Chapter

Lys63-Linked Polyubiquitination of Transforming Growth Factor β Type I Receptor (TβRI) Specifies Oncogenic Signaling

Jie Song and Maréne Landström

Abstract

Transforming growth factor β (TGFβ) is a multifunctional cytokine with potent regulatory effects on cell fate during embryogenesis, in the normal adult organism, and in cancer cells. In normal cells, the signal from the TGFβ ligand is transduced from the extracellular space to the cell nucleus by transmembrane serine–threonine kinase receptors in a highly specific manner. The dimeric ligand binding to the TGFβ Type II receptor (TβRII) initiates the signal and then recruits the TGFβ Type I receptor (TβRI) into the complex, which activates TβRI. This causes phosphorylation of receptor-activated Smad proteins Smad2 and Smad3 and promotes their nuclear translocation and transcriptional activity in complex with context-dependent transcription factors. In several of our most common forms of cancer, this pathway is instead regulated by polyubiquitination of TβRI by the E3 ubiquitin ligase TRAF6, which is associated with TβRI. The activation of TRAF6 promotes the proteolytic cleavage of TβRI, liberating its intracellular domain (TβRI-ICD). TβRI-ICD enters the cancer cell nucleus in a manner dependent on the endosomal adaptor proteins APPL1/APPL2. Nuclear TβRI-ICD promotes invasion by cancer cells and is recognized as acting distinctly and differently from the canonical TGFβ-Smad signaling pathway occurring in normal cells.

Keywords: TRAF6, APPL1/2, TGFβ, biomarkers, cancer

1. Introduction

Ubiquitination is a crucial biological process both in normal homeostasis and in diseases including cancer and immunity-related disorders. In cancers, ubiquitination of various signaling molecules acts to both promote and suppress tumors [1]. In this chapter, we will focus on the tumor-promoting role of TRAF6 in different cancers.

1.1 Ubiquitination and TRAF6

Within the lifespan of proteins, it is difficult for them to avoid post-translational modifications, which determine their localization and function. Protein ubiquitination was discovered in the early 1980s, and is a dynamic post-translational

modification regulating many cellular processes. The best known role for ubiquitination is targeting proteins for their destruction by the proteasome. In recent years, however, nonproteolytic functions of ubiquitination, including in signal transduction, cell division and differentiation, endocytosis, and the DNA damage response, have been rapidly discovered [2].

Ubiquitin is a highly conserved protein of 76 amino acids that becomes covalently attached to the ε-amino group of lysine (Lys) residues of target proteins. There are three types of ubiquitination: mono-ubiquitination, multi-mono-ubiquitination, and polyubiquitination. Polyubiquitin chains are formed by the addition of ubiquitin residues to an ubiquitin molecule already linked to a protein and acting as an additional residue. The key features of ubiquitin are seven Lys residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) and an N-terminal Met residue, all of which can be further ubiquitinated to give rise to polyubiquitin chains of distinct linkages. Lys63-linked polyubiquitination is involved in endocytosis, signal transduction, and DNA-damage tolerance [3, 4]. During recent years has also linear ubiquitination been identified to occur through N-terminal Met residue of ubiquitin. It is created by the linear ubiquitin chain assembly complex (LUBAC), which so far is the only ubiquitin ligase known to build linear ubiquitin chains *de novo*. Linear ubiquitination is crucial for regulation of innate and adaptive immune responses, including inflammatory responses and regulation of signals leading to cell death [5–7].

Ubiquitination is catalyzed by a sophisticated enzymatic cascade involving three enzymes, an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3). E3 ligase usually determines the mechanism of ubiquitin transfer to target proteins, as it can recognize substrate and mediate the addition of ubiquitin [3, 8]. E3 ligases have been classified into three subfamilies: HECT (homologous to E6-AP C terminus) ligases, RING (really interesting new gene)/U-box ligases, and RBR (RING-between-RING) ligases [3]. TRAF6 is a Ring/U-box E3 ligase belonging to the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family.

TRAF family cytoplasmic proteins were originally identified as TNF receptor signaling adaptors that bind directly to the cytoplasmic region of TNF-R2. To date, six different TRAF family members (TRAF1–6) have been found in mammals. TRAF7 is controversially classified as a member of the TRAF family, as it lacks a TRAF homology domain and does not directly bind to any member of the TNFR superfamily, two key features used to define the TRAF family. The TRAF domain, located in the C-terminal portion of TRAF family proteins, is composed of an N-terminal less-conserved coiled-coil region (TRAF-N) and a C-terminal highly conserved subdomain (TRAF-C). The TRAF domain mediates protein–protein interactions, including association with upstream regulators and downstream effectors and homo- and hetero-dimerization of TRAF proteins. Thus, TRAF family members are involved in a variety of signal transduction pathways by interaction with receptors. These include the TNF, Toll-like receptor, NLR, TGFβ signaling pathways, and others. Through these interactions, TRAF family members participate in the regulation of a broad range of cellular processes, including proliferation, differentiation, apoptosis, and survival. With the exception of TRAF1, however, TRAFs also contain an N-terminal RING domain, indicating that they are E3 ubiquitin ligases [9, 10].

TRAF6 was isolated for the first time in 1996 in a yeast two-hybrid screen with CD40 as bait [11], and later independently found to mediate the expression of interleukin 1 (IL-1) signaling, based on a screen of an EST expression library [12]. TRAF6 is well conserved across species and broadly expressed in mammalian tissues such as brain, lung, liver, etc. As an E3 ligase, TRAF6 interacts with the E2 complex Ubc13-Uev1A and participates in a number of signal transduction

pathways, including those of nuclear factor kappa B (NF-κB), toll-like receptor 4 (TLR4), and TGFβ, the last of which is further discussed later in this chapter. Knockdown of TRAF6 or inhibition of TRAF6 E3 ligase activity *in vitro* suppresses the proliferation, survival, migration, invasion, and metastasis of many human epithelial cell lines [10].

$TRAF6^{-/-}$ mice, with a complete lack of normal T and B cell areas, exhibit perinatal or postnatal death due to severe splenomegaly, osteopetrosis, lymph node deficiency, and thymic atrophy [9]. All these findings indicate the critical and highly various roles of TRAF6 in cytokine signaling, innate immune responses, and perinatal and postnatal survival [9, 13].

1.2 The TGFβ signaling pathway and its role in cancer

Cells communicate by sending and receiving signals through cytokines and membrane-associated proteins. Among the secreted growth factors and cytokines, the TGFβ family attracts a lot of attention because it controls cell fate decisions during embryonic development, tissue homeostasis, and regeneration. All cells in the developing embryo and the adult can respond to TGFβ, as it regulates proliferation, differentiation, adhesion, movement, and apoptosis in a cell-context–dependent manner. Perturbation of TGFβ signaling is often seen in inflammatory diseases, fibrotic diseases, and cancers [14, 15].

1.2.1 Basics of TGFβ signaling

The TGFβ superfamily consists of more than 30 members in humans, and they are grouped into different subfamilies based on sequence similarity and functional criteria, including TGFβ isoforms, activins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activin, nodal, and anti-mullerian hormone (AMH). The TGFβ subfamily comprises three different isoforms: TGFβ1, TGFβ2, and TGFβ3. All of them act in an autocrine, paracrine, and sometimes endocrine manner [14, 16].

Mammalian genomes encode two subfamilies of TGFβ receptors, seven type I (TβRI) and five type II (TβRII) serine/threonine kinase receptors, which are classified by their structures and functions. Both types of receptors are single-pass transmembrane kinases and share structural similarities: they have an N-terminal cysteine-rich extracellular domain, an α-helical transmembrane domain, a short juxtamembrane sequence, and a C-terminal cytoplasmic kinase domain with 11 subdomains organized in an N-lobe and a C-lobe. A conserved glycine/serine-rich sequence, the GS domain, is present in the juxtamembrane domain only in TβRI [17, 18].

The most-studied mediators of TGFβ signaling pathways are Smad proteins. TGFβ signaling pathways include canonical Smad-dependent and non-canonical Smad- independent pathways [15, 19].

1.2.2 Smad-dependent TGFβ signaling pathways

Smad proteins are named after two proteins: *small body size (Sma)* in *Caenorhabditis elegans* and *mothers against decapentaplegic (Mad)* in *Drosophila melanogaster*. The mammalian genome encodes eight Smads which form three subfamilies based on their structures and functions: receptor-activated Smads (R-Smads; Smad 1, 2, 3, 5, and 8); a single common mediator of Smad (Co-Smad; Smad4); and two inhibitory Smads (I-Smads; Smad6 and Smad7). Smad2 and

Smad3 act as signal transducers for TGFβ, activin, and nodals, whereas Smad1, Smad5, and Smad8 mediate signals by BMPs and GDFs.

Upon TGFβ ligand binding, the two types of receptors are brought together and induce the formation of a heterotetrameric complex. The constitutively active type II receptor phosphorylates the type I receptor in its highly conserved GS domain, leading to the activation of its kinase. The active serine/threonine type I receptor propagates signaling by phosphorylating R-Smads, which in turn form a trimeric complex with Smad4 and then translocate to the nucleus. In the nucleus, the Smad complex works together with other transcription factors, coactivators, and corepressors to regulate the expression of genes such as snail family transcriptional repressor 1 (Snail1), plasminogen activator inhibitor type 1(PAI1), and matrix metallopeptidase 2 (MMP2). In summary, canonical Smad-dependent TGFβ signaling pathways regulate cell proliferation, apoptosis, and the epithelial-mesenchymal transition (EMT) [20, 21].

1.2.3 Smad-independent TGFβ signaling pathways

TGFβ non-canonical signaling pathways include the c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol-3′-kinase (PI3K), and extracellular signal-regulated kinase (Erk) signaling pathways [19].

TGFβ-activated kinase-1 (TAK1) is a serine/threonine kinase and member of the mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK) family. TRAF6 associates with a conserved consensus motif in TβRI. Upon TGFβ stimulation, the interaction of TRAF6 and TβRI is important for the autoubiquitination of TRAF6 and subsequent Lys63-polyubiquitination and activation of TAK1. Once activated, TAK1 phosphorylates protein mitogen-activated kinase kinase 3/6 (MKK3/6), activating the JNK and p38 signaling pathways to drive apoptosis or EMT [22, 23].

1.2.4 TGFβ receptor endocytosis

Endocytosis is a process by which cells internalize extracellular materials and portions of their plasma membrane together with cell surface receptors. It has been divided into two categories, clathrin-dependent and clathrin-independent endocytosis [24]. TGFβRs can be internalized via both clathrin-dependent and clathrin-independent caveolae-mediated endocytosis [14, 25].

Both TβRII and TβRI appear to undergo rapid internalization in the presence and absence of ligand stimulation. After internalization, TGFβRs are found in the phosphatidylinositol-3-phosphate (PI3P)-enriched and early endosome antigen (EEA1)-positive endosomes, which recruit Smad anchor for receptor activation (SARA) to facilitate phosphorylation of R-Smads. Phosphorylated R-Smads in endosomes then dissociate from SARA and the receptors, and translocate to the nucleus together with Smad4 to regulate target gene expression [26].

In caveolae-mediated endocytosis, TGFβ signaling is turned off by the interaction between TGFβRs and Smad7-Smurf2, which leads to the degradation of TGFβRs [27].

1.2.5 TGFβ signaling in cancer

TGFβ signaling in cancer is a double-edged sword, acting as both a tumor suppressor in normal and pre-malignant cells and as a tumor promoter in malignant cells. The response to TGFβ is context dependent. TGFβ is produced by cancer cells

or stromal cells in large amounts within the cancer microenvironment, influencing not only on the cancer cells but also non-tumor cells, such as fibroblasts and immune cells [15, 28].

In the early malignant stage, TGFβ suppresses tumor progression by inducing apoptosis and inhibiting proliferation. However, malignant cells always escape this tumor-suppressive response through loss of the core TGFβ pathway or its suppressive arms, thereby turning TGFβ into a stimulator of cancer progression. As a tumor promoter, TGFβ is involved in angiogenesis, tumor growth, evasion of immune surveillance, migration, invasion, and metastasis [15, 29].

1.3 PI3K/AKT pathway

The PI3K pathway is one of the most commonly activated pathways in human cancers, regulating cell proliferation, survival, metabolism, and vesicle trafficking. This pathway's activation is initiated by various molecules, such as insulin, glucose, growth factors, and cytokines [30, 31]. PI3Ks are classified into three classes based on sequence homology and substrate specificity. Class I PI3Ks have two subfamilies, IA and IB, classified according to their different regulatory mechanisms. Class IA PI3K, a heterodimer, consists of a p110 catalytic subunit and a p85 regulatory subunit. Class I PI3K generates PtdIns [3,4,5]P_3 (PIP_3) from PtdIns [4,5]P_2 (PIP_2) *in vivo*. PIP_3 acts as a second messenger to activate downstream signaling pathways, including AKT/mTOR (mechanistic target of rapamycin kinase) pathways. Class IA PI3Ks are the focus of this chapter [31, 32].

The primary structure of p85 includes an N-terminal Src homology 3 (SH3) domain, a RhoGap homology region located between two proline-rich domains, and two SH2 domains (nSH2 and cSH2 domains) separated by a p110-binding iSH2 domain [33]. Upon binding to an activated receptor tyrosine kinase (RTK) or G protein-coupled receptor (GPCP), p85 interacts with receptors directly or indirectly via the SH2 domains, which mediate the translocation of the p85-p110 complex to the cell membrane. This induces a conformational change and activates the catalytic activity of p110 to phosphorylate PIP_2 to generate PIP_3 [30, 33].

The serine/threonine protein kinase AKT has three isoforms, AKT1, AKT2, and AKT3. PIP_3 binding induces a conformational change in AKT that recruits phosphoinositide-dependent kinase (PDK1) to phosphorylate AKT on Thr308. The mTOR complex 2 (mTORC2) phosphorylates AKT on Ser473, fully activating AKT. All three isoforms are activated in the same manner [31, 34]. In addition to phosphorylation, other post-translational modifications regulate the activity of AKT. These include dephosphorylation, glycosylation, acetylation, ubiquitination, and SUMOylation. Lys48-linked polyubiquitination of AKT is mediated by multiple E3 ligases, such as BRCA1, Chip, MULAN, and TTC, and has been shown to promote proteasome-dependent degradation. By contrast, Lys63-linked polyubiquitination, which is mediated by TRAF6, Skp2, and NEDD4, is implicated in the membrane localization and phosphorylation of AKT [34, 35]. After activation, AKT regulates downstream signaling pathways by phosphorylating protein targets, including protein kinases, transcription factors, metabolic enzymes, cell cycle proteins, and others [34].

It has been reported that TGFβ can activate the PI3K signaling pathway directly or indirectly. Of note, upon TGFβ stimulation, the phosphorylation of AKT acts in a Smad-independent manner [36–38]. Moreover, p85 constitutively interacts with TβRII and binds to TβRI after TGFβ stimulation [39]. The crosstalk between the PI3K/AKT and TGFβ signaling pathways attracts a lot of attention, as both of them play important roles in cancer.

1.4 APPL proteins

APPL1 was first identified as an AKT2-binding protein in a yeast two-hybrid screen in 1999 [40]. APPL1 was initially called DIP-13α (DCC-interacting protein 13α), as it interacts with the tumor suppressor protein DCC (deleted in colorectal cancer) [41]. APPL proteins, which include APPL1 and APPL2, are named after their unique structure, the multifunctional adaptor proteins that contain a pleckstrin homology (PH) domain, phosphotyrosine binding (PTB) domain, and leucine zipper motif [40]. APPL1 and APPL2 share 54% sequence identity and many identical binding partners. Both are found only in eukaryotes [42]. Briefly, APPL1 consists of the N-terminal Bin1/amphiphysin/rvs167 BAR domain (originally identified as the leucine zipper motif), followed by a pleckstrin homology domain (PH domain), a BPP (region "between PH and PTB domains") domain, a PTB domain, and a C-terminal CC domain [42, 43]. The BAR, PH, and PTB domains are the key functional domains. The BAR and PH domains usually act as a unit involved in sensing and stabilizing membrane curvature and anchor the host proteins to membrane compartments. The PTB domain interacts with phospholipids, receptors such as DCC, and signaling molecules including AKT2. In summary, APPL proteins regulate important physiological processes via their unique domains [44].

APPL1 is a marker of early endosomes that are precursors of classical PI3P-positive endosomes [45]. Depletion of PI3P by PI3K inhibitors leads to the reversion of EEA1-positive endosomes to the APPL1 stage, enlargement of APPL1 endosomes, and enhanced growth factor signaling [45]. APPL proteins are implicated in signaling pathways such as the EGF [46], NF-κB [47], and TGFβ signaling pathways [48]. Through its roles in endocytosis and signal transduction, APPL1 has been reported to mediate proliferation, apoptosis, and migration [44, 49].

2. TGFβ causes Lys63-linked polyubiquitination of TβRI by TRAF6, inducing the formation of the intracellular domain of TβRI (TβRI-ICD), which promotes tumor invasion by inducing the transcription of target genes in the nucleus

We identified the intracellular domain of TβRI by using two different TβRI antibodies: v22, which recognizes the C-terminal part of TβRI; and H100, which was raised against the N-terminal part of TβRI. Upon TGFβ stimulation, the C-terminal fragment of TβRI accumulates in the nucleus. However, the N-terminal part of TβRI still localizes mainly to the cell membrane [50].

We have previously shown that TRAF6 interacts with a consensus binding site in TβRI [22]. Interestingly, TRAF6 is known to cause Lys63-linked polyubiquitination of TβRI, as well as the generation of TβRI-ICD. It has been reported that TNFα-converting enzyme (TACE) induces the cleavage of TβRI through the ERK MAP-kinase pathway [51]. We confirmed that TACE cleaves TβRI by using both an activator of protein kinase C (PKC), which can activate TACE, and an inhibitor of TACE. The TACE cleavage site in TβRI is the Gly-Leu bond at position 120–121, which is close to the transmembrane domain. The G120I mutant has intact kinase activity but does not accumulate in the nucleus in response to TGFβ [50]. PKCζ, which interacts with TRAF6 [52], is required for the formation and nuclear translocation of TβRI-ICD [50].

By immunofluorescence and co-immunoprecipitation, TβRI-ICD has been shown to associate with p300 in the nucleus in a PKCζ-dependent manner. Moreover, p300 mediates the acetylation of TβRI-ICD [50]. In the nucleus,

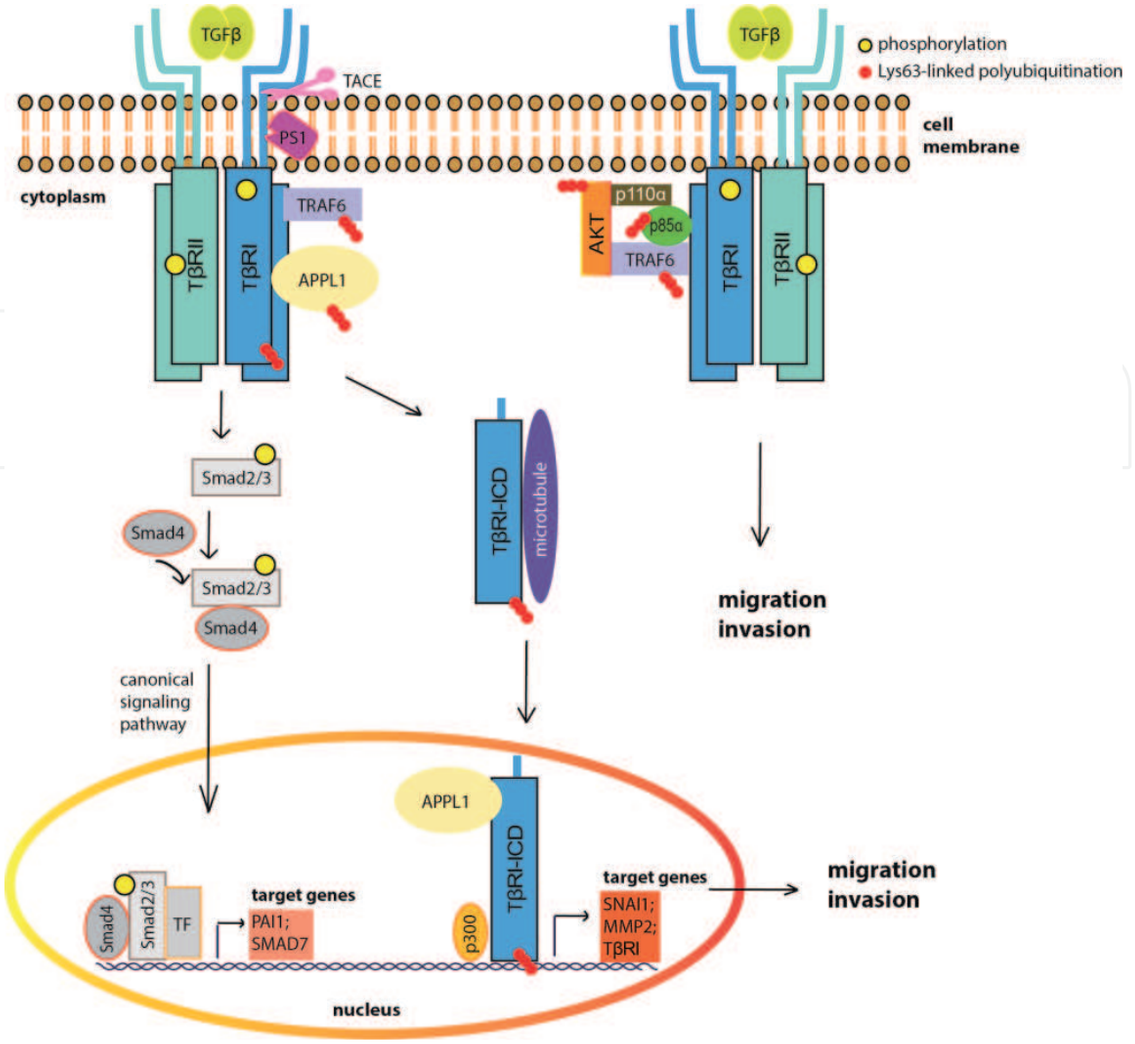

Figure 1.
Proposed model for canonical and TRAF6-mediated non-canonical TGFβ signaling pathways. Upon TGFβ stimulation, constitutively TβRII activates TβRI, leading to the phosphorylation of Smad2 and Smad3. R-Smads, which form a trimeric complex with Smad4, translocate to the nucleus for target genes expression, such as PAI1 and Smad7. In response to TGFβ, TRAF6 induces the formation of TβRI-ICD, which is generated by the proteolytic enzymes TACE and PS1. APPL proteins are necessary for the nuclear translocation of TβRI-ICD. In the nucleus, TβRI-ICD interacts with p300 and promotes tumor invasion indirectly or directly by inducing the transcription of target genes, such as SNAI1, MMP2, and TβRI. TRAF6 also causes the polyubiquitination of p85α, leading to the activation of the PI3K-AKT signaling pathway.

TβRI-ICD regulates the transcription of target genes, such as SNAI1 and MMP2, promoting the invasiveness of cancer cells. Interestingly, the cleavage of TβRI occurs only in malignant prostate cancer cells (PC-3 U), but not in normal primary human prostate epithelial cells. Nuclear accumulation of TβRI-ICD is also observed in prostate cancer, breast cancer, and bladder cancer, suggesting that preventing nuclear translocation of TβRI-ICD could be a new target in cancer treatment [50] (**Figure 1**).

3. TRAF6 induces Lys63-linked polyubiquitination and activation of PS1, leading to the cleavage of TβRI and promoting tumor invasion

Presenilin 1 (PS1) is the catalytic core of the γ-secretase complex, which mediates the cleavage of many cell surface type I transmembrane receptors, such as APP, Notch, and CD44 [53]. TRAF6 is reported to interact with PS1, which enhances the

autoubiquitination of TRAF6 [54]. To further investigate the molecular mechanism of TβRI cleavage, we examined the possible involvement of PS1.

TGFβ stimulation enhances the abundance and activity of PS1. PS1 interacts with TβRI in a TRAF6-dependent manner. TRAF6 causes Lys63-linked polyubiquitination of PS1 in response to TGFβ, leading to the activation of PS1. After the initial cleavage of TβRI by TACE, activated PS1 mediates a second cleavage between Val129 and Ile 130 in the transmembrane domain of TβRI, leading to the generation and nuclear translocation of TβRI-ICD [55].

In the nucleus, TβRI-ICD induces its own gene expression to promote cell invasion (**Figure 1**). Experiments using γ-secretase inhibitors showed that PS1 is required for TGFβ-induced cell invasion *in vitro*. Furthermore, γ-secretase inhibitors also reduce the generation of TβRI-ICD and tumor growth in a prostate cancer xenograft model *in vivo*, suggesting a novel therapeutic strategy for cancers [55].

4. Lys178 in TβRI is the acceptor lysine of Lys63-linked polyubiquitination by TRAF6, which is involved in TGFβ-induced invasion and cell cycle regulation

In *in vitro* and *in vivo* ubiquitination assays, TβRI Lys178, the only lysine close to the TRAF6 consensus binding site, has been identified as the acceptor lysine in polyubiquitination by TRAF6. Overexpression of HA-TβRI-K178R inhibits the formation and nuclear translocation of TβRI-ICD in response to TGFβ. The HA-TβRI-K178R mutant has no effect on the kinase activity of TβRI, indicating that it does not interfere with the phosphorylation of Smad2. However, transfection of cells with HA-TβRI-K178R does alter p38 activation [56].

We identified additional genes targeted by nuclear TβRI-ICD by using qRT-PCR. Overexpression of HA-TβRI-K178R changes the expression of genes implicated in invasiveness and cell cycle regulation, such as Vimentin, Twist1, N-cadherin, CCND1, and p73. As expected, the expression of PAI1 is unchanged, due to the intact kinase activity of HA-TβRI-K178R. Fewer cells enter G1 from G0 in HA-TβRI-K178R-transfected cells compared with HA-TβRI-transfected cells after incubation with TGFβ for 48 hours, as CCND1 is poorly regulated in the mutant-transfected cells. PC-3 U cells expressing HA-TβRI-K178R were less invasive than cells expressing HA-TβRI. In summary, the polyubiquitination of TβRI on Lys178 influences both cell cycle regulation and invasion [56].

5. APPL proteins are required for the nuclear translocation of the TGFβ type I receptor intracellular domain

Next, we started to investigate the mechanism of nuclear translocation of TβRI-ICD. As APPL proteins are involved in cargo trafficking from the endosomal membranes to the nucleus after EGF stimulation [46], we considered the possibility that APPL proteins play the same role in the translocation of TβRI-ICD.

The nuclear accumulation of TβRI-ICD in response to TGFβ decreased after APPL1/2 expression was silenced. Moreover, APPL1 overexpression increased the nuclear translocation of TβRI-ICD, indicating that APPL proteins are necessary for the transport of TβRI-ICD into the nucleus. Interestingly, APPL proteins also affect the activation of Smad2 and p38, suggesting that APPL1/2 may play a role in both canonical and non-canonical TGFβ signaling [48].

Using co-immunoprecipitation and an *in vitro* binding assay, we confirmed that APPL1, through its C-terminus, interacts directly with TβRI. TGFβ stimulation

enhances the formation of the APPL1-TβRI complex. Moreover, treatment with PI3K inhibitors such as LY294002 and wortmannin enlarges APPL1 early endosomes and prevents the maturation of APPL1 endosomes to EEA1-positive endosomes, and causes increased association of APPL1 with TβRI. In contrast, TβRI kinase activity is not necessary for the interaction between APPL1 and TβRI. Furthermore, endogenous APPL1 has been shown in a nuclear fractionation assay to interact with TβRI-ICD in the nucleus after TGFβ stimulation [48].

It has been reported that APPL1 undergoes Lys63-linked polyubiquitination mediated by TRAF6 in response to insulin in primary mouse hepatocytes [57]. We found that TRAF6 also causes Lys63-linked polyubiquitination of APPL1 after TGFβ stimulation of human prostate (PC-3 U) cells. Of note, TRAF6 is required for both the formation of the APPL1-TβRI complex and the interaction between APPL1 and β-tubulin. In summary, we conclude that APPL proteins are required for the nuclear translocation of TβRI-ICD, possibly via the microtubule system [48] (**Figure 1**).

Nuclear TβRI-ICD promotes the invasion of various cancer cells by inducing the transcription of pro-invasion genes, such as MMP2 and MMP9 [50]. After silencing the expression of APPL1/2, TGFβ-induced invasion is reduced, probably due to a decline in the nuclear accumulation of TβRI-ICD, in both a prostate cancer cell line (PC-3 U) and a breast cancer cell line (MDA-MB-231). MMP2 and MMP9 gene expression also decreases after APPL1/2 knock-down. We also found that APPL1 staining is correlated with a high Gleason Score (indicating the tumor invasiveness and bad prognosis), consistent with previous reports [48, 58]. Interestingly, using an *in situ* proximity ligation assay, we found more APPL1–TβRI-ICD complexes in high-Gleason Score patients. In summary, APPL1–TβRI-ICD is a potential prognostic marker for prostate cancer patients [48].

6. TGFβ activates the PI3K/AKT signaling pathway by TRAF6-mediated polyubiquitination of p85α

It has been reported that TGFβ can activate AKT. However, the detailed mechanism is still unclear. We found that, upon TGFβ stimulation, TβRI forms a complex with AKT and the phosphorylation of AKT correlates with its interaction with TβRI and TRAF6 [59]. As TRAF6 causes Lys63-linked polyubiquitination and activation of AKT upon IGF-1, LPS, and IL-1β stimulation [60], we investigated whether TRAF6 plays the same role in the TGFβ signaling pathway. Using an *in vivo* ubiquitination assay in PC-3 U cells, we demonstrated that TGFβ induces Lys63-linked polyubiquitination of AKT, which is mediated by TRAF6. TGFβ stimulation induces recruitment of the activated-AKT–TRAF6–TβRI complex to cell membrane ruffles. The interaction between TβRI and AKT does not require TβRI kinase activity, but depends on the regulatory subunit of PI3K, p85α. Furthermore, p85α is also involved in the activation and ubiquitination of AKT [59].

The interaction between TRAF6 and p85α is enhanced after TGFβ stimulation. TGFβ induces the Lys63-linked polyubiquitination of p85α in a TRAF6-dependent manner (**Figure 1**). The kinase activities of TβRI and TβRII are not involved in p85α ubiquitination. p85α was found to associate with TβRI upon TGFβ stimulation, but not with TβRII, and TβRI kinase activity is not necessary for the interaction between p85α and TβRI. We found that TGFβ induces PI3K activity in a TRAF6-dependent manner, but independently of TβRI kinase activity, and that TGFβ promotes cell migration and invasion via the PI3K pathway and TRAF6 [59]. Using mass spectrometry and an *in vivo* ubiquitination assay, we identified Lys513 and/or

Lys519 in the iSH2 domain as the major residue(s) of Lys63-linked polyubiquitination of p85α. Overexpression of a K513/K519 double mutant not only suppresses PI3K activity and AKT phosphorylation, but also inhibits cell migration and invasion. Finally, using an *in situ* proximity ligation assay performed in prostate cancer tissue samples, we found that polyubiquitination of p85α is correlated with the aggressiveness of the prostate cancer, suggesting that the polyubiquitination of p85α could be a prognostic marker for this disease [59]. As both the TGFβ and PI3K pathways are deregulated in cancers, finding the link between these two pathways will be important for future cancer research [61].

7. Conclusions

Ubiquitination regulates a broad spectrum of physiological processes, including cell proliferation, apoptosis, differentiation, and others [1, 2]. We have shown that, upon TGFβ stimulation, TRAF6 causes Lys63-linked polyubiquitination of p85α, leading to the activation of the AKT signaling pathway [59]. Moreover, TGFβ, via TRAF6, causes Lys63-linked polyubiquitination of TβRI and its PKCζ-dependent cleavage by TACE [50]. After this initial cleavage by TACE, PS1 is activated by TRAF6-mediated polyubiquitination, which results in a second cleavage of TβRI, by PS1 [55]. APPL proteins are involved in the nuclear translocation of TβRI-ICD [48]. In the nucleus, TβRI-ICD promotes the transcription of pro-invasion genes, such as SNAI1, MMP2, and TβRI itself [50, 55]. TβRI-ICD can be found in cancer cell lines, but not in normal prostate epithelial cell lines or in the normal prostate epithelium [50]. Inhibitors of γ-secretase, which prevent the generation of TβRI-ICD, suppress cell invasion *in vitro* and tumor growth *in vivo*, indicating a possible novel therapeutic target in cancer [55].

Acknowledgements

Fund: CAN 2017/544, Swedish Medical Research Council (2019-01598), Prostatacancerförbundet, King Gustaf V and Queen Victoria's Foundation of Freemasons, Novo Nordic Foundation and Lions Cancer Research Foundation, Umeå University, the County of Västerbotten (RV 933125, RV 73891). The funders did not play a role in manuscript design, data collection, data analysis, data interpretation, or writing of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

APPL1	adaptor protein phosphotyrosine interaction, PH domain, and leucine zipper containing 1
APPL2	adaptor protein phosphotyrosine interaction, PH domain, and leucine zipper containing 2
EEA1	early endosome antigen 1
ICD	intracellular domain
MAPK	mitogen-activated protein kinase

MAPKKK	mitogen-activated protein kinase kinase kinase
MKK	mitogen-activated protein kinase kinase
MMP	matrix metallopeptidase
NF-κB	nuclear factor kappa B
PC-3 U	prostate cancer-3-Uppsala
PH domain	pleckstrin homology domain
PI3K	phosphatidylinositol-3′-kinase
PKC	protein kinase C
PS1	presenilin 1
SARA	Smad anchor for receptor activation
TACE	TNFα-converting enzyme
TβRI	type I transforming growth factor β receptor
TβRII	type II transforming growth factor β receptor
TGFβ	transforming growth factor β
TNF	tumor necrosis factor alpha
TRAF6	tumor necrosis factor receptor-associated factor 6

Author details

Jie Song and Maréne Landström*
Medical Biosciences, Pathology, Umeå University, Umeå, Sweden

*Address all correspondence to: marene.landstrom@umu.se

References

[1] Mansour MA. Ubiquitination: Friend and foe in cancer. The International Journal of Biochemistry & Cell Biology. 2018;**101**:80-93. DOI: 10.1016/j.biocel.2018.06.001

[2] Chen ZJ, Sun LJ. Nonproteolytic functions of ubiquitin in cell signaling. Molecular Cell. 2009;**33**:275-286. DOI: 10.1016/j.molcel.2009.01.014

[3] Dittmar G, Winklhofer KF. Linear ubiquitin chains: Cellular functions and strategies for detection and quantification. Front Chem. 2020;**7**:Article 915. DOI: 10.3389/fchem.2019.00915

[4] Huang Q, Zhang X. Emerging roles and research tools of atypical ubiquitination. Proteomics. 2020:201900100. DOI: 10.1002/pmic.201900100

[5] Walczak H, Iwai K, Dikic I. Generation and physiological roles of linear ubiquitin chains. BMC Biology. 2012;**10**:23. DOI: 10.1186/1741-7007-10-23

[6] Iwai K, Fujita H, Sasaki Y. Linear ubiquitin chains: NF-κB signalling, cell death and beyond. Nature Reviews. Molecular Cell Biology. 2014;**15**:503-508. DOI: 10.1038/nrm3836

[7] Spit M, Rieser E, Walczak H. Linear ubiquitination at a glance. Journal of Cell Science. 2019;**132**:jcs208512. DOI: 10.1242/jcs.208512

[8] Swatek KN, Komander D. Ubiquitin modifications. Cell Research. 2016;**26**:399-422. DOI: 10.1038/cr.2016.39

[9] Xie P. TRAF molecules in cell signaling and in human diseases. Journal of Molecular Signaling. 2013;**8**:7. DOI: 10.1186/1750-2187-8-7

[10] Zhu S, Jin J, Gokhale S, Lu AM, Shan H, Feng J, et al. Genetic alterations of TRAF proteins in human cancers. Frontiers in Immunology. 2018;**9**:Article 2111. DOI: 10.3389/fimmu.2018.02111

[11] Ishida T, Mizushima S, Azuma S, Kobayashi N, Tojo T, Suzuki K, et al. Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. Journal of Biological Chemistry. 1996;**271**:28745-28748. DOI: 10.1074/jbc.271.46.28745

[12] Cao Z, Xiong J, Takeuchi M, Kurama T, Goeddel DV. TRAF6 is a signal transducer for interleukin-1. Nature. 1996;**383**:443-446. DOI: 10.1038/383443a0

[13] Dainichi T, Matsumoto R, Mostafa A, Kabashima K. Immune control by TRAF6-mediated pathways of epithelial cells in the EIME (epithelial immune microenvironment). Frontiers in Immunology. 2019;**10**:Article 1107. DOI: 10.3389/fimmu.2019.01107

[14] Yakymovych I, Yakymovych M, Heldin CH. Intracellular trafficking of transforming growth factor β receptors. Acta Biochimica et Biophysica Sinica. 2018;**50**:3-11. DOI: 10.1093/abbs/gmx119

[15] Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer. Immunity. 2019;**50**:924-940. DOI: 10.1016/j.immuni.2019.03.024

[16] Dijke PT, Arthur HM. Extracellular control of TGFβ signalling in vascular development and disease. Nature Reviews. Molecular Cell Biology. 2007;**8**:857-869. DOI: 10.1038/nrm2262

[17] Moustakas A, Heldin C-H. Mechanisms of TGFβ-induced

epithelial–mesenchymal transition. Journal of Clinical Medicine. 2016;**5**. DOI: 10.3390/jcm5070063

[18] Derynck R, Budi EH. Specificity, versatility, and control of TGF-β family signaling. Science Signalling. 2019;**12**:eaav5183. DOI: 10.1126/scisignal.aav5183

[19] Mu Y, Gudey SK, Landström M. Non-Smad signaling pathways. Cell and Tissue Research. 2012;**347**:11-20. DOI: 10.1007/s00441-011-1201-y

[20] Heldin C-H, Landström M, Moustakas A. Mechanism of TGF-β signaling to growth arrest, apoptosis, and epithelial-mesenchymal transition. Current Opinion in Cell Biology. 2009;**21**:166-176. DOI: 10.1016/j.ceb.2009.01.021

[21] Heldin C, Moustakas A. Signaling receptors for TGF-β family members. Cold Spring Harbor Perspectives in Biology. 2016;**8**. DOI: 10.1101/cshperspect.a022053

[22] Sorrentino A, Thakur N, Grimsby S, Marcusson A, von Bulow V, Schuster N, et al. The type I TGF-β receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nature Cell Biology. 2008;**10**:1199-1207. DOI: 10.1038/ncb1780

[23] Yamashita M, Fatyol K, Jin C, Wang X, Liu Z, Zhang YE. TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-β. Molecular Cell. 2008;**31**:918-924. DOI: 10.1016/j.molcel.2008.09.002

[24] Doherty GJ, McMahon HT. Mechanisms of endocytosis. Annual Review of Biochemistry. 2009;**78**:857-902. DOI: 10.1146/annurev.biochem.78.081307.110540

[25] Chen Y-G. Endocytic regulation of TGF-beta signaling. Cell Research. 2009;**19**:58-70. DOI: 10.1038/cr.2008.315

[26] Balogh P, Katz S, Kiss AL. The role of endocytic pathways in TGF-β signaling. Pathology Oncology Research. 2013;**19**:141-148. DOI: 10.1007/s12253-012-9595-8

[27] Hu CT, Wu JR, Wu WS. The role of endosomal signaling triggered by metastatic growth factors in tumor progression. Cellular Signalling. 2013;**25**:1539-1545. DOI: 10.1016/j.cellsig.2013.03.022

[28] Yu Y, Feng XH. TGF-β signaling in cell fate control and cancer. Current Opinion in Cell Biology. 2019;**61**:56-63. DOI: 10.1016/j.ceb.2019.07.007

[29] David CJ, Massagué J. Contextual determinants of TGFβ action in development, immunity and cancer. Nature Reviews. Molecular Cell Biology. 2018;**19**:419-435. DOI: 10.1038/s41580-018-0007-0

[30] Thorpe LM, Yuzugullu H, Zhao JJ. PI3K in cancer: Divergent roles of isoforms, modes of activation and therapeutic targeting. Nature Reviews. Cancer. 2015;**15**:7-24. DOI: 10.1038/nrc3860

[31] Hoxhaj G, Manning BD. The PI3K–AKT network at the interface of oncogenic signalling and cancer metabolism. Nature Reviews. Cancer. 2020;**20**:74-88. DOI: 10.1038/s41568-019-0216-7

[32] Madsen RR, Vanhaesebroeck B. Cracking the context-specific PI3K signaling code. Science Signalling. 2020;**13**:eaay2940. DOI: 10.1126/scisignal.aay2940

[33] Vallejo-Díaz J, Chagoyen M, Olazabal-Morán M, González-García A, Carrera AC. The opposing roles of PIK3R1/p85α and PIK3R2/p85β in Cancer. Trends in Cancer. 2019;**5**:233-244. DOI: 10.1016/j.trecan.2019.02.009

[34] Manning BD, Toker A. AKT/PKB signaling: Navigating the network. Cell. 2017;**169**:381-405. DOI: 10.1016/j.cell.2017.04.001

[35] Chan C-H, Jo U, Kohrman A, Rezaeian AH, Chou P-C, Logothetis C, et al. Posttranslational regulation of Akt in human cancer. Cell & Bioscience. 2014;**4**:59. DOI: 10.1186/2045-3701-4-59

[36] Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor β-mediated epithelial to mesenchymal transition and cell migration. The Journal of Biological Chemistry. 2000;**275**:36803-36810. DOI: 10.1074/jbc.M005912200

[37] Lamouille S, Derynck R. Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway. The Journal of Cell Biology. 2007;**178**:437-451. DOI: 10.1083/jcb.200611146

[38] Zhang L, Zhou F, Dijke PT. Signaling interplay between transforming growth factor-β-receptor and PI3K/AKT pathways in cancer. Trends in Biochemical Sciences. 2013;**38**:612-620. DOI: 10.1016/j.tibs.2013.10.001

[39] Yi JY, Shin I, Arteaga CL. Type I transforming growth factor β receptor binds to and activates phosphatidylinositol 3-kinase. The Journal of Biological Chemistry. 2005;**280**:10870-10876. DOI: 10.1074/jbc.M413223200

[40] Mitsuuchi Y, Johnson SW, Sonoda G, Tanno S, Golemis EA, Testa JR. Identification of a chromosome 3p14.3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2. Oncogene. 1999;**18**:4891-4898. DOI: 10.1038/sj.onc.1203080

[41] Liu J, Yao F, Wu R, Morgan M, Thorburn A, Finley RL, et al. Mediation of the DCC apoptotic signal by DIP13α. The Journal of Biological Chemistry. 2002;**277**:26281-26285. DOI: 10.1074/jbc.M204679200

[42] Liu Z, Xiao T, Peng X, Li G, Hu F. APPLs: More than just adiponectin receptor binding proteins. Cellular Signalling. 2017;**32**:76-84. DOI: 10.1016/j.cellsig.2017.01.018

[43] Li J, Mao X, Dong LQ, Liu F, Tong L. Crystal structures of the BAR-PH and PTB domains of human APPL1. Structure. 2007;**15**:525-533. DOI: 10.1016/j.str.2007.03.011

[44] Deepa SS, Dong LQ. APPL1: Role in adiponectin signaling and beyond. American Journal of Physiology. Endocrinology and Metabolism. 2009;**296**:E22-E36. DOI: 10.1152/ajpendo.90731.2008

[45] Zoncu R, Perera RM, Balkin DM, Pirruccello M, Toomre D, De Camilli P. A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. Cell. 2009;**136**:1110-1121. DOI: 10.1016/j.cell.2009.01.032

[46] Miaczynska M, Christoforidis S, Giner A, Shevchenko A, Uttenweiler-Joseph S, Habermann B, et al. APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. Cell. 2004;**116**:445-456. DOI: 10.1016/s0092-8674(04)00117-5

[47] Hupalowska A, Pyrzynska B, Miaczynska M. APPL1 regulates basal NF-κB activity by stabilizing NIK. Journal of Cell Science. 2012;**125**:4090-4102. DOI: 10.1242/jcs.105171

[48] Song J, Mu Y, Li C, Bergh A, Miaczynska M, Heldin C-H, et al. APPL proteins promote TGFβ-induced nuclear transport of the TGFβ type I receptor intracellular domain. Oncotarget.

2016;**7**:279-292. DOI: 10.18632/oncotarget.6346

[49] Diggins NL, Webb JD. APPL1 is a multi-functional endosomal signaling adaptor protein. Biochemical Society Transactions. 2017;**45**:771-779. DOI: 10.1042/BST20160191

[50] Mu Y, Sundar R, Thakur N, Ekman M, Gudey SK, Yakymovych M, et al. TRAF6 ubiquitinates TGFβ type I receptor to promote its cleavage and nuclear translocation in cancer. Nature Communications. 2011;**2**:330. DOI: 10.1038/ncomms1332

[51] Liu C, Xu P, Lamouille S, Xu J, Derynck R. TACE-mediated ectodomain shedding of the type I TGF-β receptor downregulates TGF-β signaling. Molecular Cell. 2009;**35**:26-36. DOI: 10.1016/j.molcel.2009.06.018

[52] Feng Y, Longmore GD. The LIM protein Ajuba influences Interleukin-1-induced NF-κB activation by affecting the assembly and activity of the protein kinase Cζ/p62/TRAF6 signaling complex. Molecular and Cellular Biology. 2005;**25**:4010-4022. DOI: 10.1128/mcb.25.10.4010-4022.2005

[53] McCarthy JV, Twomey C, Wujek P. Presenilin-dependent regulated intramembrane proteolysis and γ-secretase activity. Cellular and Molecular Life Sciences. 2009;**66**:1534-1555. DOI: 10.1007/s00018-009-8435-9

[54] Powell JC, Twomey C, Jain R, McCarthy JV. Association between Presenilin-1 and TRAF6 modulates regulated intramembrane proteolysis of the p75NTR neurotrophin receptor. Journal of Neurochemistry. 2009;**108**:216-230. DOI: 10.1111/j.1471-4159.2008.05763.x

[55] Gudey SK, Sundar R, Mu Y, Wallenius A, Zang G, Bergh A, et al. TRAF6 stimulates the tumor-promoting effects of TGFβ type I receptor through polyubiquitination and activation of presenilin. Science Signalling. 2014;**7**:ra2. DOI: 10.1126/scisignal.2004207

[56] Sundar R, Gudey SK, Heldin C-H, Landström M. TRAF6 promotes TGFβ-induced invasion and cell-cycle regulation via Lys63-linked polyubiquitination of Lys178 in TGFβ type I receptor. Cell Cycle. 2015;**14**:554-565. DOI: 10.4161/15384101.2014.990302

[57] Cheng KKY, Lam KSL, Wang Y, Wu D, Zhang M, Wang B, et al. TRAF6-mediated ubiquitination of APPL1 enhances hepatic actions of insulin by promoting the membrane translocation of Akt. The Biochemical Journal. 2013;**455**:207-216. DOI: 10.1042/BJ20130760

[58] Johnson IRD, Parkinson-Lawrence EJ, Keegan H, Spillane CD, Barry-O'Crowley J, Watson WR, et al. Endosomal gene expression: A new indicator for prostate cancer patient prognosis? Oncotarget. 2015;**6**:37919-37929. DOI: 10.18632/oncotarget.6114

[59] Hamidi A, Song J, Thakur N, Itoh S, Marcusson A, Bergh A, et al. TGF-β promotes PI3K-AKT signaling and prostate cancer cell migration through the TRAF6-mediated ubiquitylation of p85α. Science Signalling. 2017;**10**:eaal4186. DOI: 10.1126/scisignal.aal4186

[60] Yang W, Wang J, Chan C, Lee S, Campos DA, Lamothe B, et al. The E3 ligase TRAF6 regulates Akt ubiquitination and activation. Science. 2009;**325**:1134-1138. DOI: 10.1126/science.1175065

[61] Song J, Landström M. TGFβ activates PI3K-AKT signaling via TRAF6. Oncotarget. 2017;**8**:99205-99206. DOI: 10.18632/oncotarget.22275