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

Dissertation

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Table of Contents

List of abbreviations

α	alpha
AA	atraric acid
ADT	androgen deprivation therapy
AF	activation function
AFP	alpha-fetoprotein
AP	alkaline phosphatase
AR	androgen receptor
ARE	androgen response element
β	beta
C	carboxy
C^+	positive control
°C	degree Celsius
$CaPO_4$	calcium phosphate
Cas	casodex
CDK	Cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus
CRPCa	Castration-resistant prostate cancer
CSS	charcoal stripped serum
Δ	delta
DA	diacetate
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
DCF	dichlorofluorescein
DHT	dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DOTAP	N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methyl sulphate
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
Eco	ecotropic
EcoR	ecotropic receptor
EGFP	enhanced green fluorescent protein

EMT	epithelial-mesenchymal transition
ER	estrogen receptor
EtOH	ethanol
FBS	fetal bovine serum
g	gram
GC	guanine-cytosine
GFP	green fluorescent protein
GR	glucocorticoid receptor
GST	glutathione S-transferase
h	hour
H	histone
H	high concentration
HAT	histone acetyltransferase
HDAC	histone deacetylase
HeLa	human cervix carcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMT	histone methyltransferase
HPEC	human prostate epithelial cell
ING	inhibitor of growth
K	amino acid lysine
KD	knockdown
kDa	kilo Dalton
KO	knockout
l	liter
L	low concentration
LBD	ligand binding domain
LID	lamin interaction domain
LNCaP	lymph node cancerous prostate
luc	luciferase
LZL	leucine zipper-like
µ	micro
m	meter
m	milli
M	Manders' coefficient
M	molar

me	methyl group
MEF	mouse embryonic fibroblast
MG132	N-(benzyloxycarbonyl)leucinylleucinylleucinal
min	minutes
MMP	matrix metalloproteinase
MMTV	mouse mammary tumor virus
n	nano
N	amino
NAC	N-acetyl cysteine
NLS	nuclear localization signal
NTD	amino terminal domain
NTS	nucleolar targeting sequences
OD	optical density
p	pico
p	probability
PARP	Poly ADP ribose polymerase
PBD	partial bromo domain
PBR	polybasic region
PBS	phosphate buffered saline
Pbsn	probasin
PCa	prostate cancer
PHD	plant homeodomain
PIP	PCNA-interacting protein motif
ppRB	phosphorylated retinoblastoma protein
PR	progesterone receptor
pRB	retinoblastoma protein
PSA	prostate specific antigen
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
R1881	methyltrienolone
RNA	ribonucleic acid
R(obs)	Pearson's correlation coefficient observed
ROS	Reactive oxygen species
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute

R(rand)	Pearson's correlation coefficient following randomization
S	amino acid serine
SA-β-Gal	senescence-associated beta-galactosidase
SDS	sodium dodecyl sulfate
SELDI-MS	surface-enhanced laser desorption/ionization-mass spectrometry
SEM	standard error of the mean
seq	sequencing
sh	short hairpin
Src	sarcoma tyrosine kinase
SUMO	small ubiquitin-like modifier
TAU	transcriptional activation units
TERT	telomerase reverse transcriptase
TGF	transforming growth factor
TH	thyroid hormone
TIMP	tissue inhibitor of metalloproteinase
TR	thyroid hormone receptor
UTR	untranslated region
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

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 vielzähliger zellulärer Prozesse, darunter für die Proliferation und zellulärer Seneszenz. Eine Fehlregulierung von ING1 konnten bereits mit PCa in Verbindung gebracht werden, jedoch ist die Rolle von ING1 für die AR-Signalgebung noch unbekannt. Basierend auf den gezeigten Interaktion 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 $p27^{KIP1}$ – das hier neu identifizierte Zielgen $p27^{KIP1}$. 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 Schlüsselzielgenen 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 $p16^{INK4a}$ 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 $p27^{KIP1}$ 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 $p16^{INK4a}$ 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 site 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).

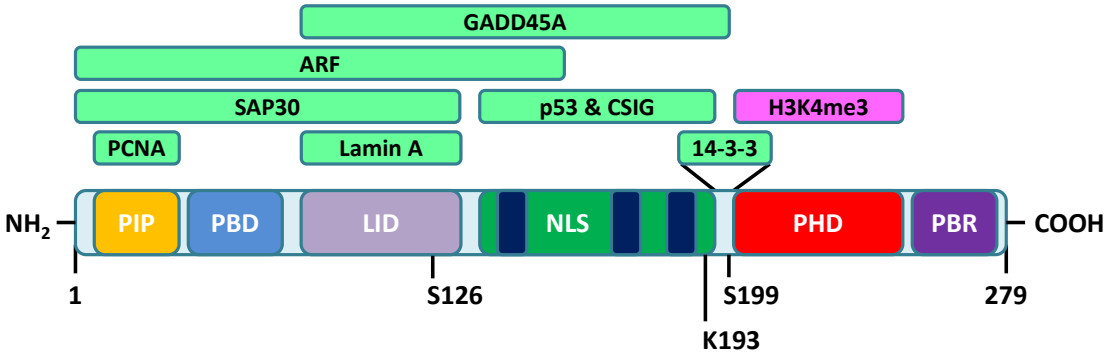


Figure 1. Human ING1b protein structure and its interacting partners. PIP: PCNA-interacting protein, PBD: Partial bromo domain (PBD), LID: lamin interaction domain, NLS: nuclear localization sequence in which nucleolar targeting sequences (NTS) were depicted in 3 dark blue boxes, PHD: plant homeodomain 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 finding suggests 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 $p19^{ARF}$-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 transavtivation 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), Dichlorofluorescin 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).

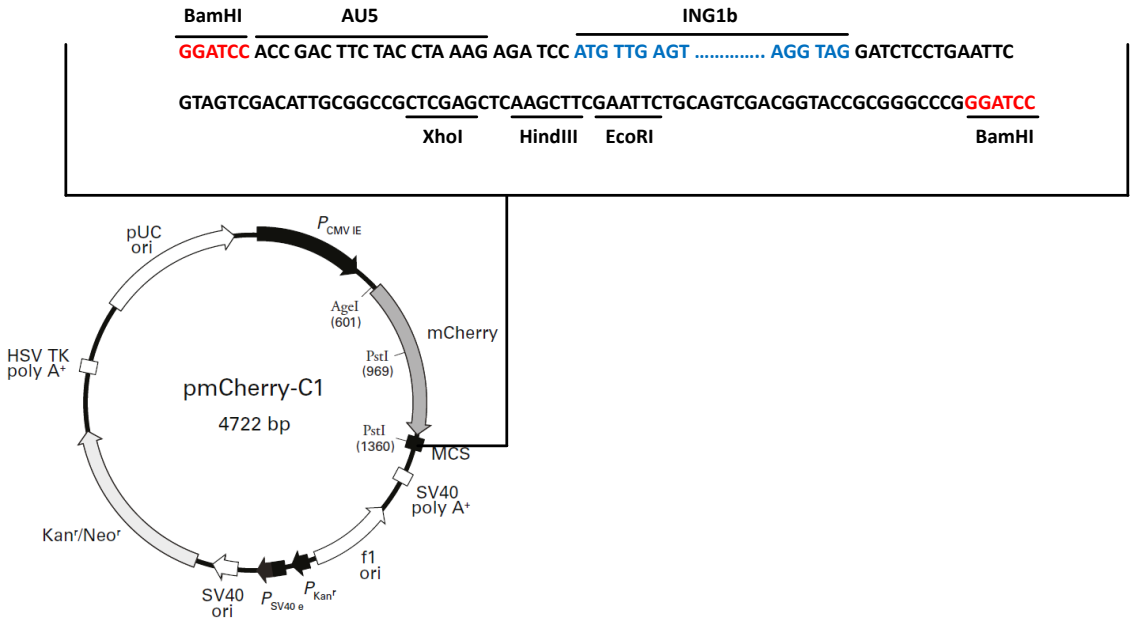


Figure 2. The schematic representation of pmCherry-ING1b construct.

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% CO_2 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 $CaPO_4$ 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 5×10^4 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 5×10^5 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 CO_2) 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 $\times 10^5$ 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 $CaPO_4$ 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 $CaPO_4$ 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 $\Delta\Delta C_t$ 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

Gene	Primer	Sequence 5' → 3'	Annealing temperature
AFP	fw	CCCGAACTTTCCAAGCCATAACTG	65°C
	rev	ATCCAGCACATCTCCTCTGCAAC	
AR	fw	TCAGCATTATTCCAGTGGATGGGC	60°C
	rev	TGGTAGAAGCGTCTTGAGCAGGAT	
BCL2	fw	CATGTGTGTGGAGAGCGTCAA	65°C
	rev	GCCGGTTCAGGTACTCAGTCA	
FKBP5	fw	GAGGAAACGCCGATGATTGGAGAC	65°C
	rev	CATGCCTTGATGACTTGGCCTTTG	
ING1b	fw	GGACTACCTGGACTCCAT	55°C
	rev	CGACTGAAGCGCTCGTA	
ING2a	fw	TGCGGGAGCTGGACAAC	60°C
	rev	TGGAGAAGCTGCTGTAGACG	
MMP9	fw	ACGACGTCTTCCAGTACCGA	55°C
	rev	TTGGTCCACCTGGTTCAACT	
MMP13	fw	CTTAGAGGTGACTGGCAAAC	55°C
	rev	TCAGAGGAGTTACATCGGAC	
NKX3.1	fw	CCGAGACGCTGGCAGAGACC	55°C
	rev	GCTTAGGGGTTTGGGGAAG	
$p14^{ARF}$	fw	CCTGGAGGCGGCGAGAAC	55°C
	rev	AGTAGCATCAGCACGAGGGC	
$p16^{INK4a}$	fw	CTTGCCTGGAAAGATACCG	55°C
	rev	CCCTCCTCTTTCTTCCTCC	
$p21^{CIP1/WAF1}$	fw	TCGACTTTGTCACCGAGACACCAC	55°C
	rev	CAGGTCCACATGGTCTTCCTCTG	
$p27^{KIP1}$	fw	GGCCTCAGAAGACGTCAAAC	55°C
	rev	ACAGGATGTCCATTCCATGA	
PSA	fw	GAGGCTGGGAGTGCGAGAAG	60°C
	rev	TTGTTCCTGATGCAGTGGGC	

RPL13a	fw rev	GTATGCTGCCCCACAAAACC TGTAGGCTTCAGACGCACGAC	60°C
TERT	fw rev	CGGAAGAGTGTCTGGAGCAA GGATGAAGCGGAGTCTGGA	60°C
TIMP1	fw rev	AAGGCTCTGAAAAGGGCTTC GAAAGATGGGAGTGGGAACA	60°C
TIMP2	fw rev	CCAAGCAGGAGTTTCTCGAC GACCCATGGGATGAGTGTTT	55°C
TMPRSS2	fw rev	CCTGCAAGGACATGGGCTATA CCGGCACTTGTGTTCAGTTTC	60°C

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

Gene	Primer	Sequence 5' → 3'	Annealing temperature
Adh1	fw rev	ACCCATCAATTTCCTGCCTGC TGCGACTTCTATGTCCTCGATG	55°C
Afp	fw rev	GGCGATGGGTGTTTAGAAAG TCTTTCCACTCCACTTTGGC	55°C
Fkbp5	fw rev	CTCAAACCCAAACGAAGGAG CATCTTCACCAGGGCTTTGT	60°C
Gstp1	fw rev	ACCATACACCATTGTCTACTTCCC GGTAAAGGGTGAGGTCTCCAT	55°C
Gus	fw rev	TGCCTGTCCCTTCTAGCTTC TGTTCCACCACATGAATCCCATTC	60°C
Hprt	fw rev	GGCCAGACTTTGTTGGATTT CAGATTCAACTTGCGCTCAT	55°C
Hsd3b6	fw rev	GGACCAGCTGGGATACAGAA ACAGTGACCCTGGAGATGGT	55°C
Ing2a	fw rev	GGGAGCTGGACAACAAATAC GGCTATTGATTAACGCTCTCTGG	60°C
Kap	fw rev	AGACAGTCTCCTCCGGCTTT ATATCCTGAATGGCAGTCGC	55°C
Nkx3.1	fw rev	AGGGAACACTCCAATTCTTCTCTGG GGCTCTCTCTAAACAGGGGAGCGG	55°C
p16^{Ink4a}	fw rev	CCGCTGCAGACAGACTGG CCATCATCATCACCTGAATCG	60°C

p19^{Arf}	fw rev	TTCTTGGTGAAGTTCGTGCGATCC ACGTGAACGTTGCCCATCATCATC	60°C
p21$^{Cip1/Waf1}$	fw rev	GACCTGGGAGGGGACAAGAG TTCTCTTGCAGAAGACCAATC	60°C
p27^{Kip1}	fw rev	CTGCTCCATTTGACTGTCTGTGTGC CGTTTGACATCTTCCTCCTCGGG	60°C
Pbsn	fw rev	GGTCATCATCCTCCTGCTCA AGCTAAGTAAATTGTTTGCCAAGG	55°C
Rhox5	fw rev	GGAGCAGGAACAAAATGAGC TGGACTCCAGTTCCCTCAGT	55°C
Rpl13a	fw rev	CTGAAGCCTACCAGAAAGTTTGC CGTCCTGTTTTCCGTAACCTCAAG	60°C
Sox9	fw rev	GGTTTCAGATGCAGTGAGGAGC CACATACAGTCCAGGCAGACC	60°C
Tnf-α	fw rev	CGATGGGTTGTACCTTGTCTACTC CTCCTGGTATGAGATAGCAAATCGG	55°C

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 Na_3VO_4, 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 glycin, 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^{TM} 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

Target	Source	Company, Ref. no.	Dilution*	Protein size in kDa
Actin	mouse	Abcam, ab6276	1:10000	42
AR	mouse	Biogenex, 256M	1:2000	110
ING1	mouse	Upstate, 05-720	1:2000	47, 33, 27, 24
ING1b	mouse	BD Biosciences, 550455	1:2000	33
ING2a	rabbit	Proteintech, 11560-1-AP	1:1000	33
p16^{INK4a}	mouse	Santa Cruz, sc-81613	1:500	16
p21$^{CIP1/WAF1}$	mouse	Cell Signaling, 2946	1:2000	21
p27^{KIP1}	mouse	Santa Cruz, sc-1641	1:2000	27
PARP	mouse	Cell Signaling, 9546	1:2000	Uncleaved: 116 Cleaved: 89
pRb	rabbit	Abcam, ab6075	1:500	105
ppRb (Ser780)	rabbit	Cell Signaling, 9308	1:2000	110
Tubulin	rabbit	Abcam, ab15246	1:2000	50
Rabbit IgG HRP§	Bovine	Santa Cruz, sc-2370	1:10000	
Mouse IgG HRP§	Goat	Santa Cruz, sc-2005	1:10000	

(*) 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 *ING1* mRNA is mainly lost or decreased in human tumors (Walzak et al. 2008, Guerillon et al. 2013). To assess the expression of *ING1* in PCa, Oncomine database was used to analyze the published microarray gene expression studies that have reported significant alteration of *ING1* mRNA level. Of those, two studies have shown downregulation of *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 ING1 in PCa, the Human Protein Atlas portal was utilized for this purpose. Interestingly, ING1 protein failed to be detected in more than 30% of the PCa specimens compared to the low level of ING1 in normal prostate tissue (Figure 3C). Similar to the data obtained for *ING1* mRNA level, there were still less than 10% of the PCa samples that displayed mild upregulation of ING1 protein. This controversial finding may be due to inverse relationships among ING1 variants (Soliman et al. 2008, Coles et al. 2007, Zhu et al. 2009). For that reason, the protein expression of major isoform 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 ING1b was detected in CRPCa cells compared to androgen-dependent ones (Figure 3D). Taken together, these data suggest that ING1 expression tends to be downregulated in PCa.

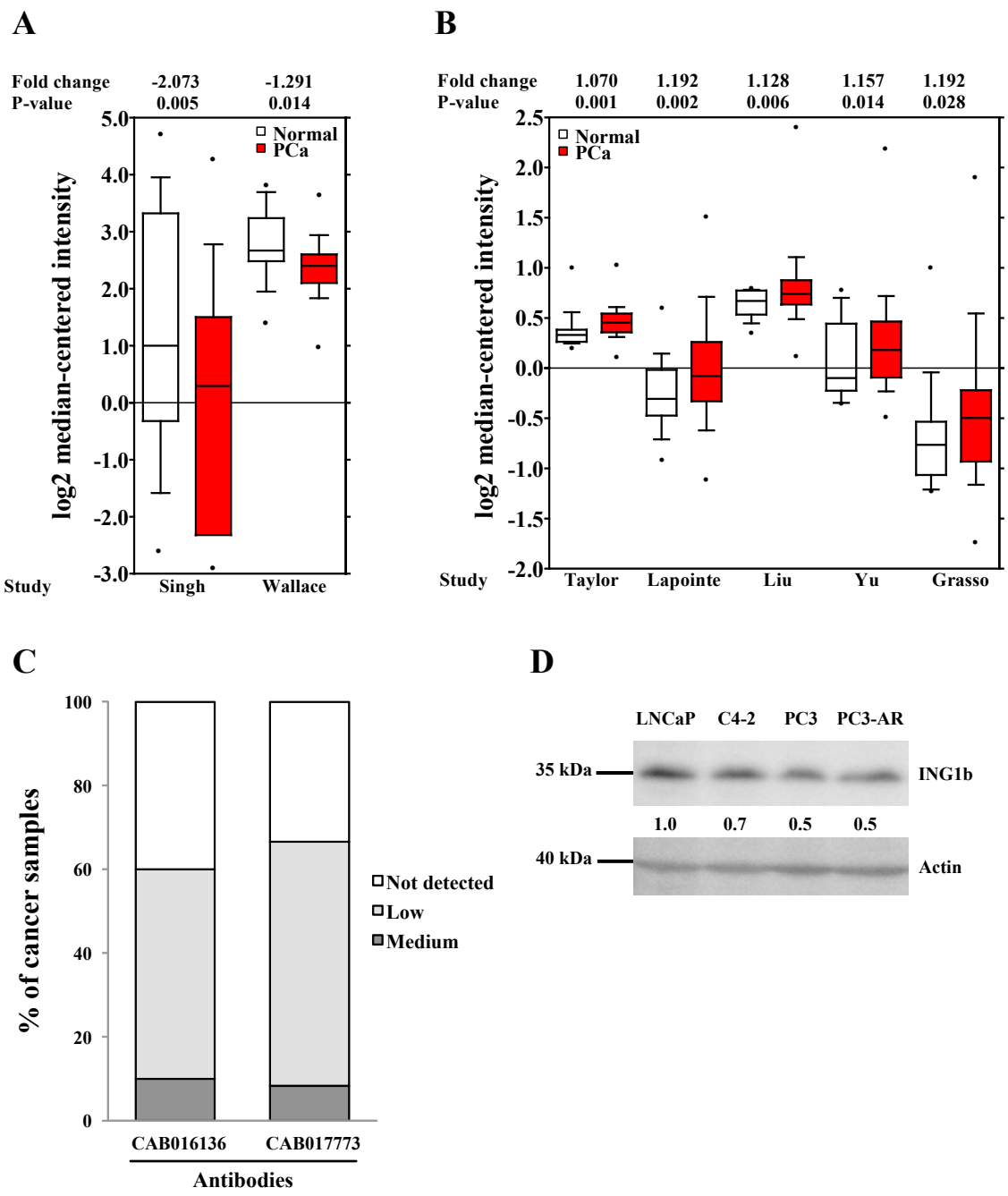


Figure 3. ING1 expression is regulated in PCa. Oncomine database analysis of *ING1* mRNA expression was performed in 7 independent datasets represented by the name of first author. (A) The studies have shown downregulation of *ING1* in PCa compared to normal prostate gland. (B) The reports have indicated 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.

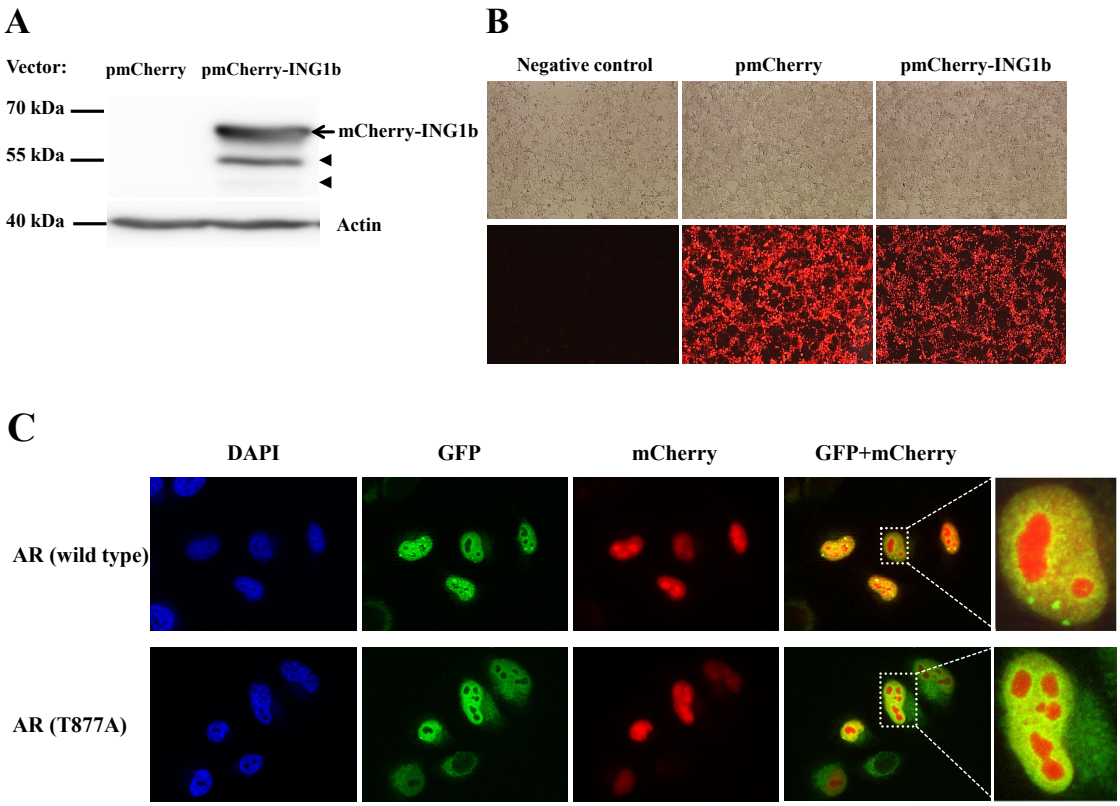


Figure 4. Intracellular colocalization of ING1b with AR. (A) Western blot analysis for the Phoenix-Eco cells transiently transfected with either empty vector (pmCherry) or pmCherry-ING1b. The tagged protein (545 amino acids) was detected by ING1 specific antibody mainly around 60 kDa. The smaller fragments indicated by arrowheads might come from internalized transcription start sites or degradation. β-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.

AR	Manders' coefficients[a]		R(obs)[b]	R(rand)[c] (mean±SD)	p-value[d]
	M1	M2			
Wild type	1.000	0.918	0.694	0.033±0.001	1.000
Mutant (T877A)	0.524	0.791	0.679	-0.002±0.001	1.000

(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.

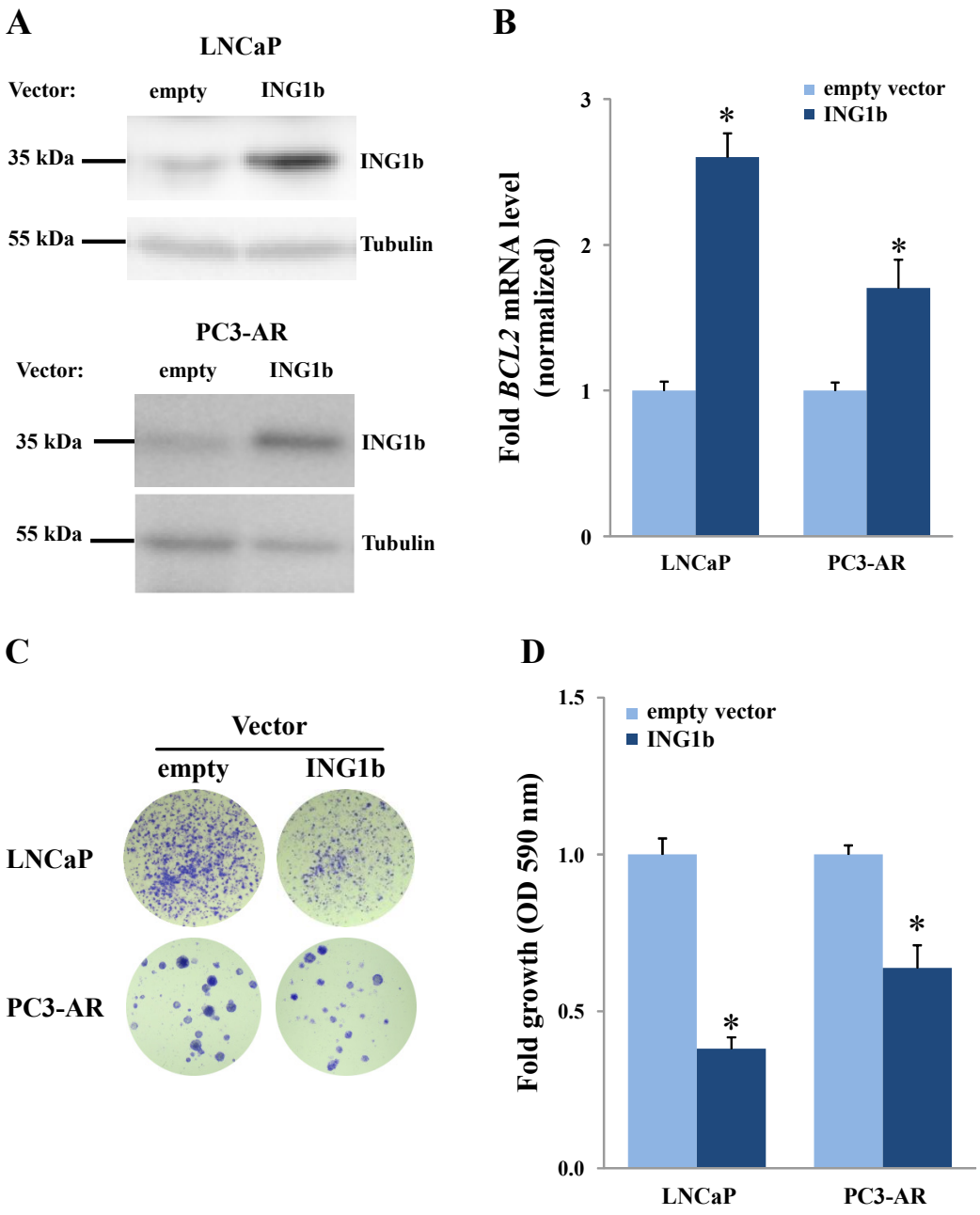


Figure 5. Stable expression of the ING1b inhibits growth of PCa cell line. (A) Western blot analysis of the ING1b protein and (B) qRT-PCR analysis of ING1b target gene *BCL2* in LNCaP and PC3-AR cells transduced with either empty vector or vector expressing ING1b. (C) Crystal violet staining of the cells 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 $p< 0.05$).

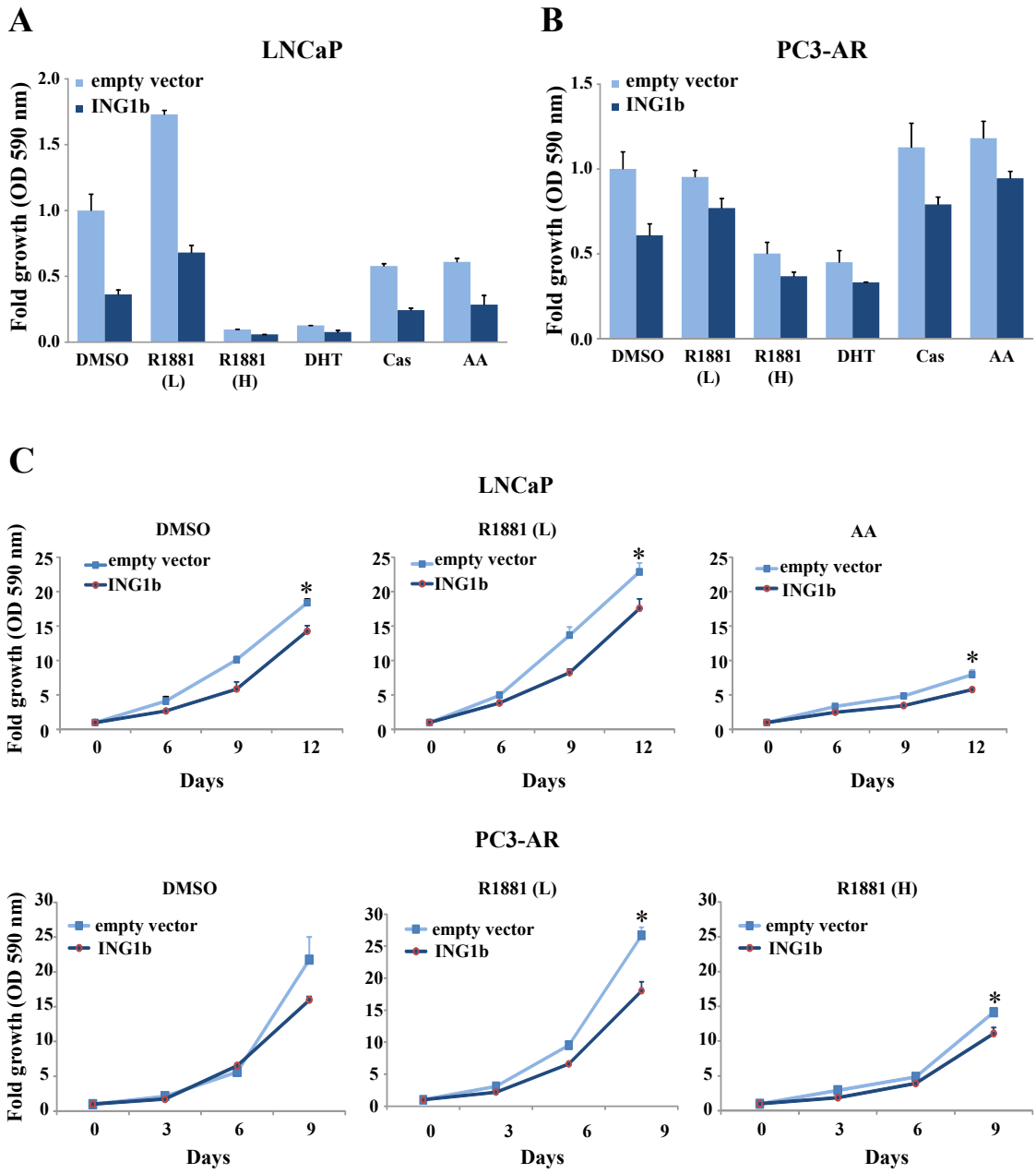
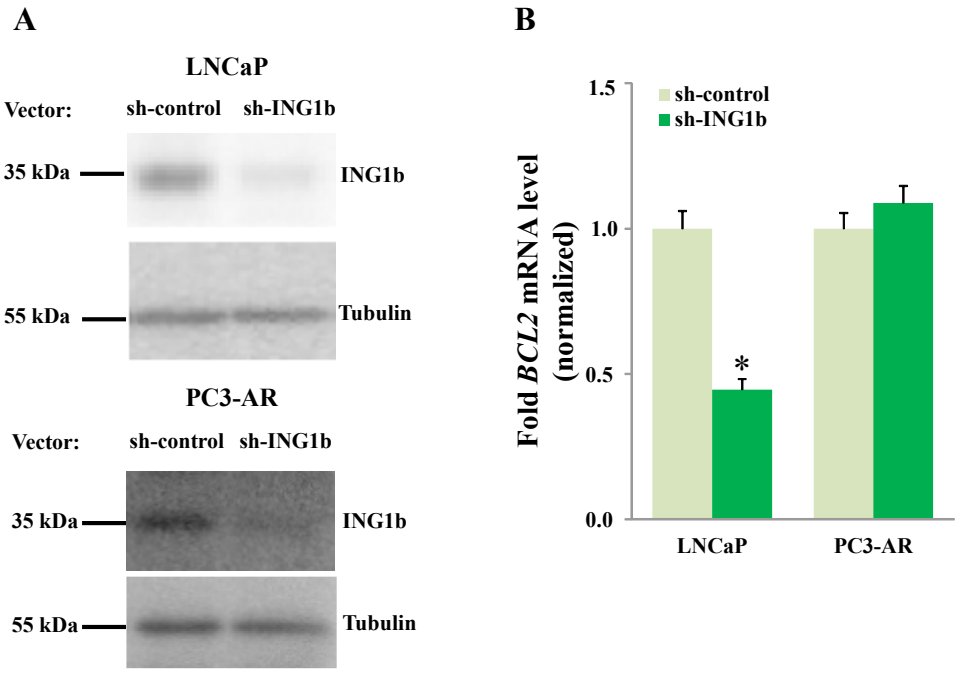


Figure 6. Different time points of stable ING1b expression inhibit growth in PCa cells. Growth assays were performed for (A) LNCaP and (B) PC3-AR cells similar to figure 5C, D under everyday antibiotic selection in the presence of different ligands: solvent control DMSO, R1881 (L) (low concentration: 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.



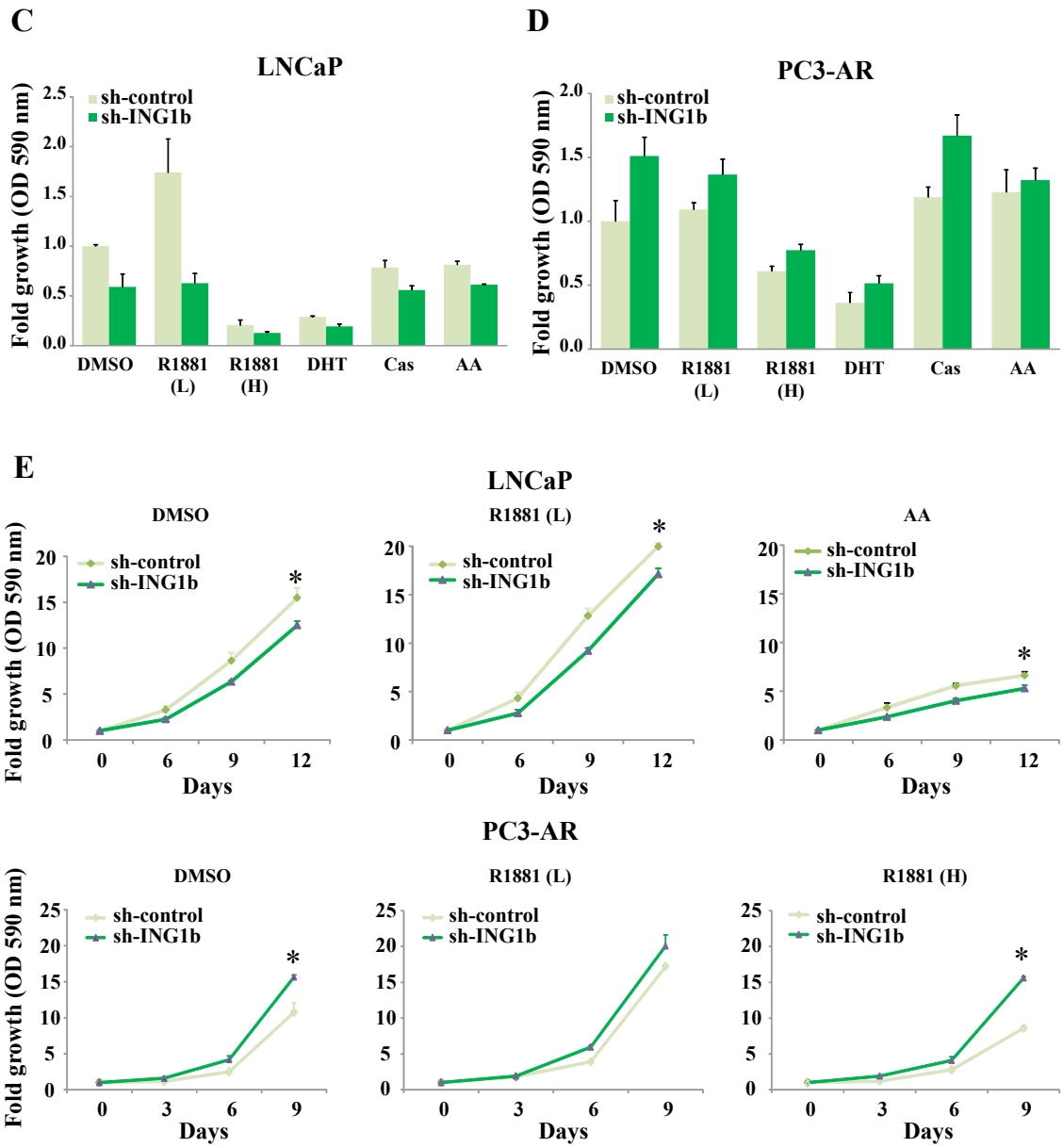


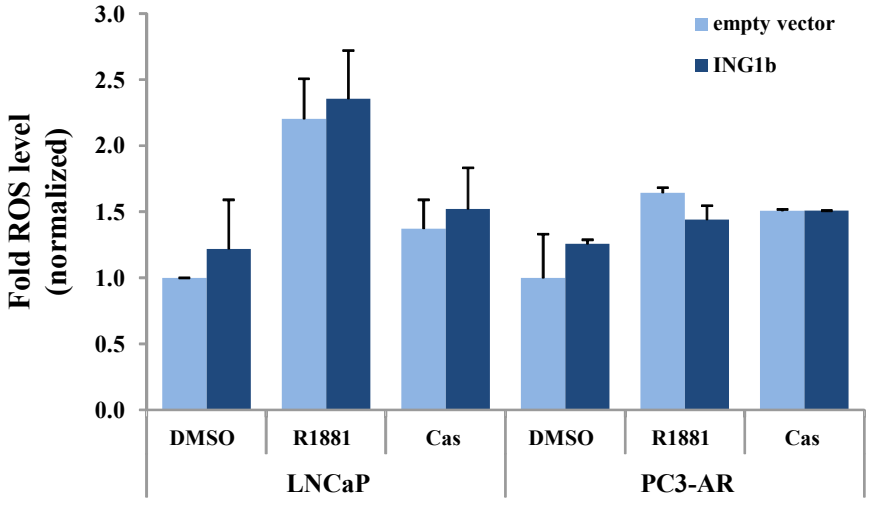
Figure 7. Stable knockdown of ING1b has opposite effects in growth behavior of PCa cell lines. (A) Western blot analysis of the ING1b protein in LNCaP and PC3-AR cells transduced with vector expressing short hairpin RNA either against firefly luciferase (sh-control) or against ING1b (sh-ING1b) (B) qRT-PCR 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.

A



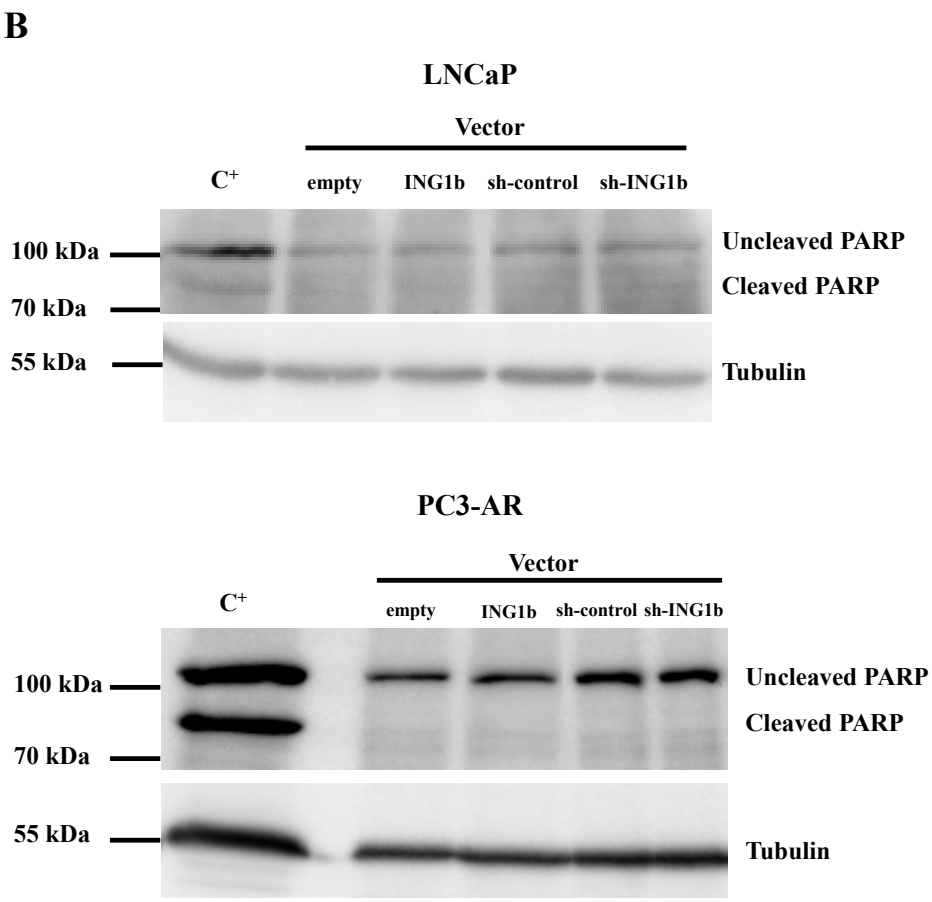


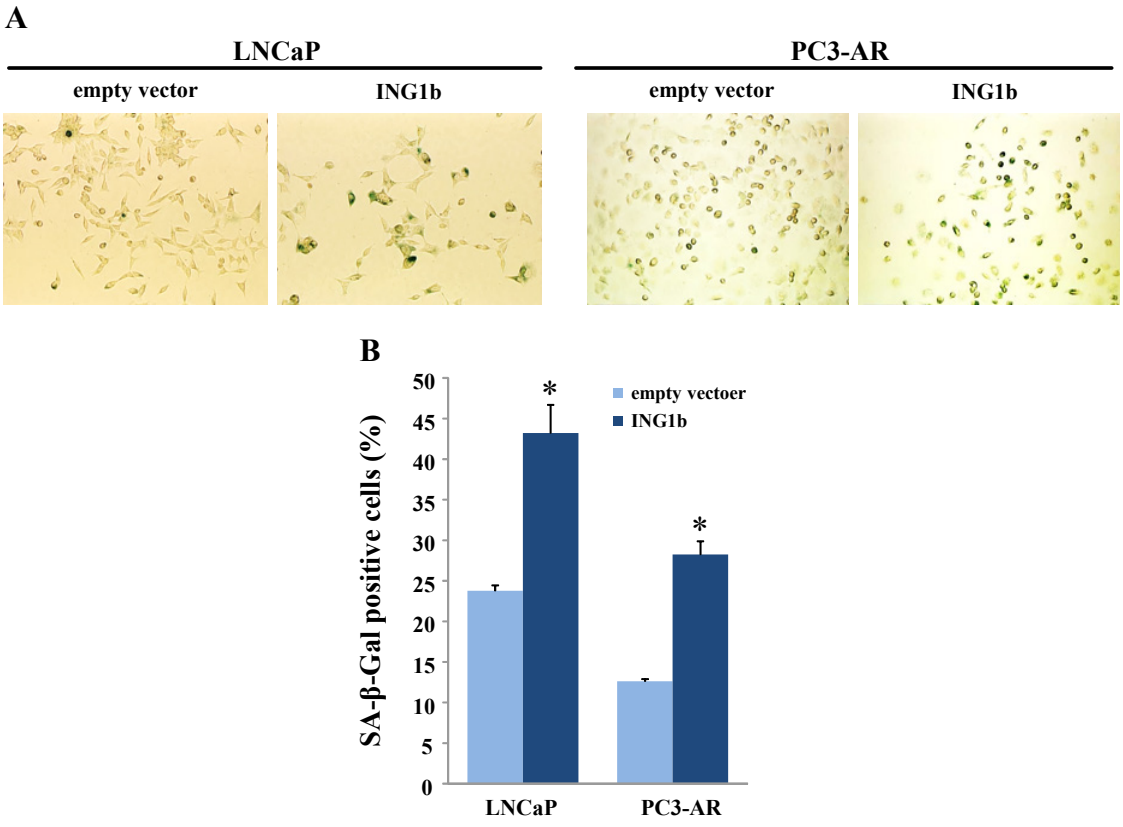
Figure 8. ROS and apoptosis are not induced by ING1b in PCa cells. (A) The stably transduced cells were treated 72h with the indicated ligands: DMSO, R1881 (1 nM) or Casodex (1 μM for LNCaP and 0.1 μM for PC3-AR). Subsequently, ROS levels were detected by DCF assay and the values obtained were normalized 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 (Schwartze 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.



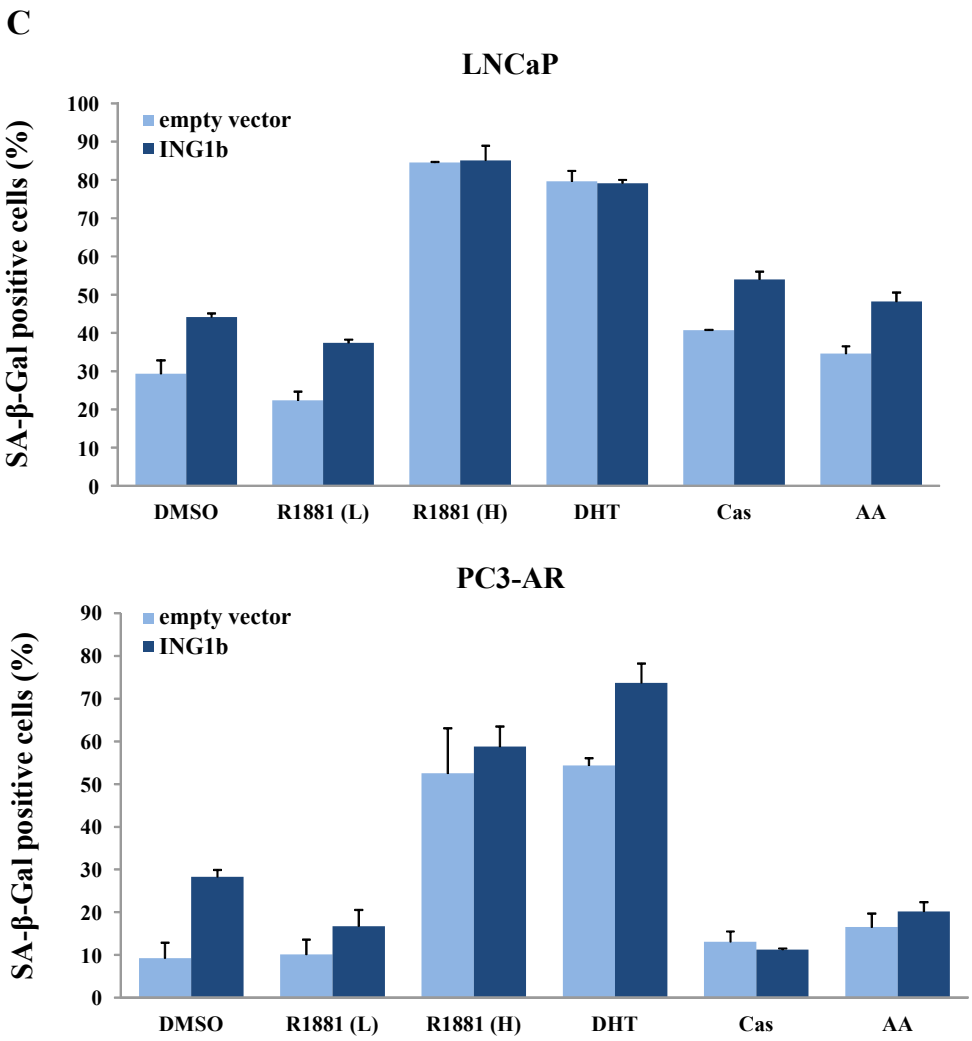


Figure 9. ING1b drives cellular senescence in PCa cells. (A) LNCaP and PC3-AR cells were transduced by indicated vectors and selected by puromycin for 15 days. Then they were stained 2 days for SA-β-Gal and visualized under brightfield microscope (100x magnification). (B) Percentage of the SA-β-Gal positive 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.

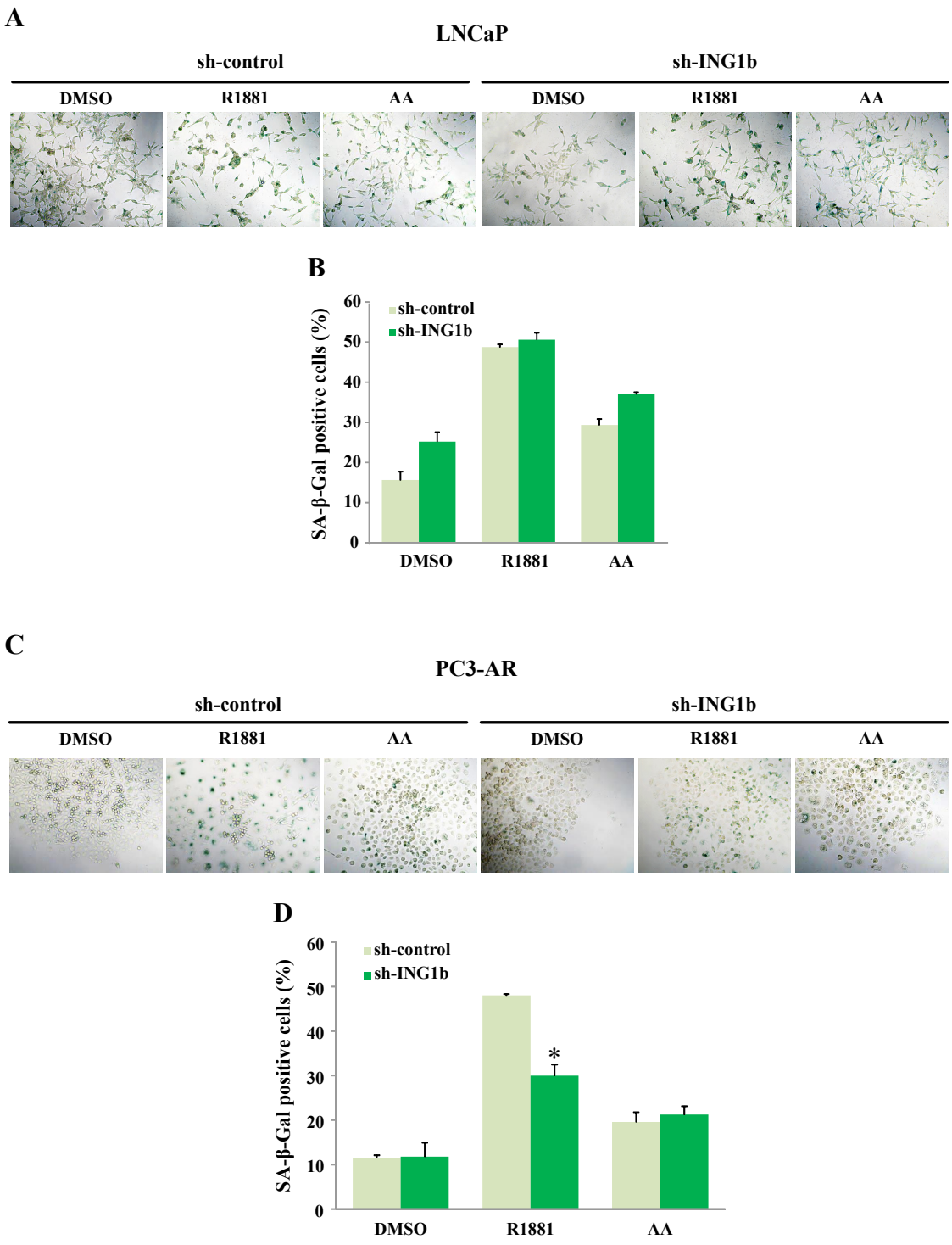


Figure 10. ING1b KD exhibits distinct effect in androgen-induced cellular senescence for LNCaP and PC3-AR cells. (A) & (C) The representative micrographs from stably transduced LNCaP or PC3-AR cells treated with DMSO or R1881 (1 nM) or AA (30 μM) for 3 days and stained for SA-β-Gal. (B) & (D) The cells 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 $p< 0.05$).

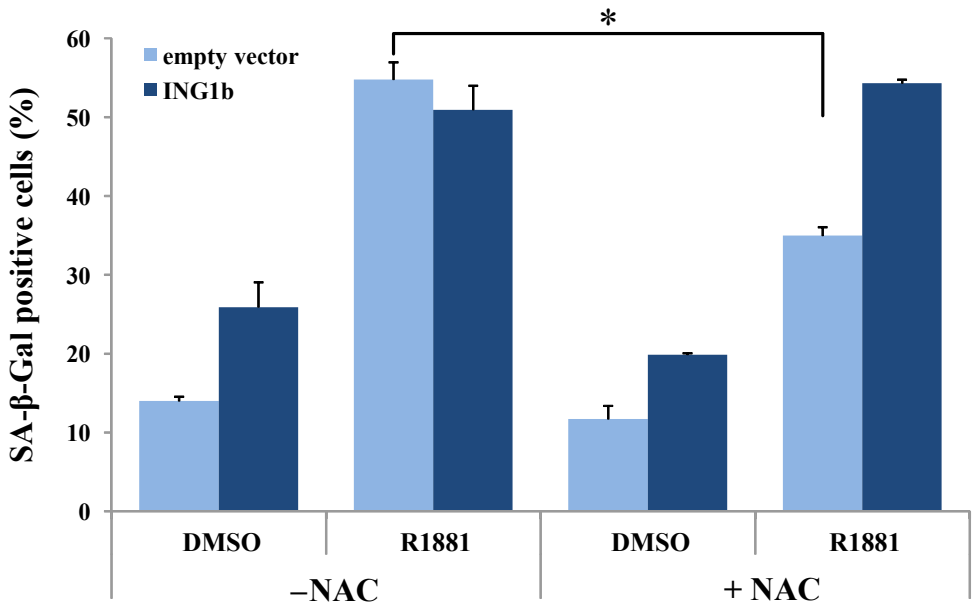


Figure 11. NAC cannot reduce the androgen-induced cellular senescence in LNCaP-ING1b cells. Stably transduced LNCaP cells were seeded on the 6-well plates. Next day, they were treated with either DMSO or R1881 (1 nM) in the presence or absence of NAC (1 mM). Three days after treatment SA-β-Gal staining 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.

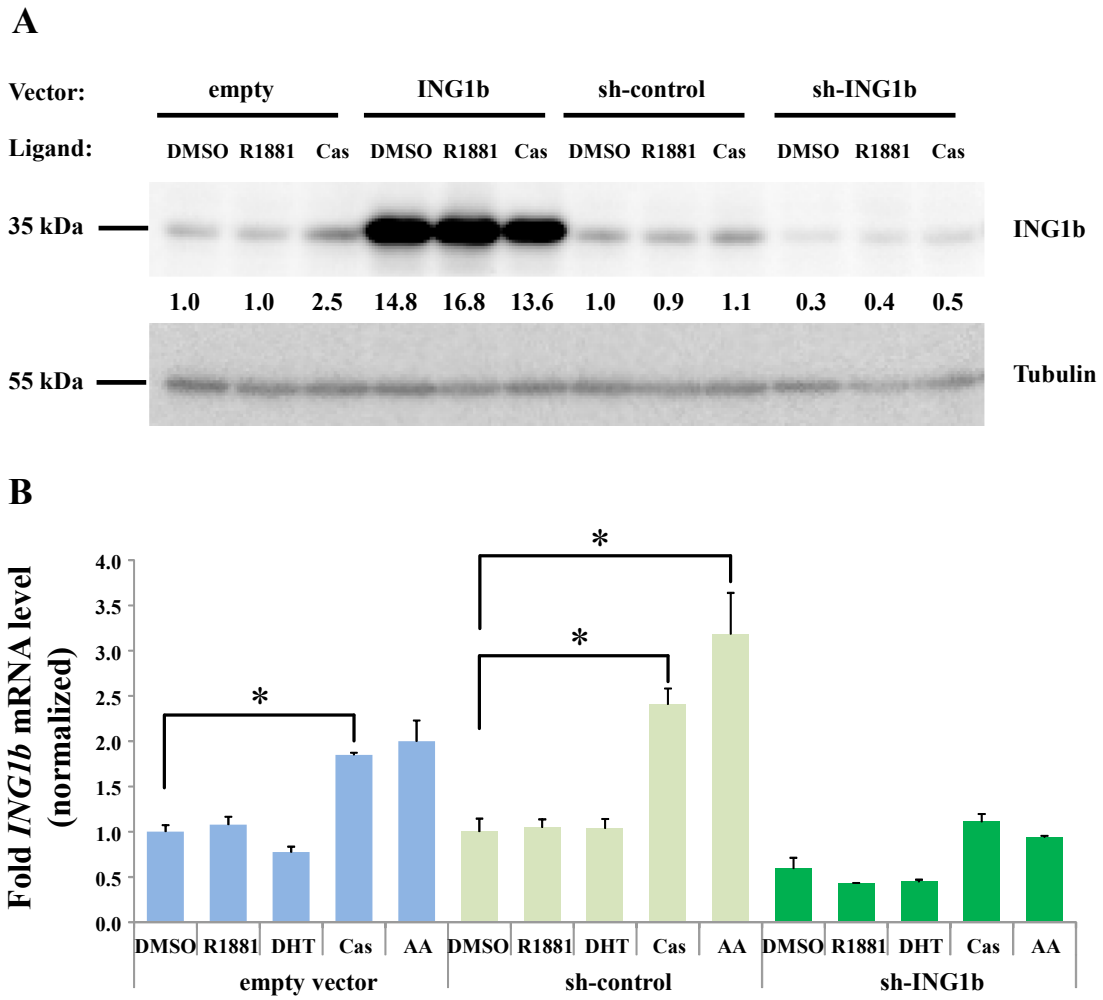


Figure 12. AR antagonists induce ING1b expression in LNCaP cells. (A) Western blot result of ING1b for the stably transduced LNCaP cells with the indicated vectors after 3 days treatment with DMSO, R1881 (1 nM) or Casodex (1 μM). The protein expression of ING1b was normalized to the loading control α-Tubulin using LabImage 1D quantification software. (B) qRT-PCR analysis of *ING1b* for the cells explained in (A) after two days treatment with indicated ligands: DMSO, R1881 (1 nM), DHT (10 nM), Casodex (1 μM) or AA (30 μM) in the depleted condition. The normalized expression level of DMSO for empty vector or sh-control cells were set arbitrarily at 1. The experiments were repeated twice. Error bars indicate SEM (* indicates $p < 0.05$).

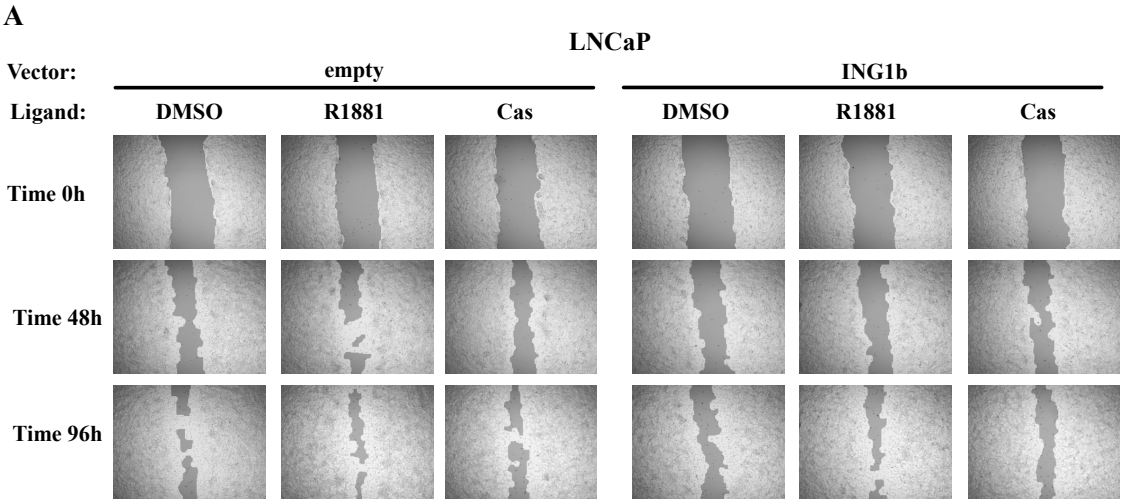
4.8 ING1b reduces cell migration and regulates migration-related genes in PCa cells

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.



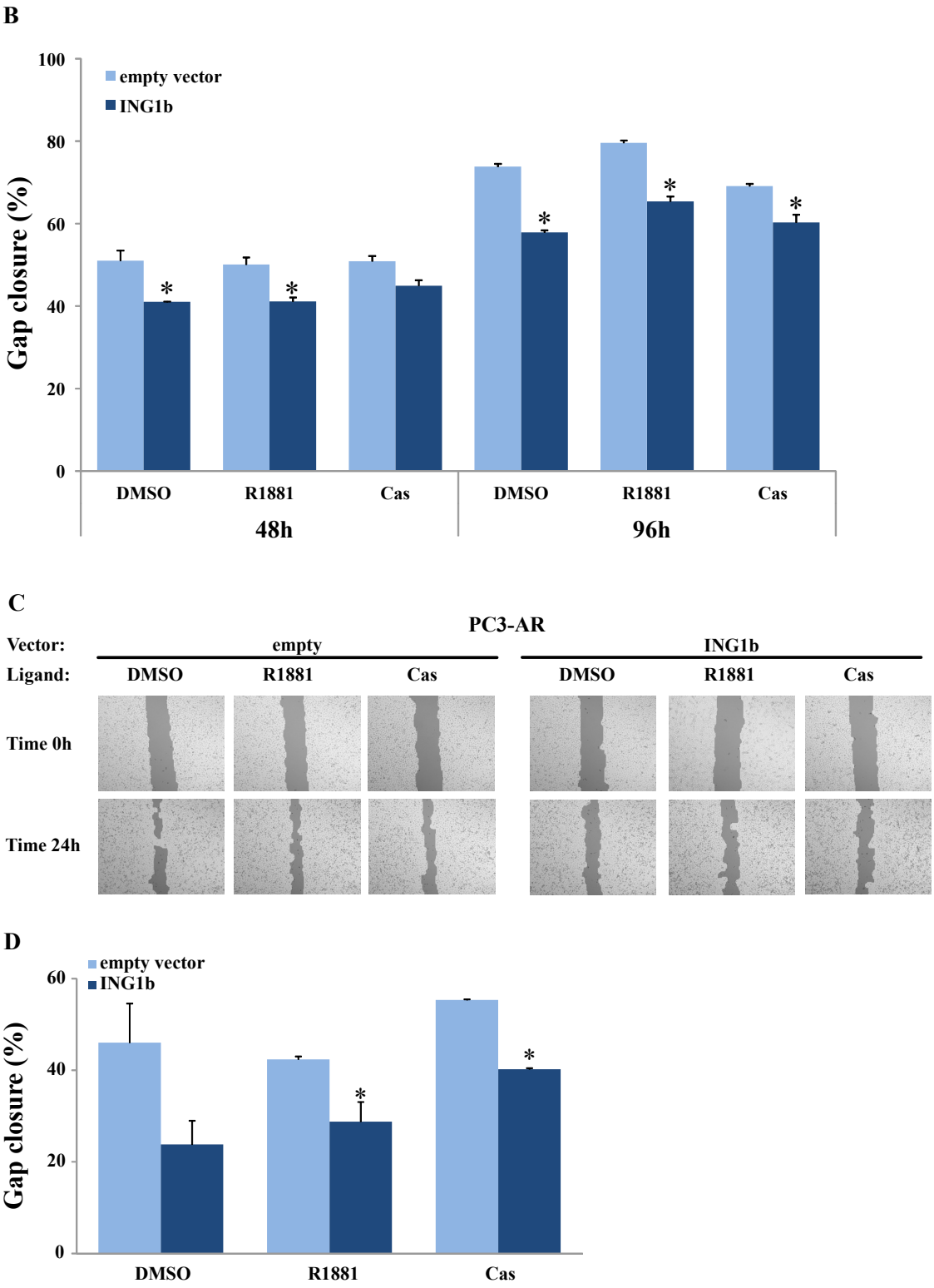
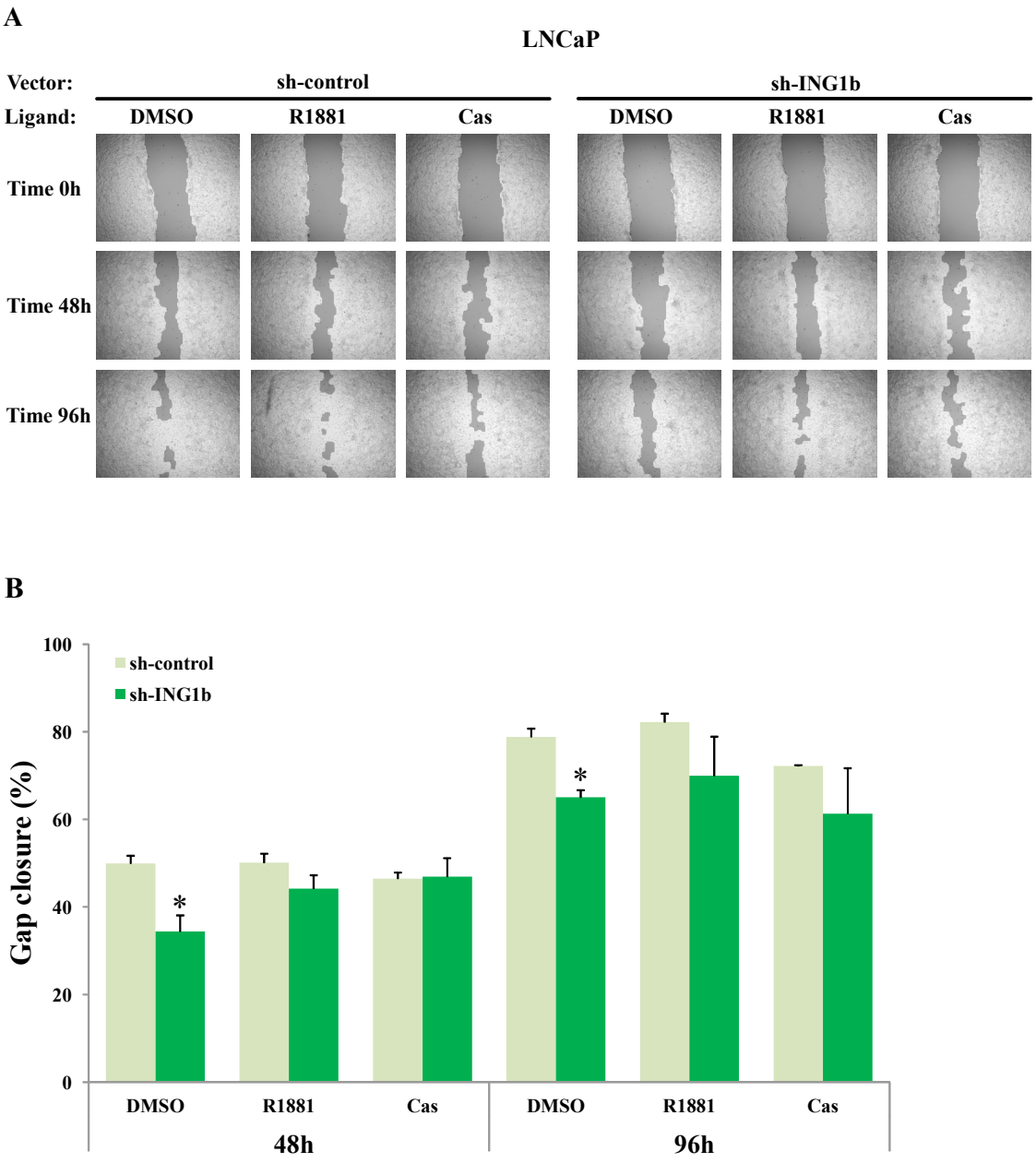


Figure 13. ING1b reduces cell migration in PCa cells. (A) Scratch assays of the stably transduced LNCaP cells under different ligand treatment: DMSO, R1881 (10 pM) or Casodex (0.1 μM). The same scratched regions were captured under the brightfield microscope at indicated time points. (B) Quatification 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) Quatification of the scratch assays for PC3-AR cells. The experiments were repeated twice. Error bars indicate SEM (* represents $p < 0.05$).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LNCaP
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DMSO
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Gap closure (%)
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60
40
20
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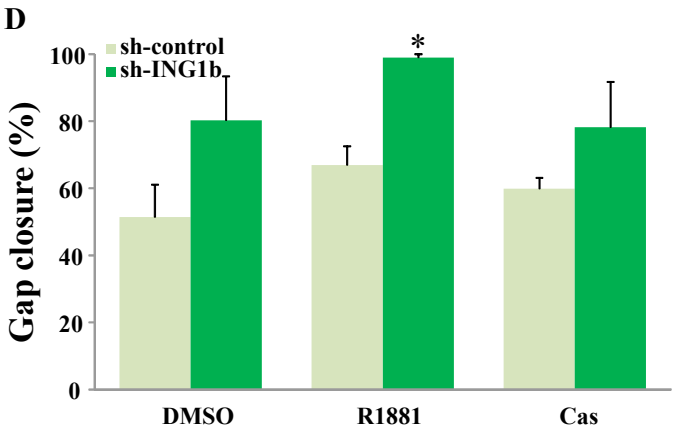


Figure 14. ING1b KD results in distinct cell migration response in LNCaP and PC3-AR cells. Scratch assays after stable knockdown of ING1b in LNCaP (A, B) and in PC3-AR (C, D) cells. The experimental setting was similar to that described in Figure 13.

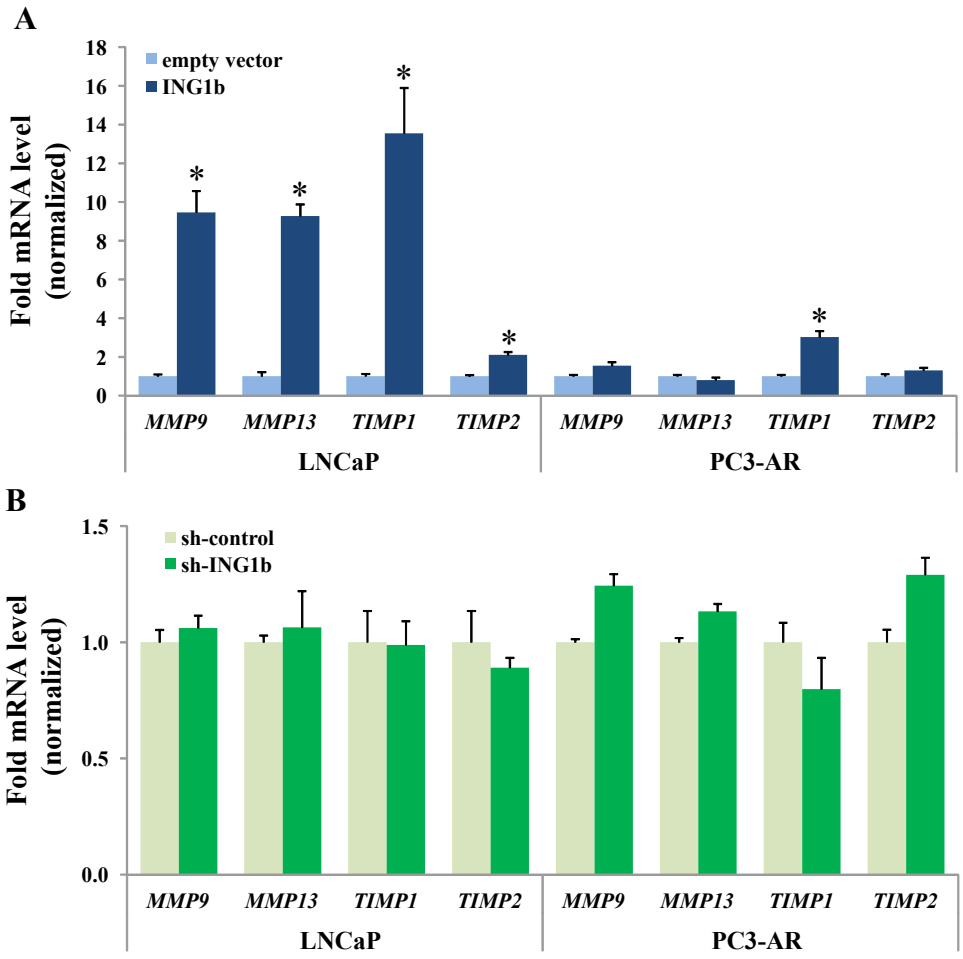
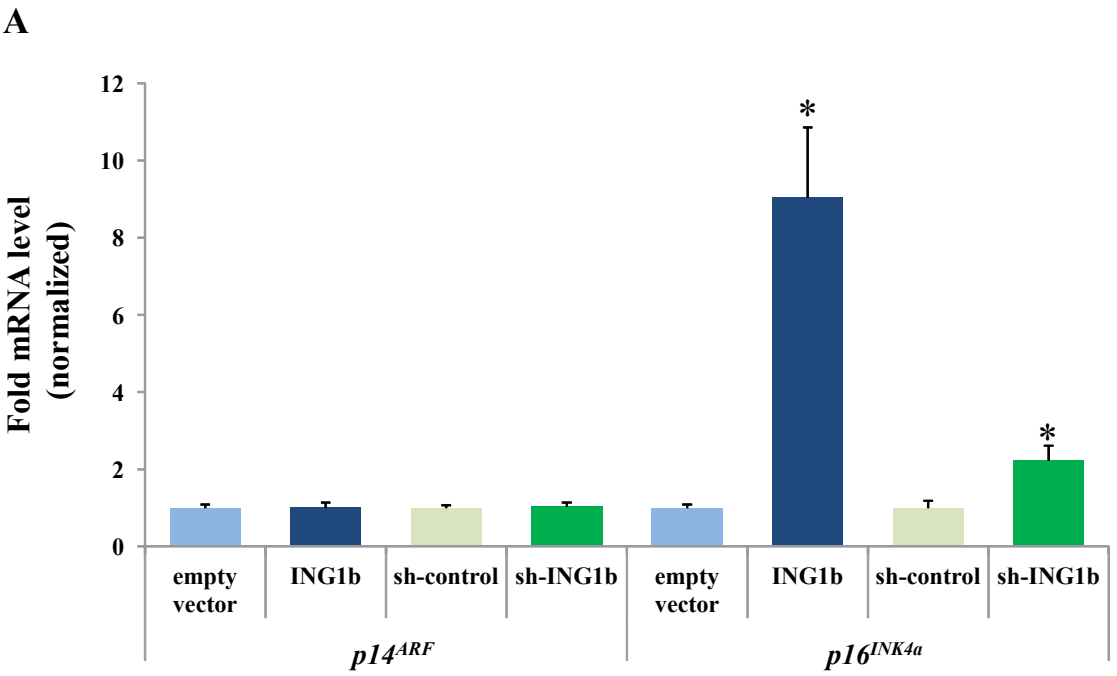


Figure 15. ING1b regulates migration-related genes. qRT-PCR analysis of the indicated migration- and invasion-related genes after overexpression (A) or knockdown (B) of ING1b in LNCaP and PC3-AR cells. The experiments were repeated twice. Error bars show SEM (* represents $p < 0.05$).

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 $p14^{ARF}$, $p16^{INK4a}$, $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ 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.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Fold mRNA level (normalized)
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10
8
6
4
2
0
*
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empty vector
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sh-control
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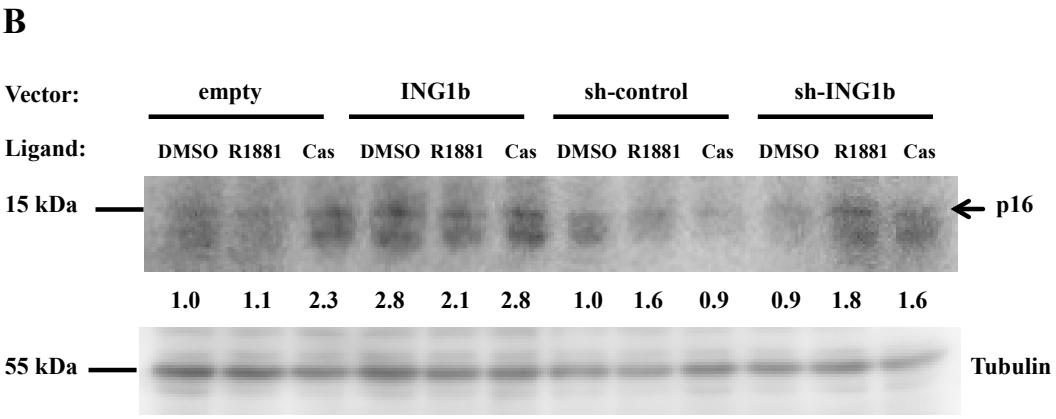
p14ARF
p16INK4a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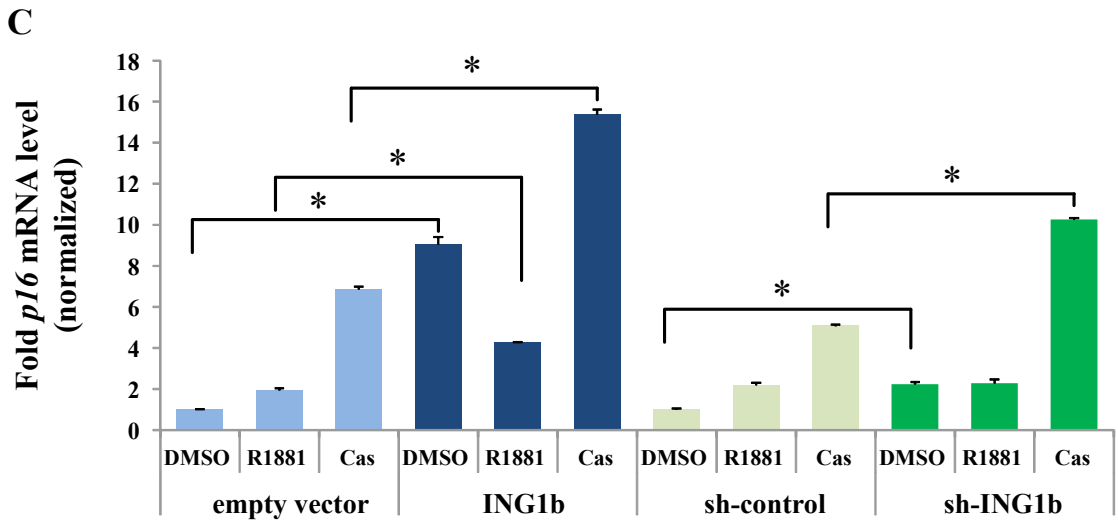
15 kDa
p16
1.0 1.1 2.3 2.8 2.1 2.8 1.0 1.6 0.9 0.9 1.8 1.6
55 kDa
Tubulin


C
Fold p16 mRNA level (normalized)
18
16
14
12
10
8
6
4
2
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empty vector
ING1b
sh-control
sh-ING1b

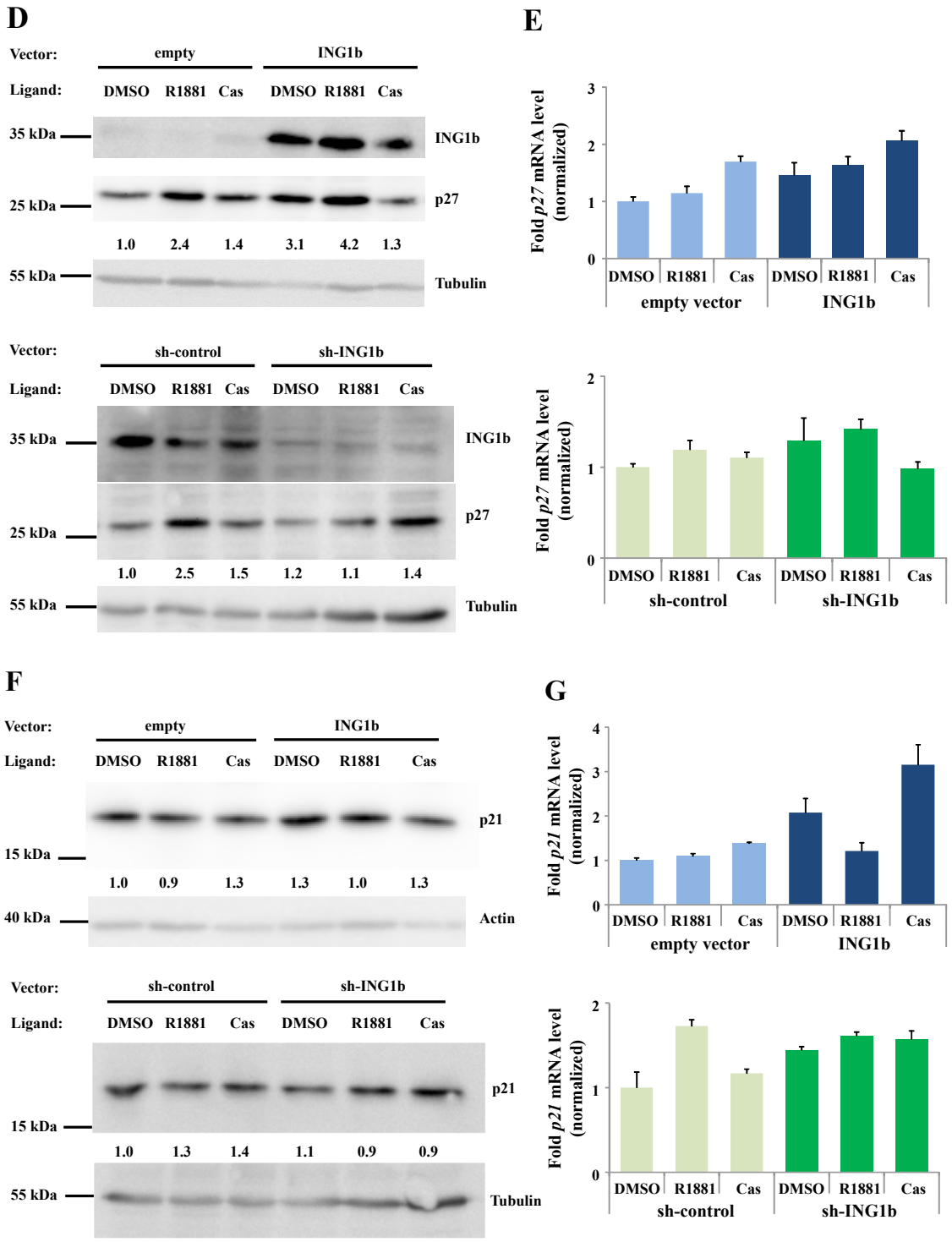


Figure 16. ING1b upregulates p16 and stabilizes p27 in LNCaP cell line. (A) qRT-PCR analysis of LNCaP cells transduced with the indicated vectors. 10 days after antibiotic selection of the transduced cells, RNA was isolated and qRT-PCR analysis was performed using target and housekeeping genes specific 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 $p < 0.05$).

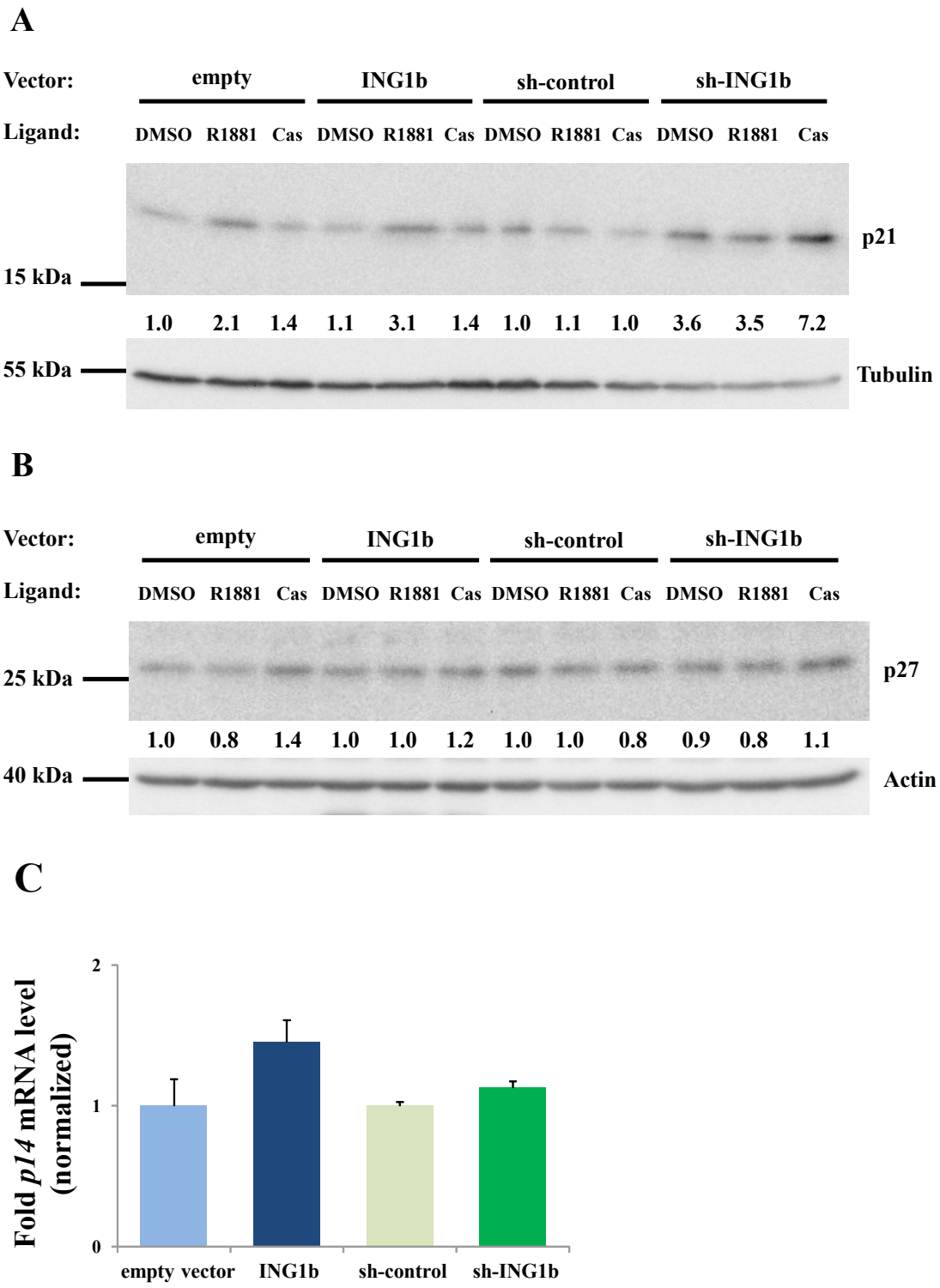
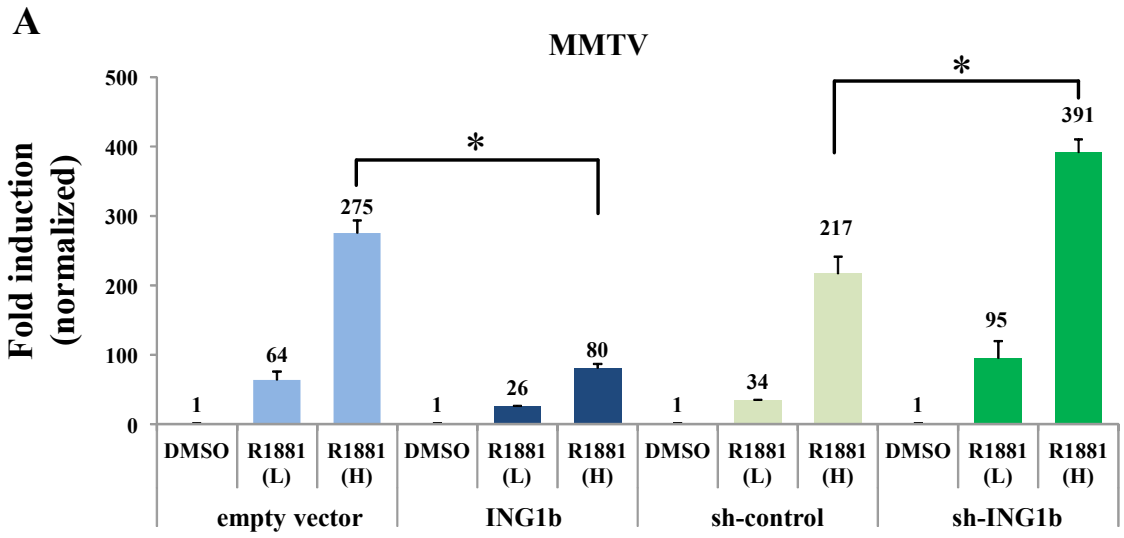


Figure 17. ING1b regulates p21 in PC3-AR cells. (A) Western blot results of p21 in PC3-AR cells stably transduced with the indicated vectors and treated with DMSO, R1881 (1 nM) or Casodex (0.1 μM). (B) Western blot result of p27 for the cells described in (A). (C) qRT-PCR analysis was performed using 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 Probain-luc promoter sequence is specific for AR (Snoek et al. 1996). These results support the ING1b role in regulating AR transactivation in LNCaP cells.



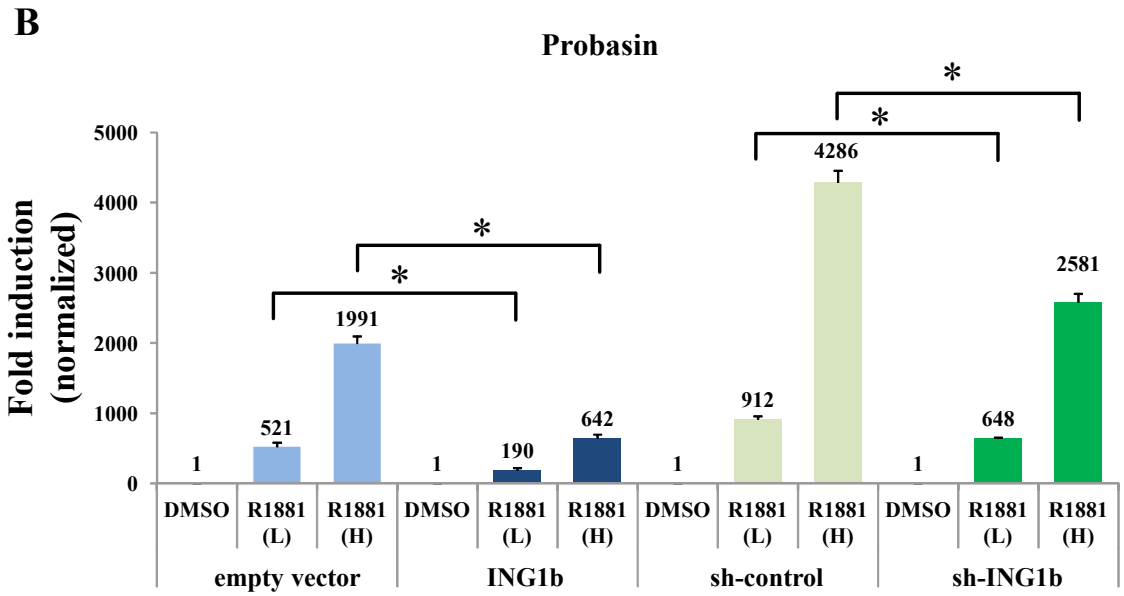


Figure 18. ING1b inhibits AR-mediated transactivation in LNCaP cells. Reporter gene assays were performed for the stable cells LNCaP-ING1b, LNCaP-ING1b KD and their corresponding control cells. The cells were seeded out in 10% CSS medium and transiently transfected with 2 µg reporter construct (A) MMTV-luc 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.

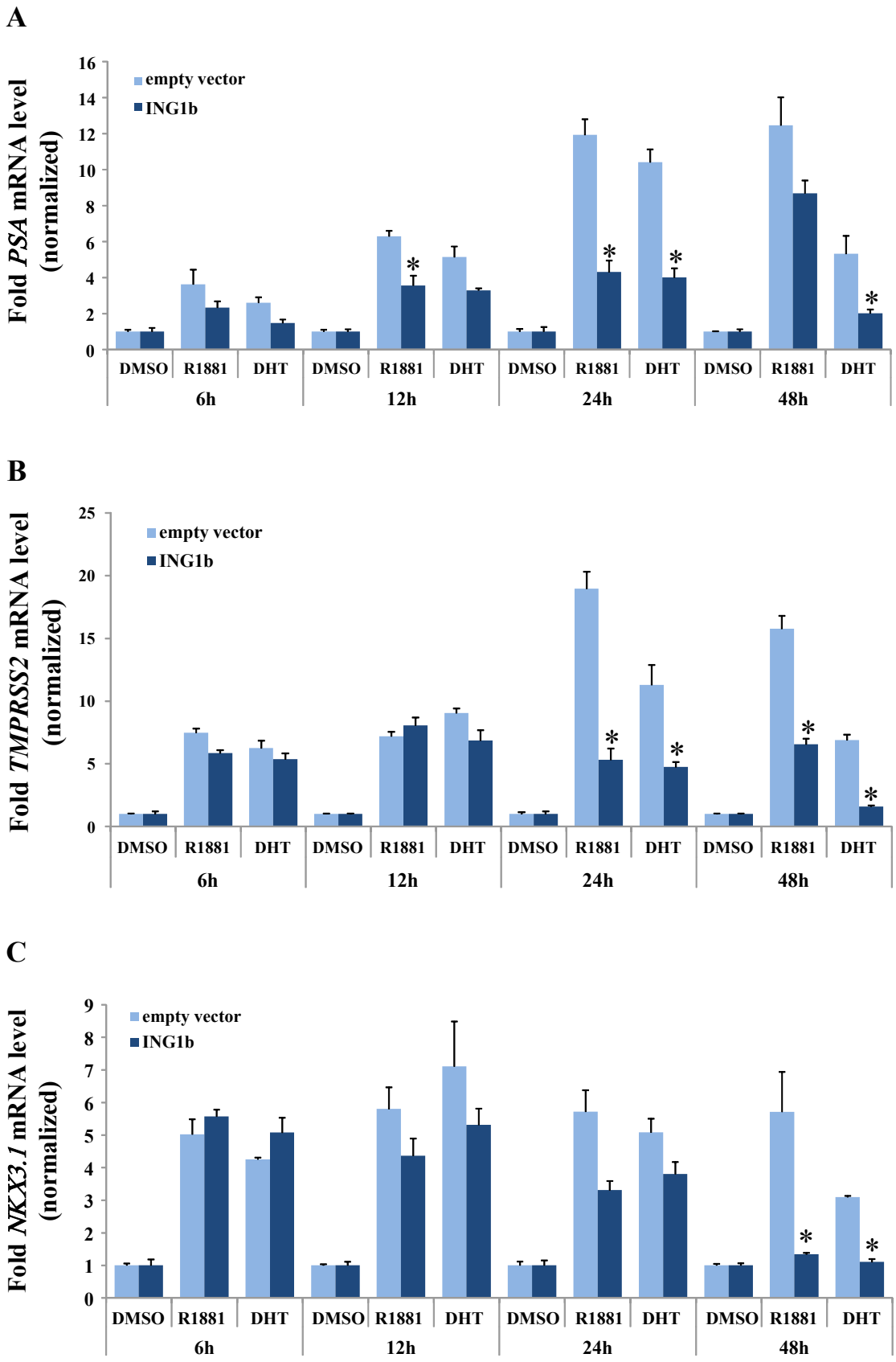


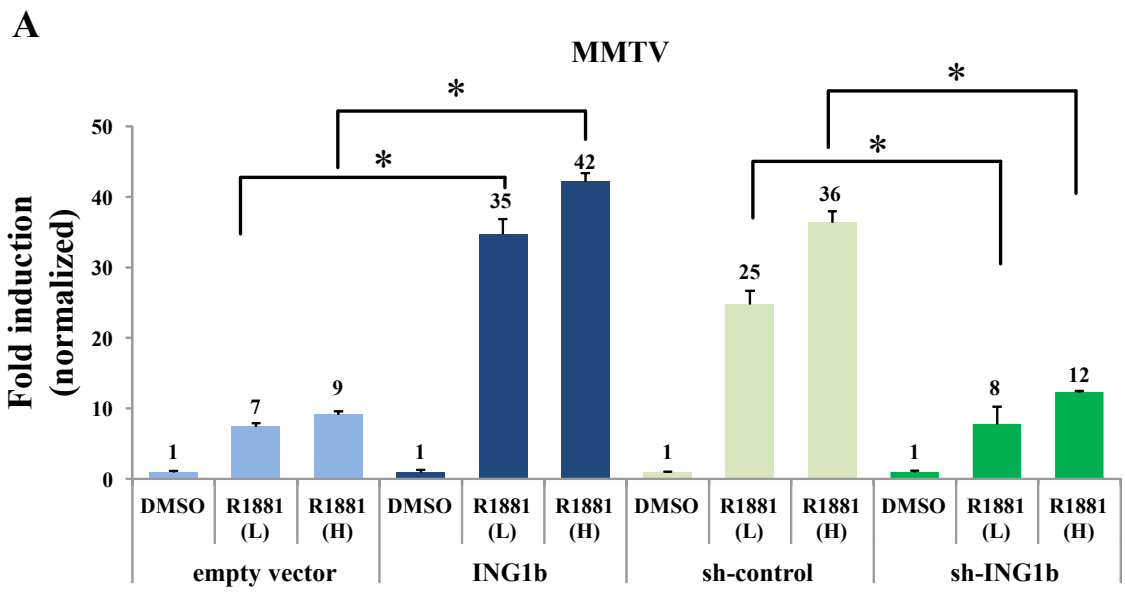
Figure 19. ING1b suppresses endogenous AR target genes in LNCaP cells. After 10 days antibiotic selection of the transduced cells with the indicated vectors, they were starved 2 days in 10% CSS medium, then the cells were treated with the solvent control DMSO or synthetic

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.



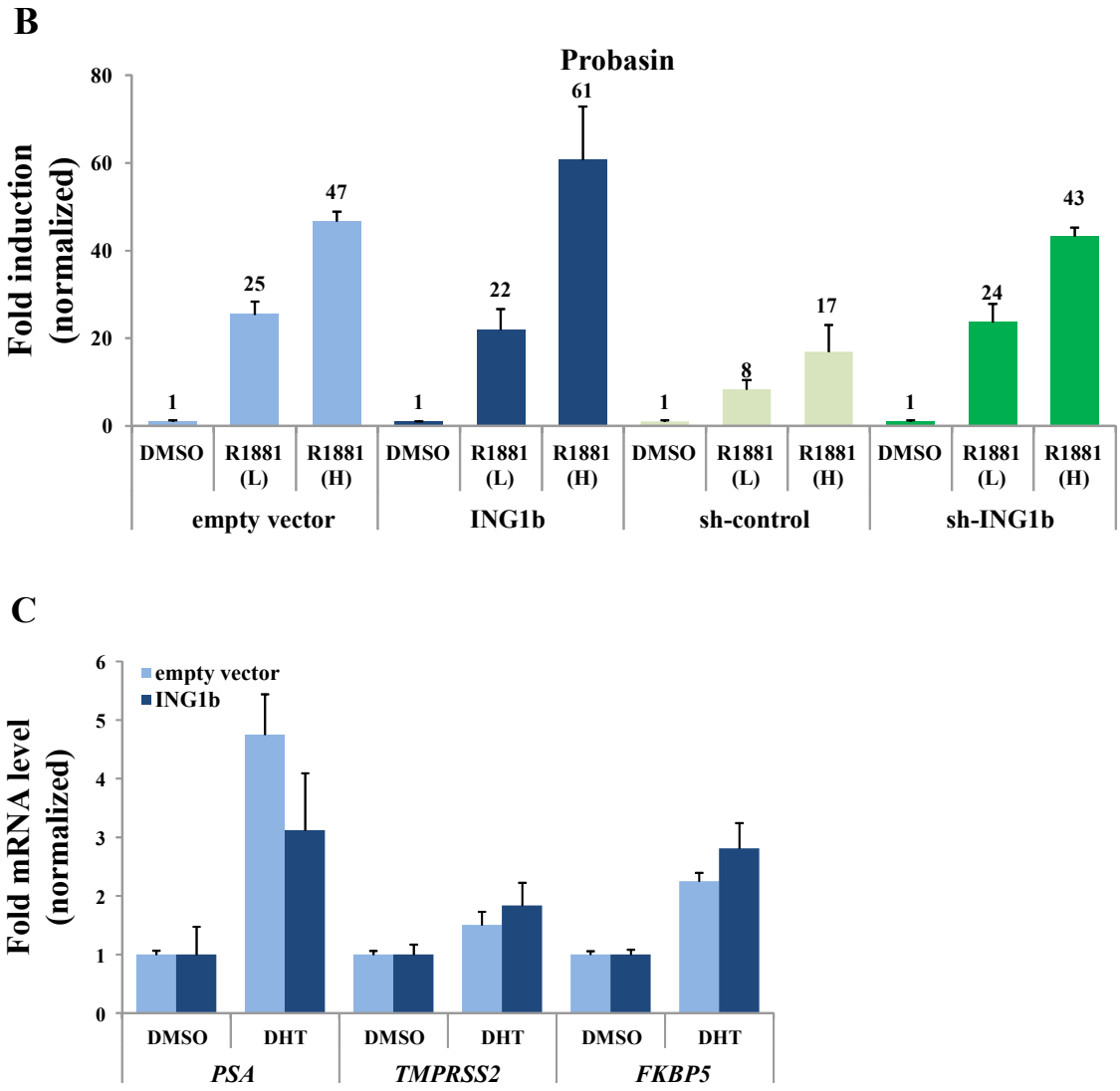


Figure 20. ING1b does not inhibit AR transactivation in PC3-AR cells. Reporter gene assay results of (A) MMTV-luc and (B) Probasin-luc for PC3-AR cells in a similar condition described in Figure 18. (C) qRT-PCR results for PC3-AR cells which were treated 48h with DMSO or DHT (10 nM) in a similar condition 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 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

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.

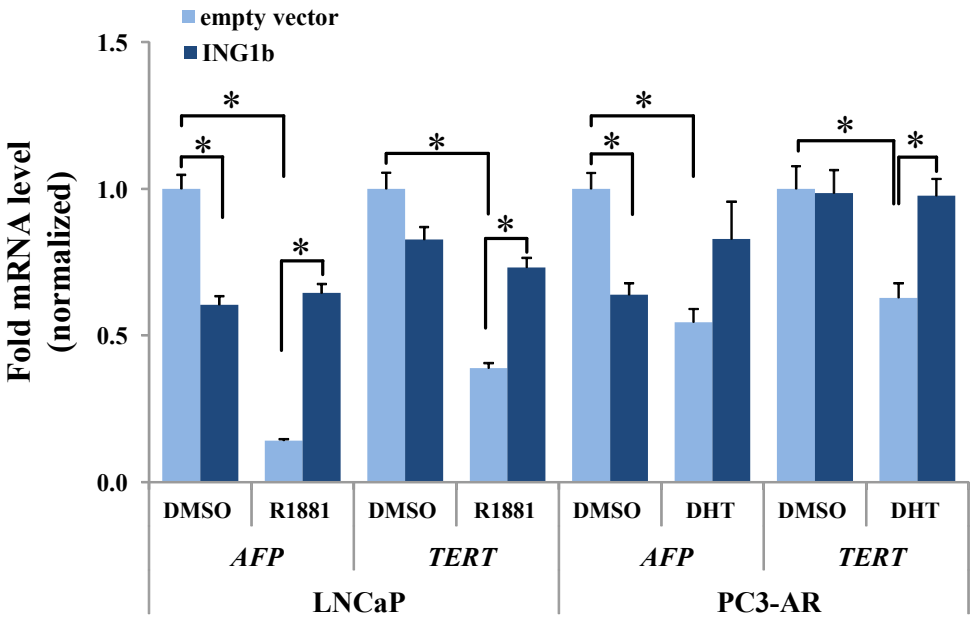


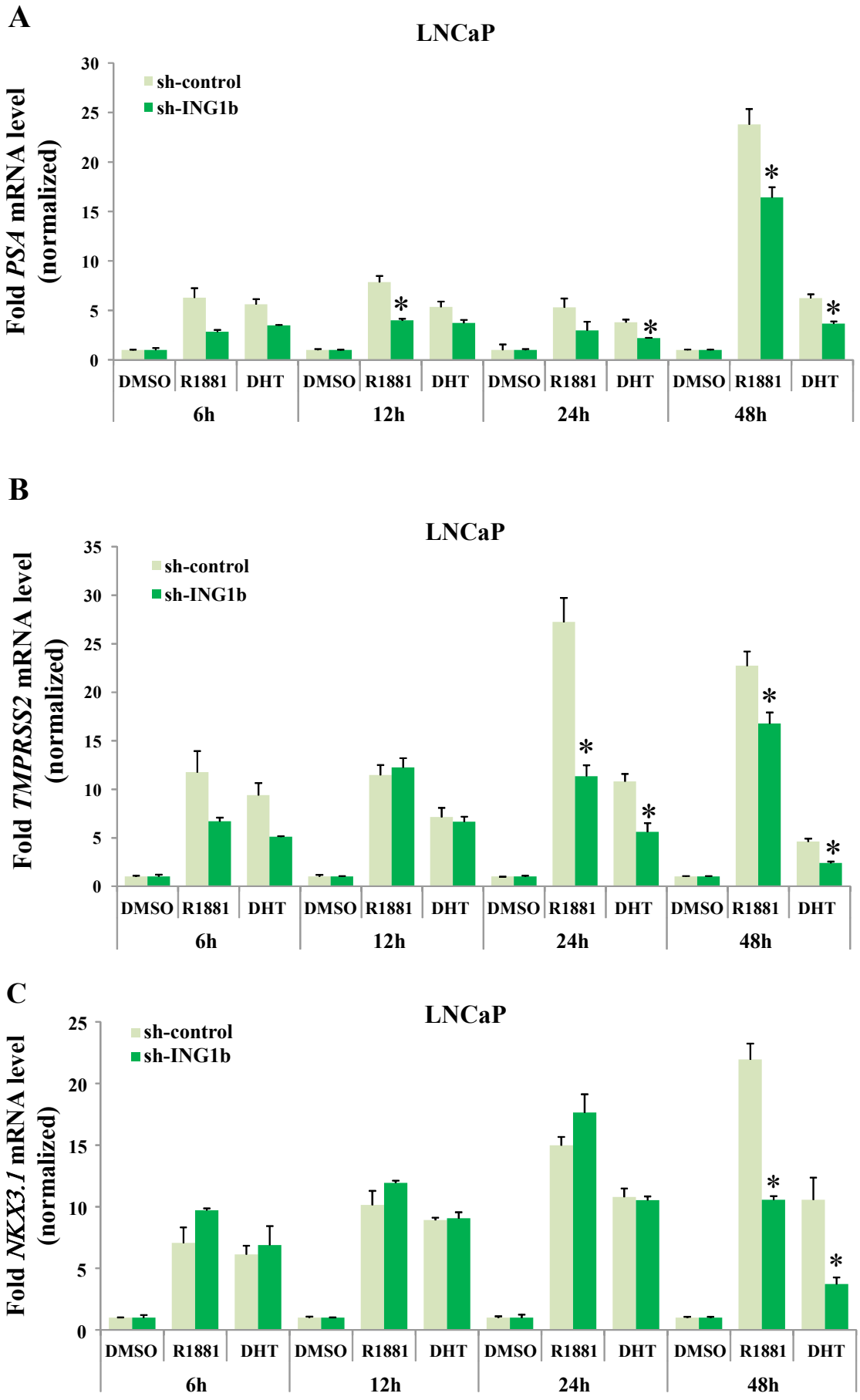
Figure 21. ING1b inhibits AR-mediated gene repression. qRT-PCR results of *AFP* and *TERT* for LNCaP and PC3-AR cells which were transduced and treated 48h with DMSO, R1881 (1 nM) or DHT (10 nM) in a similar condition explained in Figure 19. The experiments were repeated twice. Error bars represent SEM (* 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.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LNCaP
Fold PSA mRNA level (normalized)
sh-control
sh-ING1b
30
25
20
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0
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6h 12h 24h 48h
B
LNCaP
Fold TMPRSS2 mRNA level (normalized)
sh-control
sh-ING1b
35
30
25
20
15
10
5
0
DMSO R1881 DHT DMSO R1881 DHT DMSO R1881 DHT DMSO R1881 DHT
6h 12h 24h 48h
C
LNCaP
Fold NKX3.1 mRNA level (normalized)
sh-control
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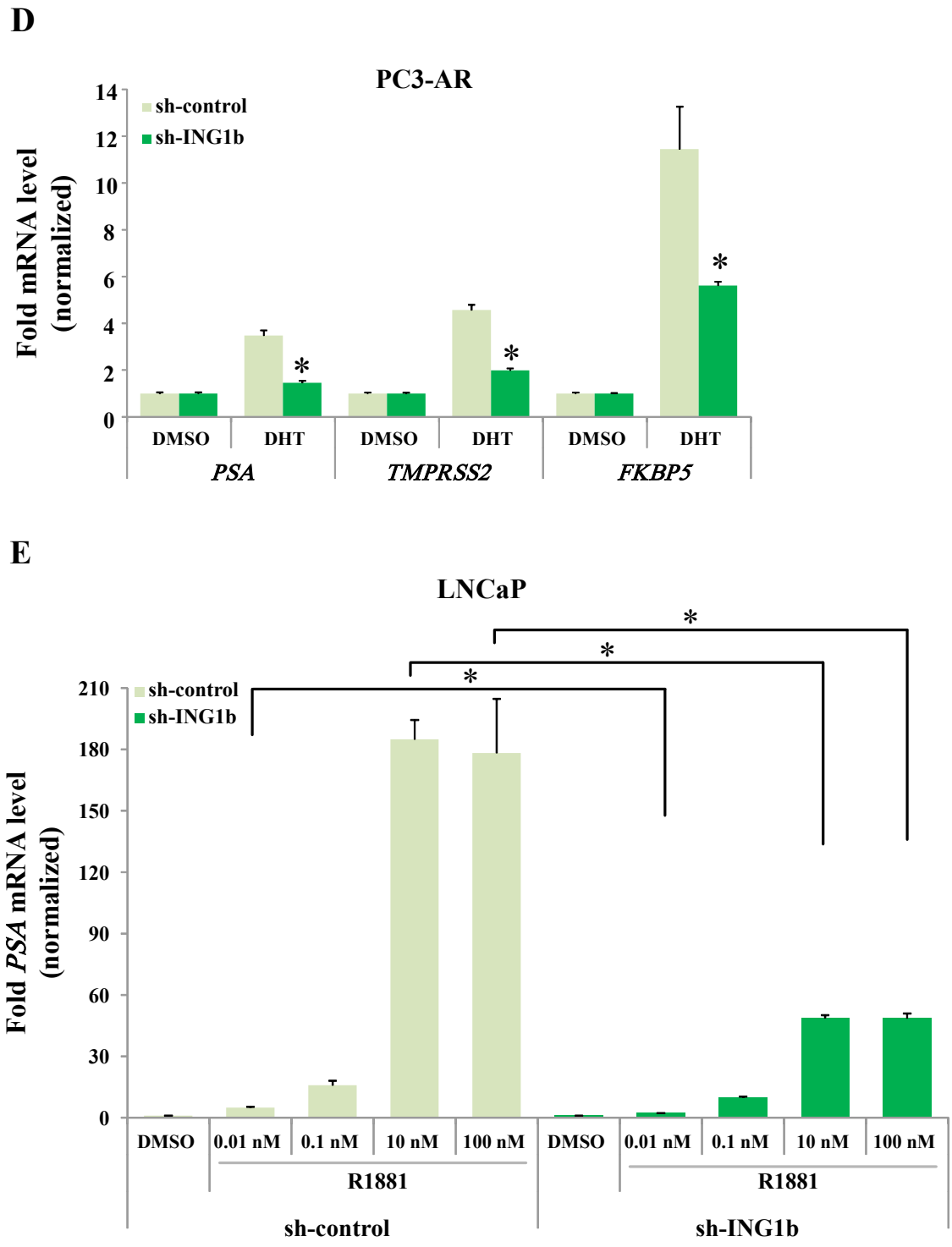


Figure 22. ING1b KD reduces androgen-induced gene expression in PCa cell lines. The stably-transduced cells with sh-control or sh-ING1b were selected 10 days against antibiotic. Then, they were starved 2 days in 10% CSS medium and treated with DMSO, R1881 (1 nM) or DHT (10 nM) for various treatment times 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 $p < 0.05$).

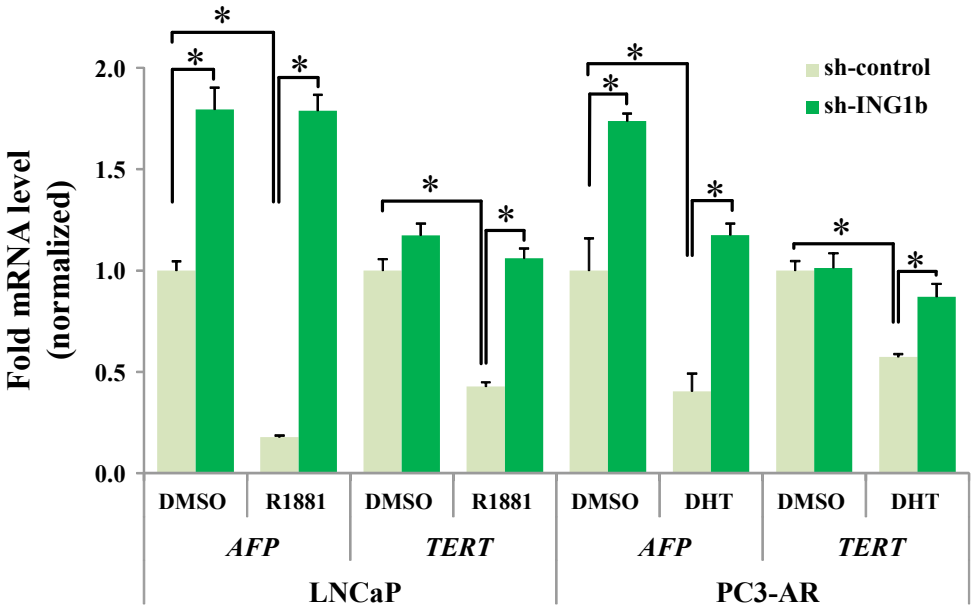


Figure 23. ING1b KD inhibits AR-mediated transcriptional represssion of endogenous genes. qRT-PCR results of *AFP* and *TERT* for LNCaP and PC3-AR cells which were transduced and treated 48h with DMSO, R1881 (1 nM) or DHT (10 nM) in a similar condition explained in Figure 22.

4.15 ING1b regulates differentially AR expression

Literatures have reported that AR-mediated transcription can be regulated with or without influencing AR protein level (Axlund 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.

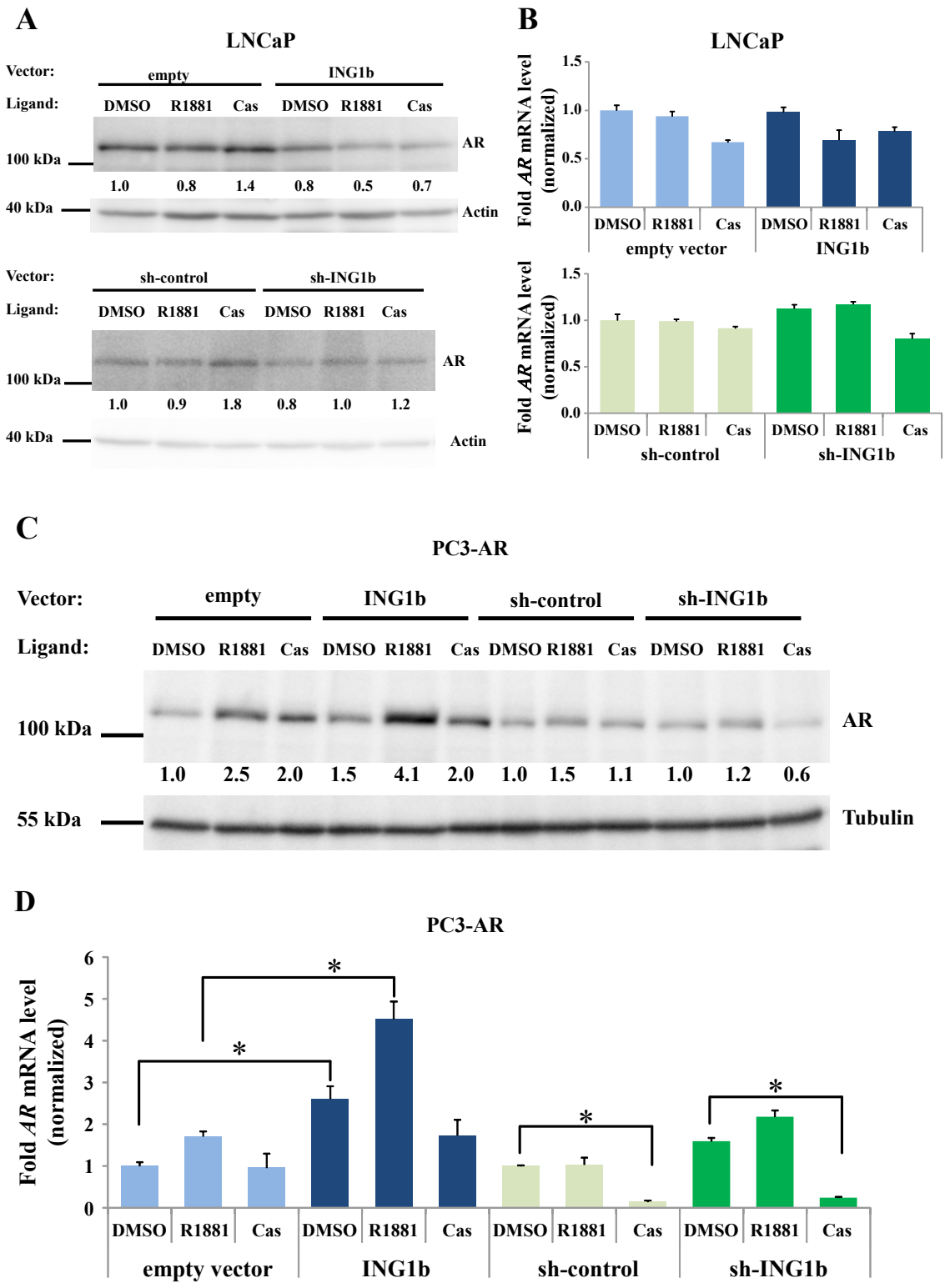
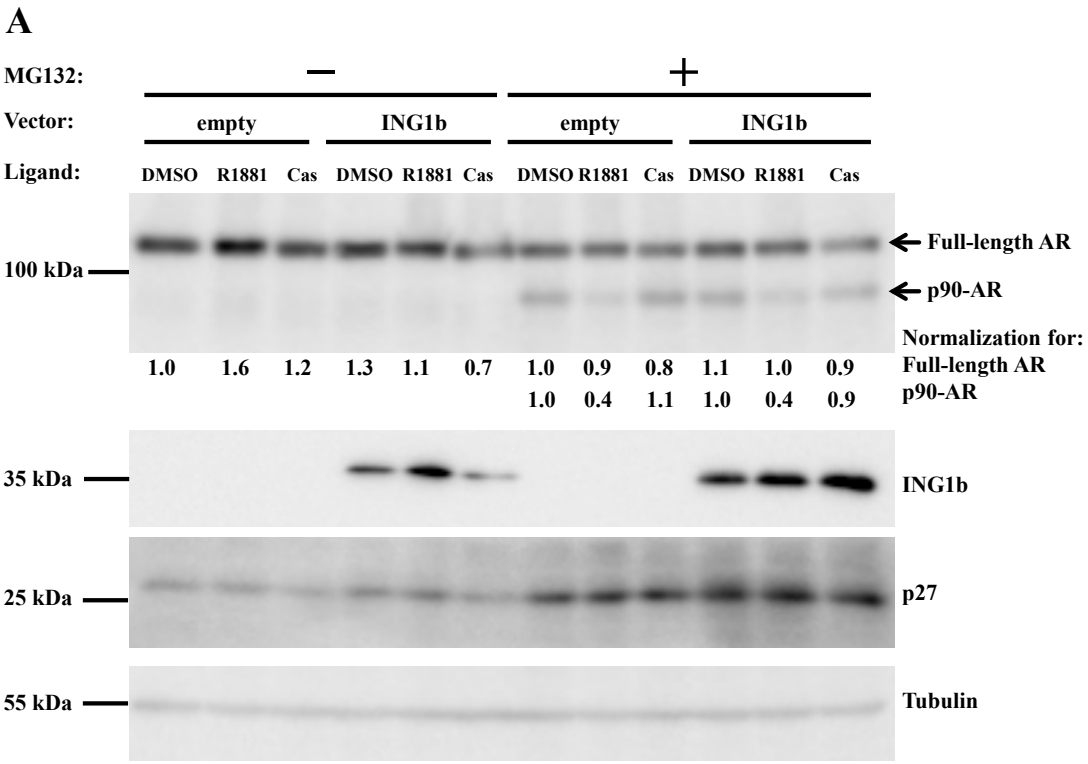


Figure 24. ING1b regulates differentially AR expression in LNCaP and PC3-AR cells. (A) Western blot analysis of AR in LNCaP cells stably transduced with the indicated vectors along with treatment with DMSO, R1881 (1 nM) or Casodex (1 μM) for 3 days. The expression AR protein was normalized to β-Actin. (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 $p < 0.05$).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Full-length AR
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Normalization for:
Full-length AR
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1.0 1.6 1.2 1.3 1.1 0.7 1.0 0.9 0.8 1.1 1.0 0.9
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35 kDa
ING1b
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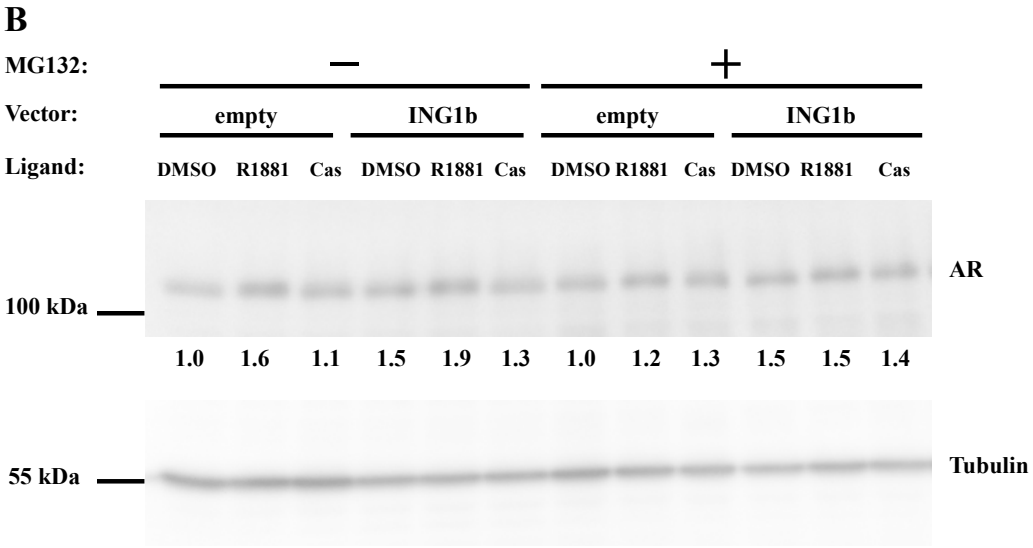
p27
55 kDa
Tubulin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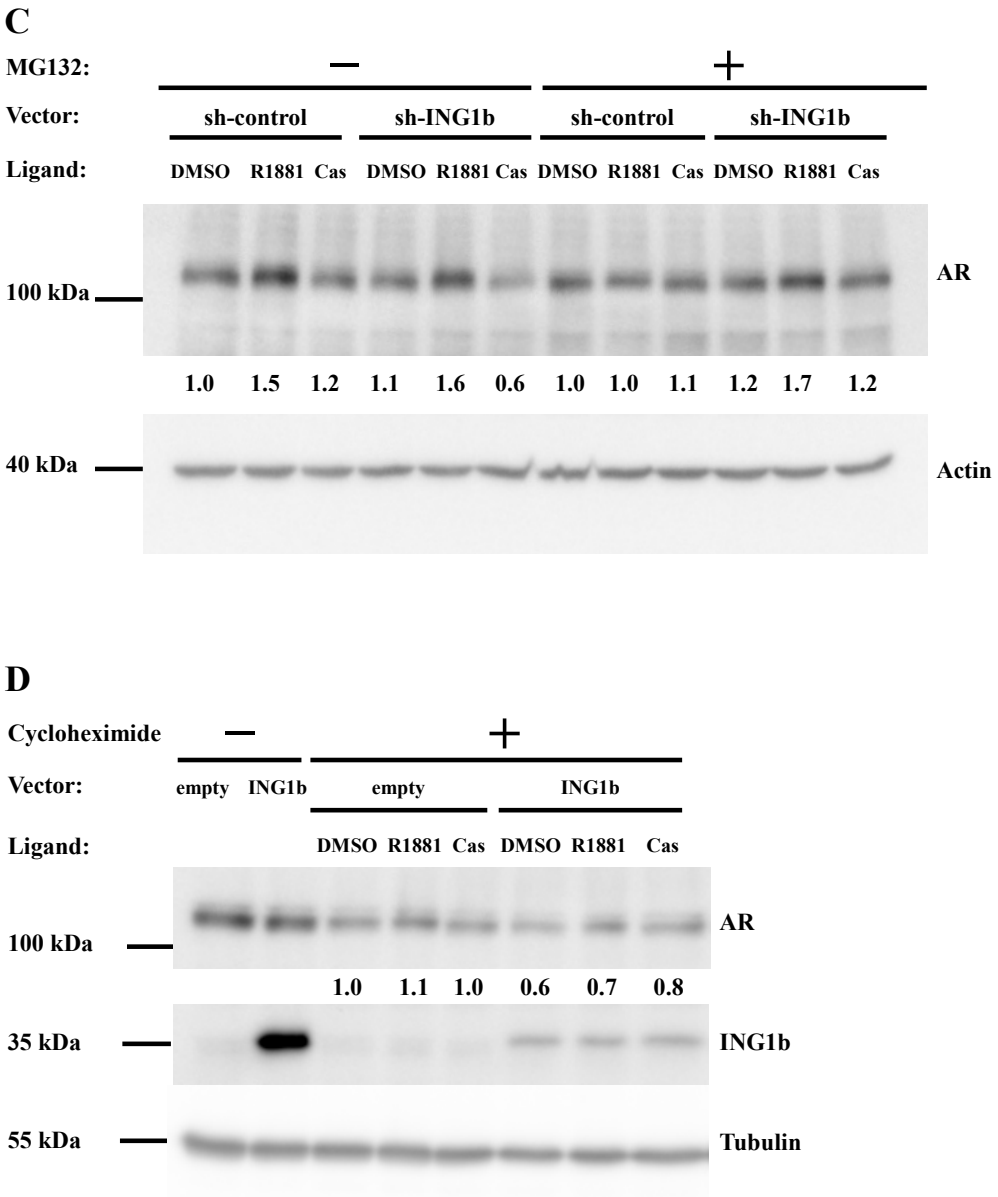


Figure 25. AR degradation by ING1b is independent of the proteasomal machinery in LNCaP cells. (A) Western blot analyses of AR, ING1b and p27 proteins in whole cell lysates from stably transduced LNCaP cells with empty vector or vector expressing ING1b after 24h treatment with DMSO, R1881 (1 nM) or Casodex (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.

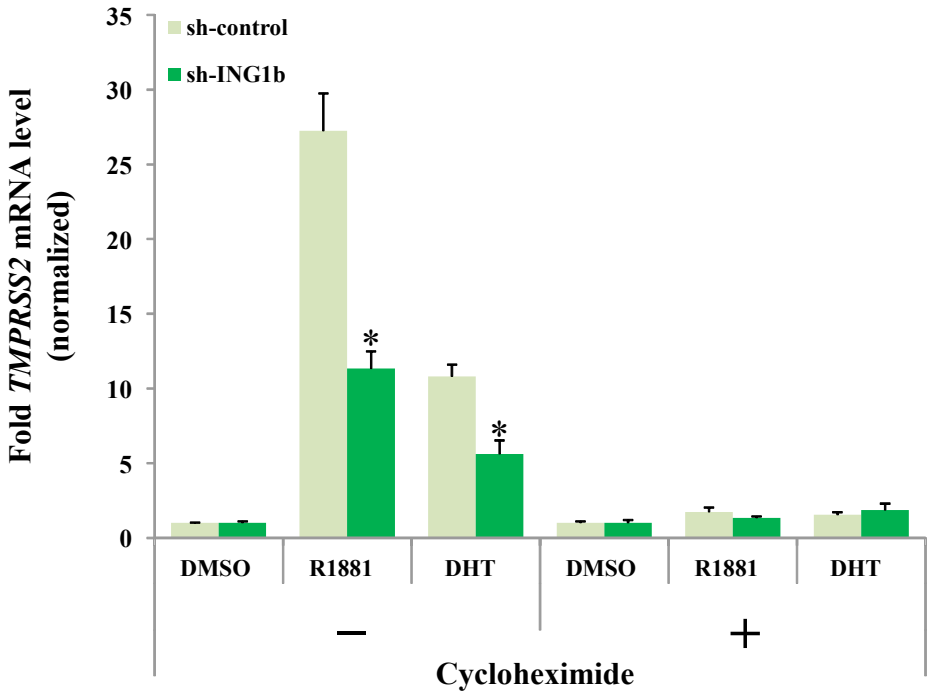


Figure 26. Cycloheximide reduces the ING1b KD effect on *TMPRSS2* mRNA level. qRT-PCR results of *TMPRSS2* for the LNCaP cells stably transduced with sh-control or sh-ING1b and treated 24h with DMSO, R1881 (1 nM) or DHT (10 nM) in the absence or presence of Cycloheximide (10 µg/ml). The normalized mRNA levels are shown as fold levels after setting the DMSO values arbitrarily at 1. Error bars represent SEM (* shows $p< 0.05$).

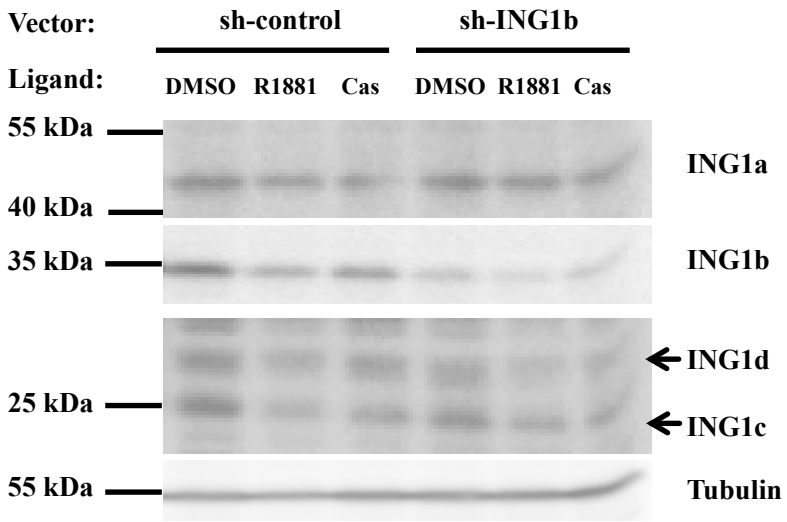


Figure 27. ING1b KD does not influence the expression of the other ING1 isoforms in LNCaP cells. Western blot analysis of ING1 protein levels in the stably transduced cells with either sh-control or sh-ING1b and treated 72h with DMSO or R1881 (1 nM) or Casodex (1 µM). α-Tubulin served as a loading control. 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.

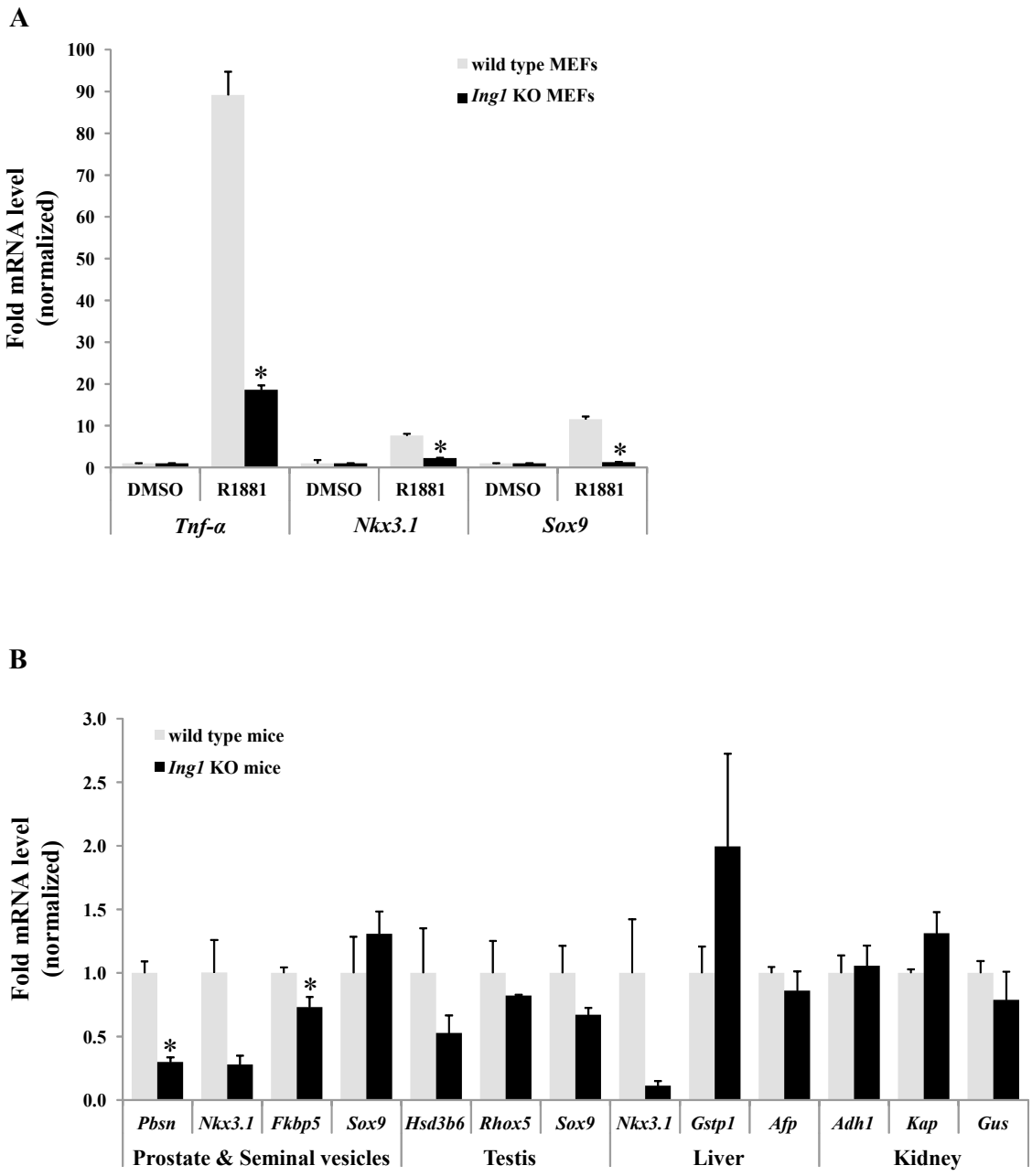


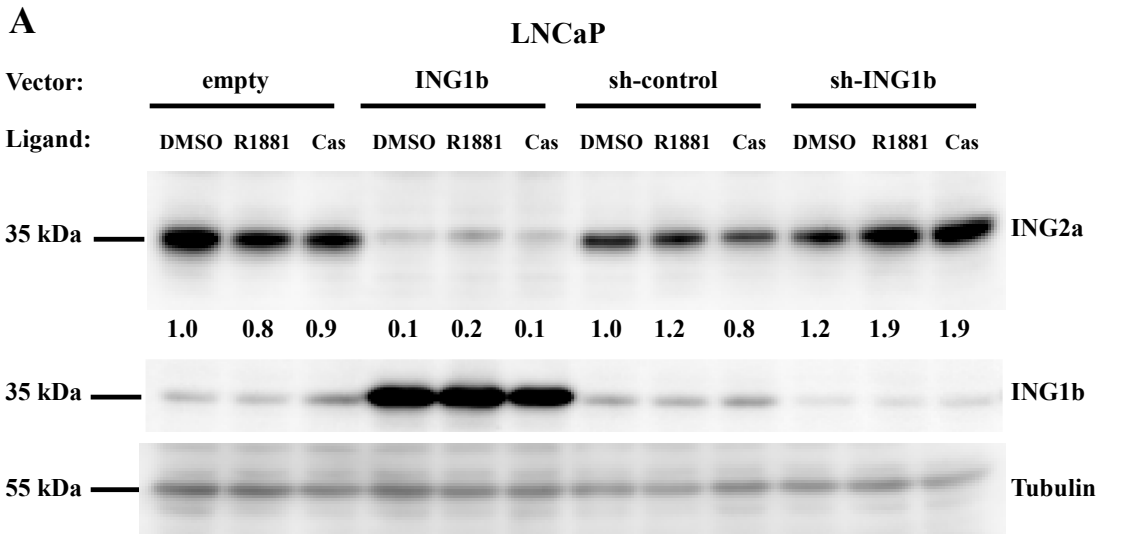
Figure 28. The mRNA expression of AR target genes is affected in *Ing1* KO mice. (A) MEFs from wild type and *Ing1* KO male mice were cultured in 10% CSS medium and treated 48h with DMSO or R1881 (0.1 nM) in the depleted condition. Thereafter RNA was isolated and qRT-PCR analysis was conducted using 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 $p < 0.05$).

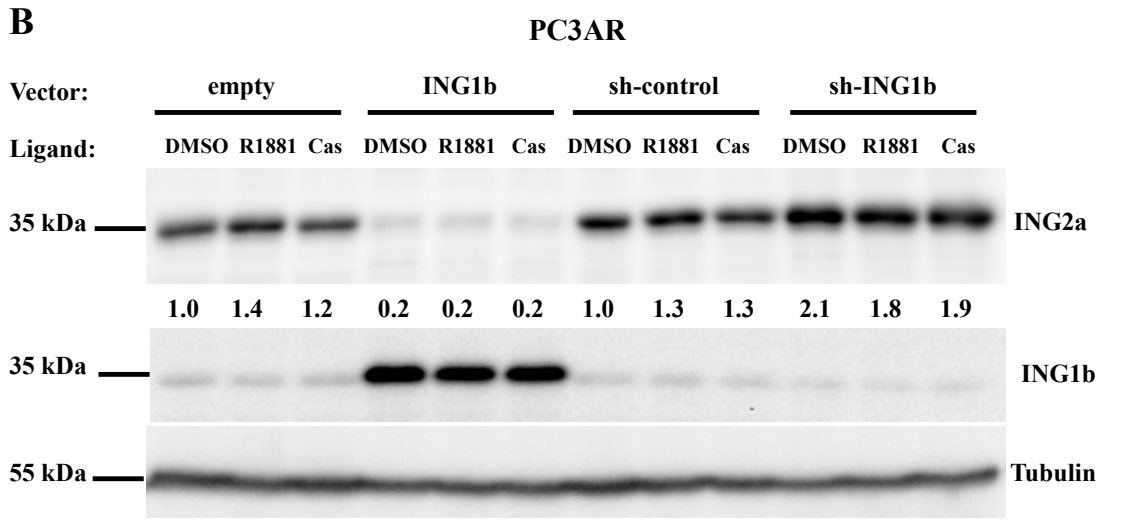
4.18 ING1b regulates the stability of ING2a protein

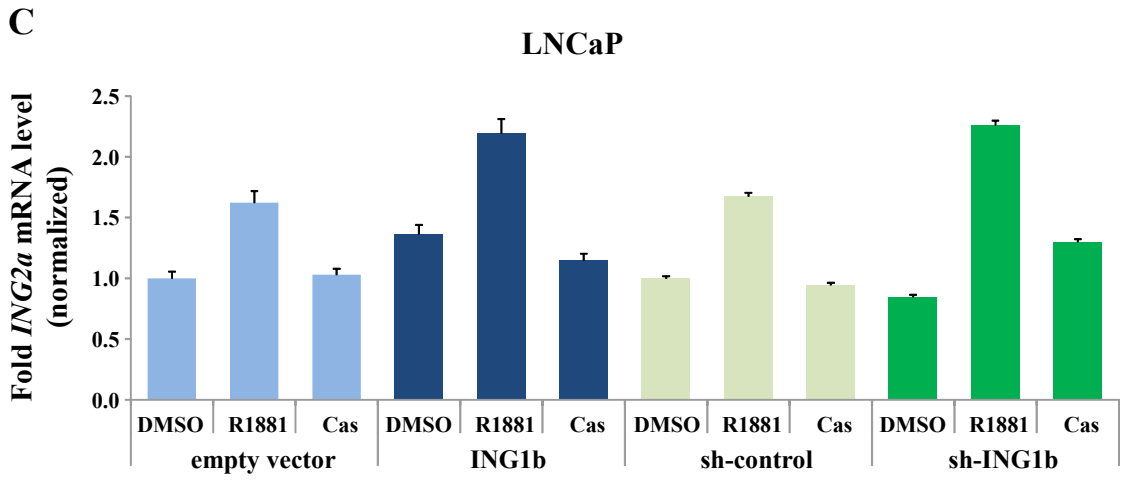
Since there are structural and functional overlaps among ING family members (Coles and Jones 2009, Jafarnejad and Li 2012), it was reasoned to analyze the expression of other ING family members in ING1 KD cells to find compensatory feedback loop. For this purpose, ING2 was selected as the closest ING 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.


A
LNCaP
Vector:
empty
ING1b
sh-control
sh-ING1b
Ligand:
DMSO R1881 Cas DMSO R1881 Cas DMSO R1881 Cas DMSO R1881 Cas
35 kDa
ING2a
1.0 0.8 0.9 0.1 0.2 0.1 1.0 1.2 0.8 1.2 1.9 1.9
35 kDa
ING1b
55 kDa
Tubulin


B
PC3AR
Vector:
empty
ING1b
sh-control
sh-ING1b
Ligand:
DMSO R1881 Cas DMSO R1881 Cas DMSO R1881 Cas DMSO R1881 Cas
35 kDa
ING2a
1.0 1.4 1.2 0.2 0.2 0.2 1.0 1.3 1.3 2.1 1.8 1.9
35 kDa
ING1b
55 kDa
Tubulin


C
LNCaP
Fold ING2a mRNA level (normalized)
2.5
2.0
1.5
1.0
0.5
0.0
DMSO R1881 Cas DMSO R1881 Cas DMSO R1881 Cas DMSO R1881 Cas
empty vector
ING1b
sh-control
sh-ING1b

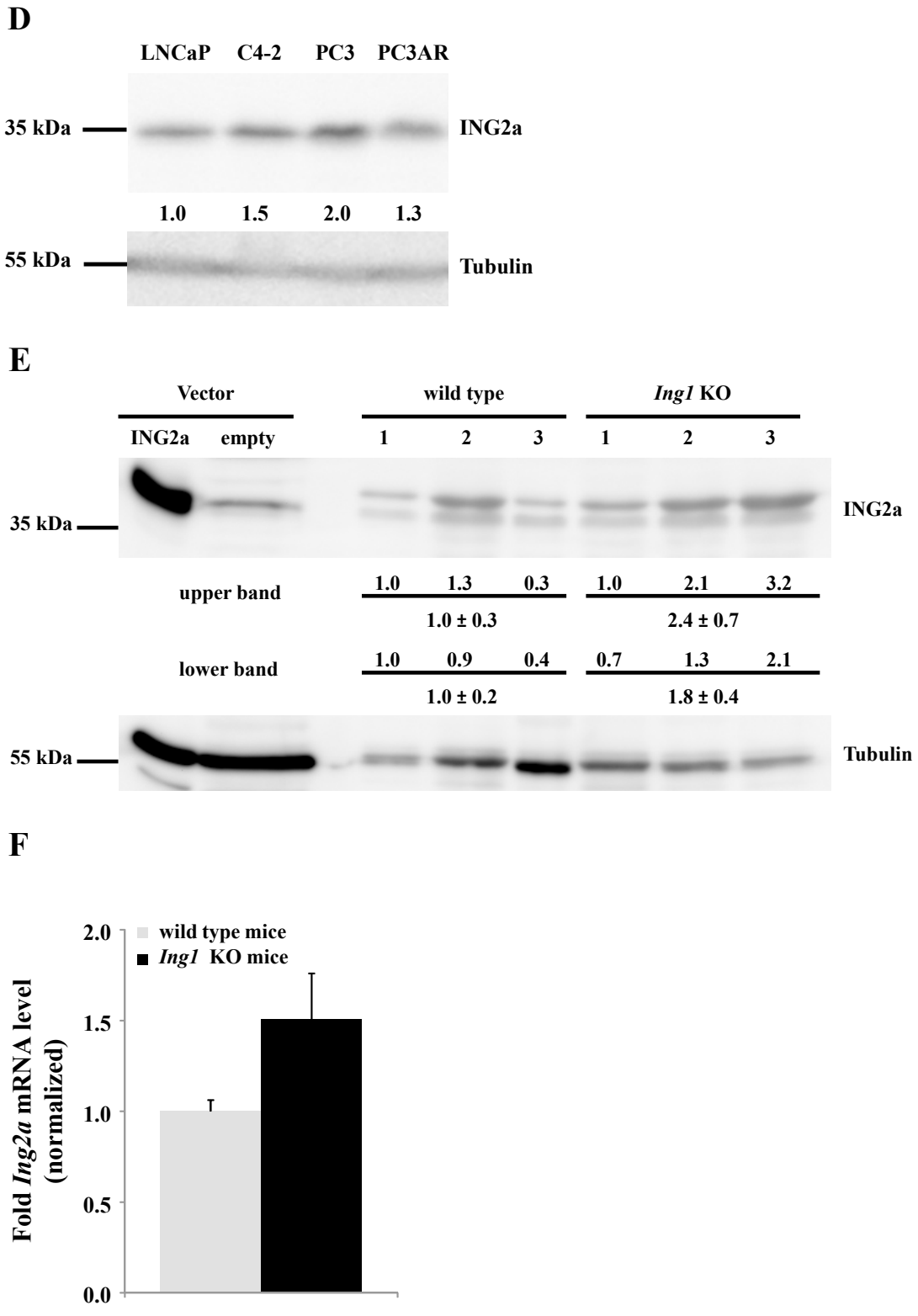


Figure 29. ING1b regulates ING2a protein level in human PCa cells and mouse prostate with seminal vesicles. Western blot analyses of the ING1b and ING2a proteins for (A) LNCaP and (B) PC3-AR cells that are explained in Figure 24. ING2a protein level was normalized to α-Tubulin. The experiments were repeated 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.

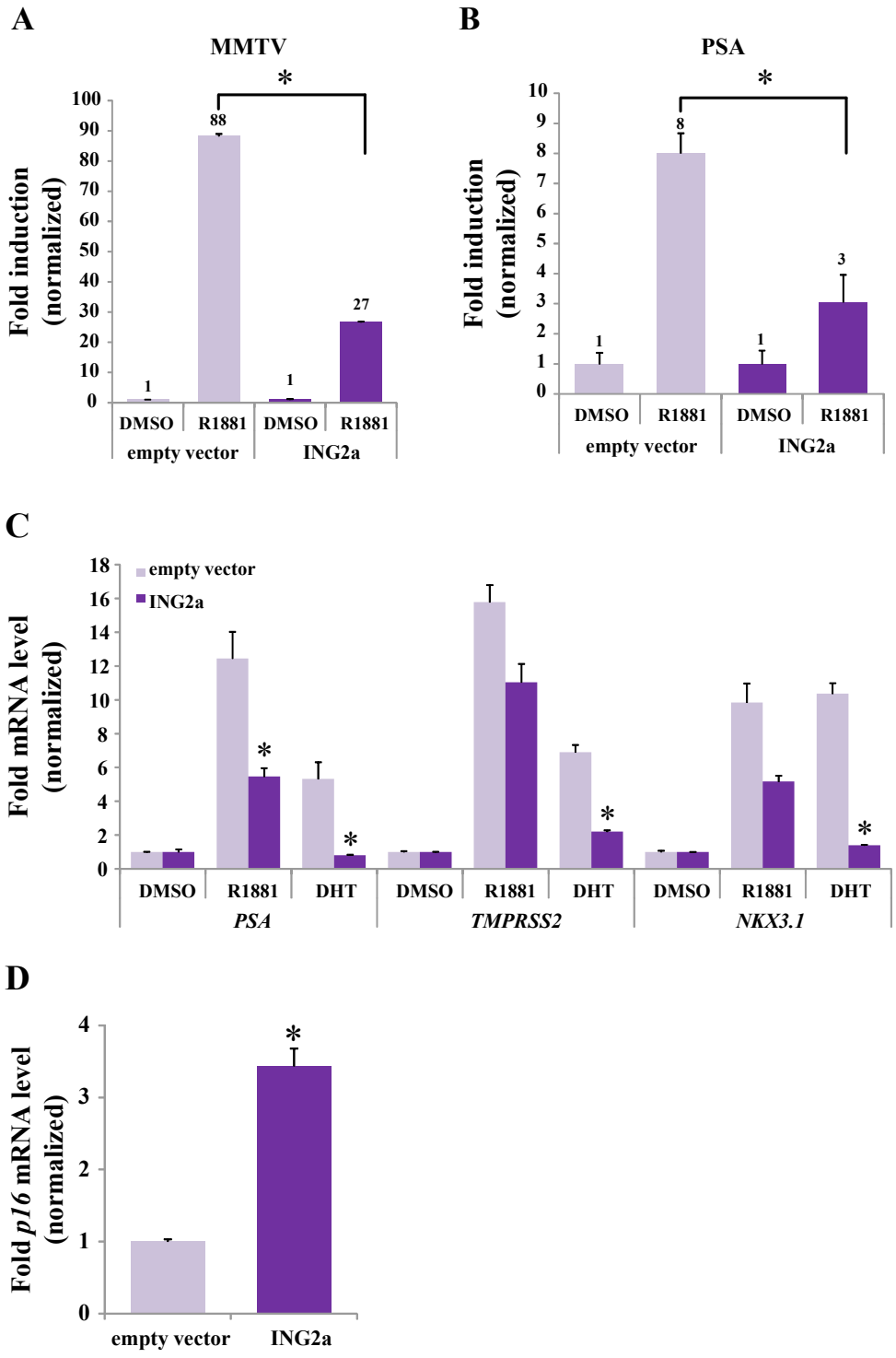


Figure 30. ING2a represses AR-mediated transactivation and upregulates *p16*. Reporter gene assays were performed for PC3-AR cells. The cells were seeded out in 10% CSS medium and transiently cotransfected with 1µg of either empty vector or vector expressing ING2a along with 1µg reporter construct (A) 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

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 *in vivo* results from *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 *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 *p14* and *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 *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 *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 *MMP9* and *MMP13* in LNCaP cells, which are responsible for promoting migration. However, it could also upregulate their inhibitors *TIMP1* and *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 *TIMP1* mRNA level has been upregulated by ING1b while no change for *MMP9*, *MMP13* and *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 *MMP9, MMP13* and *TIMP2* and decrease in *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 *PSA*, *NKX3.1*, *TMPRSS2* and *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).

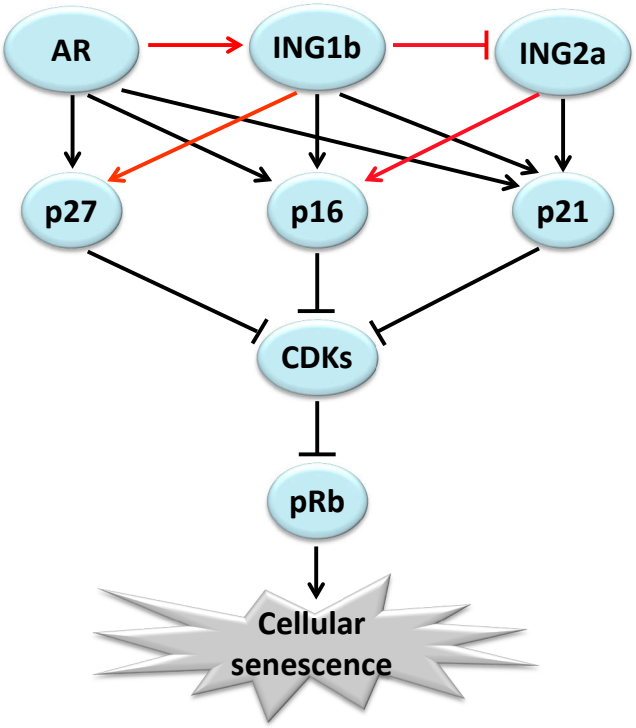


Figure 31. Model of ING1b-mediated cellular senescence in crosstalk with AR and ING2a. LNCaP cells: Agonist-bound AR induces p16 and p27 and ectopic expression of ING1b can increase p16 and p27 levels as well. Elevated level of p16 and p27 inhibits CDKs and triggers cellular senescence. Upon knockdown 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 $p19^{ARF}$ and $p21^{CIP1/WAF1}$ (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

Abad M, Menendez C, Fuchtbauer A, Serrano M, Fuchtbauer EM, Palmero I. 2007. Ing1 mediates p53 accumulation and chromatin modification in response to oncogenic stress. J Biol Chem 282(42):31060-7.

Abad M, Moreno A, Palacios A, Narita M, Blanco F, Moreno-Bueno G, Narita M, Palmero I. 2011. The tumor suppressor ING1 contributes to epigenetic control of cellular senescence. Aging Cell 10(1):158-71.

Adam-Vizi V, Chinopoulos C. 2006. Bioenergetics and the formation of mitochondrial reactive oxygen species. Trends Pharmacol Sci 27(12):639-45.

Agus DB, Cordon-Cardo C, Fox W, Drobnjak M, Koff A, Golde DW, Scher HI. 1999. Prostate cancer cell cycle regulators: response to androgen withdrawal and development of androgen independence. J Natl Cancer Inst 91(21):1869-76.

Albany C, Alva AS, Aparicio AM, Singal R, Yellapragada S, Sonpavde G, Hahn NM. 2011. Epigenetics in prostate cancer. Prostate Cancer 2011:580318.

Albritton LM, Tseng L, Scadden D, Cunningham JM. 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell 57(4):659-66.

Alimirah F, Panchanathan R, Chen J, Zhang X, Ho SM, Choubey D. 2007. Expression of androgen receptor is negatively regulated by p53. Neoplasia 9(12):1152-9.

Ao J, Meng J, Zhu L, Nie H, Yang C, Li J, Gu J, Lin Q, Long W, Dong X and others. 2012. Activation of androgen receptor induces ID1 and promotes hepatocellular carcinoma cell migration and invasion. Mol Oncol 6(5):507-15.

Asim M, Siddiqui IA, Hafeez BB, Baniahmad A, Mukhtar H. 2008. Src kinase potentiates androgen receptor transactivation function and invasion of androgen-independent prostate cancer C4-2 cells. Oncogene 27(25):3596-604.

Axlund SD, Lambert JR, Nordeen SK. 2010. HOXC8 inhibits androgen receptor signaling in human prostate cancer cells by inhibiting SRC-3 recruitment to direct androgen target genes. Mol Cancer Res 8(12):1643-55.

Balk SP, Knudsen KE. 2008. AR, the cell cycle, and prostate cancer. Nucl Recept Signal 6:e001.

Baniahmad A. 2005. Nuclear hormone receptor co-repressors. J Steroid Biochem Mol Biol 93(2-5):89-97.

Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG. 1999. The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol Cell Biol 19(12):8383-92.

Bluemn EG, Nelson PS. 2012. The androgen/androgen receptor axis in prostate cancer. Curr Opin Oncol 24(3):251-7.

Bonkhoff H, Berges R. 2010. From pathogenesis to prevention of castration resistant prostate cancer. Prostate 70(1):100-12.

Bose P, Thakur S, Thalappilly S, Ahn BY, Satpathy S, Feng X, Suzuki K, Kim SW, Riabowol K. 2013. ING1 induces apoptosis through direct effects at the mitochondria. Cell Death Dis 4:e788.

Buchanan G, Greenberg NM, Scher HI, Harris JM, Marshall VR, Tilley WD. 2001. Collocation of androgen receptor gene mutations in prostate cancer. Clin Cancer Res 7(5):1273-81.

Cai C, He HH, Chen S, Coleman I, Wang H, Fang Z, Chen S, Nelson PS, Liu XS, Brown M and others. 2011. Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1. Cancer Cell 20(4):457-71.

Campisi J, d'Adda di Fagagna F. 2007. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8(9):729-40.

Carroll AG, Voeller HJ, Sugars L, Gelmann EP. 1993. p53 oncogene mutations in three human prostate cancer cell lines. Prostate 23(2):123-34.

Chen J, Tran UM, Rajarajacholan U, Thalappilly S, Riabowol K. 2013. ING1b-inducible microRNA203 inhibits cell proliferation. Br J Cancer 108(5):1143-8.

Chmelar R, Buchanan G, Need EF, Tilley W, Greenberg NM. 2007. Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. Int J Cancer 120(4):719-33.

Claessens F, Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G, Rombauts W. 2001. Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. J Steroid Biochem Mol Biol 76(1-5):23-30.

Cleutjens KB, van der Korput HA, van Eekelen CC, van Rooij HC, Faber PW, Trapman J. 1997. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. Mol Endocrinol 11(2):148-61.

Coles AH, Jones SN. 2009. The ING gene family in the regulation of cell growth and tumorigenesis. J Cell Physiol 218(1):45-57.

Coles AH, Liang H, Zhu Z, Marfella CG, Kang J, Imbalzano AN, Jones SN. 2007. Deletion of p37Ing1 in mice reveals a p53-independent role for Ing1 in the suppression of cell proliferation, apoptosis, and tumorigenesis. Cancer Res 67(5):2054-61.

Collado M, Serrano M. 2010. Senescence in tumours: evidence from mice and humans. Nat Rev Cancer 10(1):51-7.

Cronauer MV, Schulz WA, Burchardt T, Ackermann R, Burchardt M. 2004. Inhibition of p53 function diminishes androgen receptor-mediated signaling in prostate cancer cell lines. Oncogene 23(20):3541-9.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O and others. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A 92(20):9363-7.

Dotzlaw H, Moehren U, Mink S, Cato AC, Iniguez Lluhi JA, Baniahmad A. 2002. The amino terminus of the human AR is target for corepressor action and antihormone agonism. Mol Endocrinol 16(4):661-73.

Doyon Y, Cayrou C, Ullah M, Landry AJ, Cote V, Selleck W, Lane WS, Tan S, Yang XJ, Cote J. 2006. ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. Mol Cell 21(1):51-64.

Ewald JA, Desotelle JA, Church DR, Yang B, Huang W, Laurila TA, Jarrard DF. 2013. Androgen deprivation induces senescence characteristics in prostate cancer cells in vitro and in vivo. Prostate 73(4):337-45.

Ewald JA, Jarrard DF. 2012. Decreased skp2 expression is necessary but not sufficient for therapy-induced senescence in prostate cancer. Transl Oncol 5(4):278-87.

Farla P, Hersmus R, Geverts B, Mari PO, Nigg AL, Dubbink HJ, Trapman J, Houtsmuller AB. 2004. The androgen receptor ligand-binding domain stabilizes DNA binding in living cells. J Struct Biol 147(1):50-61.

Farla P, Hersmus R, Trapman J, Houtsmuller AB. 2005. Antiandrogens prevent stable DNA-binding of the androgen receptor. J Cell Sci 118(Pt 18):4187-98.

Fegers I, Kob R, Eckey M, Schmidt O, Goeman F, Papaioannou M, Escher N, von Eggeling F, Melle C, Baniahmad A. 2007. The tumor suppressors p33ING1 and p33ING2 interact with alien in vivo and enhance alien-mediated gene silencing. J Proteome Res 6(11):4182-8.

Felder MR, Watson G, Huff MO, Ceci JD. 1988. Mechanism of induction of mouse kidney alcohol dehydrogenase by androgen. Androgen-induced stimulation of transcription of the Adh-1 gene. J Biol Chem 263(28):14531-7.

Feng X, Hara Y, Riabowol K. 2002. Different HATS of the ING1 gene family. Trends Cell Biol 12(11):532-8.

Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D, Bray F. 2013. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 49(6):1374-403.

Gao S, Lee P, Wang H, Gerald W, Adler M, Zhang L, Wang YF, Wang Z. 2005. The androgen receptor directly targets the cellular Fas/FasL-associated death domain protein-like inhibitory protein gene to promote the androgen-independent growth of prostate cancer cells. Mol Endocrinol 19(7):1792-802.

Garate M, Campos EI, Bush JA, Xiao H, Li G. 2007. Phosphorylation of the tumor suppressor p33(ING1b) at Ser-126 influences its protein stability and proliferation of melanoma cells. FASEB J 21(13):3705-16.

Garate M, Wong RP, Campos EI, Wang Y, Li G. 2008. NAD(P)H quinone oxidoreductase 1 inhibits the proteasomal degradation of the tumour suppressor p33(ING1b). EMBO Rep 9(6):576-81.

Garkavtsev I, Demetrick D, Riabowol K. 1997. Cellular localization and chromosome mapping of a novel candidate tumor suppressor gene (ING1). Cytogenet Cell Genet 76(3-4):176-8.

Garkavtsev I, Grigorian IA, Ossovskaya VS, Chernov MV, Chumakov PM, Gudkov AV. 1998. The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. Nature 391(6664):295-8.

Garkavtsev I, Kazarov A, Gudkov A, Riabowol K. 1996. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. Nat Genet 14(4):415-20.

Garkavtsev I, Riabowol K. 1997. Extension of the replicative life span of human diploid fibroblasts by inhibition of the p33ING1 candidate tumor suppressor. Mol Cell Biol 17(4):2014-9.

Gebäck T, Schulz MM, Koumoutsakos P, Detmar M. 2009. TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. Biotechniques 46(4):265-74.

Gelmann EP. 2002. Molecular biology of the androgen receptor. J Clin Oncol 20(13):3001-15.

Goeman F, Thormeyer D, Abad M, Serrano M, Schmidt O, Palmero I, Baniahmad A. 2005. Growth inhibition by the tumor suppressor p33ING1 in immortalized and primary cells: involvement of two silencing domains and effect of Ras. Mol Cell Biol 25(1):422-31.

Gomez-Cabello D, Callejas S, Benguria A, Moreno A, Alonso J, Palmero I. 2010. Regulation of the microRNA processor DGCR8 by the tumor suppressor ING1. Cancer Res 70(5):1866-74.

Gong W, Russell M, Suzuki K, Riabowol K. 2006. Subcellular targeting of p33ING1b by phosphorylation-dependent 14-3-3 binding regulates p21WAF1 expression. Mol Cell Biol 26(8):2947-54.

Gonzalez L, Freije JM, Cal S, Lopez-Otin C, Serrano M, Palmero I. 2006. A functional link between the tumour suppressors ARF and p33ING1. Oncogene 25(37):5173-9.

Grosse A, Bartsch S, Baniahmad A. 2012. Androgen receptor-mediated gene repression. Mol Cell Endocrinol 352(1-2):46-56.

Guerillon C, Bigot N, Pedeux R. 2014. The ING tumor suppressor genes: status in human tumors. Cancer Lett 345(1):1-16.

Guerillon C, Larrieu D, Pedeux R. 2013. ING1 and ING2: multifaceted tumor suppressor genes. Cell Mol Life Sci 70(20):3753-72.

Gunduz M, Ouchida M, Fukushima K, Hanafusa H, Etani T, Nishioka S, Nishizaki K, Shimizu K. 2000. Genomic structure of the human ING1 gene and tumor-specific mutations detected in head and neck squamous cell carcinomas. Cancer Res 60(12):3143-6.

Guo XB, Jing CQ, Li LP, Zhang L, Shi YL, Wang JS, Liu JL, Li CS. 2011. Down-regulation of miR-622 in gastric cancer promotes cellular invasion and tumor metastasis by targeting ING1 gene. World J Gastroenterol 17(14):1895-902.

Guseva NV, Rokhlin OW, Bair TB, Glover RB, Cohen MB. 2012. Inhibition of p53 expression modifies the specificity of chromatin binding by the androgen receptor. Oncotarget 3(2):183-94.

Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. Cell 144(5):646-74.

Harada N, Inoue K, Yamaji R, Nakano Y, Inui H. 2012. Androgen deprivation causes truncation of the C-terminal region of androgen receptor in human prostate cancer LNCaP cells. Cancer Sci 103(6):1022-7.

Harris WP, Mostaghel EA, Nelson PS, Montgomery B. 2009. Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nat Clin Pract Urol 6(2):76-85.

He GH, Helbing CC, Wagner MJ, Sensen CW, Riabowol K. 2005. Phylogenetic analysis of the ING family of PHD finger proteins. Mol Biol Evol 22(1):104-16.

Heemers HV, Tindall DJ. 2007. Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev 28(7):778-808.

Heery DM, Kalkhoven E, Hoare S, Parker MG. 1997. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature 387(6634):733-6.

Heinlein CA, Chang C. 2001. Role of chaperones in nuclear translocation and transactivation of steroid receptors. Endocrine 14(2):143-9.

Heinlein CA, Chang C. 2004. Androgen receptor in prostate cancer. Endocr Rev 25(2):276-308.

Helbing CC, Veillette C, Riabowol K, Johnston RN, Garkavtsev I. 1997. A novel candidate tumor suppressor, ING1, is involved in the regulation of apoptosis. Cancer Res 57(7):1255-8.

Helbing CC, Wagner MJ, Pettem K, Johnston J, Heimeier RA, Veldhoen N, Jirik FR, Shi YB, Browder LW. 2011. Modulation of thyroid hormone-dependent gene expression in Xenopus laevis by INhibitor of Growth (ING) proteins. PLoS One 6(12):e28658.

Hessenkemper W, Baniahmad A. 2012. Chaperones for proper androgen action - a plethora of assistance to androgen receptor function. Horm Mol Biol Clin Investig 11(1):321-8.

Hessenkemper W, Roediger J, Bartsch S, Houtsmuller AB, van Royen ME, Petersen I, Grimm MO, Baniahmad A. 2014. A natural androgen receptor antagonist induces cellular senescence in prostate cancer cells. Mol Endocrinol 28(11):1831-40.

Hoimes CJ, Kelly WK. 2010. Redefining hormone resistance in prostate cancer. Ther Adv Med Oncol 2(2):107-123.

Ikeda H, Serria MS, Kakizaki I, Hatayama I, Satoh K, Tsuchida S, Muramatsu M, Nishi S, Sakai M. 2002. Activation of mouse Pi-class glutathione S-transferase gene by Nrf2(NF-E2-related factor 2) and androgen. Biochem J 364(Pt 2):563-70.

Itoh N, Kakehi Y, Akao T, Kinoshita H, Okada Y, Yoshida O. 1997. Concomitant presence of p16/cyclin-dependent kinase 4 and cyclin D/cyclin-dependent kinase 4 complexes in LNCaP prostatic cancer cell line. Jpn J Cancer Res 88(3):229-33.

Jafarnejad SM, Li G. 2012. Regulation of p53 by ING family members in suppression of tumor initiation and progression. Cancer Metastasis Rev 31(1-2):55-73.

Jansson KH, Lynch JE, Lepori-Bui N, Czymmek KJ, Duncan RL, Sikes RA. 2012. Overexpression of the VSSC-associated CAM, beta-2, enhances LNCaP cell metastasis associated behavior. Prostate 72(10):1080-92.

Jarrard DF, Bova GS, Ewing CM, Pin SS, Nguyen SH, Baylin SB, Cairns P, Sidransky D, Herman JG, Isaacs WB. 1997. Deletional, mutational, and methylation analyses of CDKN2 (p16/MTS1) in primary and metastatic prostate cancer. Genes Chromosomes Cancer 19(2):90-6.

Jennek S. 2009. Identifikation von in vivo Protein-Protein-Interaktionen des Tumorsuppressor-Proteins p33ING1b mittels der SELDI-TOF-MS-Technik [Diploma thesis]. Jena: Friedrich Schiller University.

Jenster G, van der Korput HA, Trapman J, Brinkmann AO. 1995. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. J Biol Chem 270(13):7341-6.

Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. 1979. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Invest Urol 17(1):16-23.

Kankaanpää P, Paavolainen L, Tiitta S, Karjalainen M, Paivarinne J, Nieminen J, Marjomaki V, Heino J, White DJ. 2012. BioImageXD: an open, general-purpose and high-throughput image-processing platform. Nat Methods 9(7):683-9.

Kaspar F, Klocker H, Denninger A, Cato AC. 1993. A mutant androgen receptor from patients with Reifenstein syndrome: identification of the function of a conserved alanine residue in the D box of steroid receptors. Mol Cell Biol 13(12):7850-8.

Kataoka H, Bonnefin P, Vieyra D, Feng X, Hara Y, Miura Y, Joh T, Nakabayashi H, Vaziri H, Harris CC and others. 2003. ING1 represses transcription by direct DNA binding and through effects on p53. Cancer Res 63(18):5785-92.

Keston AS, Brandt R. 1965. The Fluorometric Analysis of Ultramicro Quantities of Hydrogen Peroxide. Anal Biochem 11:1-5.

Kichina JV, Zeremski M, Aris L, Gurova KV, Walker E, Franks R, Nikitin AY, Kiyokawa H, Gudkov AV. 2006. Targeted disruption of the mouse ing1 locus

results in reduced body size, hypersensitivity to radiation and elevated incidence of lymphomas. Oncogene 25(6):857-66.

Kim J, Wong PK. 2009. Oxidative stress is linked to ERK1/2-p16 signaling-mediated growth defect in ATM-deficient astrocytes. J Biol Chem 284(21):14396-404.

Klitzsch A. 2011. Funktionelle Interaktion zwischen dem Androgenrezeptor und dem Tumorsuppressor p33ING1b [Diploma thesis] Jena: Friedrich Schiller University.

Kuzmichev A, Zhang Y, Erdjument-Bromage H, Tempst P, Reinberg D. 2002. Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumor suppressor p33(ING1). Mol Cell Biol 22(3):835-48.

Larrieu D, Ythier D, Brambilla C, Pedeux R. 2010. ING2 controls the G1 to S-phase transition by regulating p21 expression. Cell Cycle 9(19):3984-90.

Larsson LG. 2011. Oncogene- and tumor suppressor gene-mediated suppression of cellular senescence. Semin Cancer Biol 21(6):367-76.

Leung KM, Po LS, Tsang FC, Siu WY, Lau A, Ho HT, Poon RY. 2002. The candidate tumor suppressor ING1b can stabilize p53 by disrupting the regulation of p53 by MDM2. Cancer Res 62(17):4890-3.

Li B, Lu W, Chen Z. 2014a. Regulation of Androgen Receptor by E3 Ubiquitin Ligases: for More or Less. Receptors Clin Investig 1(5).

Li B, Lu W, Yang Q, Yu X, Matusik RJ, Chen Z. 2014b. Skp2 regulates androgen receptor through ubiquitin-mediated degradation independent of Akt/mTOR pathways in prostate cancer. Prostate 74(4):421-32.

Li N, Li Q, Cao X, Zhao G, Xue L, Tong T. 2011. The tumor suppressor p33ING1b upregulates p16INK4a expression and induces cellular senescence. FEBS Lett 585(19):3106-12.

Li N, Zhao G, Chen T, Xue L, Ma L, Niu J, Tong T. 2012. Nucleolar protein CSIG is required for p33ING1 function in UV-induced apoptosis. Cell Death Dis 3:e283.

Lichtinghagen R, Musholt PB, Stephan C, Lein M, Kristiansen G, Hauptmann S, Rudolph B, Schnorr D, Loening SA, Jung K. 2003. mRNA expression profile of matrix metalloproteinases and their tissue inhibitors in malignant and non-malignant prostatic tissue. Anticancer Res 23(3B):2617-24.

Lin B, Ferguson C, White JT, Wang S, Vessella R, True LD, Hood L, Nelson PS. 1999. Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. Cancer Res 59(17):4180-4.

Lin HK, Wang L, Hu YC, Altuwaijri S, Chang C. 2002. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. EMBO J 21(15):4037-48.

Lonergan PE, Tindall DJ. 2011. Androgen receptor signaling in prostate cancer development and progression. J Carcinog 10:20.

Lu W, Xie Y, Ma Y, Matusik RJ, Chen Z. 2013. ARF represses androgen receptor transactivation in prostate cancer. Mol Endocrinol 27(4):635-48.

Ludwig S. 2012. Analyse des Einflusses des Tumorsuppressors ING1 auf die transkriptionelle Aktivität des Androgenrezeptors [Diploma thesis]. Jena: Friedrich Schiller University.

Lund SD, Miller D, Chapman V, Ganschow RE. 1988. Androgen regulation of murine beta-glucuronidase expression: identification and characterization of a nonresponse variant. Genetics 119(1):151-6.

Lv Y, Purbey BK, Huang Y, Li S, Radha G, Hao Z. 2012. Adenovirus-mediated expression of p33(ING1b) induces apoptosis and inhibits proliferation in gastric adenocarcinoma cells in vitro. Gastric Cancer 15(4):355-62.

Ma L, Chang N, Guo S, Li Q, Zhang Z, Wang W, Tong T. 2008. CSIG inhibits PTEN translation in replicative senescence. Mol Cell Biol 28(20):6290-301.

Magee JA, Chang LW, Stormo GD, Milbrandt J. 2006. Direct, androgen receptor-mediated regulation of the FKBP5 gene via a distal enhancer element. Endocrinology 147(1):590-8.

Majumder PK, Grisanzio C, O'Connell F, Barry M, Brito JM, Xu Q, Guney I, Berger R, Herman P, Bikoff R and others. 2008. A prostatic intraepithelial neoplasia-dependent p27 Kip1 checkpoint induces senescence and inhibits cell proliferation and cancer progression. Cancer Cell 14(2):146-55.

Malumbres M, Barbacid M. 2009. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer 9(3):153-66.

Mehraein-Ghomi F, Lee E, Church DR, Thompson TA, Basu HS, Wilding G. 2008. JunD mediates androgen-induced oxidative stress in androgen dependent LNCaP human prostate cancer cells. Prostate 68(9):924-34.

Melia MJ, Bofill N, Hubank M, Meseguer A. 1998. Identification of androgen-regulated genes in mouse kidney by representational difference analysis and random arbitrarily primed polymerase chain reaction. Endocrinology 139(2):688-95.

Mirochnik Y, Veliceasa D, Williams L, Maxwell K, Yemelyanov A, Budunova I, Volpert OV. 2012. Androgen receptor drives cellular senescence. PLoS One 7(3):e31052.

Miyamoto H, Marwah P, Marwah A, Lardy H, Chang C. 2003. 3 beta-acetoxyandrost-1,5-diene-17-ethylene ketal functions as a potent antiandrogen with marginal agonist activity. Proc Natl Acad Sci U S A 100(8):4440-4.

Moehren U, Papaioannou M, Reeb CA, Grasselli A, Nanni S, Asim M, Roell D, Prade I, Farsetti A, Baniahmad A. 2008. Wild-type but not mutant androgen receptor inhibits expression of the hTERT telomerase subunit: a novel role of AR mutation for prostate cancer development. FASEB J 22(4):1258-67.

Nandana S, Chung LW. 2014. Prostate cancer progression and metastasis: potential regulatory pathways for therapeutic targeting. Am J Clin Exp Urol 2(2):92-101.

Nantermet PV, Xu J, Yu Y, Hodor P, Holder D, Adamski S, Gentile MA, Kimmel DB, Harada S, Gerhold D and others. 2004. Identification of genetic pathways activated by the androgen receptor during the induction of proliferation in the ventral prostate gland. J Biol Chem 279(2):1310-22.

Nardella C, Clohessy JG, Alimonti A, Pandolfi PP. 2011. Pro-senescence therapy for cancer treatment. Nat Rev Cancer 11(7):503-11.

Nemeth JA, Yousif R, Herzog M, Che M, Upadhyay J, Shekarriz B, Bhagat S, Mullins C, Fridman R, Cher ML. 2002. Matrix metalloproteinase activity, bone matrix turnover, and tumor cell proliferation in prostate cancer bone metastasis. J Natl Cancer Inst 94(1):17-25.

Pedeux R, Sengupta S, Shen JC, Demidov ON, Saito S, Onogi H, Kumamoto K, Wincovitch S, Garfield SH, McMenamin M and others. 2005. ING2 regulates the onset of replicative senescence by induction of p300-dependent p53 acetylation. Mol Cell Biol 25(15):6639-48.

Pena PV, Davrazou F, Shi X, Walter KL, Verkhusha VV, Gozani O, Zhao R, Kutateladze TG. 2006. Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. Nature 442(7098):100-3.

Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC. 1999. Rapid signalling by androgen receptor in prostate cancer cells. Oncogene 18(46):6322-9.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9):e45.

Prieur A, Peeper DS. 2008. Cellular senescence in vivo: a barrier to tumorigenesis. Curr Opin Cell Biol 20(2):150-5.

Protopopov AI, Li J, Winberg G, Gizatullin RZ, Kashuba VI, Klein G, Zabarovsky ER. 2002. Human cell lines engineered for tetracycline-regulated expression of tumor suppressor candidate genes from a frequently affected chromosomal region, 3p21. J Gene Med 4(4):397-406.

Pungsrinont T. 2015. Interaction of ING2 and AR signaling in PCa cells [Master thesis]. Jena: Friedrich Schiller University.

Qi J, Tripathi M, Mishra R, Sahgal N, Fazli L, Ettinger S, Placzek WJ, Claps G, Chung LW, Bowtell D and others. 2013. The E3 ubiquitin ligase Siah2 contributes to castration-resistant prostate cancer by regulation of androgen receptor transcriptional activity. Cancer Cell 23(3):332-46.

Rahman M, Miyamoto H, Chang C. 2004. Androgen receptor coregulators in prostate cancer: mechanisms and clinical implications. Clin Cancer Res 10(7):2208-19.

Ramsay AK, Leung HY. 2009. Signalling pathways in prostate carcinogenesis: potentials for molecular-targeted therapy. Clin Sci (Lond) 117(6):209-28.

Reeb CA, Gerlach C, Heinssmann M, Prade I, Ceraline J, Roediger J, Roell D, Baniahmad A. 2011. A designed cell-permeable aptamer-based corepressor peptide is highly specific for the androgen receptor and inhibits prostate cancer cell growth in a vector-free mode. Endocrinology 152(6):2174-83.

Rennie PS, Bruchovsky N, Leco KJ, Sheppard PC, McQueen SA, Cheng H, Snoek R, Hamel A, Bock ME, MacDonald BS and others. 1993. Characterization of two cis-acting DNA elements involved in the androgen regulation of the probasin gene. Mol Endocrinol 7(1):23-36.

Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A, Chinnaiyan AM. 2004. ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia 6(1):1-6.

Roediger J, Hessenkemper W, Bartsch S, Manvelyan M, Huettner SS, Liehr T, Esmaeili M, Foller S, Petersen I, Grimm MO and others. 2014. Supraphysiological androgen levels induce cellular senescence in human prostate cancer cells through the Src-Akt pathway. Mol Cancer 13:214.

Roell D, Baniahmad A. 2011. The natural compounds atraric acid and N-butylbenzene-sulfonamide as antagonists of the human androgen receptor and inhibitors of prostate cancer cell growth. Mol Cell Endocrinol 332(1-2):1-8.

Rokhlin OW, Taghiyev AF, Guseva NV, Glover RA, Chumakov PM, Kravchenko JE, Cohen MB. 2005. Androgen regulates apoptosis induced by TNFR family ligands via multiple signaling pathways in LNCaP. Oncogene 24(45):6773-84.

Rubin SJ, Hallahan DE, Ashman CR, Brachman DG, Beckett MA, Virudachalam S, Yandell DW, Weichselbaum RR. 1991. Two prostate carcinoma cell lines demonstrate abnormalities in tumor suppressor genes. J Surg Oncol 46(1):31-6.

Ruthenburg AJ, Allis CD, Wysocka J. 2007. Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. Mol Cell 25(1):15-30.

Saito M, Kumamoto K, Robles AI, Horikawa I, Furusato B, Okamura S, Goto A, Yamashita T, Nagashima M, Lee TL and others. 2010. Targeted disruption of Ing2 results in defective spermatogenesis and development of soft-tissue sarcomas. PLoS One 5(11):e15541.

Santos-Rosa H, Schneider R, Bannister AJ, Sherriff J, Bernstein BE, Emre NC, Schreiber SL, Mellor J, Kouzarides T. 2002. Active genes are tri-methylated at K4 of histone H3. Nature 419(6905):407-11.

Sarkar S, Brautigan DL, Parsons SJ, Larner JM. 2014. Androgen receptor degradation by the E3 ligase CHIP modulates mitotic arrest in prostate cancer cells. Oncogene 33(1):26-33.

Sarker KP, Kataoka H, Chan A, Netherton SJ, Pot I, Huynh MA, Feng X, Bonni A, Riabowol K, Bonni S. 2008. ING2 as a novel mediator of transforming growth factor-beta-dependent responses in epithelial cells. J Biol Chem 283(19):13269-79.

Satpathy S, Guerillon C, Kim TS, Bigot N, Thakur S, Bonni S, Riabowol K, Pedeux R. 2014. SUMOylation of the ING1b tumor suppressor regulates gene transcription. Carcinogenesis 35(10):2214-23.

Schaefer A, Karaulanov E, Stapf U, Doderlein G, Niehrs C. 2013. Ing1 functions in DNA demethylation by directing Gadd45a to H3K4me3. Genes Dev 27(3):261-73.

Schneikert J, Peterziel H, Defossez PA, Klocker H, de Launoit Y, Cato AC. 1996. Androgen receptor-Ets protein interaction is a novel mechanism for steroid hormone-mediated down-modulation of matrix metalloproteinase expression. J Biol Chem 271(39):23907-13.

Schwarze SR, DePrimo SE, Grabert LM, Fu VX, Brooks JD, Jarrard DF. 2002. Novel pathways associated with bypassing cellular senescence in human prostate epithelial cells. J Biol Chem 277(17):14877-83.

Scott M, Boisvert FM, Vieyra D, Johnston RN, Bazett-Jones DP, Riabowol K. 2001. UV induces nucleolar translocation of ING1 through two distinct nucleolar targeting sequences. Nucleic Acids Res 29(10):2052-8.

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88(5):593-602.

Sharma A, Yeow WS, Ertel A, Coleman I, Clegg N, Thangavel C, Morrissey C, Zhang X, Comstock CE, Witkiewicz AK and others. 2010. The retinoblastoma tumor suppressor controls androgen signaling and human prostate cancer progression. J Clin Invest 120(12):4478-92.

Shen DH, Chan KY, Khoo US, Ngan HY, Xue WC, Chiu PM, Ip P, Cheung AN. 2005. Epigenetic and genetic alterations of p33ING1b in ovarian cancer. Carcinogenesis 26(4):855-63.

Shi X, Hong T, Walter KL, Ewalt M, Michishita E, Hung T, Carney D, Pena P, Lan F, Kaadige MR and others. 2006. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature 442(7098):96-9.

Shi XB, Tepper CG, deVere White RW. 2008. Cancerous miRNAs and their regulation. Cell Cycle 7(11):1529-38.

Shinoura N, Muramatsu Y, Nishimura M, Yoshida Y, Saito A, Yokoyama T, Furukawa T, Horii A, Hashimoto M, Asai A and others. 1999. Adenovirus-mediated transfer of p33ING1 with p53 drastically augments apoptosis in gliomas. Cancer Res 59(21):5521-8.

Siegel R, Naishadham D, Jemal A. 2013. Cancer statistics, 2013. CA Cancer J Clin 63(1):11-30.

Silverstein RA, Ekwall K. 2005. Sin3: a flexible regulator of global gene expression and genome stability. Curr Genet 47(1):1-17.

Smith JA, Jr., Soloway MS, Young MJ. 1999. Complications of advanced prostate cancer. Urology 54(6A Suppl):8-14.

Snoek R, Rennie PS, Kasper S, Matusik RJ, Bruchovsky N. 1996. Induction of cell-free, in vitro transcription by recombinant androgen receptor peptides. J Steroid Biochem Mol Biol 59(3-4):243-50.

Soliman MA, Berardi P, Pastyryeva S, Bonnefin P, Feng X, Colina A, Young D, Riabowol K. 2008. ING1a expression increases during replicative senescence and induces a senescent phenotype. Aging Cell 7(6):783-94.

Soliman MA, Riabowol K. 2007. After a decade of study-ING, a PHD for a versatile family of proteins. Trends Biochem Sci 32(11):509-19.

Stavridi F, Karapanagiotou EM, Syrigos KN. 2010. Targeted therapeutic approaches for hormone-refractory prostate cancer. Cancer Treat Rev 36(2):122-30.

Takahashi M, Seki N, Ozaki T, Kato M, Kuno T, Nakagawa T, Watanabe K, Miyazaki K, Ohira M, Hayashi S and others. 2002. Identification of the p33(ING1)-regulated genes that include cyclin B1 and proto-oncogene DEK by using cDNA microarray in a mouse mammary epithelial cell line NMuMG. Cancer Res 62(8):2203-9.

Tallen G, Riabowol K. 2014. Keep-ING balance: tumor suppression by epigenetic regulation. FEBS Lett 588(16):2728-42.

Tanner T, Claessens F, Haelens A. 2004. The hinge region of the androgen receptor plays a role in proteasome-mediated transcriptional activation. Ann N Y Acad Sci 1030:587-92.

Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP. 1995. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 332(21):1393-8.

Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, Arora VK, Kaushik P, Cerami E, Reva B and others. 2010. Integrative genomic profiling of human prostate cancer. Cancer Cell 18(1):11-22.

Thakur S, Feng X, Qiao Shi Z, Ganapathy A, Kumar Mishra M, Atadja P, Morris D, Riabowol K. 2012. ING1 and 5-azacytidine act synergistically to block breast cancer cell growth. PLoS One 7(8):e43671.

Thakur S, Singla AK, Chen J, Tran U, Yang Y, Salazar C, Magliocco A, Klimowicz A, Jirik F, Riabowol K. 2014. Reduced ING1 levels in breast cancer promotes metastasis. Oncotarget 5(12):4244-56.

Thalappilly S, Feng X, Pastyryeva S, Suzuki K, Muruve D, Larocque D, Richard S, Truss M, von Deimling A, Riabowol K and others. 2011. The p53 tumor suppressor is stabilized by inhibitor of growth 1 (ING1) by blocking polyubiquitination. PLoS One 6(6):e21065.

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R and others. 2005. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science 310(5748):644-8.

Toyama T, Iwase H, Yamashita H, Hara Y, Sugiura H, Zhang Z, Fukai I, Miura Y, Riabowol K, Fujii Y. 2003. p33(ING1b) stimulates the transcriptional activity of the estrogen receptor alpha via its activation function (AF) 2 domain. J Steroid Biochem Mol Biol 87(1):57-63.

Tsang FC, Po LS, Leung KM, Lau A, Siu WY, Poon RY. 2003. ING1b decreases cell proliferation through p53-dependent and -independent mechanisms. FEBS Lett 553(3):277-85.

Tsao CK, Galsky MD, Small AC, Yee T, Oh WK. 2012. Targeting the androgen receptor signalling axis in castration-resistant prostate cancer (CRPC). BJU Int 110(11):1580-8.

Tsihlias J, Zhang W, Bhattacharya N, Flanagan M, Klotz L, Slingerland J. 2000. Involvement of p27Kip1 in G1 arrest by high dose 5 alpha-dihydrotestosterone in LNCaP human prostate cancer cells. Oncogene 19(5):670-9.

Tyagi RK, Lavrovsky Y, Ahn SC, Song CS, Chatterjee B, Roy AK. 2000. Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. Mol Endocrinol 14(8):1162-74.

Uhlen M, Oksvold P, Fagerberg L, Lundberg E, Jonasson K, Forsberg M, Zwahlen M, Kampf C, Wester K, Hober S and others. 2010. Towards a knowledge-based Human Protein Atlas. Nat Biotechnol 28(12):1248-50.

Umesono K, Evans RM. 1989. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 57(7):1139-46.

Unoki M, Kumamoto K, Robles AI, Shen JC, Zheng ZM, Harris CC. 2008. A novel ING2 isoform, ING2b, synergizes with ING2a to prevent cell cycle arrest and apoptosis. FEBS Lett 582(28):3868-74.

Unoki M, Kumamoto K, Takenoshita S, Harris CC. 2009. Reviewing the current classification of inhibitor of growth family proteins. Cancer Sci 100(7):1173-9.

Valdes-Mora F, Clark SJ. 2014. Prostate cancer epigenetic biomarkers: next-generation technologies. Oncogene 0.

Valdez CD, Davis JN, Odeh HM, Layfield TL, Cousineau CS, Berton TR, Johnson DG, Wojno KJ, Day ML. 2011. Repression of androgen receptor transcription through the E2F1/DNMT1 axis. PLoS One 6(9):e25187.

Varisli L, Gonen-Korkmaz C, Syed HM, Bogurcu N, Debelec-Butuner B, Erbaykent-Tepedelen B, Korkmaz KS. 2012. Androgen regulated HN1 leads proteosomal degradation of androgen receptor (AR) and negatively influences AR mediated transactivation in prostate cells. Mol Cell Endocrinol 350(1):107-17.

Walzak AA, Veldhoen N, Feng X, Riabowol K, Helbing CC. 2008. Expression profiles of mRNA transcript variants encoding the human inhibitor of growth tumor suppressor gene family in normal and neoplastic tissues. Exp Cell Res 314(2):273-85.

Wang L, Hsu CL, Chang C. 2005. Androgen receptor corepressors: an overview. Prostate 63(2):117-30.

Wigler M, Pellicer A, Silverstein S, Axel R. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell 14(3):725-31.

Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S, Chung LW. 1994. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int J Cancer 57(3):406-12.

Wu M, Dickinson SI, Wang X, Zhang J. 2014. Expression and function of SIRT6 in muscle invasive urothelial carcinoma of the bladder. Int J Clin Exp Pathol 7(10):6504-13.

Wu MH, Ma WL, Hsu CL, Chen YL, Ou JH, Ryan CK, Hung YC, Yeh S, Chang C. 2010. Androgen receptor promotes hepatitis B virus-induced hepatocarcinogenesis through modulation of hepatitis B virus RNA transcription. Sci Transl Med 2(32):32ra35.

Xu K, Shimelis H, Linn DE, Jiang R, Yang X, Sun F, Guo Z, Chen H, Li W, Chen H and others. 2009. Regulation of androgen receptor transcriptional activity and specificity by RNF6-induced ubiquitination. Cancer Cell 15(4):270-82.

Xu Q, Lin HY, Yeh SD, Yu IC, Wang RS, Chen YT, Zhang C, Altuwaijri S, Chen LM, Chuang KH and others. 2007. Infertility with defective spermatogenesis and steroidogenesis in male mice lacking androgen receptor in Leydig cells. Endocrine 32(1):96-106.

Yang YC, Fu HC, Wu CY, Wei KT, Huang KE, Kang HY. 2013. Androgen receptor accelerates premature senescence of human dermal papilla cells in association with DNA damage. PLoS One 8(11):e79434.

Ythier D, Larrieu D, Brambilla C, Brambilla E, Pedeux R. 2008. The new tumor suppressor genes ING: genomic structure and status in cancer. Int J Cancer 123(7):1483-90.

Yu L, Thakur S, Leong-Quong RY, Suzuki K, Pang A, Bjorge JD, Riabowol K, Fujita DJ. 2013. Src regulates the activity of the ING1 tumor suppressor. PLoS One 8(4):e60943.

Zhou W, Wang G, Small CL, Liu Z, Weng CC, Yang L, Griswold MD, Meistrich ML. 2010. Gene expression alterations by conditional knockout of androgen receptor in adult sertoli cells of Utp14b(jsd/jsd) (jsd) mice. Biol Reprod 83(5):759-66.

Zhu ML, Kyprianou N. 2008. Androgen receptor and growth factor signaling cross-talk in prostate cancer cells. Endocr Relat Cancer 15(4):841-9.

Zhu Z, Luo Z, Li Y, Ni C, Li H, Zhu M. 2009. Human inhibitor of growth 1 inhibits hepatoma cell growth and influences p53 stability in a variant-dependent manner. Hepatology 49(2):504-12.

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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