfected with Dll1 alone and co-cultured with vector-transfected (lane 3) or ADAM12-transfected cells (lane 4). In (a) and (b), total cell lysates were analyzed by Western blotting using antibodies specific for the C termini of Dll1 or ADAM12 (top and middle panels, respectively). The extent of Dll1 cleavage was determined as the ratio of intensities of the 29- and 90-kDa Dll1 bands (mean +/- S.E., n = 3, bottom). Experiments in Fig. 3.2 were performed by Dr. Haiqing Yi.

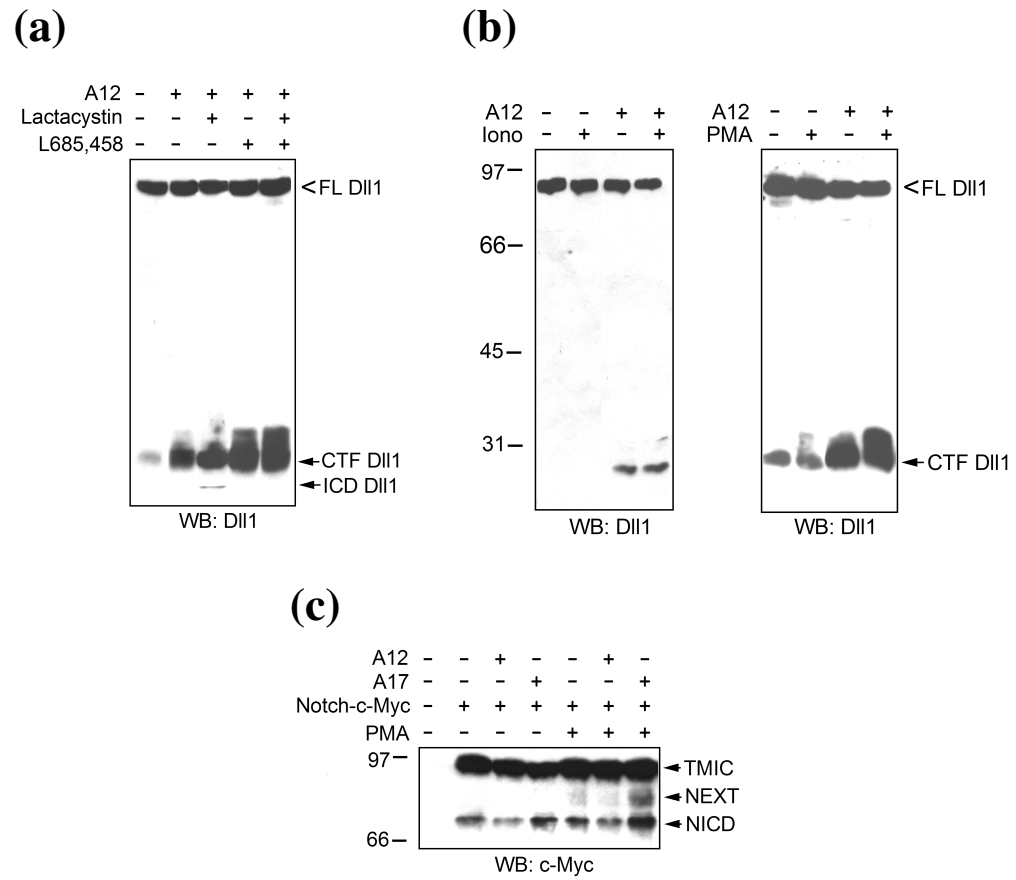

Figure 3.3: **Dll1 cleavage by ADAM12 is followed by γ-secretase cleavage (a), is not stimulated by ionomycin, and is weakly enhanced by PMA (b).** (a), COS-7 cells co-transfected with Dll1 and empty vector (lane 1) or ADAM12 (lanes 2-5) were incubated with for 3 h with 5 μM lactacystin (a proteasomal inhibitor, lanes 3 and 5) or for 6 h with 1 μM L685,458 (a γ-secretase inhibitor, lanes 4 and 5). Cell lysates were analyzed by Western blotting using anti-Dll1 antibody. ICD, intracellular domain of Dll1, is the product of γ-secretase cleavage of CTF. (b), COS-7 cells were co-transfected with Dll1 and either empty vector (lanes 1 and 2) or ADAM12 (lanes 3 and 4). Left, Twenty four hours after transfection, cells were incubated for 5 h with 5 μM L685,458 and for additional 1 h with or without 5 μM ionomycin (left) or 25 ng/ml of PMA, as indicated (right), followed by analysis of Dll1 cleavage. Cells were serum-starved for 30 min prior to and during the PMA treatment. (c) evidence for a role of ADAM17, but not ADAM12, in cleaving Notch1. COS-7 cells were transfected with Notch1 containing C-terminal c-Myc tag (lanes 2-7, Notch-c-Myc) and either empty vector (lanes 1, 2, and 5), ADAM12 (lanes 3 and 6), or ADAM17 (lanes 4 and 7). Two days after transfection, confluent cells were serum-starved for 30 min and then incubated for 1 h without (lanes 1-4) or with 25 ng/ml PMA (lanes 5-7) followed by analysis of Notch cleavage by Western blotting using anti-c-Myc antibody. TMIC, the S1 cleavage product; NEXT, Notch extracellular truncation, the S2 cleavage product; NICD, Notch intracellular domain, the S3 cleavage product. Experiment in (c) was performed by Dr. Elena Tasheva.

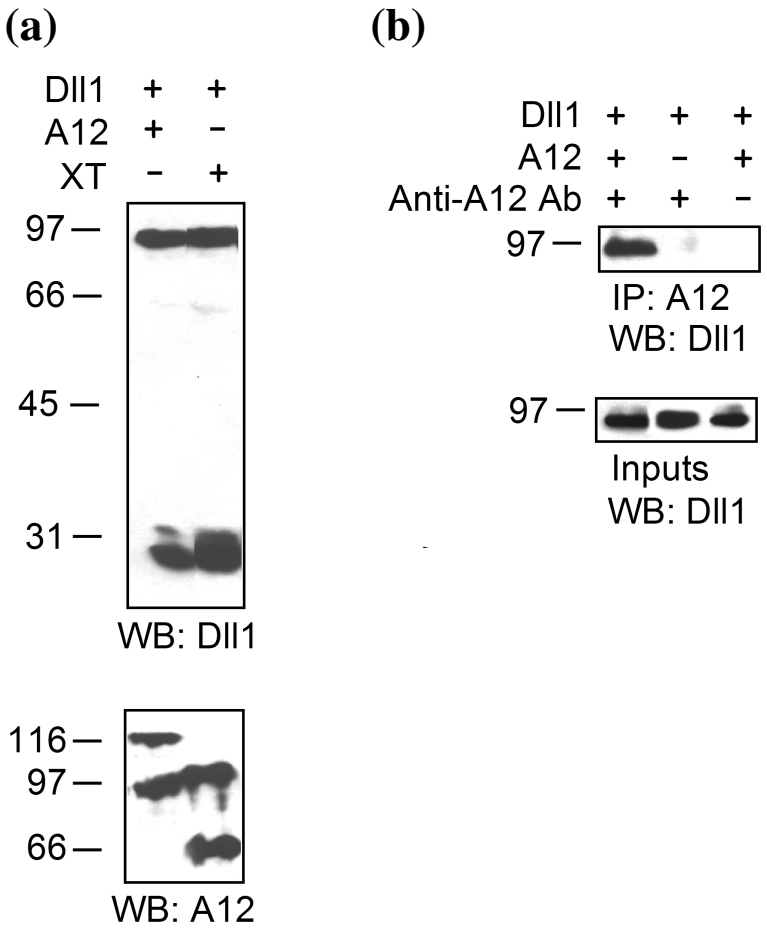

Figure 3.4: **ADAM12 forms a complex with the full length Dll1, but not with NTF or CTF**. (a) cytoplasmic domain of ADAM12 is dispensable for Dll1 cleavage. COS-7 cells were co-transfected with Dll1 and either the full-length ADAM12 (lane 1) or ADAM12 containing a 169-amino acid deletion at the C terminus (XT; lane 2). Total cell lysates were analyzed by Western blotting using antibody specific for the C terminus of Dll1 (top) or the disintegrin domain of ADAM12 (bottom). The upper bands detected with anti-ADAM12 antibody in ADAM12- and XT-transfected cells represent the nascent forms of ADAM12 or XT, respectively; the lower bands represent the mature forms lacking the prodomain. ADAM12 forms a complex with the full-length Dll1 but not with NTF or CTF. (b), Cells were co-transfected with Dll1 and ADAM12 (lanes 1 and 3) or with Dll1 and empty ADAM12 expression vector (lane 2), cell extracts were immunoprecipitated with antibody specific for the C-terminus of ADAM12 (lanes 1 and 2) or were incubated without antibody (lane 3), the immunoprecipitates (IPs) and inputs were analyzed by Western blotting using anti-Dll1 antibody. Experiment in (a) was performed by Dr. Haiqing Yi.

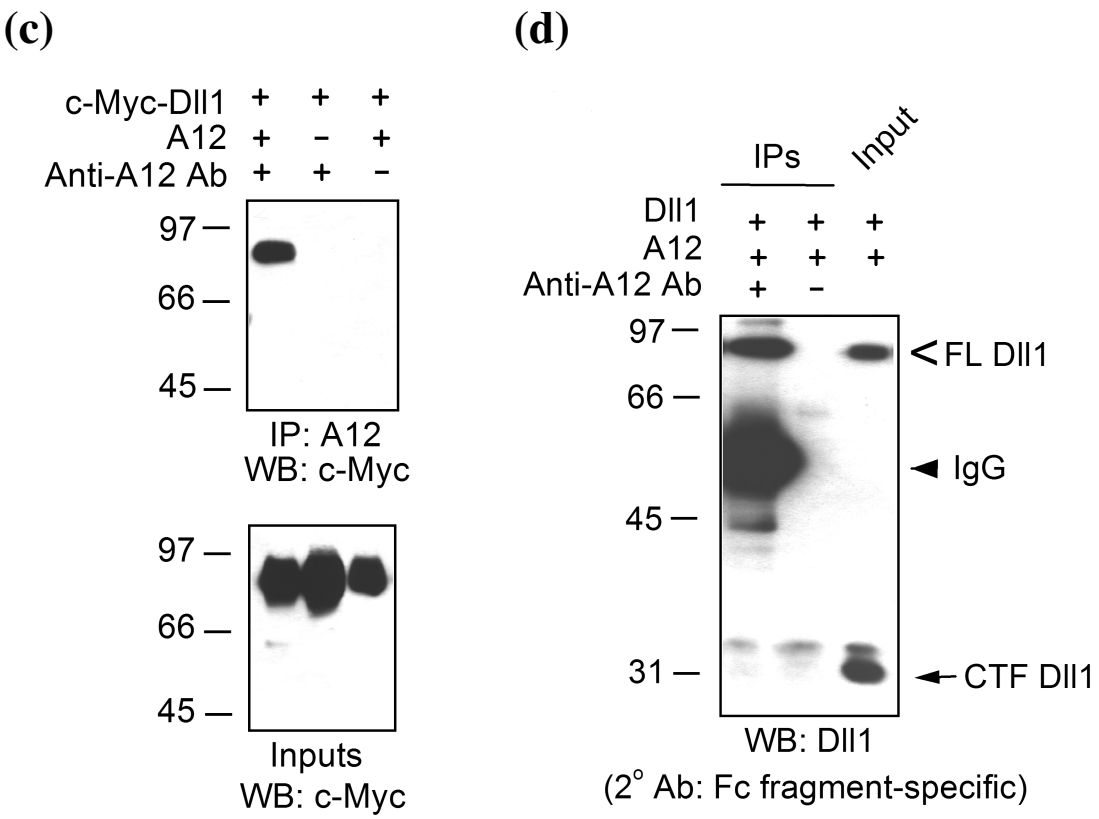

Figure 3.4: **ADAM12 forms a complex with the full length Dll1, but not with NTF or CTF (continued)**. (c), Experiment was performed as in (b) using the Dll1 construct with c-Myc tag in the N-terminal domain. The immunoprecipitates (IPs) and inputs were analyzed by Western blotting using anti-c-Myc antibody. (d), COS-7 cells were co-transfected with Dll1 and ADAM12, cell extract was immunoprecipitated with (lane 1) or without (lane 2) anti-ADAM12 antibody; immunoprecipitates (IPs) and input were analyzed by immunoblotting with anti-Dll1 antibody. The secondary antibody was anti-rabbit IgG, Fc fragment-specific, and detected only the heavy chain of anti-ADAM12 antibody used for immunoprecipitation. Notice that the relative abundance of CTF Dll1 was much higher in the input than in the immunoprecipitate.

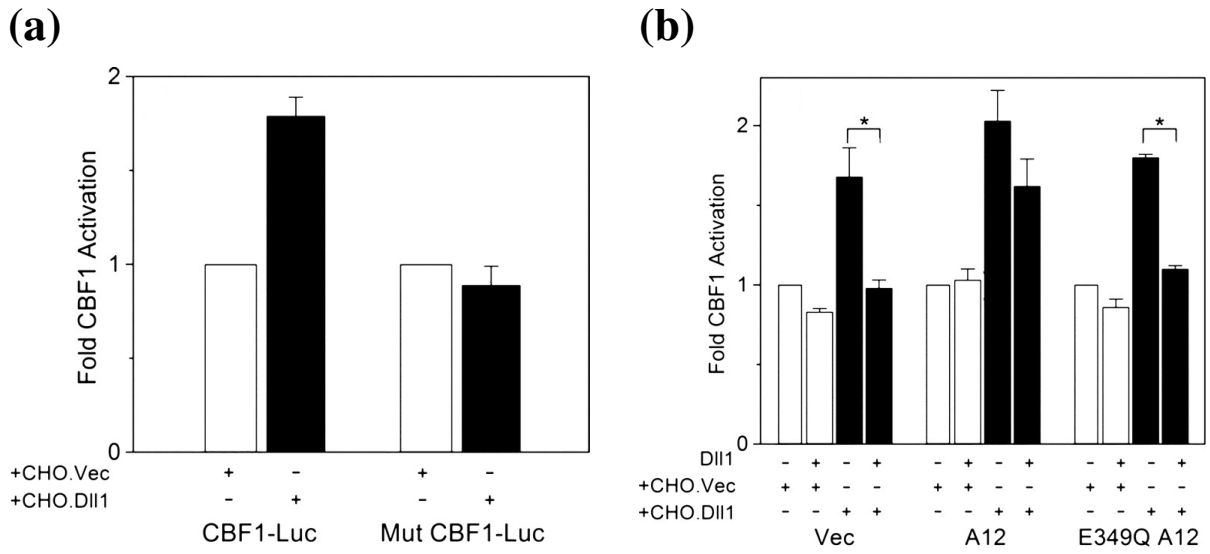

Figure 3.5: **Proteolytic processing of Dll1 by ADAM12 increases Notch signaling in a cell-autonomous manner** (a) NIH3T3 cells were co-transfected with mouse Notch1 and either a CBF1 reporter (CBF1-Luc; pJH23A) or the same reporter in which CBF1 binding sites were mutated (Mut CBF1-Luc; pJH25A). After 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). Experiments in Fig. 3.5 were performed by Dr. Danqiong Sun.

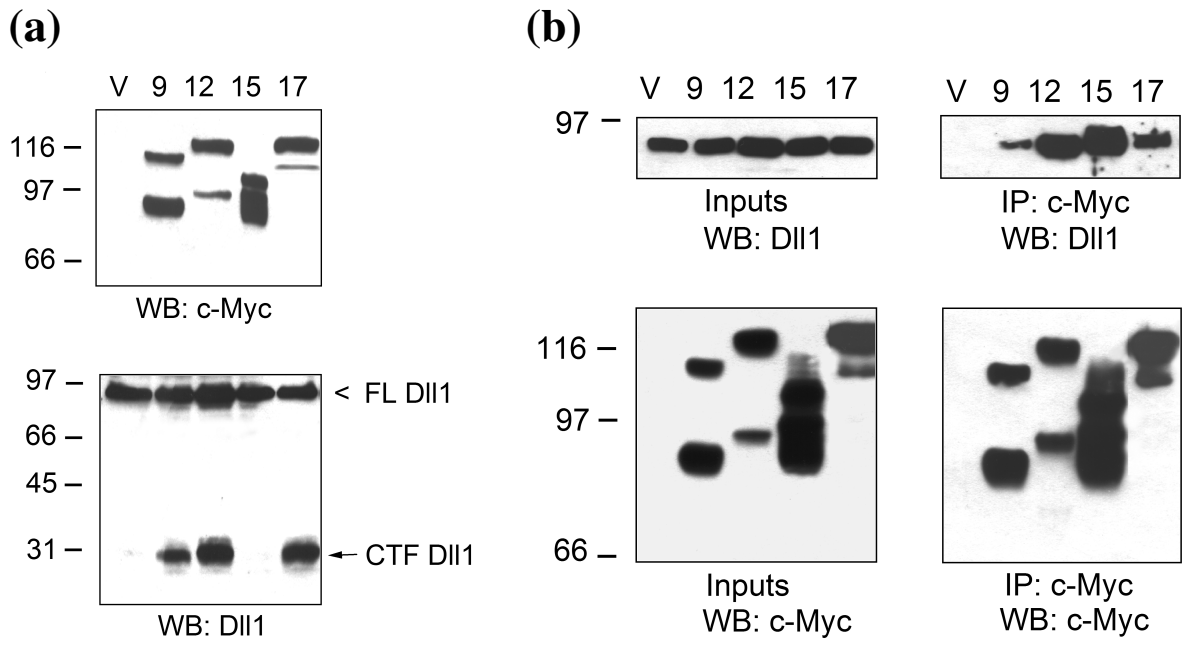

Figure 3.6: **Dll1 is a substrate for ADAM9, -12, and -17 but not for ADAM15.** (a) COS-7 cells were co-transfected with Dll1 and empty vector (V), c-Myc-tagged ADAM9, -12, -15, or -17. Cell extracts were analyzed by Western blotting using anti-c-Myc tag antibody (top) or anti-Dll1 antibody (bottom). Upper bands detected with anti-c-Myc antibody represent nascent full-length ADAM proteins, and lower bands correspond to the catalytically active forms lacking pro-domains. (b) COS-7 cells were co-transfected with Dll1 and empty vector, c-Myc-tagged ADAM9, -12, -15, or -17. ADAM proteins were immunoprecipitated with anti-c-Myc tag antibody. The inputs (left) and immunoprecipitates (right) were analyzed by anti-Dll1 (upper panels) and anti-c-Myc (lower panels) antibodies. Experiments in Fig. 3.6 were performed by Dr. Haiqing Yi.

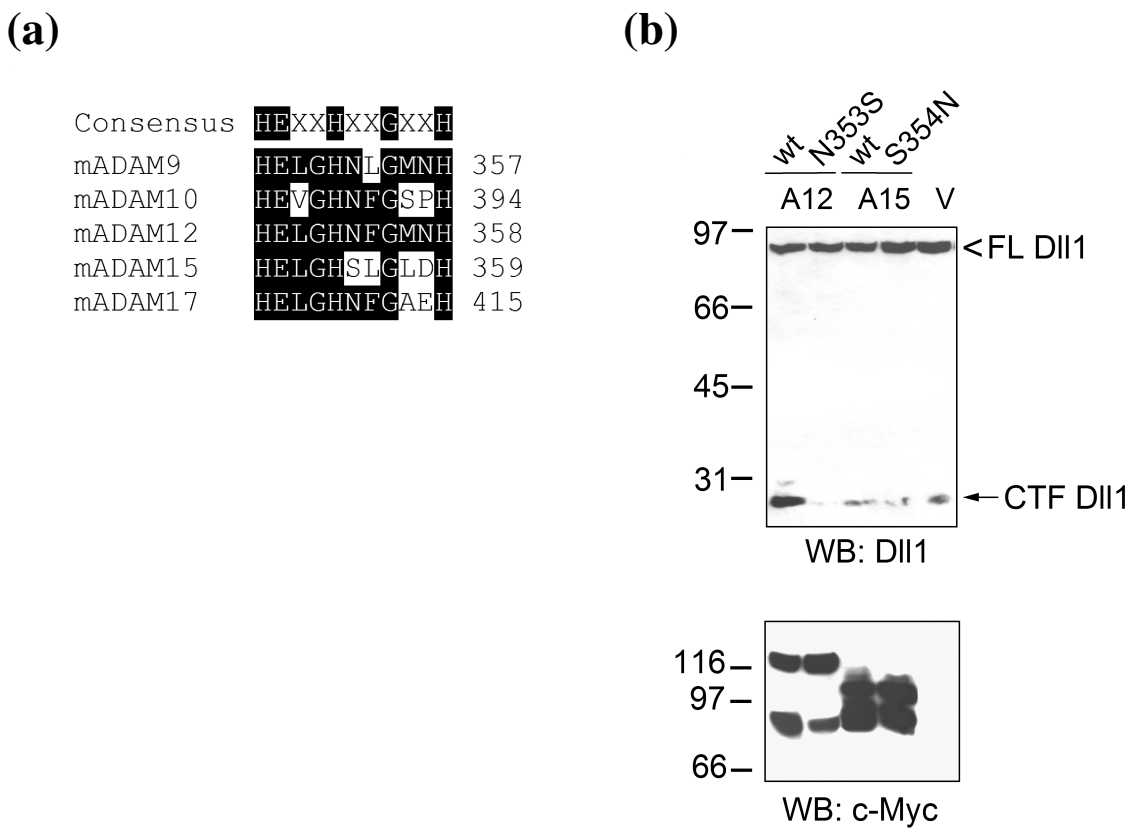

Figure 3.7: **Asn353 in ADAM12 is necessary for Dll1 cleavage.** (a), Amino acid sequence alignment of the catalytic sites of mouse ADAM9, 10, 12, 15, and 17. The consensus sequence of the catalytic site of ADAM proteases is shown on the top. (b), COS-7 cells were co-transfected with Dll1 and either wild-type or the N353S ADAM12 mutant, wild-type or S354N ADAM15 mutant, or empty vector. All ADAM constructs contained a C-terminal c-Myc tag. Cell extracts were analyzed by Western blotting using anti-Dll1 antibody (upper panel) or anti-c-Myc antibody (bottom panel).

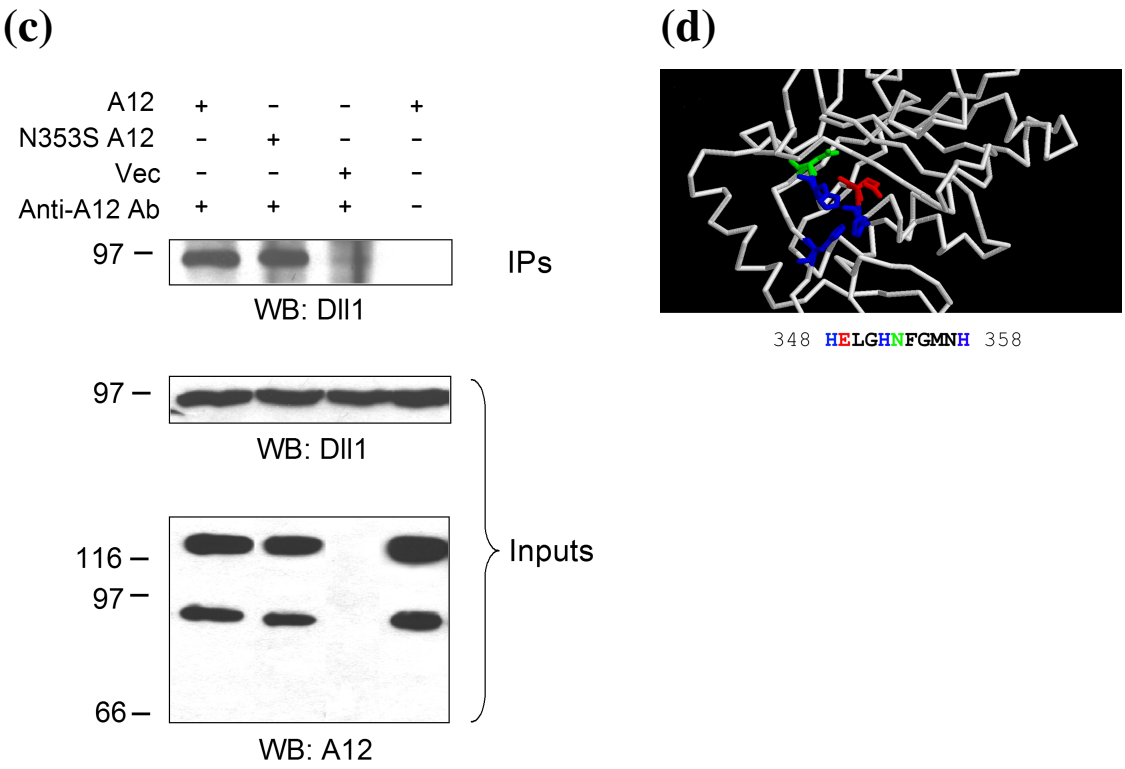

Figure 3.7: **Asn353 in ADAM12 is necessary for Dll1 cleavage (continued).** (c), COS-7 cells were co-transfected with Dll1 and wild-type ADAM12, the N353S mutant of ADAM12, or empty vector, as indicated. Cell extracts were immunoprecipitated using anti-ADAM12 antibody (lanes 1-3) or were incubated without antibody (lane 4). Immunoprecipitates (IPs) and inputs were analyzed by Western blotting with anti-Dll1 antibody, the expression of ADAM12 was verified by Western blotting of the inputs with anti-ADAM12 antibody. (d), The predicted structure of the metalloprotease domain of ADAM12, obtained by threading mouse ADAM12 sequence (amino acids 212-414) onto mouse ADAM33 (PDB structure 1R55) using the Swiss-PdbViewer software, and then submitted to the SWISS-MODEL server for refinement. Residues 339-358 are displayed as a cartoon, side chains of His348, His352, and His 358 coordinating Zn^{2+} are shown in blue, catalytic E349 is shown in red, and N353 is depicted in green.

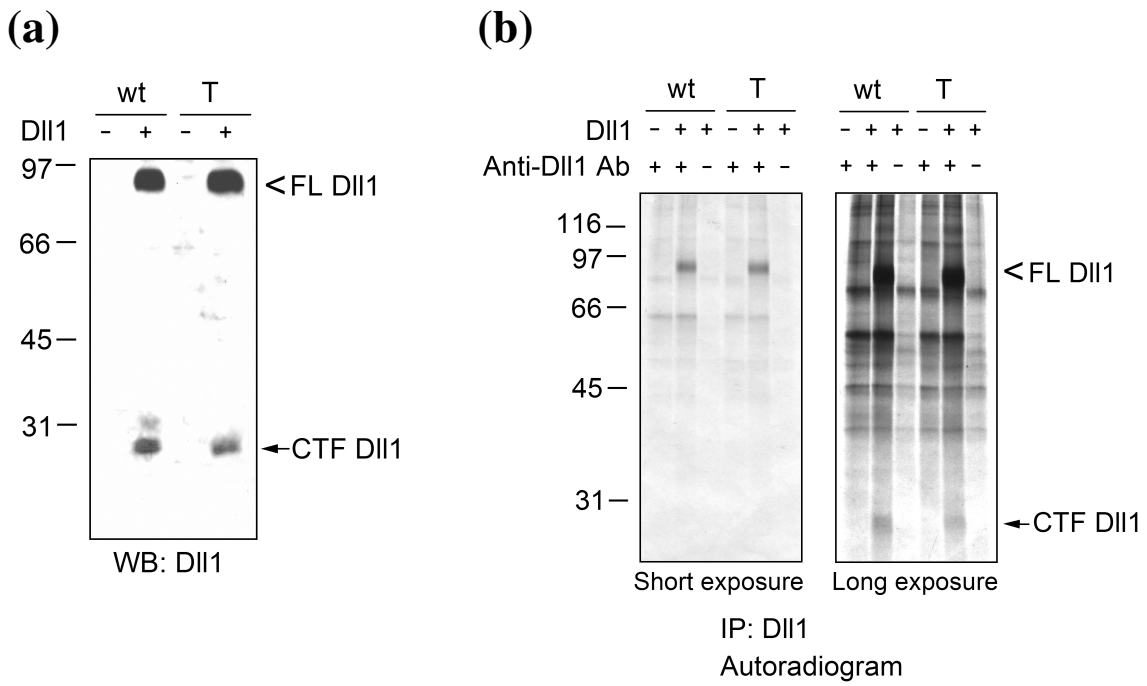

Figure 3.8: **Endogenous ADAM9 and/or ADAM12 contribute to Dll1 cleavage in MEFs.** SV40-immortalized MEFs isolated from wild type mice (wt) or from ADAM9/12/15$^{-/-}$ triple knock-out mice (T) were transiently transfected to express Dll1 as indicated. (a) after 48 h, Dll1 processing was analyzed by 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. Experiments in Fig. 3.8 were performed by Dr. Danqiong Sun.

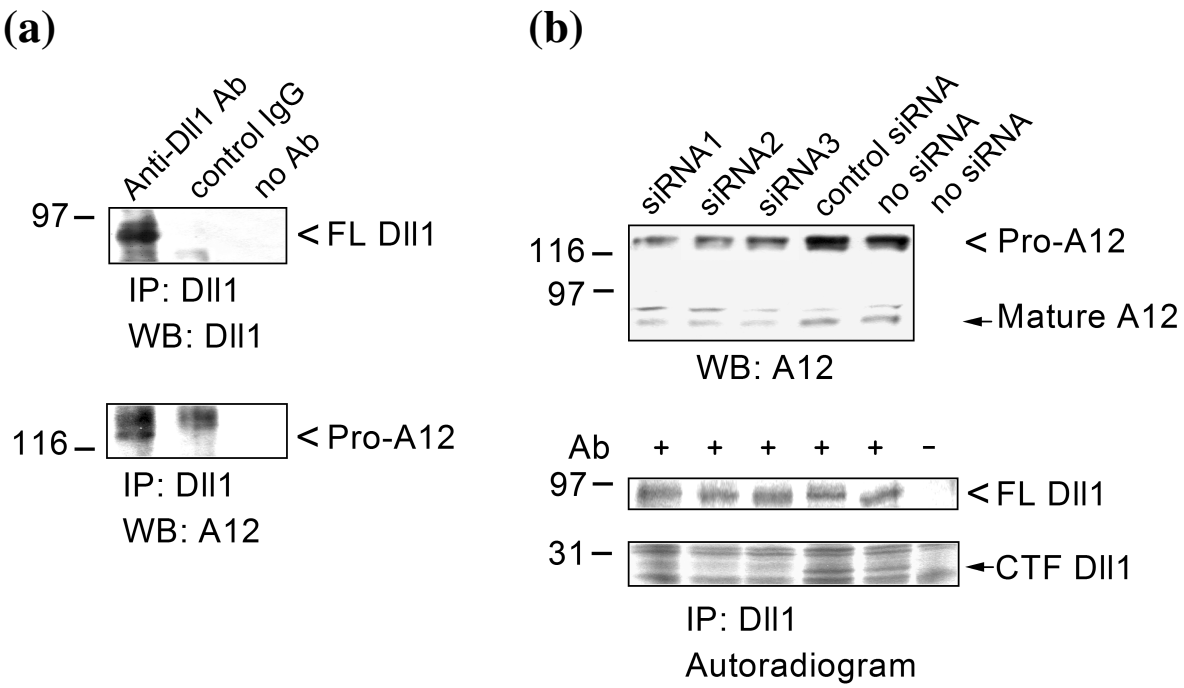

Figure 3.9: **Endogenous ADAM12 co-immunoprecipitates with Dll1 and contributes to Dll1 cleavage in primary mouse myoblasts.** (a) primary mouse myoblasts grown to confluence and incubated for 1 day in differentiation medium were subjected to immunoprecipitation with anti-ADAM12 antibody. The immunoprecipitates were analyzed by immunoblotting with anti-ADAM12 (top) or anti-Dll1 antibody (bottom). (b) primary mouse myoblasts were transfected with one of three ADAM12 siRNAs or control siRNA, as described under "Experimental Procedures." One day after transfection, cells reached confluence and were transferred to differentiation medium. After 24 h, cells were metabolically labeled with [^{35}S]methionine+cysteine for 10 h, 1 μM γ-secretase inhibitor L685,458 was added, and the labeling was continued for next 6 h. The level of endogenous ADAM12 was analyzed by Western blotting after partial purification on concanavalin A-agarose (bottom). In parallel, the endogenous Dll1 was immunoprecipitated with anti-Dll1 antibody (Ab), and the immunoprecipitates were analyzed by SDS-PAGE and direct autoradiography (top). The exposure time of the region in the autoradiogram containing the FL Dll1 was five times longer that the exposure time of the region containing CTF Dll1. Notice that the abundance of CTF Dll1 is diminished in cells transfected with ADAM12 siRNAs.

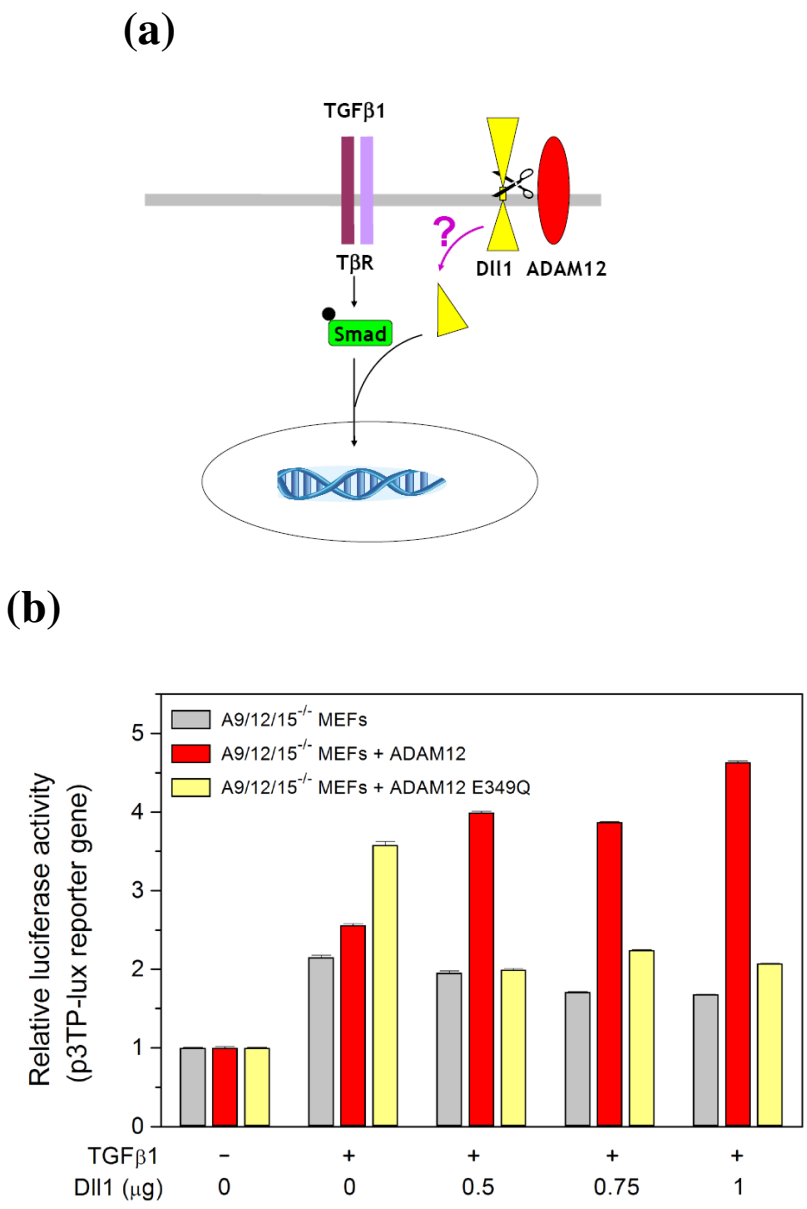

Figure 3.10: **ADAM12 enhances TGFβ1 signaling in Dll1-dependent manner.** (a) Schematic illustration of a possible cross-talk between TGFβ1 signaling and ADAM12 involving proteolytic cleavage of Dll1. (b) A9/12/15$^{-/-}$ MEFs cells were co-transfected with TGFβ1 signaling reporter (p3TPlux), wild type ADAM12 (red bars), catalytically inactive ADAM12 (ADAM12 E349Q, yellow bars), or empty vector (grey bars), and increasing amounts of Dll1. After 24 h, cells were treated with 2 ng/mL TGFβ1 or vehicle, followed by measurement of luciferase activity. Cells co-transfected with wild type ADAM12 and Dll1 show increasing TGFβ1 signaling in a dose dependent manner. This effect is not present in cells co-transfected with catalytically inactive ADAM12. Error bars represent standard error of the mean (n=3).

Chapter 4

Breast cancer-associated mutations in metalloprotease disintegrin ADAM12 interfere with the intracellular trafficking and processing of the protein

The data presented in this chapter have been published in the following journal article:

Dyczynska E., Syta E., Sun D., Zolkiewska A.

Breast cancer-associated mutations in metalloprotease disintegrin ADAM12 interfere with intracellular trafficking and processing of the protein

Int J. Cancer 122:2634-2640 (2008)

Available at

http://www3.interscience.wiley.com/cgi-bin/fulltext/117905136/PDFSTART.

Chapter 5

Summary

Members of the ADAM family can influence multiple cellular processes involved in normal development, as well as in pathogenesis. Better understanding of how ADAM12 is regulated and which signaling pathways and proteins are involved in this regulation sheds more light on proper and abnormal functions of ADAM12. Additionally, finding new substrates for this metalloprotease can expand our knowledge of processes in which ADAM12 is involved.

The TGFβ signaling pathway play an important role in many pathological processes where ADAM12 expression is dysregulated. It was shown in Chapter 2 that TGFβ upregulates ADAM12 through degradation of the SnoN repressor. Changes in the expression level of ADAM12 and SnoN, as well as increased levels of TGFβ were shown in many cancers, cardiac hypertrophy, and liver regeneration (Asakura *et al.*, 2002; Tan *et al.*, 2006; Massagué, 2008; Verrecchia & Mauviel, 2007), suggesting interplay of these factors.

Additionally, Smad2/3 have been shown to interact with E3-ubiquitin ligases and target SnoN for ubiquination and proteosomal degradation (Bonni *et al.*, 2001; Stroschein *et al.*, 2001; Levy *et al.*, 2007; Nagano *et al.*, 2007). Interestingly, Smad3-regulated proteosomal degradation is a downstream signaling event of Smad3 and can serve as a mechanism for Smad3 to cross-talk with other SnoN-dependent signaling pathways. Smad3 has been shown to interact with NICD, the intracellular domain of Notch (Blokzijl *et al.*, 2003). Our unpublished data also suggest that NICD is able to upregulate the ADAM12 gene. Whether or not this upregulation is due to degradation

of SnoN protein and releasing SnoN-mediated repression needs to be further investigated.

The Notch signaling pathway plays a crucial role in cell fate decision during embryonic development and in adulthood. Small differences in the levels of Notch ligands and receptors are amplified, and, in consequence, divide the cell population to two groups: signal-receiving and signal-sending.

Here, we expanded the list of ADAMs involved in Notch ligand cleavage. Our results presented in Chapter 3 show that ADAM12 is capable of proteolytic cleavage of Notch ligand, Dll1. Processing of Dll1 by ADAMs has three known functions: down-regulation of Notch signaling in neighboring cells, activation of Notch signaling in a cell-autonomous manner, and cross-talk with different signaling pathways through the interaction of intracellular domain (ICD) of Dll1 with transcription factors.

ADAM12 is able to modulate Notch signaling through the shedding of the Dll1 extracellular domain and activation of Notch in a cell-autonomous manner. During myogenic differentiation, cells can differentiate or stay as non-differentiate progenitor cells. Activation of Notch signaling inhibits myogenic differentiation and also functions as a positive regulator of progenitor cells (Kitzmann *et al.*, 2006). Sun *et al.* (2008) have shown that proteolytic processing of Dll1 helps achieve asymmetry in Notch signaling and this helps sustain the balance between differentiation and maintenance of undifferentiated cells.

The Notch signaling pathway plays also key roles in breast cancer progression. Adult mammary glands contain mammary stem cells, and mutations in a population of stem cells can lead to development of breast cancer (Wicha *et al.*, 2006). The Notch signaling pathway is critical for maintaining the population of self-renewing stem cells and facilitates their proliferation (Dontu *et al.*, 2004). ADAM12 expression is an increased in mammary stem cells (Dontu *et al.*, 2003), suggesting that ADAM12 can play a similar role in maintaining balance between stem cells and progenitor cells as in myogenic differentiation.

Additionally, the ICD of Dll1 was shown to translocate to the nucleus where it interacts with different transcriptional factors and cross-talks with different signaling pathways. Documented

partners for the ICD of Dll1 are Smad2/3/4. This interaction leads to an enhanced Smad-dependent signaling response to TGFβ (Hiratochi *et al.*, 2007). The ICD of Dll1 is generated by consecutive cleavage by an ADAM protease and γ-secretase. We tested whether catalytically active ADAM12 can increase TGFβ signaling through processing of Dll1 (Fig. 3.10b). ADAM12-mediated cleavage of Dll1 increased cell responsiveness to TGFβ. As shown in Chapter 2, TGFβ upregulates ADAM12, suggesting a positive feedback loop between TGFβ signaling and ADAM12 through Dll1. This situation could take place during cardiac development when Dll1 is strongly upregulated in endothelial cells, following arterial injury (Miceli-Libby *et al.*, 2008), and during ischemia-induced arteriogenesis (Limbourg *et al.*, 2007). When stimulated with TGFβ, cardiac endothelial cells promote endothelial-to-mesenchymal transition (EndMT) and contribute to accumulation of fibroblasts (Zeisberg *et al.*, 2007) which in the long term leads to cardiac fibrosis and heart failure. Thus, ADAM12 together with TGFβ signaling pathway, through cleavage of Dll1, can have an important role in cardiac remodeling and failure.

Chapter 4 focuses on the role of ADAM12 cancer-associated mutants. Our data show that ADAM12 cancer-associated mutations block generation of the mature, active form of ADAM12, leading to the loss of function at the plasma membrane. Only ADAM12 present at the cell surface is capable of cleaving of Dll1 and interacting with TβRII, suggesting that cancer-associated ADAM12 mutants are unable to modulate Notch and TGFβ signaling pathways. Additionally, both pathways are able to upregulate the ADAM12 protein leading to higher amount of misfolded proteins in ER. Further study on cancer-associated mutations should give more information on the role of ADAM12 proteolytic activity during cancer progression.

Because of ADAMs involvement in a variety of pathological events, they are attractive targets for novel therapies. ADAMs play redundant roles in substrate shedding, so further research on the regulation and control of individual ADAM activity is necessary to create more specific drugs. ADAM12 is an interesting protein due to its involvement in two major signaling pathways: Notch and TGFβ. ADAM12 has been shown to modulate TGFβ signaling through interaction with TβRII (Atfi *et al.*, 2007), as well as, Notch signaling through cleavage of Notch ligand, Dll1

(Chapter 3). Additionally, ADAM12 is also upregulated by TGFβ (Chapter 2) and by Notch (our unpublished data). ADAM12 is also the only member of the ADAM family to be mutated in breast cancer (Sjöblom *et al.*, 2006). Our characterization of ADAM12 cancer-associated mutations have shown loss of functional proteins at the cell surface (Chapter 4). Collectively, these results suggest that ADAM12 may be one of the most important ADAMs in regulating cancer progression. Elucidation of the precise role of ADAM12 in cancer is a large and important task for future studies.

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