Functional interference of androgen receptor signaling by the Inhibitor of Growth 1

Dissertation

in partial fulfilment of the requirements for the degree of doctor rerum naturalium (Dr. rer. nat.)

submitted to the Faculty Council of the School of Medicine at Friedrich Schiller University of Jena

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<tbody>
<tr>
<td>α</td>
<td>alpha</td>
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<tr>
<td>AA</td>
<td>atraric acid</td>
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<tr>
<td>ADT</td>
<td>androgen deprivation therapy</td>
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<tr>
<td>AF</td>
<td>activation function</td>
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<td>AFP</td>
<td>alpha-fetoprotein</td>
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<td>AP</td>
<td>alkaline phosphatase</td>
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<td>AR</td>
<td>androgen receptor</td>
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<td>ARE</td>
<td>androgen response element</td>
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<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>C</td>
<td>carboxy</td>
</tr>
<tr>
<td>C⁺</td>
<td>positive control</td>
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<tr>
<td>°C</td>
<td>degree Celsius</td>
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<tr>
<td>CaPO₄</td>
<td>calcium phosphate</td>
</tr>
<tr>
<td>Cas</td>
<td>casodex</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CRPCa</td>
<td>Castration-resistant prostate cancer</td>
</tr>
<tr>
<td>CSS</td>
<td>charcoal stripped serum</td>
</tr>
<tr>
<td>Δ</td>
<td>delta</td>
</tr>
<tr>
<td>DA</td>
<td>diacetate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
<td>DCF</td>
<td>dichlorofluorescein</td>
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<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DOTAP</td>
<td>N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methyl sulphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>Eco</td>
<td>ecotropic</td>
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<tr>
<td>EcoR</td>
<td>ecotropic receptor</td>
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<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GC</td>
<td>guanine-cytosine</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
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<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>H</td>
<td>histone</td>
</tr>
<tr>
<td>H</td>
<td>high concentration</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
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<tr>
<td>HeLa</td>
<td>human cervix carcinoma cell line</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HMT</td>
<td>histone methyltransferase</td>
</tr>
<tr>
<td>HPEC</td>
<td>human prostate epithelial cell</td>
</tr>
<tr>
<td>ING</td>
<td>inhibitor of growth</td>
</tr>
<tr>
<td>K</td>
<td>amino acid lysine</td>
</tr>
<tr>
<td>KD</td>
<td>knockdown</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>l</td>
<td>liter</td>
</tr>
<tr>
<td>L</td>
<td>low concentration</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>LID</td>
<td>lamin interaction domain</td>
</tr>
<tr>
<td>LNCaP</td>
<td>lymph node cancerous prostate</td>
</tr>
<tr>
<td>luc</td>
<td>luciferase</td>
</tr>
<tr>
<td>LZL</td>
<td>leucine zipper-like</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
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<tr>
<td>m</td>
<td>meter</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>M</td>
<td>Manders’ coefficient</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
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</tbody>
</table>
methyl group
mouse embryonic fibroblast
N-(benzyloxy carbonyl)leucinylleucinylleucinal
minutes
matrix metalloproteinase
mouse mammary tumor virus
nano
N-acetyl cysteine
nuclear localization signal
amino terminal domain
nucleolar targeting sequences
optical density
pico
probability
Poly ADP ribose polymerase
partial bromo domain
polybasic region
phosphate buffered saline
probasin
prostate cancer
plant homeodomain
PCNA-interacting protein motif
phosphorylated retinoblastoma protein
progesterone receptor
retinoblastoma protein
prostate specific antigen
polyvinylidene fluoride
quantitative reverse transcriptase polymerase chain reaction
methyltrienolone
ribonucleic acid
Pearson’s correlation coefficient observed
Reactive oxygen species
rounds per minute
Roswell Park Memorial Institute
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>R(rand)</td>
<td>Pearson’s correlation coefficient following randomization</td>
</tr>
<tr>
<td>S</td>
<td>amino acid serine</td>
</tr>
<tr>
<td>SA-β-Gal</td>
<td>senescence-associated beta-galactosidase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SELDI-MS</td>
<td>surface-enhanced laser desorption/ionization-mass spectrometry</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>seq</td>
<td>sequencing</td>
</tr>
<tr>
<td>sh</td>
<td>short hairpin</td>
</tr>
<tr>
<td>Src</td>
<td>sarcoma tyrosine kinase</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin-like modifier</td>
</tr>
<tr>
<td>TAU</td>
<td>transcriptional activation units</td>
</tr>
<tr>
<td>TERT</td>
<td>telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TH</td>
<td>thyroid hormone</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TR</td>
<td>thyroid hormone receptor</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside</td>
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Zusammenfassung

Der Androgenrezeptor (AR)-Signalgebung ist von entscheidender Bedeutung für das Fortschreiten von Prostatakrebs (PCa) zum kastrationsresistenten Stadium mit schlechtem klinischen Ausgang. Dysfunktionen von AR interagierenden Faktoren tragen zu kastrationsresistentem PCa (CRPCa) bei. *Inhibitor of Growth 1* (ING1) ist ein epigenetischer Regulator vieler zellulärer Prozesse, darunter für die Proliferation und zelluläre Seneszenz. Eine Fehlregulierung von ING1 konnte bereits mit PCa in Verbindung gebracht werden, jedoch ist die Rolle von ING1 für die AR-Signalgebung noch unbekannt. Basierend auf den gezeigten Interaktionen zwischen AR und ING1b stellt ING1b eine Möglichkeit dar, den AR spezifisch zu inhibieren und zelluläre Seneszenz in PCa Zellen zu induzieren.

Androgenabhängige LNCaP und CRPCa AR-exprimierende PC3-AR Zelllinien wurden verwendet, um den Einfluss von ING1 auf Wachstum, Migration, zelluläre Seneszenz und AR-vermittelte Genexpression zu analysieren. *Ing1* KO-Mäuse wurden verwendet, um die Rolle von ING1b auf AR-vermittelte Transkription *in vivo* zu analysieren.

Die Ergebnisse zeigen, dass die ING1b Expression in CRPCa Zellen im Vergleich zu androgenabhängigen Zellen herunter reguliert ist, dass ektopische ING1b Expression in beiden PCa Zelllinien die zelluläre Seneszenz induziert und die Zellmigration reduziert. Überdies induziert ING1b verschiedene CDK Inhibitoren, einschließlich p27KIP1 – das hier neu identifizierte Zielgen p27KIP1. ING1b Knockdown (KD) Experimente zeigen, dass ING1b die AR-induzierte zelluläre Seneszenz in PC3-AR Zellen vermittelt. Außerdem vermag ING1b, das androgeninduzierte Wachstum in LNCaP Zellen in ähnlichem Maße zu inhibieren wie AR Antagonisten. Interessanterweise wird die ING1b Expression durch die Stimulation mit Antagonisten hochreguliert. Die ING1b Überexpression reprimiert die AR Transaktivierung von Schlüsselzielen in LNCaP Zellen, möglicherweise durch AR Abbau. Überraschenderweise inhibiert der ING1b KD die AR-vermittelte transkriptionelle Regulation der gleichen Zielgene in beiden PCa Zelllinien, was mit Ing1 KO Mäuse *in vivo* bestätigt wurde. Dieses interessante Ergebnis konnte durch einen kompensatorischen Mechanismus mittels erhöhter ING2a Expression erklärt werden, da ektopische Expression von ING2a – ähnlich ING1b – die AR-vermittelte transkriptionelle Aktivierung hemmt. Des Weiteren deuten die Daten auf eine Induktion von p16INK4a durch ING2a in LNCaP Zellen, was zuvor noch nicht beschrieben wurde.
Zusammengenommen deutet dieser kompensatorische Mechanismus auf eine neue Wechselwirkung innerhalb den Mitgliedern der ING Familie und auch in Bezug auf die Regulation der AR-Funktionen hin und öffnet einen potentiellen Weg den AR-Signalweg in PCa zu inhibieren.
Summary

The androgen receptor (AR) signaling is critical for prostate cancer (PCa) progression to the castration-resistant stage with poor clinical outcome. Altered function of AR interacting factors contributes to castration-resistant PCa (CRPCa). Inhibitor of growth 1 (ING1) is an epigenetic regulator of various cellular processes including proliferation and cellular senescence. ING1 dysregulation and its signaling have been implicated in PCa, but its role in AR signaling is still unknown. Based on the shown interaction between AR and ING1b, specific targeting the AR by ING1b can be one possible way to inhibit the AR signaling and induce cellular senescence in PCa cells.

Androgen-dependent LNCaP and castration-resistant AR-expressing PC3-AR cell lines were used to analyze the ING1 influence on growth, migration, cellular senescence and AR mediated gene expression. To further confirm the regulatory role of ING1b on AR mediated transcription in vivo, Ing1 KO mice were used.

The results indicate that ING1b expression is downregulated in CRPCa cells compared to androgen-dependent ones. Thereby, its ectopic expression induces cellular senescence and reduces cell migration in both PCa cells. Moreover, ING1b upregulates different CDK inhibitors including p27KIP1 which is a novel target for ING1b. ING1b knockdown (KD) analysis indicates that ING1b is a downstream target of AR mediated cellular senescence in PC3-AR cells. ING1b can also inhibit androgen induced growth in LNCaP cells in a similar manner to AR antagonists. Interestingly, the expression of ING1b is upregulated upon treatment with antagonists. ING1b overexpression represses AR transactivation on key target genes in LNCaP cells possibly through AR degradation. Intriguingly, ING1b KD inhibits AR-mediated transcriptional regulation of the same target genes in both PCa cells, which could be verified in vivo using Ing1 KO mice. This interesting result could be explained by the compensatory mechanism through enhanced expression of the ING2a protein in ING1-deficient condition as ectopic expression of ING2a also hampers the AR transcriptional activation similar to ING1b. The data further suggest the induction of p16INK4a by ING2a in LNCaP cells, which has not yet been reported.

Taken together, this compensatory mechanism suggests a novel crosstalk among ING family members in regulating AR functions and opens a potential way to inhibit AR signaling in PCa.
1 Introduction

1.1 Prostate cancer

Prostate cancer (PCa) ranks as the second leading cause of male cancer-related death in the Western world (Ferlay et al. 2013, Siegel et al. 2013). Majority of localized primary prostate tumors are treated successfully by radical prostatectomy or external beam radiotherapy, however, some tumors progress to invasive form (Bluemn and Nelson 2012). Evidence shows that the androgen receptor (AR) plays a leading role not only in normal prostate development but also in promoting PCa (Balk and Knudsen 2008). Therefore, androgen deprivation therapy (ADT) through either chemical or surgical castration is applied for treatment (Bluemn and Nelson 2012). Despite the initial effectiveness, most of the patients gradually develop a metastatic hormone-refractory form so called castration-resistant prostate cancer (CRPCa) (Harris et al. 2009). However, AR is still active in CRPCa and new generation AR antagonists seem to be beneficial to inhibit AR signaling in CRPCa (Hoimes and Kelly 2010, Roell and Baniahmad 2011, Tsao et al. 2012). Furthermore, CRPCa is a multifactorial and heterogeneous disease process involving several pathways and can be treated by combining therapeutic approaches against different molecular targets (Ramsay and Leungs 2009, Stavridi et al. 2010, Bonkhoff and Berges 2010, Lonergan and Tindall 2011).

1.2 AR signaling

The AR is a nuclear transcription factor and a member of the steroid hormone receptor superfamily. It consists of four structurally and functionally distinct domains: a poorly conserved N-terminal domain (NTD), a highly conserved DNA-binding domain (DBD), a hing region and a moderately conserved C-terminal ligand-binding domain (LBD). (Gelmann 2002, Claessens et al. 2008). The NTD harbors transcriptional activation function 1 (AF1), which encompasses two transcriptional activation units (TAU): TAU-1 and TAU-5 (Jenster et al. 1995). The NTD is mainly responsible for the AR-mediated transactivation (Gelmann 2002). The DBD consists of two zinc fingers that specify gene specific nucleotide contacts within the DNA groove and facilitate homodimerization of the receptor (Umesono and Evans 1989). The LBD enables binding of the AR ligands
and contains AF2. AF1 and AF2 are responsible for the recruitment of the coregulators and transcriptional complexes to AR (Heery et al. 1997, Bevan et al. 1999). Most of the AR point mutations identified in CRPCa have been mapped to the LBD (Taplin et al. 1995, Buchanan et al. 2001).

Unliganded AR is primarily localized in the cytoplasm bound in a complex with multiple chaperones (heat shock proteins) which disable AR from entering to the nucleus (Hessenkemper and Baniahmad 2012). Upon androgen binding, AR changes its conformation and dissociates from the heat shock proteins leading to nuclear translocation and subsequent dimerization and binding to the androgen response elements (ARE) in the promoter and enhancer regions of target genes where the recruitment of coregulators and transcriptional complexes result in transactivation or inhibition of gene expression. (Heinlein and Chang 2001, Gelmann 2002, Baniahmad 2005).

There are an increasing number of the proteins which interact with and modulate AR transcriptional action. These proteins include coactivators, corepressors, chromatin remodeling proteins and also other transcription factors (Grosse et al. 2012). Unlike coactivators that enhance AR-mediated gene transcription, AR corepressors attenuate AR transactivation (Wang et al. 2005). Alterations in coregulators’ function have been postulated to contribute to CRPCa (Rahman et al. 2004, Chmelar et al. 2007, Heemers and Tindall 2007).

Furthermore, the genomic amplification and/overexpression of AR itself occur in roughly 60% of the CRPCa cases (Taylor et al. 2010). The additional mechanisms leading to AR upregulation include the factors which are responsible for the transcriptional or posttranslational regulation of AR protein (Sharma et al. 2010, Cai et al. 2011, Valdez et al. 2011, Li et al. 2014a). In this regard, posttranslational modifications and degradation of AR through various proteins have been reported as an essential biological process in maintaining the cellular homeostasis (Lin et al. 2002, Xu et al. 2009, Varisli et al. 2012, Qi et al. 2013, Li et al. 2014b, Sarkar et al. 2014).

The roles of AR in normal prostate organogenesis as well as the development of PCa on the one hand and induction of the cell cycle arrest in the PCa cells by ADT on the other hand imply that AR plays a key role in regulating cell cycle proteins (Agus et al. 1999, Heinlein and Chang 2004). The genetic pathways activated by the androgen receptor during the induction of proliferation in the ventral prostate gland have been identified
(Nantermet et al. 2004). One of the recurrent chromosomal aberrations found in PCa is the fusions between the *TMPRSS2* gene and the Ets transcription factor family members, *ERG* or *ETV1*. The androgen-responsive promoter elements of *TMPRSS2* mediate the overexpression of ETS family members in prostate cancer (Lin et al. 1999, Tomlins et al., 2005). Matrix metalloproteinases (MMP) 2 and 9, which have been found to be associated with PCa metastasis (Nemeth et al. 2002), harbor Ets binding sites on their promoters (Schneikert et al. 1996). Role of androgens and the AR in epithelial-mesenchymal transition (EMT) and invasion of prostate cancer cells has been reported (Zhu and Kyprianou 2010). Moreover, the regulatory role of AR in apoptosis (Gao et al. 2005, Rokhlin et al. 2005) and in cellular senescence (Mirochnik et al. 2012, Yang et al. 2013, Ewald et al. 2013, Roediger et al. 2014, Hessenkemper et al. 2014) have been also well established. Cellular senescence is a state of irreversible cell cycle arrest frequently in the G0/G1 phase (Campisi and d'Adda di Fagagna 2007).

### 1.3 Epigenetics and cancer

Cancer is a complex genetic disorder initiated by cells that have accumulated multiple genetic and/or epigenetic changes resulting in altered gene expression to drive malignant characteristics (Coles and Jones 2009, Tallen and Riabowol 2014). Similarly, PCa is also driven by progressive genetic and epigenetic aberrations (Albany et al. 2011). One of the hallmarks described for cancer is aberrant expression of the tumor suppressors which enable the cancer cells evading from growth suppressor mechanisms (Hanahan and Weinberg 2011). Tumor suppressor proteins have been found to regulate numerous cellular processes, including cellular senescence, DNA repair, signal transduction and apoptosis. They comprise proteins that regulate chromatin remodeling and/or modify histones to alter gene expression, including members of the inhibitor of growth (ING) family (Coles and Jones 2009).

ING family members are histone binding proteins which are also found as components of large chromatin remodeling complexes including histone deacetylases (HDAC), acetyltransferases (HAT) and methyltransferases (HMT) enzymes. HDACs, HATs and HMTs are responsible for posttranslational modifications of histones and other regulatory proteins (Coles and Jones 2009). Epigenetic alterations have been demonstrated to play critical roles in prostate carcinogenesis and metastasis (Valdes-Mora and Clark 2014).
1.4 ING family

In human, ING family of proteins consists of five known members (ING1-ING5) with various isoforms. ING family, which is evolutionary conserved from yeast to humans (He et al. 2005), regulates a wide variety of vital cellular processes (Coles and Jones 2009, Unoki et al. 2009). Initially ING family proteins were characterized as tumor suppressors, however, recent emerging evidence has now broadened this definition as epigenetic regulators due to differentially control of cell growth in different biological contexts (Unoki et al. 2009, Schaefer et al. 2013, Tallen and Riabowohl 2014). All ING proteins share a highly conserved plant homeodomain (PHD) finger at the C-terminus (He et al. 2005, Soliman et al. 2007). The PHD domain selectively binds to the lysine 4 residue of histone H3 with affinity increasing with methylation state (highest affinity for H3K4me3) (Pena et al. 2006, Shi et al. 2006, Tallen and Riabowohl 2014). H3K4me3 is preferentially located at promoters and is a histone mark associated with activated and open chromatin downstream of transcription start sites (Santos-Rosa et al. 2002, Ruthenburg et al. 2007). Interaction of ING proteins with H3K4me3 directs other complex proteins including HDACs, HATs or HMTs to regulate their target genes.

1.5 ING1 and ING2

Human ING1 was identified as first member of the ING family following searching new tumor suppressors (Garkavtsev et al. 1996). It is located on chromosome 13q14 and consists of four exons. It encodes for four known isoforms namely p47ING1a, p33ING1b, p27ING1d and p24ING1c which are generated from different promoters or as a result of alternative splicing. p33ING1b, thereafter called ING1b, is the most abundant form among ING1 isoforms (Guerillon et al. 2013). ING1b protein contains a PCNA-interacting protein motif (PIP) and a partial bromo domain (PBD) in its N-terminal part, a lamin interaction domain (LID) and a nuclear localization sequence (NLS) in its central region and a PHD and a polybasic region (PBR) in its C-terminal part (Figure 1). PHD and LID are the well conserved regions among ING proteins. NLS, which targets ING proteins to the nucleus, also contains basic nucleolar targeting sequences (NTS) in ING1 (Tallen and Riabowohl 2014).
Introduction

Figure 1. Human ING1b protein structure and its interacting partners. PIP: PCNA-interacting protein, PBD: Partial bromo domain (PBD), LID: lamin interaction domain, NLS: nuclear localization sequence in which nucleolar targeting sequences (NTS) were depicted in 3 dark blue boxes, PHD: plant homeodomain and PBR: polybasic region. Two phosphorylation sites located on serines 126 and 199 (S126, S199) have been reported to regulate the half-life and subcellular localization of ING1b, respectively. Lysine 193 (K193), which is the preferred ING1b SUMO acceptor site, regulates ING1b-mediated transcription upon SUMOylation. Upper panel shows the proteins interacting with the corresponding domains of ING1b (modified from Tallen and Riabowohl 2014).

Human ING2 is mapped at chromosome 4q35.1 and is made up of three exons resulting in two alternatively spliced isoforms: p33ING2a and p28ING2b. Both ING2a and ING2b mRNA are ubiquitously expressed, however, with much lower expression level for ING2b. Furthermore, while ING2b expression has been shown at the RNA level, no protein has ever been detected for it. ING2a is highly expressed in testis (Unoki et al. 2008, Guerillon et al. 2013). ING2a displays high amino acid sequence homology (70%) to ING1b (Guerillon et al. 2013). In contrast to ING1b, ING2a N-terminus contains a leucine zipper-like (LZL) region that can help regulate nucleotide-excision-repair-associated functions of ING2, the DNA damage response and cell differentiation (Tallen and Riabowohl 2014). Mouse Ing1 is transcribed as three known isoforms including p31ING1a, p37ING1b and p31ING1c. Human and mouse ING1b share 89% identity based on amino acid alignment. Mouse Ing2 transcription results in two isoforms: ING2a and ING2b. Human and mouse ING2a cDNAs share 90% identity (Guerillon et al. 2013).

Although two models of Ing1 knockout (KO) mice have been developed so far, they displayed similar characteristics. The first one was generated as complete Ing1 KO mouse (Kichina et al. 2006), while the other one was developed as an isoform specific ING1b-deficient mouse (Coles et al. 2007). Both KO mice are characterized by reduced body size with higher incidence of B-cell lymphomas and aberrant DNA damage response. However, difference in tumor spectrum between these two mice strains
suggests that ING1a and ING1c isoforms modulate ING1b activity in mice. The complete Ing1 KO mice have no other obvious morphological, physiological or behavioral abnormalities, indicating that Ing1 function is dispensable for the viability of mice under normal physiological conditions (Kichina et al. 2006). However, targeted disruption of Ing2 results in defective spermatogenesis in male mice and development of soft-tissue sarcomas in both genders (Saito et al. 2010). Moreover, Ing2 KO mice have displayed a decrease in acinar dilation in prostate indicating a role for Ing2 in prostate. In spite of high homology between ING1 and ING2, a different phenotype has been observed in their corresponding KO mice models (Guerillon et al. 2013).

1.6 ING1 in PCa

As expected from ING1 role as a tumor suppressor, ING1 has been found to be lost or downregulated at mRNA and/or protein expression levels in multiple malignancies (Walzak et al. 2008, Guerillon et al. 2014). Although some mutations have been reported in either the NLS or the PHD domain, mutation in ING1 is an infrequently occurring phenomenon in human tumors and cancer cell lines (Ythier et al. 2008). Analyzing ING1 expression in PCa is limited to one study indicating the low ING1 mRNA level in the prostate adenocarcinoma PC3 versus normal prostate (Walzak et al. 2008). Nevertheless, ING1b mRNA is not differentially expressed between normal prostate and prostate adenocarcinoma in this study. However, transcriptome analysis has revealed that ING1b-repressed genes associate with PCa (p= 0.044, kappa similarity score= 0.41) (Thakur et al. 2014). Another indication for ING1b role in prostate comes from the study of Schwarze et al. (2002) in which ING1b mRNA showed 1.8 fold induction in terminally senescent human prostate epithelial cells (HPECs) compared to the proliferating ones.

1.7 ING1b regulates cell growth

Apart from caretaker functions of ING1b in DNA repair, it regulates the cell proliferation, apoptosis, cellular senescence and migration as gatekeeper functions (Guerillon et al. 2013). The implication of ING1b in cell growth was supported by its expression changes during the cell cycle (Garkavtsev and Riabowol 1997). Furthermore, many studies have shown that the overexpression of the ING1b could inhibit growth of normal and cancerous cells. Initially, Garkavtsev et al. (1996) has
reported that the ectopic expression of human ING1b in Hs68 fibroblast cells caused cell cycle arrest in the G0/G1 phase. However, the mechanism by which ING1b causes cell growth inhibition seems dependent on the cellular context. ING1b not only could induce apoptosis in various cell types (Helbing et al. 1997, Shinoura et al. 1999, Lv et al. 2012, Thakur et al. 2012, Bose et al. 2013) but also could trigger cellular senescence pathway in different cells (Goeman et al. 2005, Abad et al. 2007, Abad et al. 2011, Li et al. 2011). Moreover, 8- to 10-fold higher expression of ING1b in senescent cells compared to young ones brings more evidence for involvement of ING1b in cellular senescence (Garkavtsev and Riabowol 1997). Moreover, ING1b-deficient MEFs has displayed induced Bax expression and DNA damage-induced apoptosis indicating that ING1b can negatively regulate apoptosis (Coles et al. 2007). In line with overexpression studies, suppression of ING1b expression extends the proliferative life span of normal fibroblasts (Garkavtsev and Riabowol 1997), promotes neoplastic transformation (Garkavtsev et al. 1996) and results in formation of spontaneous follicular B-cell lymphomas in ING1b-deficient mice (Coles et al. 2007).

1.8 Regulation of ING1b expression

Since mutations in ING1 gene have been rarely reported in human tumors (Ythier et al. 2008), several reasons has been postulated and studied for its downregulation in tumors. ING1 gene and flanking regions are highly GC rich (Gunduz et al. 2000), and indeed abnormally high methylation levels on the ING1 promoter have been reported to cause low ING1b mRNA expression in ovarian cancer (Shen et al. 2005) indicative of epigenetic mechanism regulating ING1 transcription. Interestingly, ectopic expression of microRNA622 promoted invasion, tumorigenesis and metastasis of gastric cancer cells where it binds to the 3’-untranslated region (3’-UTR) of ING1 transcript to post-transcriptionally repress its protein level (Guo et al. 2011). Moreover, posttranslational modifications of ING1b have been reported to modulate its stability and activity. Genotoxic stresses, like UV, induce ING1b phosphorylation at Serine 126 and extend its half-life to regulate various growth inhibitory effects (Garate et al. 2007, Garate et al. 2008). Also, phosphorylation-dependent binding of 14-3-3 to ING1b targets its subcellular localization and regulates p21^CIP1/WAF1 expression (Gong et al. 2006). Moreover, the proto-oncogene Src, a non-receptor tyrosine kinase with an important role in growth factor signal transduction, physically associates with, and phosphorylates
ING1b which results in its cytoplasmic localization and decreased stability thereby antagonizing the ability of ING1b to induce apoptosis. These findings suggest that Src may play a major role in regulating ING1 levels during tumorigenesis in those cancers in which high levels of Src expression or activity are present (Yu et al. 2013).

### 1.9 ING1b controls gene transcription

The involvement of ING1b in various cellular processes necessitates very finely regulated mechanisms by which ING1b controls the cellular pathways. Indeed one of the most important mechanisms can be regulation of the gene expression of the proteins that play key roles in cellular homeostasis. ING1b was found to associate with chromatin remodeling complexes to exert its functions (Coles and Jones 2009). It has been described that ING1 can bind with high affinity to H3K4me3 by its PHD domain and subsequent recruitment of the SIN3A/HDAC1-2/SAP30 complex results in repression of gene transcription through histone deacetylation (Kuzmichev et al. 2002, Doyon et al. 2006). Contrary to common belief about SIN3a/HDAC as a transcription repressor, it might positively regulate transcription as well (Silverstein and Ekwall 2005). Studies in both yeast and human cells have implicated ING1b in chromatin remodeling complexes containing HATs and its role in transcriptional activation (Feng et al. 2002). On the other hand, directing the growth arrest and DNA damage protein 45a (GADD45A) to H3K4me3 by ING1 causes DNA demethylation and subsequent gene activation (Schaefer et al. 2013). The recognition of the chromatin mark H3K4me3 seems to be essential for the induction of specific transcriptome signature. Mutations disrupting the ING1b PHD domain have been reported to impair the ING1b function in inducing cellular senescence and gene-specific DNA demethylation (Abad et al. 2011, Schaefer et al. 2013).

ING1b-induced senescence is associated to a specific genetic signature enriched in chemokine and cytokine signaling factors (Abad et al. 2011). In addition, ING1b has also been reported to bind to p16 promoter and upregulates $p16^{INGK4a}$ expression in 2BS fibroblasts in a p300-dependent manner that lead to induction of cellular senescence (Li et al. 2011). Moreover, in mouse mammary epithelial cells, ING1b has been found to negatively control the expression of cyclin B1 and the proto-oncogene DEK (Takahashi et al. 2002). In HepG2 cells, ING1b could stimulate $p21^{CIP1/WAF1}$ promoter (Kataoka et al. 2003). However, the ability of ING1b to cause cell-cycle arrest, p53-mediated
transactivation and induction of p21 is impaired in p19ARF-deficient primary MEFs (Gonzalez et al. 2006). Reportedly, the functional link between ING1b and p53 pathway is well established, however, ING1b can also regulate cell growth and apoptosis independently of p53 (Coles and Jones 2009, Guerillon et al. 2013).

ING1b SUMOylation on lysine 193 (K193) regulates the binding of ING1b to the ISG15 and DGCR8 promoters, consequently regulating their transcription (Satpathy et al. 2014). Indeed, interaction of ING1b with other regulatory proteins can also modulate their transactivation or transrepression properties. The ING1b protein has been found via GST-pull-down assay to associate with corepressor Alien and enhance Alien-induced gene silencing mediated by selected members of nuclear hormone receptors and E2F1 (Fegers et al. 2007). After UV irradiation, human ING1b increases its protein expression, translocates into the nucleolus and interacts with CSIG (Cellular Senescence-Inhibited Gene) and increases CSIG protein stability. CSIG is a nucleolar protein that has been involved in the regulation of cellular senescence and apoptosis (Ma et al. 2008, Li et al. 2012). Furthermore, ING1b physically associates with estrogen receptor alpha (ER-α) where it acts like a coactivator and stimulates estrogen-induced ER-α transcriptional activity (Toyama et al. 2003). Another indication of ING1b role in hormonal signaling comes from the study of Helbing et al. (2011) in Xenopus laevis in which ING1b, along with ING2a, modulate thyroid hormone (TH)-dependent responses through association with TH receptor β (TR-β) and its promoter region and enhancing TR-associated promoter activity in response to T3.

Moreover, connection between ING1b and microRNAs has been also revealed. In ING1b-deficient MEFs, the expression of Dgcr8, which encodes for a protein involved in the early steps of microRNA biogenesis, has been increased. It has been shown that ING1b contributes to the transcriptional repression of Dgcr8 by inhibiting histone acetylation through recruitment of deacetylation complexes. Consequently, a small subset of mature microRNAs that displayed statistically significant differences have been identified in ING1b-deficient MEFs relative to wild-type controls (Gomez-Cabello et al. 2010). Also, ING1b epigenetically induces microRNA203 leading to inhibition of cancer cell proliferation through coordinate downregulation of CDK6, c-Abl and Src (Chen et al. 2013). Interestingly, deregulation of the microRNA machinery is well characterized in tumors (Shi et al. 2008).

However, which mechanisms define the recruitment of activator or repressor complexes
to the target genes remains to be elusive. This issue would be more complicated when one consider what define which ING should be bound to H3K4me3 since the highly conserved PHD domain is common among ING family.
2 Objective

Continued reliance on AR signaling for survival is a hallmark of CRPCa (Bluemn and Nelson 2012). Therefore, inhibiting AR by using different strategies would be useful for PCa therapy. Cellular senescence has emerged recently as an antiproliferative tumorsuppressive mechanism (Prieur and Peeper 2008, Collado and Serrano, 2010, Nardella et al. 2011) and it can be one possible way to inhibit AR and subsequently PCa growth.

ING1b is one of the upregulated transcripts in the senescent human prostate epithelial cells (Schwarze et al. 2002) and it is also able to induce cellular senescence in other contexts (Goeman et al. 2005, Abad et al. 2007, Abad et al. 2011, Li et al. 2011). It has been reasoned that ING1b plays important role in aging process of prostate and can also induce growth arrest through cellular senescence in PCa cells. Moreover, understanding molecular mechanism of ING1b-induced cellular senescence in PCa cells is essential for further steps.

The AR can also drive cellular senescence in response to agonists and antagonists (Mirochnik et al. 2012, Roediger et al. 2014, Hessenkemper et al. 2014). Interestingly, the host group studies have shown that ING1b interacts with human AR (Diploma thesis Jennek 2009). For that reason, this study is aimed to analyze whether ING1b is the mediator of AR-driven senescence. In line with this, it should be investigated if agonists or antagonists influence ING1b expression.

It has been also indicated that ING1b can inhibit AR transactivation (Diploma theses Klitzsch 2011, Ludwig 2012). Therefore, analysis of ING1b inhibitory effect on AR signaling in PCa cells was conducted. To further confirm the regulatory role of ING1b on AR-mediated transcription in vivo, Ing1 KO mouse was used.

Taken together, functional analysis of AR-ING1b interaction in the presence of agonists and antagonists and their activity on the expression of target genes, cellular senescence, proliferation and migration will provide a potential approach for molecular-targeted therapy of CRPCa.
3 Materials and Methods

3.1 Hormones, chemicals and antibiotics

Dihydrotestosterone (DHT), Casodex (Cas), Cycloheximide, Chloroquine diphosphate, Hexadimethrine bromide (Polybrene), Dichlorofluorescein diacetate (DCF-DA) and N-acetylcysteine (NAC) were obtained from Sigma-Aldrich (Taufkirchen, Germany); Atraric acid (AA) from Merck (Darmstadt, Germany); Methyltrienolone (R1881) from Perkin Elmer (Waltham, MA, USA); MG132 from Cayman Chemical (Ann Arbor, MI, USA); Zeocin and Puromycin from InvivoGen (San Diego, CA, USA); Geneticin disulphate (G418 Sulphate) and Crystal violet from Carl Roth (Karlsruhe, Germany).

All compounds were dissolved in dimethylsulfoxide (DMSO) or ethanol (EtOH) or water and were added to the culturing medium in such a way that the final concentration of the solvent did not exceed 0.2%. Control incubations (no test compounds) were performed with the appropriate volume of DMSO or EtOH.

3.2 Plasmids

The androgen responsive pMMTV-luc plasmid, which contains a luciferase (luc) reporter gene driven by mouse mammary tumor virus (MMTV) long terminal repeats, has been already described (Kaspar et al. 1993). The pARR3-tk-luc, which expresses the luc gene driven by the Probasin promoter sequence, was kindly provided by R.J. Matusik (Nashville, TN, USA). The pPSA-luc reporter plasmid contains the PSA promoter sequence (Cleutjens et al. 1997). The pCMV-lacZ plasmid expressing β-galactosidase under control of the cytomegalovirus (CMV) promoter was employed as internal control for transfection efficiency in reporter gene assays (Dotzlaw et al. 2002). pBabe-Zeo-EcoR plasmid was obtained from Addgene (plasmid No. 10687) and used for introducing the ecotropic receptor (Albritton et al. 1989) into human PCa cells. The retroviral vector pLPC, which was used as empty vector control, the pLPC-ING1b and the pLPC-ING2a expression vectors have been previously described (Serrano et al. 1997, Goeman et al. 2005). The retroviral vectors pLMP-sh-luc and pLMP-sh-ING1b, which enable efficient production of specific shRNAs using a miR30 microRNA backbone against firefly luciferase gene and human ING1b respectively, have been described by Abad et al. (2011). The pEGFP-AR and pEGFP-AR(T877A) constructs, coding for N-terminally tagged GFP-AR fusion proteins, have been described.
previously by Farla et al. (2004, 2005) and were kindly provided by Dr. A.B. Houtsmuller (Rotterdam, The Netherlands).

The pmCherry-C1, which is a mammalian expression vector designed to express a protein of interest fused to the C-terminus of mCherry, is from Clontech Laboratories (Mountain View, CA, USA). The pmCherry-ING1b construct were subcloned as follows: The pmCherry-C1 was digested with BamHI and dephosphorylated with FastAP (Thermo Scientific, Hamburg, Germany). The linearized vector was then ligated using T4 DNA ligase (Thermo Scientific, Hamburg, Germany) with the full-length ING1b, which was already cut out from pLPC-ING1b with the same restriction enzyme to give the sticky ends. The successful cloning was verified by restriction analysis and sequencing (Figure 2).

![Diagram of pmCherry-ING1b construct](image)

**Figure 2.** The schematic representation of pmCherry-ING1b construct.

### 3.3 Cell culture

HeLa cells, a human cervix carcinoma cell line, were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml) and 25 mM HEPES (pH 7.8). MEFs, mouse embryonic fibroblasts isolated from embryonic day E15.5 male embryos, were cultured in the same medium as HeLa cells, except that FBS was 10%. MEFs from wild type and
Ing1 KO male mice were kindly provided by Dr. C. Niehrs (Mainz, Germany). Phoenix-Eco, which is a retroviral packaging cell line for the generation of helper-free ecotropic retroviruses, was cultured in the same medium as HeLa cells, except that FBS was 10% heat inactivated. The androgen-dependent human PCa line LNCaP-tet (Protopopov et al. 2002) was cultured in RPMI1640 supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 25 mM HEPES (pH 7.8) and 1% sodium pyruvate. The androgen-independent growing C4-2 cells, which are derived from the LNCaP cell line (Wu et al. 1994), were cultured in DMEM supplemented with 20% F12 medium, 10% FBS, 5 µg/ml insulin, 5 µg/ml apotransferin, 0.25 µg/ml Biotin, 25 µg/ml Adenin, penicillin (100 U/ml), streptomycin (100 µg/ml) and 25 mM HEPES (pH 7.8). PC3 cells, which are derived from advanced androgen-independent bone metastasis of PCa (Kaighn et al. 1979), were cultured in RPMI1640 supplemented with 10% heat inactivated FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 25 mM HEPES (pH 7.8). PC3-AR cells, which are a derivative of PC3 cells stably transfected with wild type AR cDNA (Peterziel et al. 1999), were cultured in the same medium as PC3 with adding 0.6 mg/ml G418. All cells were cultivated in a humidified atmosphere with 5% CO2 at 37°C. The cells were cultured in charcoal stripped serum (CSS) containing media when hormone depletion was needed.

3.4 Retroviral transduction

Retroviral gene transfer into human PCa cells was performed essentially as described by Goeman et al. (2005) with minor modifications. In brief, for the production of virus, Phoenix-Eco cells are transiently transfected with 15 µg of the retroviral vector of interest using a modified CaPO4 method (Wigler et al. 1978, Moehren et al. 2008). After 48h, the supernatant containing viral particles was filtered, diluted with fresh medium in the presence of 8 µg/ml polybrene and added to the human PCa cells, which were stably transfected with the vector pBabe-Zeo-EcoR driving the expression of the ecotropic receptor. This procedure was repeated 24h later. Successfully infected cells were selected with puromycin (0.3 µg/ml or 1 µg/ml for PC3-AR and LNCaP cells, respectively) for 10-15 days and selected cells were then pooled for the other assays.

3.5 Growth assay

The growth assay was basically carried out in 2 ways.
(I) LNCaP and PC3-AR cells were seeded on the 6-well tissue culture plates in appropriated medium at $5 \times 10^4$ and $10^4$ cells per well, respectively. Then the retroviral transduction was applied to the cells. After 48h, the medium was replaced with the ones containing 5% FBS and different AR ligands. After a further cultivation of 48h, antibiotic selection of the transduced cells in the presence or absence of AR ligands was performed for 10-15 days. The resistant cells were fixed with 1% glutaraldehyde, stained with 0.1% crystal violet as an indirect measure of cell number, and the cell associated crystal violet in dried plates was solubilized with Sørenson’s solution as described (Jansson et al. 2012). Absorbance was measured at 590 nm.

(II) The stably transduced cells were seeded in duplicate a week after the end of selection at a density of 7000 (LNCaP) and 2000 (PC3-AR) cells per well in 12-well plates in appropriate medium containing 5% FBS. After 48h, cells were fed with fresh medium and solvent control or AR ligands as indicated. At the indicated time points, cells were stained with 0.1% crystal violet after fixation, and indirect measure of cell number was performed by reading the absorbance at 590 nm. Moreover, the media were replaced with fresh one together with freshly added ligands for the other plates in these time points.

3.6 Scratch assay

The cells were plated on 6-well plates in the appropriate medium containing 5% FBS to create a confluent monolayer at following day. The required cell number for this condition was $10^6$ and $5 \times 10^5$ for LNCaP and PC3-AR, respectively. The confluent monolayer was scratched with a crystal 10 µl pipette tip. The culture medium was replaced with fresh one with or without ligands. At indicated time points, brightfield microscopic pictures were taken from the same location and the media were replaced with fresh one as described. The images acquired for each well was further analyzed quantitatively by using TScratch computing software (Gebäck et al. 2009). Cell migration is presented as percent gap closure, calculated using the following equation:

\[
\left( \frac{\text{Pre-migration area at time 0} - \text{Migration area at indicated time point}}{\text{Pre-migration area at time 0}} \right) \times 100
\]
3.7 **Senescence-associated beta-galactosidase (SA-β-Gal) staining**

The staining was performed as described earlier (Dimri et al. 1995) either directly on the transduced cells survived from antibiotic selection or on the transduced cells seeded after selection in 6-well plates at a density of 50000 (LNCaP) or 20000 (PC3-AR) cells per well. In the latter case, the cells were then treated in the presence or absence of different AR ligands for 3 days. The cells were washed with phosphate buffered saline (PBS) and fixed for 5 min in 1% glutaraldehyde. Fixed cells were washed with PBS and incubated at 37°C (no CO₂) with fresh SA-β-Gal staining solution (Dimri et al. 1995). The staining solution contains X-Gal, a galactopyranosid, which yields an insoluble blue compound when cleaved by active β-galactosidase. The percentage of stained cells was determined following counting at least 400 cells per well under light microscopy.

3.8 **Reporter gene assay**

The cells were seeded onto 6-well plates at the density of 2-5 ×10⁵ cells per well in the appropriate medium supplemented with 5% CSS medium. Next day, the cells were cotransfected with 1-2 μg reporter construct and 0.2 μg pCMV-lacZ for internal normalization. When needed, the empty vector (pLPC) or expression vector for the human ING2a (pLPC-ING2a) was added to the transfection mix. LNCaP cells were transfected using DOTAP transfection reagent (Roth, Karlsruhe, Germany) according to manufacturer’s protocol, with minor modifications (Reeb et al. 2011), whereas PC3-AR cells were transfected by using a modified CaPO₄ method (Wigler et al. 1978, Moehren et al. 2008). After 6-16 h, media were replaced with the indicated substances. Cells were harvested 72h after hormone induction to measure both luciferase and galactosidase activities. Obtained luciferase units were normalized to those of beta-galactosidase.

3.9 **Dichlorofluorescein (DCF) assay**

The assay employed the cell-permeable nonfluorescent probe 2’,7’-Dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is deacetylated by cellular esterases to non-fluorescent DCFH which is retained in the cell. DCFH is then rapidly oxidized to highly fluorescent DCF by ROS (Keston and Brandt 1965). The cells were grown in 24-well plates and treated with the respective AR ligands on the following day. 72h later, they
were washed with PBS and incubated in a loading medium consisting of pure cell growth medium with 1% FBS and 100 μM DCFH-DA for 30 min in a cell incubator. Afterwards cells were washed twice with PBS and lysed in 150-250 μl of RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% NaDeoxycholate). The fluorescence was measured in Fluoroskan Ascent (Labsystems, Helsinki, Finland) at excitation wavelength 485 nm and emission filter 538 nm. The values were normalized to the protein concentrations. The normalized values were depicted as fold ROS level after setting the DMSO values obtained for empty vector arbitrarily to 1.

3.10 Fluorescence microscopy

For colocalization studies, the HeLa cells were seeded on glass coverslips in the 5% CSS medium and transiently cotransfected with the plasmids coding for GFP-AR and mCherry-ING1b proteins using a modified CaPO4 method (Wigler et al. 1978, Moehren et al. 2008). 16h later, the cells were washed with PBS and fed with fresh 5% CSS medium for recovery and expression of the tagged proteins. After 24h, the transfected cells were treated with solvent control DMSO or R1881 (1 nM) in 5% CSS medium for 2h. Afterwards, the cells were washed three times with PBS and fixed using 4% formaldehyde solution (in PBS). Then the cells on the coverslips were mounted onto glass slides using ProLong® Gold antifade reagent with DAPI (Life Technologies, Darmstadt, Germany). The intracellular distribution of AR and ING1b was investigated with a fluorescence microscope Axio Observer.Z1 (Carl Zeiss, Jena, Germany) supplemented with ApoTome device for generation of optical sections. Colocalization was quantified using BioImageXD software (Kankaanpää et al. 2012).

3.11 Quantitative reverse transcription PCR (qRT-PCR)

RNA was isolated from the cells or mice organs using peqGOLD TriFast (Peqlab, Erlangen, Germany) according to the manufacturer’s protocol. Two-step qRT-PCR was conducted as follows: 2 μg RNA were converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Then the cDNA synthesis reaction samples were diluted 1:1 with DEPC-treated water. In the 2nd step, qRT-PCR was performed using the SsoFast™EvaGreen® Supermix (Bio-Rad, München, Germany), gene specific primers and Biorad CFX96™ Real Time PCR detection system. The primer sequences and annealing temperatures were listed in
Table 1 and Table 2. qRT-PCR results were analyzed via ΔΔCt method (Pfaffl 2001) using CFX manager software from Biorad. Among the housekeeping genes tested, human RPL13a and murine Rpl13a were found as most constant ones and used for normalization of the assays, unless stated otherwise.

Table 1. Human primers used in this study for qRT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5' → 3'</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
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<td>CCCGAACCTTTCCAAAGCCATCTACG</td>
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<tr>
<td></td>
<td>rev</td>
<td>ATCCACGACATCTCTCTGCAAC</td>
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<tr>
<td>AR</td>
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**Materials and Methods**

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- **Annealing temperature**: 60°C

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TMPRSS2
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**Table 2. Mouse primers used in this study for qRT-PCR analysis**

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</table>
Materials and Methods

3.12 Western Blotting

For preparation of total cell lysates, the cells after transfection, transduction or ligand treatment were washed with PBS, scraped in ice cold PBS and transferred to reaction tubes. To isolate the cells they were spun down (2500 rpm, 5 min, 4°C). The cell pellet was resuspended in its fivefold volume of NETN buffer (100 mM NaCl, 20 mM Tris/HCl pH 8.0, 1 mM EDTA, 0.5% NP-40) supplemented with phosphatase inhibitors (5 mM NaF, 100 μM Na₃VO₄, 10 mM β-Glycerophosphate). The cell lysis was taken place by incubating the cell suspension 10 min on ice and thereafter three cycles of freezing (in liquid nitrogen) and thawing (in 37°C water bath). The cell debris was precipitated by centrifugation (15000 rpm, 15 min, 4°C) and supernatant was collected in a fresh tube as whole cell extract used for Western blot.

Protein extraction from mice organs was performed on the organic phase remaining after RNA isolation with TriFast according to the manufacture’s protocol. Protein pellet was dissolve in 1% SDS by incubating it at 50°C. Insoluble material was then removed by centrifugation (12000 rpm, 10 min, 4°C). The protein supernatant was transferred to a fresh tube for further analysis.
40 μg of protein extracts were resolved on SDS-PAGE and wet blotted onto a PVDF membrane (Roche, Mannheim, Germany). The transfer buffer for wet tank blot consisted of 250 mM Tris, 192 mM glycine, 0.1% SDS and 10-20% methanol. Blotted PVDF membrane were washed with TBS-T buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) and blocked with 10% non-fat milk (Biomol, Hamburg, Germany) for 50 min at room temperature. The non-fat milk was solved in TBS-T buffer thereafter centrifuged (4500 rpm, 20 min) and supernatant was used for blocking. For detection of target protein, the membrane was incubated overnight at 4°C with primary antibody and afterwards with secondary antibody at room temperature for 30 min. The primary and secondary antibodies were summarized in Table 3. The light emission produced after incubation with enhanced chemiluminescence (ECL) reagent (GE Healthcare, Buckinghamshire, UK) was detected by ImageQuant™ LAS 4000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). LabImage 1D software (Kapelan Bio Imaging solutions, Leipzig, Germany) was applied for quantification of protein of interest relative to the loading control (α-Tubulin or β-Actin).

Table 3. Description of the antibodies used for Western blot

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(*) All antibodies were diluted in TBS-T.
(§) Secondary antibodies are conjugated to horseradish peroxidase (HRP).
3.13 Database and statistical analyses

For comparing ING1 mRNA expression level between normal and cancerous prostate, Oncomine platform (www.oncomine.com) (Rhodes et al. 2004) was analyzed. Likewise, the Human Protein Atlas (www.proteinatlas.org) (Uhlen et al. 2010) was used to assess the ING1 protein expression in PCa. Two-tailed unpaired Student’s t-test was performed for differential comparison between two groups using GraphPad Prism software. A value of p<0.05 was considered as statistically significant.
4 Results

4.1 ING1 expression is differentially regulated in PCa

Several studies have described that \textit{ING1} mRNA is mainly lost or decreased in human tumors (Walzak et al. 2008, Guerillon et al. 2013). To assess the expression of \textit{ING1} in PCa, Oncomine database was used to analyze the published microarray gene expression studies that have reported significant alteration of \textit{ING1} mRNA level. Of those, two studies have shown downregulation of \textit{ING1} mRNA in PCa compared to normal prostate gland (Figure 3A), however, 5 studies have indicated the opposite result (Figure 3B). It is of note that mean of fold change in downregulated studies is -1.68 versus +1.15 of that in upregulated ones. This controversial observation has been also reported for another tumor suppressor (Wu et al. 2014).

Since there was no report available on protein expression analysis of \textit{ING1} in PCa, the Human Protein Atlas portal was utilized for this purpose. Interestingly, \textit{ING1} protein failed to be detected in more than 30% of the PCa specimens compared to the low level of \textit{ING1} in normal prostate tissue (Figure 3C). Similar to the data obtained for \textit{ING1} mRNA level, there were still less than 10% of the PCa samples that displayed mild upregulation of \textit{ING1} protein. This controversial finding may be due to inverse relationships among \textit{ING1} variants (Soliman et al. 2008, Coles et al. 2007, Zhu et al. 2009). For that reason, the protein expression of major isoform \textit{ING1b} was examined in several PCa cell lines using Western blotting. The cell lines compared were the androgen-dependent LNCaP and CRPCa cell lines C4-2, PC3 and PC3-AR. A decreased protein expression of \textit{ING1b} was detected in CRPCa cells compared to androgen-dependent ones (Figure 3D). Taken together, these data suggest that \textit{ING1} expression tends to be downregulated in PCa.
Figure 3. **ING1 expression is regulated in PCa.** Oncomine database analysis of *ING1* mRNA expression was performed in 7 independent datasets represented by the name of first author. (A) The studies have shown downregulation of *ING1* in PCa compared to normal prostate gland. (B) The reports have indicated upregulation of *ING1* in PCa. For each box, the horizontal line represents the median, whereas the error bars (whiskers) represent the 90th and 10th percentile of log2 median-centered intensities. The upper and lower dots show maximum and minimum of the values, respectively. (C) The Human Atlas Protein database analysis of *ING1* protein level in PCa specimens using two different antibodies. (D) Western blot analysis for *ING1b* in total cell lysates from the PCa cell lines. The protein expression of *ING1b* was normalized to the loading control β-Actin using LabImage 1D quantification software.
4.2 ING1b colocalizes with human AR

ING1b protein localizes in the nucleus (Garkavtsev et al. 1997) and it translocates predominantly into the nucleolus upon ectopic expression or stress conditions (Scott et al. 2001). However, the AR as a transcription factor requires ligand activation for its nuclear translocation where it occupies AREs on DNA (Tyagi et al. 2000). Based on the host group’s finding regarding the interaction between ING1b and human AR detected by immunoblotting and SELDI-MS (surface-enhanced laser desorption/ionization-mass spectrometry) (Diploma thesis Jennek 2009), the colocalization analysis was employed to further verify this interaction. To this end, full-length ING1b subcloned to pmCherry vector and the expression of the mCherry-tagged ING1b was analyzed at protein level (Figure 4A) and under fluorescent microscopy (Figure 4B). Subsequently, HeLa cells were transiently cotransfected by mCherry-ING1b and GFP-AR (wild type or mutant form T877A) and treated with solvent control or the synthetic androgen R1881. Fluorescent microscopy showed that ING1b localized in the nucleus, primarily in the nucleoli, (Figure 4C) and treatment of the cells with solvent control DMSO or androgen did not affect its localization (data not shown). As expected, AR is translocated mainly into the nucleus in the presence of androgen (Figure 4C). Quantitative colocalization analysis of the images from the cotransfected cells revealed that ING1b colocalizes with AR in the nucleus excluding nucleolus regions (Figure 4C, Table 4). Ligand-induced nuclear translocation is a crucial step for AR-mediated transcription. ING1b did not prevent AR from ligand-induced import into the nucleus (data not shown). Thus, these data indicate that ING1b colocalizes with wild type and mutant form (T877A) of the AR.
**Results**

Figure 4. Intracellular colocalization of ING1b with AR. (A) Western blot analysis for the Phoenix-Eco cells transiently transfected with either empty vector (pmCherry) or pmCherry-ING1b. The tagged protein (545 amino acids) was detected by ING1 specific antibody mainly around 60 kDa. The smaller fragments indicated by arrowheads might come from internalized transcription start sites or degradation. ß-Actin served as a loading control. (B) The fluorescence microscopy of the cells explained in A. Negative control is the Phoenix-Eco cells transfected with calf thymus DNA. (C) Images of the HeLa cells cotransfected by mCherry-tagged ING1b and GFP-tagged AR and then treated with R1881 (1 nM) for 2h.

Table 4. Results of quantitative colocalization analysis between ING1b and AR based on the Costes’ randomization method.

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<td>-0.002±0.001</td>
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</table>

(a) Manders’ coefficients for channel 1 (green) and channel 2 (red) varying between 0 and +1, with 0 for no overlap and +1 for perfect overlap.
(b) Pearson’s correlation coefficient for the two selected channels varying between -1 and +1, with -1 for total negative correlation, 0 for random correlation, and +1 for perfect correlation.
(c) Pearson’s correlation coefficient for channel 1 against a number of randomized channel 2 images.
(d) Costes’ p-value of ≥0.95 indicates significant true colocalization.
4.3 ING1b inhibits growth in PCa cell lines

Previous studies have demonstrated that ING1b, as a tumor suppressor, could inhibit growth in various cellular contexts (Goeman et al. 2005, Abad et al. 2011, Lv et al. 2012, Thakur et al. 2012, Bose et al. 2013), however, this effect has not yet been studied on PCa cells. To investigate inhibitory effect of ING1b on growth of PCa cell lines, LNCaP (androgen-dependent) and PC3-AR (androgen-independent) cells were retrovirally transduced with either empty vector (pLPC) or expression vector for ING1b (pLPC-ING1b). ING1b overexpression was confirmed by Western blotting (Figure 5A) and also by upregulation of the ING1b target gene BCL2 (Figure 5B). Compared to the results seen with the empty vector, expression of ING1b reduced growth in both cell lines (Figure 5C, D). To further analyze whether interaction between ING1b and AR affect the agonist-mediated or antagonist-inhibited growth rate, different ligands were applied during different time points of growth assays. The data confirmed the inhibitory effect of ING1b on growth rate of the PCa cells in the presence of AR ligands, although no synergistic effect was seen between ING1b and AR ligands (Figure 6).

It is of note that agonists R1881 and DHT result in completely different growth response in low and high concentrations (Mirochnik et al. 2012, Roediger et al. 2014). Moreover, the antagonist Casodex at 0.1 μM exhibited no inhibitory effect on growth behavior of LNCaP cells (data not shown), therefore higher concentration (1 μM) was applied. Similarly, since AA at 30 μM had no inhibitory effect on growth behavior of PC3-AR cells (Figure 6B), therefore R1881 at high concentration (1 nM) was applied for subsequent growth assays as an growth inhibiting ligand in PC3-AR cells (Mirochnik et al. 2012) (Figure 6C). The different growth response of PC3-AR cells to low concentration of R1881 (10pM) (Figure 6B, C) might be due to low rate of R1881 inactivation and metabolization. These results show for the first time that ING1b suppresses growth of PCa cells.
Figure 5. Stable expression of the ING1b inhibits growth of PCa cell line. (A) Western blot analysis of the ING1b protein and (B) qRT-PCR analysis of ING1b target gene BCL2 in LNCaP and PC3-AR cells transduced with either empty vector or vector expressing ING1b. (C) Crystal violet staining of the cells survived after stable transduction and antibiotic selection. (D) Absorbance of the cell-associated crystal violet was then measured at 590 nm and the value obtained with the empty vector was set arbitrarily at 1. The mean±SEM values from at least two independent experiments are shown (* indicates p< 0.05).
Results

Figure 6. Different time points of stable ING1b expression inhibit growth in PCa cells. Growth assays were performed for (A) LNCaP and (B) PC3-AR cells similar to figure 5C, D under everyday antibiotic selection in the presence of different ligands: solvent control DMSO, R1881 (L) (low concentration: 10 pM), R1881 (H) (high concentration: 1 nM), DHT (10 nM), Casodex (1 µM for LNCaP and 0.1 µM for PC3-AR), AA (30 µM) and the value obtained with the empty vector treated with DMSO was set arbitrarily at 1. (C) Growth assay for the stably transduced cells after being 1-2 weeks in culture after end of selection (long term ING1b overexpression) under various treatments: DMSO or R1881 (L) (low concentration: 10 pM) or AA (30 µM) for LNCaP and DMSO or R1881 (L) (low concentration: 10 pM) or R1881 (H) (high concentration: 1 nM) for PC3-AR. The amount of OD 590 nm at each time point is represented relative to that of cells at day 0. The mean±SEM values from at least two independent experiments are shown (* indicates p<0.05).
4.4 Knockdown of ING1b results in distinct growth response in PCa cells

Given the fact that the suppression of ING1b promotes neoplastic transformation (Garkavtsev et al. 1996) and ING1b-deficient mice develop B cell lymphoma (Coles et al. 2007), it was asked whether knockdown (KD) of ING1b promotes growth in PCa cell lines. Therefore, LNCaP and PC3-AR cells were retrovirally transduced with the vectors expressing short hairpin RNA against either firefly luciferase (sh-control) or ING1b (sh-ING1b). ING1b KD was confirmed by Western blotting (Figure 7A) and also by downregulation of the ING1b target gene BCL2 in LNCaP cells (Figure 7B). The absence of BCL2 downregulation in PC3-AR cells may be due to different response to ING1b deficiency in diverse cellular contexts. Interestingly, the results from growth assays of PC3-AR cells under different ligand treatment suggested that ING1b KD promotes growth in these cells, however, LNCaP cells show unexpectedly growth inhibition upon ING1b KD (Figure 7C, D). Applying the growth assays for the cells expressing ING1b KD for longer time (20 days) showed the similar inhibition effect for LNCaP cells, while in the PC3-AR cells the growth difference is prominent at high concentration of R1881 (1 nM) compared to control cells (Figure 7E). In sum, the results indicate that ING1b KD promotes growth of PC3-AR cells and inhibits growth in LNCaP cells.
Figure 7. Stable knockdown of ING1b has opposite effects in growth behavior of PCa cell lines. (A) Western blot analysis of the ING1b protein in LNCaP and PC3-AR cells transduced with vector expressing short hairpin RNA either against firefly luciferase (sh-control) or against ING1b (sh-ING1b) (B) qRT-PCR analysis of ING1b target gene BCL2 for the cells explained in (A). (C) & (D) Growth assays were performed similar to Figure 6A, B for the cells explained in (A). (E) Growth assays were applied for the cells described in (A) in similar condition to Figure 6C. The mean±SEM values from at least two independent experiments are shown (* indicates p< 0.05).
4.5 ROS production and apoptosis are not responsible for ING1b-induced growth arrest in PCa cells

Recent study has shown that ING1b induces apoptosis through direct effects at the mitochondria (Bose et al. 2013) which are an important source of ROS within most mammalian cells (Adam-Vizi and Chinopoulos 2006). AR activation can also increase ROS in LNCaP and PC3-AR cells (Mehraein-Ghomi et al. 2008, Mirochnik et al. 2012). To investigate whether ING1b and AR interaction can synergize ROS production and mediate growth arrest, ROS production was analyzed in the PCa cell lines stably expressing ING1b along with ligand treatment. The data showed no increase in ROS level of ING1b expressing cells compared with the cells expressing empty vector, whereas the ROS accumulation upon agonist and antagonist was evident (Figure 8A). Moreover, to ask whether the different growth responses to ING1b KD in LNCaP and PC3-AR cells is due to difference in ROS induction, ROS levels were measured for ING1b KD cells under treatment with various ligands. The results from both cell lines did not exhibit any change of ROS levels in ING1 KD cells as compared to control ones (data not shown).

Likewise, the production of the cleaved PARP as a marker of apoptosis was ruled out in the cells stably expressing ING1b or sh-ING1b (Figure 8B). Moreover the microscopic observation of the explained cells during cultivation did not show any morphologically apoptotic cells (data not shown). Taken together, the data suggest that ROS production and apoptosis are not induced upon ectopic expression or knockdown of ING1b in PCa cells.
Results

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Figure 8. **ROS and apoptosis are not induced by ING1b in PCa cells.** (A) The stably transduced cells were treated 72h with the indicated ligands: DMSO, R1881 (1 nM) or Casodex (1 µM for LNCaP and 0.1 µM for PC3-AR). Subsequently, ROS levels were detected by DCF assay and the values obtained were normalized to protein concentrations. Values are shown as mean±SEM from at least two independent experiments. (B) Western blot analysis of PARP cleavage in response to the stable expression of ING1b or sh-ING1b in PCa cells. C+ is the corresponding PCa cell line treated with doxorubicin (1 µM) for induction of apoptosis as a positive control. α-Tubulin was used as a loading control in Western blotting.

4.6 **ING1b induces cellular senescence in PCa cells**

Besides induction of apoptosis, ING1b has been shown to induce cellular senescence in different primary cells (Goeman et al. 2005, Abad et al. 2011, Li et al. 2011). To test whether ING1b can play a same role in senescence status of PCa cells, the SA-β-Gal staining was applied for the stably transduced cells. The data could show the induction of cellular senescence in ING1b expressing cells compared to the control ones (Figure 9A, B). ING1b mRNA is upregulated in the senescent HPECs (Schwartz et al. 2002) and AR can also drive cellular senescence upon different ligand treatments (Mirochnik et al. 2012, Roediger et al. 2014, Hessenkemper et al. 2014). To further examine the functional consequence of AR-ING1b interaction in cellular senescence and to know whether ING1b is a mediator of AR-induced senescence, SA-β-Gal assay was
performed for the mentioned cells along with different ligand treatments. The data suggested that ING1b does not synergize with AR in fold induction of cellular senescence in the presence of different ligands (Figure 9C). Similarly, ING1b KD did not affect the ligand-stimulated cellular senescence in LNCaP cells (Figure 10A), however it impaired the agonist-induced cellular senescence in PC3-AR cells (Figure 10B).

Moreover, it has been reported that N-acetyl cysteine (NAC), ROS scavenger, can reduce androgen-mediated cellular senescence in PC3-AR cells (Mirochnik et al. 2012). To determine whether NAC can also play a same role in inhibiting androgen- or ING1b-induced senescence in LNCaP cells, the SA-β-Gal staining was performed for the stably transduced cells. As a result, NAC could attenuate the androgen-mediated cellular senescence but not ING1b-mediated senescence in response to androgen (Figure 11) which implies alternative means of senescence induction for ING1b. Thus, the data indicate that ING1b induces cellular senescence in PCa cells and it is the mediator of AR-driven senescence in response to androgens in PC3-AR cells.
Figure 9. ING1b drives cellular senescence in PCa cells. (A) LNCaP and PC3-AR cells were transduced by indicated vectors and selected by puromycin for 15 days. Then they were stained 2 days for SA-β-Gal and visualized under brightfield microscope (100x magnification). (B) Percentage of the SA-β-Gal positive cells by counting minimum 400 cells per cell type (* indicates p< 0.05). (C) Stably transduced cells were seeded on the 6-well plates. Next day, they were treated with either solvent control DMSO or R1881 (L) (low concentration: 10 pM) or R1881 (H) (high concentration: 1 nM) or DHT (10 nM) or Casodex (0.1 µM for PC3-AR and 1 µM for LNCaP) or AA (30 µM). Three days after treatment SA-β-Gal staining was performed. After 2 days, the blue/green colored cells were visualized and counted. All experiments were repeated at least two times. Error bars indicate SEM.
Figure 10. ING1b KD exhibits distinct effect in androgen-induced cellular senescence for LNCaP and PC3-AR cells. (A) & (C) The representative micrographs from stably transduced LNCaP or PC3-AR cells treated with DMSO or R1881 (1 nM) or AA (30 µM) for 3 days and stained for SA-β-Gal. (B) & (D) The cells explained in (A or C) counted and the results plotted as percentage of the SA-β-Gal positive cells. The experiments were performed three times in duplicate form. Error bars indicate SEM (* indicates p< 0.05).
Figure 11. NAC cannot reduce the androgen-induced cellular senescence in LNCaP-ING1b cells. Stably transduced LNCaP cells were seeded on the 6-well plates. Next day, they were treated with either DMSO or R1881 (1 nM) in the presence or absence of NAC (1 mM). Three days after treatment SA-β-Gal staining was performed. After 2 days, the blue/green colored cells were visualized and counted. Error bars indicate SEM (* indicates p< 0.05).

4.7 AR antagonists upregulate ING1b expression in LNCaP cells

Previous studies have suggested that the AR agonist R1881 and AR antagonist AA can induce cellular senescence in LNCaP cells (Roediger et al. 2014, Hessenkemper et al. 2014). To elucidate whether ING1b expression can be influenced by AR ligands, which could be one underlying mechanism of AR ligand-induced cellular senescence, ING1b protein and mRNA expression levels were investigated. Interestingly, transcriptional and translational expression of ING1b could be upregulated by AR antagonists Casodex and AA in LNCaP cells (Figure 12). However, this effect was not observed for PC3-AR cells (see Figure 29B in section 4.18). Thus, the data suggest that AR antagonists upregulate ING1b expression in LNCaP cells.
4.8 ING1b reduces cell migration and regulates migration-related genes in PCa cells

One of the complications in advanced prostate cancer is occurrence of metastasis which remains a significant cause of morbidity and mortality (Smith et al. 1999, Nandana and Chung 2014). ING1b as a type II tumor suppressor should have a potential of suppressing angiogenesis and cell invasion (Guerillon et al. 2013) and the reports have shown that the reduced ING1b level is associated with metastasis in breast and gastric cancers (Guo et al. 2011, Thakur et al. 2014). Moreover, ING1b overexpression could
inhibit migration and invasion of the breast cancer cell line MDA-MB231 cells, block the development of metastasis and improve survival in vivo (Thakur et al. 2014).

To evaluate the ability of ING1b to regulate migratory behavior of PCa cell line, scratch assays were conducted by using stably transduced cells under different ligand treatments. To exclude confounding effect of cellular senescence on migration, the ligands were applied at concentrations which do not induce cellular senescence. Consistent with the published data in non-PCa cells (Thakur et al. 2014), stable ING1b overexpression inhibited the ability of PCa cells to migrate and heal the wounds created in the cell monolayers. This inhibitory effect was not disturbed in the presence of the agonist or antagonist (Figure 13). However, ING1b KD resulted in distinct cell migration response in LNCaP and PC3-AR cell lines so that it promoted or decreased the cell motility in PC3-AR and LNCaP cells, respectively (Figure 14).

To identify the mechanism behind the ING1b regulatory effect on the migration behavior of the PCa cells, the migration- and invasion-related genes were analyzed by qRT-PCR (Lichtinghagen et al. 2003). ING1b overexpression could upregulate mRNA level of both migration-inducing genes (MMP9 and MMP13) and migration-inhibiting genes (TIMP1 and TIMP2) in LNCaP cells (Figure 15A), while only TIMP1 was upregulated by ING1b overexpression in PC3-AR cells (Figure 15A). ING1 KD did not significantly affect the mRNA level of the genes tested (Figure 15B). These results suggest that ING1b overexpression inhibits PCa cell migration and this inhibitory effect is mostly independent of AR ligands. Also the data indicate that ING1b regulates mRNA expression of the genes which are involved in migration and invasion.
Figure 13. **ING1b reduces cell migration in PCa cells.** (A) Scratch assays of the stably transduced LNCaP cells under different ligand treatment: DMSO, R1881 (10 pM) or Casodex (0.1 μM). The same scratched regions were captured under the brightfield microscope at indicated time points. (B) Quantification of the scratch assay by Tscratch software for the indicated time points in LNCaP cells. (C) The similar experiments as (A) for PC3-AR cells. (D) Quantification of the scratch assays for PC3-AR cells. The experiments were repeated twice. Error bars indicate SEM (* represents p<0.05).
Results

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B

![Graph](image19)

C

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Figure 14. ING1b KD results in distinct cell migration response in LNCaP and PC3-AR cells. Scratch assays after stable knockdown of ING1b in LNCaP (A, B) and in PC3-AR (C, D) cells. The experimental setting was similar to that described in Figure 13.

Figure 15. ING1b regulates migration-related genes. qRT-PCR analysis of the indicated migration- and invasion-related genes after overexpression (A) or knockdown (B) of ING1b in LNCaP and PC3-AR cells. The experiments were repeated twice. Error bars show SEM (* represents p<0.05).
4.9 ING1b upregulates p16 and stabilizes p27 in LNCaP cells

CDK inhibitors control fidelity of cell division in cell cycle checkpoints and the reduced expression of these proteins is associated with many types of cancer (Malumbres and Barbacid 2009). Reportedly, the functional link between ING1b and CDK inhibitors has been established (Garkavtsev et al. 1998, Kataoka et al. 2003, Gonzalez et al. 2006, Li et al. 2011). To understand the molecular mechanism by which ING1b induces growth arrest and cellular senescence in PCa cells, the expression of cell cycle inhibitors p14ARF, p16INK4a, p21CIP1/WAF1 and p27KIP1 were examined.

qRT-PCR and Western blot results suggested that ING1b regulates the CDK inhibitors in different manner in LNCaP and PC3-AR cells. p16 mRNA expression was upregulated in LNCaP-ING1b and unexpectedly, in LNCaP-ING1b KD cells, while no change for p14 mRNA level was detected in these LNCaP cells (Figure 16A). Since AR ligands have been shown to induce p16 (Roediger et al. 2014, Hessenkemper et al. 2014), the effect of ING1b on p16 expression under various ligands was analyzed. The results indicated that ING1b has an additive effect along with AR antagonist Casodex on p16 mRNA induction (Figure 16B, C). ING1b stabilized also p27 protein level in LNCaP-ING1b cells (Figure 16D, E). Although the p21 was not obviously influenced in LNCaP cells (Figure 16F, G), its regulation was identified in PC3-AR cells (Figure 17A). ING1b overexpression induced p21 upon agonist treatment and surprisingly ING1b KD could also upregulate this protein in PC3-AR cells (Figure 17A). However, p14 and p27 were not regulated in this cell line (Figure 17B, C) which is also known to express no detectable p16 due to hypermethylation of the promoter region (Itoh et al. 1997, Jarrard et al. 1997). Although p16/pRb axis is one of the well-known pathways for cellular senescence induction (Larsson 2011), pRb or its phosphorylation were not found to be regulated in the cells examined (data not shown). Briefly, these data suggest that ING1b upregulates different CDK inhibitors dependent on the cell type and also provide molecular evidence of how ING1b induces cellular senescence and growth arrest in PCa cells. The results further indicate that ING1b KD is also able to increases p16 and p21 expression in LNCaP and PC3-AR cells, respectively.
**Results**

**A**

![Bar graph showing fold mRNA level for p14ARF and p16INK4a.](image)

**B**

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**C**

![Bar graph showing fold p16 mRNA level.](image)
Figure 16. ING1b upregulates p16 and stabilizes p27 in LNCaP cell line. (A) qRT-PCR analysis of LNCaP cells transduced with the indicated vectors. 10 days after antibiotic selection of the transduced cells, RNA was isolated and qRT-PCR analysis was performed using target and housekeeping genes specific primers. (B) Western blot result of p16 for the cells explained in (A) after 3 days treatment with DMSO, R1881 (1 nM) or Casodex (1 µM). (C) qRT-PCR result of p16 for the cells explained in (A) after 2 days starvation and subsequent 2 days treatment with the ligands mentioned in (B) in the depleted condition. (D) Western blot analysis of ING1b and p27 for the cells in similar condition to (B). (E) qRT-PCR result of p27 in similar condition described in (C). (F) Western blot and (G) qRT-PCR analysis of p21 like (B) and (C), respectively. The normalized protein expression levels have been shown. The experiments were repeated two times. Error bars show SEM (* indicates p< 0.05).
Figure 17. ING1b regulates p21 in PC3-AR cells. (A) Western blot results of p21 in PC3-AR cells stably transduced with the indicated vectors and treated with DMSO, R1881 (1 nM) or Casodex (0.1 µM). (B) Western blot result of p27 for the cells described in (A). (C) qRT-PCR analysis was performed using target and housekeeping genes specific primers. The normalized protein expression levels have been displayed. The experiments were repeated two times. Error bars show SEM.
4.10 ING1b represses AR responsive promoters in LNCaP cells

Based on the obtained data in the host group, overexpression of ING1b can repress the transactivation of exogenous human AR (wild type and mutant form) in CV1 (green monkey kidney cell line) and NIH 3T3 (mouse embryonic fibroblast cell line) cells (Diploma theses Jennek 2009, Klitzsch 2011, Ludwig 2012). To determine whether ING1b can inhibit the transactivation of endogenous AR in PCa cells, the stable cell lines LNCaP-ING1b and LNCaP-ING1b KD cells were transiently transfected by MMTV-luc and Probasin-luc androgen responsive reporter plasmids. The LNCaP-ING1b cells exhibited diminished AR-mediated promoter activity of both reporters (Figure 18A, B). However, the LNCaP-ING1b KD cells showed surprisingly opposite effect between two reporters (Figure 18A, B). It is of note that MMTV promoter sequence contains several general binding elements for nuclear hormone receptors including AR, GR and PR (Miyamoto et al. 2003) whereas Probasin-luc promoter sequence is specific for AR (Snoek et al. 1996). These results support the ING1b role in regulating AR transactivation in LNCaP cells.
Results

**Figure 18.** ING1b inhibits AR-mediated transactivation in LNCaP cells. Reporter gene assays were performed for the stable cells LNCaP-ING1b, LNCaP-ING1b KD and their corresponding control cells. The cells were seeded out in 10% CSS medium and transiently transfected with 2 µg reporter construct (A) MMTV-luc or (B) Probasin-luc, and pCMV-lacZ (0.2 µg) which is expression vector for β-galactosidase and was used as an internal control for normalization. 6h later, the cells were treated with DMSO or low (L) or high (H) concentration of R1881 (0.1 or 1 nM, respectively) for 3 days in 10% CSS medium. Cells were then lysed and the measured luciferase values were normalized to β-galactosidase activity. The normalized luciferase values are shown as fold induction after setting the DMSO values arbitrarily at 1. The experiments were repeated at least twice. Error bars represent SEM (* shows p< 0.05).

4.11 ING1b represses endogenous AR target genes in LNCaP cells

In light of the above results regarding ING1b inhibition of AR transactivation, it was asked whether ING1b represses endogenous target genes of AR. For that purpose, LNCaP cells were stably transduced with ING1b or empty vector control and then examined for the mRNA level of AR target genes PSA, TMPRSS2 and NKX3.1. qRT-PCR analysis showed that the stable overexpression of ING1b decreases the transcription of positively regulated AR target genes in response to androgens (Figure 19A, B, C). This repression starts early for PSA, but later for TMPRSS2 and NKX3.1. The similar result was obtained for another AR target gene FKBP5 under 48h treatment (data not shown). Taken together, the data suggest that ING1b functions as a repressor in regulating the expression of positively regulated AR target genes in LNCaP cells.
Figure 19. ING1b suppresses endogenous AR target genes in LNCaP cells. After 10 days antibiotic selection of the transduced cells with the indicated vectors, they were starved 2 days in 10% CSS medium, then the cells were treated with the solvent control DMSO or synthetic
agonist R1881 (1 nM) or natural agonist DHT (10 nM) for various treatment times in the depleted condition. Thereafter RNA was isolated and qRT-PCR analysis was performed using specific primers for AR target genes (A) *PSA*, (B) *TMPRSS2* or (C) *NKX3.1*. The normalized mRNA levels are shown as fold levels after setting the DMSO values arbitrarily at 1. The experiments were repeated at least two times. Error bars represent SEM (* indicates p< 0.05).

**4.12 ING1b represses neither AR responsive promoters nor endogenous AR target genes in PC3-AR cells**

To determine whether this inhibitory effect of ING1b on the AR-mediated transactivation and gene expression can be reproduced in another AR expressing cell line, the similar experiments using reporter gene assays and qRT-PCR were applied to PC3-AR cells. However, in PC3-AR cells ING1b could increase the androgen-induced AR activity of MMTV-luc promoter in contrary to the repressive effect seen in LNCaP cells (Figure 20A). Probasin-luc reporter did not exhibit any change in ING1b expressing PC3-AR cells (Figure 20B).

In addition, the ING1b KD cells exhibited opposite effects between two reporters in this cell line (Figure 20A, B). Analyzing endogenous genes *PSA*, *TMPRSS2* and *FKBP5* verified that ING1b overexpression could not inhibit the positively regulated AR target genes (Figure 20C). Thus, the results indicate that ING1b does not reduce AR-mediated transactivation or gene expression of positively regulated endogenous genes in PC3-AR cells.
**Figure 20. ING1b does not inhibit AR transactivation in PC3-AR cells.** Reporter gene assay results of (A) MMTV-luc and (B) Probasin-luc for PC3-AR cells in a similar condition described in Figure 18. (C) qRT-PCR results for PC3-AR cells which were treated 48h with DMSO or DHT (10 nM) in a similar condition explained in Figure 19. The experiments were repeated twice. Error bars indicate SEM (* shows p< 0.05).

### 4.13 ING1b prevents AR-mediated transcriptional repression of endogenous genes

AR as a ligand-controlled transcription factor regulates gene expression by recruiting a wide variety of coactivators, corepressors, chromatin remodeling proteins and also other transcription factors at DNA binding sites. Therefore, AR can not only activate but also represses its target genes (Grosse et al. 2012). To identify whether ING1b as a chromatin remodeling factor can regulate AR repressive activity, the mRNA expression levels of α-fetoprotein (*AFP*) and catalytic subunit of human telomerase reverse transcriptase (*TERT*) were examined. It has been demonstrated that there is a link...
Results

between hepatocellular carcinoma marker AFP and AR activity (Ao et al. 2012, Wu et al. 2010). AFP was also found as a repressed target gene for AR in our experiments. ING1b can also repress AFP expression (Kataoka et al. 2003).

The qRT-PCR data indicated that overexpression of ING1b could repress the basal level of AFP mRNA in both PCa cell lines, and AR repressed AFP upon androgen treatment (Figure 21). Interestingly, ING1b prevented androgen-mediated transcriptional repression of AFP (Figure 21). To validate this inhibitory effect of ING1b on the AR-repressed genes, TERT mRNA level was analyzed. Although preferentially wild type AR can repress TERT in response to androgens (Moehren et al. 2008), in our experimental setting for 48h androgen treatment the repression of TERT was also detected in LNCaP cells which express mutant AR (T877A). Similarly, ING1b could inhibit androgen-mediated repression of TERT (Figure 21). As seen, ING1b had no effect on basal level of TERT expression. Taken together, these results suggest that ING1b functions as an inhibitor of AR transrepressive function in PCa cells.

Figure 21. ING1b inhibits AR-mediated gene repression. qRT-PCR results of AFP and TERT for LNCaP and PC3-AR cells which were transduced and treated 48h with DMSO, R1881 (1 nM) or DHT (10 nM) in a similar condition explained in Figure 19. The experiments were repeated twice. Error bars represent SEM (* shows p< 0.05).
4.14 Knockdown of ING1b counteracts the expression of endogenous AR target genes

Based on the results achieved for LNCaP cells, ING1b could reverse AR transcription function and it is expected that ING1b KD cells show the opposite effect. However, the growth, scratch and SA-β-Gal assays displayed no reversed action upon ING1b depletion in LNCaP cells. To test whether ING1b KD increases the AR-mediated gene expression, qRT-PCR analysis of the endogenous AR target genes were conducted. Unexpectedly, agonist-stimulated gene expression was repressed upon knockdown of ING1b in both LNCaP and PC3-AR cells (Figure 22). In LNCaP cells this repressive effect was validated for several AR target genes at different treatment times, however, each gene reacted at different time points more prominently to this repression (Figure 22A-C). PC3-AR cells showed the similar reduction in expression of AR target genes (Figure 22D), although ING1b overexpression had no effect in these cells. Moreover, ING1b KD could reduce PSA transcription upon applying increasing concentration of R1881 in LNCaP cells (Figure 22E).

To obtain additional evidence for the distinct effect of ING1b KD on the AR target gene expression, the negatively regulated genes, AFP and TERT mRNA level were analyzed. As expected, the ING1b KD upregulated basal level of AFP mRNA in both cell lines (Figure 23). Of interest, the data suggested that the ING1b KD could counteract the AR transrepressive effect on its target genes in LNCaP and PC3-AR cells (Figure 23). Thus, these results indicate that ING1b KD inhibits AR-mediated transcriptional activation and repression.
Results

A

LNCaP

Fold PSA mRNA level (normalized)

sh-control
sh-ING1b

B

LNCaP

Fold TMPRSS2 mRNA level (normalized)

sh-control
sh-ING1b

C

LNCaP

Fold NKX3.1 mRNA level (normalized)

sh-control
sh-ING1b
Figure 22. ING1b KD reduces androgen-induced gene expression in PCa cell lines. The stably-transduced cells with sh-control or sh-ING1b were selected 10 days against antibiotic. Then, they were starved 2 days in 10% CSS medium and treated with DMSO, R1881 (1 nM) or DHT (10 nM) for various treatment times in the depleted condition. Subsequently qRT-PCR analysis was performed on the extracted RNA from LNCaP cells using specific primers for AR target gene (A) PSA, (B) TMPRSS2 or (C) NKX3.1. The similar experimental setting was applied to (D) PC3-AR and (E) LNCaP cells which were treated 48h with the indicated agonists: DHT (10 nM) or R1881 (as shown). Normalized mRNA levels are shown as fold levels after setting the DMSO values arbitrarily at 1. The experiments were repeated three times. Error bars indicate SEM (* shows p< 0.05).
Results

Figure 23. ING1b KD inhibits AR-mediated transcriptional repression of endogenous genes. qRT-PCR results of \( AFP \) and \( TERT \) for LNCaP and PC3-AR cells which were transduced and treated 48h with DMSO, R1881 (1 nM) or DHT (10 nM) in a similar condition explained in Figure 22.

4.15 ING1b regulates differentially AR expression

Literatures have reported that AR-mediated transcription can be regulated with or without influencing AR protein level (Axland et al. 2010, Varisli et al. 2011, Lu et al. 2013, Li et al. 2014). To test whether the AR expression levels was also regulated by ING1b, qRT-PCR and Western blot analyses of AR mRNA and protein levels were performed in stably ING1b or ING1b KD expressing LNCaP or PC3-AR cells and compared with those of the corresponding control cells. The data suggested that ING1b leads to degradation of AR protein in LNCaP cells, since mRNA level of \( AR \) was not regulated (Figure 24A, B). This degradation was more prominent in the presence of AR agonist R1881 or AR antagonist Casodex indicating that this effect is independent of ligand type. However, ING1b KD did not affect neither protein nor mRNA level of AR (Figure 24A, B). Contrary to LNCaP cells, ING1b upregulated AR in the presence of DMSO or R1881 in PC3-AR cells (Figure 24C). qRT-PCR of \( AR \) mRNA level in this cell line was partially in concordance with protein level (Figure 24D). However, the difference between protein and mRNA levels of AR in the presence of antagonist Casodex is evident. The mRNA level of \( AR \) was reduced by Casodex but there was no such a reduction in its protein level suggesting a role for Casodex in regulating the stability of AR protein.
To identify whether ING1b degradation of AR in LNCaP cells is mediated through the ubiquitin proteasome pathway, LNCaP-ING1b cells were administered with proteasome inhibitor MG132 for 24h along with the ligands. The Western blot results proposed that AR degradation by ING1b is independent of proteasomal machinery (Figure 25A). The upregulation of ING1b and p27 in MG132-treated cells validated its action in inhibiting proteasomal degradation. Furthermore, MG132 treatment caused the accumulation of ~90 kDa truncated AR (Figure 25A) which is produced by a serine protease and degraded by proteasome system (Harada et al. 2012). Since there was no difference between control and ING1b cells in producing p90-AR (Figure 25A), the possibility of protease-mediated degradation of AR by ING1b is also unlikely.

It is also reported that MG132 inhibits AR degradation in time- and concentration-dependent manners (Tanner et al. 2004). For this reason, the similar experiment was conducted by applying 6h MG132 treatment. The obtained data confirmed that ING1b leads to degradation of AR independent of proteasome system (Figure 25B).

To test a potential synergistic effect of ING1b KD and MG132 treatment on AR stability, the similar experiment was performed with the LNCaP-ING1 KD and control cells. Only slight upregulation of AR protein level was observed in ING1 KD cells upon R1881 treatment with MG132 (Figure 25C). As seen from the latest Western blot results (Figure 25B, 6h untreated with MG132), ING1b had no measurable degradation effect on AR in this short time period. This case might be due to rapid AR replacement via de novo translation. To test this assumption and to find out how fast this degradation occurs, the treatment of the cells with cycloheximide for inhibiting protein synthesis was performed. The data suggested that the degradation of AR by ING1b starts as early as 6h (Figure 25D). Taken together, the data suggest that ING1b leads to degradation of AR in LNCaP cells in a proteasome- or protease-independent manner. These data also show that ING1b upregulates the AR expression in PC3-AR cells.
Figure 24. ING1b regulates differentially AR expression in LNCaP and PC3-AR cells. (A) Western blot analysis of AR in LNCaP cells stably transduced with the indicated vectors along with treatment with DMSO, R1881 (1 nM) or Casodex (1 µM) for 3 days. The expression AR protein was normalized to β-Actin. (B) qRT-PCR analysis of AR for the cells explained in (A). (C) & (D) The similar experiments (except Casodex 0.1 µM) were applied to PC3-AR cells as explained in (A) and (B), respectively. The normalized protein expression levels have been shown. The experiments were repeated twice. Error bars represent SEM (* shows p< 0.05).
### Results

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**Note:**
- **MG132:** Treatment with MG132.
- **Vector:** Expression of the vector.
- **Ligand:** Treatment with DMSO, R1881, or Cas.
- **Full-length AR:** Full-length androgen receptor.
- **p90-AR:** p90 androgen receptor.
Results

Figure 25. AR degradation by ING1b is independent of the proteasomal machinery in LNCaP cells. (A) Western blot analyses of AR, ING1b and p27 proteins in whole cell lysates from stably transduced LNCaP cells with empty vector or vector expressing ING1b after 24h treatment with DMSO, R1881 (1 nM) or Casodex (1 µM). The proteasome inhibitor MG132 (10 µM) was applied together with the ligands. (B) Western blot analysis for the cells explained in (A) after 6h treatment with the same concentration of ligands and MG132. (C) The same experimental setting described in (B) for the LNCaP cells stably transduced with vectors expressing either sh-control or sh-ING1b. (D) Western blot analysis for the cells explained in (A) under 6h treatment of translation inhibitor cycloheximide (10 µg/ml) together with the ligands. Protein from the cells untreated with cycloheximide was extracted at time zero before starting treatment. In all experiments AR protein expression was normalized to the loading controls (α-Tubulin or β-Actin) expression using LabImage 1D quantification software. The experiments were repeated twice.
4.16 ING1 KD does not affect the expression of other ING1 isoforms

Contrary to our expectation, ING1b KD could inhibit the positively- and negatively-regulated AR target genes in a similar trend as observed with ING1b overexpression (see section 4.14). AR degradation by ING1b might be a possible underlying molecular mechanism by which ING1b reduces the AR transcriptional activity. However, AR expression levels were not affected by ING1b KD (see previous section). Therefore, one hypothesis could be that knocking down of ING1b leads to enhanced expression of a putative protein, which is responsible for suppressing AR target genes in knockdown cells.

To examine this hypothesis, inhibition of the protein synthesis could serve the clue. Intriguingly, cycloheximide reduced the ING1b KD repressive effect on TMPRSS2 mRNA level in response to androgens (Figure 26).

Since ING1 isoforms are differentially expressed during cellular senescence (Soliman et al. 2008) and the ING1b-deficient mouse has displayed upregulation of ING1a and/or ING1c isoforms (Coles et al. 2007), it was asked whether ING1b deficiency in PCa cells is compensated by upregulation of other ING1 variants. Nevertheless, the results of Western blot analysis did not show any upregulation of other ING1 isoforms in LNCaP-ING1b KD cells (Figure 27) based on the protein quantification (data not displayed). Similar results were attained for PC3-AR cells in which ING1b was knockdowned (data not shown). These data suggest that a putative protein other than ING1 isoforms might be responsible for suppressing AR target genes in ING1b KD cells.
Figure 26. Cycloheximide reduces the ING1b KD effect on TMPRSS2 mRNA level. qRT-PCR results of TMPRSS2 for the LNCaP cells stably transduced with sh-control or sh-ING1b and treated 24h with DMSO, R1881 (1 nM) or DHT (10 nM) in the absence or presence of Cycloheximide (10 µg/ml). The normalized mRNA levels are shown as fold levels after setting the DMSO values arbitrarily at 1. Error bars represent SEM (* shows p< 0.05).

Figure 27. ING1b KD does not influence the expression of the other ING1 isoforms in LNCaP cells. Western blot analysis of ING1 protein levels in the stably transduced cells with either sh-control or sh-ING1b and treated 72h with DMSO or R1881 (1 nM) or Casodex (1 µM). α-Tubulin served as a loading control. The experiment was repeated twice.
4.17 *In vivo* mouse model rules out involvement of other ING1 isoforms in repressing AR target genes

To validate our previous finding *in vivo*, *Ing1* KO mouse could be a good candidate to analyze the role of ING1 in AR-mediated gene expression since all ING1 isoforms are absent (Kichina et al. 2006). This mouse strain is characterized by reduced size, hypersensitivity to radiation and elevated incidence of lymphomas. However, there are no other obvious morphological, physiological or behavioral abnormalities in *Ing1* KO mouse, indicating that *Ing1* function is dispensable for the viability of mice under normal physiological conditions (Kichina et al. 2006).

qRT-PCR results of AR target genes in primary mouse embryonic fibroblasts (MEFs), which were generated from male *Ing1* KO mice, suggest that *Ing1* KO MEFs has less potential to induce the AR target genes in response to androgens (Figure 28A). In line with this, AR target genes *Pbsn* and *Fkbp5* (Rennie et al. 1993, Magee et al. 2006) exhibit reduced expression in male *Ing1* KO mice (Figure 28B). qRT-PCR analysis of various organ-specific AR target genes (Felder et al. 1988, Lund et al. 1988, Melia et al. 1998, Ikeda et al. 2002, Xu et al. 2007, Zhou et al. 2010) in *Ing1* KO mice suggest that ING1 regulatory function on AR target gene expression is prominent in prostate with seminal vesicles (Figure 28B).

Taken together, these *in vivo* data corroborate results from the PCa cells and consistent with findings that ING1 deficiency downregulates AR target genes. The data further confirm that a protein other than ING1 isoforms might be responsible for inhibiting AR transcriptional activity in ING1b KD PCa cells and in prostate with seminal vesicles of *Ing1* KO mice.
Figure 28. The mRNA expression of AR target genes is affected in Ing1 KO mice. (A) MEFs from wild type and Ing1 KO male mice were cultured in 10% CSS medium and treated 48h with DMSO or R1881 (0.1 nM) in the depleted condition. Thereafter RNA was isolated and qRT-PCR analysis was conducted using specific primers for mouse AR target genes Tnf-α, Nkx3.1, and Sox9. Error bars represent SEM. (B) Different organs were taken from wild type and Ing1 KO male mice (n=3 mice per group, 6 months old). RNA was extracted from these organs and the expression of organ-specific AR target genes was analyzed by qRT-PCR. Rpl13a and Hprt were used as housekeeping genes for normalization. The normalized mRNA levels are shown as fold levels after setting the wild type values arbitrarily at 1. Error bars indicate SEM from 3 mice per group (* indicates p< 0.05).
4.18 ING1b regulates the stability of ING2a protein

Since there are structural and functional overlaps among ING family members (Coles and Jones 2009, Jafarnejad and Li 2012), it was reasoned to analyze the expression of other ING family members in ING1 KD cells to find compensatory feedback loop. For this purpose, ING2 was selected as the closest ING family member to ING1. Human ING2 has 2 known isoforms, however, only ING2a isoform with a molecular weight of 33 kDa has been experimentally detected at protein level. The amino acid sequence of human ING2a displays more than 70% homology with ING1b and it shares many of the functional characteristics of ING1b (Guerillon et al. 2013). For analyzing ING2 protein and mRNA expression levels, Western blot and qRT-PCR were performed in stably ING1b or ING1b KD expressing LNCaP or PC3-AR cells and compared with those of the corresponding control cells. Interestingly, ING1b overexpression downregulated strongly ING2a protein level and in line with this ING1b KD upregulates ING2a in both LNCaP and PC3-AR cells (Figure 29A, B). Analysis of ING2a mRNA level suggested that this regulation occurred mainly at protein level (Figure 29C). Contrary to PC3-AR cells, the upregulation of ING2a in the LNCaP cells was observed in a ligand-dependent manner (Figure 29A, B).

It is of note that this inverse correlation between ING1b and ING2a protein expressions was detected in different PCa cell lines (Figure 3D, Figure 29D). Accordingly, elevated mRNA level of ING2a in PC3 cells compared to LNCaP cells has been reported (Unoki et al. 2008).

To validate this result in vivo, ING2a protein expression was investigated in prostate with seminal vesicles of Ing1 KO mice as these organs showed the downregulation of AR target genes. The results displayed the upregulation of ING2a protein level in Ing1 KO mice (Figure 29E, F), although this result should be repeated with more mice per group to enhance its statistical significance. The upper band in ING2a Western blot might be a phosphorylated from of this protein since applying phosphatase to the protein extracts from ING2a-overexpressed LNCaP cells removed this band in another experimental setting (Master thesis Pungsrinont, 2015). In sum, ING1b negatively regulates ING2a protein level suggesting a novel crosstalk between ING1 and ING2.
Results

A

Vector: empty ING1b sh-control sh-ING1b

Ligand: DMSO R1881 Cas

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B

Vector: empty ING1b sh-control sh-ING1b

Ligand: DMSO R1881 Cas

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C

Fold ING2a mRNA level (normalized)

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Figure 29. ING1b regulates ING2a protein level in human PCa cells and mouse prostate with seminal vesicles. Western blot analyses of the ING1b and ING2a proteins for (A) LNCaP and (B) PC3-AR cells that are explained in Figure 24. ING2a protein level was normalized to α-Tubulin. The experiments were repeated twice. (C) qRT-PCR analysis of ING2a for the LNCaP cells described in (A). (D) Western blotting for ING2a in total cell lysates from the PCa cell lines. The protein expression of ING2a was normalized to the loading control α-Tubulin. (E) Western blot analysis of the ING2a protein in prostate and seminal vesicles of wild type and Ing1 KO mice (n=3). The normalized ING2a protein values from three mice per group are
shown as means±SEM. LNCaP cells transduced with either ING2a expressing vector or empty vector were loaded as positive controls at left side. (F) qRT-PCR of Ing2a in prostate and seminal vesicles of wild type and Ing1 KO mice. The normalized mRNA values obtained for wild type mice were set arbitrarily at 1. Error bars represent SEM.

4.19 ING2a represses the AR transactivation and upregulates p16

To confirm whether the upregulation of ING2a is responsible for reduced AR target gene expression in ING1 KD cells, the role of ING2a was investigated in PCa cell lines. To this end, PC3-AR cells were transiently cotransfected with either empty vector or vector expressing ING2a along with AR responsive promoter constructs MMTV-luc or PSA-luc. Of interest, ectopic expression of ING2a could attenuate AR transactivation of both reporters in response to androgen (Figure 30A, B). To determine whether ING2a can also inhibit the endogenous AR target genes, LNCaP cells were stably transduced with ING2a or empty vector and then the mRNA expression of PSA, TEMPRSS2 and NKX3.1 was studied. qRT-PCR results provided more evidence that ING2a could repress the androgen-induced expression of endogenous AR regulated genes (Figure 30C).

It has been previously reported that ING2a is able to induce p21 independent of p53 status (Larrieu et al. 2010), and this is consistent with the results obtained upon ING1b KD in PC3-AR cells where the concomitant upregulation of ING2a and p21 was observed in p53 null context (Figure 17A, Figure 29B). However, it seems the p21 induction by ING2a is dependent on the cellular context since LNCaP-ING1 KD cells showed no detectable change for p21 expression, but for p16 (Figure 16). Therefore, it was reasoned that ING2a can induce p16 in LNCaP cells. Expectedly, qRT-PCR result of p16 in LNCaP-ING2a cells confirmed this hypothesis (Figure 30D). However, mRNA level of CDK inhibitors p16, p19, p21 and p27 were not found to be differentially regulated in prostate and seminal vesicles of Ing1 KO mice (data not shown). Thus, the data indicate that ING2a represses AR-mediated transactivation in PCa cells and induces the expression of various CDK inhibitors dependent on the cellular context.
Figure 30. ING2a represses AR-mediated transactivation and upregulates p16. Reporter gene assays were performed for PC3-AR cells. The cells were seeded out in 10% CSS medium and transiently cotransfected with 1µg of either empty vector or vector expressing ING2a along with 1µg reporter construct (A) MMTV-luc or (B) PSA-luc, and pCMV-lacZ (0.2 µg) which is expression vector for β-galactosidase and was used as an internal control for normalization. 16h later, the cells were treated with solvent control DMSO or R1881 (1 nM) for 3 days in 10% CSS medium. The normalized luciferase values are shown as fold induction after setting the DMSO values arbitrarily at 1. (C) qRT-PCR analysis with the experimental conditions similar to Figure 19 except the vectors which were empty or ING2a expressing ones. (D) p16 mRNA level was
Results

detected by qRT-PCR for the LNCaP cells described in (C) without any ligand treatment. The experiments were repeated twice. Error bars show SEM (* represent p< 0.05).

Taken all results together, the ectopic expression of ING1b inhibits growth of PCa cells by induction of cellular senescence through upregulation of different CDK inhibitors in both androgen-dependent LNCaP and CRPCa PC3-AR cells. ING1b can also reduce cell migration in both cell lines by regulating the expression of migration-related genes. Moreover, ING1b overexpression represses AR transactivation on key target genes in LNCaP cells, however, this repressive effect is absent in PC3-AR cells. This differential repressive effect might be due to cell specific degradation of AR by ING1b in LNCaP cells. However, ING1b can function as an inhibitor of AR transrepressive function in both PCa cells suggesting a mechanism other than AR degradation for control of the negatively regulated AR target genes.

Unexpectedly, knocking down of ING1b in LNCaP and PC3-AR cells result in distinct responses on growth, migration and cellular senescence between two cell lines. ING1b KD impairs the agonist-induced cellular senescence in PC3-AR cells only. Interestingly, ING1b KD inhibits AR-mediated transcriptional activation and repression on key target genes in both PCa cells in a similar manner to ING1b overexpression in LNCaP cells, which is in accordance with the \textit{in vivo} results from \textit{Ing1} KO mice. Analyzing potential mechanisms suggests that these unexpected findings might be due to a compensatory mechanism through upregulation of ING2a protein. The data indicate that ING2a can also inhibit key target genes of AR similar to ING1b. This compensatory mechanism suggests a novel crosstalk among ING family members in regulating various AR functions. Thus, the obtained findings provide evidence for functional interaction of ING1b with AR.
5 Discussion

PCa is the second most common cause of cancer mortality of men in western countries. The risk of developing PCa increases with age (Siegel et al. 2013). With nowadays aging society it is inevitable PCa to be investigated further to develop more effective therapeutic approaches especially for the cases with CRPCa. Since AR signaling is still active in CRPCa, its inhibition seems to be beneficial for patients (Bluemn and Nelson 2012). ING1 is an epigenetic regulator of different cellular processes including proliferation and cellular senescence (Tallen and Riabowohl 2014). Given the interaction between AR and ING1b, the aims of the study were to analyze whether ING1b is the mediator of the AR-induced cellular senescence and also to get benefit of cellular senescence in inhibition of PCa cell growth by using ING1b.

5.1 ING1 and PCa

Consistent with proposed tumor suppressor role for ING1, it was found to be frequently downregulated in several cancers (Walzak et al. 2008, Guerillon et al. 2013). However, little is known about the role of ING1 in PCa. For this purpose, ING1 mRNA expression was compared between normal and cancerous prostate from published microarray gene expression studies using Oncomine tool. Controversially, the results show both downregulation and upregulation of ING1 mRNA level in PCa tissues compared to the normal prostate. Therefore, the protein expression of ING1 was analyzed using Human Protein Atlas portal. Interestingly, the data indicate that the protein level of ING1 is lost in more than 30% of the PCa specimens.

Moreover, the protein expression of major isoform ING1b is decreased in CRPCa cells C4-2 and PC3 compared to androgen-dependent LNCaP cells. Interestingly, Src, which has been shown to decrease ING1b stability (Yu et al. 2013), is upregulated in C4-2 and PC3 cells compared to LNCaP cell line (Asim et al. 2008). These findings suggest that Src may play a role in regulating ING1b levels during PCa progression. These results along with upregulation of ING1b in the senescent HPECs (Schwarze et al. 2002) support more likely the notion that ING1b has a negative impact on the development and progression of PCa.
5.2 ING1b regulates growth, cellular senescence and migration

The presenting data have shown for the first time that the ectopic expression of ING1b can inhibit growth of the androgen-dependent LNCaP and CRPCa PC3-AR cells similar to previous reports in other cancer cell lines (Shinoura et al. 1999, Tsang et al. 2003, Lv et al. 2012, Thakur et al. 2012, Bose et al. 2013). At first glance it seems ING1b inhibits growth in a p53-independent manner as PC3-AR cell line is p53 null (Rubin et al. 1991, Carroll et al. 1993). This finding is comparable to the previous studies which have shown that ING1b inhibits growth of non-PCa cells, independently of p53 (Coles and Jones 2009, Guerillon et al. 2013). However, the fold of growth reduction for PC3-AR cells is less than that for LNCaP cells suggesting that certain factors needed for the ING1b full functionality are absent in PC3-AR cells. Apart from p53 null status, PC3-AR cell line is also known to express no detectable p16 due to hypermethylation of the promoter region (Itoh et al. 1997, Jarrard et al. 1997). Reportedly, ING1b stabilizes p53 protein (Leung et al. 2002), represses the transcription of the certain genes dependent on p53 (Kataoka et al. 2003) and induces growth arrest via upregulation of p16 (Li et al. 2011). Therefore, one can suggest that ING1b fails to exert its complete function through p53 and/or p16 in PC3-AR cells. Since ING1b can still repress the growth of PC3-AR cells, it indicates that ING1b uses alternative pathways for inhibiting growth in PC3-AR cells. This growth inhibitory effect of ING1b is ligand-independent because no synergistic effect was seen between ING1b and AR ligands suggesting that ING1b inhibits growth independent of its interaction with AR.

The data demonstrate that ING1b induces cellular senescence in LNCaP and PC3-AR cells. On the other hand, ROS production and the apoptosis marker, cleaved-PARP, could not be found to be upregulated upon ING1b overexpression. It means that the induction of cellular senescence is a possible pathway by which ING1b reduces growth in PCa cells, similar to the findings reported in primary cells (Goeman et al. 2005, Abad et al. 2007, Abad et al. 2011, Li et al. 2011). However, it is contrary to the studies indicating that apoptosis is responsible for ING1b-induced growth inhibition in different non-PCa cancer cells (Shinoura et al. 1999, Tsang et al. 2003, Lv et al. 2012, Thakur et al. 2012, Bose et al. 2013). The upregulation of the ING1b target gene BCL-2 excludes the possibility of apoptosis induction by ING1b in PCa cells due to the well-known anti-apoptotic function of BCL-2. Furthermore, ING1b-deficient MEFs have displayed induced Bax expression and DNA damage-induced apoptosis indicating that ING1b can...
negatively regulate apoptosis (Coles et al. 2007). More studies are needed to clarify how ING1b affects various cancer cells dependent on their genetic context.

Liganded AR has been also shown to induce cellular senescence (Mirochnik et al. 2012, Roediger et al. 2014, Hessenkemper et al. 2014). Since ING1b does not synergize with AR for fold induction of cellular senescence in the presence of agonists, it seems that ING1b and AR induce the cellular senescence through the same pathway or the level of senescence reaches to a plateau. However, inhibiting ROS-induced senescence by NAC in LNCaP cells could attenuate the androgen-mediated cellular senescence but not ING1b-mediated senescence in response to androgen meaning that ING1b utilizes alternative pathway to induce the cellular senescence. Therefore, reaching to a plateau level of cellular senescence in the presence of agonists is more likely.

Although a logical expectation might be to see increased growth rate upon ING1b KD, this has been not observed for LNCaP cells. Actually, LNCaP-ING1b KD cells show unexpectedly growth inhibition, however, ING1b KD promotes growth in PC3-AR cells as expected. This growth promotion is more prominent with the high concentration of agonist. Interestingly, ING1b KD does not affect the ligand-stimulated cellular senescence in LNCaP cells, however it impairs the agonist-induced cellular senescence in PC3-AR cells. This can explain in part the growth response differences and emphasis that ING1b is the mediator of AR-induced senescence in response to agonist in PC3-AR cells. The growth inhibition in LNCaP-ING1 KD cells may not be attributed to downregulation of BCL-2 and induction of apoptosis because the apoptosis maker, cleaved-PARP, was not detected in these cells.

Consistent with the reported role for ING1b in inhibiting cell migration and invasion in breast cancer cells (Thakur et al. 2014), ING1b can also inhibit the migration of PCa cell lines. This effect is independent of AR ligands. Nevertheless, ING1b KD has been found to result in distinct responses in LNCaP and PC3-AR cells. In line with ING1b KD or KO studies (Garkavtsev et al. 1996, Coles et al. 2007, Guo et al. 2011, Thakur et al. 2014), ING1b deficient PC3-AR cells migrate more quickly than the control cells. Contrary to the expectation, LNCaP cells migrate slower after ING1b KD, although AR ligands abrogate this effect suggesting a functional interaction of AR with ING1b in controlling PCa cell migration.
5.3 ING1b controls gene expression

ING1b is believed to exert cellular functions primarily by altering gene expression through epigenetic mechanisms, specifically by binding to and targeting the protein complexes to the H3K4Me3 mark (Tallen and Riabowohl 2014).

5.3.1 ING1b regulates CDK inhibitors

The results demonstrate that ING1b targets specific genes in different genetic context. ING1b upregulates \textit{p16} transcription in LNCaP cells as reported previously in other cell type (Li et al. 2011). Also the data have shown for the first time that ING1b stabilizes p27 posttranscriptionally. The specificity of the ING1b in gene regulation of cell cycle factors comes from this fact that expression of \textit{p14} and \textit{p21} has not been influenced by ING1b in LNCaP cells. However, in the PC3-AR cells p21 protein is a target for ING1b that is also upregulated in response to AR agonist while no regulation for \textit{p14} mRNA and p27 protein has been found (Figure 31).

Thus, it seems that ING1b induces growth arrest and cellular senescence via upregulation of CDK inhibitors in PCa cells, although more experiments are needed to identify the underlying reasons for basal level of growth repression and senescence induction in PC3-AR cells. Furthermore, in LNCaP cells ING1b and AR cooperate around additively to upregulate \textit{p16} mRNA expression only in the presence of the AR antagonist. This cooperation has been found for p27 protein there only in the presence of AR agonist. Since the ING1b is still able to induce higher percentage of senescent cells in LNCaP cell line in the presence of antagonists but not agonists as compared with empty vector, p16 probably is more involved than p27 to induce senescence. The simultaneous induction of ROS, p16 and cellular senescence has been found in LNCaP cells similar to the previous reports in which p16 expression is induced by ROS (Kim and Wong 2009, Larsson 2011). Therefore, it is expected by inhibiting ROS and subsequent impairment of p16-induced cellular senescence, the level of agonist-mediated cellular senescence reduces. And this is the case observed after treating the LNCaP cells by ROS scavenger, NAC. However, stabilization of p27 upon agonist treatment could keep induced level of senescence compared to solvent control.

The p27-mediated senescence is probably the alternative pathway through which ING1b-overexpressed cells keep their senescence in the presence of agonist even they treated with NAC. The CDK inhibitor p27 is induced by external growth inhibitory
signals like transforming growth factor (TGF)-β and inhibits cyclin E/CDK2 leading to growth arrest via induction and maintenance of cellular senescence (Larsson 2011). Furthermore, the role of p27 in DHT-mediated growth arrest in LNCaP cells (Tsihlias et al. 2000) and AKT-induced senescence in the murine prostatic intraepithelial neoplasia have been reported (Majumder et al. 2008). ING2, a close family member to ING1, mediates TGF-β cellular responses in epithelial cells (Sarker et al. 2008). Therefore, the detailed studies are required for establishing functional link among ING1, p27 and TGF-β to better understand mechanisms responsible for ING1b-mediated cellular senescence in PCa cells.

5.3.2 ING1b regulates migration-related genes

Contrary to expectation, ING1b upregulates the mRNA level of \(\text{MMP9} \) and \(\text{MMP13} \) in LNCaP cells, which are responsible for promoting migration. However, it could also upregulate their inhibitors \(\text{TIMP1} \) and \(\text{TIMP2} \) there. Since the migration behavior of the LNCaP cells has been reduced by ING1b, it seems the effect of MMP inhibitors more potent or other migration-related genes are regulated by ING1b in LNCaP cells. A relatively higher association has been described for reduced TIMPs mRNA expression with malignant prostatic tissues (Lichtinghagen et al. 2003) indicating an important role for TIMPs. In PC3-AR cells only \(\text{TIMP1} \) mRNA level has been upregulated by ING1b while no change for \(\text{MMP9} \), \(\text{MMP13} \) and \(\text{TIMP2} \) has been detected fitting to the diminished migration of the cells upon ING1b overexpression.

Interestingly, ING1b KD has no statistically significant effect on the migration-related genes, although an increase in \(\text{MMP9} \), \(\text{MMP13} \) and \(\text{TIMP2} \) and decrease in \(\text{TIMP1} \) mRNA levels were indeed noted in response to ING1 KD in PC3-AR cells, which is partially in agreement with their higher migration rate. Analyzing protein level of the mentioned genes and measuring MMPs enzymatic activity would be helpful for better interpretation of the results.

5.3.3 ING1b controls gene expression of the AR target genes

The results demonstrate that ING1b represses positively regulated AR target genes \(\text{PSA}, \text{NKX3.1}, \text{TMPRSS2} \) and \(\text{FKBP5} \) in response to androgens in LNCaP cells, however, this repressive effect is absent in PC3-AR cells. The data indeed indicate that one level at which ING1b affects AR signaling is through the regulation of AR stability. ING1b
leads to the degradation of AR only in LNCaP cells in which the diminished AR transcriptional activity was detected. Conversely, ING1b increases AR expression in PC3-AR cells and the consequent expectation would be a higher AR transcriptional activity. As mentioned, however, no increased AR signaling has been detected in this cell line. Although p53 negatively regulates the mRNA and protein expression of AR in prostate epithelial and cancer cells (Alimirah et al. 2007) and ING1b can stabilize p53 (Leung et al. 2002, Thalappilly et al. 2011), ING1b does not downregulate AR mRNA level in LNCaP cells.

The obtained data from LNCaP cells show that the degradation of AR is independent of the proteasome and protease systems. Interestingly, LNCaP-ING1b KD cells do not show upregulation of AR protein level. More studies are required for finding the proteins or mechanism for explaining reduced AR protein level in LNCaP cells and its upregulated expression in PC3-AR cells upon ectopic expression of ING1b.

Ligand-induced nuclear translocation is a crucial step for AR-mediated transcription. The translocation studies exclude the possibility that ING1b represses AR activity by inhibiting its nuclear shuttling.

The differential repressive effect of ING1b on AR transactivation between LNCaP and PC3-AR cells can be also explained by the fact that PC3-AR cells are p53 null (Rubin et al. 1991, Carroll et al. 1993) and inhibition of p53 leads to diminished AR signaling (Cronauer et al. 2004, Guseva et al. 2012). p53 KD studies in LNCaP cells will unravel this point. Moreover, the different genetic context between LNCaP and PC3-AR leads to the expression of various sets of corepressor/coactivators in these cells and it can be also considered for this difference.

However, ING1b prevents AR-mediated transcriptional repression of the endogenous genes AFP and TERT in both cell lines suggesting a mechanism other than AR degradation for control of the negatively regulated AR target genes. The repression of AFP expression by ING1b in both PCa cell lines indicates that ING1b represses AFP independent of p53 which is consistent with the report of Kataoka et al. (2003) in non-PCa cells. Similarly, AR downregulates the AFP and TERT expression in both cell lines suggesting a p53-independent regulation, although one can conclude a partial dependence on p53 for AFP due to a stronger repression in LNCaP cells. Therefore, ING1b can prevent AR transrepressive role as well in PC3-AR.
5.4 Crosstalk between ING1b and ING2a

As ING1b downregulates the AR-mediated transcription in response to androgens, one would predict that the inhibition of endogenous ING1b should upregulate the AR transactivation in PCa cells. Unexpectedly, agonist-stimulated gene expression was repressed upon knockdown of ING1b in both LNCaP and PC3-AR cells. This result, which was validated for several AR target genes in different time point of agonist treatment, is especially interesting in PC3-AR cells in which ectopic expression of ING1b did not regulate AR transcriptional activity. In addition, ING1b KD counteracts AR-mediated transcriptional repression of endogenous genes AFP and TERT in both cells. Consistently, LNCaP-ING1 KD cells grow slower than control cells and migrate less in the absence of AR ligands. In addition, cellular senescence marker, SA-β-Gal, has not been reduced and indeed increased slightly in absence or presence of antagonist AA upon ING1b depletion in LNCaP cells. Also, the data demonstrate the upregulation of CDK inhibitors p16 upon knockdown of ING1b, in similar tendency observed for ING1b overexpression in LNCaP cells. Since AR expression is not regulated in ING1KD cells compared to control ones, it should not be responsible for downregulation of AR target genes in ING1 KD context. To find a possible underlying mechanism, the inhibition of protein synthesis has reduced the ING1b KD repressive effect on the AR target gene TMPRSS2 mRNA level suggesting that a putative protein is responsible for suppressing AR target genes in ING1b KD cells. While ING1 isoforms are differentially expressed during cellular senescence (Soliman et al. 2008) and ING1b-deficient mice have displayed upregulation of ING1a and/or ING1c isoforms (Coles et al. 2007), the obtained results however show no upregulation of other ING1 isoforms in ING1b KD cells. Moreover, Ing1 KO MEFs exhibit a reduced AR target gene expression in response to androgens. In line with this, the prostate-specific AR target genes Pbsn and Fkbp5 have been revealed to be downregulated in Ing1 KO mice confirming that ING1 isoforms are not the candidate protein.

Intriguingly, ectopic expression of ING1b downregulates strongly ING2a protein level and accordingly ING1b KD upregulates ING2a in LNCaP and PC3-AR cells. This inverse correlation between ING1b and ING2a protein expressions could be also detected in C4-2 and PC3 cell lines. Moreover, ING2a upregulation in prostate and seminal vesicles of Ing1 KO mice verifies the results in vivo. ING2a, like ING1b, is a tumor suppressor with gatekeeper and caretaker functions. It displays high amino acid
sequence homology with ING1b. Both ING1b and ING2a are stable components of the SIN3A/HDAC1-2/SAP30 complex and thus both regulate gene transcription. Since ING1b and ING2a do not coprecipitate together, they are exclusive components of the SIN3A/HDAC1-2/SAP30 complex (Guerillon et al. 2013). Apart from reduced size and higher incidence of lymphomas, Ing1 KO mice exhibit no other obvious morphological, physiological or behavioral abnormalities, indicating that Ing1 function is dispensable for the viability under normal physiological conditions (Kichina et al. 2006) and Ing2 might play a compensatory role in Ing1 depleted condition. Analyzing young (6-week-old) Ing1 KO mice has revealed no abnormalities with fertility and prostate phenotype (Kichina et al. 2006). Since the obtained results exhibit a reduced expression of prostate-specific AR target genes at 6-month-old Ing1 KO mice, the fertility and prostate phenotype need to be rechecked in detail with older Ing1 KO mice. Ing2 KO mice are characterized by defective spermatogenesis in males and higher incidence of soft tissue sarcomas (Saito et al. 2010). Interestingly, the major tumor type observed in Ing2 KO mice was histiocytic sarcoma which showed increased incidence preferentially in males for currently unknown reasons. Moreover, Ing2 KO mice have displayed a decrease in acinar dilation in prostate indicating a role for Ing2 in the prostate.

Ectopic expression of ING2a not only could attenuate AR transactivation in response to androgen in PC3-AR cells but also could repress the androgen-induced expression of endogenous AR regulated genes in LNCaP cells. This effect may be the underlying molecular mechanism explaining the repression of the AR target genes in ING1 KD cells. Also, analysis of the publicly available dataset (GSE18610 at http://www.ncbi.nlm.nih.gov/geo/) from Ing2 KO mice (2-3 months old) (Saito et al. 2010) has shown the significantly increased expression of testis-specific AR target genes Rhox5 and Sox9 in testes of Ing2 KO mice compared to wild type ones.

Although ING1b cannot inhibit AR target genes in PC3-AR cells, it is not the case for ING2a suggesting different mechanism for ING2a in repressing AR target genes. In addition, ING2a induces p16 mRNA level in LNCaP cells which explain the enhanced level of p16 in LNCaP-ING1 KD cells with elevated ING2 expression. This finding along with the proved role of ING2a in inducing cellular senescence (Pedeux et al. 2005), might be the molecular reason why there is no reduction of SA-β-Gal activity in LNCaP-ING1b KD cells. This compensatory mechanism suggests a novel crosstalk between ING1b and ING2a (Figure 31).
Figure 31. Model of ING1b-mediated cellular senescence in crosstalk with AR and ING2a.

**LNCaP cells:** Agonist-bound AR induces p16 and p27 and ectopic expression of ING1b can increase p16 and p27 levels as well. Elevated level of p16 and p27 inhibits CDKs and triggers cellular senescence. Upon knockdown of ING1b, ING2a is stabilized and induces p16 restoring the cellular senescence in the absence of ING1b. AR antagonists increase the expression of ING1b, however, compensatory role of ING2a might be responsible for restored level of cellular senescence in ING1b KD cells. **PC3-AR cells:** AR or ING1b induces p21 upon agonist treatment and causes senescence. AR-induced senescence in response to androgens is impaired in ING1b KD cells indicating that ING1b is the mediator by which AR induces senescence upon agonist treatment. Although knockdown of ING1b stabilizes ING2a and subsequently upregulates p21, it is not enough to completely restore the cellular senescence suggesting that ING2a and its partner p21 require ING1b to actively induce senescence in PC3-AR cells in response to agonist. The novel links are shown in red.

It has been previously reported that ING2a is able to induce p21 expression independent of the p53 status (Larrieu et al. 2010). This is consistent with the results obtained upon ING1b KD in PC3-AR cells in which the concomitant upregulation of ING2a and p21 were observed in p53 null context. Knocking down of ING1b in PC3-AR cells impairs, but not completely abolishes, the agonist-induced cellular senescence in PC3-AR cells suggesting that ING2a and its partner p21 require ING1b to actively induce senescence in PC3-AR cells in response to androgens. Likewise, ING1b-deficient MEFs display a defective senescence-like phenotype in response to an activated Ras oncogene, even though they express elevated level of p19ARF and p21CIP1/WAF1 (Abad et al. 2007). Double knockdown of p21 and ING1b in PC3-AR cells will elucidate this point. Furthermore, one can assume that AR-driven senescence in response to androgens is mediated by different CDK inhibitors dependent on the cellular context.
6 Conclusions and perspectives

Recently the relevance of cellular senescence as a physiological barrier against tumor initiation and progression is well established and various senescence-inducing compounds were applied for PCa therapy (Nardella et al. 2011, Ewald and Jarrard 2012). However, cancer cell-specific targeted therapy is an important point to reduce the toxicity that is associated with traditional therapies (Nardella et al. 2011). Since AR signaling is still active in CRPCa, specific targeting the AR by ING1b can be one possible way to inhibit the AR signaling and induce cellular senescence in PCa cells. Moreover, understanding molecular mechanism of ING1b-mediated cellular senescence in PCa cells helps to design better therapeutic strategies.

The recent studies have revealed that overexpression of ING1b prevents growth and invasion of the breast cancer cells, block the metastasis and improve survival in vivo (Thakur et al. 2012, Thakur et al. 2014). The obtained data could show for the first time that ING1b can inhibit growth and migration of both androgen-dependent and castration-resistant PCa cells. This inhibitory effect is due to repressing the expression of AR target genes and upregulating cellular senescence-inducing factors such as p16\(^{INK4a}\) and p27\(^{KIP1}\) in LNCaP cells suggesting that ING1b represses AR signaling. Considering this newly identified link between ING1b and p27\(^{KIP1}\), analyzing the role of p27\(^{KIP1}\) in ING1b-induced cellular senescence is of interest. Moreover, the degradation of the AR by ING1b could be observed in LNCaP cells, which indicates a possible underlying mechanism to repress AR signaling. Although the growth inhibitory function of ING1b in PC3-AR cells remains to be more analyzed, CDK inhibitor p21\(^{CIP1/WAF1}\) plays a key role in AR- and ING1b-induced cellular senescence in response to androgens in this cell line. Interestingly, the ING1b KD studies could demonstrate that ING1b is the mediator through which AR induces cellular senescence in response to androgens in PC3-AR cells. Analyzing ING1b KD cells along with Ing1 KO mice suggests a dual role for ING1b in crosstalk with the other ING family member ING2a that is also involved in cellular senescence. Therefore, it is necessary to study the role of ING2 in prostate cancer. Indeed, the recent evidence broadened the definition of ING family proteins as epigenetic regulators due to their dual roles in differentially control of cell growth in different biological contexts (Schaefer et al. 2013, Tallen and Riabowohl 2014). Based on the altered AR specific gene expression in prostate with seminal vesicles of Ing1 KO mice and reduction of prostatic acinar dilation in Ing2 KO mice,
more investigation on the cellular senescence markers in prostate of old Ing1 KO and Ing2 KO mice would be interesting. Besides, generating an inducible double KO mouse for Ing1 and Ing2 specifically in prostate will provide a model for studying their crosstalk in prostate.

AR antagonists can upregulate ING1b expression in LNCaP cells suggesting that ING1b might be responsible for antagonist-induced cellular senescence. Due to compensatory effect of ING2a, double knockdown of ING1b and ING2a will clarify this point.

Taken together, the findings obtained provide evidence for functional interaction of ING1b with AR in crosstalk with ING2, and suggest ING1 as a novel potential target to inhibit AR signaling. Analysis of the chromatin modifications on the AR regulated genes in the presence and absence of ING1 and/or ING2 will further confirm these functional interactions. Moreover, to get deep insight into genomic action and to detect which genes are commonly regulated by AR and ING1 or ING2, it is necessary to perform ING1 or ING2 chromatin immunoprecipitation-sequencing (ChIP-seq) for the human PCa cells treated with AR ligands and then compare the results with the published AR ChIP-seq data. RNA-seq experiments with ING1 KD, ING2 KD and double ING1/ING2 KD human PCa cells will elucidate that how ING1 and/or ING2 deficiency affect the AR regulated transcriptome.

Analyzing AR ChIP-seq and RNA-seq data between wild type and Ing1 KO mice prostate will provide more evidence of how ING1 deficiency influences AR chromatin binding and differential gene expression in the mice prostate.
7 References


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Referencens


8 Appendix

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Dr. Aria Baniahmad und Florian Kraft,

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, 27.07.2015

Mohsen Esmaeili
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