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Titolo della tesi

NF-κB DEREGULATION IN PROSTATE CARCINOMA: IDENTIFICATION OF TARGET GENES AS POTENTIAL THERAPEUTIC TARGETS

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Summary

NF-κB family represents a group of inducible transcription factors, which regulates a large number of genes involved in inflammation[1], immunity[2], cell growth, proliferation[3], and survival[4]. Because so many crucial processes are regulated by these factors, is not surprising that dysregulation of this pathway is involved in the pathogenesis of several disease such as inflammatory conditions[5], autoimmunity[6], and cancer[7]. Indeed, constitutive and enhanced activation of NF-κB factors is a hallmark of many types of tumours including lymphoid malignancies[8], hepatocellular carcinoma[9], breast cancer[10], and colorectal cancer[11]. Since NF-κB target genes include regulators and inhibitors of apoptosis, and other factors that promote resistance to chemotherapeutic drugs used in conventional anti-cancer therapies[12], in recent years, many efforts have been made to develop inhibitory drugs that specifically target this pathway. However, although offering great clinical potential, inhibition of NF-κB *in vivo* can result in severe side effects, therefore, the main goal is to find a safe way to specifically target this pathway without interfering with general homeostasis. In this respect, a potential target gene is *GADD45B*. Indeed, the protein GADD45B is induced by NF-κB and plays a protective role towards JNK pathway-induced apoptosis[13]. The ability of GADD45B in inhibiting JNK activity is due to its ability to bind and block the upstream kinase MKK7 thus preventing JNK activation[14]. Tornatore *et al*. have shown the pro-survival role of GADD45B in multiple myeloma and effective targeting of this pathway, without significant side effects, using the D-tripeptide (DTP3), which interfere with GADD45B-MKK7 complex thus resulting in JNK-induced apoptosis[14]. The aim of this project was to assess the NF-κB expression in human prostate carcinoma and its activation status in prostate carcinoma cell models, and to determinate the role of NF-κB-dependent *GADD45B* anti-apoptotic gene in prostate carcinoma in order to evaluate the inhibition of its activity as a potential therapeutic strategy for this tumor.

1. Introduction

1.1. Etiopathogenesis and molecular biology of prostate cancer

Prostate cancer is one of most common types of cancer in men in industrialised countries[15]. If detected early, overall survival rate at five year is about 100%, by contrast, because in most cases it grows slowly and has no symptoms in early stages, prostate cancer is often diagnosed rather late and the survival rate drops to about 32%. Prostate carcinoma occurs in the prostate gland, which is an exocrine gland located in the pelvis of men, inferior to the bladder, and anterior to the rectum. The prostate is characterized by the presence of tubulo-alveolar glands lined with columnar epithelium, androgen-independent basal cells with putative stemness properties[16], and neuroendocrine cells that regulate the homeostasis of the prostatic epithelium, possibly through a paracrine mechanism[17]; prostate glands branch out into a fibromuscular stroma consisting of fibroblasts, muscle cells, and blood vessels. The prostate gland can be subdivided into five zones: anterior fibromuscular stroma, a periurethral gland region, a transition zone (TZ), a central zone (CZ), and a peripheral zone (PZ). In some cases, this zonal description is more convenient than classical lobar subdivision because many prostate diseases show a zonal distribution[18] (Fig 1-1). Approximately, 70% of adenocarcinomas arise in the PZ, 20% in the TZ and 10% in the CZ. By contrast, benign prostatic hyperplasia

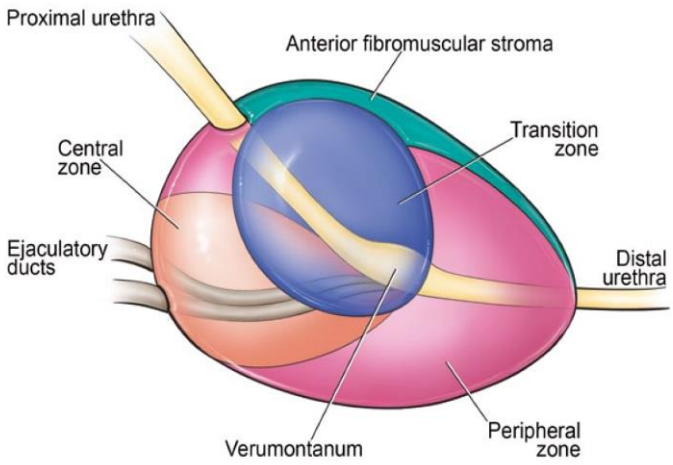

Figure 1-1. ***The zonal anatomy of the prostate*** [19]**.**

(BPH) mainly arise in the periurethral gland and TZ. About 95% of prostate cancers are adenocarcinomas[20] and, likewise many other types of tumours[21], they develop from an initially benign lesion through a multiphasic progression. The primitive, premalignant lesion is a low-grade prostatic intraepithelial neoplasia (PIN) and it represents the precursor of the prostatic adenocarcinoma[22,23]. Histologically, PIN is a dysplastic lesion and may be classified into PIN grades I, II, and III, depending on the disease severity. Whereas PINs grade I are not necessarily related to carcinomas development, PINs grade II and III are considered high grade PINs (HGPIN), and represent precursors of prostate carcinoma. Moreover, depending on PIN progression, several alterations may be found, such as nuclear enlargement, and altered nucleolar morphology[24].

Age is the most common risk factor for prostate carcinoma. Indeed, although it was observed that many primitive lesions can be identified at the age of 20-30[25], due to its slow onset, the incidence reach a peak at 70-74 years of age. Other risk factors include race/ethnicity, family history, and diet; regarding the role of diet, for example, the increase of incidence seems to be related to saturated fat and red meat consumption[26]. Moreover, recently, it was highlighted an important role of inflammation in prostate cancer pathogenesis; chronic inflammation would results in so-called proliferative inflammatory atrophy (PIA) histological lesions and subsequent transition from PIA to PIN[27]. PIAs consist in atrophic lesions, characterized by the presence of inflammatory infiltrates and active proliferation areas in response to cell injury and oxidative stress; they are very common in elderly subjects and can affect large regions of prostatic tissue. Pro-inflammatory stimuli that contribute to PIA development include infectious[28,29] and environmental agents[30–32], chemical and physical trauma, and urine reflux[33,34].

With the regard to the molecular events occurring in prostate cancer development, several mutations in proto-oncogenes and tumour suppressor genes, as well as epigenetic alteration were found (Fig 1-2). A most frequent and early epigenetic alteration in prostate cancer is the hypermethylation of CpG island in the promoter region of the glutathione S-transferase P1 gene, leading to its transcriptional repression[35,36]. GSTP1 is considered a “caretaker” gene carrying out an essential role in detoxifying cell towards carcinogens and free radicals and thus,

its inactivation, could be related to susceptibility towards dietary carcinogens and pro-inflammatory stimuli[37]. GSTP1 promoter hypermethylation is found in 90% of prostatic adenocarcinomas and it is also frequent in PIA lesions. Moreover, this event occurs frequently in PINs, suggesting that it could represent an early event in disease pathogenesis[38,39]. In addition, the presence of this alteration in PIA could be due to the inflammatory environment as it was observed for FMR1 and HPRT genes in response to nitric oxide production induced by interleukin 1 beta[40]. In general, hypermethylation seems to be a relevant mechanism in prostate cancer pathogenesis, indeed, DNA methyltransferase inhibition with decitabine is associated with a retarded PIN progression in TRAMP mice[41].

Additional alterations in prostate carcinoma are tumour suppressor genes mutations; one common deregulation affect NKX3.1 homeobox gene expression. NKX3.1 encodes for a prostate-specific transcription factor that plays a key role in prostate gland homeostasis and development[42], however, although it is regulated by androgens, its expression precedes androgens production during prostate development suggesting the existence of an alternative pathway for its expression[43].

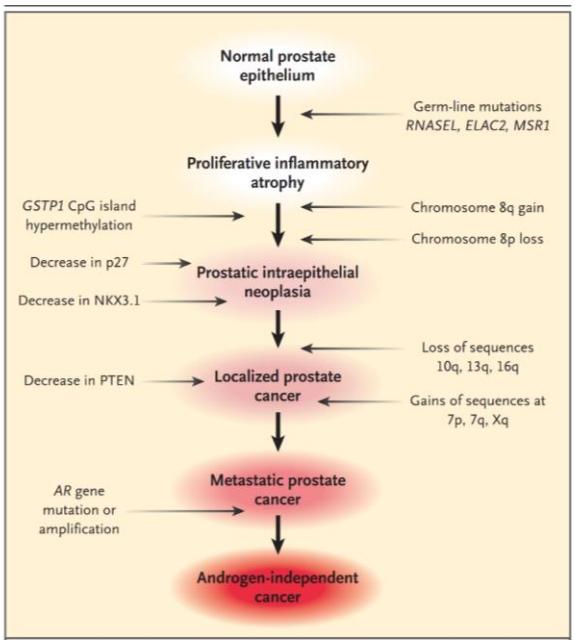

Figura 1-2. ***Molecular pathogenesis of prostate cancer*** [44]

In prostate cancer, the chromosome 8p21 region, where NKX3.1 gene is located, is often in heterozygous condition and this alteration seems to represent an early event[45–47]; moreover, NKX3.1 doesn't follow Knudson's "two-hit hypothesis" because even the loss of a single gene is able to promote PINs development inducing a state of haploinsufficiency[48]. However, in mouse models, although NKX3.1 heterozygous condition is able to induce PINs formation, it seems not to be sufficient for the development of invasive prostate carcinoma[48]. In any case, NKX3.1 activity loss seems to be related to cancer progression[49] and with the development of androgen-independent prostate cancer[45] (Fig. 1.2). PTEN is another important tumour suppressor gene that plays a key role in prostate carcinoma pathogenesis; this gene is located on human chromosome 10q23 and regulates PI3K/AKT/mTOR axis by inhibiting phosphatidylinositol 3-kinase (PI3K). In prostate carcinoma, PTEN is often mutated, deleted[50–52], or downregulated via hypermethylation of its promoter[53] and its heterozygous condition induces a state of haploinsufficiency promoting tumours development in mouse models[54,55]. Moreover, although it represents a late event[56], loss of PTEN activity is also frequent and shows synergistic effects with NKX3.1 loss of function[57]. PTEN role in prostate cancer pathogenesis was studied in mouse models[58]; it was observed that the gene dosage is very important for disease progression and that, although it plays a key role in tumour initiation, heterozygous condition is not sufficient to promote an invasive phenotype[59]. However, combination of different gene mutations or complete PTEN loss of function can induce the development of an aggressive phenotype. The key role of PTEN is also confirmed by conditional knockout studies in which homozygous mutations in p53[60], Rb[61], and NKX3.1[48] promote PINs formation but never adenocarcinomas. Others frequent alteration found in prostate carcinoma are mutations in tumour suppressor gene CDKN1B that encodes the cyclin-dependent kinase inhibitor $p27^{KIP1}$. Even then, in mouse models, heterozygous condition lead to haploinsufficiency[62] and its downregulation or loss of function correlate with high grade tumours development and lead to a poor prognosis[63,64]. In addiction, $p27^{KIP1}$ is also regulated by PI3K/AKT/mTOR axis via PI3K which downregulate its expression, therefore, PTEN loss of function reduces $p27^{KIP1}$ protein levels. Indeed, although a reduction in $p27^{KIP1}$ activity is only

associated with BPH development is mouse models, concurrent PTEN loss of function leads to development of invasive prostate tumours[65]. Other two important genes in prostate cancer pathogenesis are KLF6 e ATFB1. KLF6 encodes for a zinc-finger transcription factor that interacts with GC box promoters elements[66] and it is able to upregulate p21 protein in p53-independent manner[67]. Moreover, it interacts with cyclin D1 inhibiting Rb phosphorylation[68]. Genomic 10p15 region, at which gene is located, is deleted in 55% of adenocarcinomas[69,70], and a loss of heterozygosity was found in about 20% of tumours. On the other hand, ATFB1 encodes for a transcription factor that is able to downregulate α-fetoprotein expression[71] and to enhance p21 activity[72]. Alterations in ATFB1 including deletions and missense mutation[73] and their contribution in prostate cancer pathogenesis were studied in mouse models[74]. Other mutations include alterations in tumour suppressor genes RB1 and TP53. Rb protein plays a key role in cell cycle control by binding E2F transcription factor and its mutation seems to be an early event in prostate cancer[75]. However, although Rb mutations do not directly promote tumorigenesis in mouse models, Rb LOH is frequently observed in many carcinomas[75–77] suggesting that it could take place during tumour progression. Mutations and deletions in TP53 gene are also frequent although they are mainly found in advanced and recurrent tumours[78] and could therefore be considered late events in tumour progression[79]; moreover, p53 accumulation revealed by immunohistochemical analysis is associated with a poor prognosis[80]. Another peculiar alteration in prostate cancer is a chromosomal rearrangement that involves androgen-regulated gene TMPRSS2 with ETS-related gene (ERG and ETV1)[81]. The most common variant is TMPRSS2-ERG and it occurs in the majority of prostate cancers that overexpress EGR[82]. The resulting fusion protein drives EGR transcription factor overexpression under androgen responsive TMPRSS2 promoter[83] and is frequently found in HGPIN and advanced tumours. Although it is unable to induce the development of adenocarcinomas, TMPRSS2-ERG expression promotes PINs formations in mouse models and associations with alterations in other pathways could play a key role in the transition to an aggressive phenotype[83]. Another gene involved in cancer pathogenesis is c-Myc, which encodes for a transcription factor implicated in cell transformation and

proliferation[84]; this oncogene is often amplified or overexpressed both in primary tumours and in metastasis and is associated with poor prognosis[85,86]. Finally, androgen signaling plays a pivotal role in prostate cancer and is mediated by androgen receptor (AR). AR protein belongs to nuclear transcription factors receptors family and its activity is crucial in prostate development and prostate epithelial cell survival[87]. Androgens relevance in prostate cancer is confirmed by the results obtained following androgen deprivation therapy (ADT), however, although ADT leads to disease remission in 90% of cases, cancer often progress to a castrate-resistant prostate cancer (CRPC) type that is refractory to treatment[88]. Anyway, despite being defined as "androgen-independent" tumour, CRPC still maintains a responsiveness to androgens[89]. Underlying mechanisms include AR gene amplification[90] and mutations capable of enhancing receptor sensitivity[91]. Another mechanism is ligand-independent activation of AR; many overespressed growth factors in tumour microenvironment are able to mediate AR activity inducing androgen-responsive gene expression also in absence of androgens[92].

1.2. NF-κB transcription factors: an overview

NF-κB (nuclear factor-kB) transcription factors family was discovered for the first time in 1986 as regulators of κB light chain in mature B and plasma cells[93]. NF-κB target genes are regulators of many key processes such as proliferation, immune response, apoptosis, and can be induced by inflammatory cytokines and upon physical stresses[94,95] (Fig. 1-3). NF-κB family includes five structurally related members that associate to each other to form homodimers and heterodimers with different transcriptional activities[96] and bind the consensus κB site 5'-GGGPuNNPyPyCC-3'[97]. Based on functional activity and molecular structure, NF-κB transcription factors can be divided into two main classes: class I factors that includes proteins p50 (NF-κB1) and p52 (NF-κB2), and class II factors that includes p65 (RelA), c-Rel, and RelB (Fig 1-5). All members have a 300 amino acid long domain called Rel Homology Region (RHR); this domain is located in the N-terminal and is responsible for dimerization, DNA binding, interaction with inhibitory IkBs proteins, and nuclear translocation[97–100].

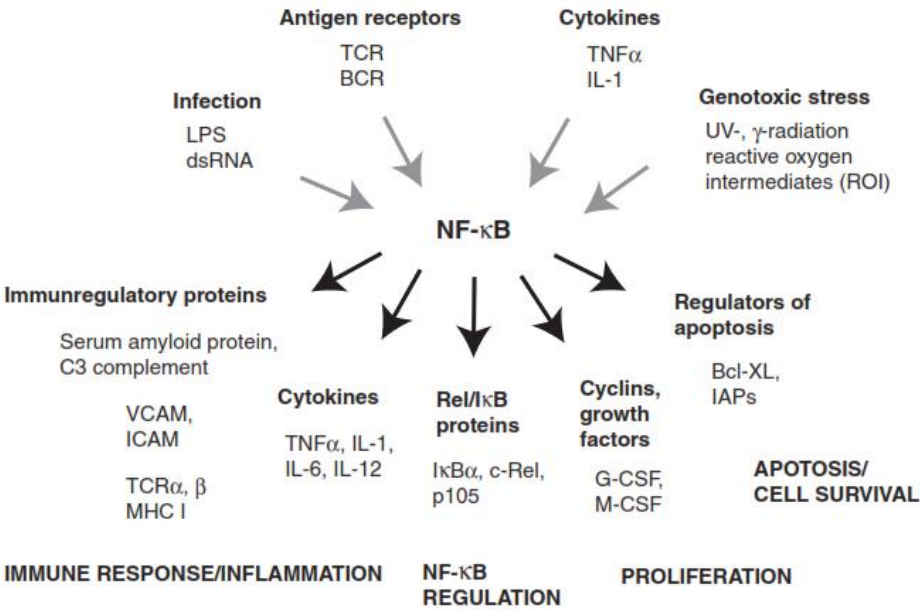

Figure 1-3. ***NF-κB activators and regulated factors*** [95].

The RHR domain, in turn, presents an N- and C-terminus, and a dimerization domain; C-terminus of RHR also contains a nuclear localization signal (NLS)[101]. The main difference between the two classes of proteins is the presence or absence of carboxy-terminal transactivation domains (TAD) (Fig 1-4); indeed, class I factors do not presents TAD and they are therefore not able to activate gene transcription unless forming heterodimers with class II factors. Moreover, class I factors p50 and p52 are generated by processing of the precursor proteins p105 and p100[102,103], respectively, and the formation of p50-p50, p52-p52 homodimers can repress gene transcription[104]. Among various homodimers and heterodimers, p50-p65 complex clearly represent the most abundant, most likely due to its increased stability compared to others dimers such as p50-p50 and p65-p65[105]. Because of the large number of genes regulated by these family of transcription factors, the activity of NF-κB pathway is finely regulated at multiple levels.

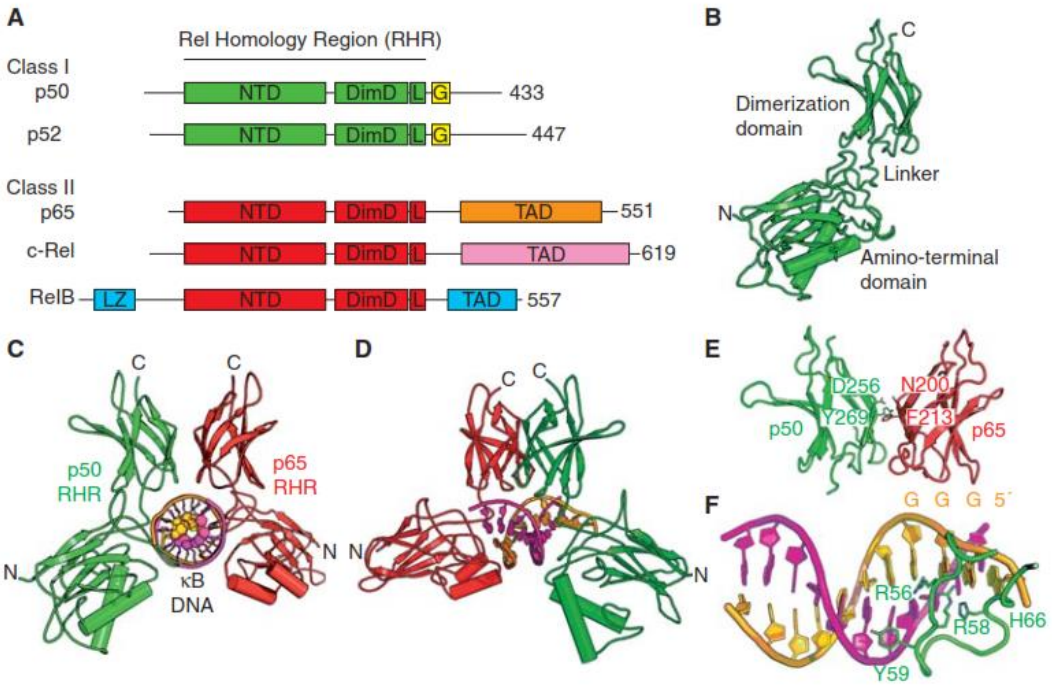

Figure 1-4. ***NF-κB transcription factors of Class I and Class II.*** **A)** Schematic representation of the two class of factors. NTD (N-terminus), DimD (dimerization domain), G (glycine rich region), L (nuclear localization domain), TAD (transactivation domain, LZ (leucine-zipper domain). **B)** Ribbon diagram representation of RHR from p50, and **C) D)** from p50-p65 heterodimer. **E) F)** NF-κB p50-p65 heterodimer dimerization domains with key amino acid side chains and key base-contacting amino acid residues labelled [106].

One mechanism of regulation is represented by inhibitory IκB proteins; indeed, in unstimulated cells, NF-κB dimers are retained in an inactive form in the cytosol through their interaction with IκB (inhibitor of kappa B) proteins[107]. IκB proteins include "classical" IκBα, IκBβ, IκBε, precursors proteins p100 and p105, and "nonclassical" IκBs Bcl-3, IκBζ, IκBNS (IκBδ) (Fig. 1-5). Although they show different structures, all of them present a common ankyrin repeat domain (ARD) element. The IκBs p100 and p105 also contain a glycine rich region (GRR) that is necessary for processing and generation of p52 and p50[108]. ARD contains six ankyrin repeats (ANK) which, in turn, consist of 33 amino acid sequence[109]; furthermore, an ARD can contain from six to nine ANK, depending of protein type, and it is responsible for the interaction and activity of IκBs towards NF-κB transcription factors. Classical IκBs IκBα, IκBβ, and IκBε also contain an N-terminal signal responsive region (SRR), whereas IκBα, IκBβ and Bcl-3 contain a proline-, glutamic acid-, serine-, and threonine-rich region (PEST) involved in protein turnover[110] and inhibiting NF-κB DNA binding[111] (Fig. 1-5). Moreover, SRR region is essential for protein activity because contains ubiquitination, phosphorylation, and nuclear export signal[112].

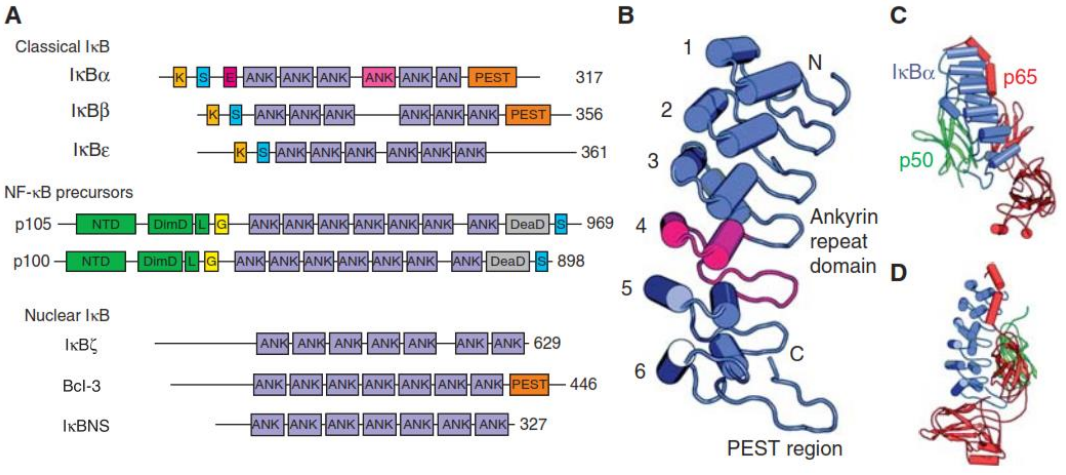

Figure 1-5. ***IkB proteins*****. A)** Schematic representation of Ikb proteins; K (ubiquitination signal), S (phosphorylation sites), E (nuclear export signal), ANK (ankyrin repeat), PEST (proline-, glutamic acid-, serine-, and threonine-rich region. **B)** IκBα ribbon diagram representation. **C) D)** Ribbon diagram of the NF-κB-IκBα complex [106].

Nonclassical IκBs p100 and p105 show a different mechanism of action; unlike classical IκBs that form 1:1 complexes with NF-κB factors, p100 and p105 present an oligomerization domain and can assembly into larger multiprotein complexes inhibiting more than one NF-κB dimer[113]. Finally, other nonclassical IκB proteins Bcl-3, IκBζ, IκBNS are defined nuclear IκBs because of their predominant nuclear localization; these proteins preferentially interact with p50 monomer and they are able both to activate and to repress gene transcription[104,114,115].

The other class of molecule involved in the regulation of NF-κB pathway are upstream IKKs (IκB kinase) kinases IKKα, IKKβ e IKKγ (NEMO) (Fig. 1-6); upon phosphorylation they assemble into multiproteic complexes and promote the release of NF-κB dimers by IκBs phosphorylating them and thus inducing their ubiquitination[116]. IKKα and IKKβ possess an N-terminal kinase domain, a leucine-zipper domain required for dimerization, and a C-terminal helix-loop-helix domain involved in kinase activity[117]; C-terminus also possesses a binding site for the protein IKKγ (NEMO)[118] that act as a scaffold-protein (Fig.1-6). The role of IKKγ as adapter subunit of IKK complex is conferred by the presence of an N-terminal coiled-coil domain (CC1) that mediates interaction with IKKα and IKKβ[119].

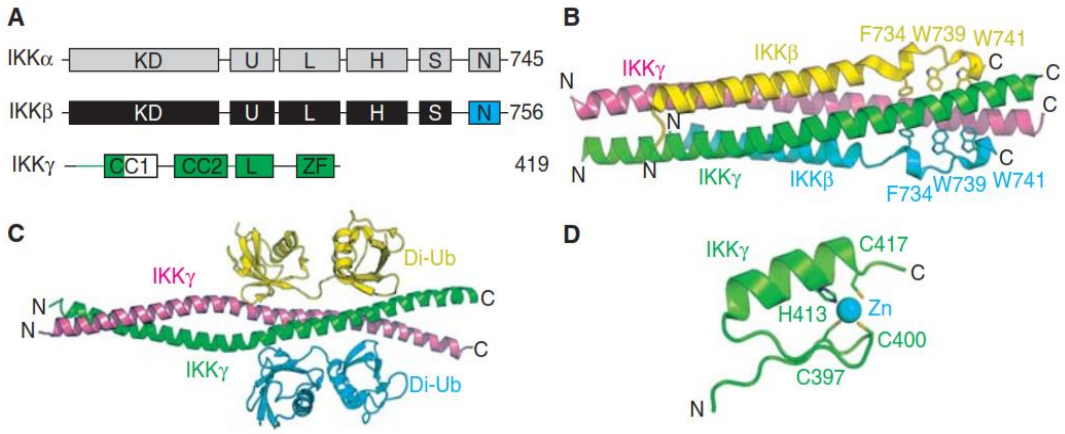

Figure 1-6. ***IKK proteins.*** **A)** Schematic representation of IKK proteins. KD (kinase domain), U (ubiquitin-like domain), L (leucine-zipper domain), H (helix-loop-helix domain), S (serine-rich region), N (IKKγ/NEMO binding site), CC (coiled-coil domain), ZF (zinc finger domain). **B)** Ribbon diagram representation of IKKγ-IKKβ complex. **C)** Ribbon diagram representation of ubiquitin- IKKγ complex and **D)** zinc finger domain [106].

Furthermore, IKKγ also possesses a C-terminal IκBα binding domain[120], and a second coiled-coil domain (CC2) that, together with a leucine zipper domain form the ubiquitination binding site[121].

There are currently two known mechanisms of NF-κB activation: canonical and non-canonical pathways[122] (Fig 1-7). The canonical pathway is triggered by stimulus such as lipopolysaccharide (LPS), inflammatory cytokines such as tumour necrosis factor α (TNF-α), and results in the activation of IKK complex formed by IKKα, IKKβ and IKKγ. Upon phosphorylation, IKKβ phosphorylates members of IκB family, such as IκBα and p105, which sequester NF-κB factors in the cytoplasm. IKK complex can also phosphorylate NF-κB transcription factors modulating their activity[123]; for instance, a key phosphorylation in canonical pathway is represented by phosphorylation of p65 Ser536 that enhances p65 transactivation capabilities[124]. These events result in IκBs ubiquitination and proteasomal degradation and the consequent release and nuclear translocation of NF-κB transcription factors[125] (Fig 1-7).

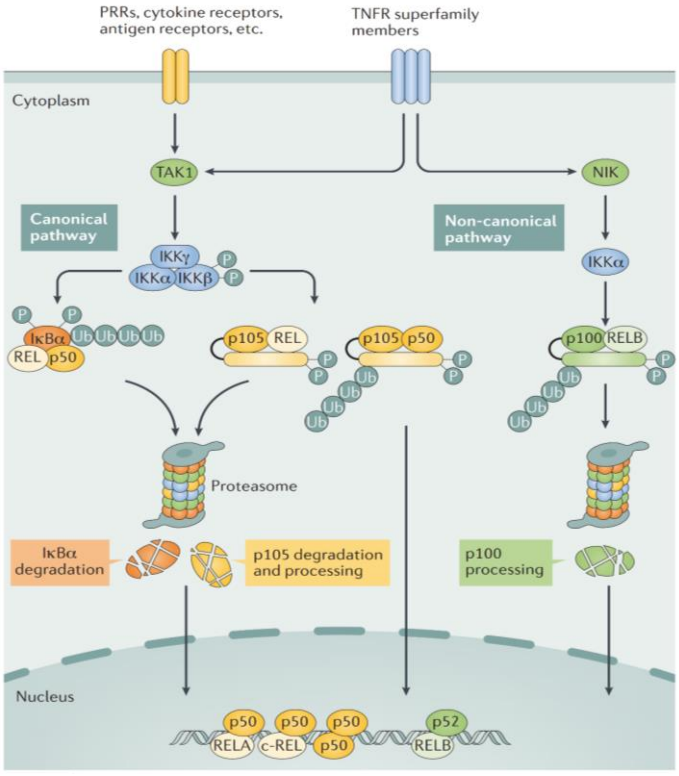

Figura 1-7. ***NF-κB canonical and non-canonical pathways.*** REL is RelA (p65) or c-Rel. [126]

By contrast, the activation of non-canonical pathway is strictly dependent on p100 processing, is induced by a subset of tumour necrosis factor receptor (TNFR) superfamily members, such as BAFF, lymphotoxin B, and is mainly active in B cells. Upon stimulation, activated NF-κB-inducing kinase (NIK) phosphorylates and activates IKKα, which in turn phosphorylates p100, promoting degradation of the C-terminal IκB-like structure of p100 and leading to the generation of p52 and the nuclear translocation of p52 and RelB[127].

1.3. NF-κB in prostate cancer

NF-κB activation is frequent event in many tumours as well as in prostate cancer. Overexpression and subcellular localization of p65 transcription factor were studied by immunohistochemical analysis and correlate with tumour grade, PSA serum levels, disease recurrance[128], biochemical relapse[129], and metastasis formation[130]. NF-κB activation was observed in PINs[131] and although it is also presents at low level in normal prostate tissue, NF-κB activity increases during tumour progression suggesting that high constitutive activation represents a late event[132]. Moreover, a reciprocal relationship between transcription factor p65 and androgens signaling exists[133,134] since AR activates NF-κB in absence of androgens and it is able to downregulate it when androgens are present[135], whereas, in turn, NF-κB can upregulate AR expression because AR gene is one of its transcriptional target[136]. However, it was also observed that androgen stimulation is able to positively regulate NF-κB through non-canonical pathway activation[137], and that, in absence of androgens, activation of the non-canonical pathway is able to promote androgen-dependent cell line growth[138]. It appears that androgens independence is sustained by an aberrant AR activation through a ligand-independent mechanism mediated by p52[139]. Furthermore, as noted in mouse models, also canonical NF-κB activation is involved in androgens independence development[140].

As mentioned above, NF-κB increases inflammatory response and, in communication with cells of immune system, establishes a positive feedback mechanism promoting carcinogenesis and tumour progression through proinflammatory cytokines release[141]. It also exists a positive feedback mechanism between NF-κB and ETS transcription factor ESE1/ELF3 mediated by IL-1β; this cytokine is strongly involved in prostate inflammation[142] and it induces ESE1/ELF3 p65-mediated transcription that in turn is able both to regulate p50 transcription and to physically interact with p50 and p65, thus promoting their nuclear translocation[143]. Even oncogenic TMPRSS2/ERG fusion protein isoform induces NF-κB mediated transcription; it was observed that TMPRSS2/ERG enhances p65 activity through its phosphorylation at Ser536[144].

NF-κB activation also correlates with metastatic phenotype acquisition; it was demonstrated that NF-κB inhibition, both *in vitro* and *in vivo*, blocks

proangiogenic factors VEGF, IL-8 and MMP-9 production thus reducing angiogenic process, invasive growth, and metastasis formation[145]. Moreover, NF-κB is involved in neoplastic cell adhesion promoting endothelial transmigration through CXCL12 chemokine and CXCR4 receptor upregulation[146]; this mechanism has been related to bone metastasis formation[147], indeed, *in vivo* inhibition of NF-κB activity reduces PC3 cell growth in bone, whereas, on the other hand, NF-κB activation promotes LNCaP cell growth[148]. Other mechanisms in bone metastasis formation include NF-κB mediated production of bone morphogenetic proteins (BMPs)[149] and alterations in IKKα. Indeed, it was observed that IKKα inactivating mutations generate a slow growth phenotype an inhibit bone metastasis formation in TRAMP mouse models; this process seems to be related to maspin expression that is downregulated by IKKα-NF-κB activation and, conversely, upregulated by receptor activator of nuclear factor kappa-B ligand (RANKL). Maspin inhibition, in fact, correlates with metastasis development[150].

NF-κB also plays an important role in cell survival and apoptosis resistance; indeed, bcl-2 antiapoptotic factor expression is upregulated by NF-κB activity[151].

1.4. MAPK pathway

c-Jun N-terminal kinases (JNKs) along with extracellular signal-regulated kinases (ERKs) and p38 kinases are members of the mitogen-activated protein kinases (MAPKs) family and are involved in proliferation, survival and differentiation[152]. Moreover, JNKs are also known as stress-activated MAP kinase (SAPKs) because they mediate the response to pro-inflammatory cytokines, genotoxic and environmental stresses[153–155]. JNKs are coded by JNK1, JNK2, and JNK3 genes, furthermore, differential splicing results in multiple isoforms of the JNK1, JNK2 and JNK3 and each has two short form (46 kDa) and long form (54 kDa)[156]. Moreover, JNK1 and JNK2 are ubiquitous proteins, whereas JNK3 expression is restricted mainly to the brain, testis, and heart.

Each MAPK is activated by dual phosphorylation of a Thr-Xaa-Tyr tripeptide motif (TXY) by specific MAPKs kinases (MKKs or MAP2K) that in turn are activated through phosphorylation at specific serine or threonine residues by a MAPKKs kinases (MKKKs or MAP3K)[157] (Fig. 1-8 A). JNK activation occurs via phosphorylation at threonine 183 and tyrosine 185 and requires a series of docking interactions mediate by scaffold proteins[158] (Fig. 1-8 B). Indeed, as well as other MAPKs, JNKs has a highly conserved negatively charged amino acids C-terminal region referred as common docking domain (CD)[159,160]. However, although is involved in the interactions with substrates, MKKs, and phosphatases, CD is not sufficient in determining docking specificity[160] but it requires the presence of so-termed ED domain[161]; CD along with ED form the docking groove[161] (Fig. 1-8 B). In addition to these domains, there is also another conserved region termed domain for versatile docking (DVD). DVD is found in C-terminus of MKKs including MKK4/7 and it is important in controlling MKKs-MKKKs interactions[162].

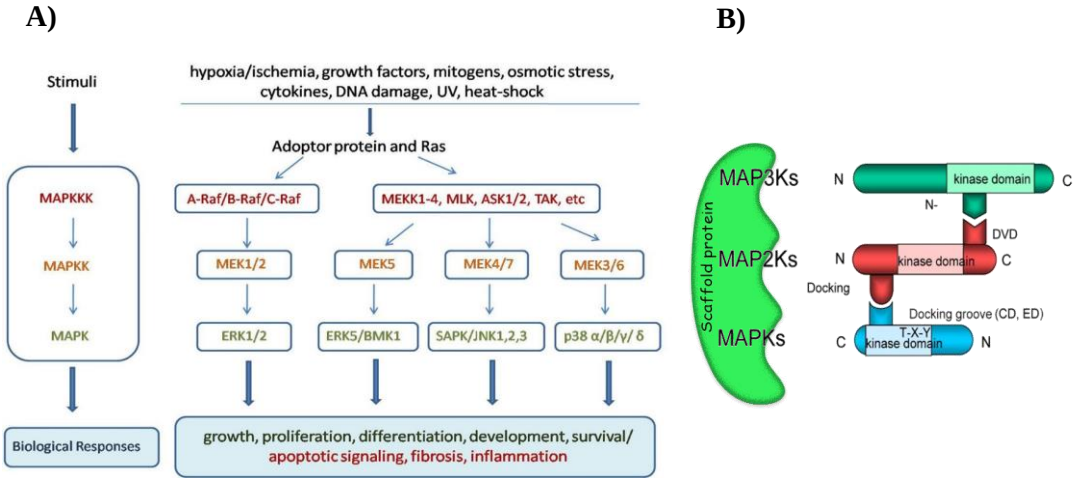

Figure 1-8. ***Mitogen-activated protein kinases (MAPKs) cascades.*** **A)** Schematic diagram of proteins involved in MAPKs signaling and their role in several biological processes[163]. **B)** Representation of MKKKs, MKKs, and MAPKs docking interactions[164].

Two major JNK activators are MKK4 and MKK7. Both kinases are activated by stresses and proinflammatory cytokines, however, whereas MKK4 is primarily activated by environmental stresses, MKK7 is primarily activated by cytokines[165,166] (e.g., TNF-α and IL-1) and is essential for JNK activation in response to this kind of stimuli[155]. Indeed, upon TNF-α stimulation, it was observed a complete loss of JNK activation in mkk7−/− murine embryo fibroblasts (MEF) against a 50% of loss observed in mkk4−/− MEF[155]. Moreover, whereas MKK4 is able to activate p38 MAPKs too, although in a lesser extent than MKK3 and MKK6[167], MKK7 is a specific JNK activator[168]. Importantly, MKK4 and MKK7 show a non-redundant function because they preferentially phosphorylate JNK on Tyr and Thr respectively and thus appear to be both essential for optimal JNK activation[169–171]. Several MKK4 and MKK7 isoforms have been identified; MKK4 isoforms differ in N-terminus whereas MKK7 isoforms differ in both C-terminus and N-terminus. Although little is known about MKK4 isoforms origin, it was observed that several transcript variants are differentially expressed in TNF-treated lymphatic cells[172]. On the other hand, it was demonstrated that murine MKK7 isoforms result from alternative splicing and transcription at different promoters sites[173]. Moreover, Tournier *et al*. well characterized different MKK7 isoforms and

investigated on the relative biochemical properties (Fig. 1-9). They identified three different isoforms in N-terminal region (α, β, and γ isoforms) and two different isoforms in C-terminal region (isoforms 1 and 2), which have a molecular weight of 38 and 52 kDa and, importantly, exhibit different capabilities on JNK activation. Specifically, MKK7α isoforms are about 40 fold less active than MKK7β/γ isoforms[173] (Fig. 1-9), most probably due to the lack of one of the three docking domain in N-terminus of MKK7α isoforms[174]. Another important issue in JNK pathway concerns the subcellular localization of MKKs. Interestingly, in contrast with ERK MAPK activator MKK1 that appear restricted to cytoplasmic compartment when inactive, MKK4 and MKK7 were found both in cytoplasm and in the nucleus, with a preferential nuclear accumulation also in absence of stimulation[173]. MKK1 cytoplasmic retention is indeed due to the presence of a nuclear exclusion signal (NES), and although the fusion of this NES with MKK4 and MKK7 increased their cytoplasmic retention, importantly, both NES-MKK4 and NES-MKK7 showed similar capabilities as MKK4 and MKK7 in JNK activation and nuclear translocation[173]. Subcellular distribution of MKKs could also

A)

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                1                                                          70
MKK7-γ2         MAASSLEQKLSRLEAKLKQENREARRRIDLNLDISPQRPRPIIVITLSPAPAPSQRAALQLPLANDGGSR
MKK7-γ1         M.......................................................................
MKK7-β2         M.....................................T-----------------.................
MKK7-β1         M.....................................T-----------------.................

               71                                                         140
MKK7-γ2         SPSSESSPQHPTPPTRPRHMLGLPSTLFTPRSMESIEIDQKLQEIMKQTGYLTIGGQRYQAEINDLENLG
MKK7-γ1         .......................................................................
MKK7-β2         .......................................................................
MKK7-β1         .......................................................................
MKK7-α2                         M.....................................................
MKK7-α1                         M.....................................................

               141  I                  II              III          IV          210
MKK7-γ2         EMGSGTCGQVWKMRFRKTGHIIAVKQMRRSGNKEENKRILMDLDVVLKSHDCPYIVQCFGTFITNTDVFI
MKK7-γ1         .......................................................................
MKK7-β2         .......................................................................
MKK7-β1         .......................................................................
MKK7-α2         .......................................................................
MKK7-α1         .......................................................................

               211 V                                          VI            VII 280
MKK7-γ2         AMELMGTCAEKLKKRMQGPIPERILGKMTVAIVKALYYLKEKHGVIHRDVKPSNILLDERGQIKLCDFGI
MKK7-γ1         .......................................................................
MKK7-β2         .......................................................................
MKK7-β1         .......................................................................
MKK7-α2         .......................................................................
MKK7-α1         .......................................................................

               281                VIII              IX                       350
MKK7-γ2         SGRLVDSKAKTRSAGCAAYMAPERIDPPDPTKPDYDIRADVWSLGISLVELATGQFPYKNCKTDFEVLTK
MKK7-γ1         .......................................................................
MKK7-β2         .......................................................................
MKK7-β1         .......................................................................
MKK7-α2         .......................................................................
MKK7-α1         .......................................................................

               351 X                                                      420
MKK7-γ2         VLQEEPPLLPGHMGFSGDFQSFVKDCLTKDHRKRPKYNKLLEHSFIKHYEILEVDVASWFKDVMAKTES
MKK7-γ1         .......................................................................
MKK7-β2         .......................................................................
MKK7-β1         .......................................................................
MKK7-α2         .......................................................................
MKK7-α1         .......................................................................

               421                                            467
MKK7-γ2         PRTSGVLSQHHLPFFSGSLEESPTSPPSPKSFPLSPAIPQAQAEWVSGR#
MKK7-γ1         ..............R#
MKK7-β2         ..............................................#
MKK7-β1         ..............R#
MKK7-α2         ..............................................#
MKK7-α1         ..............R#
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B)

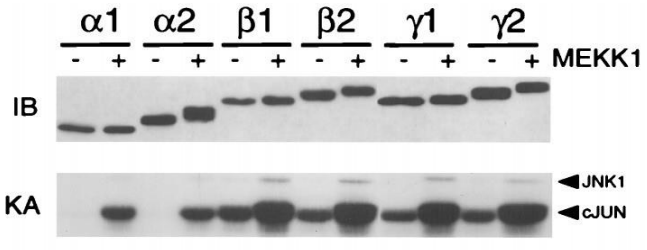

Figure 1-9. ***MKK7 isoforms.*** **A)** Primary structure of MKK7 isoforms with deletions (-) and stop codons. **B)** Western blot (IB) showing different MKK7 isoforms; biochemical activity of MKK7 isoforms was analysed by a kinase assay (KA) by assessing JNK1-mediated activation of downstream c-Jun transcription factor in presence or absence of upstream kinase MEKK1[173].

be cell type dependent; for example, in neurons, it was observed that MKK4 was present in equal extent both in nucleus and in cytoplasm, whereas MKK7 was exclusively localized in nucleus[175], on the other hand, MKK7 was found mainly in cytoplasmic compartment in resting 293T and NIH3T3 cell lines[176]. Moreover, MKK7 appeared to have a predominant cytoplasmic localization in non-malignant cells but showed a more marked nuclear presence in malignant cells such as Jurkat cells[176] or in cellular stress conditions[177]. Interestingly, it was also observed that

MKK7 works as a cytoplasmic anchoring protein for JNK1 since knockdown of MKK7 promotes JNK1 nuclear entry of endogenous JNK1[176]. Moreover, because anchoring mechanism could be defective in malignant cells as reported for Jurkat cells, and JNK subcellular localization has no influence on its activation, MKK7 defective cytoplasmic anchoring of JNK can lead to a constitutive JNK activation inducing apoptosis resistance and promoting tumorigenesis[176,178]. Another important issue concerns levels of MKKs expression and although it seems to be relevant in cancer, more effort should be made to elucidate its role. To date, there are controversial evidences since, for example, it was observed a high expression of MKK4 and MKK7 in high stage prostate cancer[179] whereas, in other cases, MKK4 was found to work as metastatic suppressor[180].

1.5. JNK activation: a matter of life and death

Activation of JNK pathway is involved in a multitude of biological processes including cell proliferation, survival, differentiation, and apoptosis. Upon phosphorylation and activation, JNKs are indeed able to interact with several molecular targets both in the nucleus and in the cytoplasm and the biological outcome is dictated by duration and extent of JNK activation as well as types of stimuli. It was observed, for example, that prolonged activation induces apoptosis, whereas transient JNK activation promotes cell survival[13,181].

One of the major targets of JNKs is c-Jun (Jun) protein that along with c-Fos (Fos) and other factors including JunB, JunD and Fra-1 form activator protein 1 (AP-1). Upon activation, JNK is able to phosphorylate c-Jun at Ser-63 and Ser-73 enhancing its transcriptional activity[182] and c-Jun, for its part, seems to play a key role in AP-1 transcriptional activity regulation[183]. AP-1 complex is largely the most important effector of JNK pathway and its composition in terms of subunits appears to be crucial in determining the biological response (Fig. 1-10)[184]. For example, it was observed that c-Jun overexpression in breast cancer can alter the expression of other AP-1 regulated genes JunB and Fra-1[185]. In particular, JunB was found downregulated whereas Fra-1 was found upregulated; due to their inhibitory actions towards c-Jun[186,187], the resulting qualitative and quantitative change in AP-1 composition enhances transcriptional activity and promotes the development of a tumorigenic and hormone resistant breast cancer phenotype[185]. Moreover, Ras induces transformation through c-Jun phosphorylation on the same sites of JNK and mutations of these sites suppresses tumorigenicity[188]. c-Jun plays also an important role in cell cycle regulation and proliferation in sever cell types

including fibroblasts, liver cells and keratinocytes[189–191] and is required for proliferation and survival of B-lymphoma cells[152].

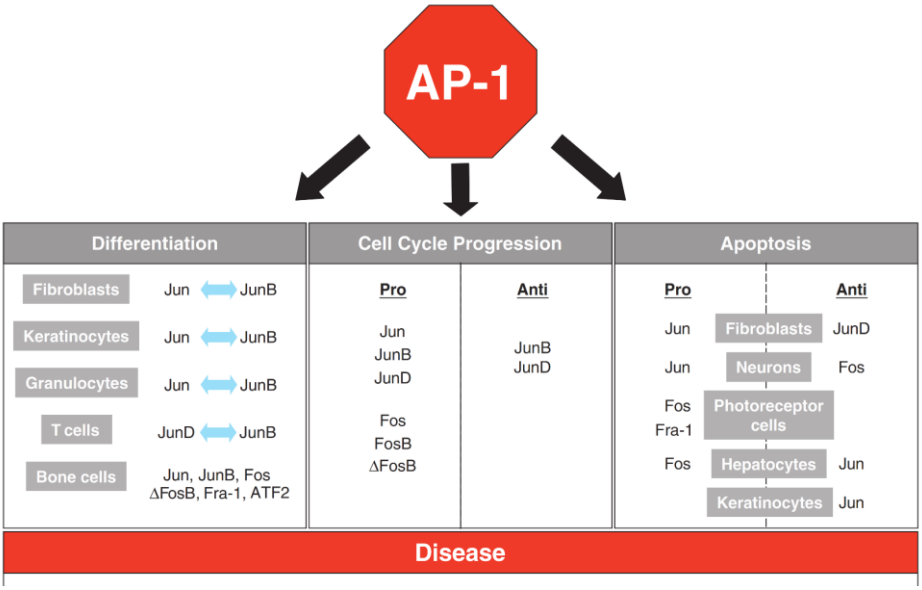

Figura 1-10. ***AP-1 functions in different cellular processes and diseases.*** AP-1 complex with different proteins affecting several biological processes such as differentiation, cell cycle progression and apoptosis. Antagonistic roles are indicated by the double-headed arrows[184].

JNK role in cell survival is also confirmed by its capability of inhibiting apoptosis process. It was indeed observed that c-Jun is able to phosphorylate and inactivate proapoptotic Bcl-2 family protein BAD thus inhibiting apoptosis cascade[192]. Although there are many evidences about JNK role in survival and proliferation, activation of JNK is also strongly related to induction of apoptosis. These seemingly paradoxical roles of JNK are, in fact, context and cell type depending. Moreover, in addition to crucial role of qualitative and quantitative status of AP-1 transcription factor and the nature and duration of the stimuli, the resulting biological outcome is also dependent by different JNKs isoforms that appear to show a non-redundant function[193,194]. For example, JNK1 seems to be involved in cell survival, whereas JNK2 mediates apoptosis[195]. JNK1 and JNK2 also show different actions in cell cycle regulation; JNK2−/− fibroblasts entered in S phase earlier than wild type cells assuming an opposite behaviour than JNK1−/−[196]. Again, due to key role of c-Jun

in cell cycle regulation[197], JNK1 and JNK2 appear to show opposite functions in some contexts, since JNK1 deletion decrease c-Jun levels inhibiting fibroblasts proliferation, whereas JNK2 deletion increase c-Jun levels inducing cellular proliferation[198]. Furthermore, in apparently controversial view but in agreement with non-redundant JNK isoforms functions are the findings that JNK1 is involved in apoptosis signaling, whereas JNK2 is involved in cell survival. In this respect, it was indeed observed that JNK1 is required for tumour necrosis factor-α (TNF-α) induced apoptosis[199] and that its deficiency results in decreased c-Jun phosphorylation and resistance to UV-induced cell death, whereas lack of JNK2 enhances cell sensitivity to UV[200].

In light of these findings, what is the role and mechanisms by which JNK acts in apoptosis process? Apoptosis cascade is classified into two main pathways: extrinsic and intrinsic pathways. Extrinsic pathway is initiated by death receptors through interaction with ligands such as TNF-α, Fas ligand (FAS-L), and TNF-related apoptosis-inducing ligand (TRAIL) (Fig. 1-11A), whereas intrinsic pathway is initiated from mitochondria[201] (Fig. 1-11B). Despite different stimuli and molecular mechanisms involved, all three JNK isoforms play important roles in both apoptosis pathways. A crucial event is undoubtedly represented by c-Jun activation; indeed, upon activation by MKKs, JNK translocates to nucleus where phosphorylates c-Jun promoting formation of AP-1 complex[202] that, in turn, is able to drive transcription of pro-apoptotic genes such as Fas-L, TNF-α[203], Bim and Bak[204], Bax[205,206], other than death receptors such as death receptor 5 (TRAIL-R2)[207]. Moreover, JNK-mediated c-Jun phosphorylation appears to be a key event in UV-induced apoptosis in fibroblasts cells because mutations on c-Jun phosphorylation sites protect fibroblasts from apoptosis[208]. Nuclear JNK signaling is also important in neuronal cell death since the expression of nuclear dominant negative inhibitors of JNK prevents apoptosis, whereas cytosolic inhibitors only interferes with physiological JNK functions[209]. In addition to nuclear signaling, JNK contributes to apoptosis through other mechanisms; for example, JNK is able to phosphorylate and thus modulate the activity of several members of Bcl-2 family.

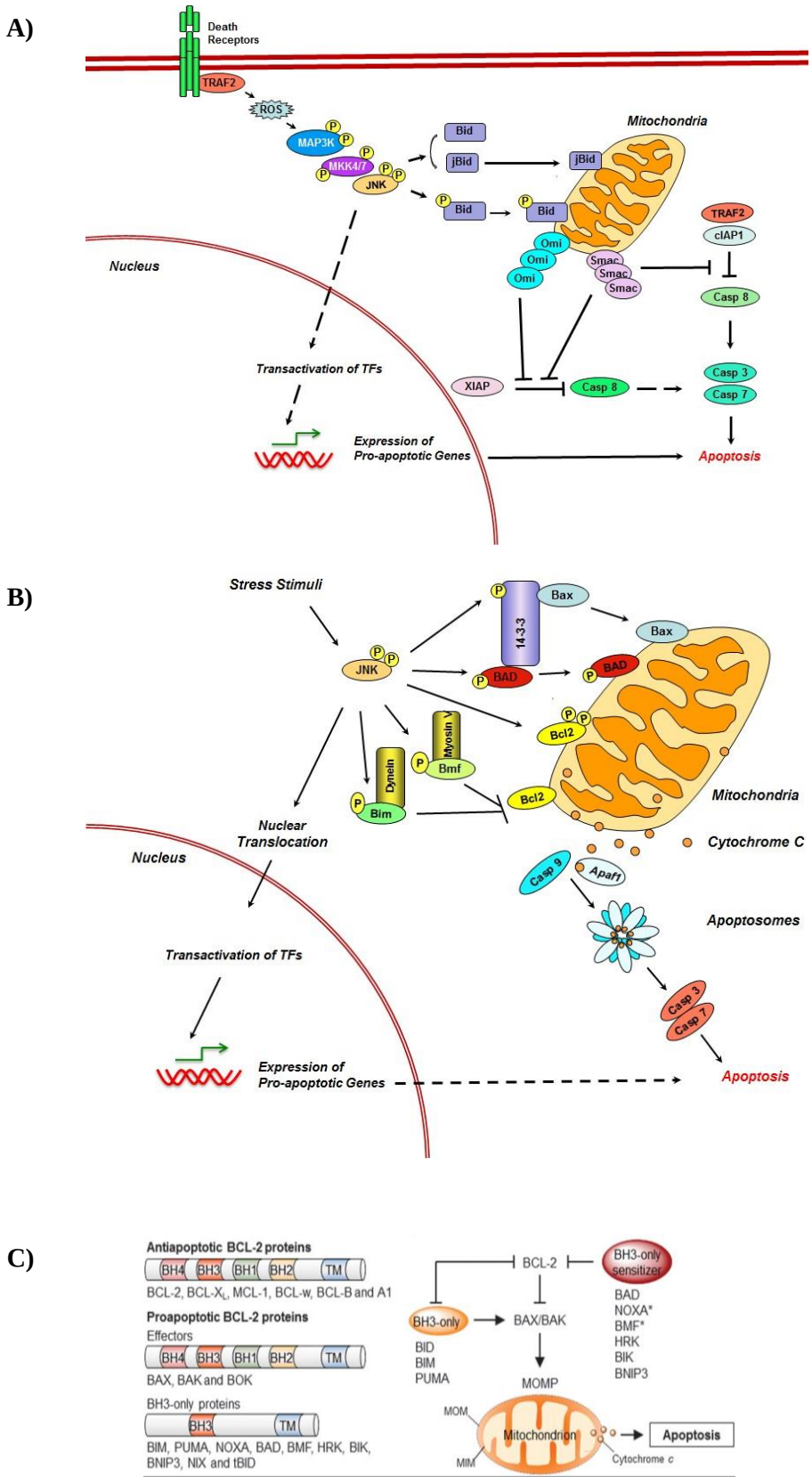

Figure 1-11. ***JNK signalling and apoptosis.*** **A)** Extrinsic pathway **B)** Intrinsic pathway **C)** Bcl-2 family proteins and their role in apoptosis process[210, 211]

Bcl-2 family proteins (Fig. 1-11C) are master regulators in apoptosis and include both proapoptotic and antiapoptotic factors. Proapoptotic action of several Bcl-2 family proteins is due to their capabilities in regulating mitochondria outer membrane permeabilization (MOMP) that promotes cytochrome c release and other factors such as SMAC/DIABLO and OMI/HTRA2 leading to apoptosome formation and caspases activation (Fig. 1-11A-B). Effectors of MOMP are Bax and Bak and are positively regulated by a subset of Bcl-2 proteins termed BH3-only proteins because they present only the BH3 domain (Fig. 1-11C). Moreover, among BH3-only proteins, several proteins such as Bid, Bim and Puma are considered direct activator of Bax and Bak, whereas other proteins such as Bad and Bmf are denominated "sensitizers" because they competitively interact with antiapoptotic Bcl-2 proteins that usually sequester proapoptotic BH3-only proteins thus suppressing apoptosis[212] (Fig. 1-11C). In this scenario, JNK is able to regulate apoptotic process at multiple levels. For example, in extrinsic pathway, it was observed that JNK can modulate the activity of BH3-only protein Bid; Bid exists in a weaker apoptogen full length form and in a more potent truncated form tBid following cleavage by caspase 8[213]. In Fas-L and TNF-α extrinsic pathway initiation, upon cleavage and activation by caspase 8, tBid translocates to mitochondria and triggers cytochrome c release[213]. JNK is able both to phosphorylate full-length form[214] and to cleave full-length form generating a different short form of Bid termed jBid[215]. Interestingly, whereas tBid promotes cytochrome c release, JNK-dependent generation of jBid induces preferential release of SMAC/DIABLO and OMI/HTRA2 factors thus inhibiting inhibitors of caspases XIAP and cIAP1 and promoting apoptosis (Fig. 1-11A). Moreover, phosphorylation of full-length Bid at Thr59 by JNK inhibits its caspase 8-mediated cleavage and, although prevents tBid form generation, it protects Bid from subsequent proteasomal degradation[216] promoting full-length form accumulation and leading to a slower full-length Bid-mediated apoptotic response[214].

JNK is also essential for activation of proapoptotic proteins Bak and Bax that represents a crucial event in intrinsic pathway of apoptosis. Indeed, upon activation, Bax and Bak lead to MOMP and promote cytochrome c release from mitochondria that in combination with Apaf-1 and caspase-9 form the apoptosomes

thus activating caspases cascade [217]. In this context, in addition to drive directly Bax expression via c-Jun activation[218], JNK promotes Bax translocation to mitochondria via phosphorylation of Bax-anchor proteins 14-3-3 thus leading to Bax-14-3-3 complex dissociation and release of Bax[219] (Fig. 1-11C). Moreover, JNK is also able to promote apoptosis through modulation of BH3 only-proteins; for example, JNK can phosphorylate Bad at Ser128 and enhance its proapoptotic activity[220]. Again, JNK-mediated phosphorylation of Bim and Bmf can both activate directly Bax[221] and counteract Bcl-2 antiapoptotic activity[222]. Furthermore, JNK is also capable to repress directly Bcl-2 activity through its phosphorylation at Ser70[223] (Fig. 1-11C).

Other important JNK-mediated proapoptotic mechanisms concern the interaction with p53 and p73 tumour suppressors. For example, JNK was found to interact and to phosphorylate p53 in context specific manner; in resting cells, JNK is able to bind p53 and promote its ubiquitin-mediated degradation, whereas, in stressed cells, JNK-mediated p53 phosphorylation at Ser6 inhibits ubiquitination thus stabilizing p53 and enhancing its proapoptotic function[224]. Moreover, JNK pathway can also regulate p53 at transcription level since c-Jun is able to repress p53 promoter. It was indeed observed that c-Jun deficiency promotes p53 expression and that, conversely, c-Jun overexpression represses p53 transcription thus mimicking a p53 loss condition[225]. Finally, several findings showed that JNK could function differently in normal and tumour cells based on p53 status[226]. JNK is also able to regulate the activity of tumour suppressor p73, a member of p53 family. Specifically, in response to DNA damage, JNK can bind, phosphorylate, and stabilize p73 thus promoting p73-madiated expression of proapoptotic genes Bax and Puma[227].

1.6. GADD45 family and its role in stress response

GADD45 (Growth arrest and DNA-damage) family genes encode for the highly conserved proteins GADD45A (GADD45), GADD45B (MyD118) and GADD45G (CR6), which were identified in response to stresses associated with growth arrest[228]. *GADD45* genes products are small in size (18 kDa) and highly acid (isoelectric point 4.0-4.2), and are mainly localized in nuclear compartment. GADD45 proteins are involved in several biological pathways associated with stress response and regulation of cell growth by interacting with proteins such as p21, PCNA, cdc2/cyclinB1, p38 and JNK kinases[229]. Specifically, through these interactions, GADD45 proteins are able to modulate DNA repair[230], cell cycle arrest[231], apoptosis and cell survival[13,232,233]. *GADD45A* was the first gene discovered[234]; its induction was observed in response to ionized radiation and was found to be regulated at transcriptional level by p53 transcription factor[235]. Subsequently, also *GADD45B* and *GADD45G* were identified as inducible genes upon IL-6[236] and IL-2[237] stimulation respectively. Despite their high degree of homology, GADD45 proteins are able to play different roles based on cellular context and in response to different stimuli.

Regarding their role in DNA repair, after DNA damage all three proteins are induced [238]. Both GADD45A and GADD45B are involved in DNA excision repair due to interaction with PCNA protein[239,240] and GADD45A plays also a role in global genomic repair (GGR)(Figure 1-12). Moreover, GADD45A deficiency significantly reduces DNA repair mechanism and promotes carcinogenesis by chemicals[241]. All GADD45 proteins are involved in G2/M checkpoint that blocks mitosis in presence of DNA damage[242,243] and genes silencing with antisense nucleotides leads to an impairment in G2/M checkpoint[244]. Specifically, GADD45A and GADD45B proteins promote cell cycle arrest through interaction with the CDK1/Cyclin B1 complex, thus disrupting the complex, whereas GADD45G inhibits kinase activity of the complex without disrupting molecular interactions. GADD45A plays also a role in S-phase of cell cycle displacing PCNA from the cyclin D1 complex and inhibiting DNA replication[245]. All GADD45 proteins also interact with the cyclin-dependent kinase inhibitor p21 although their exact function remains to be elucidated.

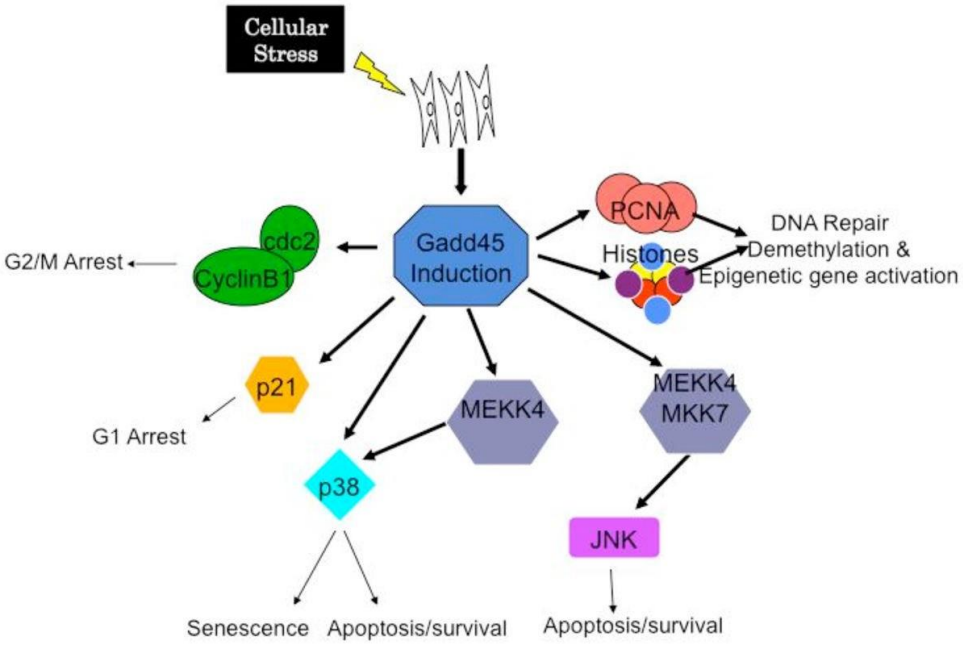

Figure 1-12. ***Gadd45 proteins signaling*.**[229]

Another important role of GADD45 proteins concerns the involvement in apoptosis and the interaction with MAPKs components in stress response (Figure 1-12). Indeed, it was observed that GADD45 proteins are able to interact with MEKK4, the upstream kinase that mediates the activation of p38/JNK kinases fallowing stressful stimuli. In particular, all three proteins were found to interact with N-terminal domain of MEKK4[246] and since MEKK4 N-terminal domain inhibits C-terminal kinase domain, binding and disruption of N-C terminal domains interaction by GADD45 proteins promotes p38/JNK kinases activation[247]. Moreover, overexpression of GADD45 proteins was reported to activate MEKK4 and to induce apoptosis[246]. Again, impairment in JNK activation and UV-induced apoptosis was observed in GADD45A null mouse skin and keratinocyte cell lines[233] and *GADD45B* gene silencing through antisense expression delayed TGF-β-

induced cell death due to a reduction in p38 activation[248]. Also GADD45G is involved in apoptosis and MAPKs activation since it is required for caspase activation in renal tubular cells[249] apoptosis and is able to activate p38 in Th1 cells[250]. On the other hand, it was observed that GADD45 proteins, especially GADD45B, play a protective role towards apoptosis. It was indeed reported that NF-κB activation contributes to inhibition of apoptosis through c-myc-mediated repression of *GADD45A* and *GADD45G* genes expression[251] (Figure 1-13A). Moreover, Gupta *et al*.[252] showed that GADD45A and GADD45B cooperate to prevent apoptosis in haematopoietic cells following UV irradiation. In particular, GADD45A was found to activate p38 pathway that, in turn, was able to promote phosphorylation of IκB and activation of NF-κB pathway. For its part, upon induction, GADD45B was found to interact and block MKK4 thus inhibiting JNK signaling. Moreover, it was demonstrated that, upon TNF-α stimulation, NF-κB-induced GADD45B expression suppressed JNK activity and that GADD45B acts by specifically targeting MKK7 kinase[13,253,254] (Figure 1-13B). Again, GADD45B deficiency in bone marrow cells increases their sensitivity to UVC, daunorubicin and etoposide[232] and GADD45B overexpression protects fibroblasts cells from apoptosis[255]. These findings make clear that NF-κB regulation of GADD45 proteins plays a key role in apoptosis inhibition; indeed, if on the one hand NF-κB activation leads to c-myc-mediated GADD45A and GADD45G repression and subsequent MEKK4 inhibition, on the other promotes GADD45B expression thus preventing MKK7 activation. Furthermore, NF-κB activation acts in synergistic manner in inhibiting MAPKs response thus inhibiting JNK response and promoting cell survival (Figure 1-13A and 1-13B). In summary, we can say that regulation of GADD45 proteins is essential both in normal and cancer cells and that survival or apoptosis responses are most probably dictated by GADD45 proteins balance and levels that are cell type and context dependent.

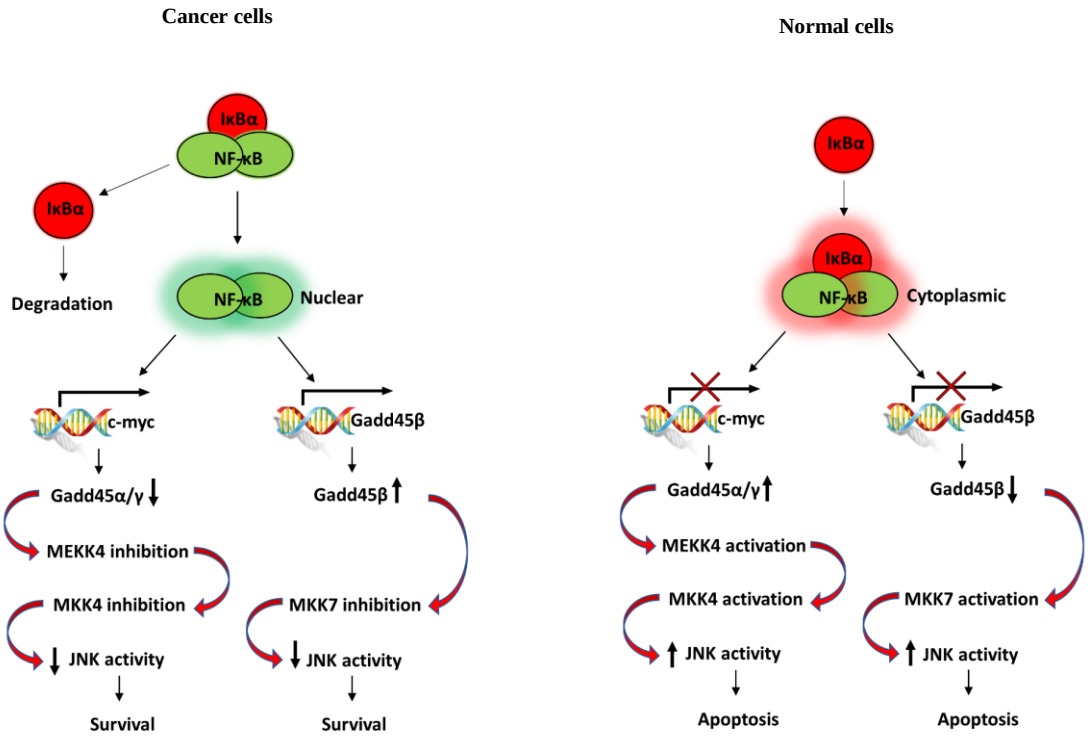

Figure 1-13. ***Regulation of GADD45 proteins via NF-κB.*** In cancer cells, constitutive NF-κB activation promotes survival through inhibition of GADD45A/G and induction of GADD45B (left panel); in normal cells inactivation of NF-κB increases sensitivity to apoptotic cell death (right panel).

1.7. Targeting MKK7-JNK axis through GADD45B inhibition

Due to their key role in regulating programmed cell death, specific targeting of GADD45B could represent a precise therapeutic strategy to treat also apoptotic resistant tumours by exploiting direct activation of JNK kinases. Moreover, many cells do not express high levels of GADD45B[256] restricting the effects of its targeting to overexpressing cells such as cancer cells. Indeed, although many NF-κB inhibitors are currently available, they show several side effects due to their unintended inhibition of NF-κB physiological functions including inflammatory reactions, liver damage and immunological disorders. To date, there are several examples of pharmacological modulation of GADD45 proteins; for instance, Jiang *et al*. showed that synthetic retinoid CD437 was able to induce apoptosis in ovarian carcinoma cells through GADD45A induction[257]. Again, Hirose *et al*. revealed that trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, promoted growth arrest at the G1 and/or G2/M phases through GADD45A[258].

GADD45B interaction with MKK7 kinase was amply demonstrated[259]; GADD45B lacks phosphatase activity and thus inhibits the kinase activity by binding specific regions of MKK7. Specifically, structure of GADD45B consists of five α-helices and two acidic loops that surround a central four-stranded β-sheet core; Papa *et al*. demonstrated that α3 region is involved in docking of GADD45B to MKK7 and that loop 1 and α4-loop 2 regions are required for kinase inhibition due to their interaction with MKK7 catalytic pocket preventing ATP binding and stabilizing kinase in an inactive conformation[259]. Moreover, Larsen *et al.* also indicated that a mechanism by which GADD45B inhibits MKK7 activity is represented by prevention of its phosphorylation[260]. Interestingly, although α3 region is highly conserved among GADD45 proteins and could thus represent a common docking kinase domain, loop 1 and α4-loop 2 regions are less conserved among GADD45 factors suggesting that these loop regions are probably involved in selective inhibition of specific enzymes.

In order to activate JNK cascade and apoptosis in cancer, Tornatore *et al.* developed the DTP3 molecule, a potent GADD45B inhibitor[14]. By screening a combinatorial library of L-tetrapeptides, they first identified two acetylated (Ac) L-tetrapeptides, Ac-LTP1 and Ac-LTP2, that were able to block MKK7 activity and

then, in order to increase bioavailability, developed DTP3 molecule. DTP3 showed a high selectivity and affinity for MKK7 (Kd=65 nM) and, importantly, was able to bind kinase both in isolation and in complexed status with GADD45B revealing its capabilities in disrupting MKK7-GADD45B complex. The use of DTP3 in highly expressing GADD45B multiple myeloma cell lines induced apoptosis and ablated myeloma xenograft through activation of MKK7-JNK axis. Indeed, DTP3 showed no significant or apparent side effects. These findings revealed thus a novel mechanism of NF-κB inhibition at different level (Figure 1-14) and formed the basis for a new therapeutic approach for selectively targeting NF-κB pathway.

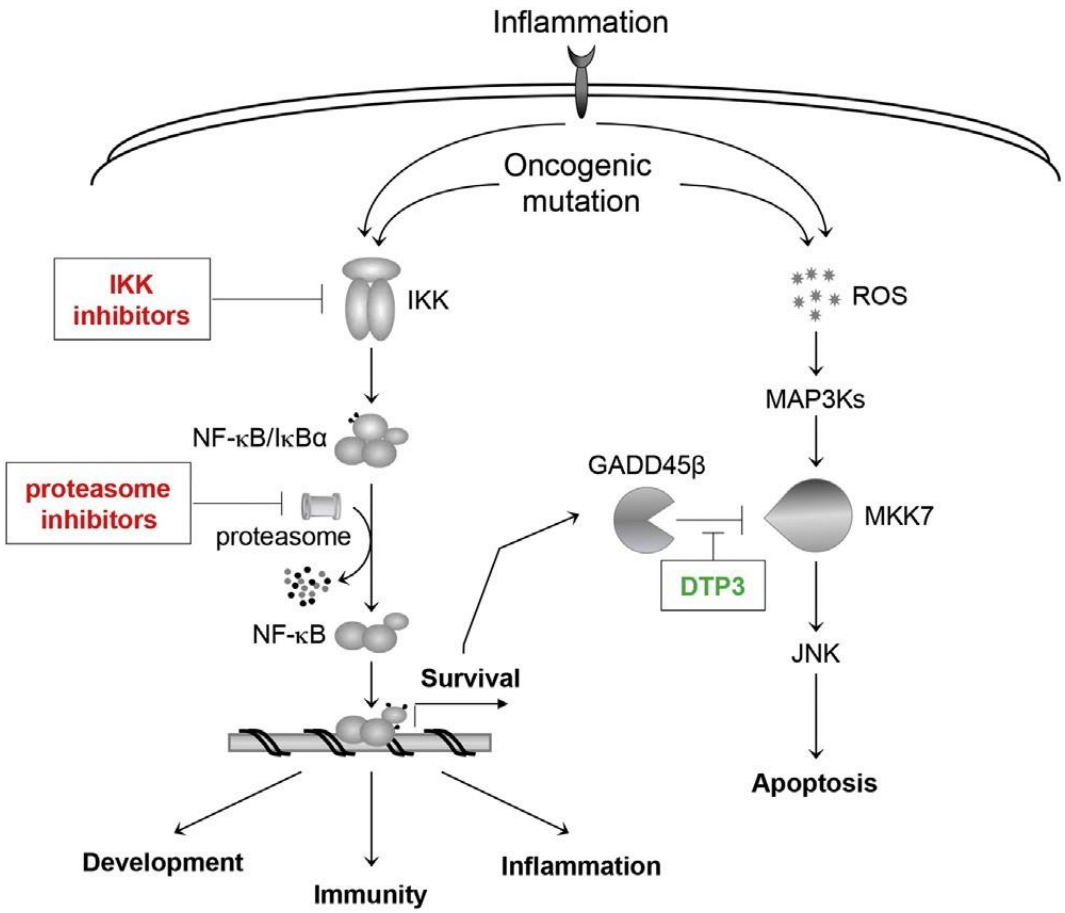

Figure 1-14. ***NF-κB inhibition strategies*****.** Schematic representation of the main (red) or potential (green) strategies able to inhibit the NF-kB pathway in cancer[14].

2. Materials and methods

Cell cultures and reagents

Human prostate cancer cell lines DU145 and PC3 were cultured in RPMI 1640 (Roswell Park Memorial Institute) and supplemented with 10% FBS (Fetal Bovine Serum) (GIBCO, Carlsbad, CA, USA); 22Rv1 cell line was cultured in DMEM (Dulbecco's Modified Eagle's Medium) with 10% FBS. LnCap cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% Hepes and 1% Sodium Pyruvate. All media were supplemented with L-glutamine 2mM (GIBCO, Carlsbad, CA, USA), Penicillin-StreptoMycin 50U/mL (GIBCO, Carlsbad, CA, USA). DTP3 molecule and TNF-α for treatments were purchased from Selleck Chemicals and Cell Signaling, respectively.

RNA Extraction and Quantitative Real-Time Polymerase-Chain Reaction (Q-PCR) Analysis

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications and 1μg of RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Inc Foster City, CA, USA). Q-PCRs were carried out using Taq Man Universal Master Mix and ABI 7500 Fast real-time PCR machine (Life Technologies) and the following predesigned TaqMan® Gene Expression Assays: 18s Human Hs99999901_s1 FAM, Gadd45b Human Hs04188837_g1 FAM. Ct values were normalized to 18S gene expression.

Evaluation of NF-κB Activity

Nuclear protein extracts were assayed by using the TransAM NF-κB p65 protein assay (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol and NF-κB activity was evaluated measuring the absorbance at 450 nm using a μ-Quant plate-reader (Bio-Tek Instruments, Winooski, VT, USA).

Western blot analysis

Total cell extracts were prepared in RIPA buffer (1X phosphate buffered saline, 1 % IGEPAL, 0,5 % sodium deoxycholate, 0,1 % SDS, 150 mM NaCl, 0,6 mM PMSF, 10 µg/ml aprotinine, 5 mM Na3VO4, 10 mM NaF) containing complete mini EDTA-free protease inhibitors (Roche Molecular Biochemicals, Basilea Svizzera) and protein concentration was assessed by the BCA assay kit (Pierce, Thermo Fisher Scientific Carlsbad, CA, USA). Nuclear and cytoplasmic fractionation extracts were prepared as follows: briefly, 24 hours prior extraction, cells were plate in 100 mm culture dishes and scraped on next day at specific time points. Plasmatic membrane was disrupted in specific subcellular fractionation buffer (Hepes 10 mM, sucrose 350 mM, KCl 10 mM, MgCl2 1,5 mM, EDTA 1 mM, IGEPAL 0,2 %, 0,6 mM PMSF, 10 µg/ml aprotinine, 5 mM Na3VO4, 10 mM NaF); after 30 minutes of incubation nuclei were isolated by centrifugation at 700g for 10 minutes, lysed in RIPA buffer and protein concentration of both cytoplasmic and nuclear phases were assessed by the BCA assay kit (Pierce,Thermo Fisher Scientific Carlsbad, CA, USA). Primary antibodies used were: NF-κB p65 (D14E12) XP (8242 Cell Signaling), Phospho-NF-κB p65 (93H1) (Ser536) (3033 Cell Signaling), Phospho-MKK7 (Ser271/Thr275) (4171 Cell Signaling), MKK7 (4172 Cell Signaling), Phospho-SEK1/MKK4 (Ser257/Thr261) (9156 Cell Signaling), SEK1/MKK4 (9152 Cell Signaling), Phospho-SAPK/JNK (Thr183/Tyr185) (9251 Cell Signaling), SAPK/JNK (9252 Cell Signaling), GADD45B (PA537114, Life Technologies), Actin (C-11) (sc-1615 Santa Cruz Biotechnology), α Tubulin (AA13) (sc-58668 Santa Cruz Biotechnology), Lamin A/C (E-1) (sc-376248 Santa Cruz Biotechnology), Cleaved Caspase-3 (Asp175) (5A1E) (9664 Cell Signaling). Western blot detection was performed using ECL (Amersham).

Lentiviral infection and RELA or GADD45B silencing

RELA or GADD45B silencing was performed using MISSION shRNA Lentiviral Transduction Particles (Clone ID: NM_015675.1-226s1c1, Sigma; Clone ID: NM_021975.1-2282s1c1, Sigma) according to the manufacturer's specifications. Briefly, 4,5 x 10^5 cells were plated in 60 mm culture dishes 24 hours prior to

transduction. The next day, cells were inoculated with shGadd45β/shRelA particles or with a scramble sh particles as negative control, centrifugated at 1800 rpm for 45 minutes at 32 °C and then incubated for 24 hours. After 72 hours post-infection, cells were grown in selection media containing 1 µg/ml puromycin. Appropriate puromycin concentration was determined by performing a kill curve experiment in 96 well plate using concentrations ranging from 0.5 –5 µg/ml.

Cell viability assays

Cell viability assays were carried out using CellTiter-Glo® 3D Cell Viability Assay reagent (G9681 Promega) according to the manufacturer's protocol. Counting was performed using Cyto Smart cell counter (Corning). Cell treatment were performed as follows: cells were counted and plated in 96 well plates a day before treatment. Next day cells were treated daily for 120 hours. At each time point, cells were lysed in CellTiter-Glo® 3D reagent and luminescence was determined with Packard Lumicount Microplate Reader BL10000.

Human cancer datasets

The human dataset of Prostate adenocarcinoma acinar type (PRAD) is part of The Cancer Genome Atlas (TCGA) program and was downloaded from the UCSC Cancer Genomic Browser. The gene profiling data were downloaded together with clinical information. Patients were stratified into two groups based on *RelA* mRNA expression levels. In both case 66^{th} percentile was used as thresholds.

Statistical Analyses

The PFI of Prostate cancer patients were calculated using the Kaplan-Meier method and comparison between two groups were analyzed using the long-rank test. P value < 0.05 as the level of significance.

3. Results

3.1. High NF-κB activity is associated with a worse progression free interval and correlates with androgen-independent phenotype

To explore the role of NF-κB in prostate cancer disease we first analyzed publicly available prostate cancer patient datasets. As shown in Figure 3-1 A, high expression of transcription factor RelA (p65) is associated with a lower progression free interval. Moreover, similar results were found analyzing the same dataset for patients with high Gleason score (7-10) (Figure 3-1 B). These data confirmed previous published data indicating a role for NF-κB in prostate cancer progression and demonstrated that, in human prostate cancer, NF-κB expression correlates with a worse prognosis.

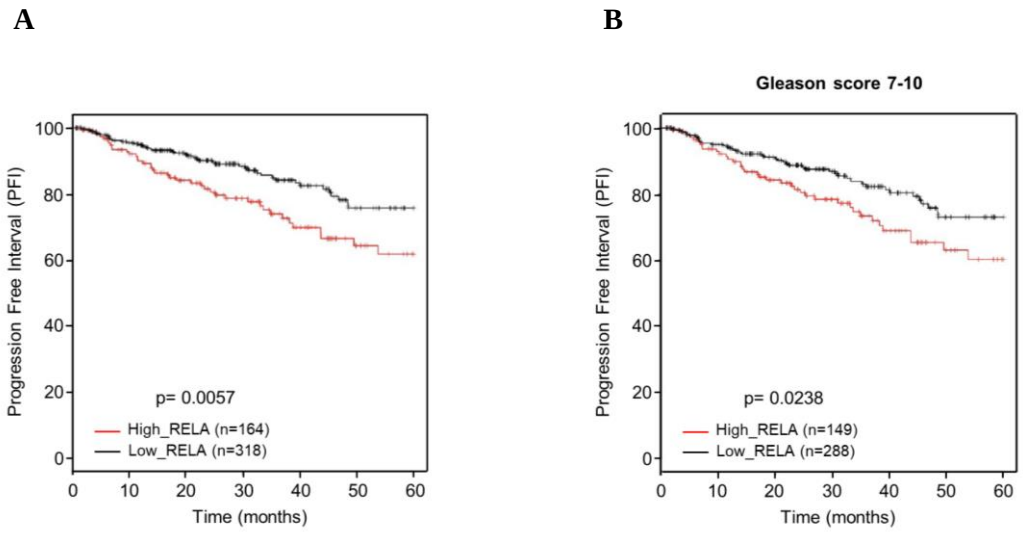

Figure 3-1. ***Correlation between RELA and poor prognosis in prostate cancer patients.*** **A)** Progression free interval (PFI) in prostate cancer patients from TCGA dataset (PRAD; n=482) stratified on *RelA* mRNA expression. **B**) PFI in prostate cancer patients with Gleason score 7-10 from the same dataset stratified on high or low *RelA* mRNA expression. Low and High *RelA* mRNA levels were stratified based on the 66th percentile. Comparison between groups were analysed by the log-rank test.

Due to extremely important role of NF-κB in cancer progression, and based on data showed in Fig. 3-1 which correlates high NF-κB expression with a lower progression free interval, we chose *in vitro* cell models of androgen-dependent and androgen-independent prostate cancer to evaluate the NF-κB activity level in prostate cancer progression. Cell lines used to this purpose were DU145 and PC3, as androgen-insensitive cell models, and 22Rv1 and LNCaP cell lines as androgen sensitive cell models. Firstly, we evaluated the expression level of both total and phosphorylated form on Ser536 of p65 protein, the NF-κB transcription factors member most widely expressed, by western blot. Phosphorylation on Ser536 of p65 protein is necessary for its activation. As shown in Figure 3-2 A, we found that p65 total form expression was similar in all four cell lines, as expected. However, expression of active form p-p65 on Ser536 was highly expressed only in androgen-independent cell lines DU145 and PC3. In particular, DU145 exhibited the highest expression level of p-p65 denoting a strong activation of NF-κB canonical pathway in this cell line. To investigate further on the p65 activity we subsequently carried out a DNA binding assay to determinate the relative amount of DNA-bound protein (Figure 3-2 B). Again, according to western blot data, we found that DU145 cell line showed the highest level of nuclear p65-DNA interaction followed by PC3 cell line. 22Rv1 and LNCaP cell lines not appeared to express p-p65 in western blot analysis and they do not showed significant levels of NF-κB activity in p65 DNA-binding assay. Taken together, these data demonstrated that NF-κB plays an important role in prostate cancer and that its activity is much more evident in androgen independent cell lines, suggesting a crucial role for this transcription factor in prostate cancer progression towards the androgen independent phenotype.

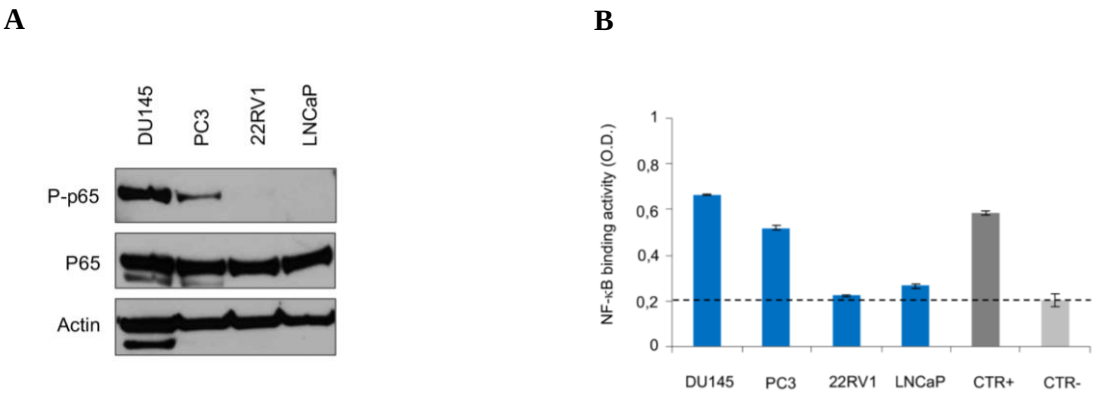

Figure 3-2. ***NF-κB expression in androgen-independent prostate cancer cell lines.*** **A)** Western blot showing total and phosphorylated (P) p65 in prostate cancer cell lines at basal level. Actin is shown as loading control. **B)** DNA binding assay showing relative amount of DNA-bound p65.

3.2. GADD45B expression correlates with NF-κB activity and cancer progression

GADD45B represents an important survival factor that is tightly regulated by NF-κB. Indeed, as mentioned earlier, *GADD45B* is a NF-κB target gene and is able to suppress pro-apoptotic JNK signaling in response to TNF-α[13] by forming a molecular complex with MKK7 and inhibiting its activity. To investigate on the role of GADD45B in prostate cancer cell survival, we first evaluated GADD45B endogenous levels in all four cell lines through both Real Time PCR and Western blot analysis; as shown in Figure 3-3 A, GADD45B mRNA resulted more expressed in androgen independent DU145 and PC3 cell lines, with the highest expression in DU145 cells. These expression data were also confirmed by Western Blot analysis as shown in Figure 3-3 B and it correlated with NF-κB activation (see Fig. 3-2). Moreover, due to crucial role of subcellular localization of MAPKs components cascade, and more specifically of MKKs, and considering that the pro-survival function of GADD45B relies on its ability to bind and inhibit MKK7, we also evaluated the subcellular localization of GADD45B protein. As expected, due to its mainly nuclear localization, nuclear expression levels of GADD45B was observed in all four cell lines. However, interestingly, high expression of cytoplasmic GADD45B was detected in androgen independent DU145 and PC3 cell lines. Importantly, this cytoplasmic GADD45B expression correlates with prostate cancer progression suggesting that could be correlated with its cytoprotective function in androgen-independent prostate cancer cells. Interestingly, it has been previously shown that MKK7 can also localize in the nuclear compartment, thus supporting the idea that nuclear fraction of GADD45B could protect against apoptosis by inhibiting nuclear MKK7 activation. We also confirmed that GADD45B expression is strictly dependent by NF-κB in prostate cancer cell lines; indeed, silencing of transcription factor p65 (RELA) downregulated GADD45B expression in DU145 cells (Figure 3-3 D). Finally, a correlation between RELA and GADD45B was also observed in prostate cancer patients datasets described in Figure 3.1 (Figure 3-4).

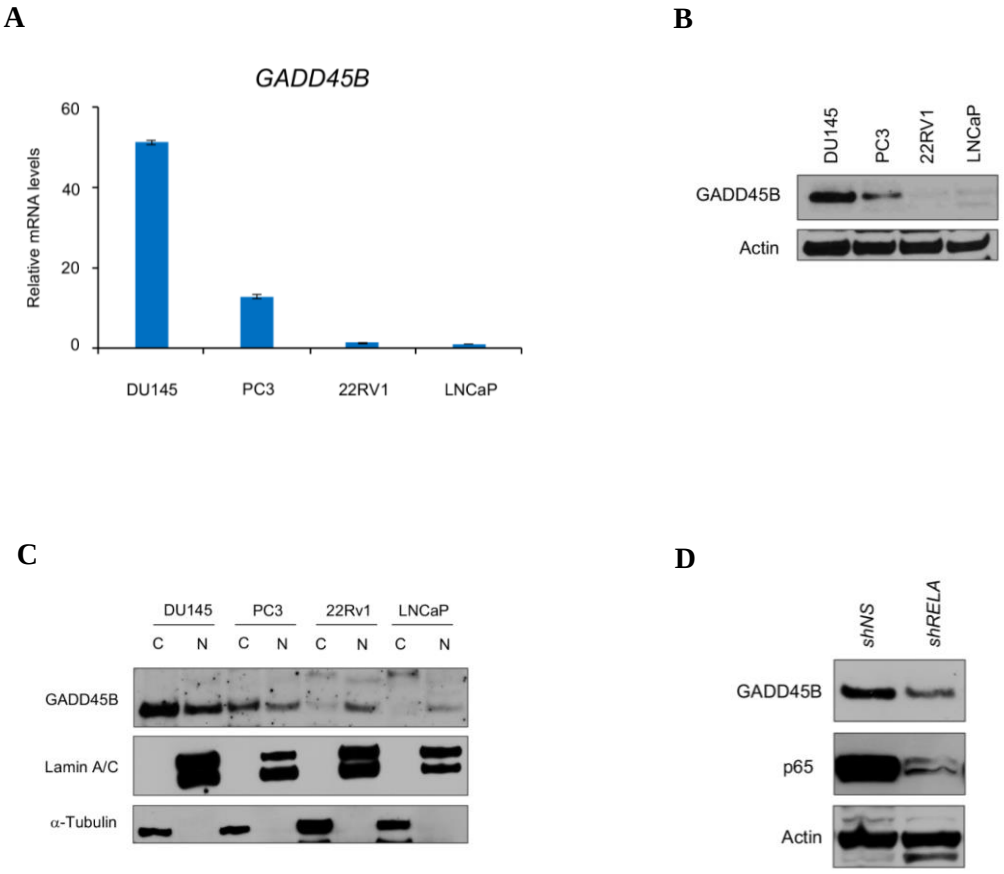

Figure 3-3. ***GADD45B expression levels in prostate cancer cell lines.*** **A)** q-RTPCR showing is the relative GADD45B mRNA levels in prostate cancer cell lines. **B-C)** Western Blot analysis showing GADD45B protein in whole protein extracts **(B)**, and nuclear (N) and cytoplasmic (C) fractions **(C).** Actin, Lamin A/C and α-Tubulin are used as loading control. **D)** Western Blot analysis showing downregulation of GADD45B expression after p65 (RELA) silencing in DU145 cell line

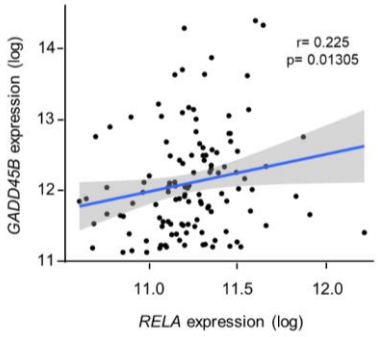

Figure 3-4. ***Correlation between GADD45B and RELA expression in prostate cancer patients dataset.*** Shown is the correlation between *GADD45B* and *NF-κB RELA* expression in the PRAD dataset. Low and high *GADD45B* mRNA levels were stratified based on the 75th percentile. Gene values are expressed as Z-score. r, Pearson correlation coefficient.

3.3. "Breaking the brake on apoptosis" through GADD45B silencing

As first demonstrated by De Smaele et al.[253] GADD45B represents an important anti-apoptotic factor suppressing TNF-α-induced prolonged JNK activation and thus inhibiting the proapoptotic JNK signaling. Therefore, in order to verify whether in cell survival of androgen-independent cell lines was dependent on the over-expression of GADD45B, we performed a lentiviral infection of a specific *GADD45B* short hairpin in DU145 cell line. *GADD45B* silencing was demonstrated both at mRNA and protein levels by Real Time PCR (Figure 3-5 A) and by Western Blot analysis (Figure 3-5 B), respectively. The scramble *COO2* short hairpin was used as negative control. As shown in Figure 4-1 B, GADD45B inhibition induced a strong JNK activation and, importantly, an activation of the apoptotic pathway denoted by caspase 3 cleavage. No JNK and caspase 3 activation were observed in sh scramble cells (Figure 3-5 B). Moreover, as highlighted by CellTiter Glo viability assay, we also observed a significant reduction of viability in sh *GADD45B* compared to sh scramble cells (Figure 3-5 C). Taken together, these data demonstrate that GADD45B over-expression in advanced prostate cancer cells is pivotal for tumor cell survival and reduction of its expression is sufficient to induce a JNK-mediated apoptotic pathway. Thus, pharmacological interfering with the NF-κB pathway in advanced prostate cancer could be achieved by molecular targeting of GADD45B.

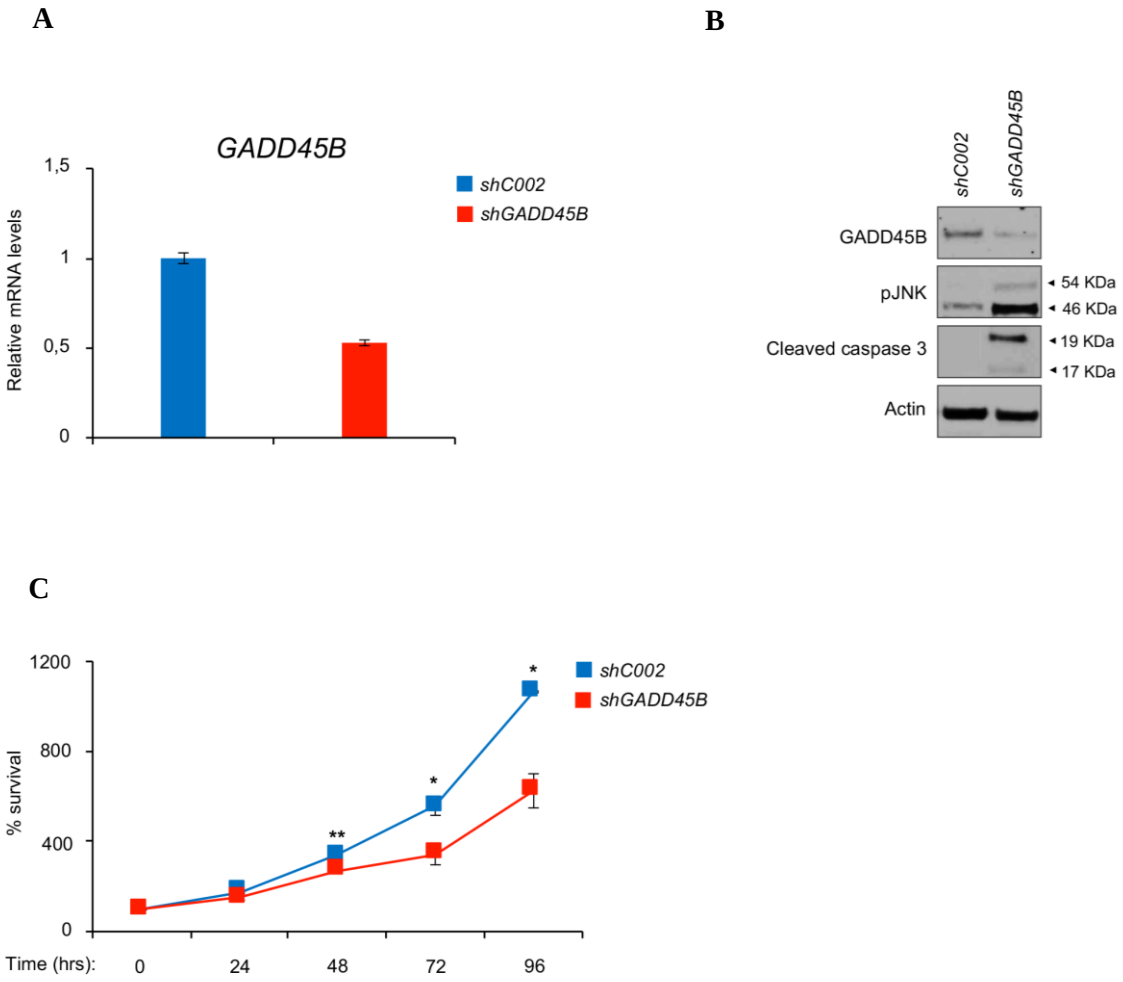

Figure 3-5. ***DU145 cell survival relies on GADD45B expression.*** **A)** Real Time PCR showing *GADD45B* mRNA levels in DU145 cell line following lentiviral infection of non-specific (*shC002*) and *GADD45B*-specific (*shGADD45B*) shRNAs. **B)** Western blot analysis showing the protein levels of GADD45B, p-JNK and cleaved-caspase 3 in the same cell line. **C)** Cell-Titer Glo viability assay showing the viability of the same cell lines after lentiviral infection at the indicated time points. Experiment was conducted in triplicate. Values are mean ± S.D.

3.4. GADD45B over-expression correlates with MKK7 phosphorylation in androgen-independent prostate cancer cells

Due to the crucial role of GADD45B in blocking MKK7 signaling, we investigated on MAPKs signaling in androgen-independent prostate cancer cell lines DU145 and PC3, and in androgen-dependent prostate cancer cell lines 22Rv1 and LNCaP by western blot analysis. Interestingly, as shown in Figure 3-6, we observed two MKK7 isoforms (see total MKK7) and two MKK4 isoforms (see total MKK4) (Fig. 3-6). Interestingly, the shorter MKK7 isoform was found highly phosphorylated specifically in androgen independent DU145 cells and at less strengthen in PC3 cell line (see Fig. 3-6, P-MKK7 panel, 37 KDa band). Low levels of phosphorylated full-length form of MKK7 was found only in 22Rv1 cell line (see Fig. 3-6, P-MKK7 panel, 49 KDa band) whereas no significant MKK7 phosphorylation was observed in LNCaP at basal condition. Previous studies have demonstrated that MKK7 shorter isoforms have a lower activity than the full length MKK7 protein, whereas they have also higher inducibility[173]. Importantly, despite constitutive phosphorylation of MKK7 shorter isoform, we did not observe any JNK activity neither in DU145 nor in PC3 cell lines, suggesting that GADD45B over-expression in these cells plays a key prosurvival role by inhibiting MKK7 activity and thus avoiding prolonged JNK activation. Also 22Rv1 cells did not show any JNK activation despite of a very slight p-MKK7 band (Fig. 3-6)

It is known that MKK4 activation correlates with progression from androgen-sensitivity to androgen-independent status; in fact, MKK4-JNK signaling has been shown to play a key role in inhibiting androgen receptor activity and in promoting androgen-independent phenotype[261]. Here, we found a marked phosphorylation of the shorter isoform in DU145 androgen independent cells (Fig. 3-6). This shorter isoform was evident only in this cell line (see total MKK4, Fig 3-6). The precise role of this MKK4 shorter isoform is not known, however, the presence of an in-frame start codon in MKK4 mRNA at docking N-terminal region suggests that it could exhibit similar behavior than shorter MKK7 isoform described above. Considering that GADD45B has been shown to specifically bind MKK7, these results suggest also that in DU145 cells another inhibitor might be express that specifically blocks MKK4 activity.

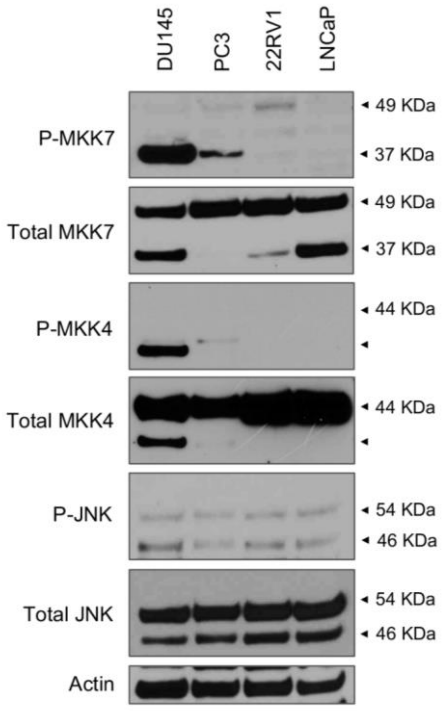

Figure 3-6. ***MAPK signaling in prostate cancer cell lines.*** Western blot analysis showing total and phosphorylated (P) proteins in prostate cancer cell lines at basal level. Shown are short isoform and full-lenght isoform of MKK7 and MKK4. Actin is shown as loading.

3.5. MKK7-JNK response is inhibited in androgen-independent cell lines

To further investigate on MKK7-JNK response in androgen-independent and androgen-dependent prostate cancer cells, we performed experiments on MAPKs molecular dynamics upon stimulation with TNF-α, a cytokine able to induce NF-kB signaling and MKK7-dependent JNK activation. Indeed, this cytokine is often secreted by several cells in tumor microenvironment, therefore it is extremely relevant to understand the mechanisms that promote or inhibit TNF-α-induced cell death. Among used cell lines, DU145, PC3 and 22Rv1 cells are resistant to TNF-α treatment, whereas LNCaP cells resulted TNF-α sensitive and showed sign of apoptosis (data not shown). All cell lines were treated with TNF-α 20 ng/ml for specific time points and nuclear-cytoplasmic fraction were analyzed by Western Blot. As shown in fig. 3-7, treatment with TNF-α rapidly induced p65 phosphorylation in all cell lines.

DU145 cells respond to TNF-α stimulation with a rapid and transient JNK activation; at 30 minutes after treatment, we observed a rapid phosphorylation of full-length MKK7 isoform. Moreover, an increase of the shorter MKK7 isoform in the nuclear fractions were observed. A slight increase in phosphorylation was observed also for MKK4 short isoform, whereas no activation of full-length MKK4 was observed. Finally, no nuclear JNK translocation was detected. These data reveal that DU145 cells show a transient MKKs-JNK response and that TNF-α signaling transduction is mediated by MKK7 full-length isoform activation and involve also shorter MKKs isoforms.

TNF-α response of PC3 cell line displayed a different scenario (Figure 3-7 B). Surprisingly, despite the activation of full-length MKK7 isoform we observed no significant activation of JNK proteins. A low and transient JNK activation was only detected at 12h after treatment and coincides with activation of both MKK4 full-length and short isoforms. Interestingly, at this time, shorter MKK4 isoforms activation was detected in nuclear compartment. We also observed a transient activation and nuclear translocation of MKK7 shorter isoform. Again, these data suggest that, despite the activation of MKK7 kinase, a prolonged JNK activation is not triggered, highlighting the existence of a block in MKK7-JNK signaling.

Compared to DU145 cells and PC3, 22Rv1 cell line show a different response upon TNF-α stimulation. Indeed, as shown in Figure 3-7 C, at 30 minutes after treatment, we observed an increase in phosphorylation status of MKK7 full-length isoform with a correspondent progressive activation in JNK proteins. Interestingly, we also observed an induction of shorter MKK7 isoform suggesting further a possible role in mediating TNF-α signaling. However, unlike MKK7, no significant MKK4 activation was detected.

TNF-α response in LNCaP cell line is shown in Figure 3-7 D. LNCaP cells respond to TNF-α with a strong JNK activation at 12-24 hours after treatment. Importantly, we observed a progressive activation of both MKK4 and MKK7 full-length isoforms. We also detected nuclear phosphorylation of MKK7 shorter isoform whereas no nuclear MKK4 signaling was observed. Moreover, at 24 hours we detected the cleavage of caspase 3 (data not show) denoting activation of apoptosis.

Taken together, these findings reveal that MKK7 is induced by TNF-α in all prostate cell lines but JNK activation showed different kinetic of induction, depending on Gadd45β expression. Indeed, despite MKK7 activation is present, androgen-independent DU145 and PC3 cell lines do not show a significant JNK activation and when present it appears transient or not sufficient to induce an apoptotic response. These cell lines are also the two whose GADD45B expression was higher (Fig. 3-3 B-C). Androgen-sensitive 22Rv1 cells show an intermediate response; they responded to TNF-α stimulation with a progressive MKK7 and JNK activation whereas no significant MKK4 activation was detected. This cell line showed low GADD45B expression level (Fig. 3-3 B-C). Finally, LNCaP cells, that showed lower levels of GADD45B expression, showed a constant over time activation of both MKK7 and MKK4 kinases and responded to TNF-α stimulation undergoing apoptosis.

In addition, although further studies are needed to fully understand the specific function, we identified the involvement of MKKs shorter isoforms in modulating TNF-α signaling in prostate cancer cells and highlighted the presence of nuclear-cytoplasmic shuttling of these shorter isoforms upon stimulation. The

greater understanding of these isoforms and their shuttling will be very useful to the development of subcellular specific therapeutic strategies.

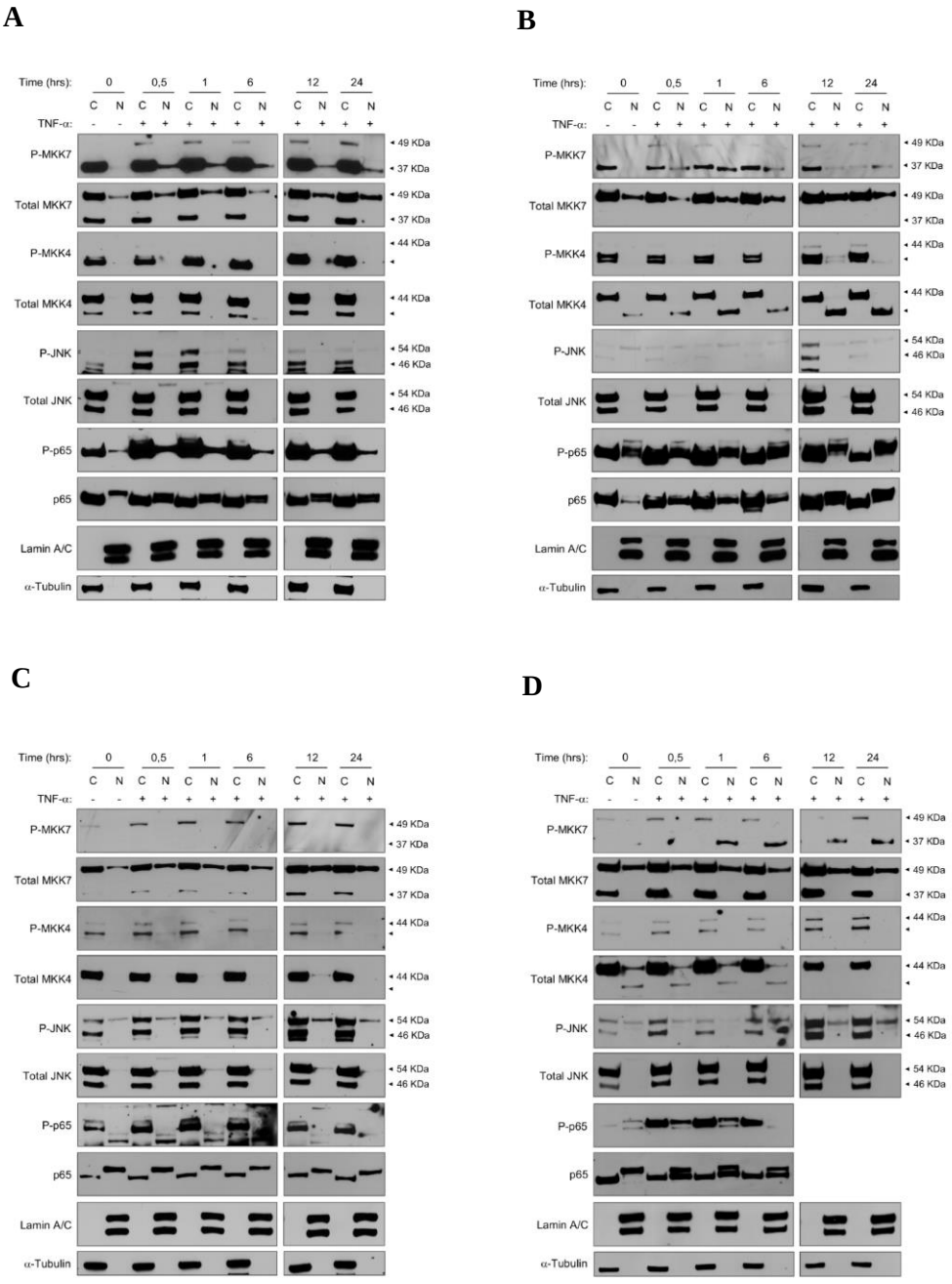

Figure 3-7. ***MAPKs signalling activation by TNFα in prostate cancer cell lines.*** (A-D) Western blot showing total and phosphorylated (P) proteins in nuclear (N) and cytoplasmic (C) fractions of DU145 (A), PC3 (B), 22Rv1 (C) and LNCaP (D) following stimulation with TNF-α (20 ng/ml) at the indicated time point. α-Tubulin and Lamin A/C are shown as loading controls.

3.6. Efficacy of MKK7-GADD45B inhibitory molecule DTP3 for prostate cancer treatment

Considering that androgen-independent cell lines were demonstrated to express constitutive activation of NF-kB (Fig. 3-2), high levels of GADD45B (Fig. 3-3) with pro-survival activity (Fig. 3-5) and constitutive activation of the short isoform of pMKK7 with no activation of JNK (Fig. 3-6), we thought to assess the therapeutic potential of DTP3 in our models of prostate cancer cells. As mentioned earlier, DTP3 molecule is able to interfere with GADD45B-MKK7 complex leading to MKK7 activation and thus promoting JNK-mediated apoptosis. To this end, we treated all four cell lines with DTP3 at 500 µM for different time points (Fig.3-8). LNCaP cell line, having a faint GADD45B expression, was considered as a negative control. As expected, we observed a significant reduction in cell survival of DU145, PC3 and 22Rv1 cells upon treatment with DTP3, whereas LNCaP which showed, as discussed previously, no MKK7 constitutive activation and low GADD45B did not showed any sensitivity to DTP3 induced death. Importantly, these responses reflect the expression levels of GADD45B. In fact, scaling down the DTP3 doses (Fig. 3-9) we observed that DU145 cells, having the high level of GADD45B and expressing significant amount of active MKK7, respond only at dose of DTP3 500 µM (Figure 3-9 A); PC3 cells, which express high levels of GADD45B but lower than DU145, are responding at dose of DTP3 250-500 µM (Figure 3-9 B); whereas 22Rv1, expressing both GADD45B and active MKK7 at lower levels showed a sensitivity also at DTP3 50 µM (Figure 3-6 C). All together, these data demonstrated that disrupting the binding of GADD45B to MKK7 by means of DTP3 molecule might be an efficacy strategy for advanced prostate cancer treatment.

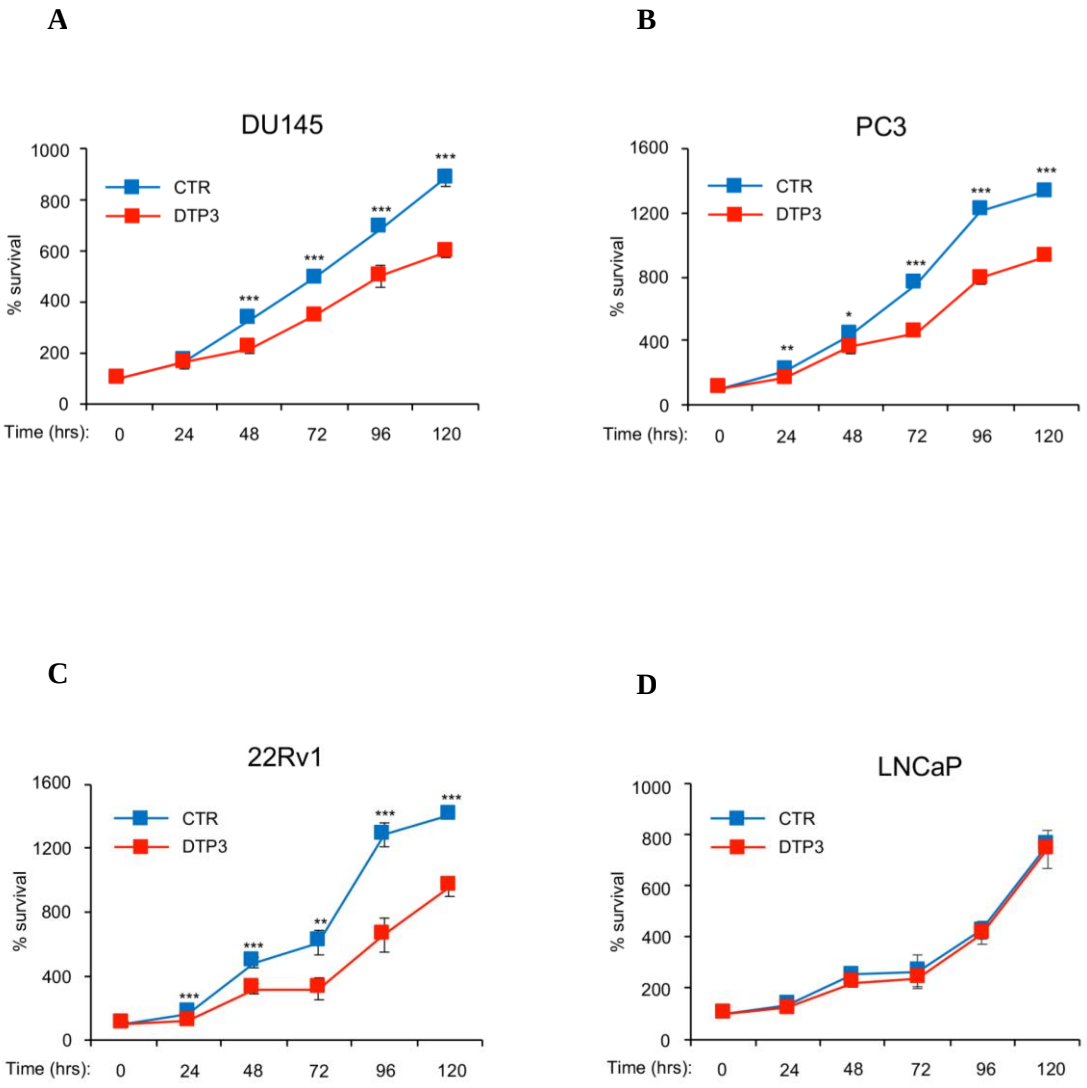

Figure 3-8 ***The potential activity of DTP3 in prostate cancer cell lines.*** **(A-D)** Cell-Titer Glo showing the viability of DU145 **(A)**, PC3 **(B)**, 22Rv1 (**C)** and LNCaP (**D)** following 5-day treatment with 500 µM of DTP3. Experiment was conducted in triplicate. Values are mean ± S.D.

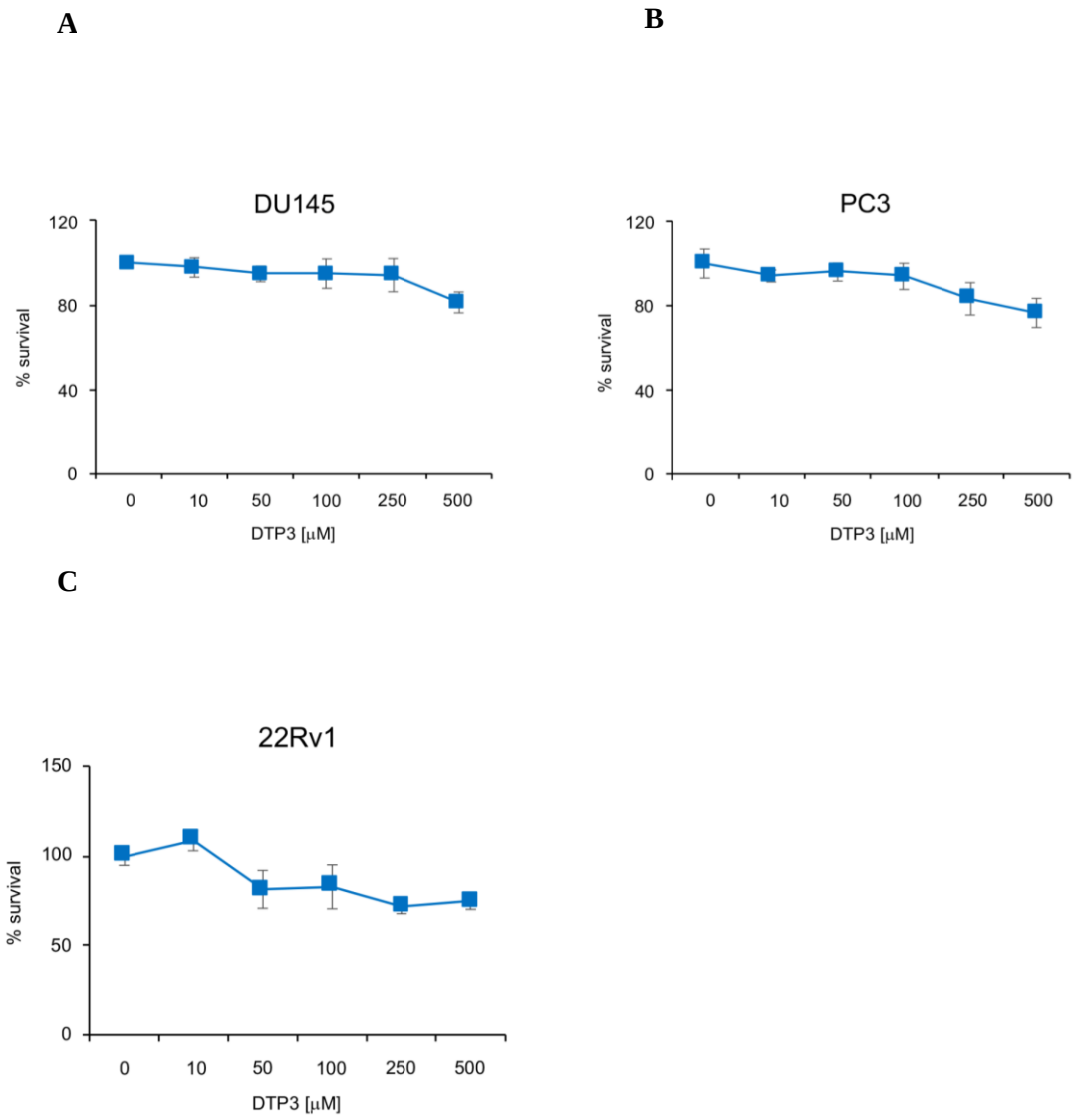

Figure 3-9 ***The therapeutic activity of DTP3 in prostate cancer cell lines.*** **(A-C)** Cell-Titer Glo showing the viability of DU145 **(A)**, PC3 **(B)**, and 22Rv1 (**C)** following 5-day treatment with the indicated concentration of DTP3. Experiment was conducted in triplicate. Values are mean ± S.D.

4. Discussion

Prostate cancer represents one of most frequent cancer in men in industrialised countries[15]. Despite standard treatments for localized disease such as radiotherapy, prostatectomy, and androgen deprivation therapy (ADT), a significant number of men will develop disease recurrence and ultimately metastatic cancer, and many of them develop lethal prostate cancer. Although ADT is highly effective at inducing apoptosis in prostate cancer cells and delays disease progression, patients will inevitably over time develop lethal, androgen-independent metastatic prostate cancer. In the last few years, important progress has been made in understanding the molecular events occurring during prostate cancer progression. The further understanding of the role of dysregulation of pathways and proteins expression is fundamental for improving novel strategies of molecular target therapy.

NF-κB deregulation frequently correlate with cancer development and progression and is associated with cancer cells survival, chemotherapeutic drug resistance and lack of apoptotic response. In this work, by analyzing the human dataset of Prostate adenocarcinoma acinar type (PRAD) of The Cancer Genome Atlas (TCGA), we demonstrated that NF-κB over-expression statistically correlates with a worst prognosis in human prostate cancer. Therefore, using DU145, PC3, 22Rv1 and LNCaP cell lines as models, we demonstrated a constitutive activation of canonical NF-κB signaling in prostate cancer that correlates with cancer progression. For this purpose, we evaluated by Western Blot the expression level of phosphorylated form of transcription factor p65 at Ser536 and performed a DNA-binding assay to quantify the relative amount of p65-DNA bound levels. Highest expression of p-p65 and highest p65-DNA binding levels were detected in androgen independent DU145 and PC3 cells. Subsequently, due to pro-survival role of GADD45B and its close relationship with NF-κB, we investigated on the possible role of GADD45B in prostate cancer. We showed that GADD45B expression correlates with androgen-independent phenotype and NF-κB activation because highest levels of expression were detected in DU145 and PC3 cell lines. Moreover, a large fraction was observed in cytoplasm compartment of androgen-independent cell lines suggesting a potential role in inhibiting MKK7-JNK signaling. Due to the

role of GADD45B as a linker protein between NF-κB and JNK pathways, we also investigated on MAPKs pathway. We observed a constitutively active MAPKs system without significant JNK activation and identified the existence and activation of shorter MKKs isoforms that correlate with cancer progression; although further studies are needed to fully understand their specific function, Tournier *et al*.[173] revealed that these low molecular weight isoforms show a lower capabilities in activating JNK proteins although possess a greater inducibility upon stimulation compared to full-length isoforms. Despite these MKK7 isoforms activation in DU145 and PC3 cell lines, any phosphorylation of MKK7 full-length isoform was observed in basal condition. On the other hand, we demonstrated a slight constitutive activation of MKK7 full-length isoform in 22Rv1 cell line that showed lower GADD45B levels compared to DU145 and PC3 cells. Finally, LNCaP cell line, that showed the lowest activation of MAPKs system, also showed the lowest level of GADD45B expression with a mainly nuclear localization. These findings suggest that the high GADD45B expression, which correlates with an high constitutive MKK7 activity, is probably necessary to prevent JNK hyperactivation thus protecting tumor cells from apoptosis. Indeed, inhibition of GADD45B in these cells is sufficient to induce JNK-mediated apoptosis.

We further investigate on MKK7-JNK signaling in prostate cancer cell lines treated with TNF-α. Upon TNF-α stimulation, we also established the presence of an inhibition of MKK7-JNK axis that correlates with cancer progression that could represent the cause of TNF-induced cell death resistance. We also highlighted the potential role of shorter MKK7 isoforms in modulating JNK signaling in TNF-α-stimulated conditions due to their inducibility and nuclear-cytoplasmic shuttling upon stimulation, thus revealing that TNF-α signaling involves both cytoplasmic and nuclear signaling in prostate cancer.

Finally, in light of these findings, we evaluated DTP3 as potential therapeutic molecule. Over the past 25 years it has been a priority to pursue a strategic approach for therapeutically targeting NF-κB pathway in cancer. Nevertheless, despite numerous attempts by the pharmaceutical industry, no specific NF-κB inhibitors have been approved for clinical use, due to the preclusive toxicities associated with the systemic suppression of NF-κB. Thus, the innovative

research turned towards the development of a new generation of NF-κB inhibitors, able to target either upstream signaling mechanisms that regulates NF-κB activation or downstream effectors of the NF-κB tumorigenic functions. The role of GADD45B in cancer cell survival has been well established in the context of Multiple Myeloma, a tumor in which NF-κB is stably activated by recurrent genetic alterations of its upstream regulators. The recently developed D-tripeptide inhibitor of the GADD45B and MKK7 interaction, DTP3, was shown to exhibit a potent and cancer-cell selective capacity to induce JNK-dependent apoptosis of Multiple Myeloma cell lines and primary cells from patients, both *in vitro* and *in vivo*, without any toxicity to healthy tissues. In keeping with these preclinical results, an initial evaluation of the first-in-human, phase I/IIa clinical study of GADD45B-targeting agents in patient with Multiple Myeloma has demonstrated clinical safety and tolerability at all dose levels investigated thus far, alongside a cancer-selective pharmacodynamic response. These results identified the GADD45B/MKK7 complex as an essential survival module downstream of NF-κB and a novel therapeutic target. Interestingly, here we showed that DTP3 is also effective in prostate cancer cell lines over-expressing GADD45B and showing constitutive activation of MKK7 without any constitutive JNK activity. Indeed, analyzing drug titration curves we observed that the effective dose is proportional to GADD45B expression.

In conclusion, we identified GADD45B as a pro-survival factor in advanced prostate cancer and as a molecular target for therapeutic intervention with DTP3. Further experiments in mouse preclinical models will be useful to define the DTP3 therapeutic efficacy *in vivo*.

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