Non-canonical TGFβ signaling pathways in prostate cancer

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Cover: Immunostaining of GFP-APPL1 (green) and pp38 (red) in PC-3U cells, which were treated with TGFβ for 2 hours.
To my family
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**Abstract**

Prostate cancer is the second leading cause of cancer-related death in men in the Western world. Deregulation of transforming growth factor β (TGFβ) signaling pathway is frequently detected in prostate cancer and contributes to tumor growth, migration, and invasion. In normal tissue and the early stages of cancer, TGFβ acts as a tumor suppressor by regulating proliferation, differentiation, and apoptosis. In later stages of cancer, TGFβ acts as a tumor promoter by inducing angiogenesis, tumor invasion, and migration. Thus, it is important to investigate the molecular mechanisms behind the tumor-promoting effects of TGFβ, which is the topic of this thesis.

The tumor necrosis factor receptor--associated factor 6 (TRAF6) controls non-canonical TGFβ signals due to its enzymatic activity, causing polyubiquitination of the cell membrane-bound, serine/threonine kinase TGFβ type I receptor (TβRI) and its subsequent cleavage in the extracellular domain by tumor necrosis factor α–converting enzyme (TACE) in a protein kinase Cζ (PKCζ)-dependent manner. TRAF6 also recruits the active γ-secretase complex to the TβRI, resulting in a second cleavage in the transmembrane region and the liberation of the TβRI intracellular domain (TβRI-ICD), which enters the nucleus, where it associates with the transcriptional co-regulator p300. In **Paper I**, the aim was to elucidate by which mechanisms TβRI-ICD enters the nucleus. We found that the endocytic adaptor protein APPL1 interacts with TβRI and PKCζ. APPL proteins are required for TβRI translocation from endosomes to the nucleus via microtubules in a TRAF6-dependent manner. Moreover, APPL proteins are important for TGFβ-induced cell invasion, and high levels of APPL1 are detected by immunohistochemistry in prostate cancer. Finally, we demonstrated that the APPL1–TβRI complex visualized with the *in situ* proximity ligation assay (PLA) correlates with Gleason score, indicating that it might be a novel prognostic marker for aggressive prostate cancer. In **Paper II**, the aim was to explore by which mechanisms TGFβ causes activation of the AKT pathway, which regulates migration and therapy resistance of cancer cells. We found that the E3 ligase activity of TRAF6 induces Lys63-linked polyubiquitination of p85α upon TGFβ stimulation, resulting in plasma membrane recruitment, Lys63-linked polyubiquitination, and subsequent activation of AKT. Moreover, the TRAF6 and PI3K/AKT pathway were found to be crucial for the TGFβ-induced migration. Importantly, we demonstrated, by PLA, a correlation between Lys63-linked polyubiquitination of p85α and aggressive prostate cancer in tissue sections from patients with prostate cancer. In **Paper III**, the aim was to investigate the mechanisms for TGFβ-induced activation of PKCζ and the role of PKCζ in tumor regression. We found that TRAF6 caused Lys63-linked polyubiquitination of PKCζ. By using two novel chemical compounds that inhibit PKCζ, we demonstrated that PKCζ is crucial for prostate cancer cell survival and invasion. In **Paper IV**, the aim was to investigate further the target genes for the nuclear TβRI-ICD–APPL1 complex identified in Paper I. We provide evidence that APPL proteins and the TGFβ signaling pathway are important for cell proliferation. TβRI regulates cell mitosis and cytokinesis by binding to AURKA in the centrosome and AURKB in the midbody. APPL1 also interacts with AURKB and survivin. TβRI kinase inhibitor suppresses the activation of AURKA and AURKB. In summary, the results reported in this thesis suggest the potential usefulness of the identified signaling components of the tumor-promoting effects of TGFβ as drug targets and biomarkers for aggressive prostate cancer.
Original Articles

This thesis is based on the following paper and manuscripts:


III. Yabing Mu, **Jie Song**, Guangxiang Zang, Linlin Gao, Timothy C. Gahman, Maréne Landström. TGFβ-induced activation of PKCζ confers invasive prostate cancer growth. *Manuscript*.

IV. **Jie Song**, Chunyan Li, Carl-Henrik Heldin, Maréne Landström. TGFβ type I receptor and endosomal APPL regulate AURKB during mitosis and cytokinesis. *Manuscript*.

(* indicates that these authors contributed equally to the work)

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Related Articles

1. Mikkel Roland Holst, Maite Vidal-Quadras, Jie Song, Jeanette Blomberg, Magnus Lundborg, Madlen Hubert, Maréne Landström, Richard Lundmark. Clathrin-independent endocytosis suppresses cancer cell blebbing and invasion. *Cell Reports (under revision).*
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>anti-mullerian hormone</td>
</tr>
<tr>
<td>AP-2</td>
<td>adaptor protein complex-2</td>
</tr>
<tr>
<td>APC/C</td>
<td>anaphase-promoting complex/cyclosome</td>
</tr>
<tr>
<td>APPL1</td>
<td>adaptor protein phosphotyrosine interaction, PH domain, and leucine zipper containing 1</td>
</tr>
<tr>
<td>APPL2</td>
<td>adaptor protein phosphotyrosine interaction, PH domain, and leucine zipper containing 2</td>
</tr>
<tr>
<td>AURKA</td>
<td>Aurora kinase A</td>
</tr>
<tr>
<td>AURKB</td>
<td>Aurora kinase B</td>
</tr>
<tr>
<td>AURKC</td>
<td>Aurora kinase C</td>
</tr>
<tr>
<td>BAD</td>
<td>B cell lymphoma associated death</td>
</tr>
<tr>
<td>BIR</td>
<td>baculovirus inhibitor of apoptosis protein repeat</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CIE</td>
<td>clathrin-independent endocytosis</td>
</tr>
<tr>
<td>CPC</td>
<td>chromosomal passenger complex</td>
</tr>
<tr>
<td>CRPC</td>
<td>castration-resistant prostate cancer</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EEA1</td>
<td>early endosome antigen 1</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial–mesenchymal transition</td>
</tr>
<tr>
<td>FOXO</td>
<td>forkhead box O</td>
</tr>
<tr>
<td>GDF</td>
<td>growth and differentiation factor</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein–coupled receptor</td>
</tr>
<tr>
<td>GS domain</td>
<td>glycine/serine domain</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia-inducible factor</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>INCENP</td>
<td>inner centromere protein</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</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>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKFeK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MCAK</td>
<td>mitotic centromere-associated kinesin</td>
</tr>
<tr>
<td>MKK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MH</td>
<td>Mad homology</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export signal</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NuRD</td>
<td>nucleosome remodeling and histone deacetylase</td>
</tr>
<tr>
<td>ICD</td>
<td>intracellular domain</td>
</tr>
<tr>
<td>Par6</td>
<td>partitioning defective 6</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PC-3U</td>
<td>prostate cancer-3-Uppsala</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PH domain</td>
<td>pleckstrin homology domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3'-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA</td>
<td>proximity ligation assay</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate-specific antigen</td>
</tr>
<tr>
<td>PS1</td>
<td>presenilin 1</td>
</tr>
<tr>
<td>PTB domain</td>
<td>phosphotyrosine binding domain</td>
</tr>
<tr>
<td>PtdIns(4,5)P₂(PIP₂)</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P₃(PIP₃)</td>
<td>phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>Rab5</td>
<td>Rab-protein 5</td>
</tr>
<tr>
<td>RBD</td>
<td>Ras-binding domain</td>
</tr>
<tr>
<td>RING</td>
<td>really interesting new gene</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad anchor for receptor activation</td>
</tr>
<tr>
<td>SBE</td>
<td>Smad binding element</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SHIP</td>
<td>SH2 domain-containing inositol-5'-phosphatase</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>Smad</td>
<td>Sma- and Mad-related protein</td>
</tr>
<tr>
<td>TACE</td>
<td>TNFα-converting enzyme</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGFβ-activated kinase 1</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TRAF6</td>
<td>tumor necrosis factor receptor (TNFR)-associated factor 6</td>
</tr>
<tr>
<td>TβRI</td>
<td>type I transforming growth factor β receptor</td>
</tr>
<tr>
<td>TβRII</td>
<td>type II transforming growth factor β receptor</td>
</tr>
<tr>
<td>USP4</td>
<td>ubiquitin-specific peptidase 4</td>
</tr>
</tbody>
</table>
Introduction

Prostate cancer

Prostate cancer is the most common cancer and the leading cause of cancer-related death in men in Sweden. In 2014, a total of 10,985 new cases of prostate cancer were diagnosed in the country (The National Board of Health and Welfare, Sweden). About 99% of cases occur in men those over the age of 50, but the median age at diagnosis is falling.

The incidence of prostate cancer has increased, probably because of the introduction of the prostate-specific antigen test (PSA) since the early 1990s. However, the PSA test leads to over-treatment and has not decreased the death rate from prostate cancer (Djulbegovic et al., 2010), which has led to further screening for new diagnostic and prognostic markers. Recently, it has been reported that altered expression of early endosomal markers in prostate cancer provides a new focus on biomarker studies (Johnson et al., 2014, 2015; Song et al., 2016). Currently, the most common grading system for prostate cancer is the Gleason grading system, which is used to indicate how likely it is that a tumor will spread based on its microscopic appearance (Gleason and Mellinger, 1974). This system uses a scale from 1 to 5, where 5 represents the more aggressive tumor pattern. Two grades are given, one to the most common area and the other to the second most common area, respectively. Then the pathologist adds together the two grades to obtain the Gleason score (GS). The GS ranges from 2 to 10 and has a very strong prognostic value as a predictor of death from prostate cancer. Patients with a high GS (8–10) have worse survival outcomes. In 2014, a new grading system was proposed that separated a GS of 7 into two different groups: GS 3+4=7 and GS 4+3=7, according to the different associated prognoses (Kryvenko and Epstein, 2016).

Advanced prostate cancer is usually accompanied by metastasis to the bone, but it can also metastasize to liver, lungs, and lymph nodes. The outcome of prostate cancer is mainly determined by the presence or absence of metastasis (Jemal et al., 2010). The situation-dependent treatments for men with prostate cancer include watchful waiting or active surveillance, surgery, radiotherapy, hormone therapy, and chemotherapy. Unfortunately, many tumors treated with castration therapy relapse within a few years into what is termed castration-resistant prostate cancer (CRPC), which is an incurable stage with a mean survival time of around only 1–2 years (Kirby et al., 2011).
Thus, a main focus for scientists is to develop new drugs to prolong survival with improved quality of life.

In prostate cancer, high levels of transforming growth factor β (TGFβ) have been detected and are associated with a poor prognosis (Jones et al., 2009). TGFβ acts as a tumor suppressor or a promoter depending on the cell context. It is the main bone-derived factor responsible for cancer metastasis in bone (Juarez and Guise, 2011; Massagué, 2012). Targeting the TGFβ pathway is a potential therapeutic strategy for prostate cancer, including use of antisense oligonucleotides, neutralizing antibodies, and small molecule inhibitors (Jones et al., 2009; Lahn et al., 2005).

**TGFβ**

**TGFβ superfamily**

TGFβ was isolated and characterized three decades ago (Massagué, 1990). Members of the TGFβ family have important roles in the regulation of embryogenesis and in tissue homeostasis in adults, as well as in cancer progression, through regulation of proliferation, differentiation, apoptosis, migration, and epithelial-mesenchymal transition (EMT) (Massagué, 2012; Moustakas and Heldin, 2016).

**TGFβ ligand**

TGFβ family cytokines comprise 33 members in humans, including activins, bone morphogenetic proteins (BMPs), TGFβ isoforms, growth and differentiation factors (GDFs), activin, nodal, and anti-mullerian hormone (AMH). They are usually divided into three major subfamilies: BMPs, TGFβs, and activin/inhibin/nodal, according to their similarities in structure and functional activities.

In mammals, TGFβ consists of three isoforms: TGFβ1, TGFβ2 and TGFβ3. They can act in autocrine, paracrine, and sometimes endocrine manners (Dijke and Arthur, 2007). TGFβ1 null mutation in mice results in severe
multiorgan inflammation and early death (Kulkarni et al., 1993). TGFβ2 knock out mice exhibit perinatal death and multiple development defects, including cardiac, craniofacial and urogenital defects (Sanford et al., 1997). TGFβ3 null mutation mice die within 20 hours of birth, due to abnormal lung development and cleft palate (Kaartinen et al., 1995). TGFβ is synthesized within cells as a precursor molecule, and before secretion through the Golgi apparatus, the C-terminal domain of the precursor is cleaved off. After secretion, however, TGFβ remains non-covalently bound to the latency-associated peptide (LAP), which prevents its association from signaling receptors. The complex of TGFβ and LAP is called the small latent complex (SLC). The mechanisms of activation of latent TGFβ are related to the perturbation of the association between TGFβ and LAP by thrombospondin-1, integrins, and proteinases (such as the matrix metalloproteinases MMP2/MMP9). SLC can link to a large secretory glycoprotein known as latent TGFβ-binding protein (LTBP) to form the large latent TGFβ complex (LLC). LTBP, which interacts with fibrillin microfibrils, is important for LLC binding to the extracellular matrix (ECM). Activation of TGFβ requires the liberation of LLC from the microfibrils and ECM (Dijke and Arthur, 2007).

**TGFβ receptors**

The TGFβ receptor family is classified into two subfamilies according to their structures and functions, i.e. type I and type II serine/threonine kinase receptors. Both types of receptor are single-pass transmembrane proteins, organized into an N-terminal cysteine-rich extracellular domain that binds the ligand, an α-helical transmembrane domain, and a C-terminal intracellular serine/threonine kinase domain. A conserved glycine/serine-rich domain (GS domain) (the SGSGSG sequence), which is present only in the type I receptor, is located immediately upstream of the kinase domain (Figure 1). Humans express seven different type I receptors (ALKs 1–7) and five type II receptors (ActR1I-A, ActR1I-B, BMPRII, AMHRII, TβRII) (Heldin and Moustakas, 2016).

**TGFβ signaling pathways**

The most-studied and best-known mediator proteins of the TGFβ signaling pathway are Smads, named from two proteins: small body size
Figure 1. TβRI structure.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>TGFβ subfamily</th>
<th>BMP subfamily</th>
<th>Co-Smad</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type II receptor</td>
<td>TβRI</td>
<td>ActRII-A,B</td>
<td>ActRII-A,B</td>
</tr>
<tr>
<td>Type I receptor</td>
<td>TβRI (ALK5)</td>
<td>ALK4.7</td>
<td>ALK1,2,3,6</td>
</tr>
<tr>
<td>R-Smad</td>
<td>Smad2/Smad3</td>
<td>Smad1/Smad5/Smad8</td>
<td></td>
</tr>
<tr>
<td>I-Smad</td>
<td>Smad7</td>
<td>Smad6/Smad7</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. The TGFβ superfamily.

(Sta) in Caenorhabditis elegans and mothers against decapentaplegic (Mad) in Drosophila melanogaster. Eight Smads encoded in the human genome are divided into three subfamilies based on structure and function: receptor-regulated Smads (R-Smads; Smads 1, 2, 3, 5, and 8); the common mediator of Smad (Co-Smad), Smad4; and inhibitory Smads (I-Smads; Smad6 and Smad7). Smad1, 5, and 8 principally propagate signals by BMPs and GDFs, while Smad2 and 3 serve in signaling by TGFβ, activin, and nodal. Smad4 is
a common binding partner of all of the R-Smads. Smad6 and Smad7 control TGFβ signaling in a negative feedback loop (Table 1) (Heldin et al., 2009).

R-Smads and Co-Smad include an N-terminal Mad-homology 1 (MH1) domain, a linker region, and a C-terminal Mad-homology 2 (MH2) domain. I-Smads lack the structures of an N-terminal MH1 domain but contain the MH2 domain (Figure 2). The MH1 domain is a DNA-binding segment, that is enabled by a β-hairpin structure and stabilized by a zinc atom. The linker region contains a PPXY (PY) motif that can recognize and associate with the WW domain of ubiquitin ligases Smurf1 and Smurf2, leading to ubiquitination and degradation of R-Smads through the proteasome. The serine and threonine residues in the linker region can be phosphorylated by kinases such as mitogen-activated protein kinases (MAPKs), glycogen synthase kinase-3β (GSK-3β) and cyclin-dependent kinases (CDKs). Smad4 contains a nuclear export signal (NES) but lacks the PY motif. The MH2 domain provides sites for the interaction with receptors, co-activators, co-repressors, transcription factors, and other Smads (Massagué et al., 2005; Schmierer and Hill, 2007).

**Figure 2.** Structure of the Smad family.

TGFβ cytokines initiate signaling through bringing together two types of receptors, inducing the formation of a heterotetrameric complex. The constitutively active type II receptor phosphorylates several serine and threonine residues in the strictly conserved GS domain of the type I receptor, giving rise to its conformational changes and activation. Activated type I receptor propagates signaling by phosphorylating the C-terminal Ser-X-Ser
(SXS) motif of R-Smads, which translocate and accumulate in the nucleus, regulating target genes together with Smad4 (Massagué et al., 2005). Smad2 activation and nuclear accumulation also require an intact microtubule network (Batut et al., 2007). Because of the 30 amino acid residues encoded by exon 3, Smad2 cannot bind directly to DNA, in contrast with other R-Smads and Smad4. The DNA sequences binding with Smads are designated as Smad binding elements (SBEs), and the minimal SBE includes only four base pairs, 5’-AGAC-3’ (Schmierer and Hill, 2007). Phosphorylation of Smad3 in the MH1 domain by protein kinase C (PKC) inhibits its binding to DNA, suggesting a regulatory role of PKC in Smad-mediated transcription (Yakymovych et al., 2001). As genomes are enriched in SBEs, Smad-dependent TGFβ signaling pathways regulate expression of approximately 300 genes, controlling cell proliferation, apoptosis, and EMT (Heldin et al., 2009).

TGFβ also signals via non–Smad signaling pathways, such as Erk, c-Jun N-terminal kinase (JNK), the p38 MAPK pathways, and the phosphatidylinositol 3′-kinase (PI3K) pathway, which will be discussed later.

The role of TGFβ signaling pathways in cancer

The hallmarks of cancer include maintaining proliferative signaling, resisting growth suppressors, evading cell death, inducing limitless replicative potential, developing blood vessels (angiogenesis), and enabling invasion and metastasis. In 2011, two other hallmarks were proposed: abnormal metabolism and evading the immune system (Hanahan and Weinberg, 2011).

In normal and premalignant cells, TGFβ acts as a tumor suppressor by regulating proliferation, differentiation, apoptosis, and the cellular microenvironment. However, cancer cells always lose the TGFβ-suppressive response by loss of the core pathway (such as TβRII) or loss of the suppressor arm. Thus, TGFβ can act as a tumor promoter by evading immune surveillance, activating angiogenesis, and inducing tumor growth, invasion, and migration (Massagué, 2008).

TGFβ induces cell cycle arrest in G1 by suppressing the expression of the transcription factor Myc and inducing the expression of the CDK inhibitors p15Ink4b, p21Cip1, and p27Kip1 (Heldin et al., 2009). TGFβ also induces apoptosis by inhibiting the PI3K/AKT/survivin pathway (Wang et al., 2008).
Although TGFβ inhibits proliferation of most epithelial cells, it stimulates proliferation of certain mesenchymal and cancer cell lines, such as smooth muscle cells and glioma cells. TGFβ is an inducer of EMT, which promotes tumor invasion and dissemination due to the cell junction-free, motile phenotype that the cells obtain (Massagué, 2008). The polarity complex protein partitioning defective 6 (Par6) can be phosphorylated by TβRII, leading to the degradation of RHOA and tight junction disassembly. TGFβ regulates the expression of SNAI1, SNAI2, ZEB1, and ZEB2 to repress the expression of E-cadherin, resulting in the disruption of adherence junctions. TGFβ-induced expression and activation of MMP2 and MMP9 are important for the enhancement of migratory and invasive properties of endothelial cells for angiogenesis (Moustakas and Heldin, 2016).

TGFβ receptors endocytosis

Endocytosis

Endocytosis is a process for the uptake of extracellular materials as well as the internalization of cell surface receptors surrounded by an area of plasma membrane, which then invaginates and buds off inside the cell to form a vesicle containing the ingested molecules (Sorkin and von Zastrow, 2002). Endocytotic pathways can be subdivided into two categories: clathrin-dependent and clathrin-independent endocytosis. Some of these pathways can be stimulated by specific signals or ligands (such as epidermal growth factor receptor (EGFR)), whereas others are constitutive (for example, transferrin receptor) (McMahon and Boucrot, 2011). Ligand-induced endocytosis was initially thought to negatively regulate signaling by attenuating the number of receptors in the plasma membrane and inducing receptor degradation, but later it was found to play an important role in regulating the signals in time and space (Sorkin and von Zastrow, 2009). Endocytosis also controls ligand availability and activity, such as the endocytosis of ligands in DSL (Delta, Serrate and LAG-2) family, required for activating Notch signaling in Drosophila and mammals (Scita and Fiore, 2010).

Clathrin-dependent endocytosis is the best-characterized pathway and is responsible for the entry of nutrients, antigens, pathogens, growth factors, and receptors into cells (Le Roy and Wrana, 2005). In clathrin-mediated
endocytosis, the receptor is internalized into clathrin-coated pits, followed by coat assembly, membrane invagination, and vesicle scission. After uncoating, released vesicles fuse with tubule-vesicular compartments, designated as ‘early endosomes.’ The cargos can be degraded in lysosomes or sent back to the cytoplasm to be reused after sorting in early endosomes or multi-vesicular bodies (McMahon and Boucrot, 2011; Mosesson et al., 2008). Endosomes are the key center for the signals because of the dual role they have: They assist signals by providing a platform for receptors, ligands, and certain lipids and proteins, or they inhibit signals by maintaining the receptors and ligands in them (Scita and Fiore, 2010). Clathrin-dependent endocytosis is used by many cell surface receptors, including EGFR, G protein–coupled receptors (GPCRs), and insulin receptors. Adaptor proteins (such as adaptor protein complex-2 (AP-2)) and accessory proteins are required in this pathway because clathrin cannot bind directly to the membrane or cargo receptors (McMahon and Boucrot, 2011).

Consensus does not exist around classification of clathrin-independent endocytosis (CIE) because many different factors control it, such as dynamin and GTPase. Caveolae-mediated CIE is well characterized pathway and regulated by dynamin, PKC and Src kinase. Caveolae are defined as 50–80-nm flask-shaped invaginations of the plasma membrane marked by the presence of a member of the caveolin family (Mayor and Pagano, 2007; Parton and Simons, 2007). Caveolae bud off from the plasma membrane to form endocytic caveolar carriers. The caveolar unit can recycle back to the plasma membrane or fuse with the caveosome in a Rab5-independent manner or with the early endosome in a Rab5-dependent manner (Parton and Simons, 2007). It has been reported that Wnt and Notch receptors undergo CIE, as well as the cellular entrance of virus (Sorkin and von Zastrow, 2009).

**TGFβR endocytosis**

TGFβRs can be internalized via clathrin- and caveolae-mediated endocytosis. In the clathrin-dependent endocytosis, TGFβRs are internalized from the plasma membrane to the phosphatidylinositol-3-phosphate (PI3P)-enriched and early endosome antigen 1 (EEA1)-positive early endosomes, where they interact with the Smad anchor for receptor activation (SARA) and hepatocyte growth factor–regulated tyrosine kinase substrate (HRS) to induce the canonical Smad-dependent signaling pathways (Chen, 2009; Le Roy and Wrana, 2005). SARA binds to Smad2 directly and facilitate the phosphorylation of Smad2 by TGFβ receptors, inducing dissociation of
Smad2 from SARA. Then the Smad2–Smad4 complex translocates to the nucleus, regulating the target genes expression (Tsukazaki et al., 1998). From the early endosome, TGFβRs can recycle back to the cell surface by entering into Rab11-positive endosomes. Recently, it has been reported that CIN85 (Cbl-interacting protein of 85 kD), which interacts with TβRI, is implicated in Rab11-dependent recycling of TβRI to the plasma membrane (Yakymovych et al., 2015). AP2 associates with TβRI and TβRII with the β2 subunit to facilitate the internalization of TGFβRs (Yao et al., 2002). The kinase activity of TβRI may not be required for the receptor internalization (Anders et al., 1998). It is still unclear whether clathrin-mediated endocytosis is required for TGFβ signaling because Smad signaling can take place initially from the plasma membrane, but it has been reported that inhibition of clathrin-mediated endocytosis reduces the nuclear accumulation of Smad2 and abrogates Smad2-mediated transcriptional responses (Runyan et al., 2005).

In caveolae-mediated endocytosis, TGFβRs are internalized into caveolin-1–positive vesicles together with Smad7 and Smurf2, leading to ubiquitin-dependent degradation of the receptors (Le Roy and Wrana, 2005).

Recently, it has been reported that a portion of clathrin-coated vesicles and caveolar vesicles fuse after mediating TβRI internalization. Rab5 regulates targeting of the fused vesicles to the early endosome to deliver the internalized TβRI to the EEA1 and caveolin-1 double-positive early endosomes (caveolin-1–positive early endosome), which may act as a multifunctional device for TGFβ signaling and sorting center for TβRI (He et al., 2015). APPL endosomes, which are the precursors of PI3P endosomes (Zoncu et al., 2009), are also involved in the nuclear translocation of TβRI-ICD (intracellular domain) in a TRAF6-dependent manner (Song et al., 2016).

**Endosomal proteins**

The proteins in endosomes that regulate TGFβ signal transduction include Rab5 and three FYVE domain-containing proteins, EEA1, SARA, and endofin. Rab5 is a member of the Ras-like small G protein family (Rab proteins), which controls budding, tethering, fusion, and motility by cycling between the active guanosine-5’-triphosphate (GTP)-bound form and the inactive guanosine diphosphate (GDP)-bound form (Chen, 2009; Sorkin and von Zastrow, 2002). Rab5 is also a key regulator of early endosome
trafficking and sorting. Studies of overexpressing wild-type Rab5 or the constitutively active mutant Rab5Q79L indicate that stimulation of Rab5 activity leads to formation of enlarged early endosomes, which is consistent with a role for Rab5 in fusion of clathrin-coated endosomal structures. Expression of high levels of Rab5 also results in a redistribution of early endosomes from punctate structures dispersed throughout the cytoplasm to the juxtanuclear region of the cell (Bucci et al., 1992; Stenmark et al., 1994). These observations can be explained by the fact that Rab5 stimulates the binding of early endosomes with microtubules and regulates endosome motility towards the minus ends of microtubules (Nielsen et al., 1999).

EEA1 is a Rab5 effector, which mediates tethering of early endosomes, a process that precedes their fusion. EEA1 forms a parallel coil-coiled homodimer and contains a FYVE domain, which binds PI3P (Zerial and McBride, 2001). Treatment of cells with wortmannin, an inhibitor of PI3K, leads to dissociation of EEA1 from membranes (Simonsen et al., 1998). EEA1 also contains two Rab5 binding domains: One is the Ras-binding domain (RBD), which interacts with GTP-bound Rab5 on the C-terminus, and the other is a zinc finger domain on its N-terminus. The RBD domain is located immediately upstream of the FYVE finger, which is essential for associating with the early endosome membrane (Christoforidis et al., 1999; Simonsen et al., 1998). Because Rab5 is necessary for fusion of donor and acceptor membranes, EEA1 acts as a bridge to bring the two membranes together (Zerial and McBride, 2001).

Endoﬁn binds to TβRI and Smad4 and therefore potentiates TGFβ signaling by assisting the formation of Smad2-Smad4 complex (Chen et al., 2007).

**Non-Smad signaling**

**TRAF6**

Protein ubiquitination is a dynamic post-translational modification regulating a broad of cellular processes, including signal transduction, endocytosis, DNA damage response, cell division, and differentiation. Ubiquitin is a highly conserved 76-amino acid protein, and ubiquitination initiates by the covalent attachment of a single ubiquitin to the lysine (Lys) residue of target proteins. The formation of polyubiquitination chains results in the addition
of further ubiquitin residues to the protein-attached ubiquitin. Ubiquitination is achieved by a sophisticated three-step enzymatic cascade catalyzed by a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3). E3 usually determines the type of ubiquitin modification because it provides substrate specificity and mediates the addition of ubiquitin to target proteins. E3 has been classified into two subfamilies: those that contain the HECT (homologous to E6-AP C terminus) domain E3 ligases, which transfer ubiquitin from E2s to substrates directly, and those that contain the RING (really interesting new gene) domain E3 ligases, which bind to E2s and substrates and enable ubiquitination of target proteins by E2s (Chen and Sun, 2009; Swatek and Komander, 2016).

Ubiquitin has 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 form polyubiquitination chains of distinct linkages. Lys48-linked polyubiquitination usually mediates the degradation of target proteins by the proteasome, whereas Lys63-linked polyubiquitination performs nonproteolytic functions including DNA damage tolerance, endocytosis, and signal transduction (Chen and Sun, 2009; Swatek and Komander, 2016).

The tumor necrosis factor (TNF) receptor–associated factor (TRAF) family was originally identified as signal transducers of TNF receptors by directly binding to the cytoplasmic tail of TNF-R2 (Xie, 2013). Currently, six different types of TRAFs (TRAF1–6) have been found in mammals. The designation of TRAF7 is still controversial because it lacks the TRAF homology domain that defines the TRAF family. All members of the TRAF family contain a common C-terminal TRAF domain composed of an N-terminal less conserved coiled-coil region (TRAF-N) and a C-terminal highly conserved subdomain (TRAF-C) (Xie, 2013). The TRAF domain mediates association with upstream receptors, downstream effectors and homo- and heterodimerization of TRAF proteins. All TRAF proteins, with the exception of TRAF1, contain an N-terminal RING finger domain, followed by a different number of zinc finger repeats (Figure 3). As a majority of E3 ubiquitin ligases have a RING finger domain, TRAFs play
important roles not only in signal transduction as adaptor proteins but also as E3 ubiquitin ligases to regulate signaling (Xie, 2013).

TRAF6 was initially isolated by yeast two-hybrid screening with CD40 as bait (Ishida et al., 1996) and later independently identified as a signaling adaptor to activate NF-κB by interleukin 1 (IL-1) using screening of an EST expression library (Cao et al., 1996). Later, TRAF6 was reported to be involved in the TGFβ and lipopolysaccharide (LPS) signaling pathway. As an E3 ubiquitin ligase, TRAF6 interacts with the E2 complex Ubc13-Uev1A to catalyze non-degradative Lys63-linked polyubiquitination. TRAF6-/- mice exhibit perinatal or postnatal death because of severe osteopetrosis, splenomegaly, and thymic atrophy (Xie, 2013).

TGFβ-activated kinase-1 (TAK1) was initially identified as a mediator in the TGFβ signaling pathway, but later it was reported to be involved in the IL-1 pathway. TAK1 is a serine/threonine kinase, a member of the mitogen-activated protein kinase MAPK kinase (MAPKKK) family (Landström, 2010). The TAK1 is activated by TRAF6-mediated Lys63-linked polyubiquitination, which in turn phosphorylates and activates the MKK (MAPK kinase) family, resulting in the activation of JNK and p38 kinases. The activated TAK1 also phosphorylates IKKβ (IκB kinase β) in the activation loop, leading to IKK activation (Wang et al., 2001). TRAF6 associates with a consensus motif in TβRI, causing the autoubiquitination of TRAF6 and subsequent Lys63-linked polyubiquitination of TAK1. Then TAK1 phosphorylates members of the MKK family, activating the JNK and p38 pathways, leading to apoptosis or EMT (Sorrentino et al., 2008; Yamashita et al., 2008). The activation of TAK1 by TRAF6 is not dependent on TβRI kinase activity (Sorrentino et al., 2008). Upon TGFβ stimulation, TRAF6 causes Lys63-linked polyubiquitination of TβRI, leading to the cleavage of TβRI by TNFα-converting enzyme (TACE). The intracellular domain of TβRI (TβRI-ICD) then translocates to the nucleus where it induces gene involved in tumor invasion, such as MMP2, SNAI1, and p300 (Mu et al., 2011). TRAF6 also regulates the expression of presenilin 1 (PS1), which promotes the second cleavage of TβRI in the transmembrane region (Gudey et al., 2014). The TβRI undergoes Lys63-linked polyubiquitination induced by TRAF6 at Lys178, contributing to the nuclear translocation of TβRI-ICD (Sundar et al., 2015). The TβRI-ICD binds to the TβRI gene to promote its own expression (Gudey et al., 2014).
APPL1

APPL1 and APPL2 are Rab5 effectors, and APPL1 was initially identified as an AKT2-binding protein in a yeast two-hybrid screen (Miaczynska et al., 2004; Mitsuuchi et al., 1999). These proteins are named after a unique structure, the multifunctional Adaptor proteins that contain a Pleckstrin homology (PH) domain, Phosphotyrosine binding (PTB) domain, and Leucine zipper motif (Mitsuuchi et al., 1999). APPL1 and APPL2 share 54% sequence identity and are found only in eukaryotes (Miaczynska et al., 2004).

APPL proteins bind to the GTP-bound active form of Rab5 via the Bar domain (originally identified as the leucine zipper motif) and PH domain. The Bar (Bin1/amphiphysin/rvs167) domain drives the membrane curvature, and the PH domain, which is adjacent to the Bar domain, may increase the lipid specificity of the Bar domain to anchor the host proteins to membrane compartments (Deepa and Dong, 2009). The PTB domain is localized in the C-terminal of the APPL isoforms (Figure 4). In general, PTB domains function as multifunctional adaptors or scaffolds to regulate spatiotemporal organization of signaling pathways involved in extensive physiological processes, such as neural development, tissue homeostasis, and cell growth (Uhlik et al., 2005). APPL proteins interact with AKT2, DCC (deleted in colorectal cancer protein), and adiponectin receptor 1 (AdipoR1) via the PTB domain (Deepa and Dong, 2009).

APPL1 is an early endosome marker, and APPL1 endosomes act as precursors of classical PI3P-positive endosomes: They mature to EEA1 endosomes directly or via WDFY2 (WE repeat and FYVE domain containing 2) endosomes. Depletion of PI3P results in the reversion of Rab5-positive endosomes to the APPL1 endosomes, expansion of the APPL1 compartments, and enhanced EGF signaling (Zoncu et al., 2009). Recently,
it has been reported that APPL1 endosomes are stable and consist of tubule-vesicular structures. APPL1+EEA1 double-positive endosomes are an important subpopulation of early endosomes for cargo trafficking (Kalaidzidis et al., 2015).

APPL proteins are involved in several signaling pathways, including EGF (Miaczynska et al., 2004), Wnt/β-catenin (Rashid et al., 2009), and NF-κB pathways (Hupalowska et al., 2012). Upon EGF stimulation, Rab5 hydrolyzes its bound GTP, releasing APPL1 from early endosomes and allowing APPL1 translocation to the nucleus, where it interacts with components of nucleosome remodeling and histone deacetylase complexes (NuRD/MeCP1) (Miaczynska et al., 2004). APPL1 and APPL2 act as activators of Wnt/β-catenin by relieving Reptin-mediated transcriptional repression. Both APPL1 and APPL2 associate with Reptin via the PH domain (Rashid et al., 2009). In the NF-κB pathway, APPL1 triggers p65 nuclear translocation through its interaction with TRAF2, which prevents NIK degradation by reducing its association with the degradative complex (Hupalowska et al., 2012).

In summary, APPL proteins have been reported to mediate cell proliferation (Miaczynska et al., 2004), survival (Schenck et al., 2008), chromatin remodeling (Miaczynska et al., 2004), and endosomal localization of proteins (Deepa et al., 2011). Given all of these varied functions, APPL1 has emerged as a new potential biomarker in prostate cancer (Johnson et al., 2014, 2015; Song et al., 2016), and the expression of APPL1 and Rab5a is increased in lung adenocarcinoma (Bidkhori et al., 2013).

**PI3K/AKT pathway**

*Phosphatidylinositol 3’-kinase (PI3K) family*

Phosphatidylinositol 3’-kinases (PI3Ks) are lipid kinases that phosphorylate the 3’-hydroxyl group of phosphatidylinositol and phosphoinositides (Engelman et al., 2006). This reaction controls many physiological functions and cellular processes, including cell survival, proliferation, growth, motility, metabolism, and vesicle trafficking (Engelman et al., 2006; Thorpe et al., 2015). PI3Ks are classified into three classes according to their sequence homology and substrate preference (Figure 5). In mammals, class I PI3Ks
are divided into the subfamilies IA and IB due to their different regulatory mechanisms. Class IA PI3K is a heterodimer that contains a p110 catalytic subunit and a p85 regulatory subunit. The highly similar p110 catalytic isoforms p110α, p110β, and p110δ are encoded by three genes, PIK3CA, PIK3CB, and PIK3CD, respectively. The regulatory isoforms p85α (and its splice variants p55α and p50α), p85β, and p85γ are encoded by PIK3R1, PIK3R2, and PIK3R3, respectively. The class IB PI3K is a heterodimer composed of the catalytic subunit p110γ (encoded by PIK3CG) and the regulatory subunit p101 (encoded by PIK3R5) or p87 (encoded by PIK3R6) (Engelman et al., 2006; Thorpe et al., 2015). Class I PI3Ks generate PtdIns(3,4,5)P3 (PIP3) from PtdIns(4,5)P2 (PIP2) in vivo. Class II PI3Ks are monomers that consist of a single catalytic subunit. Their substrates are phosphatidylinositol and phosphatidylinositol-4-phosphate (PtdIns(4)P). Class III PI3Ks consist of a catalytic subunit VPS34 and a regulatory subunit VPS15. They produce only PI3P from PtdIns, which is important for membrane trafficking and endocytosis (Thorpe et al., 2015). Class IA PI3Ks are the focus of this study.

The highly similar homologous p110 catalytic isoforms consist of five domains: an N-terminal p85 binding domain, a Ras binding domain (RBD domain), a putative membrane binding domain (C2 domain), a helical domain, and a catalytic domain (Thorpe et al., 2015). p85 contains an N-terminal SH3 domain, a GAP (GTPase-activating protein) domain, two SH2 (Src homology 2) domains (nSH2 and cSH2 domains), and a p110-binding domain iSH2. p55 and p50 consist only of two SH2 domains and an iSH2 domain. The nSH2 and iSH2 domain inhibit the catalytic activity of the p110 subunit (Mellor et al., 2012). In the absence of activating signals, the preformed p85–p110 complex is inactive and cytosolic. Following the upstream activation signals, such as receptor tyrosine kinase (RTK) or GPCR, p85 associates with receptors directly or indirectly via the SH2 domain, which recruits the p85–p110 complex to the cell membrane, relieves the inhibition of p110 catalytic activity, and phosphorylates PIP2 to generate PIP3. PIP3 acts as a second messenger that activates downstream signaling pathways, including AKT/mTOR (mammalian target of rapamycin) pathways (Thorpe et al., 2015). PIP3 levels are tightly controlled in mammalian cells by the action of several proteins, including PIP3 phosphatase PTEN, SH2 domain-containing inositol-5’-phosphatase (SHIP)1/2 (Vivanco and Sawyers, 2002). PTEN, which is short for phosphatase and tensin homolog, can remove the 3’-phosphate from PIP3 to regenerate PIP2. The nuclear PTEN can trigger the degradation of Aurora kinases by activating the E3 ligase activity of anaphase-promoting
complex/cyclosome (APC/C) (Song et al., 2011). SHIP1 and SHIP2 dephosphorylate at the D5 position to produce PtdIns(3,4)P$_2$ (Vivanco and Sawyers, 2002).

**Figure 5.** The structure, *in vivo* substrate, and product of the PI3K family. p110$\gamma$ contains a RBD domain, a C2 domain, a helical domain, and a catalytic domain. The structures of p101 and p87 are not fully known. The C-terminus of p101 is shown to bind to G$_{\beta\gamma}$ (G$_{\beta\gamma}$ binding domain (G$_{\beta\gamma}$BD)). Class II PI3K isoforms have an RBD domain, two C2 domains, a helical domain, a catalytic domain, and a Phox homology (PX) domain. VPS34 includes a C2 domain, a helical domain, and a catalytic domain. VPS15 consists of a catalytic domain, HEAT domain, and WD40 repeats.

Several serine and tyrosine residues in p85 can be phosphorylated, some of which regulate PI3K activity (Mellor et al., 2012). Casitas B-lineage lymphoma-b (Cbl-b), an E3 ubiquitin ligase, interacts with p85 and induces p85 ubiquitination, which does not lead to degradation (Fang et al., 2001). The iSH2 domain of p85 is identified to associate with $\alpha/\beta$-tubulin (Kapeller et al., 1995). In response to insulin, the iSH2 domain of p85 binds to $\gamma$-tubulin, which is a marker of the centrosome (Kellogg et al., 1994). But the role of p85 in the centrosome is still unknown.

**AKT pathway**
The serine/threonine protein kinase AKT comprises three closely related isoforms, AKT1, AKT2, and AKT3, which are encoded by \( PKB\alpha, PKB\beta, \) and \( PKB\gamma \), respectively. They share a highly conserved structure: an N-terminal PH domain, a central kinase domain, and a C-terminal regulatory domain that contains the hydrophobic motif (HM) (Figure 6) (Hers et al., 2011). AKT1 is widely expressed in tissues and involved in cell growth and survival. AKT2 is highly expressed in adipocytes, liver, and muscle and is important for glucose homeostasis. AKT3 is mainly expressed in the testis and brain and implicated in mitochondrial biogenesis and postnatal brain development (Hers et al., 2011; Wright et al., 2008).

![Figure 6. AKT structure.](image)

The intramolecular interaction between PH and the catalytic domain maintains AKT in an inactive state. A conformational change in AKT, which is induced by the association between the PH domain and PIP\(_3\), can recruit phosphoinositide-dependent kinase 1 (PDK1) to reach the activation loop and phosphorylate AKT on Thr308. mTORC2 phosphorylates AKT on Ser473 in the hydrophobic motif, which maximizes AKT activity (Hers et al., 2011). AKT can also be phosphorylated on other sites in addition to these two well-known phosphorylation residues. Cdk2/cyclin A can phosphorylate AKT1 in its C-terminal region on Ser474/Thr467, which is important for cell cycle progression (Pengda et al., 2014). In addition to phosphorylation, other post-translational modifications are involved in AKT activity regulation, including dephosphorylation, ubiquitination, SUMOylation, glycosylation, and acetylation (Chan et al., 2014). Lys48-linked polyubiquitination of AKT, which is mediated by BRCA1, CHIP, TTC3, and MULAN ubiquitin ligases, is important for the degradation and termination of AKT (Chan et al., 2014). AKT also undergoes Lys63-linked polyubiquitination, which is mediated by TRAF4, TRAF6, and Nedd4. Interestingly, Lys63-linked polyubiquitination of AKT is involved in AKT phosphorylation and membrane recruitment.
instead of degradation (Chan et al., 2014). After activation and dissociation from the membrane, AKT regulates the downstream signaling pathways by phosphorylating distinct AKT protein substrates in the cytoplasm and nucleus (Hers et al., 2011).

**PI3K/AKT pathway in cancer**

Over-activation of the PI3K/AKT pathway is one of the most frequent events in many types of cancer. Loss of the PTEN gene by mutation within the sequence, gene deletion, or epigenetic alterations is the most common mechanism of increasing PI3K/AKT signaling. The most frequent point mutations in p110α, E545 and H1047, can activate AKT without growth factor stimulation (Mellor et al., 2012). The mutation hot spot on p85α is the iSH2 domain, which associates with and inhibits the activity of p110, suggesting tumor suppressor roles for p85α in certain tissues. Increased expression of p110α and decreased expression of p85α have been reported in prostate cancer (Thorpe et al., 2015). The details of tumorigenic mechanisms of PI3K/AKT are as follows:

Cell growth and proliferation: AKT stimulates cell growth by activating mTORC1. AKT phosphorylates and inactivates tuberous sclerosis complex 2 (TSC2), a well-known negative regulator of mTORC1 (Manning and Cantley, 2007). AKT also phosphorylates p27Kip1 and p21Cip1, leading to cytosolic translocation, and attenuates their cell cycle inhibitory effects in the nucleus. GSK3 isoforms can be phosphorylated by AKT on the conserved N-terminal regulatory site, resulting in the degradation of GSK3 in the proteasome. Through the inhibition of GSK3, AKT stabilizes cyclin D, cyclin E, c-jun, and c-myc, which all promote G1-to-S phase cell cycle transition (Engelman et al., 2006; Manning and Cantley, 2007).

Cell survival: AKT promotes cell survival by phosphorylating pro-apoptotic substrate proteins, causing inhibition of apoptosis. AKT phosphorylates mouse double minute 2 homolog (MDM2) in the nucleus, which results in polyubiquitination and degradation of p53. AKT also phosphorylates B cell lymphoma associated death (BAD), which binds 14-3-3 protein, leading to release of BAD from the target protein. The phosphorylation of forkhead box O (FOXO) proteins by AKT releases them from targeted genes including FasL and Bim, and induces their export from the nucleus (Manning and Cantley, 2007). By phosphorylating IKKα, PI3K/AKT leads
to activation of NF-κB survival signaling (Ozes et al., 1999). AKT inhibits JNK/p38 apoptotic signaling by phosphorylating its upstream protein apoptosis signal-regulating kinase 1 (ASK1) (Kim et al., 2001).

Cell migration and invasion: It has been reported that AKT1 inhibits mammary epithelial cell migration and EMT but stimulates mouse embryonic fibroblast migration. AKT2 stimulates migration of human breast cancer cells but inhibits it in a mouse embryonic fibroblast model, indicating the different roles for AKT isoforms in metastasis, and their functions are therefore regulated in a context-dependent manner (Manning and Cantley, 2007).

Metabolism: In insulin-responsive tissues, AKT2 is implicated in glucose uptake by translocating glucose transporter 4 (Glut4) to the plasma membrane upon insulin stimulation (Hers et al., 2011). Phosphorylation of GSK3 by AKT stimulates glycogen synthesis. Activation of AKT leads to the enhancement of the rate of glycolysis partly by increasing the expression of glycolytic enzymes through HIFα (hypoxia-inducible factor α) (Manning and Cantley, 2007).

Crosstalk between the PI3K/AKT pathway and the TGFβ signaling pathway

It has been reported that PI3K can be activated by TGFβ and that phosphorylation of AKT occurs in a Smad-independent manner (Bakin et al., 2000; Lamouille and Derynck, 2007; Yi et al., 2005). In addition, TβRII constitutively binds to p85, and TGFβ stimulation is required for the association between TβRI and p85 (Yi et al., 2005). AKT can also be activated by TGFβ indirectly. First, the activation of PI3K by TGFβ is mediated by TGFβ-induced synthesis of TGFα promoting EGF receptor phosphorylation. Second, the expression of miR-216a/217 by TGFβ contributes to the TGFβ-induced AKT phosphorylation. Smad7 and PTEN are two functional targets of miR-216a/217, which is associated with early tumor recurrence and poor survival. On the other hand, it has been reported that TGFβ downregulates the PI3K/AKT signaling pathway by Smad-induced expression of the lipid phosphatase SHIP. AKT activity is inhibited by SHIP through its function of dephosphorylating PIP3 (Zhang et al., 2013).

The PI3K/AKT pathway has been reported to inhibit TGFβ-induced cytostatic responses and apoptosis in several ways. AKT directly associates with unphosphorylated Smad3, which prevents nuclear accumulation of
Smad3 and inhibits Smad3-mediated transcription, leading to the protection of cells from TGFβ-induced apoptosis (Conery et al., 2004; Remy et al., 2004). Moreover, FOXO, a substrate of AKT, binds to the Smad3/Smad4 complex in the nucleus in response to TGFβ, inducing expression of the p21cip1 gene, encoding a cell cycle inhibitor. AKT also phosphorylates FOXO, resulting in the nuclear export of the FOXO and impairing the TGFβ-induced cytostatic response (Seoane et al., 2004). TGFβ induces apoptosis by inhibiting the PI3K/AKT/survivin pathway (Wang et al., 2008).

In addition, PI3K/AKT is involved in TGFβ-mediated EMT, invasion, and migration. SNAI1, which suppresses the expression of E-cadherin (CDH1), can be phosphorylated by GSK-3β, leading to cytoplasmic colocalization and degradation of SNAI1. Thus, AKT induces EMT through stabilizing SNAI1 by phosphorylating and inactivating GSK-3β (Zhou et al., 2004). In addition, the activation of NF-κB induced by AKT stimulates the expression of SNAI1 and consequently represses expression of E-cadherin to promote EMT (Julien et al., 2007). Furthermore, phosphorylation of TWIST1 (twist family bHLH transcription factor 1) by AKT is important for invasion and metastasis of breast cancer by upregulating the expression of TGFβ2, which in turn maintains hyperactivation of the PI3K/AKT pathway (Xue et al., 2012). Also, mTOR, a downstream effector of AKT, is involved in the TGFβ-mediated migration and invasion by regulating protein synthesis via phosphorylation of S6 kinase 1 (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (Lamouille and Derynck, 2007). Finally, ubiquitin-specific peptidase (USP) 4 directly associates with TβRI and stabilizes TβRI at the plasma membrane as a deubiquitinating enzyme. USP4 is predominantly located in the nucleus, and the phosphorylation of USP4 by AKT redirects its subcellular localization to the cytoplasm and plasma membrane. High expression of USP4 is found in many cancers, and it is involved in TGFβ-induced EMT (Zhang et al., 2012). In summary, the PI3K/AKT pathway collaborates with TGFβ signaling pathways to contribute to tumor progression.

**PKCζ**

PKC is a family of serine/threonine kinases that belong to the ‘AGC’ superfamily together with cAMP-dependent kinase (PKA) and cGMP-dependent kinase (PKG) (Hirai and Chida, 2003). The PKC family is grouped into three subfamilies: conventional or classical PKCs (cPKCs) α,
βI, βII, and γ; novel PKCs (nPKCs) δ, ε, η, and θ; and atypical PKCs (aPKCs) ζ and λ/ι (human PKCι and mouse PKCλ are orthologs). The cPKC subfamily members consist of a pseudosubstrate (PS) motif and four conserved (C1–C4) domains and are activated by calcium, phosphatidylserine, and diacylglycerol (DAG) or phorbol esters. The nPKCs lack a C2 domain and do not need calcium for activation. aPKC subfamily members possess four domains and motifs, including an N-terminal PB1 domain, a PS motif, a C1 domain, and a C-terminal kinase domain with C3 and C4 domains (Hirai and Chida, 2003; Xiao and Liu, 2013). The C1 domain of aPKCs differs from that of cPKCs and nPKCs because it cannot be activated by DAG and phorbol esters. C3 is an ATP-binding domain (Figure 7). The complete and stable activation of PKCζ mainly involves two events: direct interaction of PKCζ with PIP3, which causes release of PS-mediated auto-inhibition, and phosphorylation of PKCζ at Thr410 in the activation loop by PDK1 (Hirai and Chida, 2003; Xiao and Liu, 2013).

PKCζ is implicated in cell polarity. The PAR3–PAR6–PKCζ complex is involved in the formation of tight junctions in mammalian epithelial cells (Hirai and Chida, 2003). Polarization is an important step for cell migration, and the Par6–PKCζ complex can be found at the leading edge of migrating cells in the plasma membrane. Moreover, PTEN loss results in enhanced cell invasiveness, which can be suppressed by PKCζ inhibitor (Xiao and Liu, 2013). Finally, PKCζ can promote cell invasion by inducing the expression of MMP9 (Xiao and Liu, 2013).
aPKC can be phosphorylated and activated by TGFβ (Gunaratne et al., 2013; Mu et al., 2011). Moreover, the TGFβR, Par6, PKCζ have been reported to localize at the leading edge of migrating cells (To et al., 2008). TGFβ-induced EMT can be impaired by aPKC deficiency (Gunaratne et al., 2013).

**Aurora kinases**

The Aurora kinases, which include Aurora kinase A (AURKA, encoded by AURKA), Aurora kinase B (AURKB, encoded by AURKB), and Aurora kinase C (AURKC, encoded by AURKC), are highly conserved serine/threonine kinases with essential roles in many aspects of mitosis and/or meiosis (Carmena and Earnshaw, 2003; Goldenson and Crispino, 2015). Three Aurora kinases share a high sequence identity, with an N-terminal regulatory domain and a C-terminal catalytic kinase domain. AURKA contains destruction box (D-box) and D-box–activating motif, which mediate its degradation at the end of mitosis. Human AURKA and AURKB share 71% sequence identity in their C-terminal catalytic domains. However, the N-terminals of Aurora kinases, which are responsible for protein–protein interactions, are not very similar in sequence (Figure 8) (Carmena and Earnshaw, 2003; Goldenson and Crispino, 2015).

![Figure 8](image.png)

Figure 8. The structure of the Aurora kinase family. The KEN motif, which is involved in the degradation of other mitotic proteins, is not crucial for AURKA degradation.

**AURKA**

The autophosphorylation of AURKA at Thr288 in the kinase activation loop is the main factor in triggering and maintaining the activity of AURKA in
mitosis. The partner proteins involved in this phosphorylation include TPX2 (targeting protein for Xenopus kinesin-like protein 2), NEDD9 (neural precursor cell expressed, developmentally down-regulated 9), Ajuba/JUB, and BORA (Nikonova et al., 2013). Of note, NEDD9 is also involved in the focal adhesions regulating invasion, migration, and ciliary resorption (Shagisultanova et al., 2015). The levels of AURKA mRNA are low in G1/S but peak at G2/M, following protein expression peaking later (Goldenson and Crispino, 2015). The degradation of AURKA starts at the end of mitosis via association with APC/C by the proteasome. APC/C specificity factors Cdc20 and Cdh1 are also involved in the degradation of AURKA. GSK-3β mediates the ubiquitination and degradation of AURKA through the F-box protein FBXW7. The loss of PTEN in tumors leads to the stabilization of AURKA through the AKT/GSK-3β pathway (Nikonova et al., 2013).

The subcellular localization of AURKA is in accordance with its function during the cell cycle. AURKA, which is essential for centrosome maturation and separation, starts to associate with centrosomes during S phase. Centrosome maturation requires the recruitment of different proteins including γ-tubulin, centrosomin, and LATS2, which is impaired in the absence of AURKA (Marumoto et al., 2005; Nikonova et al., 2013). In late G2 phase, AURKA is activated, which is important for the recruitment of the inactive CDK1/cyclin B complex to the centrosome, where the complex can be activated and allow mitotic entry. AURKA controls the construction and assembly of the bipolar spindle through the Ran–TPX2 pathway, which also causes phosphorylation of AURKA (Marumoto et al., 2005). AURKA regulates chromosome alignment by phosphorylating CENP-A, a conserved kinetochore-specific histone H3 variant. Both inhibition and overexpression of AURKA result in the failure of cytokinesis and the formation of multinucleated cells, indicating that accurate control of activation and inactivation of AURKA is essential for proper cytokinesis (Marumoto et al., 2005; Nikonova et al., 2013).

Selective inhibition of AURKA results in defects in centrosome maturation, mitotic spindle assembly, chromosomal segregation, and microtubule stability. AURKA-deficient or -overexpressing cells can escape spindle checkpoint-mediated arrest in mitosis and exit mitosis with chromosomal segregation error or extra centrosomes, respectively. Then the escaped cells undergo the post-mitotic G1 checkpoint, which is dependent on the p53. In short, cells with functional p53 arrest in G1 undergo apoptosis, whereas cells with deficient p53 continue through the cell cycle, leading to genomic instability (Goldenson and Crispino, 2015; Marumoto et al., 2005).
AURKB and chromosomal passenger complex (CPC)

AURKB provides serine/threonine catalytic kinase activity to the CPC. The CPC also consists of non-enzymatic subunits, including inner centromere protein (INCENP), borealin, and survivin, which regulate kinase activity, targeting, and localization (Goldenson and Crispino, 2015; Lens et al., 2010). INCENP was first identified in the complex and functions as a scaffold protein that associates with other members of the complex. The N-terminus of INCENP is involved in CPC localization to centromeres. The highly conserved IN box at C-terminal of INCENP associates with AURKB (Carmena et al., 2012; Ruchaud et al., 2007). AURKB binding of INCENP initially activates low levels of kinase activity; in turn, AURKB phosphorylates a TSS (Thr-Ser-Ser) motif in the C-terminal of INCENP and induces Thr232 in the T-loop residue of its kinase domain, which further activates AURKB in a positive feedback loop.

![Survivin structure](image)

**Figure 9.** Survivin structure.

Survivin, encoded by *BIRC5*, is a highly conserved and the smallest member of the inhibitor of apoptosis protein (IAP) family. It consists of a single baculovirus IAP repeat (BIR) that is responsible for dimerization of survivin and the hallmark of all IAPs and a C-terminal helical extension (Figure 9) (Altieri, 2008a, 2008b; Carmena et al., 2012). The BIR domain is required for the correct localization of CPC in anaphase. Survivin interacts with the other three CPC members and is phosphorylated by AURKB. However, survivin was initially identified as an inhibitor of apoptosis protein that suppresses apoptotic and non-apoptotic cell death. It antagonizes cell death by regulating upstream of effector caspases. Survivin is undetectable in most adult tissues but is overexpressed in many human cancers. The subcellular localizations of survivin include the mitotic apparatus, which is discussed later in detail, the nuclei, cytosol, and mitochondria. The function of survivin in the nuclei of interphase cells and its role in cancer are still unknown and
need to be further investigated. The mitochondrial survivin, especially in the intermembrane space, is required for its anti-apoptotic effect. The cytosolic survivin comes from nuclear export controlled by trafficking pathways or mitochondria in response to cell death stimuli (Altieri, 2008a, 2008b; Carmena et al., 2012).

During late S phase, CPC is first seen on pericentromeric heterochromatin. In early G2 phase, histone H3 phosphorylation of Ser10 and Ser28 by AURKB is first visualized near centromeres and required for chromosome condensation during prophase. CPC accumulates at the inner centromere during (pro)metaphase, where it has a central role regulating chromosome bi-orientation by detaching erroneous kinetochore–microtubule attachments. AURKB phosphorylates the mitotic centromere-associated kinesin (MCAK) and inhibits its microtubule depolymerizing activity for spindle assembly. CPC ensures the correct chromosomal alignment on the spindle equator and is required for spindle checkpoint. In anaphase, the CPC transfers to the spindle midzone and equatorial cortex. Here, it contributes to central spindle formation. In telophase, CPC accumulates at the cleavage furrow and then concentrates at midbody, where it is involved in cytokinesis. In summary, the dynamic localization of the CPC during cell division enables its multifunctionality (Carmena et al., 2012; Horst and Lens, 2014; Ruchaud et al., 2007; van der Waal et al., 2012).

The inhibition of AURKB impairs the phosphorylation of H3 for chromosome condensation, results in defect in bi-oriented chromosomes, and perturbs cytokinesis. It generates tetraploid cells with two centrosomes and no apoptosis is observed. However, the loss of AURKB does not activate G1 check point. The tetraploid cells will keep dividing (Giet et al., 2005; Lens et al., 2010).

**Aurora kinases: oncogenes or not?**

Aurora kinases are overexpressed in many types of cancer, including colorectal, breast, and prostate cancer. However, whether they are oncogenes is still a question. Overexpression of AURKA induces oncogenic transformation in immortalized rodent fibroblasts NIH3T3 and rat-1 cells, and these transformed cells successfully develop tumors when implanted in nude mice. On the other hand, overexpression of AURKA in the mammary glands cannot induce malignant tumors in either p53 functional and mal-functional mice, indicating that AURKA overexpression alone is not
sufficient for tumorigenesis (Giet et al., 2005; Lens et al., 2010). The contributions of AURKA to carcinogenesis include its role in chromosome segregation, spindle checkpoint, and inhibition of p53. The binding of p53 to AURKA inhibits its oncogenic activity; however, AURKA regulates p53 by phosphorylating p53 at Ser215 and Ser315 to suppress its transcriptional activity and activate its degradation, respectively (Goldenson and Crispino, 2015; Marumoto et al., 2005).

In the case of AURKB, it is also overexpressed in many cancers. Its overexpression is related to metastasis after implantation in nude mice and poor prognosis in glioblastoma patients. However, it cannot induce oncogenic transformation in immortalized rodent fibroblasts when it is overexpressed. High levels of AURKB lead to tumors by inducing tetraploidy and genomic instability due to failure of cytokinesis. p53-deficient cells overexpressing AURKB develop more aggressive tumors. In summary, proficient p53 seems to inhibit both AURKA and AURKB oncogenic effects by stabilizing polyploidy (Giet et al., 2005; Goldenson and Crispino, 2015; Lens et al., 2010).
Aims of the thesis

The aim of this study was to investigate the roles of APPL1/APPL2, PI3K/AKT, and PKCζ in TGFβ signal transduction in cancer and the effects of APPL proteins and TβRI in mitosis and cytokinesis.

Specific Aims

Paper I: To explore the potential role of APPL1 and APPL2 in the nuclear translocation of TβRI-ICD and the role of APPL1 in prostate cancer.

Paper II: To investigate the molecular mechanism of PI3K/AKT activation by TGFβ.

Paper III: To determine the molecular mechanism of the activation of PKCζ by TGFβ and its role in prostate cancer.

Paper IV: To demonstrate target genes of the nuclear TβRI-ICD–APPL1 complex and the roles of APPL proteins and TβRI in mitosis and cytokinesis.
Materials and methods

Cell culture

The human prostate cancer cell line PC-3U (Franzén et al., 1993) and human neuroblastoma cell line KELLY (Fransson et al., 2015) were grown in RPMI-1640 containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Franzén et al., 1993). The breast cancer cells MDA-MB-231 were purchased from ATCC and grown in L15 media with 15% FBS and 2 mM L-glutamine. Human embryonic kidney HEK293T cells, TRAF6−/− MEF cells, and HaCaT cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS. p85α−/− p85β−/− MEF cells were a kind gift from Prof. Giorgio Scita (Innocenti et al., 2003) (University of Milan, School of Medicine, Italy) and are referred to as p85−/− MEF cells, which were grown in DMEM with 15% FBS. Mouse macrophage RAW264.7 cells were purchased from ATCC and grown in DMEM supplemented with 10% FBS. Zinc finger PKCζ mutants 9a and 26a were cultured in the same way as PC-3U cell lines and were made according to the manufacturer’s protocol (Sigma-Aldrich). For TGFβ stimulation, TGFβ (5–10 ng/ml) was added in medium containing 1% FBS to cells that had been starved for 12–18 hours. In Paper II, the cells were starved in serum-free medium for at least 20 hours. A total of 10 ng/ml EGF or 100 ng/ml PDGF-BB were added to stimulate cells.

All cells were incubated at 37°C in 5% CO2 in a humidified chamber, except for p85−/− MEF cells, which were cultured in the presence of 10% CO2.

Plasmid transfection

The transient transfection of HEK293T cells was performed with the calcium phosphate method. PC-3U and RAW264.7 cells were transfected with FUGENE HD (Roche) following the manufacturer’s instructions.

siRNA transfection

siRNA was transfected into cells using Oligofectamine Transfection Reagent (Invitrogen) according to the manufacturer’s protocol. A non-targeting RNA was used as a control.

Protein analysis

After stimulation, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold RIPA buffer. After centrifugation, the supernatants were collected and the protein concentrations measured using the BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein
from each total cell lysate were subjected to SDS-PAGE, followed by western blotting (Edlund et al., 2003).

**Immunoprecipitation**
Cell lysates were incubated overnight with the indicated antibody at 4°C. The next day, Protein G sepharose beads were added and incubation was prolonged for 1 hour at 4°C. Then the protein G beads with protein complexes were washed with RIPA lysis buffer, followed by addition of SDS sample buffer with reducing agent and then heated at 95°C for 10 minutes.

**Nuclear fractionation assay**
After starvation and stimulation with TGFβ1, cells were washed twice with ice-cold PBS and harvested in ice-cold PBS. After centrifugation, the supernatant was discarded. The pellets were treated with ice-cold lysis buffer (Buffer I) containing 10 mM morphine ethanesulfonic acid (pH 6.2), 10 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 5 mM dithiothreitol (DTT), 1% Triton X-100, and protease inhibitors. After a second centrifugation step, the supernatant with the cytoplasmic part was removed and centrifuged. The remaining nuclear pellet was washed with Buffer I, and nuclear proteins were obtained by adding an ice-cold extraction buffer (Buffer II) (25 mM Tris-HCl, pH 10.5, 1 mM EDTA, 0.5 M NaCl, 5 mM β-mercaptoethanol, 0.5% Triton X-100) (Edlund et al., 2005).

**In vivo ubiquitination assays**
Cells were transfected as indicated. After stimulation, the cells were washed once in cold PBS, scraped in 1 ml PBS, and centrifuged. Non-covalent protein interactions were dissociated with PBS/SDS and boiled for 10 minutes. Then samples were added to PBS/NP40 buffer and protease inhibitors. After centrifugation, the supernatants were subjected to immunoprecipitation, followed by immunoblotting.

**Immunofluorescence**
In brief, cells were seeded on coverslips and fixed in 4% paraformaldehyde for 30 minutes, permeabilized with 0.2% Triton X-100 in PBS for 5 minutes at room temperature, and then blocked in 10 mM glycine. Incubation with the primary antibodies was performed for 1 hour at room temperature. After being washed in PBS, the cells were incubated with the secondary antibodies and mounted using mounting medium with 4′,6-diamidino-2-phenylindole (DAPI). Photomicrographs were obtained using a confocal microscope.
**Immunohistochemistry**
The tissue slides were deparaffinized in dimethylbenzene, rehydrated through graded alcohols, incubated for antigen retrieval, and blocked by endogenous peroxidase. After blocking, the sections were incubated overnight at 4°C with primary antibody, followed by horseradish peroxidase polymer. The sections were developed with DAB under microscopic control and then stained with hematoxylin, dehydrated, and mounted. Images were acquired with Pannoramic 250 Flash.

**In situ proximity ligation assay (PLA)**
Cells were fixed using 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. For brightfield PLA analysis, the tissue sections were pre-treated with deparaffinization, retrieval, and permeabilization. *In situ* PLA was performed according to the manufacturer’s instructions with the Duolink Detection Kit (Sigma). Photomicrographs for immunofluorescence were taken with an AX10 microscope or confocal microscope (Carl Zeiss). Images for brightfield were acquired with Pannoramic 250 Flash.

**Invasion assay**
For invasion assays, we used the Corning® BioCoat™ Matrigel® Invasion Chamber (Corning, Discovery Labware, Bedford, MA, USA). After rehydration by adding 500 µl of warm serum-free medium to the inner compartment, the cells were plated into each insert in serum-free medium. The lower well of the invasion plate was filled with 500 µl of medium containing 10% FBS. After incubation for 24 hours, non-invasive cells were removed. Invasive cells were stained and photographed with a microscope. Each insert was incubated with Extraction Solution. Optical density at 560 nm was measured in a plate reader.

**Quantitative real-time PCR (qRT-PCR)**
Total RNA was isolated from PC-3U cells with an RNeasy Mini Kit (Qiagen). cDNA was synthesized by Thermoscript RT-PCR (Invitrogen). Power SYBR Green (Applied Biosystems) with appropriate forward and reverse primers was used to perform real-time PCR.

**Statistical analysis**
Statistical analyses were performed with SPSS 22 or SPSS 24 software for Windows. The Mann–Whitney U test and Student’s t test were used to analyze differences between two independent groups.
Results and Discussion

Paper I

**APPL proteins promote the TGFβ-induced nuclear transport of the TGFβ type I receptor intracellular domain**

Our previous study showed that upon TGFβ stimulation, TRAF6 promotes the proteolytic cleavage of TβRI by TACE in a PKCζ-dependent manner. TRAF6 also recruits PS1 to TβRI, leading to the second cleavage of TβRI in the transmembrane region. Therefore, the liberated intracellular domain (ICD) translocates to the nucleus to induce gene promoting cell invasion, such as Snail1, MMP2, PAI1, and TβRI (Gudey et al., 2014; Mu et al., 2011). However, the precise molecular mechanism of TβRI-ICD nuclear translocation was still unknown. In this report, we showed that APPL1 associated with TβRI and PKCζ and that APPL proteins were involved in the nuclear translocation of TβRI-ICD and TGFβ signaling pathways. TRAF6 acted upstream of APPL1 and as a regulator of the APPL1–TβRI complex, promoting Lys63-linked polyubiquitination of APPL1. Of note, the tumor invasion was suppressed after APPL proteins were knocked down.

We also showed that APPL1 expression was different in normal prostate and prostate cancer. We detected elevated levels of APPL1–TβRI complexes in high Gleason scores prostate cancers. Based on these data together with other reports (Johnson et al., 2014, 2015), we suggested that APPL1–TβRI may be a new prognostic biomarker of aggressive prostate cancer.
Paper II

TGFβ promotes cancer cell migration via TRAF6-specific ubiquitination of p85α causing activation of the PI3K/AKT pathway

Deregulation of the PI3K/AKT pathway occurs in many types of cancer. Overexpression of p-AKT is related to poor prognosis in cancer patients because AKT regulates migration and therapy resistance in cancer cells (Thorpe et al., 2015; Wegiel et al., 2008). Even though it has been reported that TGFβ activates PI3K followed by the phosphorylation of AKT (Bakin et al., 2000; Lamouille and Derynck, 2007; Yi et al., 2005), the precise mechanism was still unknown.

We report here that TGFβ stimulation, via TRAF6, caused the Lys63-linked polyubiquitination of p85α, leading to activation of the PI3K pathway. p85α associated with TβRI via one of its SH2 domains in a TRAF6-dependent manner. The activation of p85α recruited AKT to the plasma membrane and Lys63-linked polyubiquitination and activation of AKT. Moreover, AKT bound to TRAF6 upon TGFβ simulation. The activation of AKT by TGFβ was not affected by TβRI kinase but was dependent on PI3K activity. Furthermore, the PI3K/AKT pathway and TRAF6 were found to be involved in TGFβ-mediated cell migration. Finally and importantly, we found a positive correlation between Lys63-linked polyubiquitination of p85α and higher Gleason score of prostate cancer by in situ proximity ligation. In summary, our findings revealed the detail mechanism of the activation of PI3K/AKT by TGFβ.
Paper III

TGFβ-induced activation of PKCζ confers invasive prostate cancer growth

Ubiquitination, a post-translational modification, affects many different cellular processes, such as cell cycle regulation, endocytosis, protein degradation, and gene regulation (Chen and Sun, 2009; Swatek and Komander, 2016). TRAF6-mediated Lys63-linked polyubiquitination is involved in activation of some intracellular kinases, including TAK1 after TGFβ stimulation (Sorrentino et al., 2008; Yamashita et al., 2008), and TβRI-ICD nuclear translocation is regulated in a PKCζ-dependent manner (Mu et al., 2011).

In this study, we showed that TRAF6 induced Lys63-linked polyubiquitination of PKCζ. TGFβ-induced AKT activation in the cell membrane was dependent on PKCζ. We also generated PKCζ-deficient PC-3U cell lines by zinc finger nuclease (ZFN) technology and investigated the effect of two different PKCζ inhibitors on cell survival and invasion. We observed lower cell numbers and invasion cells in the PKCζ mutant cell lines and PKCζ inhibitor treatment groups. Our results suggested that PKCζ might be a novel potent anti-cancer target for clinical treatment of prostate cancer.
Paper IV

**TGFβ type I receptor and endosomal APPL regulate AURKB during mitosis and cytokinesis in cancer cells**

Cancer cells circumvent growth inhibition and apoptosis by various mechanisms (Hanahan and Weinberg, 2011). Among them, TGFβ has been proven to be a tumor promoter in prostate cancer. The genomic instability caused by mitosis and cytokinesis errors is important for tumorigenesis. However, how tumor cells escape normal growth control regulated by TGFβ and the role of TGFβ in mitosis and cytokinesis were still unclear. Aurora kinases are important in multiple steps of mitosis, including centrosome maturation and separation, spindle assembly and checkpoint, chromosome alignment, and cytokinesis. AURKA and AURKB are overexpressed in many types of cancer, and the inhibitors are being examined in clinical trials (Carmena and Earnshaw, 2003; Goldenson and Crispino, 2015).

In this study, we reported that APPL proteins regulated cell viability, which is in line with previous papers (Miaczynska et al., 2004; Schenck et al., 2008). We performed microarray analyses and found that APPL proteins control the expression of genes encoding AURKB and the pro-survival protein survivin. We identified APPL1 as an AURKB- and survivin-associated protein. We also found that TβRI colocalized with AURKA and AURKB in the centrosome and midbody, respectively. The TβRI kinase inhibitor could affect the activation and phosphorylation of AURKA and AURKB. In summary, our results indicated that TβRI promotes mitosis and cytokinesis by AURKA and AURKB. TβRI inhibitors could thus have additional, previously unknown effects as potent anti-cancer drugs via suppressing the expression of Aurora kinases.
Conclusions

The following conclusions drawn in this thesis describe novel insights into the regulation of non-canonical TGFβ signaling pathways (Figure 10).

- TGFβ activates PI3K/AKT via TRAF6.
- TRAF6 causes Lys63-linked polyubiquitination of APPL1, PKCζ, p85α, and AKT.
- TRAF6 mediates the interaction of APPL1 and β-tubulin, AKT and TβRI, and p85α and TβRI.
- APPL proteins affect the TGFβ signaling pathway.
- APPL proteins facilitate the nuclear translocation of TβRI-ICD.
- APPL1 associates with TβRI via its C-terminus.
- APPL1 associates with PKCζ, β-tubulin, AURKB, and survivin.
- APPL proteins are linked to cell survival by regulating the expression of AURKB and survivin.
- TβRI colocalizes with mitosis regulators AURKA and AURKB in the centrosome and midbody, respectively.
- TβRI kinase inhibitor suppresses the activation of AURKA and AURKB.
- APPL1 proteins and the APPL1–TβRI-ICD complex correlate with the Gleason score of prostate cancers.
- TβRI-ICD–mediated cell invasion requires APPL1 proteins, TRAF6, and the PI3K/AKT signaling pathway.
- Lys63-linked polyubiquitination of p85α is correlated with the aggressiveness of prostate cancer.
- PKCζ may be a novel potent anti-cancer drug target.
Figure 10. Illustrated summary of Papers I, II, III, and IV.
Future perspectives

Paper I

We have shown that APPL proteins are required for TβRI-ICD translocation from endosomes to the nucleus via microtubules. Moreover, TRAF6 is involved in the formation of APPL1–TβRI-ICD and APPL1–β-tubulin. In addition, APPL proteins facilitate TβRI-ICD–induced cell invasion by regulating the expression of MMP2 and MMP9. We also report that APPL1 expression is different in the normal prostate and prostate cancer, which is in accordance with other reports (Johnson et al., 2014, 2015).

Open questions remain to be resolved, however. Where and when does the cleavage of TβRI-ICD happen, in the cell membrane or in the endosomes after internalization of full-length receptor? How does the TβRI-ICD enter the nuclear pore – together with APPL proteins or just alone? It has been reported that the full-length receptor enters the nucleus with the help of importin β1 (Chandra et al., 2012). Is there any other role of APPL in the nucleus beyond interacting with the NuRD complex (Miaczynska et al., 2004)? Which part of TβRI interacts with APPL1? Future investigations should address these questions.

Paper II

We found that p85α interacts with TβRI and TRAF6 via one of its SH2 domains, and that TRAF6 induces Lys63-linked polyubiquitination of AKT and p85α upon TGFβ stimulation. It would be interesting to know which part of TRAF6 and TβRI interacts with p85α. We have determined the lysine residues in p85α that accept the Lys63-linked polyubiquitination chains; further analyses of the functional properties of the ubiquitination-deficient p85α mutants are highly warranted. It remains to be determined whether these mutants affect the motility of the cells because PI3K/AKT and TRAF6 are required for TGFβ-mediated cell migration.

Paper III

We have identified TRAF6 as an E3 ligase that causes the ubiquitination and activation of PKCζ. We have generated two PKCζ mutant cell lines by zinc finger nuclease technology and showed the suppressive effects of PKCζ-deficient cell lines and PKCζ inhibitors on aggressive prostate cancer cell survival and invasion. We are currently doing in vivo experiments with
mutant cell lines and PKCζ inhibitors and recently received one PKCζ inhibitor that can be used in in vivo experiments. We plan to perform microarray analysis to investigate PKCζ-regulated genes with both in vitro and in vivo materials. The relationship between PKCζ and AKT still needs to be further investigated. Finally, the importance of PKCζ for TβRI-ICD nuclear translocation is still unclear.

Paper IV

We have investigated the role of APPL and the TGFβ signaling pathway in proliferation and apoptosis. We also found that AURKB binds to APPL1 and TβRI in the midbody during cytokinesis. AURKA interacted with TβRI in the centrosome. In addition, TβRI kinase inhibitor suppressed the activation and phosphorylation of AURKA and AURKB.

We will investigate further the role of TβRI in the midbody and whether TβRI can phosphorylate AURKB. We will also perform PLA in prostate tissues to identify a possible correlation between TβRI–AURKB and Gleason score. Given that PKCζ is activated by AURKA in sensory organ precursor cells (Wirtz-Peitz et al., 2008) and is localized in the centrosome in neurite elongation of cells (Mori et al., 2009), it will be interesting to investigate the roles of PKCζ, TβRI, and AURKA in the centrosome.
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