TRAF6 stimulates TGFβ-induced oncogenic signal transduction in cancer cells

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It’s never easy to achieve the Best in Life, without patience and hard work.

To my family
Abstract

Prostate cancer is one of the leading causes of cancer-related deaths in men worldwide, with 10,000 new cases/year diagnosed in Sweden. In this context, there is an urgent need to identify new biomarkers to detect prostate cancer at an initial stage for earlier treatment intervention. Although how prostate cancer develops has not been fully established, the male sex hormone testosterone is a known prerequisite for prostate cancer development. High levels of transforming growth factor-β (TGFβ) are prognostically unfavorable in prostate cancer patients.

TGFβ is a multifunctional cytokine that regulates a broad range of cellular responses. TGFβ signals through either the canonical Smad or the non-Smad signaling cascade. Cancerous cells develop different strategies to evade defense mechanisms and metastasize to different parts of the body. This thesis unveils one such novel mechanism related to TGFβ signaling.

The first two articles provide evidence that TGFβ receptor type I (TβRI) is ubiquitinated by tumor necrosis factor receptor-associated factor 6 (TRAF6) and is cleaved at the ectodomain region by tumor necrosis factor alpha converting enzyme (TACE) in a protein kinase C zeta type-dependent manner. After TβRI is shed from the ectodomain, it undergoes a second cleavage by presenilin 1 (PS1), a γ-secretase catalytic subunit, which liberates the TβRI intracellular domain (TβRI-ICD) from the cell membrane. TRAF6 promotes TGFβ-dependent Lys63-linked polyubiquitination and recruitment of PS1 to the TβRI complex, and facilitates the cleavage of TβRI by PS1 to generate a TβRI-ICD. The TβRI-ICD then translocates to the nucleus, where it binds with the transcriptional co-activator p300 and regulates the transcription of pro-invasive target genes such as Snail1. Moreover, the nuclear translocated TβRI-ICD cooperates with the Notch intracellular domain (NICD), a core component in the Notch signaling pathway, to drive the expression of invasive genes. Interestingly, treatment with γ-secretase inhibitors was able to inhibit cleavage of TβRI and inhibit the TGFβ-induced oncogenic pathway in an in vivo prostate cancer xenograft model.

In the third article, we identified that Lysine 178 is the acceptor lysine in TβRI that is ubiquitinated by TRAF6. The TβRI K178R mutant was neither ubiquitinated nor translocated to the nucleus, and prevented transcriptional regulation of invasive genes in a dominant negative manner.

In the fourth article, we show that TGFβ utilizes the E3-ligase TRAF6 and the p38 mitogen-activated protein kinase to phosphorylate c-Jun. In turn, the phosphorylated c-Jun activates p21 and Snail1 in a non-canonical Smad-independent pathway, and thereby promotes invasion in cancerous cells.

In summary, we elucidate a new mechanism of TGFβ-induced oncogenic signal transduction in cancer cells in which TRAF6 plays a fundamental role. This opens a new avenue in the field of TGFβ signaling.
List of Papers

This thesis is based on the following papers:


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Populärvetenskaplig sammanfattning


Celler signalerar via olika mekanismer och signalerna överförs av specifika proteiner som är viktiga beståndsdelar i cellerna. Vissa proteiner fungerar som receptorer, de överför signaler från den extracellulära miljön till den intracellulära. Ett exempel på en sådan signalsubstans är cytokinet Transforming Growth Factor β (TGFβ), som kan fungera som en tillväxt-faktor för vissa celltyper. TGFβ stimulerar cellmembranbundna receptorer, som när de är aktiverade överför signalen till cellkärnan via Smad proteiner eller icke-Smad proteiner, som aktiverar specifika gener som svar på TGFβ.

I avhandlingen rapporteras om de molekylära mekanismer genom vilka den varvid den cellmembranbundna type I TGFβ receptor (TβRI) klyvs av proteolytiska enzymer. Den första klyvningen utförs av ett enzym som heter tumor necrosis factor alpha converting enzyme (TACE) i den extracellulära delen av TβRI. Den andra klyvningen sker via ett annat enzym; presenilin1 (PS1) som utgör en viktig beståndsdel i gamma-secretase-komplexet, i den transmembrana delen av TβRI. Detta leder till att den intracellulära delen (ICD) av TβRI, frigörs och transporteras in i cellkärnan, där den aktiverar gener som gör cancercellerna invasiva. Tumor necrosis factor receptor-associated factor 6 (TRAF6) är ett E3 ubiquitin ligas som ubiquitinerar och aktiverar PS1. TRAF6 rekryterar PS1 till TβRI- komplexet, varvid aktivt PS1 klyver TβRI. Genom att mutera TβRI och behandla prostatacancer celler in vitro med olika gamma-secretase inhibitorer (GSI), har vi kunnat hämma inhibera klyvning av
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AβP</td>
<td>Amyloid-beta peptide</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti-mullerian hormone</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitinating enzyme</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506 binding protein</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth and differentiation factor</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to the E6-AP Carboxyl Terminus</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
</tr>
<tr>
<td>iCLiP</td>
<td>Intramembrane cleaving protease</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent TGFβ binding protein</td>
</tr>
<tr>
<td>MAML</td>
<td>Mastermind-like</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MH</td>
<td>Mad homology</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NTF</td>
<td>N-terminal fragment</td>
</tr>
<tr>
<td>PC-3U</td>
<td>Prostate cancer line-3</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin1</td>
</tr>
</tbody>
</table>
PS2  Presenilin2
RING  Really Interesting New Gene
RIP  Regulated intramembrane proteolysis
SARA  Smad anchor for receptor activation
SBE  Smad binding element
siRNA  Small interfering RNA
Smurf  Smad ubiquitylation regulatory factor
SREBP  Sterol regulatory element-binding protein
SUMO  small ubiquitin-like modifier
TACE  TNFα converting enzyme
TAK1  TGFβ-activated kinase 1
TGFβ  Transforming growth factor-β
TNFα  Tumor necrosis factor alpha
TRAF  Tumor necrosis factor receptor-associated factor
TβR  TGFβ receptor
TβRI/TβRII  TGFβ receptor types I/II
UBP  ubiquitin-specific processing proteases
UCH  Ubiquitin C-terminal hydrolase
Introduction

Cells receive signals from its environment that regulate their growth, survival, differentiation and migration. Such important signals come from soluble growth factors and cytokines that bind to specific cell surface receptors. Signal transduction events are initiated when a signal is triggered by a certain stimulus; this signal is sensed by a surface-bound receptor, which communicates the signal from outside to inside the cell and leads to a change in a particular cell function. Signal transduction is a evolutionarily conserved mechanism. Disruptions, aberrations, or discrepancies in signal transduction can result in improper signal processing and transmission, which in turn may cause disease conditions such as cancer. In fact, this mechanism occupies a central role in cancer biology (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011; Weinberg, 2007).

Extracellular signals are perceived by receptors that are present on the cell surface. Most signal transduction mechanisms are primarily channeled through these cell surface receptors. The cell surface receptors are activated by stimuli (e.g., growth factors, cytokines, neurotrophins) upon ligand binding. This thesis focuses mainly on the cytokine transforming growth factor-β (TGFβ) and its downstream signaling cascades, with a primary focus on non-Smad signaling pathways. The present investigation has two aims: to unravel the molecular mechanisms involved in the cleavage of TGFβ receptor type I (TβRI) and its role in oncogenesis; and to investigate the role of the ubiquitin ligase TRAF6 in the regulation of c-Jun and p21.
1. Prostate cancer

Prostate cancer is the fifth leading cause of cancer-related death in men and the most diagnosed cancer in men (Ferlay et al., 2012; Garcia et al., 2007). In Europe, an estimated 382,000 cases occurred in 2008; the observed rates were highest in Tyrol, Austria and lowest in Serbia (Bray et al., 2010; Ferlay et al., 2010). The incidence of prostate cancer in European countries is nearly 214 cases per 1000 men (Heidenreich et al., 2011). In Sweden, prostate cancer is one of the most common cancers, and nearly 10,000 new cases are diagnosed each year. It strikes older men; approximately one-half of men diagnosed with prostate cancer are older than 70 years of age, and none are younger than 40 years of age.

Although how prostate cancer develops has not been fully established, the male sex hormone testosterone is a known prerequisite for prostate cancer. Additional factors, including environment, lifestyle, and heredity, also appear to play an important role in the development of prostate cancer. Two of the major pathways implicated in prostate cancer initiation and progression are the androgen and TGFβ pathways (Diener et al., 2010; Felgueiras et al., 2014).

One major problem with prostate cancer is that the lack of specific prognostic biomarkers makes it difficult to predict prognosis at an early stage. Prostate cancer is detected using a prostate-specific antigen (PSA) test, by palpating the prostate via the rectum, or by a histological examination of a prostate tissue sample (biopsy). Available treatment options include the surgical removal of the prostate gland, radiation, hormonal therapy, and cytotoxic drugs (chemotherapy) (Stavridis et al., 2010).

Androgen deprivation therapy, which was originated by Huggins and Hodge, is one of the most commonly used therapeutic interventions and is used as a first-line treatment for metastatic prostate cancer (Huggins, 1942; Huggins, 1943; Huggins, 1944). Chemical castration agonists,
such as gonadotrophin-releasing hormone (GnRH), which inhibits testicular androgen synthesis in combination with androgen receptor antagonists (biculatamide), are initially administered to patients to decrease androgen production (Klotz et al., 2000; Lukka et al., 2006). However, at later stages prostate cancer cells often metastasize to the lymph nodes, bone, and lungs. After 2-3 years, the recurrence of a more advanced metastatic stage of prostate cancer known as metastatic castration-resistant prostate cancer (CRPC) develops in these patients. The androgen receptors are reactivated at this stage, which results in a vicious loop in which the tumors produce their own androgens, stimulate their own androgen receptors, and maintain appropriate androgen levels to support their own growth. In turn, androgen receptors are activated by the circulating androgen receptor ligand, which promotes nuclear import of the androgen receptor 5α-dihydrotestosterone (DHT). In the nucleus, the androgen receptor dimerizes and binds to androgen responsive elements (ARE). DHT then interacts with co-activators, such as histone acetyl transferases CBP/p300 and p160, and regulates the transcription of genes such as Kallikrein 3 and probasin (Augello et al., 2014; Ni et al., 2013; Sung and Cheung, 2014).

Recent advances have provided us with a better understanding of prostate cancer. However, the lack of biomarkers that can predict prostate cancer prognosis at initial stages constitutes a large scientific and medical void that is waiting to be filled. The development of such predictive biomarkers would increase the quality of care for patients by enabling earlier treatment of prostate cancer.
2. The relationship between prostate cancer and TGFβ

In prostate cancer patients, the overexpression or deregulation of components in the TGFβ signaling pathway has been associated with poor prognosis and survival. For example, the overproduction of TGFβ1 in tumor epithelial cells has been commonly observed leading to angiogenesis, metastasis, and short cancer-specific survival (Wikstrom et al., 1998). In vitro studies with prostate cancer cell lines suggest that low-level TGFβ1 expression induces autocrine signaling by regulating its own expression (Yu et al., 2010).

The loss of TβRI and/or TGFβ receptor type II (TβRII) expression has been correlated with a high Gleason score (Kim et al., 1996). Moreover, loss of TβRI expression alone (but not loss of TβRII expression) is prognostically unfavorable in prostate cancer patients (Kim et al., 1998). This finding provides a strong clue regarding how prostate cancer cells counteract the TGFβ-induced growth inhibitory effect by downregulating TGFβ receptor (TβR) expression (Guo et al., 1997). In addition, the tumor-suppressive effect of TβR is impaired by epigenetic modifications, such as the methylation of TβRI/TβRII promoters in cancerous cells (Zhang et al., 2005a).

Loss of heterozygosity on chromosome 18q is frequently observed in prostate cancer, and results in the depletion of related tumor-suppressor genes (Padalecki et al., 2003; Yin et al., 2001). In rat experiments, silencing Smad2 expression induced tumor growth (Yang et al., 2009). However, no Smad2 mutations were reported in prostate cancer cells, which suggests that its dominant role is in TGFβ-induced apoptosis and gene expression (Kubiczkova et al., 2012; Latil et al., 1999).
3. TGFβ

3.1. TGFβ history and family members

TGFβ is a multifunctional cytokine that is involved in many critical cellular functions (e.g., growth arrest, differentiation, and apoptosis) that are crucial during embryogenesis, angiogenesis, and the epithelial-mesenchymal transition (EMT) (Heldin et al., 2009; Massagué, 2008). As TGFβ has such panoramic and multifunctional roles, any disturbances of its family members may lead to abnormal signaling, which in turn can result in a broad range of pathologic consequences. The pathogenesis and progression of numerous human cancers has been linked to the deregulation of TGFβ signaling by aberrations such as mutations, loss of heterozygosity, and the microsatellite instability of TβRI and TβRII or Smad2 and Smad4 in the TGFβ signaling pathway (Levy and Hill, 2006; Padua and Massagué, 2009).

The TGFβ family is composed of 33 members in humans (Massagué, 1998; Moustakas and Heldin, 2009), including bone morphogenetic proteins (BMPs), activins, TGFβ, growth and differentiation factors (GDFs), Nodal, and anti-Mullerian hormone (AMH) (Padua and Massagué, 2009). The TGFβ subfamily consists of three isoforms, designated as TGFβ1, TGFβ2, and TGFβ3 (Khalil, 1999). The name ‘transforming growth factor’ (TGF) was coined in 1980 because of the ability of this malicious cytokine to transform rat fibroblasts. Later, TGF was reported to exist in two forms as TGFα and TGFβ (Anzano et al., 1983; Moses et al., 1981; Roberts et al., 1980). TGFβ signaling is highly conserved throughout evolution from the ancestral Trichoplax adhaerens (a primitive organism of phylum Placozoa) to modern chordates (Herpin et al., 2004; Huminiecki et al., 2009). Members of TGFβ superfamily regulate the fate of development in an organism from the process of gastrulation.
to organ morphogenesis and adult tissue homeostasis (Heldin et al., 2009; Massague and Gomis, 2006). Furthermore, TGFβ is known to act as a double-edged sword in tumor biology: although it initially exerts its effects as a tumor suppressor by limiting epithelial proliferation and arresting pre-malignant growth in specific contexts, TGFβ also exerts pro-tumorigenic effects on the microenvironment and tumor cells by promoting cell invasion, migration, and evasion of immunity, resulting in the enhancement of tumor progression and metastasis (Heldin et al., 2012; Massague, 2012; Padua and Massagué, 2009; Pickup et al., 2013). This enigmatic switching of roles from a tumor suppressor to a tumor promoter has stimulated enormous research interest, generating nearly 50,000 articles.

3.2. Mechanism of TGFβ ligand activation

The TGFβ ligands are secreted as precursor proteins that contain a long N-terminal pro-peptide and a short C-terminal mature polypeptide. The precursor proteins form dimer pairs through intermolecular disulfide linkages through conserved cysteine residues in the pro-peptide and mature peptide sequences (Moustakas and Heldin, 2009). The precursor is N-glycosylated and the pro-domain is proteolytically cleaved at a consensus site (RK)-X-X-(RK) by furin-like proteases to generate a C-terminal fragment that binds to latency-associated peptide to form the small latent TGFβ complex (SLC). Later, the SLC interacts with large latent binding protein (LTBP) to constitute the large latent TGFβ complex (LLC), enabling its association with extracellular matrix (ECM) proteins (e.g., fibrillin1 and fibronectin). The mature C-terminal dimeric ligands are activated by several proteases, such as elastase (cleaves fibrillin1), bone morphogenetic protein 1 (BMP1; cleaves LTBP at the hinge region, resulting in the release of LLC), and matrix metalloproteases (MMP-2 cleaves LAP to release mature TGFβ). The mature TGFβ ligand binds to the TβRs, resulting in the activation of TGFβ signaling (ten Dijke and Arthur, 2007).
3.3. The TGFβ receptor superfamily

TGFβ exerts its cellular effects by recruiting two pairs of type I and type II serine/threonine kinase receptors to form a heterotetrameric complex (Huang et al., 2011; Wrana et al., 1992). TβRI is also known as activin receptor-like kinase-5 (ALK5), and is one of the seven different type I serine/threonine kinase receptors (ALKs 1-7). TβRII is one of five type II serine/threonine kinase receptors (ActR-IIa, ActR-IIIB, BMPRII, AMHRII, and TβRII) (Franzen et al., 1993; ten Dijke et al., 1994). They are classified into five categories according to their respective ligand binding (Table 1). Additionally, TGFβ type III receptors, such as betaglycan and endoglin, assist in the process of presenting ligands (TGFβ2) that have low intrinsic affinity to bind TβRII for efficient signaling (Yamashita et al., 1994a). The TGFβ1 ligand signals mainly through TβRII and TβRI (ALK5) (Moustakas and Heldin, 2009; Padua and Massagué, 2009; ten Dijke and Arthur, 2007).

<table>
<thead>
<tr>
<th></th>
<th>TGFβ1, 2, 3</th>
<th>Activin</th>
<th>BMP/GDF</th>
<th>AMH</th>
</tr>
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<tbody>
<tr>
<td><strong>Type I receptor</strong></td>
<td>TβRI (ALK5)</td>
<td>ALK4, 7</td>
<td>ALK1, 2, 3, 6</td>
<td>ALK2, 3, 6</td>
</tr>
<tr>
<td><strong>Type II receptor</strong></td>
<td>TβRII</td>
<td>ActRII-A, B</td>
<td>BMPRII, ActRII-A, B</td>
<td>AMHRII</td>
</tr>
<tr>
<td><strong>R-Smad</strong></td>
<td>Smad2, Smad3</td>
<td></td>
<td>Smad1, Smad5, Smad8</td>
<td></td>
</tr>
<tr>
<td><strong>Co-Smad</strong></td>
<td>Smad4</td>
<td></td>
<td>Smad4</td>
<td></td>
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<tr>
<td><strong>I-Smad</strong></td>
<td>Smad7</td>
<td></td>
<td>Smad6, Smad7</td>
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</table>

**Table 1.** Members of the TGFβ family. Corresponding ligands, receptors, and their downstream targets: the receptor-activated Smads (R-Smads), common-mediator Smad (co-Smad), and inhibitory Smad (I-Smad)
3.4. TGFβ receptor activation

TβRs are transmembrane serine/threonine kinase cell surface glycoproteins. They contain an extracellular ligand binding domain, a transmembrane domain, and an intracellular serine/threonine kinase domain (Figure 1) (Derynck and Zhang, 2003). Although TβRs are expressed ubiquitously in most tissues, they are expressed at lower levels in normal and transformed mammalian and avian cells (Massague et al., 1990). TβRI was identified as a serine/threonine kinase receptor with a molecular mass of 53 kDa (Franzen et al., 1993; Laiho et al., 1991; Wrana et al., 1992), and TβRI is similar to the TβRII (70 kDa) serine/threonine kinase receptor (Massagué, 1998). TβRI has a very weak affinity for binding to the ligand directly, but has a very high specificity for TβRII (Wrana et al., 1992; Yamashita et al., 1994b). Ligand binding to the constitutively active TβRII generates 300-fold enhanced affinity of binding between TβRII and TβRI to form a heterotetrameric complex (Franzen et al., 1993; Yamashita et al., 1994b). Ligand binding to TβRII alone (but not to TβRI) is sufficient to form a heterotetrameric complex and phosphorylate ligand-free TβRI (Vivien and Wrana, 1995). The ligand binding is a key step, as it leads to TβRII phosphorylating TβRI at a 30-amino-acid region rich in serine and threonine residues termed as the GS (glycine/serine) domain (owing to SGSGSG repeats) (Massagué, 1998). Upon ligand binding, the TβRI is phosphorylated at the juxtamembrane part of the cytoplasmic domain region at the TTSGSGSGG sequence (Franzen et al., 1995; Okadome et al., 1994; Wieser et al., 1995; Wrana et al., 1994). The GS domain is regarded as the catalytic core of the TβRI, as its phosphorylation leads to the transfer of generated signals to the downstream effectors. Consequently, ligand-induced activation of the receptor complex leads to the phosphorylation of R-Smads at their extreme C-terminus, thereby enhancing their ability to transfer to the nucleus and activate gene transcription to drive TGFβ-induced responses (Abdollah et al.,...
Figure 1. The TGFβ receptor I consists of an extracellular ligand binding domain, a transmembrane domain, and an intracellular serine/threonine kinase domain.
3.5. The Smads

The name ‘Smad’ was derived from the discovery of the characteristic gene homology shared by these proteins, which were known as Small body size (Sma) in *Caenorhabditis elegans* and Mothers against decapentaplegic (Mad) in *Drosophila melanogaster* (Raftery and Sutherland, 1999; Savage et al., 1996; Sekelsky et al., 1995). Eight Smad proteins have been uncovered in humans (Itoh et al., 2000). The Smads are classified into three different groups. The receptor-activated Smads (R-Smads) comprise Smads 1, 2, 3, 5, and 8, of which only Smad2 and Smad3 are responsive to TGFβ ligand signaling, while Smads 1, 5, and 8 act in BMP signaling. The second (single-member) class of the Smad family is the common mediator Smad (Co-Smad), or Smad4, which acts as a partner for the R-Smads and shuttles between the cytoplasm and the nucleus (Abdollah et al., 1997; Kretzschmar et al., 1997; Souchelnytskyi et al., 1997). The final class of Smads is the inhibitory Smads (I-Smads), Smad6 and Smad7. Smad7 negatively regulates TGFβ/BMP signaling and is also a direct target gene of the TGFβ signaling cascade (Afrakhte et al., 1998; Hayashi et al., 1997; Nakao et al., 1997; Shi and Massagué, 2003).

The constitutively active TβRIIs bind to and transphosphorylates TβRIIs upon ligand binding and stimulate their protein kinase activity. The activated TβRIIs then phosphorylate Smad2 or Smad3, which later binds to Smad4. The resulting Smad complex of Smad2 or Smad3 with Smad4 translocates to the nucleus, where it associates with transcription factors and acts as a modulator to regulate the transcription of certain TGFβ target genes (Ross and Hill, 2008).
4. TGFβ signaling

4.1. Canonical Smad signaling mechanisms: Smad activation, nucleocytoplasmic shuttling, and transcriptional regulation

The best-characterized model of Smad structure indicates that Smads consist of three domains: an N-terminal Mad homology1 (MH1) domain, a linker domain, and a C-terminal Mad homology 2 (MH2) domain. The MH1 domain (130 amino acid residues) facilitates interaction with other proteins and promotes nuclear translocation owing to the presence of the nuclear localization signal (NLS) and a DNA binding domain. The middle linker domain is enriched with prolines and phosphorylatable serine or threonine residues that assist interaction with ubiquitin ligases and the formation of homo-oligomers. The C-terminal MH2 domain (200 amino acid residues) is responsible for binding to TβRIs and other proteins, mediating the homo- and hetero-oligomerization and transactivation of nuclear Smad complexes (Massague and Gomis, 2006; Moustakas and Heldin, 2009).

Canonical Smad signaling is initiated by the phosphorylation of R-Smads at their extreme serine residues (Ser-X-Ser) in the C-terminal motif by the TβRI (Abdollah et al., 1997; Kretzschmar et al., 1997; Macias-Silva et al., 1996; Souchelnytskyi et al., 1997). Although the Smads are localized in the cytoplasm in an inactive state (absence of phosphorylation), Smad4 shuttles between the nucleus and the cytoplasm. In the cytoplasm, the R-Smads bind to SARA (Smad anchor for receptor activation), a double zinc finger or FYVE domain-containing protein that is predominantly localized in early endosomes. Upon phosphorylation, the active R-Smads lose their attraction for cytoplasmic proteins such as SARA and bind to the Co-Smad (Smad4), forming a trimeric complex (Itoh et al., 2002; Tsukazaki et al., 1998; Xu et al., 2000). In general, the trimeric complex is comprised of homo- or heteromeric R-Smads
and a co-Smad; it can be a Smad2/Smad2/Smad4 complex, a Smad3/Smad3/Smad4 complex, or a Smad2/Smad3/Smad4 complex. The trimeric complex later translocates into the nucleus and binds to chromatin (Lönn et al., 2009; Ross and Hill, 2008). Recent investigations suggest that the TGFβ-mediated nuclear accumulation of Smads 2, 3, and 4 is mediated by their interactions with importins 7 and 8 or with nucleoporins (Nup214/153) or exportin 4 to cross the nuclear pore barrier. However, it must be kept in mind that the R-Smads and Smad4 each possess a nuclear localization signal (NLS) in their respective MH1 domain (Heldin et al., 2009). After entering the nucleus, the active Smad complexes bind to promoters of different genes and regulate their transcription. DNA binding and mutation analysis studies have revealed that the TGFβ-activated PAI-1 promoter binds to the Smad3 and Smad4 DNA binding element (known as a Smad-binding element, or SBE) specifically to the AGAC or GTCT sequence (Dennler et al., 1998; Zawel et al., 1998). Furthermore, the TGFβ/Smad pathway determines processes such as differentiation and apoptosis through selective transcriptional regulation with various transcriptional coactivators, such as CREB binding protein (CBP), histone acetyltransferases (HATs), and p300; corepressors, such as Ski, SnoN, and c-Myc; and transcription factors, such as AP-1, p53, and forkhead transcription factors (e.g., FOXH1) (Roberts, 1999; Ross and Hill, 2008). Therefore, the TGFβ-mediated canonical Smad pathway acts centrally in TGFβ signal transduction.

4.2. Non-Smad signaling

TGFβ also transmits its signals through other non-Smad partners, such as the extracellular signal-regulated kinases (Erks), c-Jun N-terminal kinases (JNKs), and p38 mitogen-activated protein kinase (MAPK), although the precise mechanisms that describe how these pathways operate remain to be explored further (Derynck and Zhang, 2003; Edlund et al., 2003; Engel et al., 1999; Landstrom, 2010; Moustakas and Heldin, 2009). Here is a brief summary of some
of the well-studied signaling partners of the TGFβ family. One of the mysteries regarding how TGFβ activates Erk MAP kinases was partially solved when Lee and colleagues reported that the TβRI phosphorylates serine and tyrosine residues in the adaptor protein ShcA and activates the Erk MAP kinases by inducing the association between growth factor receptor-bound protein 2 (Grb2), Ras guanine exchange factor, and son of sevenless (Lee et al., 2007).

Par6, an epithelial cell polarity regulator, is another important protein implicated in non-Smad signaling. Par6 interacts with TβRI/II and is phosphorylated by the constitutively active TβRII. Phosphorylation of Par6 is a key event in this context, because it controls the interaction of Par6 with the E3 ubiquitin ligase known as Smurf1, which in turn targets the degradation of guanosine triphosphatase (GTP) Rho, leading to a loss of tight junctions (Ozdamar et al., 2005).

Tumor necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase, associates with the TβRI via a specific consensus motif in TβRI. The TβRI-TRAF6 interaction is crucial for the TGFβ-induced autoubiquitination of TRAF6, which leads to subsequent activation of the TAK1-p38/JNK pathway and promotion of apoptosis (Sorrentino et al., 2008). TRAF6 has also been suggested to mediate activation of JNK and p38 by TGFβ in a Smad-independent manner, resulting in the EMT (Yamashita et al., 2008).
4.3. Post-translational modifications of the TGFβ receptors

Research during the last 20 years has provided a deep understanding of the molecular mechanisms related to TGFβ-activated Smad and non-Smad signaling. TGFβ receptor availability and its function are determined by post-translational modifications. Some of the well-studied post-translational modifications that the TGFβ receptors often encounter are phosphorylation, sumoylation, and ubiquitination (Kang et al., 2009).

4.4. TGFβ receptor phosphorylation

Phosphorylation of the TGFβ receptors sets the stage for signal propagation to downstream targets, such as R-Smads (Massagué, 1998); (Moustakas et al., 2001). The mechanism of TGFβ receptor phosphorylation is initiated when the ligand binds to the TβRII and entices TβRI to form a heterotetrameric complex, which results in the phosphorylation of multiple serine and threonine residues in the GS domain of the TβRI (Wieser et al., 1995; Wrana et al., 1994; Yamashita et al., 1994b). However, it is noteworthy that the TβRII forms homooligomers independent of ligand binding (Chen and Derynck, 1994; Henis et al., 1994). The phosphorylation of TβRI is a key event in TGFβ signal propagation, as it activates TβRI kinase activity to phosphorylate R-Smads. Moreover, the phosphorylation of TβRI results in a conformational change that prevents the binding of FK506 binding protein 12 (FKBP12), thereby allowing access to activation by TβRII. FKBP12 is an immunophilin and acts as an inhibitor of TGFβ signaling (Huse et al., 1999). In the absence of phosphorylated TβRI, FKBP12 binds to the unphosphorylated GS regions of the TβRI, blocking the ligand-independent phosphorylation of TβRI by TβRII (Chen et al., 1997; Okadome et al., 1996; Wang et al., 1994). Crystallographic studies suggest that phosphorylation at the GS domain activates TβRI by converting the GS region into an efficient recruitment site for a proper
substrate (Huse et al., 2001). Apart from the phosphorylation of the serine and threonine residues of the TGFβ receptors, they are also phosphorylated at tyrosines. For instance, the TβRII cytoplasmic domain is autophosphorylated at three tyrosine residues (Lawler et al., 1997); in addition, the TβRII can also be phosphorylated by Src and will thereafter recruit Grb2 and Shc, resulting in activation of the p38 MAPK pathway (Galliher and Schiemann, 2007). The TβRI interacts with Shc and phosphorylates Shc at tyrosine and serine residues, resulting in the recruitment of Grb2 and SOS to Shc, which then activates the Erk MAP kinase pathway (Lee et al., 2007).

4.5. Ubiquitination

Ubiquitination is a reversible post-translational modification that affects diverse cellular processes, such as protein degradation, progression through the cell cycle, DNA repair, gene transcription, receptor trafficking, autophagy, and endocytosis (Emmerich et al., 2011; Haglund et al., 2003; Haglund and Dikic, 2005; Hershko and Ciechanover, 1998). Ubiquitin is a conserved 76-amino-acid molecule with a molecular mass of 8.5 kDa. The name ubiquitin is derived from the native term ‘ubiquitous immunopoietic polypeptide’ (Ciechanover et al., 1978; Goldstein et al., 1975). The process of marking a protein for ubiquitination consists of a series of enzymatic reactions that involves an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-ligating enzyme (Ciechanover et al., 1980a; Ciechanover et al., 1980b; Hershko et al., 1980). These enzymatic reactions create a covalent linkage between the c-terminal glycine residue in a ubiquitin molecule and a lysine (K) residue or the N-terminus of the target protein (Deribe et al., 2010; Hershko et al., 1981; Kirkin and Dikic, 2007). Recent advances in the ubiquitin field suggest that ubiquitin molecules also have a binding preference for methionine, cysteine, serine, and threonine residues in the acceptor molecules (Hochstrasser, 2009).
A Ubiquitin molecule comprises of seven lysine residues at position number 6, 11, 27, 29, 33, 48 and 63 (Dikic et al., 2009; Kravtsova-Ivantsiv and Ciechanover, 2012). The process of marking a protein for ubiquitination results in modification of a target site by a single ubiquitin (referred to as monoubiquitination) or by a series of ubiquitin-linked chains (referred to as polyubiquitination) (Hochstrasser, 2009). Recent reports suggest that monoubiquitination is a major process that favors receptor endocytosis and DNA damage repair, while ubiquitination through Lys6, 11, 27, 29, 33, and 48-linked polyubiquitin chains results in protein degradation; especially Lys48 favors protein degradation by the proteosome. Moreover, Lys63-linked polyubiquitination plays a major role in the signal transduction of various proteins (Deribe et al., 2010; Kravtsova-Ivantsiv and Ciechanover, 2012; Kravtsova-Ivantsiv et al., 2013; Pickart and Fushman, 2004).

Ubiquitination has been suggested to regulate the stability of the TβRI and the heteromeric TβR complex (Ebisawa et al., 2001; Kavsak et al., 2000). Smad7, an I-Smad and a direct target gene of TGFβ, acts as an adaptor protein that binds to the TβRI and competitively inhibits Smad2/3 activation (Hayashi et al., 1997; Nakao et al., 1997). Smurfs are members of the Homologous to the E6-AP Carboxyl Terminus (HECT) family of E3 ubiquitin ligases and induce the ubiquitination and degradation of various TGFβ family associates (e.g., BMP-specific Smads 1 and 5). Smurf1/2 utilizes the adaptor protein Smad7 to degrade TβRI. Initial reports suggest that the ubiquitin ligase Smurf2 associates with Smad7 to target the TβRI for degradation, in which Smad7 helps to recruit Smurf2 to the activated receptor complex, highlighting the role of Smurf2 in Smad7 inhibitory function. Thus, the Smurf2-Smad7 interaction functions as an auto-regulatory feedback loop to mediate the rapid degradation of TGFβ receptors (Kavsak et al., 2000). Smad7 interacts with Smurf1 in the nucleus, and the complex or Smad7 are later exported to the cytoplasm where Smad7 interacts with TβRI and
acts as an adaptor protein to recruit Smurf1, resulting in the degradation and rapid turnover of TβRI (Ebisawa et al., 2001). The E3 ligase Arkadia preys on the negative regulators of TGFβ signaling (Ski, SnoN, Smad7) for proteosomal degradation, and thereby boosts a positive feedback loop (Koinuma et al., 2003).

The role of other E3 ligases in the context of non-Smad signaling has been described recently. The E3 ligase TRAF6 was found to interact with a consensus motif in the TβRI, and the TβRI-TRAF6 interaction is required for the TGFβ-induced autoubiquitination of TRAF6 and subsequent activation of the TAK1-p38/JNK pathway, leading to apoptosis (Sorrentino et al., 2008) and EMT (Yamashita et al., 2008).

Ubiquitination is a reversible process. Another enzyme type, known as deubiquitinating enzymes (DUBs), free the substrate of ubiquitins by specifically cleaving the isopeptide bond at the C-terminus of the ubiquitin molecule, releasing the substrate to its normal state (Clague et al., 2012). Most DUBs belong to either the ubiquitin-specific processing protease (UBP) or the ubiquitin C-terminal hydrolase (UCH) family of cysteine proteases. UCH37 deubiquitinates and stabilizes the TβRI by competitively binding to the N-terminal half of Smad7 to reverse Smurf-mediated ubiquitination (Wicks et al., 2005).

### 4.6. Sumoylation

Sumoylation is another post-translational modification that primarily targets nuclear and perinuclear proteins, such as transcription factors. The process of sumoylation is initiated by the covalent attachment of a small ubiquitin-like modifier (SUMO) protein to the lysines of the substrate proteins. Despite the name, SUMO proteins share only approximately 18% sequence identity with ubiquitin (Muller et al., 2001). SUMO proteins are approximately 11 kDa in molecular mass. They binds to the lysine residue of the substrate with the consensus
sequence $\psi$KxE, where $\psi$ corresponds to a large hydrophobic amino acid, K is a lysine residue, x is any amino acid and E is a glutamic acid residue (Hannoun et al., 2010). Attachment of SUMO proteins to cell surface receptors for growth factors is a very rare phenomenon. However, a recent report suggests that the TβRI is sumolyated by Ubc9, resulting in enhanced recruitment and phosphorylation of Smad3 (Kang et al., 2008). This report also suggests that the kinase activities of TβRI and TβRII are a prerequisite for sumoylation, which is favored by the exposure of the sumoylation site at the surface of the kinase domain. The attached SUMO protein faces the same direction as the GS domain and L45 loop that specify the interaction between the TβRI and Smad2 or Smad3. Therefore, TβRI sumoylation enhances the recruitment of Smad2 or Smad3 and results in enhancement of the transcription of TGFβ target genes (Kang et al., 2009).
5. Proteins involved in non-Smad signaling

5.1. The TRAF dynasty

The tumor necrosis factor receptor-associated factors (TRAFs) belong to a class of adapter proteins that are centrally involved in the ligand-induced activation of various signaling cascades, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK), JNK, and p38 activation initiated by tumor necrosis factor (TNF) receptors (Inoue et al., 2000; Landstrom, 2010; Mu et al., 2012; Song et al., 1997). The TRAFs are highly conserved among different species and exist in seven different types in mammals (TRAF1-7) (Wajant et al., 2001). However, the expression of the TRAF members is restricted in a cell-type-specific manner. TRAF2, TRAF3, and TRAF6 are expressed ubiquitously (Cao et al., 1996; Ishida et al., 1996a; Rothe et al., 1994), while TRAF1, TRAF4, and TRAF5 are expressed less broadly (Ishida et al., 1996b; Mosialos et al., 1995; Nakano et al., 1996). TRAFs are predominantly located in the cytoplasm and function as adapter proteins in signal transduction.

All TRAF family members contain a highly conserved C-terminal TRAF domain that is divided into two sub-regions: a C-terminal region and an N-terminal region (Figure 2). The C-terminal region is highly conserved among various species and facilitates interaction with various cell surface receptors and forms both TRAF homo- and hetero-oligomers (Dempsey et al., 2003).

With the exception of TRAF1, the TRAF family members also contain a less conserved N-terminal region that consists of a RING (Really Interesting New Gene) finger domain (Figure 2) (Zapata and Reed, 2002). The RING finger domain is rich in cysteines and histidines that coordinate the binding of Zn$^{2+}$ ions (Borden et al., 1995); deletion of this region leads to
dominant negative TRAF mutants (Arch et al., 1998). The RING domain binds to ubiquitination enzymes and their substrates to promote Lys63-linked polyubiquitination and activation of the signaling partners. For example, TRAF6 mediates TGFβ-induced Lys63 polyubiquitination of TGFβ-activated kinase 1 (TAK1) and activation of p38-MAPK signaling (Adhikari et al., 2007; Thakur et al., 2009).

**Role of TRAF6 in TGFβ signaling**

TRAF6 was initially isolated from an EST expression library by employing CD40 as bait for a yeast two-hybrid screen. It was characterized for its role as an adaptor protein in the activation of NF-κB signaling by interleukin 1 (Cao et al., 1996; Ishida et al., 1996a). TRAF6-deficient mice die prematurely because of defects in bone formation (Lomaga et al., 1999; Naito et al., 1999). TRAF6 functions as an E3 ligase, as it interacts with the E2 conjugating enzyme Msm2, which consists of Ubc13 and Uev1A. Moreover, TRAF6 has been implicated in the promotion of Lys63-linked ubiquitin chains to the target proteins, thereby promoting activation of various proteins and their downstream targets (Mu et al., 2012; Sundar, 2012; Wang et al., 2012). TRAF6 binds to the TβRI at a conserved consensus motif (basic residue-X-P-X-E-X-X-aromatic/acidic residue) in the TβRI, and the TβRI-TRAF6 interaction is required for TGFβ-induced autoubiquitination of TRAF6 and subsequent Lys63-linked polyubiquitination of TAK1. In turn, activated TAK1 activates MKK3/6, leading to p38 activation (Sorrentino et al., 2008). TβRI kinase activity is required for activation of the Smad pathway; in contrast, the E3 ubiquitin ligase activity of TRAF6 has been demonstrated to regulate the activation of TAK1 in a receptor-kinase-independent manner. TGFβ specifically activates TAK1 through the interaction of TβRI with TRAF6 (Landstrom, 2010). In line with the above report, Yamashita et al. demonstrated that TGFβ causes Lys63-linked polyubiquitination of TAK1 to promote the EMT (Yamashita et al., 2008).
Figure 2. The general structure of TRAF6 comprising of an N-terminal zinc-binding domain and a C-terminal TRAF domain.

Role of TRAF4 in TGFβ signaling

TRAF4, also known as CART1, was identified as a TRAF family member as a result of a cDNA library screen of lymph nodes of metastatic tumor cells (Regnier et al., 2002; Regnier et al., 1995). TRAF4 has been implicated in the regulation of cell polarity and embryonic development (Mathew et al., 2009). Recent reports suggest that TRAF4, like TRAF6, interacts with TβRI in a TGFβ-dependent manner and promotes the Lys63-linked polyubiquitination of TRAF4 and TAK1, as well as activation of the downstream target p38-MAP kinase and NF-κB pathways. Interestingly, the transcriptional regulation of Snail1 and interleukin 11 are inhibited in TRAF4-deficient breast cancer cells (Zhang et al., 2013).

5.2. c-Jun

c-Jun is an Activator protein-1 (AP-1) transcription factor that regulates cellular responses such as proliferation, differentiation, cell death, and cellular response to stress (Mechta-Grigoriou et al., 2001). c-Jun forms homo- or heterodimers with Fos family members or other Jun proteins to comprise the AP-1 transcription factor (Hess et al., 2004). c-Jun mediates transcriptional responses to stress, a unique function that is conserved among Jun family members from yeast to mammals (Shaulian and Karin, 2001; Toone et al., 2001). c-Jun is
composed of a C-terminal dimerization domain, a DNA-binding domain, and an N-terminal transactivation domain (Drosatos et al., 2007). It is activated by phosphorylation at its N-terminal serine residues 63 and 73 by the c-Jun N-terminal kinase (JNK) family (Adler et al., 1992; Kallunki et al., 1996; Smeal et al., 1992). The functional role of c-Jun was identified in c-Jun siRNA experiments that resulted in the blockade of transition of cells from G1 to S phase. This finding suggested that c-Jun is a positive regulator of the cell cycle (Kovary and Bravo, 1991; Smith and Prochownik, 1992). Moreover, fibroblasts deficient in c-Jun exhibited a severe proliferation defect, suggesting that poor activation of cyclin D1 hampered the progression of cells from G1 to S phase (Wisdom et al., 1999). Experiments performed in cells lacking c-Jun resulted in increased expression of the tumor suppressor gene p53 and its target gene, the CDK inhibitor p21. In contrast, overexpression of c-Jun results in repression of p53 and p21 expression, as well as accelerated cell proliferation. These findings imply that c-Jun regulates p53 expression (Schreiber et al., 1999). Previous reports suggest that TGFβ1 and BMP-2 induce transcription of p21 in a Smad1-dependent manner (Pardali et al., 2000). Furthermore, p21 is a common target for all TGFβ superfamily pathways (Pardali et al., 2005). Liberati and colleagues demonstrated that Smad3 and Smad4 bind to Jun family members (JunB, c-Jun, and JunD) and that endogenous c-Jun interacts with Smad3/4 and is phosphorylated rapidly upon exposure of cells to TGFβ (Liberati et al., 1999). Finally, overexpression of c-Jun has been shown to inhibit TGFβ- and Smad3-induced transactivation of the Smad-specific promoter construct SBE4-Lux (Verrecchia et al., 2001).

5.3. Presenilins

The presenilins are polytransmembrane proteins. They were initially identified for their roles in generating amyloid precursor protein (APP) from amyloid-beta peptides (AβP) in Alzheimer’s disease and in releasing the transcriptionally active domain of the Notch receptor.
Presenilins exist as presenilin1 (PS1) and presenilin2 (PS2) and are expressed ubiquitously in most human and mouse tissues. However, PS1 mRNA is expressed at significantly higher levels in developing brain tissue (Lee et al., 1996). PS1-/- mice have severe birth defects (e.g., impaired axial skeleton formation, cortical dysplasia, impaired neurogenesis, and impaired neuronal survival) and die shortly after birth (Hartmann et al., 1999; Shen et al., 1997). In addition, PS1-/- embryos exhibit defects in somite segmentation and differentiation (Wong et al., 1997). Previous reports suggest that PS1 is found in the endoplasmic reticulum (ER), Golgi apparatus, ER/Golgi intermediate compartments, endosomes, lysosomes, phagosomes, plasma membrane, and mitochondria (Brunkan and Goate, 2005; De Strooper et al., 1997; Vetrivel et al., 2006). Although the structure of PS1 is controversial according to information obtained from different studies, a recent model proposes that PS1 contains nine transmembrane domains and that the N-terminus and a hydrophilic loop are located in the cytosol, while the C-terminus is located in the lumen/extracellular space (Laudon et al., 2005). However, in the context of the present study we will consider the widely accepted PS1 topology model, which consists of eight transmembrane domains with cytosol-facing N- and C-termini and an intracellular, hydrophilic loop between transmembrane segments 6 and 8 (Figure 3) (Doan et al., 1996; Li and Greenwald, 1996; Li and Greenwald, 1998).

**Figure 3.** The widely accepted structure of presenilin1.

![Diagram of Presenilin1 Structure](image-url)
The inactive PS1 holoprotein (42-43 kDa) is an aspartyl protease. It undergoes endoproteolytic cleavage by an unknown presenilinase to generate an N-terminal fragment (NTF, 27-28 kDa) and a C-terminal fragment (CTF, 16-17 kDa) at a stoichiometric ratio of 1:1 (Esler et al., 2000; Levitan et al., 1996; Li et al., 2000). Furthermore, the CTF is subjected to an alternate caspase-3-mediated cleavage at aspartate 345 and serine 246 to generate a 14-kDa PS1-CTF (Grunberg et al., 1998; Kim et al., 1997; Loetscher et al., 1997). The discovery that PS1-deficient mice exhibit accumulation of CTFs of APP and a 5-fold drop in the production of AβP led to the conclusion that presenilins function as the catalytic core of the γ-secretase complex (De Strooper et al., 1998). The active γ-secretase complex facilitates the cleavage of various cell surface type I transmembrane receptors (e.g., APP, Notch, CD44, and interleukin 1) (McCarthy et al., 2009). The association of PS1 with nicastrin, presenilin enhancer 2 (pen-2), and the anterior pharynx defective-1 (Aph-1) proteins generates an active γ-secretase complex (Edbauer et al., 2003; Wakabayashi and De Strooper, 2008), and PS1 functions as its catalytic subunit (De Strooper et al., 1998; Takasugi et al., 2003). Nicastrin, a type I membrane protein, contributes to the proper assembly of the γ-secretase complex within the ER, its intracellular transport to the plasma membrane, and the initial recognition of the short N-terminal stubs of the substrates and subsequent recruitment to the γ-secretase complex (Shah et al., 2005; Zhang et al., 2005b). Nicastrin is also involved in locking the substrates into the lipid bilayer, thereby exposing it to substrate processing by presenilin, which in turn results in the generation of the fragment by the γ-secretase complex cleavage event. Anterior pharynx defective-1 (Aph-1), the other component of the γ-secretase complex, is involved in the stabilization and glycosylation of nicastrin by scaffolding nicastrin to the immature γ-secretase complex (LaVoie et al., 2003). Aph-1 has been reported to associate with nicastrin; this interaction occurs very soon after Aph-1 synthesis. Aph-1 is processed by endoproteolysis and upon co-expression of nicastrin with Aph-1, resulting in the generation of
a stable C-terminal fragment that associates with nicastrin (Fortna et al., 2004). The final component of the γ-secretase complex is presenilin enhancer 2 (Pen-2), which assists in the generation of presenilin NTF/CTF heterodimers by endoproteolysis (Luo et al., 2003). All four components associate with each other to function as an active complex (Steiner et al., 2002). Co-expression of the four components leads to increased presenilin heterodimerization, full glycosylation of nicastrin, and enhanced γ-secretase activity (Kimberly et al., 2003).

PS1 undergoes various post-translational modifications, including phosphorylation of serine residues at the PS1-CTF region to form oligomers along with PS1-NTF (Seeger et al., 1997). PS1 is also subjected to ubiquitination. PS1 holoprotein is reportedly polyubiquitinated and undergoes proteasomal degradation, as treatment with MG132 or lactacystin inhibitors prevents PS1 holoprotein degradation. However, PS1-CTF and PS1-NTF have been reported as the stable forms of PS1 (Marambaud et al., 1998; Steiner et al., 1998). Moreover, SEL-10, a member of the SCF (Skp1-Cdc53/CUL1-F-box protein) E2-E3 ubiquitin ligases, forms a complex with PS1, which facilitates PS1 ubiquitination and promotes the degradation of PS1-NTF and PS1-CTF (Li et al., 2002). PS1 has been reported to contain a conserved TRAF6 binding motif. PS1 association with TRAF6 leads to enhanced TRAF6 autoubiquitination and promotes ubiquitination of the p75 neurotrophin receptor (p75NTR) (Powell et al, 2009).
Regulated intramembrane proteolysis

Cells trigger signal transduction responses through various mechanisms. One such mechanism is regulated intramembrane proteolysis (RIP). RIP of substrates regulates a variety of cellular processes, including cell adhesion, gene transcription, protein activation, lipid metabolism, release of transcription factors, degradation of protein fragments, transport of viral proteins during infection, neuronal survival, and differentiation (De Strooper and Annaert, 2010; Hass et al., 2009; Kopan and Ilagan, 2004; Lal and Caplan, 2011).

With the increasing evidence that has accumulated more recently, RIP is now considered another mechanism by which both intercellular and intracellular signals are transmitted (Kang et al., 1987; McCarthy et al., 2009). The evolutionarily conserved mechanism of RIP has been reported throughout the phylogenetic kingdom, in plants (Physcomitrella patens), primitive slime mold (Dictyostelium discoideum), eubacteria (Enterococcus faecalis), and eukaryotes (An et al., 1999; Brown et al., 2000; Hass et al., 2009; Khandelwal et al., 2007; McMains et al., 2010).

RIP is a two-step process. The first step is the cleavage of the membrane-spanning, lipid-anchoring type I or type II protein substrates at the ectodomain region by proteases belonging to the “a disintegrin and metalloproteinase” (ADAM) family of sheddases (ADAM-10, ADAM-17) (Hayashida et al., 2010; Weber and Saftig, 2012; Zolkiewska, 2008). These sheddases specifically hydrolyze the peptide bonds of the target protein close to the transmembrane region with or without ligand stimulation. This cleavage releases the extracellular part of the target protein into the extracellular space, while the intracellular part remains tethered to the membrane (Edwards et al., 2008).
The tethering intracellular portion of the target protein, commonly referred to as the intracellular domain (ICD), is further processed by intramembrane cleaving proteases (iCLiPs) and released into the cytosol, where it binds to other proteins to activate signaling (E-cadherin), or to inactivate intracellular signaling (Polycystin-1) or the proteosomal degradation of other proteins (N-cadherin) (Lal and Caplan, 2011; Marambaud et al., 2003; Reiss et al., 2005). Alternatively, it may be translocated to the nucleus to activate gene transcription (Notch1, TβRII) (De Strooper et al., 1998; Gudey et al., 2014).

Ectodomain shedding is considered a prerequisite for some receptors. The initial recognition and priming of substrates to undergo ectodomain shedding is operated by a subset of proteins that belong to the ADAM family of proteases or matrix metalloproteinases (MMPs) (Hayashida et al., 2010). These zinc proteases are commonly referred to as sheddases based on their function. Moreover, the ectodomain cleavage by these sheddases is dependent on the structural conformation of the cleavage site and the distance of the substrates from the plasma membrane (Hayashida et al., 2010; Seals and Courtneidge, 2003). ADAMs and MMPs are activated upon removal of their prodomain. In the case of ADAMs, the prodomain is removed at the trans-Golgi by furin-like pro-protein convertases, and the remaining portion moves to the plasma membrane where it is catalytically active (Seals and Courtneidge, 2003). The process of ectodomain shedding is activated by different stimuli, including phorbol esters-phorbol myristate acetate, growth factors, cytokines, and protein kinase C (PKC) isozymes. Peptide hydooxamates are one of first reported inhibitors to prevent the shedding of substrates (e.g., TNFα) (Mohler et al., 1994). Moreover, substitutions of amino acids in the juxtamembrane region by proline or glycine prevents shedding of the receptors (e.g., APP, TGFα) (Brachmann et al., 1989; Sisodia, 1992; Wong et al., 1989). Interestingly, human prostatic cancer cell lines exhibit strong protein expression of ADAM-17/TNFα converting
enzyme (TACE), and the examination of clinical tumor sections has revealed that all the tumor samples analysed had high expression of ADAM-17 compared to the specimens of benign prostatic hyperplasia that showed 30% of TACE expression (Karan et al., 2003). The role of TACE in the context of TGFβ signaling has been elucidated recently. In this report, TACE cleaves TβRI, resulting in decreased surface availability of TβRI and the downregulation of TGFβ signaling (Liu et al., 2009).

Some of the well-characterized substrates that are targeted by these sheddases are listed in Table 2.

**Table 2.** Sheddases and their targeted substrates

<table>
<thead>
<tr>
<th>Sheddase</th>
<th>Shedded substrate</th>
<th>Unable to shed these substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>TACE/ADAM-17</td>
<td>TNFR, ErbB4, Notch1, APP, TGFα, HB-EGF, TβRI</td>
<td>ACE, TRANCE, Syndecan-1, TβRII</td>
</tr>
<tr>
<td>ADAM-9/ADAM-10</td>
<td>HB-EGF</td>
<td></td>
</tr>
<tr>
<td>ADAM-10</td>
<td>N-Cadherin, E-Cadherin</td>
<td></td>
</tr>
<tr>
<td>ADAM-8/ADAM-15/ADAM-28</td>
<td>CD23</td>
<td></td>
</tr>
<tr>
<td>MMP-7</td>
<td>TNFα, HB-EGF</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>E-cadherin</td>
<td></td>
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</tbody>
</table>

The second step of RIP is defined by the iCLiP family of proteases. In humans, 13 different iCLiPs have been reported. They are classified into three groups based on their substrate topology: metalloproteases, aspartyl proteases, and serine proteases. Each iCLiP has its own substrate specificity. For example, presenilins are aspartyl proteases with high specificity to cleave type I membrane proteins, while signal peptide peptidase (SPP), an aspartyl protease cleaves only type II membrane proteins. Among iCLiPs, PS1 has been well studied for its role.
in cleaving various membrane receptors. Table 3 describes the family of iCLiPs, their substrates, and their biological significance.

**Biological significance of RIP**

The mechanism of regulated intramembrane proteolysis has been under intense scrutiny since its emergence. It was intriguing to understand how a proteolytic event takes place in hydrophobic conditions within the lipid bilayers (Sannerud and Annaert, 2009). The first protease identified was site-2 protease (S2P), which was implicated in the cleavage of sterol regulatory element-binding proteins (SREBPs) (Rawson et al., 1997). The role of RIP of the substrates is being investigated across the broad phylogenetic spectrum, from plants and protozoans to metazoans. RIP of substrates leads to a variety of functional aspects. The ICD generated by this mechanism has various functions depending on localization. For example, translocation of the ICD to the nucleus affects gene transcription (Notch1, ErbB4, TβRI) (Carpenter and Liao, 2013; Gudey et al., 2014; De Strooper et al., 1999). If it is localized in the cytoplasm, it may form a complex with other proteins to promote proteosomal degradation (N-cadherin); if present at the extracellular space, it may assist in intercellular signal transduction events by binding to the cell surface receptors of neighboring cells (Carpenter and Liao, 2013; Gudey et al., 2014; Lal and Caplan, 2011; Lee et al., 2001; Urban et al., 2001). Interestingly, in ancient eubacteria, the signal peptide of the cAD1 precursor is cleaved off to release the mature cAD1 pheromone (An et al., 1999). RIP has also been implicated in the release of the transcription factor domain of SREBP for nuclear translocation (Duncan et al., 1998; Rawson et al., 1997; Ye et al., 2000).

The list of substrates targeted to undergo RIP are increasing rapidly, with nearly 66 type I transmembrane protein substrates recorded to date (McCarthy et al., 2009).
Table 3. Proteases, their substrates, and the function of the released intracellular domains

<table>
<thead>
<tr>
<th>Protease type</th>
<th>Protease specificity</th>
<th>Substrate specificity</th>
<th>Substrate</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metallo protease</td>
<td>Site-2-protease (S2P)</td>
<td>Transcription factor</td>
<td>SREBP</td>
<td>Released SREBP domain effects transcription</td>
<td>(Duncan et al., 1998; Rawson et al., 1997; Ye et al., 2000)</td>
</tr>
<tr>
<td>Aspartyl protease</td>
<td>Presenilin1 (PS1)</td>
<td>Type I membrane proteins</td>
<td>APP</td>
<td>ICD effects transcription</td>
<td>(De Strooper et al., 1998; Haass et al., 1992; Shoji et al., 1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Notch</td>
<td>ICD effects transcription</td>
<td>(De Strooper et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ErbB4</td>
<td>ICD effects transcription</td>
<td>(Ni et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TβRI</td>
<td>ICD effects transcription</td>
<td>(Gudey et al., 2014; Mu et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N-Cadherin</td>
<td>ICD promotes proteosomal degradation</td>
<td>(Marambaud et al., 2003; Reiss et al., 2005)</td>
</tr>
<tr>
<td>Aspartyl protease</td>
<td>Signal peptide peptidase (SPP)</td>
<td>Type II membrane proteins</td>
<td>MHC Class I molecules</td>
<td>N-terminal fragment transported along with TAP and presented on the cell surface by HLA-E</td>
<td>(Lemberg et al., 2001)</td>
</tr>
<tr>
<td>Serine protease</td>
<td>Rhomboid protease (RHBDL2)</td>
<td>Type I membrane proteins</td>
<td>EGFR</td>
<td>ICD generated is localized to membrane and nuclear fractions</td>
<td>(Liao and Carpenter, 2012)</td>
</tr>
</tbody>
</table>
Notch signaling

Notch signaling plays a fundamental role at various stages of metazoan development, starting with embryogenesis, cell lineage progression, and differentiation (Andersson et al., 2011; Capaccione and Pine, 2013; Hansson et al., 2004; Lardelli et al., 1995). Notch was the prima facie of notched wing phenotype in *Drosophila melanogaster* in which the *Notch* gene was mutated (Mohr, 1919). The legacy of Notch discovery is nearing a century since its genesis in the early 1990s (Mohr, 1919), and slowly the enigma of Notch is being demystified.

In mammals, Notch signaling operates upon the binding of one of five Notch ligands (serrate-like ligands (Jagged 1, Jagged 2) or delta-like ligands (DLL-1, DLL-3, and DLL-4) to one of four transmembrane Notch receptors. This combination of ligand and receptor binding creates a cohort of signaling outputs (Andersson et al., 2011). Notch homologs have been identified in a wide variety of phylogenetic spectra, including but not limited to *Drosophila melanogaster*, to mammals (Ellisen et al., 1991; Kopan and Weintraub, 1993; Lardelli and Lendahl, 1993).

Notch receptor proteolysis involves three important steps. The initial step takes place at the trans-Golgi network, where the receptors are cleaved constitutively by furin-like convertases to form bipartite Notch receptors that translocate to the cell surface; this initial step is termed S1 cleavage (Blaumueller et al., 1997; Logeat et al., 1998). After the initial cleavage, Notch ligands from the neighboring cells bind to the bipartite Notch receptors, triggering ectodomain shedding by TACE. This ectodomain shedding leaves a truncated form of the Notch receptor called Notch extracellular truncation fragment (NEXT); this second event is referred to as S2 cleavage (Brou et al., 2000). In the third step, the NEXT fragment is processed by the γ-secretase machinery. PS1 (the catalytic subunit of the γ-secretase machinery) cleaves the
truncated Notch receptor at the transmembrane region, generating the Notch intracellular domain (NICD); this cleavage event is termed S3 cleavage (De Strooper et al., 1999). The third cleavage event liberates NICD from the plasma membrane, enabling translocation of the NICD to the nucleus, where it can regulate gene expression by binding to the CSL protein complex (CBF1/RBP-Jk in mammals, Su(H) in *Drosophila* and Lag-1 in *Caenorhabditis elegans*) in combination with other transcriptional co-activators or inhibit transcription by binding to repressors (Capaccione and Pine, 2013; Roy et al., 2007).

In the presence of an active transcriptional co-activator (e.g., p300 or MAMLs) bound to CSL and NICD, the Notch-induced transcriptional machinery regulates the expression of genes such as human hairy and enhancer of split 1 (HES1), hairy/enhancer-of-split related with YRPW motif families (HEY1), NF-κB, c-Myc, and Slug (Capaccione and Pine, 2013; McElhinny et al., 2008).

One of the master regulators of transcription in the Notch context is the Mastermind-like (MAML) family of proteins, which were initially identified in *Drosophila* (Smoller et al., 1990; Yedvobnick et al., 1988). Three mammalian MAML isoforms have been identified: MAML1, MAML2, and MAML3 (Wu et al., 2000; Wu et al., 2002). The MAML proteins do not bind to DNA. However, they act as co-activators by binding to the CSL and recruiting the NICD to this complex, thereby promoting gene transcription. MAML1 reportedly recruits p300/CBP and functions in a synergistic manner. MAML1 interaction with p300 leads to p300 phosphorylation, and this interaction also promotes MAML1 acetylation by p300 (Fryer et al., 2002; Saint Just Ribeiro et al., 2007). A number of co-repressors (e.g., N-CoR/SMRT, CIR, and SPEN) are also centrally involved in the Notch signaling cascade (McElhinny et al., 2008).
Crosstalk between Notch and TGF-β signaling

The conjunction of the Notch and TGF-β signaling paradigms dictates crucial decisions regarding cell fate in various organisms. Crosstalk has been reported between various downstream members of these pathways. TGFβ induces the expression of the Notch target gene Hes1 both in vitro and in vivo, and the synergistic effect of both TGFβ and Notch1 leads to enhanced expression of Hes1 (Blokszijl et al., 2003). Moreover, TGFβ stimulation enhances the interaction between the NICD and the R-Smad Smad3 (Blokszijl et al., 2003). TGFβ was also reported to activate the transcriptional repressor Hey1 and the Notch ligand- Jagged 1, and TGFβ activates complex formation between Smad3, Smad4, and the Hey1 promoter region, thereby promoting TGFβ-induced EMT (Zavadil et al., 2004). Interestingly, during heart development, collaboration of Notch signaling and TGFβ2, is required for the Snail1-induced endocardial EMT (Timmerman et al., 2004). Finally, both the Notch and TGFβ pathways cooperate at the transcriptional level by inducing Jagged 1, the transcriptional co-regulator CSL, and the cell cycle CDK inhibitor p21 to promote cytostasis (Niimi et al., 2007).
Present Investigation

Aims

The aim of this thesis was to investigate the roles of TRAF6, TACE, and PS1 in the cleavage of TβRI, as well as their biological significance. The particular aims of each paper were as follows.

**Paper I:** To elucidate the molecular mechanisms underlying the formation of an ICD of the TβRI, as well as the roles of TRAF6, TACE, and protein kinase C zeta type (PKCζ) in this process.

**Paper II:** To demonstrate the molecular mechanisms involved in the transmembrane cleavage of the TβRI by PS1 and the role of TRAF6 in the cleavage.

**Paper III:** To identify the possible acceptor lysine in TβRI that is ubiquitinated by TRAF6 and the functional role of this event in tumor promotion.

**Paper IV:** To investigate whether TGFβ utilizes the E3 ligase TRAF6 to regulate the potential downstream targets c-Jun and p21, and to assess their biological significance.
Materials and Methods

Cell Culture

The following cell lines were used in the experiments.

Human prostate cells (PC3U cells originating from PC3 cells) derived from a metastatic site (bone, grade IV adenocarcinoma), human prostate cells (LnCaP) derived from a metastatic site (left supraclavicular lymph node), and human lung cancer carcinoma cells (A549) were used in the study. Each of the above cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin and streptomycin (PEST). Cells were starved for 18 hours in RPMI-1640 medium supplemented with 1% FBS, 1% L-glutamine, and 1% PEST.

Human embryonic kidney (HEK) cells containing the SV40 T-antigen (293T). MDA-MB-231 cells derived from human mammary gland/breast from a metastatic site (pleural effusion, adenocarcinoma), genetically ablated TRAF6−/− mouse embryonic fibroblasts (MEFs), or WT TRAF6+/+ MEFs, genetically ablated PS1−/− MEFs and WT PS1+/+ MEFs were grown in Dulbecco’s modified essential medium (DMEM) supplemented with 10% FBS, 1% L-glutamine, and 1% PEST. HEK 293T and MDA-MB-231 cells were starved for 18 hours in DMEM medium supplemented with 1% FBS, 1% L-glutamine, and 1% PEST. MEFs were starved for 18 hours in DMEM medium supplemented with 0.5% FBS, 1% L-glutamine, and 1% PEST.

C57BL/6 mouse prostate epithelial cells (TRAMPC2) were cultured in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.005 mg/ml bovine insulin, 10 nM dehydroisoandrosterone, 5% FBS, 5% Nu-Serum IV,
and 1% PEST. Cells were starved for ≥18 hours in DMEM supplemented with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 0.005 mg/ml bovine insulin, 10 nM dehydroisoandrosterone, 1% FBS, 1% Nu-Serum IV, and 1% PEST.

All cells were incubated at 37°C, 5% CO₂ in a humidified chamber.

**Transfection**

**Plasmid transfections**

PC3U and HEK 293T cells were transfected with Fugene6 transfection reagent with plasmids encoding various constructs following the manufacturer’s instructions.

**siRNA transfections**

PC3U cells were transfected with oligofectamine transfection reagent with a non-targeting control RNA or a specific short interfering RNA (siRNA) following the manufacturer’s instructions.

**Protein analysis**

**Protein preparation**

After cells were stimulated for the indicated time periods with TGFβ1 (10 ng/ml), cells were harvested and washed once with PBS. Cells were then lysed in RIPA lysis buffer. Protein concentration was measured with a bicinchoninic acid protein assay kit (BCA) and equal amounts of protein was loaded onto SDS-PAGE gels.

For immunoprecipitation assays, cell lysates were immunoprecipitated with the protein of interest and incubated overnight at 4°C. The next day, protein G Sepharose beads were added
to the lysates and incubated for 1 hour at 4°C. The lysates were centrifuged and the protein G beads containing the protein complexes were washed with RIPA lysis buffer. Proteins were released from the protein G beads upon the addition of SDS sample buffer with reducing agent.

**Western blotting**

Equal amounts of protein were loaded onto SDS-PAGE gels. Proteins were transferred to a nitrocellulose membrane using an iBlot transfer machine. Membranes were probed with specific antibodies, as described in each experiment. Membranes were developed using ECL chemiluminescence and autoradiography. Biorad Quantityone software was used to quantify the intensity of the bands on the Western blots.

**Gene expression profiling by quantitative real-time RT-PCR**

RNA was prepared from cells using the Qiagen RNA isolation kit following the manufacturer’s instructions. Two micrograms of RNA were used to prepare cDNA using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis kit. One microgram of cDNA was used in a mixture containing water, SYBR green mix, and forward and reverse primers to perform real-time (RT)-PCR.

**Immunofluorescence**

After the cells were stimulated, they were harvested, washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 2% Triton X-100, blocked in 5% BSA, and incubated with primary and secondary antibodies, respectively. Finally, cells were mounted with mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI) and the co-localization of various proteins was visualized with confocal microscopy.
Ubiquitination assays

**In vivo ubiquitination assays**

Cells were harvested and washed with PBS. Next, PBS/SDS solution was added to the cells and they were boiled at 95°C, followed by the addition of PBS/NP40 buffer and protease inhibitors. After centrifugation, the slimy layer was removed and incubated overnight with an antibody raised against the protein of interest. Protein G sepharose beads were added and incubated for 1 hour. Next, samples were subjected to centrifugation and the supernatant was removed. Then the beads were washed with PBS/NP40 buffer, and sample buffer containing reducing agent was added. Samples were boiled at 95°C. Samples were loaded and SDS-PAGE electrophoresis was performed. Membranes were probed with specific primary antibodies (ubiquitin or Lys63-ubiquitin), incubated with secondary antibodies, and were developed using chemiluminescence. Detailed information is provided in the articles (Gudey et al., 2014; Sorrentino et al., 2008).

**In vitro ubiquitination assays (Paper III)**

Reaction mixtures containing E1, E2 (Ubc13-Uev1A), GST-TβRI, or GST-K178R-TβRI proteins (approximately 0.1 µg each) in the presence or absence of recombinant GST-TRAF6 (approximately 0.1 µg) with 20 mM Tris (pH 7.4), 50 mM NaCl, 10 mM MgCl₂, 10 mM Dithiothreitol (DTT), 10 mM ATP, 2.5 µM ubiquitin, and 100 µM MG132 were incubated at 37°C for 1 hour. The reaction was stopped with non-reducing sample buffer. The samples were loaded onto an SDS-PAGE gel and immunoblotted with antisera against TβRI (V22) (Santa Cruz Biotechnology), TRAF6 (Invitrogen), and ubiquitin (Cell signaling). Reaction mixtures with or without E1 and with or without E2 were used as negative controls.
Chromatin immunoprecipitation assays (Papers I, II)

Chromatin immunoprecipitation assays were performed according to the supplier’s protocol (Abcam). DNA and proteins were cross-linked with formaldehyde; cells were sheared; DNA was immunoprecipitated with the protein of interest and reverse cross-linked; and then DNA was purified. The purified DNA segments were amplified using qRT-PCR in triplicate with their respective primers.

Invasion assays

Invasion assays were performed using the CytoSelect Cell Invasion assay kit (Cell Biolabs). RPMI 1640 medium supplemented with 1% FCS, 1% L-glutamine, and 1% PEST was used to rehydrate the upper chamber. Next, cell suspension was seeded into the upper chamber with RPMI 1640 medium with or without TGFβ1 treatment and the lower chamber was filled with RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine, and 1% PEST. Non-invasive cells were removed from the upper chamber, and the invasive cells that protruded through the membrane into the lower chamber were stained with crystal violet staining solution. Invasive cells were photographed, and calorimetric quantification was performed with a spectrophotometer at an absorbance of 560 nm.

Proximity ligation assays

Proximity ligation assays (PLAs) were performed according to the manufacturer’s instructions (Sigma-Aldrich). After TGFβ1 treatment, PC3U cells were fixed, permeabilized, blocked, and incubated with primary antibodies. Secondary antibodies conjugated with PLA probes were added and incubated. Next, the slides were mounted and confocal microscopy
was used to visualize proteins in close proximity. The corresponding signals were quantified using Blob-Finder software.

**Nuclear cytoplasmic fractionation assays**

Cells were collected after treatment with TGFβ1, washed with PBS, and centrifuged; the pellet was collected. Buffer 1 [20 mM Tris HCl (pH 7.0), 10 mM KCl, 2 mM MgCl₂, 0.5% NP40, 1 mM aprotinin, 1 mM Pefabloc] was added to the pellet. Next, cells were sheared with a syringe and needle. After shearing, samples were centrifuged and the supernatant (the cytoplasmic fraction) was collected. Buffer 2 (buffer 1 with 0.5 M NaCl) was added to the pellet and the mixture was centrifuged. Supernatants were collected from this fraction (the nuclear fraction).

**Mutagenesis**

**Paper I**

Constitutively active TβRI tagged with green fluorescent protein (GFP-caTβRI) was constructed by incorporating full-length protein encoding constitutively active TβRI between the CMV promoter and the enhanced green fluorescent protein (EGFP) coding sequence. Constitutively active TβRI that was C-terminally tagged with hemagglutinin (HA-caTβRI) was mutated to G120I mutant caTβRI by site-directed mutagenesis. Mutagenesis was confirmed by sequencing the corresponding plasmids.

**Paper II**

HA-caTβRI was mutated to VI129AA mutant caTβRI using site-directed mutagenesis. Mutagenesis was confirmed by sequencing the corresponding plasmids.
Amino acid residues 1 to 178 were deleted from the full-length caTβRI to generate HA-TβRI-ICD. This region was cloned into a pcDNA3 vector, and an HA-tag was ligated to the C-terminus of the TβRI-ICD cDNA.

**Paper III**

HA-caTβRI was mutated to K178R mutant caTβRI and GFP-caTβRI was mutated to GFP-K178R mutant caTβRI using site-directed mutagenesis. Mutagenesis was confirmed by sequencing the corresponding plasmids.

**FACS analysis (Paper III)**

After the cells were stimulated for the respective time periods, cells were collected and fixed with 2% paraformaldehyde, permeabilized with Triton X-100 and blocked with 5% BSA. After blocking, cells were incubated with primary antibody, followed by incubation with the corresponding secondary antibody. Next, cells were incubated with propidium iodide solution and further analyzed using the FACS LSRII system (BD Biosciences).

**Immunohistochemistry (Paper IV)**

Tissue sections were deparaffinised and pre-treated. Next, the slides were incubated with hydrogen peroxide and blocked. Next, slides were incubated with primary antibody, and then incubated in HRP polymer. Finally, slides were counterstained with hematotoxylin, dehydrated with alcohol, and mounted onto a coverslip. The tissue sections were visualized using a bright field microscope.
**Animals and treatment (Paper II)**

We used C57BL/6 mice in the xenograft experiments. The local animal review board (Umea, Sweden) approved an ethical permit for these experiments (Approval ID: A110-12; Date: 21 August 2012). TRAMPC2 cells (4.2×10^5/ml) were injected subcutaneously into the mice. When palpable tumor volume was reached, initial tumour measurements were recorded. Next, mice were administered an intraperitoneal injection of DMSO (control) or \( \gamma \)-secretase inhibitor (DBZ) once daily for a period of 10 days. After completion of treatment, mice were sacrificed, and tumors were collected, weighed, and freezeed in liquid nitrogen. The tissue was further processed for RNA and protein analysis.

**Patient material**

In paper I, we used tumor tissues from prostate, kidney, and urinary bladder cancers. In paper IV, we used normal prostate tissues and prostate cancer tissues. The Uppsala Ethical Review Board granted ethical permits to use the tumor tissues and generate tissue slides in accordance with the Swedish Ethical Review Act.

**Statistical analysis**

**Papers I, III, and IV**

Student’s two-tailed \( t \)-test or one-way ANOVA was used for statistical analysis. \( P<0.05 \) was considered statistically significant. Statistitica software was used for statistical analysis.

**Paper II**

The Mann-Whitney U test (two-tailed) was primarily used for comparison between groups. IBM SPSS statistics 20 software was used to perform statistical analysis.
Results and Discussion

Paper I

TRAF6 ubiquitinates the TGFβ type I receptor to promote its cleavage and nuclear translocation in cancer

The ectodomain shedding of various transmembrane receptors by proteases has been reported in an increasing number of substrates (Hayashida et al., 2010). This mechanism results in either the generation of an ICD that has transcriptional significance or the desensitization of the signalling cascade. It was recently reported that a metalloprotease TACE cleaves the TβRI in an Erk MAP-kinase-dependent manner, resulting in the deregulation of TGFβ signalling (Liu et al., 2009). However, our data suggest that TGFβ stimulation causes Lys63-linked polyubiquitination of the TβRI by TRAF6, which promotes cleavage of the TβRI by TACE. We observed that ectopic expression of WT PKCζ promoted the expression and cleavage of the constitutively active TβRI and kinase dead TβRI, suggesting that cleavage occurs independent of the kinase activity of the TβRI. To further substantiate the role of PKCζ in the context of TβRI and TACE, experiments conducted using a PKCζ pseudosubstrate prevented TGFβ-induced colocalization of the TβRI and TACE.

Our results, which indicate that the ICD was generated as a result of the cleavage, favor its association with the transcriptional co-activator p300. We also found that TRAF6 is required for the interaction of the ICD with p300. qRT-PCR experiments indicate that WT TβRI regulates the transcription of genes involved in tumor invasion, such as Snail1 and MMP-2, in a TRAF6-dependent manner. Chromatin immunoprecipitation assays reveal binding of the TβRI-ICD to the endogenous Snail1 promoter in a TGFβ-dependent manner in PC3U cells. Our findings suggest that the TβRI is cleaved by TACE and an ICD is generated that
translocates to the nucleus, where it associates with p300 in nuclear promyelocytic leukemia (PML) bodies and drives invasion only in cancerous cells.

**Paper II**

**TRAF6 stimulates the tumor-promoting effects of the TGFβ type I receptor through the polyubiquitination and activation of Presenilin1**

The γ-secretase machinery is involved in the cleavage of various cell surface receptors. To date, reports indicate that approximately 66 different transmembrane receptors are cleaved in the transmembrane region (McCarthy et al., 2009). Cleavage of some receptors leads to the generation of an ICD. For example, the cleavage of Notch receptors at the transmembrane region generates an ICD that translocates to the nucleus and activates the transcription of target genes. Presenilins are regarded as the catalytic core of the γ-secretase machinery because they cleave various transmembrane receptors.

TβRII is constitutively active; upon ligand binding, it phosphorylates TβRI, triggering either the Smad signaling cascade or non-Smad signaling partners and leading to the transcription of certain TGFβ target genes (Massagué, 1998; Moustakas and Heldin, 2009). Previous reports suggest that the TβRI is cleaved by TACE at the ectodomain region (Liu et al., 2009). We have demonstrated that TRAF6 has an important role in the activation of TACE, and generates an ICD that translocates to the nucleus (Mu et al., 2011). However, the molecular mechanism underlying the liberation of the intracellular region remains unclear.

In this study, we provide evidence that the TβRI is cleaved in the transmembrane region in a fashion similar to the receptor of Notch. Our findings suggest that TGFβ regulates PS1 expression and that PS1 cleaves the TβRI at the transmembrane region, generating an ICD. TRAF6 mediates the cleavage by activating PS1 through TGFβ-dependent Lys63-linked polyubiquitination and recruiting PS1 to the TβRI complex, thus promoting the cleavage of
TβRI. Moreover, mutation of the TβRI at the cleavage site inhibits the cleavage of TβRI and obstructs its nuclear translocation. In the nucleus, the TβRI binds to its own promoter and drives its own gene expression. The nuclear translocated TβRI-ICD cooperates with the NICD, a core component in the Notch signaling pathway, to drive the expression of invasive genes such as Snail1. We also demonstrate that this TGFβ-induced oncogenic pathway functions in a prostate cancer xenograft in vivo model and that treatment with γ-secretase inhibitors was able to inhibit cleavage of TβRI.

Paper III

Identification of Lys178 as the acceptor lysine of TGF-beta type I receptor poly-ubiquitination

Ubiquitination is a reversible post-translational modification. During the process of ubiquitination, a covalent bond is created between the glycine of the ubiquitin molecule and the lysine of the target protein. Identifying the acceptor lysine in the target protein is crucial because the modification of this region of the protein could provide novel insights into biological functions.

Building on our previous observation that TβRI is ubiquitinated by TRAF6 in a Lys63-dependent manner, we investigated the acceptor lysine of TβRI that is prone to be ubiquitinated. We identified Lysine 178 as the acceptor lysine in the TβRI that is ubiquitinated by TRAF6; point mutation of lysine to arginine inhibited ubiquitination both in vivo and in vitro. Furthermore, the ubiquitination-deficient TβRI K178R mutant is unable to translocate to the nucleus, and is therefore unable to activate the transcription of invasive genes such as Snail1.
Paper IV

TGFβ engages TRAF6 and p38 to regulate c-Jun activity and the ability of prostate cancer cells to invade

TGFβ also signals by activating members of the MAPK family (e.g., Erk, JNK or p38). Our group has shown that the E3 ligase TRAF6 is autoubiquitinated upon the binding of TGFβ to its receptors (Sorrentino et al., 2008). Moreover, TRAF6 binds to a consensus motif in the TβRI sequence and is activated, which in turn polyubiquitinates its downstream target TAK1 in a Lys63-dependent manner, and thereby activates MKK3/6, which in turn activates the p38 pathway, leading to apoptosis. However, it is unclear which downstream targets of p38 signalling are, in the TGFβ-TRAF6 pathway.

As a continuation of the above observation, the present study demonstrates that TGFβ utilizes TRAF6 and the p38 MAPK to cause phosphorylation of the transcription factor c-Jun. We found that TRAF6 regulates the expression of both c-Jun and p21. Phosphorylation of c-Jun at Ser63 leads to its transactivation, whereby c-Jun regulates its own gene expression and activates p21 in a non-canonical, Smad-independent pathway in which TRAF6 and p38 play an integral role. Furthermore, c-Jun binds to the Snail1 promoter in a TRAF6-dependent manner and promotes invasion in PC3U cells.
Conclusions

Our data support the following conclusions to describe TGFβ signal transduction in cancerous cells (Figure 4):

✓ TGFβ induces the TRAF6-mediated Lys63-polyubiquitination of TβRI.

✓ TβRI is cleaved at the ectodomain region by TACE and at the transmembrane region by PS1. In combination, these cleavage events generate a cytosolic TβRI-ICD that translocates to the nucleus.

✓ TRAF6 promotes the cleavage of TβRI by recruiting PS1 into a complex with TβRI and promotes the Lys63-polyubiquitination of PS1.

✓ The nuclear translocated TβRI-ICD interacts with NICD in a TGFβ-dependent manner and promotes the expression of Jagged1, Snail1, and TβRI mRNAs.

✓ The nuclear localized TβRI-ICD binds to the transcriptional co-activator p300 and to the promoters of the genes encoding Snail1 and TβRI to drive the expression of EMT-related genes Snail1 and MMP2.

✓ Nuclear translocation of TβRI-ICD leads to invasion in cancerous cells in vitro (human prostate, breast, and lung carcinoma cells).

✓ In an in vivo prostate cancer xenograft model, treatment with the inhibitor γ-secretase inhibited generation of the TβRI-ICD, as well as the pro-invasive expression of the Snail1 gene.

✓ The TβRI nuclear translocation mechanism is a characteristic feature observed only in cancerous cells, not in normal prostate epithelial cells.
✓ TβRI-K178R in TβRI is the potential acceptor lysine that is ubiquitinated by TRAF6.

✓ TGFβ engages TRAF6 and p38 to cause activation of c-Jun, thereby promoting the c-Jun-dependent transcriptional regulation of p21 and Snail1 in a non-canonical, Smad-independent pathway.

**Figure 4.** A model of TGFβ signal transduction in cancerous cells.
Future Perspectives

Tumorigenesis is a multistep process in which normal cells transform into malignant tumor cells by acquiring certain characteristics that lead to metastasis, such as self-sufficiency in growth signals, escaping antigrowth signals, evading apoptosis, and tissue invasion. These characteristics are considered the hallmarks of cancerous cells (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). Once these characteristic features are evolved by epithelial tumor cells, they start the EMT process, which involves invasion, intravasation, systemic dissemination, extravasation, dormancy, and colonization, eventually establishing a secondary tumor at a site distant from its origin (Kalluri and Weinberg, 2009; Scheel and Weinberg, 2012; Valastyan and Weinberg, 2011).

The potent role of TGFβ in various biological effects, including embryogenesis, wound healing, differentiation, and proliferation, is evident from the massive amount of data accumulated about this multifunctional cytokine. Several transmembrane receptors are proteolytically processed in order to gain intracellular access to bind or recruit partners and relay biological signals that promote invasion and lead to tumorigenesis. However, the dual role of TGFβ as both a tumor promoter and a tumor suppressor is highly complex, and the switching of these roles is still unclear (Heldin et al., 2009; Massagué, 2008).

The components of this thesis try to address some of the hallmarks that characterize these cancerous cells in the context of TGFβ signaling. The thesis describes a novel molecular mechanism of TGFβ signaling in cancerous cells: TGFβ induces the Lys63-polyubiquitination of TβRI by TRAF6 and its cleavage by TACE and PS1 to release the TβRI-ICD, which then translocates to the nucleus. The nuclear localized TβRI-ICD binds to the transcriptional co-activator p300 and to the promoters of the genes encoding Snail1 and TβRI. It also drives the
expression of EMT-related genes Snail1 and MMP2, leading to invasion by cancerous cells. The nuclear translocated TβRI-ICD interacts with NICD in a TGFβ-dependent manner and promotes the mRNA expression of Jagged1, Snail1, and TβRI. Mutagenesis of the TβRI at the transmembrane region inhibited nuclear translocation. Moreover, we provide evidence that treatment with γ-secretase inhibitor prevented the generation of TβRI-ICD and the expression of pro-invasive gene Snail1 in an in vivo prostate cancer model, effectively inhibiting tumor growth and invasion (Gudey et al., 2014; Mu et al., 2011). This nuclear translocation mechanism is a characteristic feature observed only in cancerous cells (prostate, breast, and lung carcinoma cells), not in normal prostate epithelial cells, which suggests a potent positive feedback mechanism by which cancerous cells continuously innovate alternate mechanisms to increase their numbers.

Our findings contribute to the understanding of how TGFβ promotes tumor progression and invasion. However, a number of questions must be addressed in greater depth. For example, how does TβRI-ICD translocate to the nucleus; do importins act in the transport of the TβRI-ICD, in the report by Chandra et al., in which they claim that the TβRI holoreceptor translocates to the nucleus with the aid of importin β1 (KPNB1) (Chandra et al., 2012)? As we have reported that TβRI binds to its own promoter and also to the Snail1 promoter, it would be interesting to investigate whether the TβRI contacts the DNA directly; alternatively, this process may require other transcription factors. From a clinical perspective, it is also important to know at what stage of tumorigenesis the nuclear translocation of the TβRI takes place. In this newly discovered pathway, identifying the cleaved TβRI product that is responsible for the invasive nature of cancer cells might produce a possible biomarker.

Interestingly, RanBPM (Ran-binding protein in the microtubule-organizing center) has been reported to block the nuclear translocation of the TβRI by inhibiting an interaction between
TRAF6 and the TβRI (Zhang et al., 2014). In this context, it would be worthwhile to know the detailed biological significance of competitive binding of RanBPM to the TβRI, apart from blocking the nuclear translocation of the TβRI.

More broadly, one might also speculate whether other partners that promote metastasis (e.g., fibroblasts, endothelial cells, stromal components, microenvironmental components, or other inflammatory cytokines) may also be involved in the promotion of this paradigm. As Notch is one prime molecule being investigated in relation to cancer, might the reported interaction between TβRI-ICD and NICD be a molecular target for therapy?

Our findings mean that it is of prime importance to design molecular targets and traps to block nuclear translocation, because the illustrated mechanistic pathway elucidates a positive feedback loop that is initiated by the TβRI in order to maintain its required gene pool in cancerous cells and promote the expression of EMT-related genes to enhance the formation of distant metastases. With the current knowledge of γ-secretase inhibitors, which were used successfully in the in vivo prostate cancer xenograft model, it would be interesting to test such reagents in metastatic tumor models (it must be kept in mind that there are no proper metastatic prostate cancer models available). Because the cleavage event does not depend on TβRI kinase activity, designing small molecule inhibitors and monoclonal antibody therapy might be another way to block nuclear translocation of the TβRI. The emerging knowledge of nanobodies may also be used to target TβRI.

With the success of trastuzumab, a monoclonal antibody that is used to target the HER2/neu receptor in the treatment of breast cancers, the TβRI may be an emerging drug target that requires detailed investigation.
We have identified K178 as the acceptor ubiquitin lysine in TβRI. Because the mutagenesis of lysine to arginine blocked the invasive property in cancerous cells. It will also be interesting to explore the structural consequences of this mutant K178R TβRI receptor in detail. In addition, noting the convergence of the pathways that cause cleavage of the TβRI and the non-Smad signaling operated by the TRAF6-TAK1-p38 signaling cascade at the Snail level. It would be interesting to identify other co-regulators of Snail1 in the context of non-Smad signaling.
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