Molecular mechanisms for activation of non-canonical TGFβ pathways and their importance during prostate cancer progression

ANAHITA HAMIDI
Abstract

Prostate cancer is the most common invasive cancer diagnosed in men and a major and growing health problem in Western countries. Deregulation of different pathways has been implicated in progression of prostate cancer, namely nuclear factor kappa enhancer binding protein (NF-κB), transforming growth factor β (TGFβ), phosphoinositide 3'-kinase/AKT (PI3K/AKT) and Src kinase pathways. However, the detailed mechanisms by which TGFβ activates these pathways to contribute in tumorigenesis and invasive behavior of prostate cancer cells have not been elucidated.

We have demonstrated (paper I) that the E3 ligase activity of TRAF6 is crucial for recruitment of the regulatory subunit of PI3K, p85α, to TβRI and for TGFβ-induced Lys63-linked polyubiquitination of p85α. TRAF6 is required for the TGFβ-induced recruitment of AKT to the complex of PI3K and TβRI, where the polyubiquitination and activation of AKT occurs. When activated, AKT promotes TGFβ-induced cell migration which is dependent on p85 and PI3K activity, as well as on TRAF6, but not on TβRI kinase activity. Thus, TGFβ-induced activation of PI3K/AKT induces cell motility contributing to the progression of cancer.

We have demonstrated (paper II) a pivotal role of TAK1 polyubiquitination in three different pathways, including TNFR, IL-1R, and TLR4 signaling. Lys63-linked polyubiquitination of TAK1 at Lys34 is essential for downstream signaling to NF-κB-mediated target gene expression in both cancer and immune cells. These findings are of importance for the understanding of the mechanism of activation of NF-κB in inflammation and may aid in the development of new therapeutic strategies to treat chronic inflammation and cancer.

We have also shown (paper III) that TGFβ activates the tyrosine kinase Src via formation of a complex between TβRI and Src. The E3 ligase TRAF6 promotes the formation of the complex in a manner not dependent on its ubiquitin ligase activity, suggesting that TRAF6 acts as an adaptor. Moreover, the activation of Src is not dependent on the kinase activity of TβRI. On a functional level, Src activity was found to be necessary for TGFβ-induced chemotaxis.

In conclusion, we have elucidated molecular mechanisms whereby TGFβ activates non-Smad pathways, i.e. PI3K and Src. Our findings shed light on the pro-tumorigenesis mechanisms of TGFβ. In addition, we have demonstrated how the activation of TAK1, an important component of the TGFβ non-Smad pathway, by TGFβ and other stimuli leads to the activation of NF-κB and thereby induction of inflammation which likely contributes to prostate cancer progression.

Keywords: TGFβ, AKT, PI3K, p85α, TRAF6, TAK1, NF-κB, Src, prostate cancer, PC-3U, cell migration, inflammation

Anahita Hamidi, Ludwig Institute for Cancer Research, Box 595, Uppsala University, SE-75124 Uppsala, Sweden.

© Anahita Hamidi 2015

ISSN 1651-6206
ISBN 978-91-554-9301-1
urn:nbn:se:uu:diva-259224 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-259224)
To my families especially my parents who taught me to learn eagerly and to be patient and persistent
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Reprints were made with permission from the respective publishers.
# Contents

Introduction ................................................................................................... 11

1. TGFβ signaling .......................................................................................... 12  
   1.1 TGFβ family of ligands and receptors .................................................. 12  
   1.2 TGFβ signaling pathways ...................................................................... 12  
      1.2.1 TRAF6-TAK1 pathway ...................................................................... 14  
   1.3 TGFβ signaling in cancer ....................................................................... 15

2 The phosphatidylinositol 3′-kinase/AKT pathway ...................................... 17  
   2.1 Effect of AKT on cell signaling and proliferation .................................... 20  
   2.2 Role of the PI3K/AKT pathway in cancer .............................................. 21  
   2.3 Links between PI3K/AKT and TGFβ pathways ....................................... 21

3 The NF-κB signaling pathway ........................................................................ 23  
   3.1 NF-κB proteins and genes ...................................................................... 23  
   3.2 Inhibitors of NF-κB (IκB) ...................................................................... 23  
   3.3 NF-κB signaling pathways ..................................................................... 24  
   3.4 The canonical NF-κB signaling pathway ............................................... 25  
   3.5 Non-canonical NF-κB signaling pathway .............................................. 25  
   3.6 TAK1 and NF-κB signaling .................................................................... 26  
   3.7 NF-κB signaling, inflammation and cancer ............................................ 26

4 Ubiquitination ............................................................................................... 28  
   4.1 Proteasomal degradation of ubiquitinated proteins ................................. 29  
   4.2 Non-canonical functions of ubiquitination ............................................. 30

5 Src signaling .................................................................................................. 31  
   5.1 Regulation of Src activity ....................................................................... 32  
   5.2 Substrate effectors of Src ....................................................................... 33

Present investigations ..................................................................................... 35  
   Paper I: TGFβ promotes cancer cell migration via TRAF6-specific  
   ubiquitination of p85α causing activation of the PI3K/AKT pathway ......... 35  
   Paper II: Polyubiquitination of transforming growth factor β (TGFβ)-  
   associated kinase 1 mediates nuclear factor-κB activation in response  
   to different inflammatory stimuli ................................................................. 36  
   Paper III: Mechanism of regulation of Src kinase by transforming  
   growth factor β ............................................................................................. 37
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABD</td>
<td>adaptor binding domain</td>
</tr>
<tr>
<td>ALK1-7</td>
<td>activin receptor-like kinase 1-7</td>
</tr>
<tr>
<td>AP1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>BAD</td>
<td>B cell lymphoma associated death</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell activating factor</td>
</tr>
<tr>
<td>BCL-XL</td>
<td>B cell lymphoma-XL</td>
</tr>
<tr>
<td>BH domain</td>
<td>breakpoint cluster homology domain</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>CAS</td>
<td>Crk-associated substrate</td>
</tr>
<tr>
<td>Cbp</td>
<td>Csk binding protein</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>COUP-TFII</td>
<td>COUP transcription factor II</td>
</tr>
<tr>
<td>Csk</td>
<td>C-terminal Src kinase</td>
</tr>
<tr>
<td>CYLD</td>
<td>cylindromatosis</td>
</tr>
<tr>
<td>DUB</td>
<td>deubiquitination enzyme</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>Erk</td>
<td>extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>Fz</td>
<td>frizzled</td>
</tr>
<tr>
<td>GS domain</td>
<td>glysine-serine domain</td>
</tr>
<tr>
<td>GSK3β</td>
<td>glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney 293</td>
</tr>
<tr>
<td>HECT</td>
<td>homologous to E6AP COOH-terminus</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of NF-κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1β</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>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTβ</td>
<td>lymphotoxin β</td>
</tr>
<tr>
<td>LTBP</td>
<td>latent TGFβ-binding protein</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MDM2</td>
<td>mouse double minute 2 homolog</td>
</tr>
</tbody>
</table>
MMP2/9 matrix metalloproteinase 2/9
mTORC2 mammalian target of rapamycin complex 2
NEMO NF-κB essential modulator
NF-κB nuclear factor kappa enhancer binding protein
NIK NF-κB-inducing kinase
PDK1 phosphoinositide-dependent kinase 1
PEP proline-enriched tyrosine phosphatase
PI3K phosphatidylinositol 3′-kinase
PKB protein kinase B
PtdIns(4,5)P2 (PIP2) phosphatidylinositol 4, 5-bisphosphate
PtdIns(3,4,5)P3 (PIP3) phosphatidylinositol 3, 4, 5-trisphosphate
PTEN phosphatase and tensin homolog deleted on chromosome 10
PTHRP parathyroid hormone related protein
PTPα protein tyrosine phosphatase-α
RBD Ras-binding domain
RHD Rel-homology domain
RHEB RSD homolog enriched in brain
RING really interesting new gene
R-Smads receptor-activated Smads
RTKs receptor tyrosine kinases
SCFβTrCP Skp1, Cdc53/Cullin 1, F-box protein βTrCP
SHIP1 SH2 domain-containing inositol 5′-phosphatase 1
Skp2 S-phase kinase-associated protein 2
Smad Sma- and Mad-related protein
Src Rous sarcoma virus oncogene
STAT3 Signal transducer and activator of transcription 3
TABs TAK1 binding proteins
TAK1 TGFβ-associated kinase 1
TβRI/II TGFβ receptor type I/II
TGFβ transforming growth factor β
TNFα tumor necrosis factor α
TRA跛 TNF Receptor Associated Factors
TRAIL tumor necrosis factor-related apoptosis ligand
TSC2 tuberous sclerosis complex 2
USP4 ubiquitin specific protein 4
VASP vasodilator-stimulated phosphoprotein
Wnt Wingless/Int
Introduction

The proliferation, motility, migration and death of cells are regulated by signals they receive from their microenvironment. The signaling cascade triggered by a stimulus is controlled strictly to sustain the homeostasis of the cells. Deregulation of different signaling pathways has been implicated in tumor progression and cancer. Prostate cancer is the most common invasive cancer diagnosed in men and a major and growing health problem in Western countries (Assinder, Dong et al. 2009). It is one of the leading causes of cancer-related death in men. In spite of the growing knowledge of the biological mechanisms which results in initiation and progression and resistance to therapies, there are no efficient treatment (Assinder, Dong et al. 2009). The most common treatment strategy is androgen blockade, but it is effective only initially. Most patients become insensitive to this treatment as the tumor switches the phenotype from being androgen-dependent to androgen-independent. The patients relapse and die from hormone-refractory metastatic prostate cancer (Wegiel, Bjartell et al. 2008; Sarker, Reid et al. 2009). Therefore, it is important to understand the detailed mechanisms underlying development and progression of prostate cancer. In this study, we have investigated how the stimulus transforming growth factor β (TGFβ) activates signaling pathways linked to prostate cancer, namely the phosphoinositide 3′-kinase (PI3K)/Akt pathway (Assinder, Dong et al. 2009) and the Src pathway (Varkaris, Katsiampoura et al. 2014). We also studied the activation of nuclear factor kappa enhancer binding protein (NF-κB) which is regulated by TGFβ associated kinase-1 (TAK1).
1. TGFβ signaling

1.1 TGFβ family of ligands and receptors

TGFβ is the prototype of the TGFβ superfamily ligands which includes 33 members in mammals as well as in other species, like frogs, fish, flies and worms (Moustakas and Heldin 2008). Three isoforms of TGFβ (TGFβ1, TGFβ2 and TGFβ3) are synthesized in the endoplasmic reticulum, transferred to the Golgi apparatus and secreted from cells in a large complex in a latent form. The latent form is bound to proteins of the extracellular matrix mediated by the cleaved proregion of the TGFβ precursor (the latency-associated peptide; LAP) or latent TGFβ-binding proteins (LTBPs) to control the ligand access to their receptors (Weiss and Attisano 2013). Active TGFβ is a dimeric molecule which is stabilized by hydrophobic interactions and a disulfide bond (Heldin, Landstrom et al. 2009). The TGFβ ligand transmits signaling by formation of a heteromeric receptor complexes which consists of two type I (TβRI) and two type II (TβRII) transmembrane dual specificity kinase receptors, capable of phosphorylating tyrosine (Lee, Pardoux et al. 2007) as well as serine/threonine residues (Mu, Gudey et al. 2012). TGFβ binds first to TβRII with higher affinity and thereafter TβRI is docked into the complex (Heldin, Landstrom et al. 2009). There are 7 type I receptors traditionally known as activin receptor-like kinases (ALK1-ALK7) and 5 type II receptors. The whole family of TGFβ receptors have similar structure; they all have a short cysteine-rich extracellular domain which bind the ligand, a transmembrane α-helical domain and a cytoplasmic serine/threonine kinase domain with phospho-acceptor sites and docking sites for adaptors or signaling proteins (Moustakas and Heldin 2008). There is a conserved glysine-serine (GS) domain located just upstream of the kinase domain in type I, but not in type II, receptors (Weiss and Attisano 2013). Ligands of the TGFβ family signal through different combinations of type I and type II receptors (Shi and Massagué 2003).

1.2 TGFβ signaling pathways

It is well established that TβRI and TβRII form non-signaling homodimers already during trafficking to the plasma membrane. TGFβ binds TβRII to make a complex to which TβRI is recruited to make a heterotetramer. In this
heterotetrameric complex, the constitutively active TβRII trans-phosphorylates several serine residues in the GS domain of TβRI which increases the catalytic activity of TβRI due to conformational changes (Moustakas and Heldin 2008; Mu, Gudey et al. 2012).

The activated TβRI phosphorylates downstream signaling effectors such as members of the Smad family. The Smad family includes 3 different groups; receptor-activated Smads (R-Smads; Smad2 and Smad3 activated by TGFβ and Smad1 and Smad5 activated by BMPs), common-mediator Smads (co-Smad; Smad4) and inhibitory Smads (I-Smads; Smad6 and Smad7) (Weiss and Attisano 2013). Activated TβRI propagates the signal to R-Smads by phosphorylating a conserved motif (Ser-Ser-X-Ser) at their extreme C-terminals, which leads to a conformational change of the protein promoting oligomerization with themselves or other R-Smads and Smad4, to act as the transcriptional co-regulators in the nucleus (Moustakas and Heldin 2008). Besides signaling through the canonical Smad-dependent pathway, additional signaling effectors downstream TGFβ receptors are involved (Figure 1). Examples of the so-called non-canonical or non-Smad pathways are Erk1/2 and JNK/p38 MAPK pathways, the phosphatidylinositol 3'-kinase/AKT pathway and the Src pathway (Moustakas and Heldin 2008; Zhang 2009; Massague 2012).

Figure 1. TGFβ signaling pathways; Smad vs. non-Smad pathways. [Mu Y, Gudey SK, Landström M, Cell Tissue Res. 2012 Jan;347(1):11-20. Image was received by personal communication from the author, Dr. M. Landström]
1.2.1 TRAF6-TAK1 pathway

TNF-receptor-associated factors (TRAFs) play an important role in the ligand-induced activation of various signaling cascades, such as mitogen activated protein kinase, NF-κB and c-Jun N-terminal kinase (JNK) (Song, Regnier et al. 1997). TRAF6 is a member of the TRAF family, which are really-interesting-new-gene (RING) E3 ubiquitin ligases catalyzing Lys63-linked polyubiquitination (Skaug, Jiang et al. 2009). To catalyze polyubiquitination, TRAF6 interacts with the E2-conjugating enzyme Msm2 which consists of Ubc13 and Uev1A (Adhikari, Xu et al. 2007). It was initially identified as a signaling adaptor in the CD40 ligand-CD40 receptor pathway in B cells and cancer cells (Elgueta, Benson et al. 2009). CD40 receptor is a cell surface receptor which belongs to the TNF-R family. Later TRAF6 was found to play roles on other signaling pathways such as those induced by TGFβ, interleukin-1β (IL-1β) and lipopolysaccharide (LPS). TRAF6 has recently been implicated in the TGFβ-dependent activation of p38 by TAK1 (Sorrentino, Thakur et al. 2008; Yamashita, Fatyol et al. 2008). Besides TRAF6, other types of TRAFs were identified in mammals (TRAF1-7) (Wajant and Scheurich 2001). TRAFs contain a conserved C-terminal TRAF domain, which interacts with receptors and form TRAF homo- and hetero-oligomers (Dempsey, Doyle et al. 2003). Besides this domain, they contain a less conserved N-terminal subdomain consisting of the RING region (except TRAF1) (Zapata and Reed 2002).

TAK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) belonging to the family of serine/threonine kinases. The MAPKKKs connect the MAPK module to cell surface receptors or external stimuli by phosphorylating and activating MAPKKs, which in turn phosphorylate and activate MAPKs (Yamaguchi, Shirakabe et al. 1995). TAK1 plays various roles in immunological responses and development. It was found to regulate differentiation and apoptosis in Drosophila melanogaster, Xenopus laevis (Shibuya, Iwata et al. 1998; Takatsu, Nakamura et al. 2000) and mouse (Jadrich, O'Connor et al. 2006) during embryonic development. On the other hand, it is also implicated in inflammatory responses in mammalian cells in vivo (Dong, Liu et al. 2006). TAK1 is able to undertake these different biological roles as it is involved in several signaling pathways, namely TGFβ/BMP, Wnt/Fz, JNK/p38 and NF-κB pathways. Depending on the biological context, TAK1 makes complexes with different proteins such as TAK1-binding proteins (TABs) and TRAFs, which results in diverse roles of TAK1. TAK1 has a crucial role in regulating JNK/p38 MAPKs and IkB kinase (IKK) signaling pathways, thus activating the transcription factors AP-1 in response to TGF-β/BMP and NF-κB in response to inflammatory stimuli (Delaney and Mlodzik 2006). TAK1 was initially identified as a TGFβ responsive kinase, hence the name (Yamaguchi, Shirakabe et al. 1995), while it was shown later that TAK1 is also activated in response to
IL-1, tumor necrosis factor-α (TNF-α) (Sakurai, Nishi et al. 2002) and lipopolysaccharide (LPS) (Irie, Muta et al. 2000). TGFβ-induced assembly of the TβRI/TβRII-complex, induces oligomerization of TRAF6 promoting its catalytic activity because TRAF6 associates with TβRI via a conserved consensus motif (basic residue-X-P-X-E-X-X-aromatic/acidic residue) in the juxtamembrane region of TβRI. In response to TGFβ, autoubiquitination of TRAF6 and subsequent Lys63-linked polyubiquitination of TAK1 occurrs, which is not dependent on the kinase activity of TβRI (Sorrentino, Thakur et al. 2008). TAK1 polyubiquitination leads to its autophosphorylation and activation, which in turn causes the activation of mitogen-activated protein kinase kinase 3/6 (MKK3/6) and p38 (Sorrentino, Thakur et al. 2008).

There have been reports linking TRAF6 to tumor formation, growth and proliferation of cancer cells as well as angiogenesis. TRAF6 is upregulated in several tumors including pancreatic cancer (Rong, Wang et al. 2014), colon cancer (Sun, Li et al. 2014), lung cancer (Starczynowski, Lockwood et al. 2011) and gastric cancer (Sun, Ye et al. 2012).

1.3 TGFβ signaling in cancer

TGFβ signaling has multifunctional roles in many cellular functions, such as regulation of cell proliferation, survival, apoptosis, growth arrest, autophagy, differentiation and migration. Therefore, TGFβ is important during embryogenesis and in adult tissue homeostasis. Impairment of TGFβ signaling has been implicated in diseases, such as fibrosis, autoimmune disease and cancer (Heldin, Vanlandewijck et al. 2012). During tumorigenesis TGFβ has both suppressing and promoting effects. TGFβ serves as a tumor suppressor in several types of carcinomas including pancreatic, colorectal and ovarian cancers, in which inactivating genetic mutations of TGFβ receptors or Smad4 occur. TGFβ-mediated growth inhibition is mediated by Smad-dependent downregulation of c-MYC, CDC25A expression and upregulation of cyclin-dependent kinase (CDK) inhibitors such as p21CIP1 and p15INK4B (Heldin, Vanlandewijck et al. 2012). In premalignant states, TGFβ suppresses inflammation and expression of mitogens by cells in the stroma of tumors (Siegel and Massagué 2003). However, TGFβ exerts tumor promoting effects in the progression of advanced breast, prostate and colorectal cancers, where TGFβ is overexpressed and cancer cells takes advantage of the TGFβ-rich environment (Ikushima and Miyazono 2010). TGFβ enhances tumorigenesis by activating epithelial-mesenchymal transition (EMT) which makes tumor cells migratory and more invasive. In addition, TGFβ acts on stromal cells of the tumor by suppressing immune surveil lance, promoting angiogenesis and recruiting inflammatory cells to the tumor microenvironment (Heldin, Vanlandewijck et al. 2012). In particular,
TGFβ plays an important role in metastasis of breast and prostate cancers to bone, because TGFβ promotes the expression of IL-11 and parathyroid hormone related protein (PTHRP) which are important for the vicious cycle of bone resorption and osteolytic metastasis (Cook, Shay et al. 2014).
2. The phosphatidylinositol 3′-kinase/AKT pathway

AKT, also known as protein kinase B (PKB), is part of AGC (PKA, PKG and PKC related kinase) subfamily of the protein kinase superfamily. PKB is a homologue of v-AKT, which is a murine oncogene in AKT8 retrovirus. AKT is a family of 3 homologous isoforms (AKT-1, AKT-2 and AKT-3) (Hanada, Feng et al. 2004; Nelson, Evans et al. 2007). AKT-1 and AKT-2 are expressed in most tissue types whereas AKT-3 is expressed in the heart, kidney, brain, testes, lung and skeletal muscle (Steelman, Abrams et al. 2008). The signaling through AKT-1 and AKT-2 plays important roles in cell proliferation and survival (Yang, Wang et al. 2009). It has become evident that the role of AKT-3 is different from that of the other isoforms; it functions in the regulation of mitochondrial biogenesis (Wright, Maroulakou et al. 2008). AKT-1 is the main oncogenic isoform and is often referred to as AKT (Assinder, Dong et al. 2009). AKT consists of a pleckstrin homology (PH) domain, a serine/threonine kinase domain and a C-terminal hydrophobic motif (HM) (Figure 2) (Hanada, Feng et al. 2004).

Figure 2. Domain Structure of AKT isoforms. Adapted from Hanada M, Feng J, Hemmings BA, Biochim Biophys Acta. 2004 Mar 11;1697(1-2):3-16.

17
Activation of AKT occurs by its recruitment to the membrane and phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) on Thr308 in the activation loop, and by mammalian target of rapamycin complex 2 (mTORC2) on Ser473 in the hydrophobic motif (Assinder, Dong et al. 2009). Phosphatidylinositol 3, 4, 5-trisphosphate (PtdIns(3,4,5)P3, or simply PIP3) provides a platform in the membrane to which the PH domain of AKT binds whereafter it is phosphorylated. PIP3 is produced through phosphorylation of phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P2 or simply PIP2) by phosphatidylinositol 3’-kinase (PI3K) (Assinder, Dong et al. 2009). In addition to the two established phosphorylation events required for full AKT activation, there are other phosphorylation events reported in the kinase and C-terminal domains. AKT undergoes other post-transcriptional modifications besides phosphorylation, i.e. ubiquitination, sumoylation, acetylation and glycosylation. Lys48-linked ubiquitinations of AKT by CHIP, BRCA1, TTC3 and MULAN ubiquitin ligases are important for its proteasomal-dependent degradation and inactivation. In addition, Lys63-linked ubiquitination of AKT has been observed which does not trigger AKT degradation, but induces recruitment of AKT to the membrane, and promotes its serine/threonine phosphorylation and activation (Chan, Jo et al. 2014). Yang and colleagues showed that TRAF6 is the E3 ubiquitin ligase for AKT in response to insulin-like growth factor-1 (IGF-1) stimulation (Yang, Wang et al. 2009). Later Nedd4 was reported to be another E3 ubiquitin ligase for AKT, activated by IGF-1 stimulation (Fan, Lum et al. 2013). It was also reported that S-phase kinase associated protein 2 (Skp2) or TRAF4 triggers Lys63-linked ubiquitination and activation of AKT upon EGF stimulation (Chan, Li et al. 2012; Li, Peng et al. 2013). These groups illustrated that deficiency in Skp2, TRAF6 or TRAF4 in genetic mouse models restrains in vivo cancer development which reinforces involvement of distinct E3 ubiquitin ligases in AKT-mediated cancer progression (Chan, Jo et al. 2014).

PI3Ks which phosphorylate the 3’-hydroxy group of phosphatidylinositides, can be classified into three types depending on their structures and substrate specificities. Class IA PI3Ks are further divided into subclasses IA and IB based on how they are regulated. Class IA PI3K enzymes are heterodimeric proteins consisting of a 110-kDa catalytic subunit (p110α, p110β or p110δ) and a p85-type regulatory subunit (p85α and its splicing variants p55α and p50α, p85β and p55γ) (Duronio 2008; Thorpe, Yuzugullu et al. 2015). Class IB PI3Ks are heterodimers of the p110γ catalytic subunit and p101 or p87 regulatory subunits. Whereas the substrate of class I PI3Ks is PtdIns(4,5)P2 to produce PtdIns(3,4,5)P3, class II and III PI3Ks generate PtdIns(3)P from PtdIns. Class II PI3Ks are monomeric kinases which do not have a regulatory subunit, while the single class III PI3K is a constitutive dimer of the VPS34 catalytic subunit and the VPS15 regulatory subunit (Thorpe, Yuzugullu et al. 2015). Members of the class IA PI3K enzymes are the focus of this study and are referred to as PI3K hereafter. The catalytic
subunit isoforms contain a PI3K signature motif which is composed of a C2 domain, which binds membrane, a helical domain and a catalytic kinase domain. In addition, p110 has an N-terminal adaptor binding domain (ABD), which binds p85, and a Ras-binding domain (RBS). Regulatory subunit isoforms have two Src homology 2 domains (nSH2 and cSH2) and an intervening domain which binds p110 (iSH2) (Jean and Kiger 2014). Additionally, p85α and p85β have N-terminal Src homology 3 (SH3) and breakpoint cluster homology (BH) domains (Figure 3) (Thorpe, Yuzugullu et al. 2015).

It is well established that PI3K is activated by recruitment to the plasma membrane through the interaction with phosphorylated YXXM motifs on receptor tyrosine kinases (RTKs) or their adaptors upon stimulation of RTKs (Sarker, Reid et al. 2009; Thorpe, Yuzugullu et al. 2015). In addition, upon G-protein coupled receptor (GPCR) activation, PI3K interacts with GPCR-associated Gβγ subunits. At the plasma membrane, PI3K generates PIP₃ which is a second messenger activating AKT-dependent and AKT-independent signaling pathways, which regulate cell growth, metabolism, motility and survival. (Figure 4) (Thorpe, Yuzugullu et al. 2015). PI3K signaling is inactivated by two lipid phosphatases; phosphatase and tensin homolog deleted on chromosome 10 (PTEN) which removes the 3'-phosphate from PIP₃ (Thorpe, Yuzugullu et al. 2015) and SH2 domain-containing inositol 5'-phosphatase 1 (SHIP1) which removes 5'-phosphate from PIP₃ (Laurent, Severin et al. 2014).
2.1 Effect of AKT on cell signaling and proliferation

Activated AKT phosphorylates many proteins to regulate proliferation and survival of the cell (Carnero 2010). AKT promotes cell survival by inhibiting proapoptotic proteins. It phosphorylates B cell lymphoma associated death (BAD) which then, binds 14-3-3 proteins that protect BAD from the inhibitory effect of B cell lymphoma XL (BCL-XL); thereby cytochrome C is released and apoptosis is inhibited. AKT also phosphorylates certain members of the FOXO family which induce their translocation from the nucleus to the cytoplasm; thus, the transcription of the apoptosis-promoting genes mediated by FOXO proteins is blocked. By phosphorylating the E3 ligase mouse double minute 2 homolog (MDM2), AKT induces its translocation to the nucleus, where MDM2 polyubiquitinates p53 and marks it for degradation. Another AKT substrate is IκB kinase α (IKKα), the activation of which leads to NFκB activation (Carnero 2010; Zhang, Zhou et al. 2013).

AKT induces cell proliferation by targeting proteins involved in cell cycle regulation, thus, phosphorylation of the CDK inhibitors p21CIP1/WAF1 and p27Kip1 prevents their inhibitory effects. AKT also phosphorylates glycogen synthase kinase 3β (GSK3β), thereby blocking its kinase activity which results in increased levels of cyclin D1 and β-catenin. These events promote cell proliferation by positively regulating G1/S cell cycle progression. Furthermore, AKT has an important role in increasing cell mass by phosphorylating and inhibiting tuberous sclerosis complex 2 (TSC2) which relieves TSC-inhibition of RSD homolog enriched in brain (RHEB). RHEB is a GTPase activating the mTORC1 kinase, which in turn phosphorylates 4E-BP1 and P70S6K to stimulate protein translation initiation and elongation (Carnero 2010; Zhang, Zhou et al. 2013).
2.2 Role of the PI3K/AKT pathway in cancer

Hyperactivating mutations in components of the PI3K/AKT pathway have been identified in many types of cancer (Zhang, Zhou et al. 2013). Particularly, elevated level of pAKT was found to correlate with poor prognosis of prostate cancer (Assinder, Dong et al. 2009). Mutations, deletions or promoter methylation silencing of the PTEN gene causes inactivation of PTEN and increased levels of PIP3 in several types of cancer (Carnero 2010). PTEN is mutated and not functional in LNCaP, and deleted in PC-3, prostate cancer cell lines (Assinder, Dong et al. 2009). The most frequent oncogenic mutations (E542K, E545K and H104R) in the gene coding for p110α lead to enhanced catalytic activity of PI3K which constitutively activates AKT in human tumors. Increased expression of p110α has been reported in prostate cancer. Most of the mutations in the gene encoding for p85α in different cancers are in the iSH2 domain, the domain with which it binds to p110. These mutants show reduced ability to inhibit p110, thereby promoting increased PI3K activity. Reduction of expression of p85α has been reported in some cancers, including prostate cancer (Thorpe, Yuzugullu et al. 2015). Taniguchi et al showed that p85α−/− mice develop an aggressive hepatocellular carcinoma, suggesting that p85α has tumor suppressor function in certain tissues (Taniguchi, Winnay et al. 2010). A somatic mutation in the lipid-binding pocket of AKT1, E17K, was reported in breast, colorectal and ovarian cancers. E17K mutated AKT forms new hydrogen bonds with phosphatidylinositol ligand in the plasma membrane, inducing a pathological activation of downstream signaling which promotes leukemia in mice (Carpten, Faber et al. 2007). Activation or overexpression of PI3K and AKT without any mutation has been observed in several cancers (Carnero 2010).

2.3 Links between PI3K/AKT and TGFβ pathways

There are only two reports showing that TGFβ activates PI3K/AKT but different mechanisms were proposed (Yi, Shin et al. 2005; Lamouille and Derynck 2007). Despite the fact that Lamouille et al showed that inhibition of the kinase activity of TβRI by the chemical inhibitor, SB431542 resulted in the downregulation of pAKT (Ser473) as well as pSmad3, they concluded that the induction of AKT phosphorylation by TGFβ is not dependent on Smads (Lamouille and Derynck 2007). On the other hand, Yi et al showed that AKT phosphorylation is Smad-dependent since Smad7 blocked the stimulation of PI3K activity induced by constitutively active TβRI (Yi, Shin et al. 2005). In addition, there are studies suggesting that activation of PI3K/AKT by TGFβ is through indirect mechanisms. TGFβ induces miR-216a/217, which in a positive feedback loop upregulates PI3K/AKT and TGFβ signaling by inhibiting PTEN and Smad7, respectively. These events
lead to early tumor recurrence, poor disease-free survival and an EMT phenotype in liver cancer and glomerular mesangial cells. Moreover, TGFβ induces miR-21, which by targeting PTEN, upregulates AKT/mTORC1 signaling resulting in mesangial cell hypertrophy and matrix protein synthesis. In contrast, TGFβ also promotes the Smad-dependent expression of SHIP and inhibition of PI3K/AKT signaling. This response may regulate the duration of TGFβ-induced AKT activation (Zhang, Zhou et al. 2013).

PI3K/AKT pathway antagonizes the tumor suppressive effects of the TGFβ/Smad pathway. In premalignant states, AKT blocks TGFβ/Smad-mediated induction of p15INK4B/p21CIP1 by binding to Smad3 and sequestering it from phosphorylation, complexing with Smad4 and entering the nucleus (Song, Cornelius et al. 2003). Moreover, AKT phosphorylates FOXO transcription factors which consequently sequesters FOXO in the cytoplasm. Therefore, the formation of a Smad/FOXO complex is required for efficient transcription induction of p15INK4B/p21CIP1, whereby TGFβ-mediated apoptosis and cytostatic response are impaired (Seoane, Le et al. 2004). In the premalignant state in prostate, PTEN loss causes hyperactivation of the PI3K/AKT pathway. When PTEN loss is coupled with inactivation or genetic deletion of Smad4, prostate cancer growth and metastatic progression is advocated. Smad4 is inactivated by interaction with COUP transcription factor II (COUP-TFII) (Ding, Wu et al. 2011; Qin, Wu et al. 2013).

PI3K/AKT activation enhances the switch of TGFβ signaling from tumor-suppressive to tumor-promoting by inducing the EMT program. Activated AKT phosphorylates and inactivates GSK3β. One of the substrates of GSK3β is SNAIL, a transcription factor which acts as a key inducer of EMT. Phosphorylated SNAIL is confined in the cytoplasm and degraded. Once GSK3β is inactive, SNAIL therefore, accumulates in the nucleus, E-Cadherin is suppressed and EMT is induced. Furthermore, activated AKT promotes SNAIL transcription and protein stabilization by activation of NF-κB. AKT also promotes EMT by phosphorylating and activating TWIST1, another inducer of EMT (Zhang, Zhou et al. 2013).

PI3K/AKT activation promotes TGFβ signaling towards non-Smad pathways to drive tumorigenesis in breast cancer cells by modulating ubiquitin-specific protein 4 (USP4). AKT associates with and phosphorylates USP4 which translocates to the plasma membrane where it in complex with USP11/USP15 deubiquitinates and stabilizes TβRI (Zhang, Zhou et al. 2012).

These studies suggest that the integration of TGFβ/Smad and PI3K/AKT pathways contributes to cancer progression.
3. The NF-κB signaling pathway

3.1 NF-κB proteins and genes

NF-κB was discovered as a factor binding to the transcriptional enhancer of the immunoglobulin κ light chain gene in B cells (Hoffmann and Baltimore 2006). Despite the name, NF-κB is not a B-cell specific nuclear factor, but has been shown to be a pivotal family of transcription factors regulating a variety of physiological and pathological processes. It regulates many genes involved in the immune response, cellular stress responses, cell growth, adhesion, differentiation, proliferation and survival, as well as angiogenesis and apoptosis (Hoffmann, Levchenko et al. 2002; Sun and Zhang 2007). The NF-κB family comprises five homologous genes, i.e. nfkb1, nfkb2, rela, relb and crel. The gene nfkb1 encodes p105 and p50 proteins. P105 is a non-DNA-binding cytoplasmic protein while p50 is a DNA-binding protein. The gene nfkb2 encodes p100 and p52 proteins. The p50 and p52 proteins are produced by proteolytic processing of the precursor proteins p105 and p100, respectively. The gene rela encodes p65 or RelA. The gene relb encodes RelB and the crel gene codes for cRel protein. NF-κB proteins share the Rel-homology domain (RHD), which is responsible for DNA-binding, dimerization and association with inhibitor of NF-κB (IκB) protein. cRel and p65 have transcriptional activation domains. Five NF-κB family proteins form 15 homo- and heterodimeric transcription factors which can be divided to 3 groups; transcriptional activators, dimers lacking transcriptional activation domains and transcriptional repressors lacking DNA-binding ability. Dimers that are present in a cell depends on the type and environment of the cell. While the predominantly expressed species are p65 dimers in many cell types, cRel dimers are mainly expressed in mature lymphoid cells. (Hoffmann, Levchenko et al. 2002; Sun and Zhang 2007).

3.2 Inhibitors of NF-κB (IκB)

The IκB family of proteins is comprised of five proteins with inhibitory roles in NF-κB signaling. These proteins are IκBα, IκBβ, IκBε, as well as the precursor proteins p105 and p100. The C-terminal parts of p105 and p100 give rise to IκBγ and IκBδ, and the N-terminal parts to p50 and p52 (Hoffmann
These proteins share the ankyrin repeat domain which is responsible for the interaction with NF-κB proteins.

3.3 NF-κB signaling pathways

NF-κB is retained inactive in the cytoplasm by interaction with inhibitory IκB proteins under basal conditions. In response to different stimuli, IκB proteins are phosphorylated on specific serine residues by the IκB kinase (IKK) complex (Sun and Zhang 2007). This marks IκB for K48-linked ubiquitination by the SCF βTrCP E3 ligase complex and subsequent proteasomal degradation (Skaug, Jiang et al. 2009). As a result, NF-κB is released and translocated to the nucleus. This type of NF-κB activation is classified as the canonical pathway and is essential for immune responses, inflammation and promoting cell survival. There is also a non-canonical pathway for NF-κB activation, which involves the processing of precursor proteins p100 and p105 rather than inhibitor degradation. The non-canonical pathway plays an important roles in secondary lymphoid organogenesis, maturation of B-cells and adaptive humoral immunity (Sun and Zhang 2007) (Figure 5).

Figure 5. Two pathways leading to NF-κB activation. In the canonical NF-κB activation (left), stimulation of the TNF receptor (TNFR), IL-1 receptor (IL-1R), and Toll-like receptors (TLRs) leads to activation of the TAK1 complex through TRAF proteins. In the noncanonical NF-κB activation (right), stimulation of a subset of receptors, including the BAFF receptor, leads to the stabilization of the kinase NIK, followed by activation of IKKα. Image was reprinted with permission.
3.4 The canonical NF-κB signaling pathway

Stimulation of cells with pro-inflammatory factors such as pathogen-derived lipopolysaccharide (LPS), cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β), growth factors and activation of B- or T- cells by antigen receptors, activates the canonical NF-κB pathway. The signal induces the p50:p65 (RelA) NF-κB isoform (also known as classical NF-κB). Upon stimulation, the canonical IKK complex, consisting of two kinase subunits, IKKα (or IKK1) and IKKβ (or IKK2) and a structural subunit, IKKγ (or NF-κB essential modulator, NEMO), phosphorylates the canonical IkB proteins IkBα, IkBβ, IkBε on two N-terminal serine residues. The phosphorylated IkB is subsequently recognized by the SCFβTrCP E3 ligase complex, K48-linked polyubiquitinated and degraded by proteasomes. Free p50:p65 dimers activate gene transcription. IkBα is one of the early upregulated genes and exerts a feedback effect (Hoffmann and Baltimore 2006; Skaug, Jiang et al. 2009).

3.5 Non-canonical NF-κB signaling pathway

Stimulation of cells with non-inflammatory stimuli, such as B-cell activating factor (BAFF), lymphotoxin β (LTβ) and CD40 ligand, induces the non-canonical NF-κB pathway (Hoffmann and Baltimore 2006; Skaug, Jiang et al. 2009). NF-κB-inducing kinase (NIK) is required in this pathway to phosphorylate the T-loop serines of IKKα, which in turn phosphorylates p100 in an IKKβ- and NEMO-independent manner. The phosphorylation of p100 leads to recruitment of the SCFβTrCP E3 ligase complex, Lys48-linked polyubiquitination and proteasomal processing of the C-terminal part of p100 to produce p52 (Scheidereit 2006). RelA or RelB, which were retained inactive by dimerization with p100, now associate with p52 and are activated (Scheidereit 2006). P100 can also act similar to the canonical IkBs to inhibit the RelB:p50 dimer which is released after complete proteasomal degradation of p100. This event is triggered by phosphorylation of IKKα followed by stimulation (Baud and Karin 2009).

Generation of p50 is regulated by two independent mechanisms, basal-constitutive and signal-induced. In basal conditions, NF-κB proteins, such as p50 and p65, are sequestered in the cytosol by docking to p105. SCFβTrCP-dependent complete degradation of p105 occurs constitutively to maintain the level of p50 and p65 which are required by different cellular processes. On the other hand, stimulation-mediated processing of the C-terminus does not need SCFβTrCP, but yet proteasome in order for the dimer of p50:p50 or p50:p65 to be released (Cohen, Achbert-Weiner et al. 2004).
3.6 TAK1 and NF-κB signaling

Stimulation of IL-1R by IL-1β or TLR4 by LPS causes Myd88, IRAK and TRAF6 to be recruited to the receptor. TRAF6, an E3 ubiquitin ligase of the RING type, undergoes K63-linked autoubiquitination in a complex with the dimeric E2 enzyme Ubc13/Uev1A. Polyubiquitin chains of TRAF6 functioning as a scaffold, recruit TAK1 and IKK complex which leads to polyubiquitination of TAK1 (Adhikari, Xu et al. 2007; Skaug, Jiang et al. 2009). TAB2, or TAB3, is required as an adaptor protein to link TRAF6 and TAK1. TAK1 recruitment to this complex results in its subsequent autophosphorylation of two threonine residues (Thr184 and Thr187) and a serine residue (Ser192). TAB1 is also required, as an activator protein, in this step to associate with TAK1 through its N-terminal kinase domain (Chen 2005). Whether phosphorylation or ubiquitination occurs first, remains to be elucidated. Now fully active, TAK1 phosphorylates IKKβ in the IKK complex. The IKK complex consists of 3 subunits, i.e., IKKα, IKKβ and IKKγ or NEMO. NEMO is the regulatory subunit through which the IKK complex is associated with polyubiquitin chains of TRAF6. Phosphorylated IKK subsequently phosphorylates IκB which marks it for K48-linked ubiquitination by the SCFβTrCP E3 ligase complex and proteasomal degradation. Thus, NF-κB dimers which are sequestered in the cytoplasm by binding to IκB proteins under basal conditions, are free from inhibitory partners and enter the nucleus (Adhikari, Xu et al. 2007; Skaug, Jiang et al. 2009).

Stimulation of tumor necrosis factor receptor (TNFR) by its ligand, TNF-α, causes the adaptor proteins TRADD, RIP1, TRAF2, cIAP1 and cIAP2 to form a receptor-associated complex. Subsequently, RIP1 is K63-linked polyubiquitinated by TRAF2. Polyubiquitin chains of RIP1 recruit TAK1 through TAB2 or TAB3, and the IKK complex through NEMO. TAK1 activates the IKK complex by phosphorylation, leading to activation of NF-κB (Skaug, Jiang et al. 2009).

Cylindromatosis (CYLD) is a tumor suppressor and deubiquitinating enzyme (DUB) which cleaves Lys63-linked ubiquitin chains on molecules involved in NF-κB pathway, including TAK1 (Ahmed, Zeng et al. 2011; Wang, Gao et al. 2012), thus negatively regulating NF-κB signaling (Wang, Gao et al. 2012). A20 and cezanne-1 are other examples of DUBs targeting the components of the NF-κB signaling pathways (Wertz and Dixit 2010).

3.7 NF-κB signaling, inflammation and cancer

Inflammation has been implicated in cancer initiation and progression (Naugler and Karin 2008). There are a number of findings supporting the fact that NF-κB is one of the key bridges linking inflammation and cancer (Baud and Karin 2009). During persistent inflammation due to inflammatory
cytokines or during chronic infections, NF-κB becomes activated. Dysregulation of the NF-κB pathway, leading to its constitutive activation occurs in many solid and haematopoietic malignancies. It is the transcriptional activator of a variety of genes involved in different cellular functions including inflammation, proliferation, survival, angiogenesis as well as tumor promotion and metastasis. Therefore, it has a role in initiation and expansion of pre-neoplastic cells, as well as in rendering tumors resistant to proapoptotic agents, anti-cancer drugs and radiation (Basseres and Baldwin 2006). Sweeney et al. observed that NF-κB was constitutively bound to DNA in prostate cancer cell lines. They also studied prostatectomy specimens by immunohistochemistry and found that NF-κB is overexpressed in prostatic intraepithelial neoplasia and adenocarcinoma compared with the benign epithelium (Sweeney, Li et al. 2004). Zhang and colleagues demonstrated that NF-κB activity increases in prostate cancer cells which are resistant to tumor necrosis factor-related apoptosis ligand (TRAIL)- or TNF-α-induced apoptosis (Zhang, Huang et al. 2009). Lessard et al studied the level of nuclear NF-κB staining of primary tumors in patients with prostate cancer metastasis to lymph nodes as compared with patients without lymph node invasion. Their results indicate that nuclear NF-κB expression is elevated in invasive cancer epithelium and there is significant association between the extent of NF-κB staining and lymph node invasion. Therefore, the level of nuclear localization of NF-κB in primary prostate tumors can be used to predict the probability of spread of prostate cancer (Lessard, Karakiewicz et al. 2006).
4. Ubiquitination

Ubiquitin is a highly conserved 76-amino acid protein which can be covalently attached to proteins in monomeric or polymeric forms. Polyubiquitination (polyubiquitylation) of the protein was initially known to mark it for degradation by the proteasomal system (Chen 2005), but other regulatory effects have recently been shown to also occur as a consequence of ubiquitination. Ubiquitin is added to the protein in three steps. An E1 ubiquitin-activating enzyme makes a thioester bond with ubiquitin to activate it in an ATP-dependent manner. The activated ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme which in turn interacts with and transfers the activated ubiquitin to an E3 ubiquitin ligase. The E3 ligase facilitates the formation of an isopeptide bond between the ε–amino group in the side chain of a lysine residue in a target protein and the C-terminal glycine (G76) of ubiquitin (Figure 6). Further ubiquitin molecules can be linked to any of the seven lysine residues of the previous ubiquitin or to its N-terminal amino group via C-terminal G76 to make polymers. There are only two isoforms of E1 in the cell (E1a and E1b) while there are 50 different E2s. E3 ligases are even more abundant and classified in three groups according to which domain they contain, i.e. homologous to E6AP COOH-terminus (HECT), really interesting new gene (RING) domain or U-box domains (Lehman 2009).
4.1 Proteasomal degradation of ubiquitinated proteins

Lys48- and Lys11-linked chains composed of a minimum four ubiquitins, targets the protein for proteasomal degradation. The proteasome is a 26S subcellular organelle which is highly conserved from archaeabacteria to eukaryotes. It is cylindrical and composed of two subunits, the 20S catalytic subunit and the 19S regulatory subunit. The 19S components bind to polyubiquitin chains, prepare and translocate the substrate to the core while the 20S core particle contains proteolytic activity. The 20S subunit consists of four protein rings, i.e. 2 α and 2 β rings (Wertz and Dixit 2010).

In the rings of the core subunit, DUBs remove ubiquitin monomers followed by the proteolytic degradation of the target protein (Figure 7). Cleavage products are short peptides, which are exposed to further proteolysis in the cytosol, and recyclable ubiquitin molecules (Mani and Gelmann 2005; Lehman 2009).
4.2 Non-canonical functions of ubiquitination

The classical ubiquitination of proteins via Lys48-linked chains marks them for degradation by proteasomes. However, monoubiquitination at one or multiple lysine residues and Lys63-linked polyubiquitination facilitate endocytosis, protein sorting, receptor trafficking and/or activation of kinases (Mani and Gelmann 2005). Recently, other types of ubiquitin linkage have also been shown to have physiological roles, such as Lys6-, Lys29- and Lys33-linked polyubiquitin chains (Li and Ye 2008). Linear ubiquitin chains have been emerged recently. The linkage in linear ubiquitin chains is between the N-terminal amino group of methionine on a ubiquitin which is covalently bond with a target protein and the C-terminal carboxy group of the incoming ubiquitin. Linear ubiquitination has physiological roles in innate and adaptive immunity and suppression of inflammation (Walczak, Iwai et al. 2012).
v-Src is the first identified viral oncogene, and one of the best studied. v-Src is the causative agent of malignancy produced by the Rous sarcoma virus in chickens. The proto-oncogene and the cellular counterpart of this oncogene is c-Src which is poorly transforming as compared to v-Src (Yeatman 2004). c-Src, referred to as Src hereafter, is the prototypic member of the Src family kinases (SFKs) which also contains Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck and Lyn. Among the members, Src, Fyn and Yes are expressed ubiquitously. SFKs are non-receptor membrane-associated protein tyrosine kinases which by phosphorylating their substrates transduce signals from extracellular environment to pathways in the cell (Guarino 2010).

Src is a 60-kDa protein comprised of several functional domains, i.e. the N-terminal which contains a myristoylation site to allow association with the inner layer of the plasma membrane, the unique domain which renders unique function and specificity to each member of SFK, an SH3 domain important for intra- and intermolecular interactions by binding to proline-rich sequences, an SH2 domain which binds phospho-tyrosine residues on Src or other proteins, the linker domain which binds the intramolecular SH3 domain, an SH1 catalytic domain with the kinase activity containing the positive-regulatory autophosphorylation site at Tyr419 in humans (Tyr416 in chicken), and the C-terminal tail containing the negative-regulatory Tyr530 in humans (Tyr527 in chicken) (Guarino 2010).

The biological action of Src is regulated by its interaction with other proteins facilitated through specific domains in Src and also by the subcellular localization of Src. In the absence of transient activating signals, Src is maintained inactive with a juxtanuclear localization. In the inactive form, SH2/SH3 intramolecular interactions and phosphorylated Tyr530 in the C-terminal tail which binds the SH2 domain, inhibit the kinase domain. Upon stimulation, Src is dephosphorylated in the C-terminal tail and translocates to the plasma membrane at the cell-cell and cell-matrix adhesion sites through association of SH3 domain with actin filaments. At the plasma membrane, Src interacts with membrane-bound phosphotyrosine or proline-rich proteins that can compete for binding to the SH2 or SH3 domain, and thereby destabilize the low-affinity SH2/SH3 intramolecular interactions. Consequently, an open active configuration is formed and Tyr419 in the catalytic domain is exposed to phosphorylation which causes full kinase activation of Src.
Figure 8) (Varkaris, Katsiampoura et al.; Yeatman 2004; Guarino 2010; Varkaris, Katsiampoura et al. 2014).

Figure 8. Schematic representation of Src in the inactive (left) and the active (right) state. [Guarino M, J Cell Physiol. 2010 Apr;223(1):14-26. Image was reprinted with permission.]

5.1 Regulation of Src activity

Two studies have reported rare point mutations which truncates Src just C-terminal to the regulatory Tyr530. Although these mutations resulted in Src activation in colon cancer (Irby, Mao et al. 1999) and endometrial carcinoma (Sugimura, Kobayashi et al. 2000), activating mutations or gene amplification of Src is very rare in human tumors (Yeatman 2004). Since Src is often overactivated during cancer progression, identification of other mechanisms controlling Src phosphorylation by kinases or phosphatases are of importance. Tyr530 phosphorylation is performed by C-terminal Src kinase (Csk). Reduced expression or activity of Csk has been observed in hepatocellular and colorectal carcinomas. Csk is recruited to the membrane through the interaction of its SH2 domain with the transmembrane adaptor Csk binding protein (Cbp) which is phosphorylated at a tyrosine motif by Src. Therefore, Csk acts in a negative feedback loop mediated by Cbp, by phosphorylating Src to induce the inactive closed configuration (Guarino 2010). Multiple phosphatases including protein tyrosine phosphatase-α (PTPα), PTP-λ, PTP1B, SH2-containing phosphatase 1 (SHP1) and SHP2 dephosphorylate Tyr530 and stabilize the open conformation (Varkaris, Katsiampoura et al. 2014). The PTP1B level is upregulated in breast cancer cell lines and human colon cancer cells (Guarino 2010; Varkaris, Katsiampoura et al. 2014). The Tyr419 phosphate is targeted by proline-enriched tyrosine phosphatase (PEP) which also binds the SH3 domain of Csk (Ingley 2008). Another
mode of Src regulation is ubiquitination mediated by the E3 ubiquitin ligase Cbl, which leads to Src degradation by the proteasome-dependent pathway; this pathway is deregulated in cancer cells, thus promoting Src activity (Yeatman 2004; Guarino 2010). The interaction of Src with ligand-activated RTKs, such as EGFR, PDGFR, ERBB2, IGF-1R, colony-stimulating factor-1 receptor (CSF-1R), hepatocyte growth factor receptor (HGFR) and stem cell factor receptor (SCFR) also results in synergistic Src activation probably by disrupting the intramolecular interactions which hold Src in closed configuration (Yeatman 2004; Varkaris, Katsiampoura et al. 2014).

5.2 Substrate effectors of Src

Src signals inside the cell by phosphorylating tyrosine residues on substrates mainly downstream of RTKs and integrins. Upon binding of the respective ligand to RTK, receptors dimerize and autoprophosphylate on tyrosine residues of the cytoplasmic domain which recruit and activate Src. Activated Src, then, phosphorylates RTK which both enhances RTK tyrosine kinase activity and creates SH2 binding sites for the downstream proteins, such as the growth factor receptor-bound protein 2/son of sevenless homolog 1 (Grb2/Sos 1) complex, and PI3K leading to the activation of Ras/MAPK and AKT, respectively (Guarino 2010).

Src is also the downstream mediator of integrins which are transmembrane adhesion receptors for matrix molecules at focal adhesions (Guarino 2010). Integrins link components of extracellular matrix (ECM) to the cytoskeletal stress fibers containing actin and myosin via structural molecules such as paxillin, talin, vinculin, tensin, α-actinin, and signaling molecules, such as Src and focal adhesion kinase (FAK) to control the shape, the motility and invasiveness of the cell (Yeatman 2004). Activated Src and FAK promote the disassembly of the focal adhesion and stress fibers to induce cell motility and invasiveness. This happens during normal cellular migration and mitosis, as well as during transformation. When motility is not required, Csk phosphorylates and inactivates Src to maintain the integrity of focal adhesions. Substrates of Src, Crk and Nck are adaptor proteins containing SH2 and SH3 domains, which recruit proline-rich molecules important for the actin cytoskeleton reorganization. Crk-associated substrate (CAS) is another Src substrate, which is implicated in cell motility (Guarino 2010).

To promote tumor-cell motility and invasiveness, Src also interacts with the components of adherens junctions, i.e. cell-cell adhesion sites. Homotypic E-cadherin molecules on adjacent cells bind in adherens junctions. First, β-catenin is recruited to the cytoplasmic C-terminus of E-cadherin and next α-catenin binds and links this complex to actin cytoskeleton directly or indirectly via intermediate molecules, such as vinculin and vasodilator-stimulated phosphoprotein (VASP). Src and PTP1B associate with this com-
plex. When activated by PTP1B or other mechanisms, Src promotes ubiquitination of E-cadherin which leads to endocytosis of E-cadherin and disruption of adherens junctions, resulting in increased motility and invasiveness and eventually metastasis (Yeatman 2004; Varkaris, Katsiampoura et al. 2014).

FAK is a substrate of Src that after activation by Src, activates JNK signaling pathway inducing matrix metalloproteinase MMP2 and MMP9. MMPs promote the breakdown of the ECM which is required for tumor invasion of surrounding tissues (Hsia, Mitra et al. 2003). Src also activates signal transducer and activator of transcription 3 (STAT3) which induces expression of vascular endothelial growth factor (VEGF), thereby promoting angiogenesis. STAT3 activation by Src has been shown to promote tumorigenesis by enhancing cell growth and survival, and immune evasion (Yeatman 2004; Yu and Jove 2004).

There are studies demonstrated that Src and the androgen receptor (AR) physically interact and reciprocally activate each other, which is important for prostate cancer initiation and progression (Varkaris, Katsiampoura et al. 2014). Src activation was observed in castrate-naïve prostate cancer tissues. Upon androgen-binding to AR and physical interaction of Src and AR, cell proliferation is induced through Src phosphorylation and activation (Vlaeminck-Guillem, Gillet et al. 2014). On the other hand, Src activation was correlated with AR phosphorylation in metastatic castrate-resistant human samples. Src, activated by extracellular signals produced by neighboring tumor or stromal cells, such as IGF-1, EGF, neuropeptides IL-6 and IL-8, leads to tyrosine phosphorylation of AR and promoting indirect phosphorylation of AR at serine/threonine residues. Thereby, ligand-independent AR-dependent activation of gene expression programs is induced through AR translocation to the nucleus, decreased proteasomal degradation and decreased interaction with co-repressors (Vlaeminck-Guillem, Gillet et al. 2014).
Present investigations

Prostate cancer is the most common cancer and one of the leading causes of cancer-related death in men (Assinder, Dong et al. 2009). Defects in signaling pathways, such as NF-κB, PI3K/Akt and Src pathways, have been implicated in initiation and progression of prostate cancer.

The aims of this study were to investigate:

1. How PI3K/Akt is regulated in response to TGFβ resulting in the migration of the cells which is important for the progression of tumors.
2. How TAK1 becomes activated in response to stimulation by TNF-α, IL-1β and LPS and how this leads to the activation of NF-κB.
3. Whether TGFβ activates Src and explain the mechanism

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

In spite of high incidence of prostate cancer among men especially in industrialized countries, the treatment strategies are limited and not successful in every patient. There is also a need for identification of better biomarkers to predict individual prognosis (Assinder, Dong et al. 2009). The level of pAKT is elevated in many cancers especially in those with poor prognosis (Wegiel, Bjartell et al. 2008). Deregulation of PI3K and TGFβ pathways has been recognized to correlate with tumor progression of prostate cancer. The precise molecular mechanism for activation of AKT in response to TGFβ stimulation is not known.

We have demonstrated that TRAF6 is crucial for interaction of p85α with TβRI and for TGFβ-induced Lys63-linked polyubiquitination of p85α. Through one of the SH2 domains, p85α binds to TRAF6 and TβRI, and is ubiquitinated by TRAF6. This leads to the recruitment of AKT to the complex and Lys63-linked polyubiquitination and activation of AKT which requires the E3 ligase activity of TRAF6. AKT interaction with TβRI and its activation through TGFβ stimulation does not depend on the kinase activity of TβRI because pre-treatment of cells with an inhibitor of TβRI kinase did not affect the TGFβ-induced AKT phosphorylation. Moreover, the activation of AKT by TGFβ is dependent on PI3K activity. Finally, we performed
wound-healing and transwell motility assays and demonstrated that PI3K-dependent TβR1 kinase-independent polyubiquitination of p85α and AKT is necessary for the motility of cells stimulated by TGFβ, which promotes cancer progression. Our findings reveal the mechanism of activation of PI3K/AKT pathway by TGFβ.

Paper II: Polyubiquitination of transforming growth factor β (TGFβ)-associated kinase 1 mediates nuclear factor-κB activation in response to different inflammatory stimuli

It has been observed that inflammation involves activation of NF-κB which is a transcription factor that is sequestered inactive in the cytoplasm by IκB. In stimulated cells, IκB kinase (IKK) is activated through different signaling pathways and catalyzes the phosphorylation of IκB which results in its degradation and detachment from NF-κB. Free NF-κB activates many target genes in the nucleus (Karin 2006). The IKK complex is composed of three subunits, i.e. IKKα, IKKβ, IKKγ (NEMO). IKKβ is activated by phosphorylation by TAK1 (Dong, Liu et al. 2006). In the TGFβ, IL-1β and TLR4 pathways, the E3 ubiquitin ligase TRAF6 is required for activation of TAK1 (Adhikari, Xu et al. 2007). Site-specific Lys63-linked auto-ubiquitination of TRAF6 is a critical upstream mediator of IKK activation in IL-1R signaling (Lamothe, Besse et al. 2007). In the TNF-α signaling pathway, phosphorylation of TRAF2 and Lys63-linked polyubiquitination of TRAF2 induce association with TAB2 and 3 and bridge of the TAK1 complex to IKKα/β (Li, Wang et al. 2009).

We report here that polyubiquitination of TAK1 at K34 is required for its own activation and for activation of downstream components of the pathway in TNFR-β, IL-1R and TLR4-mediated signaling. The mutation of Lys34 to arginine in TAK1 inhibited the polyubiquitination of TAK1 in all the pathways addressed in this study. The phosphorylation of TAK1 or IKKα/β was impaired by the K34R mutant TAK1 in IL-1β signaling. The phosphorylation of IKKα/β was inhibited by transfection of the K34R mutant TAK1 in response to TNF-α. We also show that C70A mutant TRAF6 which lacks the E3 ligase activity, reduced TAK1 polyubiquitination and phosphorylation, as well as IKKα/β phosphorylation in response to IL-1β. Moreover, by performing NF-κB luciferase assays, we demonstrate that polyubiquitination of TAK1 at K34 was essential for appropriate NF-κB signaling in three different inflammatory pathways, i.e. signaling via TNFR, IL-1R and TLR4. The mutated K34 TAK1 impaired NF-κB activation by preventing nuclear translocation of p65 leading to downregulation of IL-8 and IL-6 production and
secretion in TNF-α- and LPS-stimulated cells, respectively. IL-6 luciferase assays confirmed that mutation of TAK1 at Lys34 diminished NF-κB activation.

Our study explains how Lys63-linked polyubiquitination of TAK1 regulates the pro-inflammatory responses.

Paper III: Mechanism of regulation of Src kinase by transforming growth factor β

Src has been implicated in tumorigenesis by promoting cell growth, migration, invasion and metastasis, and has been shown to be activated by TGFβ (Ungefroren, Sebens et al. 2011). TGFβ plays important roles in proliferation, migration and invasion of cancer cells. However, the mechanisms by which TGFβ activates Src and how this activation leads to these cellular processes are not known.

We demonstrate that TGFβ induces Src Y419 phosphorylation, reflecting its activation, in human embryonic kidney cells (HEK293T), human prostate carcinoma cells (PC3U) and mouse embryonic fibroblasts (MEF). Src was activated proportionally to the amount of the expressed HA-TβRI, in a TGFβ-dependent manner. By transfection of constitutively active (T20D) or kinase-dead (K232R) mutant HA-TβRI in HEK293T, we observed that Src was phosphorylated at Y419 and activated by TGFβ stimulation in both cell types independent of the kinase activity of TβRI. Src interacts with TβRI in a TGFβ-dependent manner and this association does not depend on the kinase activity of TβRI or Src because a kinase-deficient mutant Src (K295R, Y527F) was co-immunoprecipitated with the receptor similarly to wt Src. The amount of Src co-immunoprecipitated with the receptor increased in proportion to the amount of overexpressed TRAF6 although the E3 ligase activity of TRAF6 was not crucial. By overexpression of a mutant TβRI which cannot bind TRAF6 (E161A), we confirmed that TRAF6 is required for the interaction of Src and TβRI. TGFβ induces Src ubiquitination which is also enhanced by overexpression of E3 ligases Cbl and TRAF6. We performed the transwell migration assay to study the importance of Src for TGFβ-induced cell motility. TGFβ-enhanced motility of cells was down-regulated in Src- and Fyn-deficient MEF cells as compared to wt MEF cells.

Overall, we found the mechanism of activation of Src by TGFβ which leads to the migration of cells.
Future perspectives

Paper I
We identified TRAF6 as an E3 ligase which ubiquitinates AKT after TGFβ stimulation. The acceptor lysine for ubiquitination by TRAF6 in response to different growth factors, IGF-1, EGF and IL-1, has been identified (Yang, Wang et al. 2009). We will investigate whether TRAF6 mediates the ubiquitination of AKT at the same acceptor lysine in TGFβ-stimulated cells. We also found that p85α binds to TRAF6 and TβRI via one of the SH2 domains and to be ubiquitinated by TRAF6. By mutating the putative lysines in p85α, we can identify the lysine which accepts Lys63-linked polyubiquitin chain. We will investigate whether this mutant is able to abolish the motility of the cells. We have found that TRAF6 is tyrosine phosphorylated (data not shown). This could explain the nature of the interaction of TRAF6 with PI3K and AKT. We need to investigate this possibility by mutating the putative tyrosine and studying the interaction of wt vs. mutated TRAF6 with p85α and AKT by co-immunoprecipitation experiments.

Paper II
We elucidated that TAK1 undergoes polyubiquitination in response to ligand-induced activation of TNF-R, IL-1R, and TLR4, and that mutation of Lys-34 (K34R) in TAK1 resulted in a loss of TAK1 and IKK activation, which prevented activation and translocation of NF-κB into the nucleus. However, the kinetics for TAK1 polyubiquitination and subsequent activation in various signaling pathways differs, which might be due to the involvement of other components that are different among the pathways. This possibility remains to be further explored.

It will be interesting to investigate the mechanism which determines whether NF-κB-mediated prosurvival pathway or JNK/p38 proapoptotic pathway is pursued in response to TGFβ, since we reported that TGFβ induces NF-κB signaling by TRAF6-induced Lys63-linked polyubiquitinated TAK1 in hepatoma cells (HepG2) independent of p38, whereas it was previously reported by our group that TGFβ-induced Lys63-linked polyubiquitination of TAK1 is crucial for its activation and subsequent activation of p38 MAP kinase and induction of apoptosis (Sorrentino, Thakur et al. 2008).
Our observation that LPS/TLR4-induced activation of the NF-κB pathway in mouse macrophages (RAW264.7 cell line) resulted in IL-6 promoter activation and IL-6 secretion can provide a hint to design a treatment to prevent the vicious cycle of tumor-macrophage interplay leading to prostate tumor progression mediated by IL-6.

**Paper III**

Src is fully activated when the activating Y419 phosphorylation occurs and the inhibitory phosphate group is removed from Y530. Therefore, it will be interesting to study the Y530 phosphorylation pattern in response to TGFβ to determine the importance of the two events resulting in Src full activation.

We observed that TRAF6 enhances the TGFβ-induced Y419 phosphorylation of Src, as well as interaction of Src with TβRI. We will investigate whether TRAF6 binds Src and, if so, which domain of TRAF6 binds Src. Moreover, both Cbl and TRAF6 promotes TGFβ-induced Src polyubiquitination. Whether there is a balance between the two E3 ligases to ubiquitinate Src and the role of each type of ubiquitination for Src stability and interaction with TβRI, remain to be explored further.

Both Src and TGFβ are involved in the aggressive behavior of prostate cancer and its metastasis to bone (Cook, Shay et al. 2014; Varkaris, Katsiampoura et al. 2014). It will be of great interest to study how TGFβ/TRA6/Src axis contributes to the invasive property of prostate cancer and determine the molecular mechanism whereby the functionality of this axis is regulated.
Acknowledgments

It has been a great experience for me to do my PhD at Ludwig Institute for Cancer Research, Uppsala. This place is amazing since one gets scientific feedbacks whenever it is needed and everyone is so friendly that I have always felt working with my family. I cannot imagine how I should adjust myself to other working places. However, this is only imagination and I will move forward anyway because nothing last forever in this world. But the positive side of the fact is that I learned not only many techniques and some signaling pathways, the details of which I might forget after some time, but also how to interact with others in a scientific environment, which I will never forget. I learned how I can be a good researcher and at the same time be helpful and interactive and share my knowledge with my colleagues. This is something one cannot find at every scientific place. In my opinion, this could not be possible without such a wonderful head of the Institute, Prof. Carl-Henrik Heldin whom I really admire. He was also my co-supervisor and helped me with the projects.

The opportunity to be a PhD student at Ludwig Institute was given to me by Prof. Maréne Landström, my supervisor who has always been friendly, kind and patient, and when it comes to experiments, optimistic and encouraging and has guided me throughout this period. Dear Maréne, I can summarize describing your personality by saying “You are my idol”.

I have especial thanks to Prof. Aristidis Moustakas for giving very good feedbacks to my projects during the group meetings on Mondays.

I would like to thank those with whom I worked in the lab; Noopur Thakur, Maryia and Ihor Yakymovych and the previous members of the group; Maria Ekman, Anders Marcusson, Alessandro Sorrentino. We had very nice time together.

I enjoyed sharing the lab with Carmen, Anders Sundqvist, Ria, Masato and Olex, and previously with Johan Lennartsson’ group; Johan, Lotti, Linda, Piotr, Basia and especially Ola who is an angel.

I appreciate endless help which I got from the members of the Institute; Aive, Ulla, Aino, Anita, Lotti and Maryia.

Working was not possible without technical support of Ingegärd, Lasse and Uffe. Thank you!

I am thankful to former and current group leaders; Ulf Hellman, Paraskevi Heldin and Ingvar Ferby, and other people who are or have been working in the Institute, namely Inna, Erna, Yukihide, Aga, Berit,
Helena, Glenda, Haisha, Mahsa, Varun, Pratyusha, Natalia, Maria Tsioumpekou, Costas, Panos, Andries, Laia, Kallia, Ana Rosa, Sara, Claudia, Mari, Julia and Giulia.

I would like to thank my colleague and friend, Vanessa karen de Sá from Brazil, who was a guest researcher in the Institute for one year. Although you were here for a short time, I feel that I know you forever.

I would like to thank my kind friend, Maryam Nikpour who has always been there for me when I wanted.

I would like to show my gratitude to my parents who have guided me throughout my life as long as I was living together with them and been always there for me afterwards. Dear Dad, thank you for teaching me to love reading and learning; Dear Mom, thank you for teaching me to be patient. Without you and your pieces of advice, I would have not been standing at this position where I am now. You have been great!

I really appreciate my sister, Rosita who has always helped me with taking care of the children. More importantly, at those moments when I cannot share my feelings with anyone, I know that I can talk to you to feel strong and confident again.

I would like to show my love and gratitude to my husband, Masoud who means everything to me. My dear love, without your support, I was not able to do this!

In the end, I am thankful to my children who accepted that mamma had to work although they missed me. Dear Dariush and Arta, you might not understand this at the moment, but you will later on. Dariush, you always surprise me with your words which I expect from an older child and Arta, you are very sweet and in the meantime very naughty. It is because of both of you, sweethearts, that I feel hopeful every day.
References


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1126

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-259224