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14. ABSTRACT: The research project was planned to investigate the role of I κ B kinases (IKK) and NF- κ B in development of prostate cancer (CaP), and explore the possibility that IKK inhibitors can be used in CaP treatment. To reach this goal we employed mouse models and in vitro cell culture systems. We found neither IKK β nor IKK α were required for normal prostate development. However, IKK α plays an important role in development of advanced CaP, particularly in CaP metastasis. IKK α links inflammation to CaP metastasis by suppressing expression of the metastasis-suppressor gene maspin in response to RANK signaling. We also found that IKK β /NF- κ B activities in CaP cells were dramatically increased during development of androgen resistance. Deletion of IKK β in inflammatory cells of the tumor microenvironment significantly reduced androgen-independent CaP formation. Furthermore, we found that a putative IKK inhibitor, IT-3, could suppress the proliferation of human CaP cells in vitro. Our results suggest an important role for both IKK α and IKK β in development and progression of CaP, and inhibition of one or both of them would be an effective option for treatment of CaP, especially for prevention of metastasis and emergence of hormone refractory CaP.					
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Introduction

NF- κ B transcription factors play an important role in regulation of innate and adaptive immune responses, inflammation, and cell survival (1). By virtue of their ability to activate many anti-apoptotic genes and become activated during chronic inflammation, NF- κ B transcription factors were suggested to play a key role in cancer development and progression, especially in the context of underlying or tumor-generated inflammation (2). A wide range of stimuli activate NF- κ B, mostly through I κ B kinase (IKK)-dependent phosphorylation and subsequent degradation of I κ B inhibitory proteins (3). The freed NF- κ B dimers enter the nucleus where they enhance transcription of many genes encoding cytokines, growth factors, cell adhesion molecules, and anti-apoptotic proteins (1). The IKK complex consists of two highly homologous kinase subunits (IKK α and IKK β) and a nonenzymatic regulatory component, IKK γ /NEMO (3).

There are two NF- κ B activation pathways. The first pathway, the classical NF- κ B activation pathway, is normally triggered in response to microbial and viral infections and exposure to proinflammatory cytokines which activate the three subunit IKK complex leading to phosphorylation-induced degradation of I κ Bs. This pathway depends mainly on the IKK β catalytic subunit (4). The other pathway, the alternative pathway, leads to activation of p52:RelB dimers by inducing processing of the NF- κ B2/p100 precursor protein that binds RelB in the cytoplasm. This pathway is triggered by certain members of the tumor necrosis factor (TNF) cytokine family through selective activation of IKK α homodimers by the upstream kinase NIK (3, 5, 6).

There is considerable evidence that the two IKK/NF- κ B signaling pathways are involved in carcinogenesis, cancer progression, metastasis and drug resistance. Although certain oncoviral proteins, cancer-associated chromosomal translocations and mutations can lead to constitutive activation of NF- κ B in cancer progenitor cells (7), the most common mechanism leading to NF- κ B activation during tumorigenesis depends on autocrine and paracrine production of proinflammatory cytokines or underlying chronic infections (2). Persistent activation of NF- κ B can lead to increased production of tumor growth factors by components of the tumor stroma as well as to upregulation of anti-apoptotic genes within the cancer cell itself (2). This process was recently demonstrated to occur during two different mouse models of inflammation-associated cancer leading to development of colorectal cancer (8) and hepatocellular carcinoma (9, 10). We also provided evidence for a critical role of NF- κ B in inflammation-driven metastatic growth (11). In that model, as well, IKK-driven NF- κ B activation is responsible for production of growth and survival factors by stromal components (macrophages) and upregulation of anti-apoptotic genes within the cancer cell (11).

Prostate cancer (CaP) is one of the most common cancers in men and the second leading cause of cancer-related deaths among men in the United States. Androgens stimulate the growth of both the normal prostate epithelium and early CaP. While early CaP responds to androgen-ablation therapy, disease progression is characterized by a switch from an androgen-dependent (AD) to an androgen-independent (AI) phenotype (12). Although 80% of patients with advanced CaP initially respond to androgen ablation therapy, within 12-18 months most CaPs become AI, resulting in poor prognosis (13). The mechanisms underlying the evolution of AI cancer remain poorly understood, but their elucidation should offer ample opportunities for development of novel therapeutic measures.

It was shown that NF- κ B transcription factors can directly interact with several members of the nuclear receptor family including androgen receptor (AR) itself (14-16). Thus, NF- κ B may function as a co-activator for AR causing it to be active independently of androgen binding (14-

16). In this case, prostate epithelial cells with high NF- κ B activity are rendered resistant to androgen withdrawal. However, NF- κ B can also trans-repress ligand-bound AR or repress expression of the AR gene itself (15). Most importantly, NF- κ B activity itself is repressed by androgen treatment via AR-mediated trans-repression (14, 17) or other mechanisms. In this case, NF- κ B activity may increase in response to androgen withdrawal. Once activated, NF- κ B can stimulate production of various cytokines by prostate epithelial cells, CaP and stromal components. Specifically, IL-6, a well-known autocrine and paracrine growth factor for AI CaP cells (18, 19), is encoded by a typical NF- κ B target gene (20). The dependence of IL-6 expression on NF- κ B was demonstrated in the prostate epithelium (21). Importantly, the human AD CaP cell line, LNCaP, was shown to assume a neuroendocrine (NE) phenotype in response to IL-6 exposure (22). Although NE cancers of the prostate are rare, foci with NE-like features can be observed in nearly all prostate adenocarcinomas and extensive NE differentiation is generally considered to be of poor prognostic value (23). Indeed, NE differentiation appears more frequently in hormone-refractory cancer (23). Inhibition of IL-6 activity induces the regression of AI human CaP xenografts in mice (18). Thus, NF- κ B inhibition should result in a similar effect by inhibiting IL-6 expression. Studies performed on various CaP cell lines reveal that AI cells often display constitutive NF- κ B activity (24). Interestingly, inhibition of NF- κ B activity in such cells was achieved by treatment with ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) that most likely acts as a non-specific IKK inhibitor in a manner akin to aspirin (24). Furthermore, constitutively activated NF- κ B in three different human CaP cell lines was linked to over expression of IKK subunits, and inhibition of NF- κ B activity in these cells through expression of a non-degradable super-repressor mutant of I κ B α resulted in either spontaneous apoptosis or increased sensitivity to TNF- α (25).

In this research project we focused on the role of the two IKK catalytic subunits and NF- κ B in the development of CaP, and examined the possibility that IKK inhibitors can be used in the therapy of CaP through their ability to induce apoptosis of prostatic carcinoma cells or increase their susceptibility to apoptosis-inducing anti-cancer drugs or therapeutic radiation. These studies, which are summarized below in detail, have already resulted in one original paper accepted for publication in "*Nature*", two review articles published in "*Journal of Clinical Investigation*" and "*Journal of Clinical Immunology*", and many other interesting results that are being prepared for publication.

Body:

1. Examine the role of IKK α in prostate cancer genesis, progression and metastasis

NF- κ B activation depends on the I κ B kinase (IKK) complex, which contains two catalytic subunits: IKK α and IKK β (3, 26). Gene ablation experiments have revealed a major role for IKK β in NF- κ B activation(27). By comparison, the function of IKK α is versatile, including NF- κ B-independent control of keratinocyte differentiation(28) and attenuation of NF- κ B activation during inflammation and its resolution(29). IKK α also activates the alternative NF- κ B signaling pathway in immune cells(6) and the classical NF- κ B pathway in mammary epithelial cells(30). In the latter case, IKK α is activated upon occupancy of receptor activator of NF- κ B (RANK) by RANK ligand (RANKL), leading to induction of cyclin D1 transcription and proliferation of mammary epithelial cells during pregnancy(30).

Given that development of both the mammary and prostate glands is controlled by sex steroids, we examined whether IKK α activity is also required for development of the prostate gland and emergence of CaP. Although *Ikk α ^{AA/AA}* mice, in which IKK α activation is prevented by replacement of the two critical serines in its activation loop with alanines(30), exhibit normal prostate development and form CaP in response to a prostate-specific SV40 T antigen (Tag) transgene(31), they display very few distant organ metastases. This observation led us to

identify a new signaling pathway that depends on IKK α enzymatic activity and its translocation to the nucleus, but is independent of NF- κ B. This pathway can be triggered by occupancy of RANK, whose ligand is produced by tumor-infiltrating inflammatory cells and leads to repression of the metastasis suppressor gene maspin. The presence of activated IKK α in the nucleus also correlates with clinical progression in human CaP. The following are details of our research procedures and findings regarding the role of IKK α in prostate cancer progression and metastasis.

a) IKK α activity is required for prostate cancer metastasis

Ikk α ^{AA/AA} mutant mice were crossed with TRAMP mice(31) expressing SV40 Tag from the prostate specific probasin promoter for at least 6 generations. As previously described, WT/TRAMP mice developed CaP rather early and started dying around 22 weeks of age, but homozygosity for the *Ikk α ^{AA}* allele on the same genetic background prolonged tumor onset and delayed mortality (Fig. 1a). Whereas the entire WT/TRAMP cohort succumbed to CaP by 40 weeks of age, 50% of the *Ikk α ^{AA/AA}/TRAMP* cohort was still alive at this point. Despite reduced mortality in the *Ikk α ^{AA/AA}/TRAMP* group, no discernable histological differences in primary tumors and lymph node metastases were found between the genotypes upon necropsy (Fig. 1b-g). Despite having typical primary CaPs at the time of death whose size was similar to primary CaP in dead WT/TRAMP mice, *Ikk α ^{AA/AA}/TRAMP* mice exhibited considerably less distant site metastases (Fig. 1h-l). For instance, 87% (20/23) of dead WT/TRAMP mice had pelvic lymph node metastases (Fig. 1h), 43% (10/23) had renal lymph node metastases (Fig. 1i), 17% (4/23) had liver metastases (Fig. 1j) and 22% (5/23) had lung metastases (Fig. 1k). By contrast, only 41% (9/22) of dead *Ikk α ^{AA/AA}/TRAMP* mice exhibited pelvic lymph node metastases (Fig. 1h), 18% (4/22) showed renal lymph node metastases (Fig. 1i), and none (0/22) had liver or lung metastases (Fig. 1j-k). The average number of lymph nodes harboring metastases in *Ikk α ^{AA/AA}/TRAMP* mice was much lower than in WT/TRAMP mice (Fig. 1l). Consistent with previous observations, TRAMP mice of either genotype exhibit very few bone metastases.

b) IKK α kinase activity is not critical for early carcinogenesis

These findings suggested that IKK α plays a critical role in CaP progression, particularly in metastatic spread. To detect if IKK α affected early prostate carcinogenesis, we compared prostate size, weight, morphology and histology in three month-old male mice. No clear differences in these parameters were found and both genotypes showed similar primary tumor pathology, from high grade PIN to well differentiated CaP (Fig. 2a-b), suggesting that IKK α kinase activity is not critical for early prostate carcinogenesis.

As mentioned above, growth of the prostate and mammary epithelia depend on sex steroids and *Ikk α ^{AA/AA}* females exhibit retarded lobuloalveolar epithelial development during pregnancy. We therefore extended the analysis of prostate development to nontransgenic *Ikk α ^{AA/AA}* and WT mice, but found no differences in prostate size, weight, morphology, histology and proliferation rates (Fig. 3). We did, however, identify a proliferation defect in *Ikk α ^{AA/AA}/TRAMP* CaP of four-month-old mice. Male mice of either genotype were pulsed with 5-bromodeoxyuridine (BrdU) and BrdU incorporation, which identifies cells undergoing DNA synthesis, was examined 2.5 hrs later. Fewer BrdU-positive cells were present in CaP of *Ikk α ^{AA/AA}/TRAMP* mice than in WT/TRAMP CaP (Fig. 4a-b). However, primary tumor burden was similar at time of death in WT/TRAMP and *Ikk α ^{AA/AA}/TRAMP* mice, indicating that despite lower growth rates, primary CaP eventually reach the same size in *Ikk α ^{AA/AA}/TRAMP* mice as in WT/TRAMP mice and that accelerated proliferation in WT CaPs is partially balanced by increased death.

Progression of CaP in TRAMP mice was shown to correlate with reduced E-cadherin expression, a hallmark of EMT. The same was observed in our mice regardless of their genotype (Fig. 5a, b). CaP progression is also associated with increased expression of the

neuroendocrine marker synaptophysin both in human cancer and TRAMP mice(32, 33). Synaptophysin expression was indeed elevated in advanced CaP from 7-9 month old mice but was not influenced by the IKK α status (Fig. 5c).

c) Elevated maspin expression in primary tumors of *Ikk α ^{AA/AA}/TRAMP* mice

Tumor metastasis is governed by genetic and epigenetic factors that regulate metastasis-suppressor or metastasis-promoter genes (34). We compared expression of approximately forty such genes, including E cadherin, in primary CaP of both genotypes by quantitative (Q) RT-PCR (Fig. 6). The only gene that exhibited marked and consistent differences between *Ikk α ^{AA/AA}/TRAMP* and WT/TRAMP CaP was maspin (35), an established tumor metastasis-suppressor (Fig. 7a). Maspin was identified as a gene expressed by normal mammary epithelial cells but not by mammary carcinoma, whose forced expression can inhibit invasion and motility of breast cancer cell lines (35). Further studies confirmed that maspin is highly expressed in normal breast epithelial cells and that its expression is frequently decreased in breast cancer cells and completely lost in metastatic carcinoma. Ectopic maspin expression in mammary epithelium inhibits development of lobuloalveolar structures during pregnancy(36), resulting in a phenotype similar to the mammary phenotype of *Ikk α ^{AA/AA}* mice. Furthermore, maspin overexpression from a mammary epithelial promoter dramatically decreased metastasis of SV40 Tag-induced mammary carcinoma(37). Importantly, there are strong inverse correlations between maspin expression and metastatic potential in human CaP(38).

We examined maspin expression in prostates of WT/TRAMP and *Ikk α ^{AA/AA}/TRAMP* mice during tumor progression. Maspin was similarly expressed in cancerous prostates of both genotypes at 3 months of age (Fig. 7b). However, in WT/TRAMP mice maspin expression began to decline in CaP at 4-5 months of age but remained high in *Ikk α ^{AA/AA}/TRAMP* CaPs (Fig. 7c). At 7-8 months of age maspin was no longer detected in WT/TRAMP CaP, but primary tumors in *Ikk α ^{AA/AA}/TRAMP* mice retained high maspin expression (Fig. 7a, d). Immunohistochemistry analysis revealed that maspin was highly expressed in cytoplasm and membranes of *Ikk α ^{AA/AA}/TRAMP* CaP cells, but was barely detectable or absent in WT/TRAMP CaP cells (Fig. 7e, f). Consistent with its role as a metastasis-suppressor, maspin gene expression was extinguished in the rare lymph node metastases found in *Ikk α ^{AA/AA}/TRAMP* mice (Fig. 7a).

d) IKK α activity and maspin expression determine metastatic potential of CaP cells

To determine whether changes in maspin expression account for reduced metastasis in *Ikk α ^{AA/AA}/TRAMP* mice we examined metastatic potential of primary CaP cells from 7-8 month old mice that were cultured for 1-3 weeks. Single-cell suspensions were injected into spleens of Nude (*Nu/Nu*) male mice and 10 min later the spleens were removed. After 4 weeks, metastases to liver were enumerated and liver SV40 Tag mRNA was quantitated. While maspin-negative WT CaP cells gave rise to numerous liver metastases, very few metastases were formed by *Ikk α ^{AA/AA}* CaP cells, which expressed high amounts of maspin (Fig. 8a). Similar results were obtained by Q-PCR analysis of Tag mRNA (Fig. 8b). By contrast, the tumorigenic potential of subcutaneously transplanted *Ikk α ^{AA/AA}* CaP cells was only marginally lower than of WT CaP cells (Fig. 9). Next, we examined whether manipulation of maspin expression alters metastatic potential. Primary WT CaP cells were infected with either a maspin-expressing retrovirus or an empty retrovirus control. Transduced cells selected in zeocin for 1 week contained high levels of maspin when infected with the maspin retrovirus (Fig. 10a). Forced maspin expression greatly reduced metastatic ability measured by the same assays described above (Fig. 10b, c). We also knocked down maspin expression in *Ikk α ^{AA/AA}* CaP cells using a maspin siRNA-retrovirus. After one week selection in puromycin, cells receiving maspin siRNA contained much less maspin (Fig. 10d) and displayed substantially higher metastatic potential than mock-infected cells (Fig. 10e, f). Importantly, maspin knockdown increased the metastatic

potential of *Ikkα^{AA/AA}* CaP cells to that of WT CaP cells (Fig. 10g). Thus, differential maspin expression is a major determinant of metastatic potential in our experimental system.

To examine the effect of IKKα activation on metastatic activity we used adenoviral vectors to express either WT or a constitutively active IKKα(EE), in which the activation loop serines were replaced with phosphomimetic glutamate residues, in *Ikkα^{AA/AA}* primary CaP cells. Due to the transient nature of adenovirus infection we used the chick embryo spontaneous metastasis assay to determine metastatic activity. This model allows for spontaneous metastatic spread of cancer cells inoculated on the chorioallantoic membrane of 10 days old chicken embryos. Only cells transduced with the IKKα(EE) virus showed metastatic activity in this assay (Fig. 11). Yet, primary transplanted tumors grew at the same rate. Thus in addition to maspin, IKKα activity (which controls maspin transcription, see below) also determines metastatic potential.

e) IKKα translocates to the nucleus in prostate carcinoma and inhibits maspin transcription

Next, we examined how IKKα controls maspin gene expression. Primary *Ikkα^{AA/AA}* CaP cells expressing endogenous maspin were infected with adenoviruses encoding GFP, WT IKKα, activated IKKα(EE) or activated IKKβ(EE). Three days later, maspin protein levels were examined. IKKα(EE) significantly down-regulated maspin expression, while WT IKKα or IKKβ(EE) had little or no effect (Fig. 12a). To examine whether IKKα regulates maspin gene transcription we used a maspin-luciferase reporter containing 759 base pairs (bp) of the 5'-human maspin upstream region. Transfection of the reporter with different amounts of IKKα(EE) expression vector into *Ikkα^{-/-}* mouse embryonic fibroblasts (MEFs) resulted in dose-dependent repression, an effect not seen with WT IKKα or the inactivateable IKKα(AA) variant (Fig. 12b). No repression of maspin promoter activity was seen upon co-expression of activated IKKβ(EE) (Fig. 12c), which is a more potent activator of NF-κB-dependent transcription than IKKα(EE) (Fig. 13).

We generated human maspin promoter truncation mutants and fused them to a luciferase reporter. These constructs were co-transfected with or without IKKα(EE) expression vector into *Ikkα^{-/-}* MEFs and luciferase activity was determined. There was a sharp cutoff in responsiveness to IKKα(EE) between 316 and 267 bp of maspin 5' DNA (Fig. 14). This region of the human maspin promoter contains several putative *cis* acting elements including an androgen receptor (AR) responsive element (ARE). Although AR was reported to repress maspin transcription, we could not find any effect of castration on mouse maspin expression (Fig. 15) or inactive (unliganded) or activated AR on repression of human maspin promoter by IKKα(EE) (Fig. 16). Since the transfection experiments were conducted with the human maspin promoter, we used the normal human mammary epithelial HME cells that express maspin to examine whether ectopically introduced activated IKKα interacts with the endogenous maspin promoter in native chromatin. Chromatin immunoprecipitation (ChIP) analysis revealed that activated IKKα was recruited to the endogenous maspin promoter region (Fig. 17). The interaction was specific as IKKα did not interact with intron 2 of the maspin gene.

We noticed that endogenous IKKα was present in both the cytoplasmic and nuclear fractions of CaP cells, whereas IKKβ and the IKKγ/NEMO regulatory subunit were exclusively cytoplasmic (Fig. 18a, d). Very little, if any, nuclear IKKα was found in normal prostate epithelium and immunoblotting with phospho-specific antibody revealed that nuclear IKKα in CaP was mostly in its phosphorylated and activated form. While these results are consistent with presence of a nuclear localization sequence (NLS) in IKKα, it is noteworthy that nuclear IKKα was previously found only in differentiating keratinocytes. To examine whether IKKα acts in the nucleus to control maspin expression, the maspin-luciferase reporter (pM-759-Luc) was co-transfected with IKKα(EE), IKKα(EE)-NLS (a nuclear localization defective version of

activated IKK α) and IKK α (KM) (a catalytically inactive mutant of IKK α) expression vectors into *Ikk α ^{-/-}* MEFs. Neither IKK α (EE)-NLS nor IKK α (KM) repressed maspin promoter activity (Fig. 18b). To exclude the possibility that IKK α (EE)-NLS lost its kinase activity, IKK α (EE)-NLS or IKK α (EE) was expressed in HEK293 cells and their I κ B kinase activity was measured. IKK α (EE)-NLS was as active as IKK α (EE) in I κ B phosphorylation (Fig. 18c). Hence, the ability of IKK α to repress maspin transcription requires kinase activity and nuclear entry. As NF- κ B activation depends on I κ B phosphorylation in the cytoplasm, and IKK β (EE), which is a more potent activator of NF- κ B than IKK α (EE) (Fig. 13), did not repress maspin expression, repression of maspin by IKK α appears to be NF- κ B independent.

The presence of IKK α in the nucleus correlated with the state of CaP progression. The amount of activated nuclear IKK α was substantially higher in CaP from 7-8 month-old WT/TRAMP mice than in CaP from younger mice (Fig. 18d). Furthermore, the amount of IKK α in the nucleus was inversely correlated with maspin expression. Most importantly, the presence of activated nuclear IKK α correlated with clinical grade in human CaP and was highest in stage 4 tumors that did not express maspin (Fig. 18e). No activated IKK α was detected in the nuclear fraction of normal human prostate or benign prostate hyperplasia, whose cytoplasmic fractions contained high levels of maspin.

f) Tumor-infiltrating inflammatory cells express RANKL, an inhibitor of maspin expression

In mammary epithelial cells as well as myeloid cells IKK α is activated upon occupancy of RANK by RANKL. Another cytokine that activates IKK α is lymphotoxin (LT) $\alpha\beta$. These members of the TNF family are expressed by activated lymphoid and myeloid cells. Immunohistochemistry revealed only small amounts of such cells in early CaP of either genotype, but the amount of tumor infiltrating T cells (CD3⁺) (Fig. 19a) and macrophages (F4/80⁺) (Fig. 19b) was dramatically increased in primary prostate tumors of 7-9 month old TRAMP mice (Fig. 19). Likewise, the levels of RANKL and LT α mRNA were dramatically elevated in CaP from 7-9 month-old TRAMP mice relative to tumors of 4-5 month-old mice (Fig. 20). Given the very high increase in RANKL mRNA, which was also seen at the protein level (Fig. 21), we treated primary prostate epithelial cells with RANKL and examined maspin expression. Maspin amounts declined within 6 hrs of RANKL application to WT cells but no effect was seen in *Ikk α ^{AA/AA}* cells (Fig. 22a). These results suggest that prostate cancer metastasis could be a consequence of tumor infiltration by RANKL-expressing inflammatory cells that activate IKK α in nuclei of carcinoma cells to repress maspin transcription (Fig. 22b).

2. Examine the role of IKK β in prostate carcinogenesis and formation of hormone refractory prostate cancer

(1) Construct mice with a specific deletion of *Ikk β* in prostate epithelial cells and examine whether this deletion inhibits prostate carcinogenesis in the TRAMP model

To test the role of the IKK β subunit in CaP development, we constructed a mouse strain containing a specific deletion of the gene coding for the IKK β catalytic subunit in prostate epithelial cells. In these experiments we took advantage of *Ikk β ^{F/F}* mice, which carry a conditional loss-of-function "floxed" *Ikk β* allele. To delete IKK β in prostate epithelial cells we crossed *Ikk β ^{F/F}* mice to *PB-CRE4* transgenic mice, which express the CRE recombinase in prostate epithelial cells from the probasin promoter (25). This yields *Ikk β ^{F/+}/PB-CRE4* heterozygote mice, which were intercrossed to generate homozygote *Ikk β ^{F/F}/PB-CRE4* mice. We examined the efficiency of IKK β deletion in the prostate and in purified prostate epithelial cells from 10-12 week old male *Ikk β ^{F/F}/PB-CRE4* mice by polymerase chain reaction (PCR) and immunoblotting and found efficient deletion of the *Ikk β ^F* allele and absence of IKK β protein in

purified ventral and dorsolateral prostate gland epithelial cells from *Ikkβ^{F/F}/PB-CRE4* mice (Fig. 23). No differences in size of the prostate gland and its histological composition between *Ikkβ^{F/F}* and *Ikkβ^{F/F}/PB-CRE4* mice were observed. Thus, IKKβ is not required for normal prostate development and maintenance. Since effective and prostate-specific deletion of IKKβ has been confirmed, we crossed recombinant *Ikkβ^{F/F}/PB-CRE4* mice as well as *Ikkβ^{F/F}* mice to the *TRAMP* transgenic mouse to generate *Ikkβ^{F/F}/PB-CRE4-TRAMP* and *Ikkβ^{F/F}-TRAMP* mice. Male mice of the appropriate genotypes (*Ikkβ^{F/F}/PB-CRE4-TRAMP* and *Ikkβ^{F/F}-TRAMP*) were monitored for prostate cancer formation and progression. We did not find clear differences in either tumor incidence or tumor pathology between *Ikkβ^{F/F}/PB-CRE4-TRAMP* and *Ikkβ^{F/F}-TRAMP* mice. Thus, rather surprisingly, IKKβ is not essential for primary CaP development in *TRAMP* mice.

Primary mouse CaP cells were cultured from primary tumors isolated from 12-week-old *Ikkβ^{F/F}/TRAMP* mice. Colonies of CaP cells were formed two weeks after plating of the primary tumors, exhibiting low motility, high cell density and spherical shape (Fig 24a). These colonies were trypsinized and passed in culture to yield rapidly growing cells that formed invasive tumors when inoculated subcutaneously into immune compromised *Rag1^{-/-}* mice (Fig 24b). These cells expressed high levels of SV40 Tag, p53 protein (presumably stabilized and inactivated by Tag) and normal levels of IKKα, IKKβ and IκBα (Fig 24c). After several rounds of IL-6 treatment, these cells assumed an NE phenotype, which is characterized by rounding of cell bodies, development of long-branched neuritic-like processes and presence of secretory vesicles (Fig 25a). These transdifferentiated cells express higher levels of the NE markers: chromogranin-A (Fig 25 b and c) and synaptophysin (Fig 25b and d) and are more motile and dispersed than the original cells.

(2) Examine the role of IKKβ in evolution of androgen-independent prostate cancer

To examine whether IKK or NF-κB are activated during the evolution of AI CaP, the androgen-dependent (AD) human CaP cell line LNCaP was inoculated subcutaneously into immunocompromised SCID mice. When tumor mass reached 1 cm³, one half of the tumor-bearing cohort was sacrificed and AD tumor tissue was collected. The other half of the cohort was castrated. As a result of the castration, the tumors in these mice had regressed, but two months later when the tumors in the castrated mice re-grew, mice were sacrificed and tumor tissues were collected to yield AI tumors. Protein lysates derived from AD and AI tumors were assayed for NF-κB DNA binding activity by an electrophoretic mobility shift assay (EMSA) (Fig 26a) and for IKK activity by an immunocomplex kinase assay (Fig 26b). The results demonstrated that both NF-κB and IKK activities are markedly elevated in AI tumor tissues (Fig 26). To further confirm these results, we employed a syngeneic mouse model, in which an AD mouse prostate cell line, myc-CaP, was inoculated subcutaneously into immune competent FVB mice. When tumor mass reached 1 cm³, one half of the tumor-bearing cohort was sacrificed and AD tumor tissue was collected. The other half of the mouse cohort was castrated. When the tumors in the castrated mice re-grew, mice were sacrificed and tumor tissues were collected to yield AI tumors. We analyzed NF-κB DNA binding activity in tumor tissues by EMSA (Fig 27a) and IKK activity by an immunocomplex kinase assay (Fig 27b). Very similar to the results seen in the LNCaP model, both NF-κB and IKK activities are markedly elevated in AI tumor tissues (Fig 27). Using the *TRAMP* model, we also found that both IKK kinase activity and NF-κB DNA binding activities were increased in AI tumors of castrated mice (Fig. 28).

Prostate tumors contain many cell types, including CaP cells, stroma cells and infiltrating inflammatory cells. To detect which cell type contribute to the increased NF-κB and IKK activities in AI tumor tissues, we employed an *in vitro* co-culture system, in which AD LNCaP cells were co-cultured with differentiated human macrophages or undifferentiated human monocytes with or without dihydrotestosterone (DHT) in the culture medium. After DHT

withdrawal, both NF- κ B and IKK activities in LNCaP cells were moderately increased when cultured alone, and markedly increased when co-cultured with monocytes, suggesting that inflammatory cells in the tumor microenvironment may contribute to NF- κ B activation in carcinoma cells, and thus may play an important role in development of CaP (Fig. 29).

To delete IKK β constitutively in mature myeloid cells (macrophages, myeloid dendritic cells and granulocytes) we have crossed *Ikk β ^{F/F}* mice with *Mx1-Cre* transgenic mice in which CRE expression is inducible by interferon (IFN). To induce IFN production and CRE expression, mice bearing this transgene that are at least 4 weeks old are injected with 200 μ g/mouse of poly(IC). A single injection of poly(IC) is sufficient for inducing effective IKK β deletion in macrophages, osteoclasts and other bone marrow derivatives. To ensure long lasting deletion, injection of poly (IC) can be repeated. To examine whether IKK β in immune cells is important for androgen-independent CaP evolution, twenty-week-old *Ikk β ^{F/F}/TRAMP* and *Ikk β ^{F/F}/Mx1-Cre/TRAMP* male mice were injected with poly(IC) three times. Ten days after the last injection, the mice were castrated and tumor development was carefully monitored. We found that CaP metastases in *Ikk β ^{F/F}/Mx1-Cre/TRAMP* mice were dramatically decreased relative to *Ikk β ^{F/F}/TRAMP* mice after more than five months follow-up (Fig. 30). These results suggest that IKK β /NF- κ B signaling in immune cells may play an important role in the process of androgen-independent CaP evolution.

3. Use various strategies to inhibit IKK activity and test them for their ability to inhibit proliferation and induce apoptosis in CaP cell lines of human origin.

The experiments described above provide genetic tests for the involvement of IKK β or IKK α in the development of CaP in mice. At the same time we investigated the possible use of IKK/NF- κ B inhibitors in the treatment of human CaP. This question was addressed through the use of various IKK inhibitors, targeting either IKK α or IKK β , and examination of their effects on human CaP cell lines, both *in vitro* and in mouse xenografts.

A prototypical IKK inhibitor named IT-3 was synthesized according to the patent literature by a chemist at the UCSD Cancer Center. We examined IT-3 for its effects on the proliferation of several human CaP cell lines: DU145, PPC-1, PC3 and ALVA31. Previous work has shown that these cell lines exhibit constitutively activated NF- κ B. The different cell lines were incubated in 96-well plates and treated with or without the inhibitor for 24 hours. Cell proliferation rates were determined by the MTT assay. We found that IT3 inhibited the proliferation of all human prostate cell lines tested (Fig 31). To assess effects on NF- κ B activation, the CaP cell lines were pretreated with different concentrations of IT-3 before being stimulated with TNF- α . Cells were collected at 30 minutes after TNF- α treatment and I κ B α levels were analyzed by immunoblotting. We found that IT-3 inhibited TNF- α -induced I κ B α protein degradation in all cell lines (Fig 32), suggesting that IT-3 does inhibit IKK activity. Further assays for the specificity of IT-3 action are underway.

At the same time, other putative IKK/NF- κ B inhibitors (the macrolides erythromycin, clarithromycin, azithromycin, 10-deoxymethynolide, etc) were tested for their ability to inhibit IKK and NF- κ B activity and their effects on the proliferation of CaP cell lines, but none of them was found to be a specific IKK or NF- κ B inhibitor.

As described above, NF- κ B plays an important role in AI prostate cancer formation. To test the possibility of pre-clinical use of IKK inhibitors to treat hormone-independent prostate cancer development, we used an identified and validated IKK inhibitor, ML120B (Millennium) to test the effect of IKK β inhibition on the formation of AI prostate cancer in mice. The AD mouse prostate cell line, myc-CaP, was inoculated subcutaneously into immunocompetent FVB mice. When tumor mass reached 1 cm³, tumor-bearing mice were castrated. One half of the castrated mouse cohort was treated with ML120B every two days by gavage. The other half of the mouse cohort

was used as control. AI tumor development is currently being monitored.

Key Research Accomplishments

1. We identified a new pathway that links inflammation-activated signaling to prostate cancer metastasis. Defective activation of I κ B kinase (IKK) α inhibited distant organ metastasis in TRAMP mice, which express SV40 Tag in the prostate epithelium. Decreased metastasis correlated with elevated expression of the metastasis suppressor maspin, whose ablation restored metastatic activity. IKK α activation, which can be triggered by RANK, whose ligand is expressed by tumor-infiltrating inflammatory cells, elevated metastatic activity and repressed maspin gene transcription through a novel NF- κ B-independent mechanism requiring nuclear translocation of IKK α . The amount of nuclear IKK α correlates with the degree of progression in mouse and human prostate cancers and links inflammatory signaling to metastasis (Fig. 1-Fig. 22).
2. Specific deletion of IKK β in prostate epithelial cells was achieved by crossing *Ikk β ^{F/F}* mice to *PB-CRE4* transgenic mice to obtain *Ikk β ^{F/F}/PB-CRE4* mice. We have established mouse CaP cell lines harboring *Ikk β ^{F/F}* alleles in which IKK β can be deleted upon expression of exogenous CRE recombinase. Deletion of IKK β in prostate epithelial cells did not affect tumor incidence in TRAMP mice but reduced metastasis in a xenograft mouse model.
3. We found that neither IKK α nor IKK β are required for normal prostate development and maintenance, thus IKK β -specific or IKK α -specific inhibitors are unlikely to be toxic to the normal prostate.
4. We found that both IKK and NF- κ B activities are activated during the transition of CaP from an AD state to an AI state. Thus, IKK inhibitors may interfere with the emergence and increased aggressiveness of AI CaP.
5. We found that mouse *Ikk β ^{F/F}* CaP cells can be induced to become neuroendocrine cells after IL-6 treatment. This provides us with a system for testing the role of IKK β in CaP progression.
6. We found that inhibition of IKK β in inflammatory cells of the tumor microenvironment dramatically decreases AI tumor development in TRAMP mouse model.
7. We found that a prototypical IKK inhibitor, IT-3, can suppress the proliferation of human prostate cancer cells. This inhibitor may serve as a starting point for the development of IKK α - and IKK β -specific inhibitors for clinical applications.

Reportable Outcomes

Our paper is currently in press in Nature, a premier, general interest scientific journal: Luo, J.-L., Tan, W., Ricono, J.M., Korchynskyi, O., Zhang, M., Gonias, S.L., Cheresch, D.A., Karin, M. (2007). Inflammation and Metastasis: IKK α activation inhibits expression of the metastasis suppressor maspin to promote prostate cancer metastasis. *Nature*. *In press*.

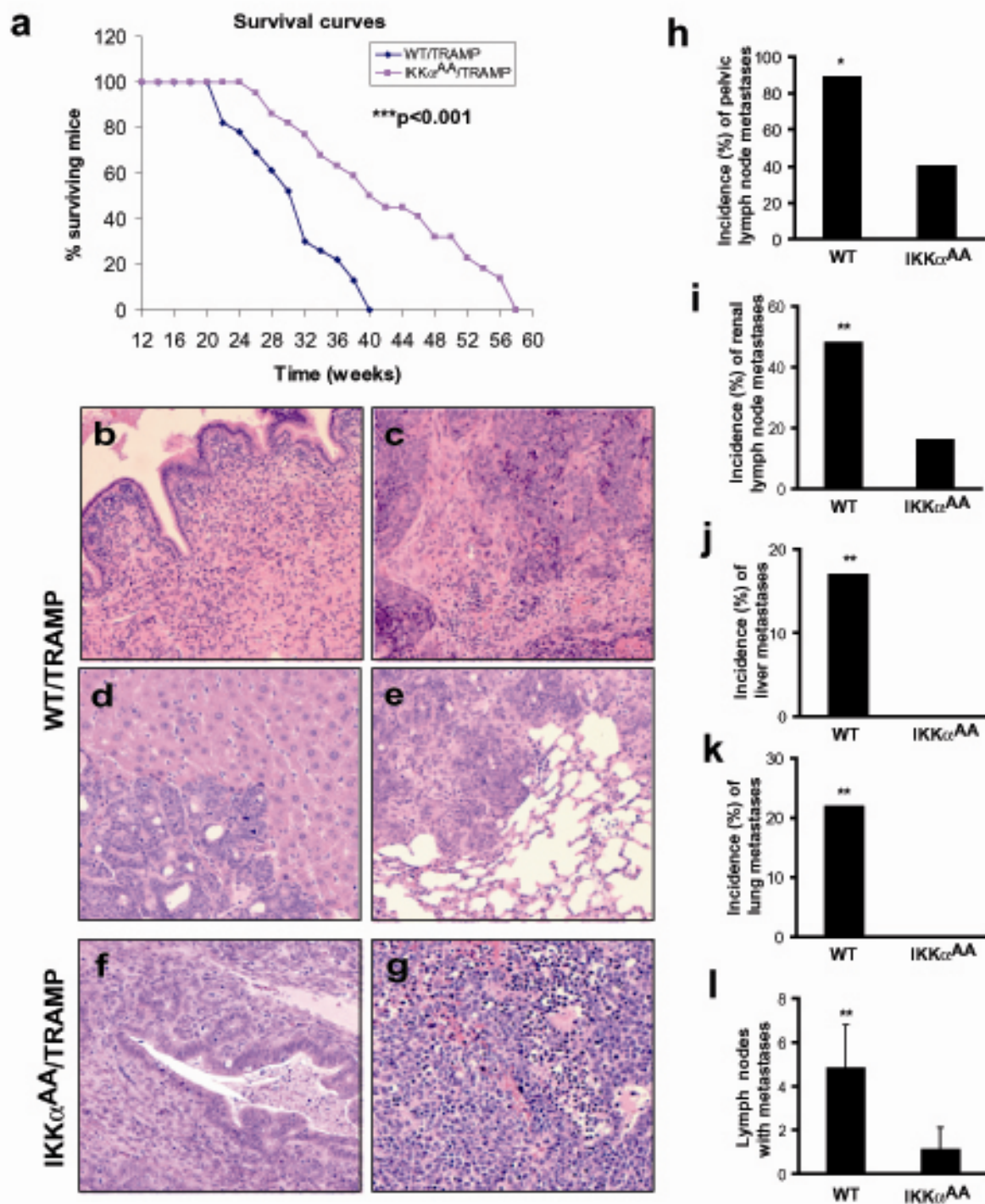


Figure 1: IKK α activity is required for prostate cancer metastasis. a, TRAMP mice were intercrossed for at least 6 generations with *Ikk α ^{AA/AA}* mice. Survival of *Ikk α ^{AA/AA}/TRAMP* and WT/TRAMP mice was compared. b-g, Histological analysis (H&E staining of paraffin embedded sections; magnification: 100) WT/TRAMP primary prostate carcinomas (b), and lymph node (c), liver (d), and lung (e) metastases, as well as *Ikk α ^{AA/AA}/TRAMP* primary prostate carcinomas (f) and a rare lymph node metastasis (g). h-k, Incidence of pelvic lymph node (h), renal lymph node (i), liver (j) and lung (k) metastases. l, Average numbers of lymph nodes harboring metastases. * $p < 0.05$, ** $p < 0.01$

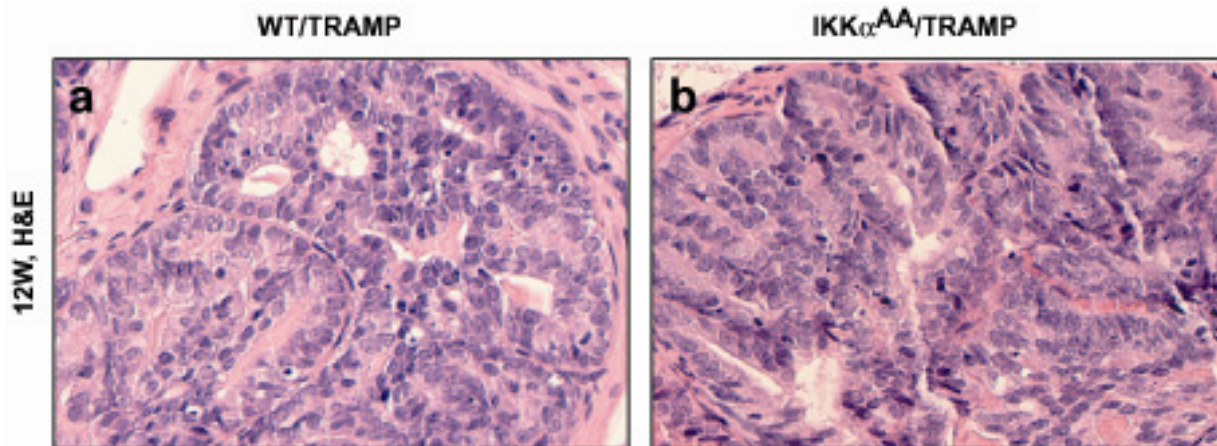


Figure 2: IKK α does not affect CaP histology. Histology of prostate adenocarcinomas from 3 months-old WT/TRAMP (a) and *Ikk α ^{AA/AA}*/TRAMP (b) mice was examined by H&E staining (magnification: 200X).

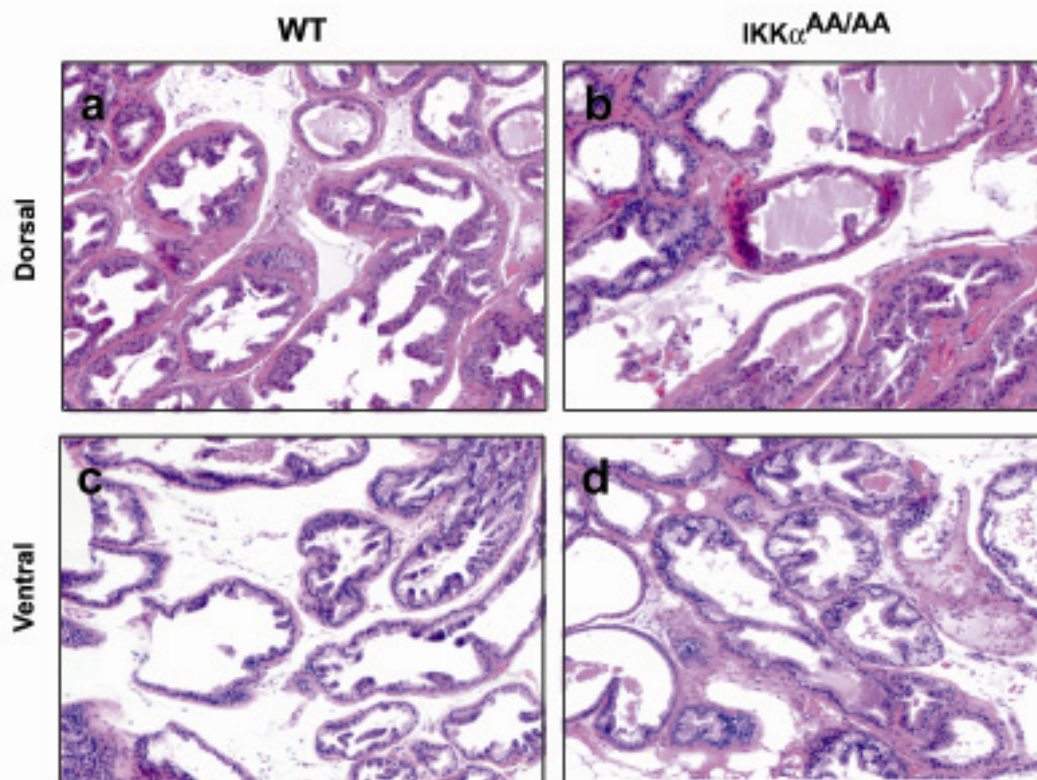


Figure 3: Histological analysis of WT and *Ikk α ^{AA/AA}* prostates. Dorsal (a, b) and ventral (c, d) prostate tissues from WT (a, c) and *Ikk α ^{AA/AA}* (b, d) mice were dissected, fixed, paraffin-embedded, sectioned and H&E stained (Magnification: 100X).

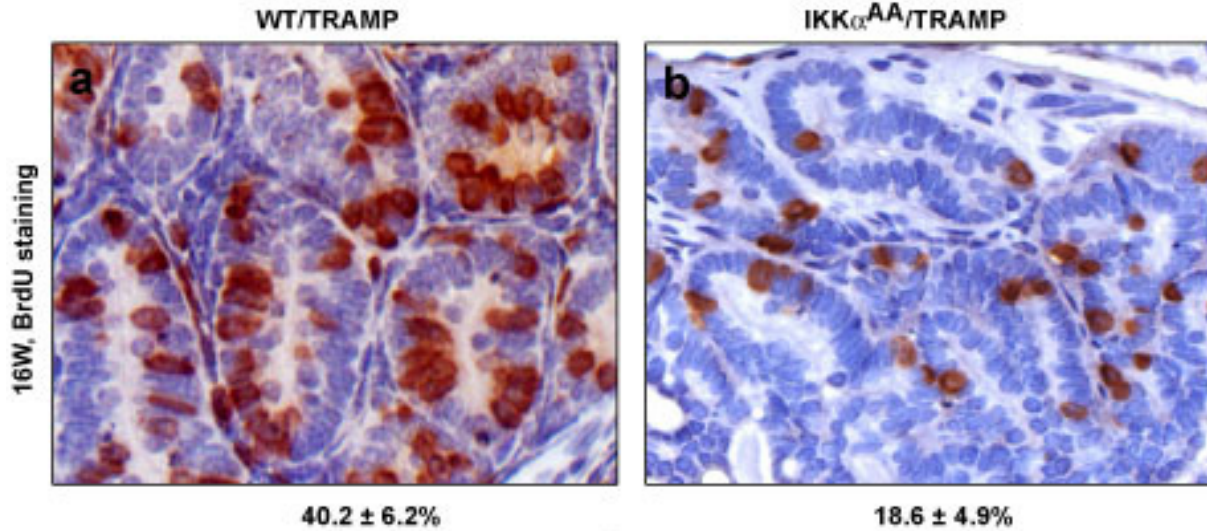


Figure 4: IKK α affects CaP proliferation. Cell proliferation in prostate carcinomas of 4 months-old WT/TRAMP (a) and *Ikk α AA/AA*/TRAMP (b) mice was examined by BrdU labeling. Percentages of BrdU-positive cells are indicated underneath.

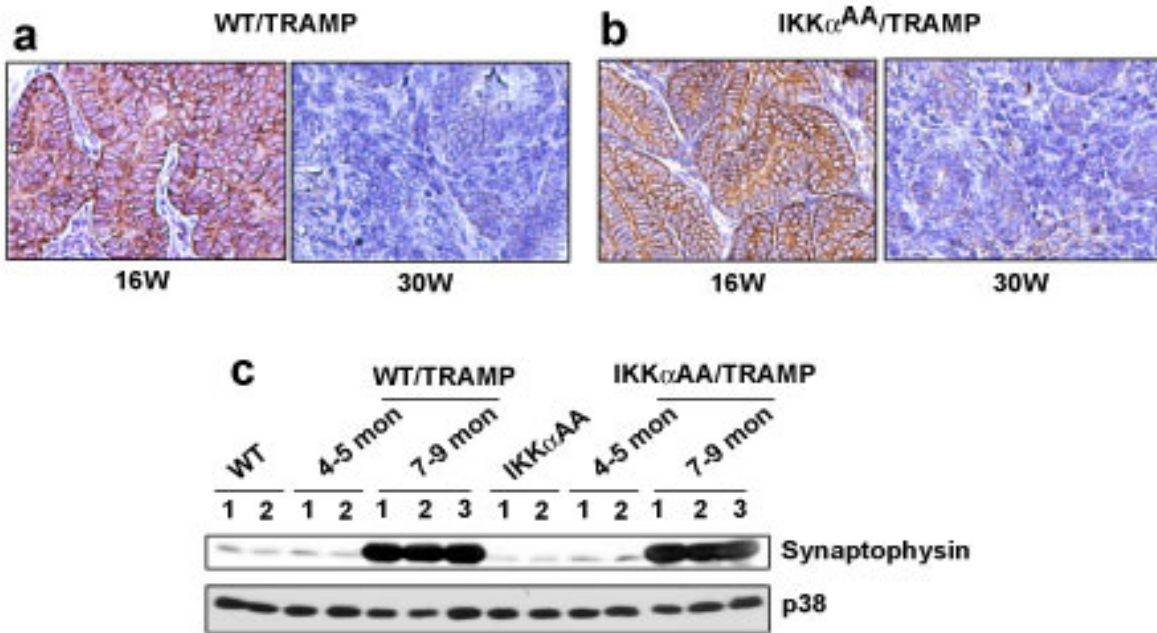


Figure 5: a, b Expression of the epithelial marker E-Cadherin was examined by immunohistochemistry in CaP from 16 week and 30 week-old WT/TRAMP (a) and *Ikk α AA/AA*/TRAMP (b) mice. c, Expression of the neuroendocrine marker synaptophysin was examined by immunoblotting of prostate extracts from the indicated mice.

Figure 6 (a1-g4)

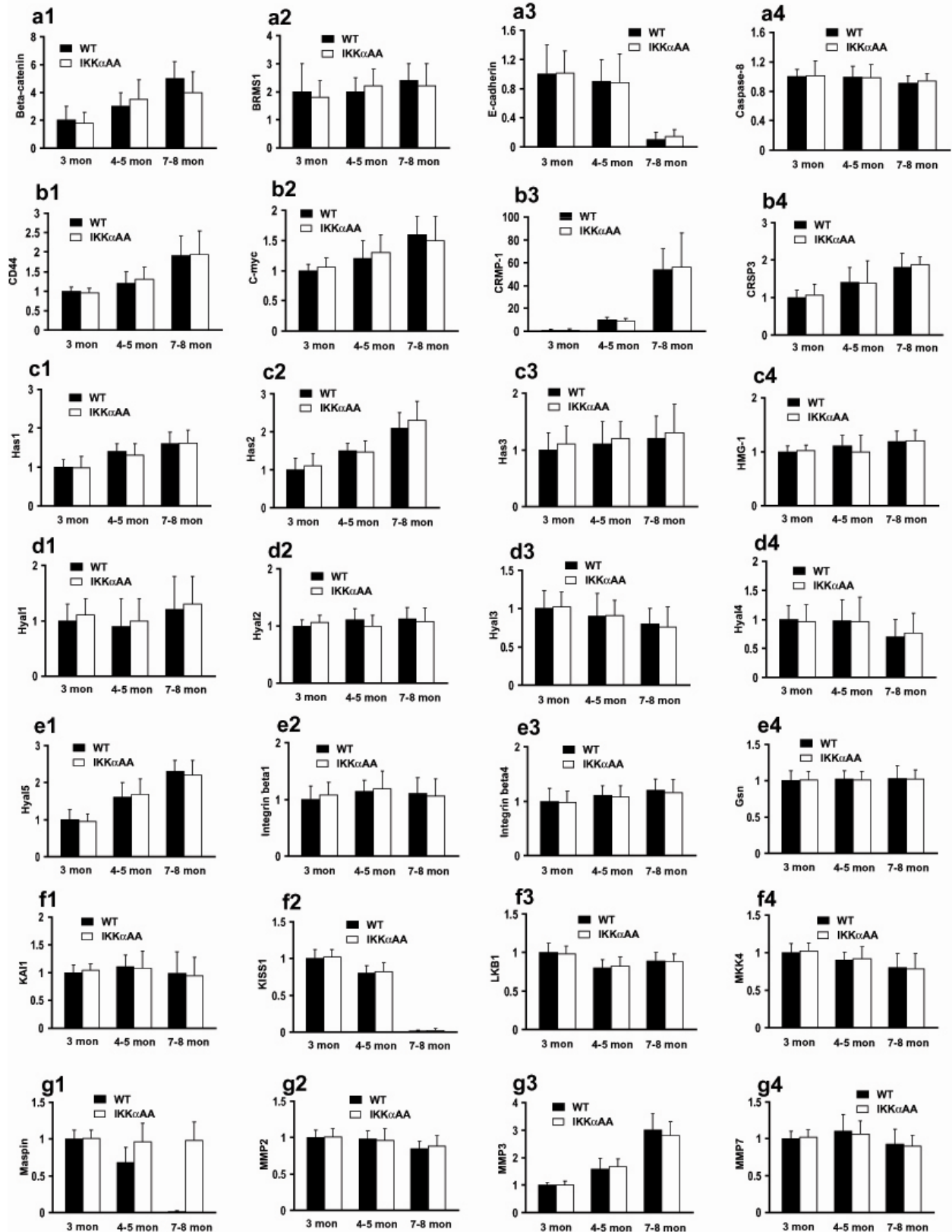
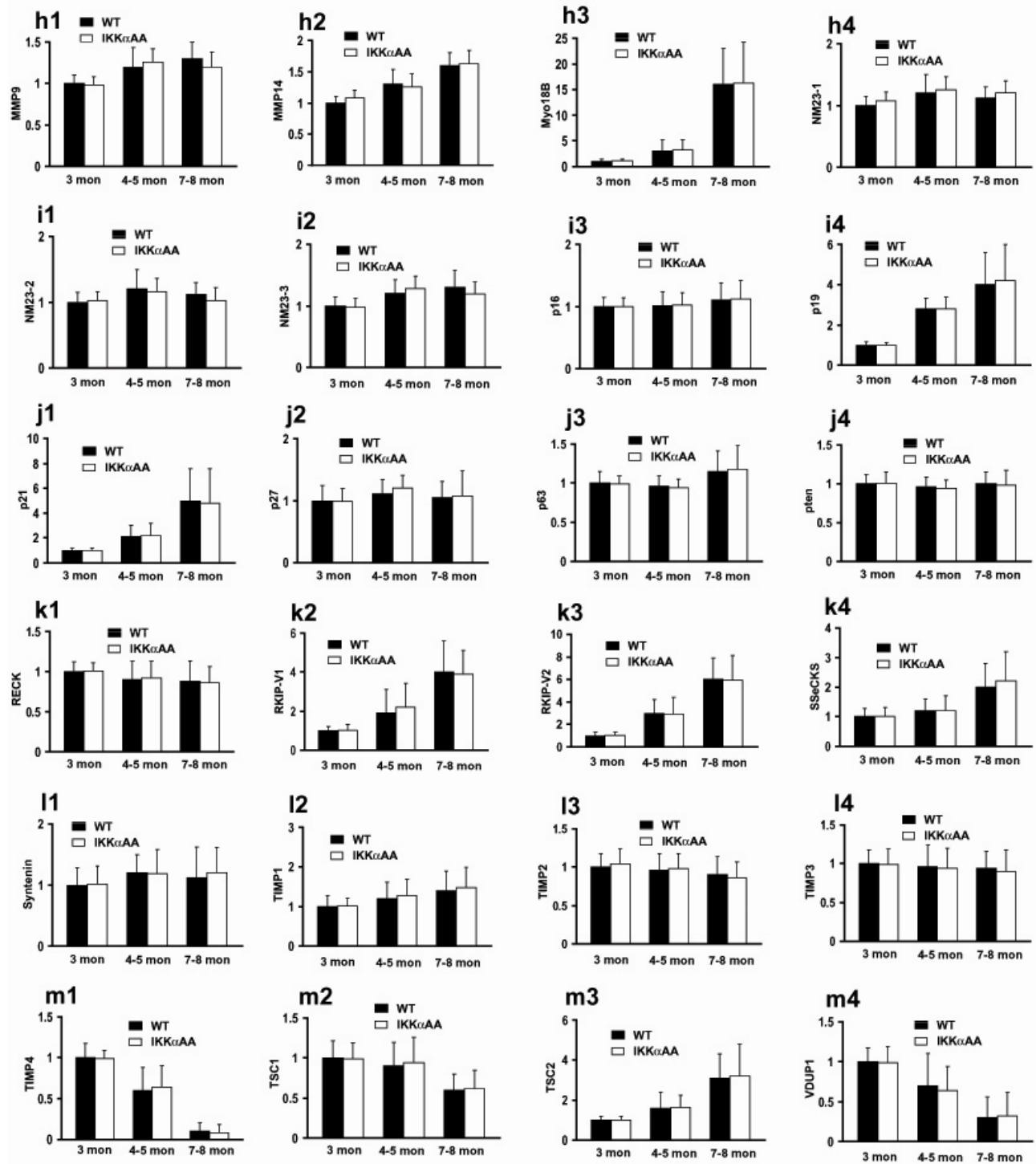


Figure 6 (h1-m4)



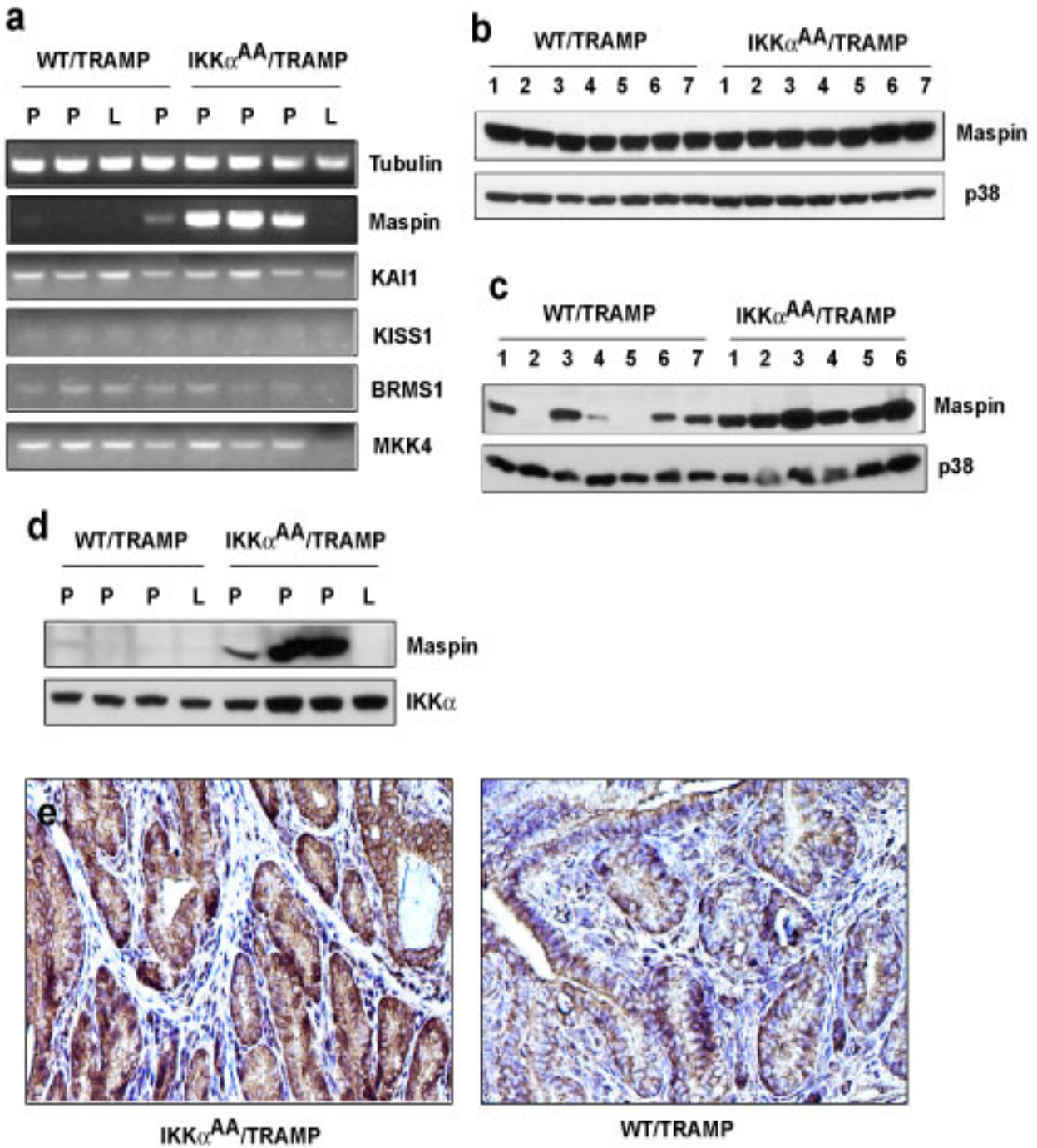


Figure 7: Maspin expression correlates with extent of metastasis. a, Expression of mRNAs encoding metastasis-suppressors was analyzed by RT-PCR in primary (P) CaPs and lymph node (L) metastases. b-d, Maspin expression was examined in primary CaPs of 3 months-old (b), 4-5 months-old (c) or 7-8 months-old (d) WT/TRAMP and *Ikk α ^{AA/AA}/TRAMP* mice by immunoblotting. e, f, Immunohistochemical staining for maspin in paraffin-embedded prostate carcinoma sections from *Ikk α ^{AA/AA}/TRAMP* (e) and WT/TRAMP (f) mice. Numbers in b and c refer to different tumor isolates.

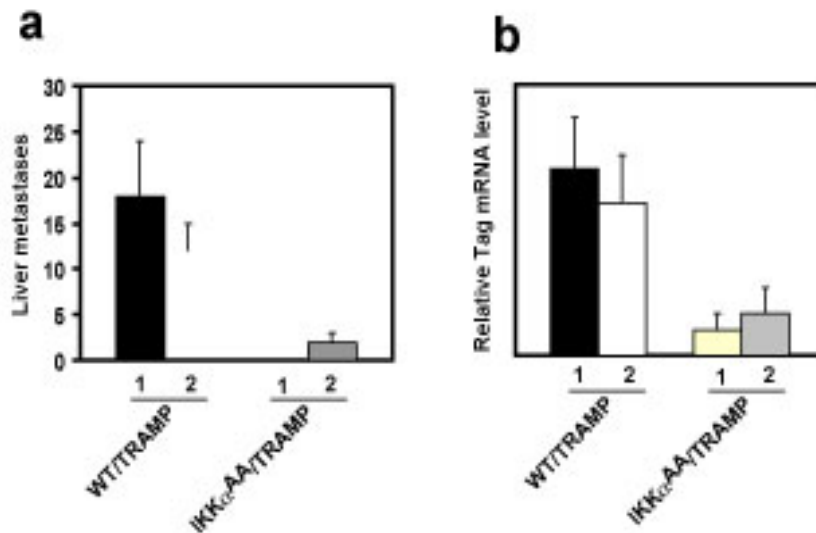


Figure 8: a, CaP cells from WT/TRAMP and $Ikk\alpha^{AA/AA}$ /TRAMP mice were isolated and cultured for 1-3 weeks. Single-cell suspensions were injected into spleens of 3-5 Nu/Nu male mice and 10 min later spleens were removed. After 4 weeks mice were sacrificed and liver metastases were counted. Numbers represent different cell isolates and cultures. b, Real-time PCR analysis of SV40 Tag mRNA in livers of Nu/Nu mice whose spleens were inoculated with cultured CaP cells.

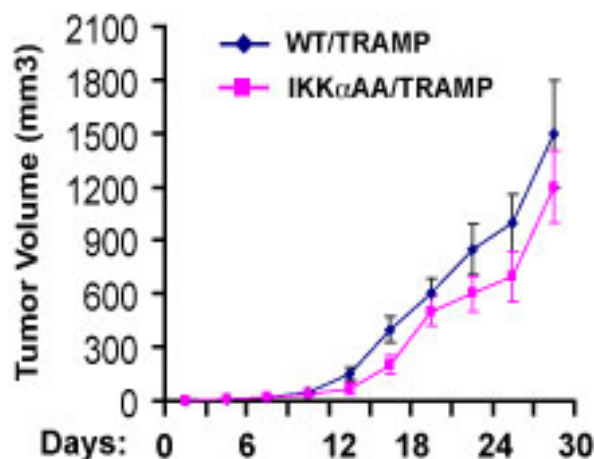


Figure 9: Primary CaP cells from WT/TRAMP and $Ikk\alpha^{AA/AA}$ /TRAMP exhibit similar tumorigenic potential. Primary CaP cells (4×10^6) from 7-8 month-old WT/TRAMP and $Ikk\alpha^{AA/AA}$ /TRAMP mice were mixed in matrigel and implanted subcutaneously into the flank of male Nude (Nu/Nu) mice. Tumor size was measured every three days using a caliper. Results are averages from three individual mice per CaP genotype.

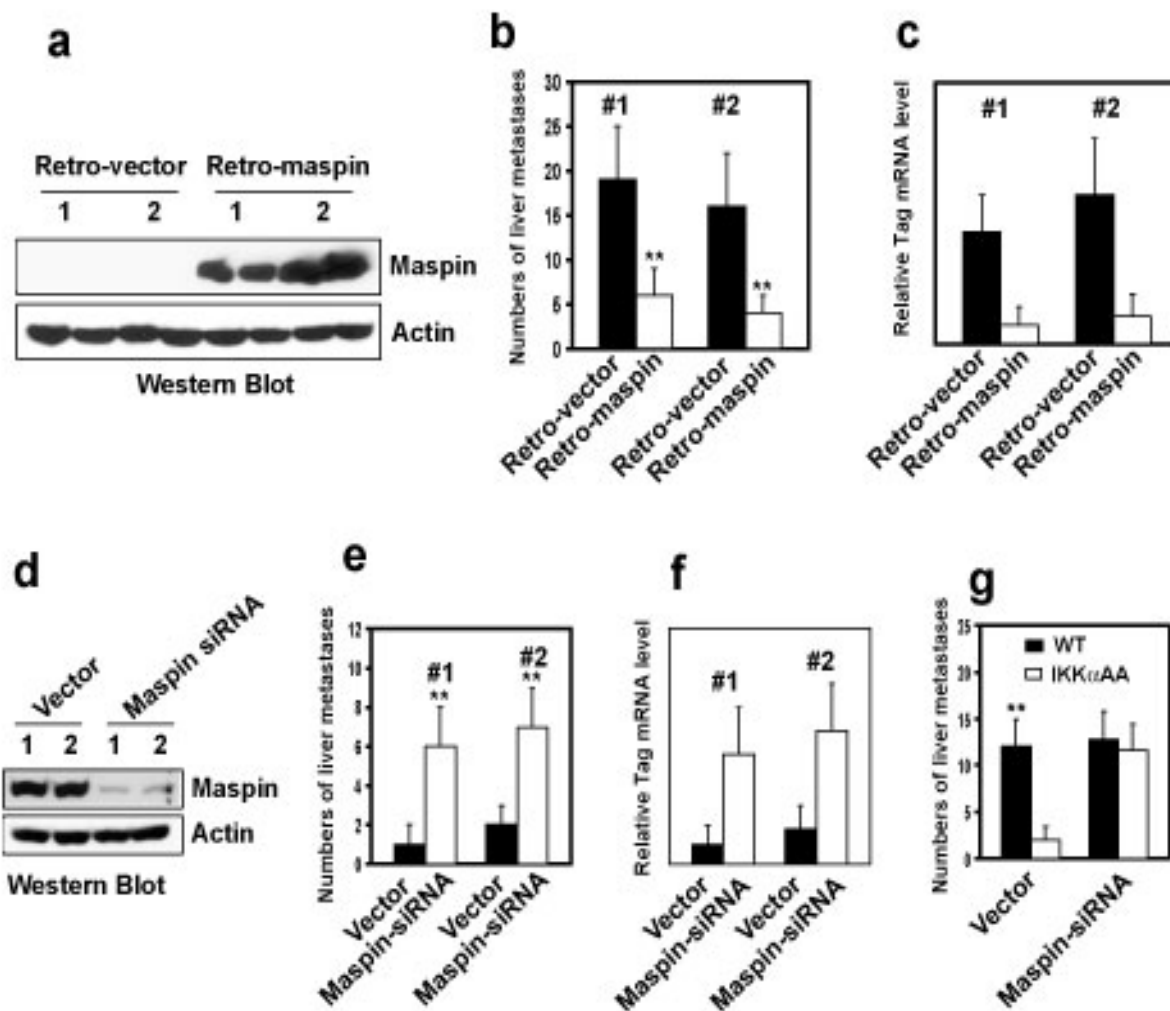


Figure 10: a, CaP cells from WT/TRAMP mice were infected with either a maspin retrovirus or an 'empty' retrovirus. Two different preparations of transduced cells per virus were selected in 100 μ g/ml zeocin for 1 week and immunoblotted for maspin expression. b, Single-cell suspensions of retrovirus-infected and zeocin-selected cells from a were injected into spleens of Nu/Nu male mice and liver metastases were analyzed as in figure 8. c, Real-time PCR analysis of SV40 Tag mRNA in livers from b. d, Primary CaP cells from *Ikk α ^{AA/AA}/TRAMP* mice expressing endogenous maspin were infected with maspin siRNA-retrovirus or an "empty" retrovirus vector. Two different preparations of transduced cells per virus were selected in 1 μ g/ml puromycin and immunoblotted for maspin expression. e, Single-cell suspensions of retrovirus-infected and puromycin-selected cells from d were injected into spleens of Nu/Nu male mice, and liver metastases were analyzed as above. f, Real-time PCR of SV40 Tag mRNA in livers from e. g, Maspin expression was silenced in WT and *Ikk α ^{AA/AA}* CaP cells and metastatic activity was examined as above.

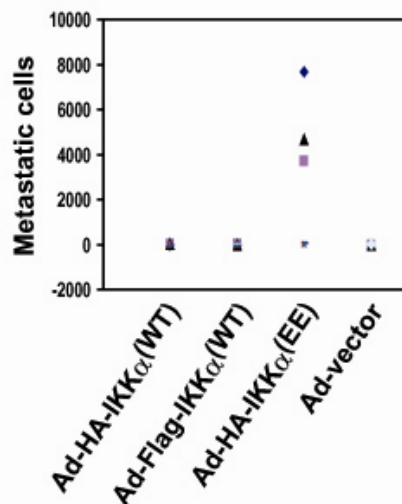


Figure 11: Four different coded samples of $Ikk\alpha^{AA/AA}$ /TRAMP CaP cells expressing endogenous maspin, infected with the indicated adenoviral vectors were inoculated on the back side of the chorioallantoic membrane of a 10-day old chick embryo. After 9 days pulmonary metastasis was quantified by detection of SV40 Tag DNA sequences within chick lung genomic DNA. The chicken GAPDH sequence was used as an internal control.

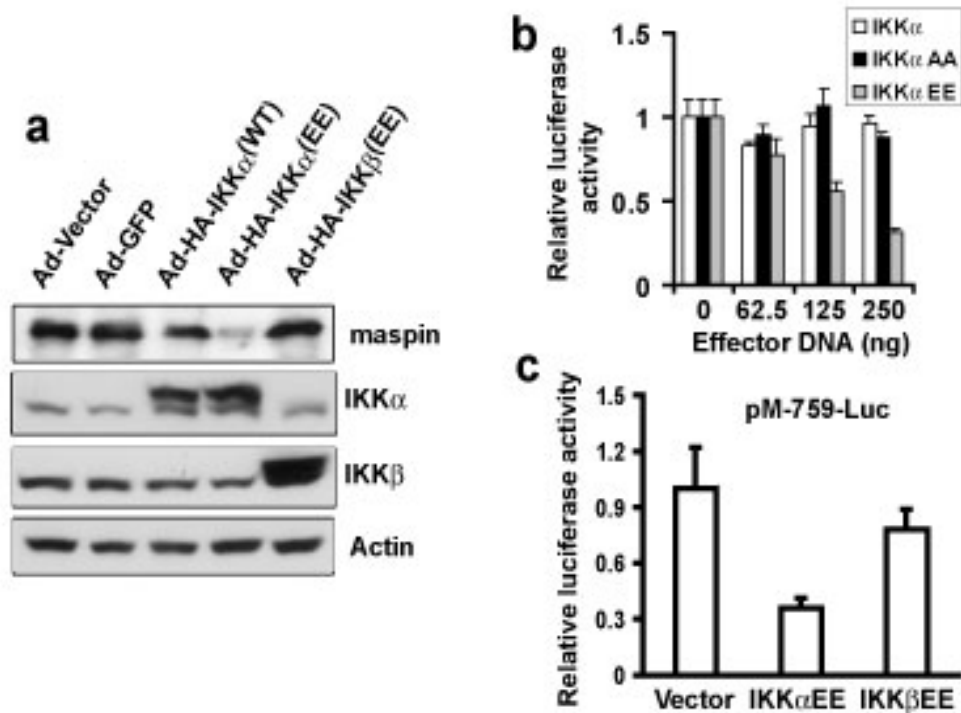


Figure 12: IKK α activation represses maspin transcription. a, Primary *Ikk α ^{AA/AA}/TRAMP* CaP cells were infected with different adenoviruses as indicated. After 3 days, maspin, IKK α , IKK β and actin levels were analyzed by immunoblotting. b, A maspin-luciferase reporter containing 759 base pairs (bp) of the human maspin 5' upstream region was co-transfected with different amounts of WT IKK α , IKK α (AA) or IKK α (EE) vectors into *Ikk α ^{-/-}* MEFs. Luciferase activity was measured and normalized to a co-transfected PRL-TK reporter. c, The maspin-luciferase reporter was co-transfected with IKK α (EE) or IKK β (EE) vectors into *Ikk α ^{-/-}* MEFs. Luciferase activity was measured and normalized as above.

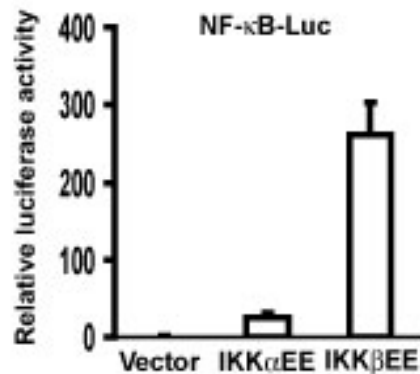


Figure 13: NF- κ B-luciferase reporter was co-transfected with IKK α (EE) or IKK β (EE) expression vectors into *Ikk α ^{-/-}* MEFs. Luciferase activity was measured after 36 hrs and normalized to that of a co-transfected internal control (pRL-TK).

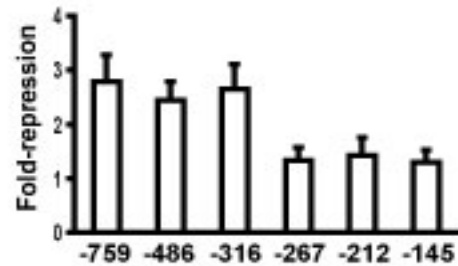


Figure 14: Different maspin promoter truncation mutants fused to a luciferase reporter were co-transfected with or without IKK α (EE) expression vector as in figure 12 and luciferase activity was measured. Results denote fold-repression by IKK α (EE) based on 3 separate experiments.

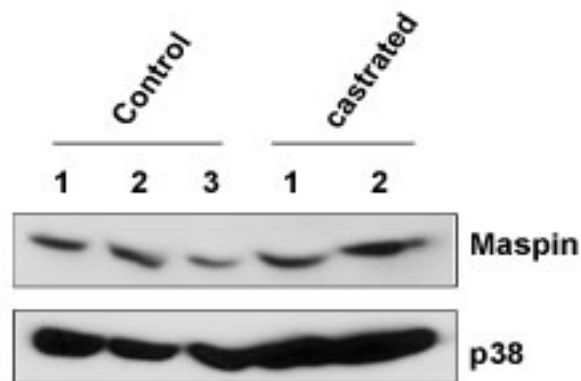


Figure 15: Four-month old male mice were divided into two groups, one group was castrated, another group was sham operated. One week later, mice were sacrificed and prostate tissues were collected and lysed for immunoblot analysis of maspin and p38.

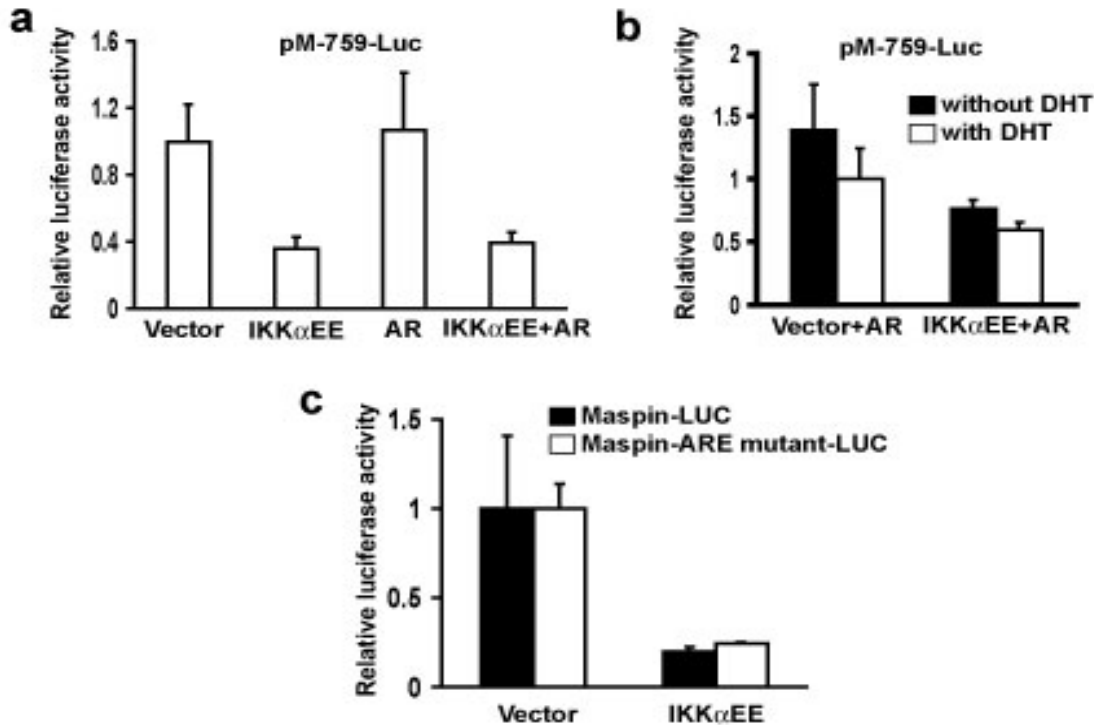


Figure 16: IKK α represses maspin transcription independency of androgen receptor (AR). a, Human maspin-luciferase reporter (pM-759-Luc) was co-transfected with empty, IKK α (EE), AR, or IKK α (EE) + AR expression vectors into *Ikk α ^{-/-}* MEFs. Luciferase activity was measured and normalized as above. b, pM-759-Luc was co-transfected with vector + AR, or IKK α (EE) + AR into *Ikk α ^{-/-}* MEFs. After 36 hrs the cells were incubated with dihydrotestosterone (DHT) and luciferase activity was determined and normalized as above. c, pM-759-Luc or ARE-mutant-pM-Luc was co-transfected with empty vector or IKK α (EE) into *Ikk α ^{-/-}* MEFs. Luciferase activity was measured and normalized as above

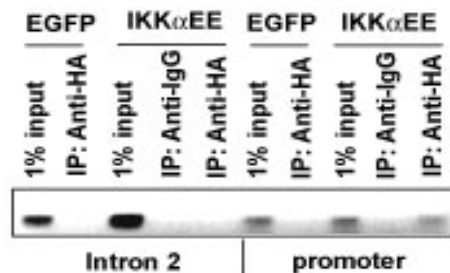


Figure 17: ChIP analysis of IKK α recruitment to the human maspin promoter. HME cells were transduced with either HA-IKK α (EE) or GFP adenovirus. After 48 hrs, proteins were crosslinked to DNA and chromatin was extracted and fragmented. Protein-DNA complexes were immunoprecipitated with HA antibody. Presence of the maspin promoter region (87 to-315) and intron 2 (+3958 to +4350) in the immunoprecipitates was examined by PCR.

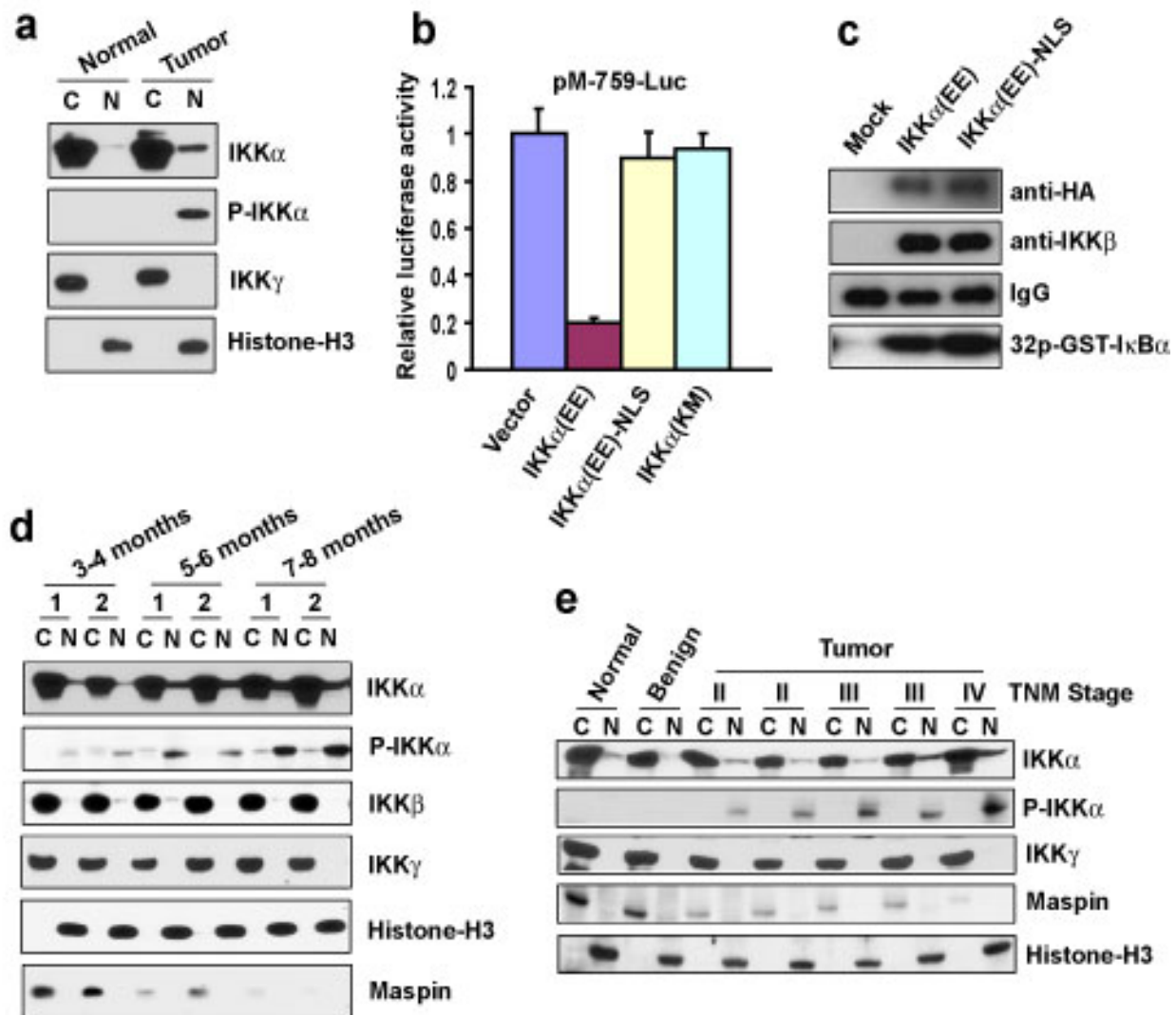


Figure 18: IKK α -mediated repression of maspin expression requires nuclear entry and kinase activity. a, Nuclear (N) and cytoplasmic (C) extracts of normal and TRAMP prostate epithelium were analyzed for IKK α , phospho(activated)-IKK α , IKK γ and histone H3 by immunoblotting. b, The maspin-luciferase reporter (pM-759-Luc) was co-transfected with empty, IKK α (EE), IKK α (EE)-NLS and IKK α (KM) vectors and luciferase activity was measured as described above. c, HEK293 cells were transfected with different expression vectors as indicated. After 36 hrs IKK complexes were immunoprecipitated with HA-specific antibody and their I κ B kinase activity and IKK β content were measured. d, CaP cells from TRAMP mice of the indicated age (1 and 2 are individual mice) were fractionated into cytoplasmic (C) and nuclear (N) extracts that were examined for IKK α , phospho-IKK α , IKK β , IKK γ , histone H3 and maspin by immunoblotting. e, Normal human prostate, benign prostatic hyperplasia and prostate tumors at different stages of progression (TNM stage) were divided into nuclear (N) and cytoplasmic (C) fractions that were examined for presence of the indicated proteins by immunoblotting.

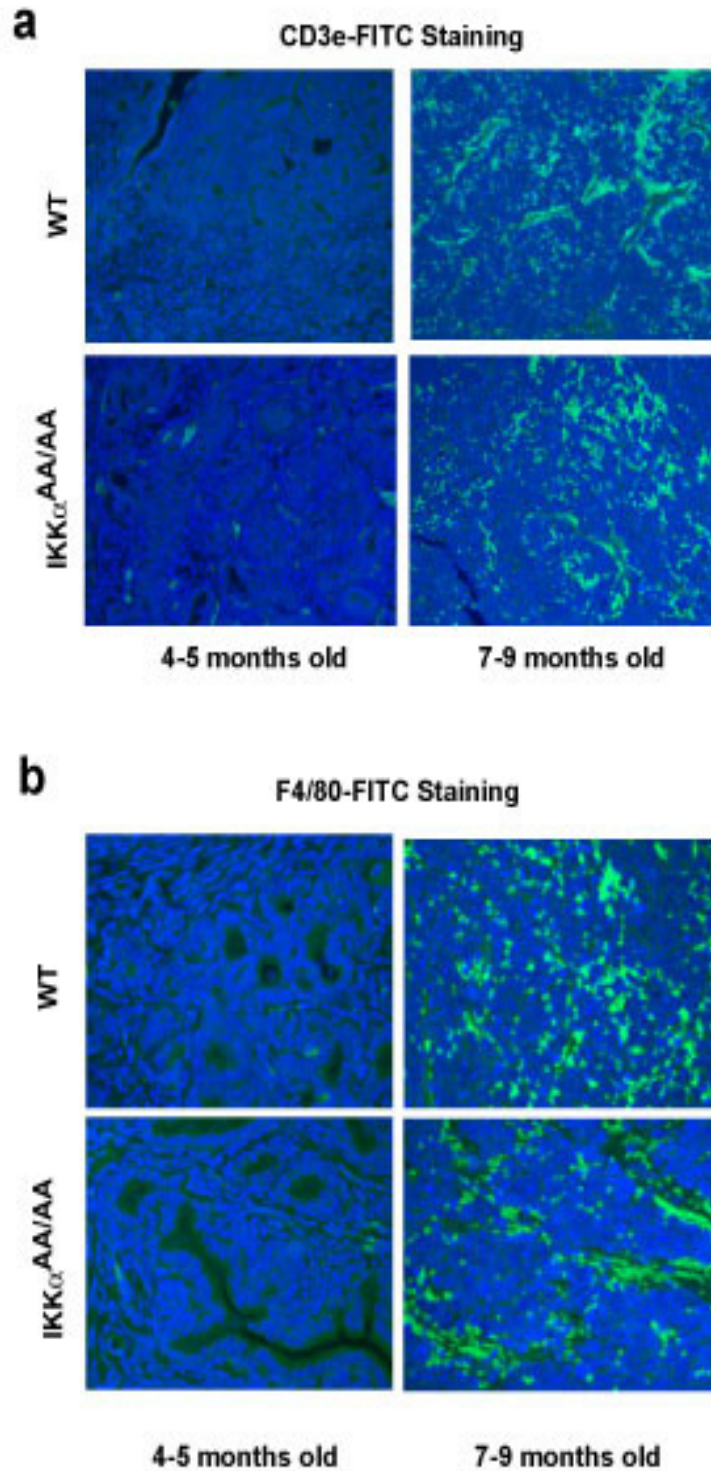


Figure 19: Prostate tumors from 4-5 and 7-9 month-old TRAMP mice of the indicated genotype were stained for the T cell marker CD3 (a) or the macrophage marker F4/80 (b).

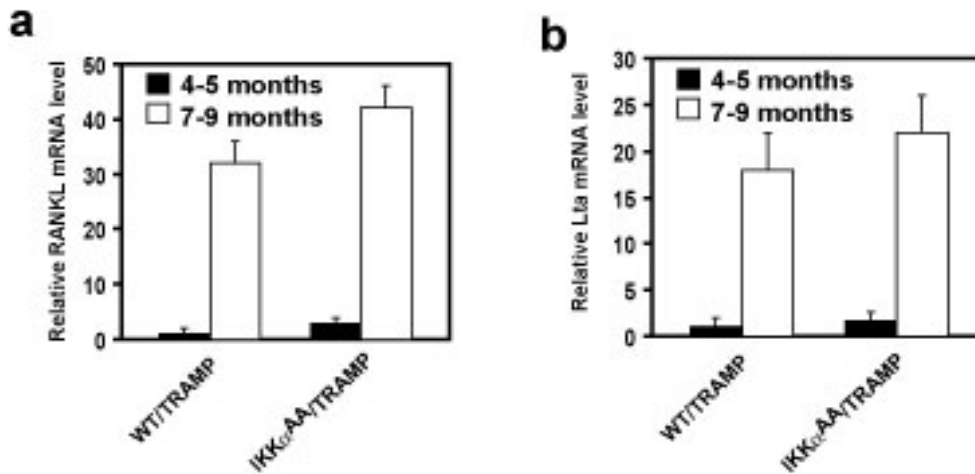


Figure 20: Prostate tumors from mice of the indicated age and genotype were analyzed by Q-PCR for expression of RANKL (b) and Lta (c) mRNAs.

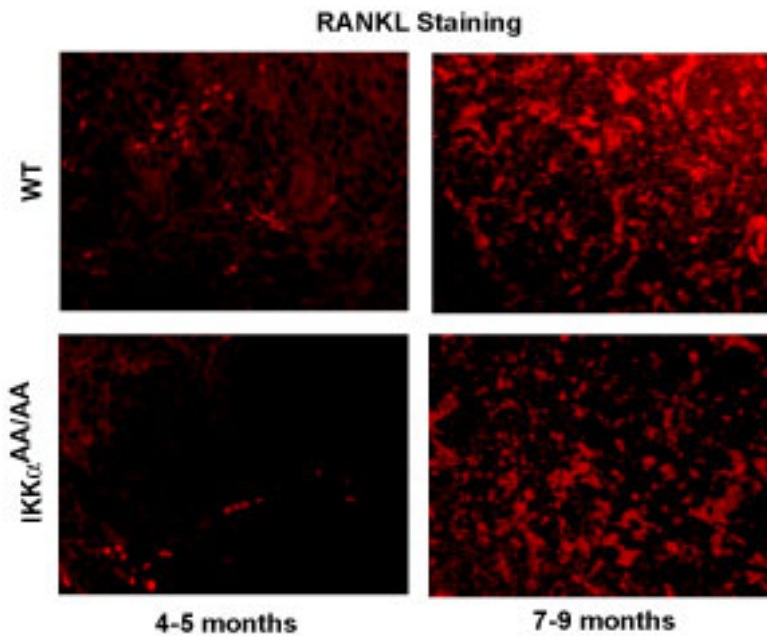


Figure 21: Expression of RANKL in prostate tumors. Tumors from TRAMP mice of the indicated age and genotype were stained with RANKL antibody and visualized as above (magnification 200X).

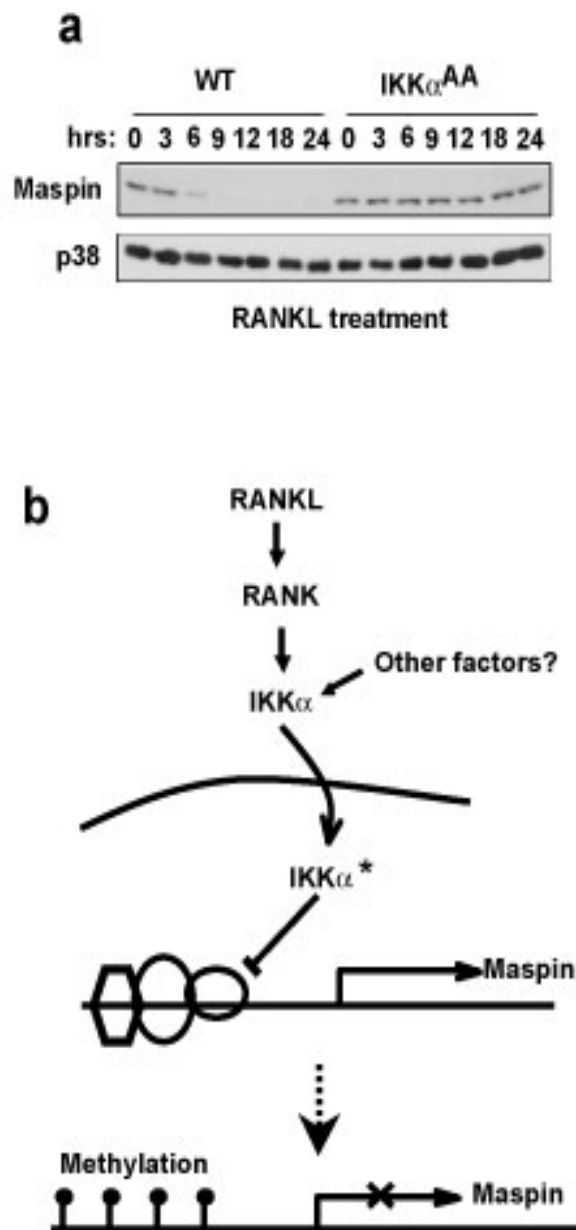


Figure 22: a, WT and $IkK\alpha^{AA/AA}$ prostate epithelial cells were cultured for 4-5 days and treated with LPS-free RANKL (200 μ g/ml). At the indicated times cell extracts were prepared and for maspin and p38a content by immunoblotting. b, A model explaining how RANK signaling leads to repression of maspin transcription. After transient $IKK\alpha$ -mediated repression, maspin transcription is likely to be permanently silenced through DNA methylation.

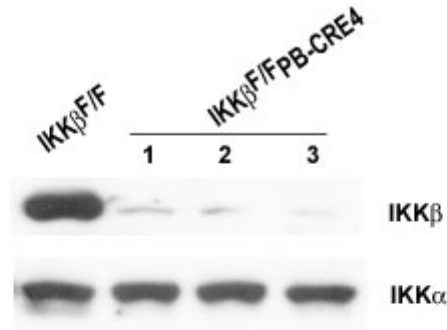


Figure 23: IKK β protein is efficiently deleted in prostate epithelial cells of IKK β ^{F/F}/PB-CRE4 mice. Purified ventral and dorsolateral prostate epithelial cells from 10-12 week old male IKK β ^{F/F} and IKK β ^{F/F}/PB-CRE4 mice were lysed for immunoblot analysis. The results show efficient deletion of IKK β in ventral and dorsolateral prostate epithelial cells from IKK β ^{F/F}/PB-CRE4 mice.

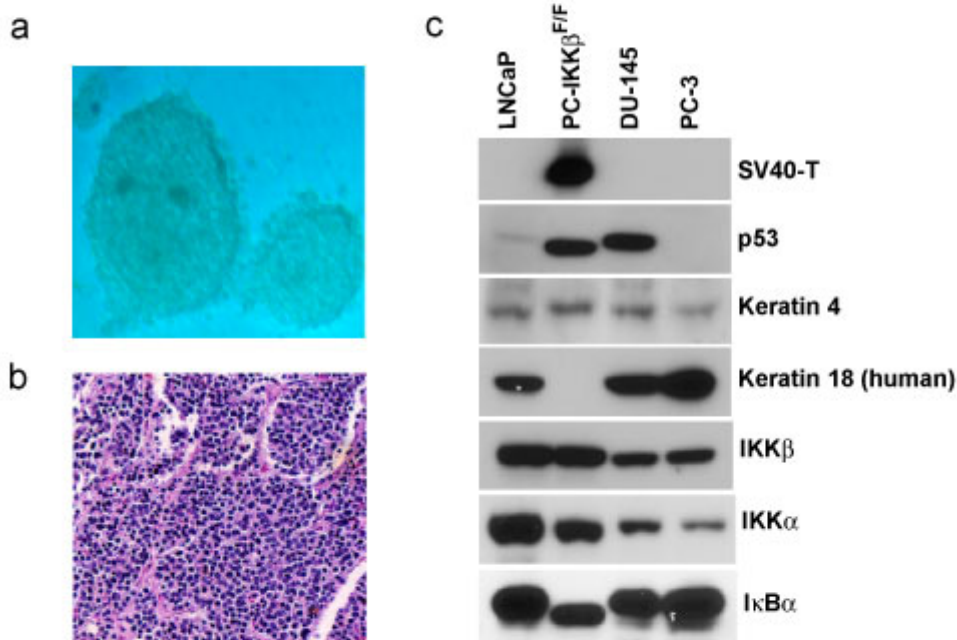


Figure 24: Primary cultures of mouse CaP cells were derived from a single primary prostate tumor isolated from IKK β ^{F/F}/TRAMP mice. Colonies were formed after two weeks. Large colonies with high cell density and spherical shape, consisting mainly of small cells, were picked up for further passage (a). These cells grew aggressively and formed invasive tumors when inoculated subcutaneously into Rag1^{-/-} mice. (b) The histology of the tumors formed by these cells. Tumors were paraffin embedded, H&E stained and examined under microscope (x 20). (c) These cells expressed high levels of Tag, p53 and normal levels of IKK β , IKK α and I κ B α relative to human CaP cell lines. Protein expression was examined by immunoblot analysis.

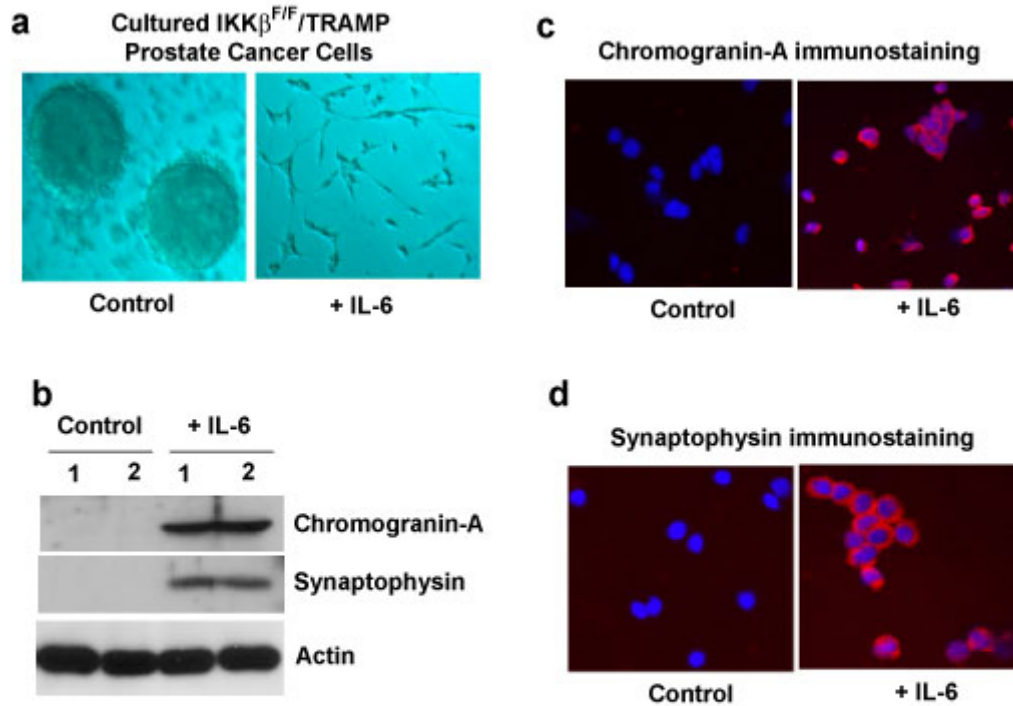


Figure 25: Cultured IKK $\beta^{F/F}$ /TRAMP CaP cells were incubated with or without mouse IL-6. After several passages, the IL-6 treated cells have assumed a neuroendocrine (NE) cell phenotype. These cells are characterized by rounding of cell bodies, development of long-branched neuritic-like processes, and the presence of secretory vesicles (a). These transdifferentiated cells express higher levels of the NE markers chromogranin-A (b, c) and synaptophysin (b, d) detected by either immunoblot analysis (b) or indirect immunofluorescence after staining with specific antibodies (c, d).

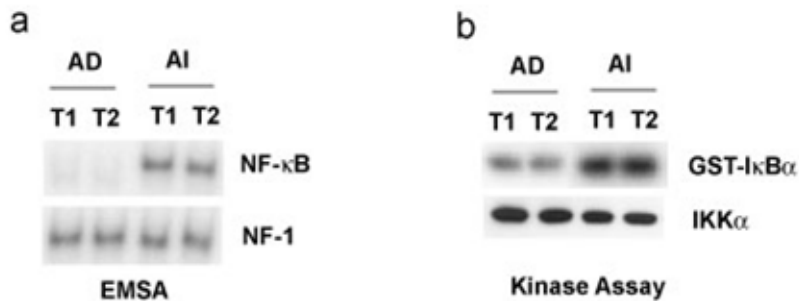


Figure 26: Human LNCaP cells were implanted subcutaneously into SCID mice. When tumor mass reached 1 cm³, half of the tumor bearing mice were sacrificed and tumor tissues were collected (androgen-dependent tumors, AD). The other half of the mice were castrated. Two months later when the tumors in the castrated mice regrew, the mice were sacrificed and tumors were collected (androgen-independent tumors, AI). Tumor extracts were used for electrophoretic mobility shift assay (EMSA) (a) and IKK kinase assay (b). Both NF- κ B DNA binding activity and IKK kinase activity were markedly elevated in AI tumors as compared to AD tumors.

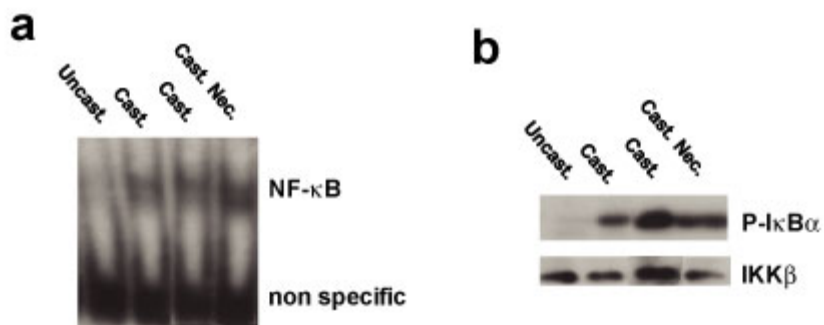


Figure 27: Myc-CaP cells (3×10^6) were injected into 8-week-old FVB male mice. When the tumor size reached 1 cm, mice were castrated or sacrificed to collect the tumor samples. When the tumors in the castrated mice regrew to 1 cm the mice were sacrificed. Protein extracts were obtained from tumor samples, and analysed by EMSA (a) or by IKK kinase assay (b).

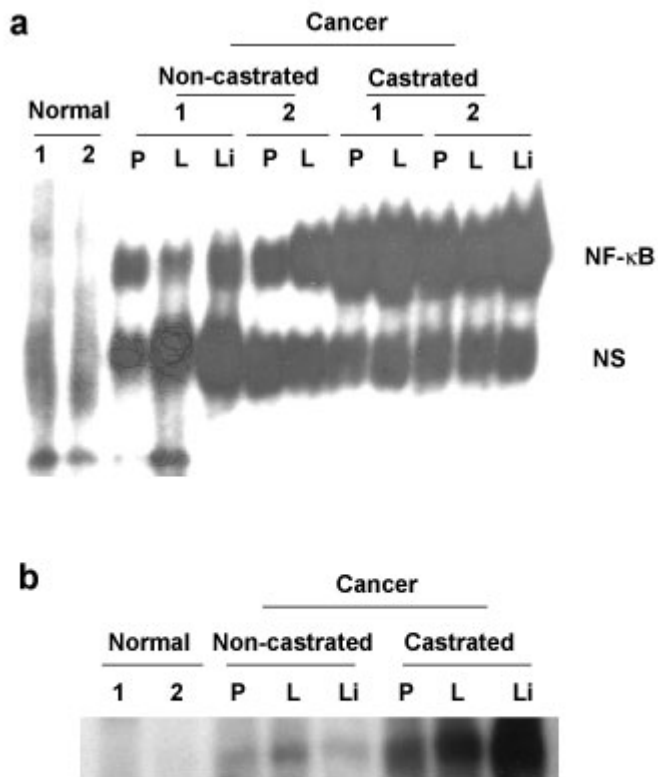


Figure 28: TRAMP mice were castrated 20 weeks of age. Fifteen to twenty weeks after castration mice were sacrificed and tumor tissues were collected for EMSA (a) and IKK Kinase Assay (b). Normal prostate tissues and prostate tumor tissues from mice with similar ages were used as control. (P = Primary tumor; L = Lymph node metastasis; Li = Liver metastasis).

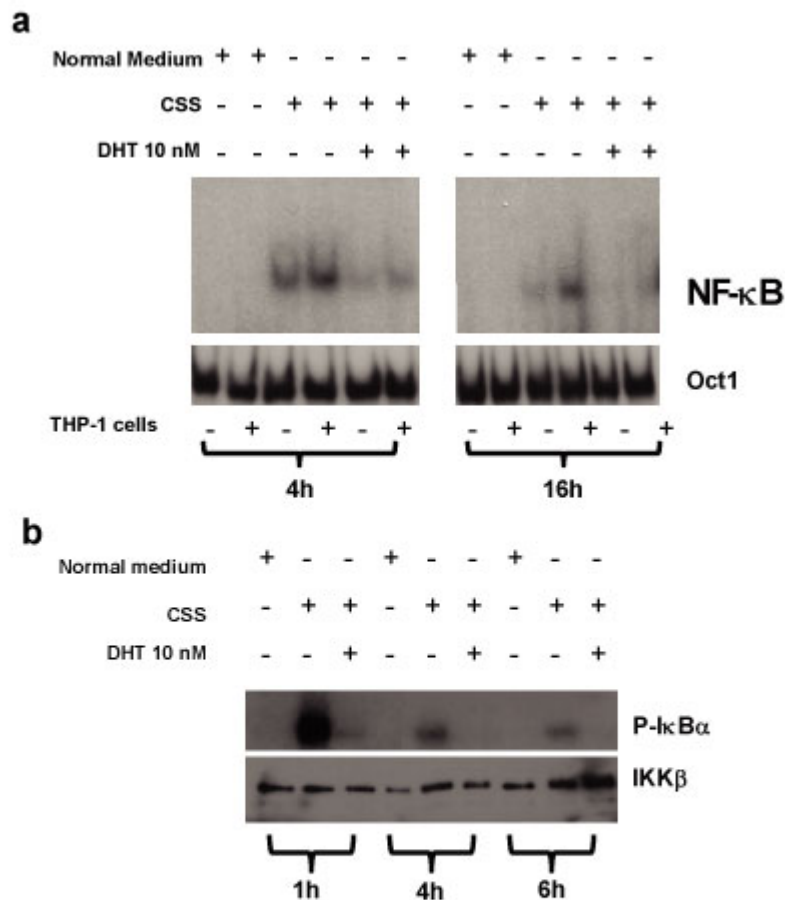


Figure 29: a, LNCaP cells were cultured in RPMI1640 plus 10% FBS (Normal Medium) or in RPMI1640 phenol red free plus 10% Charcoal Stripped Serum (CSS) alone or together with THP-1 human monocytic cells using a cell culture insert to separate the two cell lines, with or without dihydrotestosterone (DHT), for the indicated times. Protein extracts were obtained and 5 μ g were incubated with a 32 P-labelled NF- κ B or Oct-1 probes. Protein-DNA complexes were analysed by EMSA and gel was exposed to X-ray film for 15h. b, LNCaP cells were cultured in Normal Medium or CSS, with or without DHT, for the indicated time points. Protein extracts were prepared and IKK kinase activity was measured by I κ B α phosphorylation.

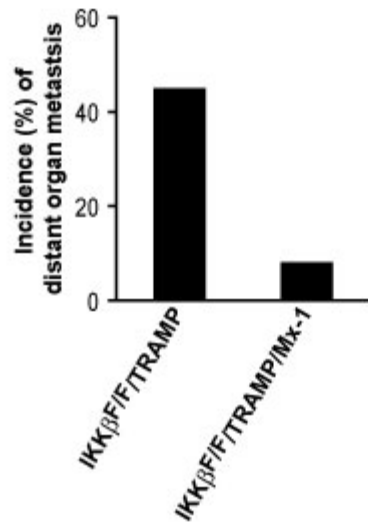


Figure 30: Twenty-week-old $Ikk\beta^{F/F}/TRAMP$ and $Ikk\beta^{F/F}/TRAMP/Mx-1$ male mice were injected poly(IC) three times. Ten days later the mice were castrated and tumor development was carefully monitored. CaP metastases in $Ikk\beta^{F/F}/TRAMP/Mx-1$ -Cre mice were dramatically decreased after more than five months follow-up.

MTT ASSAY

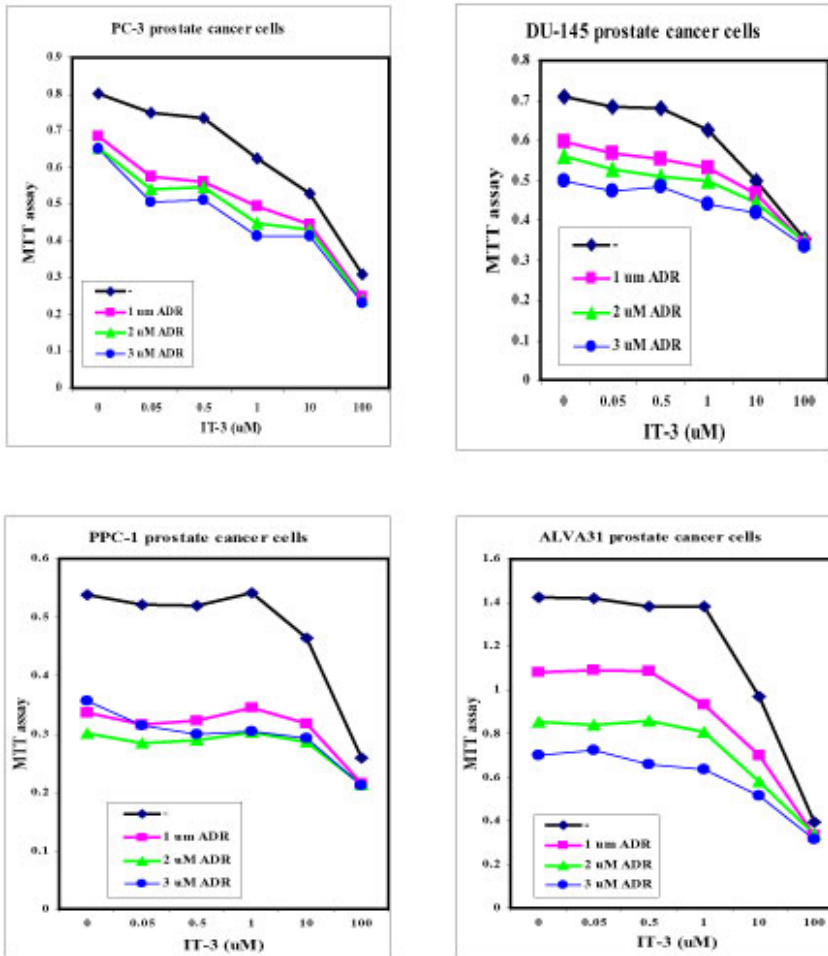


Figure 31: The IKK inhibitor IT-3 inhibits proliferation of human CaP cells and/or reduces their viability. Four human CaP cell lines (DU-145, ALVA31, PC-3 and PPC-1) as indicated were treated with IT-3, alone or in combination with adriamycin (ADR) for 24 hours. Cell viability was measured by the MTT assay. The results show that IT-3 can inhibit the proliferation or reduce the viability of human CaP cells.

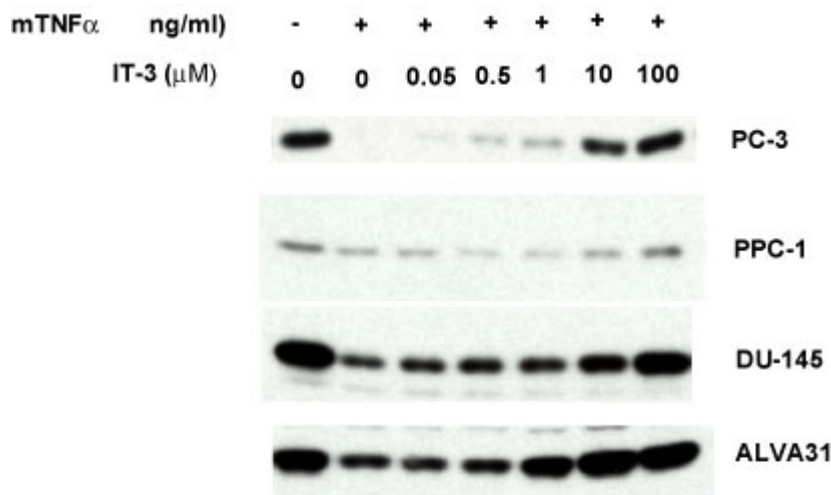


Figure 32: Immunoblot analysis of I κ B α levels in different CaP cell lines. Cells were pretreated with different concentrations of the IKK inhibitor IT-3 before TNF α stimulation. Cells were collected at 30 minutes after TNF α treatment, and I κ B α levels were examined by immunoblotting. IKK activation by TNF α induces the degradation of I κ B α and IT-3 at 10 to 100 μ M prevents this.

Conclusions

In summary, the results of this project suggest that both IKK α and IKK β play an important role in development and progression of human and mouse CaP. Nuclear IKK α links inflammation-activated signaling to metastatic progression of prostate cancer. Interference with IKK α activation can prevent distant organ metastasis in TRAMP mice. Decreased metastasis correlated with elevated expression of the metastasis suppressor maspin, whose ablation restored metastatic activity. IKK α activation, which can be triggered by RANK, whose ligand is expressed by tumor-infiltrating inflammatory cells, elevates metastatic activity and represses maspin gene transcription through a novel NF- κ B-independent mechanism requiring nuclear translocation of IKK α . By contrast, IKK β is activated during the transition of CaP from an AD to an AI state. Inhibition of IKK β activity in inflammatory cells of the tumor microenvironment significantly reduced the formation of hormone refractory prostate cancer. IKK inhibitors can inhibit the proliferation or induce the death of human CaP cell lines *in vitro*.

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1 Nuclear cytokine-activated IKK α controls prostate cancer metastasis by repressing Maspin

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Inflammation enhances tumour promotion through NF- κ B-dependent mechanisms¹. NF- κ B was also proposed to promote metastatogenesis through epithelial–mesenchymal transition². Yet, a mechanistic link between inflammation and metastasis is missing. We identified a role for I κ B kinase α (IKK α), activated by receptor activator of NF- κ B (RANK/TNFRSF11A), in mammary epithelial proliferation during pregnancy³. Owing to similarities between mammary and prostate epithelia, we examined IKK α involvement in prostate cancer (CaP) and its progression. Here we show that a mutation that prevents IKK α activation slows down CaP growth and inhibits metastatogenesis in TRAMP mice, which express SV40 T antigen in the prostate epithelium⁴. Decreased metastasis correlated with elevated expression of the metastasis suppressor Maspin⁵, the ablation of which restored metastatic activity. IKK α activation by RANK ligand (RANKL/TNFSF11) inhibits Maspin expression in prostate epithelial cells, whereas repression of *Maspin* transcription requires nuclear translocation of active IKK α . The amount of active nuclear IKK α in mouse and human CaP correlates with metastatic progression, reduced Maspin expression and infiltration of prostate tumours with RANKL-expressing inflammatory cells. We propose that tumour-infiltrating RANKL-expressing cells lead to nuclear IKK α activation and inhibition of *Maspin* transcription, thereby promoting the metastatic phenotype.

CaP, a heterogeneous disease, progresses from prostatic intra-epithelial neoplasia to locally invasive adenocarcinoma, and then to hormone-refractory metastatic carcinoma⁶. One in six men will be diagnosed with CaP, and one in thirty-three will die of metastatic disease⁶. Early CaP confined to the prostate can be treated⁷, but no effective treatments are available for metastatic disease. To understand whether signalling pathways related to NF- κ B activation, which are amenable for pharmacological inhibition⁸, are involved in metastatogenesis, we examined the role of IKK α , one of the two catalytic subunits of the IKK complex⁹, in CaP development and metastatic progression. To this end, we used *Ikk α ^{AA/AA}* mice in which the serines whose phosphorylation is required for IKK α activation are replaced with alanines³. Growth of the prostate and mammary epithelia depends on sex steroids and *Ikk α ^{AA/AA}* females exhibit retarded mammary gland growth during pregnancy³; however, *Ikk α ^{AA/AA}* mice show no defects in prostate development and composition (Supplementary Fig. 1). *Ikk α ^{AA/AA}* mice were crossed with TRAMP mice expressing SV40 Tag from the prostate specific probasin promoter⁴. As described^{4,10}, single mutant TRAMP (WT/TRAMP) mice developed CaP rather early and started dying at around 22 weeks of age, but homozygosity for the *Ikk α ^{AA}* allele prolonged tumour onset and delayed mortality (Fig. 1a). Nonetheless,

no discernible histological differences in primary tumours (Fig. 1b, c) and lymph node metastases (not shown) were found between the genotypes on necropsy. Although no size differences of primary CaP were detected at death (which was due to primary CaP),

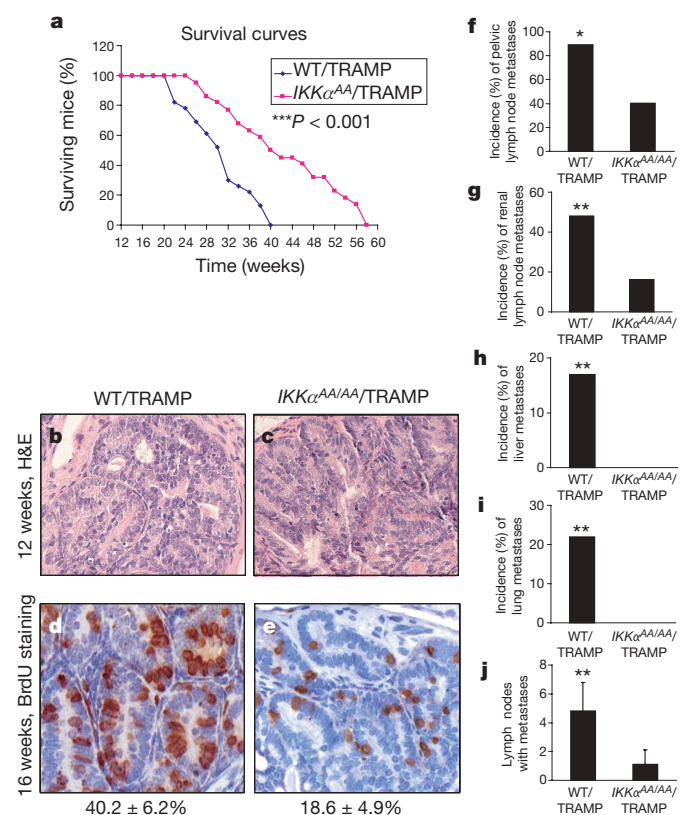


Figure 1 | IKK α activity is required for prostate cancer metastasis.

a, TRAMP mice were intercrossed for at least six generations with *Ikk α ^{AA/AA}* mice. Survival of *Ikk α ^{AA/AA}*/TRAMP (n = 22) and WT/TRAMP (n = 23) mice was compared. **b, c**, Histological analysis (haematoxylin and eosin staining) of paraffin-embedded sections; magnification, $\times 100$ of primary prostate adenocarcinomas from 3-month-old WT/TRAMP (**b**) and *Ikk α ^{AA/AA}*/TRAMP (**c**) mice. **d, e**, Cell proliferation in prostate carcinomas of 4-month-old WT/TRAMP (**d**) and *Ikk α ^{AA/AA}*/TRAMP (**e**) mice was examined by BrdU labelling. Percentages of BrdU-positive cells are indicated underneath (n = 4). **f–j**, Incidence of pelvic lymph node (**f**), renal lymph node (**g**), liver (**h**) and lung (**i**) metastases. **j**, Average numbers of lymph nodes harbouring metastases (WT/TRAMP, n = 23; *Ikk α ^{AA/AA}*/TRAMP, n = 22). * $P < 0.05$; ** $P < 0.01$; error bars, s.d.

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Ikk α ^{AA/AA}/TRAMP mice exhibited considerably fewer distant-site metastases (Fig. 1b–j).

Consistent with slow tumour growth, we observed a proliferation defect in CaP of 4-month-old *Ikk α ^{AA/AA}/TRAMP* mice pulsed with 5-bromodeoxyuridine (BrdU; identifies cells undergoing DNA synthesis; Fig. 1d, e). These changes in cell proliferation affected tumour size only at intermediate time points (Supplementary Table 1), and at death the primary tumours reached the same size in *Ikk α ^{AA/AA}/TRAMP* as in WT/TRAMP mice. Progression of CaP in WT/TRAMP mice correlates with reduced E-cadherin expression, a hallmark of epithelial–mesenchymal transition, and increased expression of the neuroendocrine marker synaptophysin¹⁰, which is also upregulated in advanced human CaP¹¹. No differences in E-cadherin or synaptophysin expression were detected between the genotypes (Supplementary Fig. 2).

Tumour metastasis is governed by genetic and epigenetic factors¹². We compared expression of approximately 40 promoters and

suppressors of metastasis in primary CaP of both genotypes by quantitative reverse transcription (qRT)–PCR (Supplementary Fig. 3). The only gene that exhibited marked and consistent differences between the genotypes was *Maspin* (*Serp1b5*), an established metastasis suppressor (Fig. 2a). *Maspin* is a gene expressed by normal mammary epithelial cells but not by mammary carcinoma, and the forced expression of *Maspin* inhibits invasion and motility of breast cancer cells⁵. Ectopic *Maspin* expression in mammary epithelium inhibits development of lobulo-alveolar structures during pregnancy¹³, resulting in a phenotype similar to the mammary phenotype of *Ikk α ^{AA/AA}* mice³. *Maspin* overexpression from a mammary epithelial promoter inhibited metastasis of SV40 Tag-induced mammary carcinoma¹⁴. Importantly, there are strong inverse correlations between *Maspin* expression and metastatic potential in human CaP^{15–17}.

Although *Maspin* was similarly expressed in cancerous prostates of both genotypes at 3 months of age (Supplementary Fig. 4), its

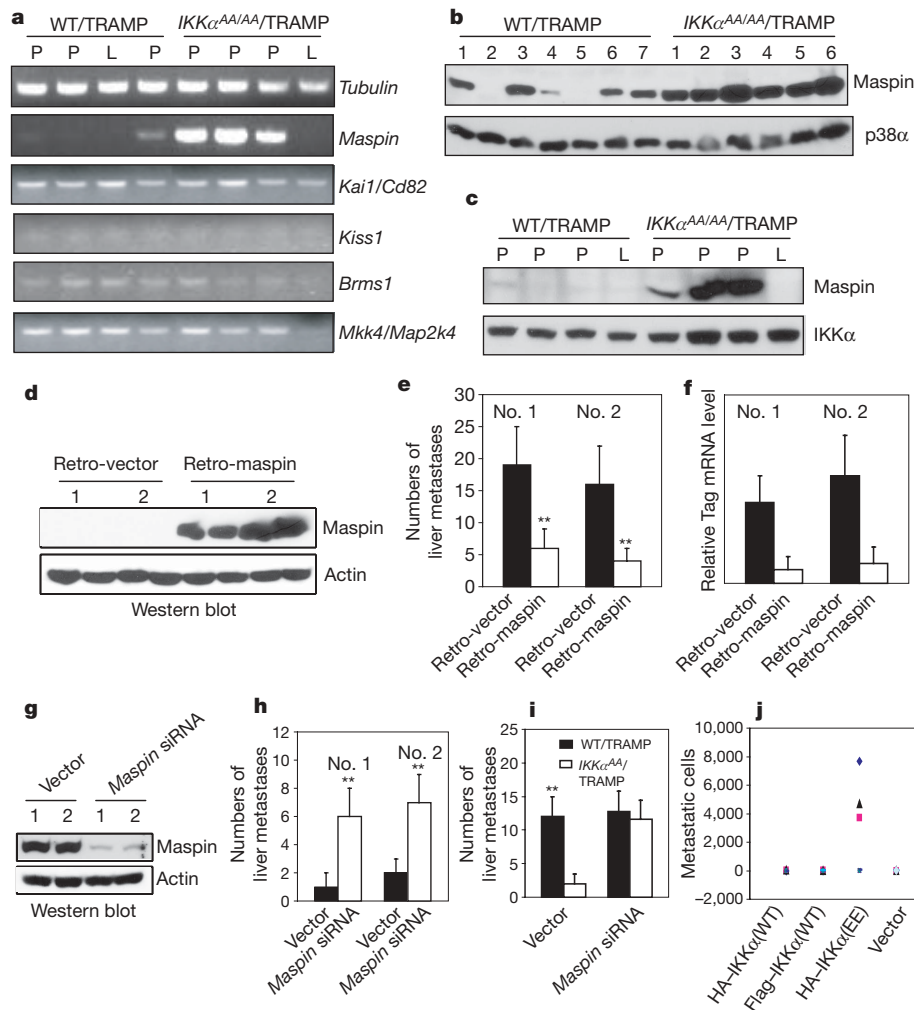


Figure 2 | Maspin expression correlates with metastatogenesis.

a, Expression of mRNAs encoding metastasis suppressors was analysed by qRT–PCR in primary (P) CaPs and lymph node (L) metastases. **b**, **c**, *Maspin* expression was examined in primary CaPs of 4–5- (**b**) or 7–8- (**c**) month-old mice by immunoblotting. **d**, CaP cells from WT/TRAMP mice were infected with either a *Maspin* retrovirus (retro-maspin) or an ‘empty’ retrovirus (retro-vector). Two preparations of transduced cells per virus were selected in $100 \mu\text{g ml}^{-1}$ zeocin for 1 week and immunoblotted for *Maspin* expression. **e**, Single-cell suspensions of cells from **d** were injected into spleens of *Nu/Nu* male mice (each group, $n = 5$) and liver metastases were counted 4 weeks later. Numbers represent different cell isolates and cultures. **f**, qRT–PCR analysis of SV40 Tag mRNA in livers from **e**. **g**, Primary CaP cells from *Ikk α ^{AA/AA}/TRAMP* mice expressing endogenous *Maspin* were infected with

Maspin siRNA retrovirus (*Maspin* siRNA) or an ‘empty’ retrovirus (vector). Two preparations of transduced cells per virus were selected in $1 \mu\text{g ml}^{-1}$ puromycin and immunoblotted for *Maspin* expression. **h**, Single-cell suspensions of cells from **g** were injected into spleens of *Nu/Nu* male mice (each group, $n = 5$) and liver metastases were counted after four weeks.

i, *Maspin* was knocked down in WT/TRAMP and *Ikk α ^{AA/AA}/TRAMP* CaP cells and metastatic activity was examined as above. **j**, Four coded samples of *Ikk α ^{AA/AA}/TRAMP* CaP cells infected with the indicated adenoviruses were inoculated on backsides of chorioallantoic membranes of 10-day-old chick embryos (each group, $n = 10$). After 9 days, pulmonary metastasis was quantified by detection of SV40 Tag DNA sequences. Chicken GAPDH DNA was used as an internal control. Error bars (**e**, **f**, **h**, **i**), s.d.

expression started declining in WT/TRAMP CaPs at 4–5 months of age but remained high in *Ikk α ^{AA/AA}*/TRAMP CaPs (Fig. 2b). At 7–8 months of age Maspin was not detectable in WT/TRAMP CaP, but primary *Ikk α ^{AA/AA}*/TRAMP CaP retained high Maspin expression (Fig. 2a, c). Immunohistochemistry revealed that Maspin was highly expressed in *Ikk α ^{AA/AA}*/TRAMP CaP cells, but was barely detectable or absent in WT/TRAMP CaP (Supplementary Fig. 5). Consistent with its role as a metastasis suppressor, *Maspin* expression was extinguished in the rare lymph node metastases found in *Ikk α ^{AA/AA}*/TRAMP mice (Fig. 2a).

To determine whether Maspin expression accounts for reduced metastasis, we examined metastatic potential of primary CaP cells from 7–8 month old mice that were cultured for 1–3 weeks. Single-cell suspensions were injected into spleens of Nude (*Nu/Nu*) male mice and 10 min later the spleens were removed. After 4 weeks, metastases to the liver were enumerated and liver SV40 Tag messenger RNA was quantified. *Maspin*-negative WT/TRAMP CaP cells gave rise to numerous liver metastases or Tag mRNA in the liver, but forced Maspin expression reduced metastatic activity (Fig. 2d–f). In contrast, *Ikk α ^{AA/AA}*/TRAMP CaP cells formed very few liver metastases, but a *Maspin* knockdown (Fig. 2g) increased metastatic potential to the level of WT/TRAMP CaP cells (Fig. 2h, i). Despite marked reduction in metastatic potential, the tumorigenic potential of *Ikk α ^{AA/AA}*/TRAMP CaP cells was only marginally lower than that of WT/TRAMP CaP (Supplementary Fig. 6). The reduced tumour growth could be due to expression of Maspin, which can inhibit cell proliferation^{16,17}.

To examine whether IKK α activation modulates metastatic activity we used adenoviruses to express either wild-type (WT) or constitutively active (EE) IKK α —in which the activation loop serines were replaced with phosphomimetic glutamate residues¹⁸—in *Ikk α ^{AA/AA}* primary CaP cells. Because adenovirus infection is transient we used the chick embryo spontaneous metastasis assay¹⁹ to determine metastatic activity. This model examines metastatic spread of cancer cells inoculated on the chorioallantoic membrane of 10-day-old chicken embryos. Only activated-*IKK α* -transduced cells showed metastatic activity (Fig. 2j). Yet, primary transplanted tumours grew at the same rate. Thus in addition to Maspin, IKK α activity also determines metastatic potential.

We examined how IKK α controls *Maspin* gene expression. *Ikk α ^{AA/AA}*/TRAMP CaP cells expressing Maspin were infected with adenoviruses encoding green fluorescent protein (GFP), IKK α (WT), activated IKK α (EE) or activated IKK β (EE). Three days later, Maspin levels were examined. IKK α (EE) significantly downregulated Maspin expression, whereas IKK α (WT) or IKK β (EE) had little or no effect (Fig. 3a). To examine whether IKK α regulates *Maspin* gene transcription, we used a *Maspin*-luciferase reporter containing 759 base pairs (bp) of the human *Maspin* 5' upstream region²⁰. Transfection of the reporter with different amounts of IKK α (EE) vector into *Ikk α ^{-/-}* mouse embryonic fibroblasts resulted in dose-dependent repression, an effect not seen with IKK α (EE) or IKK α (AA) (Fig. 3b). No repression of *Maspin* promoter activity was seen on co-expression of activated IKK β (EE) (Fig. 3c), which is a more potent activator of NF- κ B-dependent transcription than IKK α (EE) (Supplementary Fig. 7).

Because the transfection experiments were conducted with the human *Maspin* promoter, we used Maspin-expressing, normal, human mammary epithelial cells to examine whether ectopically introduced activated IKK α interacts with the endogenous *Maspin* promoter in native chromatin. Chromatin immunoprecipitation (ChIP) analysis revealed that activated IKK α was recruited to the endogenous *Maspin* promoter (Fig. 3d). The interaction was specific because IKK α did not interact with intron 2 of the *Maspin* gene.

Endogenous IKK α was present in both the cytoplasmic and nuclear fractions of CaP cells, whereas IKK β and the IKK γ /NEMO regulatory subunits were exclusively cytoplasmic (Fig. 3e). Very little, if any, nuclear IKK α was found in normal prostate epithelium and immunoblotting with phospho-specific antibody revealed that

nuclear IKK α in CaP was mostly in its phosphorylated and activated form. Although these results are consistent with the presence of a nuclear localization sequence (NLS) in IKK α , nuclear IKK α was previously found only in differentiating keratinocytes²¹. To examine whether IKK α acts in the nucleus to control Maspin expression, the *Maspin*-luciferase reporter was co-transfected with IKK α (EE), IKK α (EE)-NLS (a nuclear localization defective version of activated IKK α) and IKK α (KM) (a catalytically inactive mutant of IKK α) vectors into *Ikk α ^{-/-}* mouse embryonic fibroblasts. Neither IKK α (EE)-NLS nor

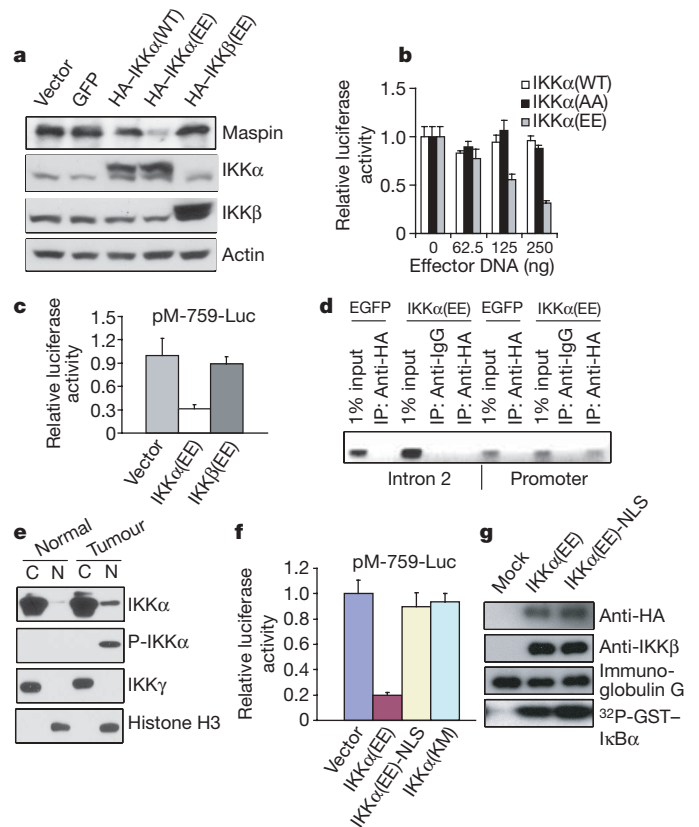


Figure 3 | Activated nuclear IKK α represses *Maspin* transcription. **a**, *Ikk α ^{AA/AA}*/TRAMP CaP cells were infected with different adenoviruses. After three days, Maspin, IKK α , IKK β and actin levels were analysed by immunoblotting. **b**, A *Maspin*-luciferase reporter containing 759 base pairs (bp) of the human *Maspin* 5' upstream region (pM-759-Luc) was co-transfected with different amounts of IKK α (WT), IKK α (AA) or IKK α (EE) vectors into *Ikk α ^{-/-}* mouse embryonic fibroblasts. Luciferase activity was measured and normalized to a co-transfected PRL-TK reporter ($n = 3$). **c**, The *Maspin*-luciferase reporter was co-transfected with IKK α (EE) or IKK β (EE) vectors into *Ikk α ^{-/-}* mouse embryonic fibroblasts. Luciferase activity was measured and normalized as above ($n = 3$). **d**, ChIP analysis of IKK α recruitment to the human *Maspin* promoter. HME cells were transfected with either HA-IKK α (EE) or enhanced GFP (EGFP) adenovirus. After 48 h, proteins were crosslinked to DNA and chromatin was extracted and fragmented. Protein–DNA complexes were immunoprecipitated with haemagglutinin (HA) or non-specific immunoglobulin G (IgG) antibody. The presence of *Maspin* promoter region (+87 to –315) and intron 2 (+3958 to +4350) in the immunoprecipitates was examined by PCR. **e**, Nuclear (N) and cytoplasmic (C) extracts of normal and TRAMP (tumour) prostate epithelium were analysed for IKK α , phospho(activated)-IKK α (p-IKK α), IKK γ and histone H3 by immunoblotting. **f**, The *Maspin*-luciferase reporter was co-transfected with 'empty' (vector), IKK α (EE), IKK α (EE)-NLS or IKK α (KM) vectors and luciferase activity was measured as described above ($n = 3$). **g**, HEK293 cells were transfected with different expression vectors as indicated. After 36 h, IKK complexes were immunoprecipitated with HA-specific antibody and I κ B kinase activity and IKK β content were measured. Error bars (**b**, **c**, **f**), s.d. 32p-GST-I κ B α , the GST (glutathione S-transferase) fusion protein containing the N-terminal 50 residues of I κ B α .

IKK α (KM) repressed *Maspin* promoter activity (Fig. 3f). To exclude the possibility that IKK α (EE)-NLS lost its kinase activity, IKK α (EE)-NLS or IKK α (EE) were expressed in HEK293 cells and their I κ B kinase activity was measured. IKK α (EE)-NLS was as active as IKK α (EE) in I κ B phosphorylation (Fig. 3g). Hence, repression of *Maspin* transcription requires nuclear IKK α kinase activity. Because NF- κ B activation depends on I κ B phosphorylation in the cytoplasm, and IKK β (EE), which is a more potent activator of NF- κ B than IKK α (EE), did not repress *Maspin* expression, then repression of *Maspin* by IKK α seems to be NF- κ B-independent.

The amount of activated nuclear IKK α correlated with CaP progression, because it was substantially higher in CaP from 7–8-month-old WT/TRAMP mice than in CaP from younger mice (Fig. 4a). Furthermore, the amount of nuclear IKK α inversely correlated with *Maspin* expression. Most importantly, activated nuclear IKK α correlated with progression of human CaP and was highest in stage 4

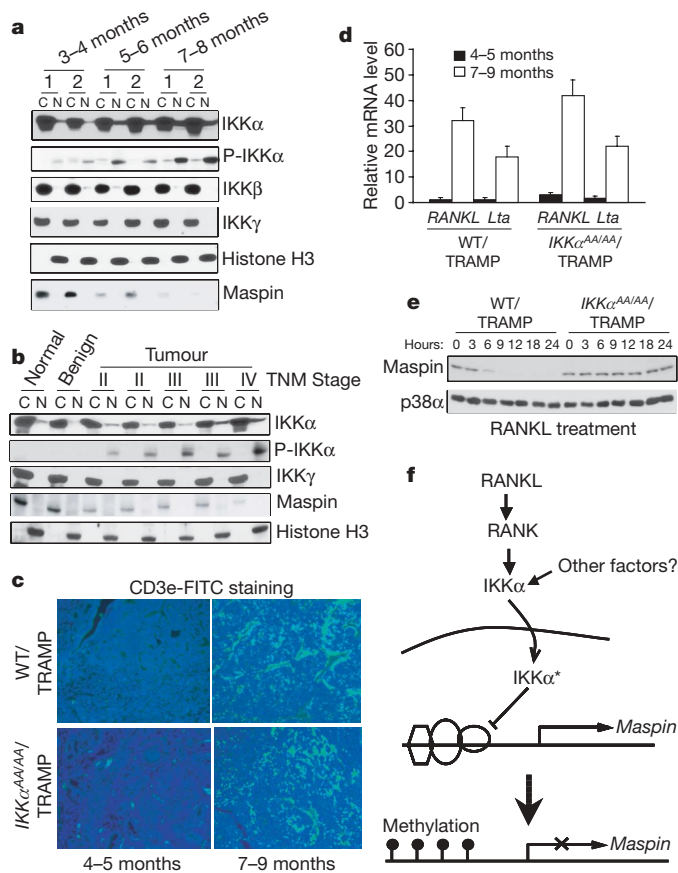


Figure 4 | Metastatic progression correlates with nuclear activation of IKK α , inflammatory cell infiltration and massive upregulation of RANKL. **a**, CaP cells from TRAMP mice of indicated ages (1 and 2 are individual mice), fractionated into cytoplasmic (C) and nuclear (N) extracts, were analysed for IKK α , phospho-IKK α , IKK β , IKK γ , histone H3 and *Maspin* by immunoblotting. **b**, Normal human prostate, benign prostatic hyperplasia and prostate tumours of different clinical stages (TNM stage) were divided into nuclear (N) and cytoplasmic (C) fractions and analysed for the indicated proteins by immunoblotting. **c**, Prostate tumours from 4–5- and 7–9-month-old TRAMP mice of the indicated genotype were stained for the T cell marker CD3. FITC, fluorescein isothiocyanate. **d**, Prostate tumours from mice of the indicated age and genotype were analysed by qRT-PCR for expression of *RANKL* and *Lta* mRNAs, (results are averages \pm s.d.; $n = 4$). **e**, WT/TRAMP and *Ikk α ^{AA/AA}*/TRAMP prostate epithelial cells were treated with lipopolysaccharide-free RANKL (200 μ g ml⁻¹). At the indicated times cell extracts were prepared and examined for *Maspin* and p38 α content by immunoblotting. **f**, A model explaining how RANK signalling leads to repression of *Maspin* transcription. After transient IKK α -mediated repression, *Maspin* transcription is likely to be permanently silenced through DNA methylation.

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tumours, which did not express *Maspin* (Fig. 4b). No activated IKK α was detected in the nuclear fraction of normal human prostate or benign prostate hyperplasia, which expressed high levels of *Maspin*.

In mammary epithelial cells³ and myeloid cells^{22,23}, IKK α is activated on occupancy of RANK by RANKL. Another cytokine that activates IKK α is lymphotoxin (LT) $\alpha\beta$ (ref. 22). These tumour necrosis factor (TNF) family members are expressed by lymphoid and myeloid cells. Immunohistochemistry revealed only small amounts of such cells in early CaP of either genotype, but the amount of tumour infiltrating T cells (CD3⁺) and macrophages (F4/80⁺) was vastly increased in primary tumours of 7–9-month-old mice (Fig. 4c; Supplementary Fig. 8). Likewise, *RANKL* and *Lta* mRNA were dramatically elevated in CaP from 7–9-month-old TRAMP mice relative to tumours of younger mice (Fig. 4d).

Given the striking increase in *RANKL* mRNA, which was also paralleled at the protein level (Supplementary Fig. 9), we treated primary prostate epithelial cells with RANKL and examined *Maspin* expression. *Maspin* expression declined within 6 h of RANKL application to WT/TRAMP cells but no effect was seen in *Ikk α ^{AA/AA}*/TRAMP cells (Fig. 4e). These results suggest that prostate cancer metastasis is a consequence of tumour infiltration by RANKL-expressing inflammatory cells that activate IKK α in the nuclei of carcinoma cells to repress *Maspin* transcription (Fig. 4f).

In recent years it has become clear that inflammation and a pro-inflammatory microenvironment make important and critical contributions to tumour development²⁴. Mechanistic studies have revealed an important tumour-promoting role for the inflammation-responsive IKK complex and its target NF- κ B, acting both within cancer (or pre-malignant) cells and inflammatory cells¹. Although inflammation is expected to enhance metastatic progression, distinct genetically established mechanisms linking inflammation and metastasis are scarce. Here we describe a novel mechanism in which IKK α activation, which can be achieved on binding of the pro-inflammatory cytokine RANKL to its receptor RANK, promotes prostate cancer metastasis (Fig. 4f). Although the *Ikk α ^{AA}* mutation also affects growth rate of primary cancer, its most pronounced effect is on metastatogenesis. Analysis of genes that enhance or suppress metastasis¹² revealed that IKK α exerted its pro-metastatic effect by repressing transcription of the *Maspin* gene. Inactivation of IKK α increased *Maspin* expression and inhibited metastasis, whereas short interfering RNA (siRNA)-mediated *Maspin* knockdown elevated the metastatic potential of *Ikk α ^{AA/AA}*/TRAMP CaP cells to that of WT/TRAMP CaP cells. Repression of *Maspin* expression requires nuclear translocation of catalytically active IKK α . An excellent correlation between active nuclear IKK α , the level of *Maspin* expression and tumour progression was observed both in mouse and human CaP.

Maspin is a member of the serpin family with well-established anti-metastatic activity in breast and prostate cancers^{5,17,25}. An excellent inverse correlation between *Maspin* expression and metastatic potential of human CaP was detected, such that metastatic CaPs express little or no *Maspin*^{15,16}. Furthermore, in metastatic human CaP the *Maspin* promoter and 5' control region are heavily methylated and treatment with DNA methyl transferase inhibitors reactivated *Maspin* transcription²⁶. Yet, how *Maspin* transcription is repressed before epigenetic silencing occurs was heretofore unknown. On the basis of a general model, in which DNA methyl transferases are recruited to gene regulatory regions through specific repressors or co-repressors, the repressive action of which may be transient²⁷, we propose that initial repression of *Maspin* transcription is mediated by IKK α activation in response to engagement of RANK or similar receptors. With time, transient repression is converted to epigenetic silencing through DNA methylation (Fig. 4f). This process commits CaP cells to a metastatic fate.

RANKL-mediated RANK activation induces migratory behaviour in breast and prostate carcinomas and promotes bone metastasis of melanomas²⁸. Our results indicate that RANK may be a general

promoter of metastatic behaviour in prostate or mammary carcinoma cells. At least in TRAMP mice, the prometastatic activity of RANK is accomplished in part through repression of *Maspin* gene transcription. Importantly, RANK is activated by RANKL, the expression of which is vastly upregulated in late stage CaP. At that time, the primary tumours are highly infiltrated with T cells and macrophages, cells that could be a major source for RANKL and related factors. Correspondingly, late stage human and mouse prostate carcinomas contain the highest levels of active nuclear IKK α and produce little or no Maspin.

METHODS

A detailed Methods section is available in Supplementary Information. Briefly, previously described *Ikk α ^{AA}* and TRAMP mice were intercrossed for six generations to generate *Ikk α ^{AA/AA}/TRAMP* and *Ikk α ^{+/+}/TRAMP* (WT/TRAMP) mice of nearly identical genetic background. Human material was obtained from the Cooperative Human Tissue Network (CHTN) along with pathology reports. Histology, gene expression and NF- κ B and IKK signalling were analysed as described^{3,29}. Metastatic activity was assayed either by injection of cancer cells into the spleen and measurement of liver metastases one month later³⁰, or using the chick embryo metastasis assay¹⁹.

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The Anti-Death Machinery in IKK/NF- κ B Signaling

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The most extensively studied function of NF- κ B is its ability to promote cell survival through induction of target genes, whose products inhibit various aspects of the apoptotic machinery in both normal and malignant cells. Recent studies, however, indicate that NF- κ B activation can also suppress programmed necrosis through induction of genes encoding anti-oxidant proteins. Since tumor cells often use NF- κ B pathway as a shield to escape the killing of conventional anti-cancer therapies, intervention of IKK/NF- κ B signaling would be a promising option to improve the efficacy of cancer treatment.

KEY WORDS: NF- κ B; IKK; cell death; cancer therapy.

THE IKK/NF- κ B PATHWAYS

The NF- κ B family contains five members: NF- κ B1 (both p105 and p50), NF- κ B2 (both p100 and p52), c-Rel, RelB, and RelA (p65). These proteins share a Rel homology domain (RHD) which mediates DNA binding, dimerization and interactions with inhibitory factors known as I κ B proteins, which retain NF- κ B dimers in the cytoplasm. A wide range of stimuli activate NF- κ B, mostly through I κ B kinase (IKK)-dependent phosphorylation and subsequent degradation of I κ B proteins. The liberated NF- κ B dimers enter the nucleus where they regulate transcription of many genes encoding cytokines, growth factors, cell adhesion molecules, as well as pro- and anti-apoptotic proteins (1, 2). The IKK complex consists of two highly homologous kinase subunits, IKK α and IKK β , and a nonenzymatic regulatory component, IKK γ /NEMO (3).

There are two distinct NF- κ B activation pathways described. The first pathway, the classical pathway, is normally triggered in response to microbial and viral infections or exposure to proinflammatory cytokines that activate the three subunit IKK complex leading to phosphorylation-induced degradation of I κ Bs. This pathway, which mainly targets p50:RelA and p50:c-Rel dimers, depends mainly on the kinase activity of the IKK β catalytic subunit (4). The other pathway, the alternative pathway, leads to selective activation of p52:RelB dimers by inducing processing of the NF- κ B2/p100 precursor protein, which is mostly found as a heterodimer with RelB in the cytoplasm. This pathway is triggered by certain members of the tumor necrosis factor (TNF) cytokine family, through selective activation of IKK α homodimers by the upstream kinase NIK (5). Both pathways regulate cell survival and death (6), but amongst the two, it is the classical NF- κ B pathway that is responsible for inhibition of programmed cell death (PCD) under most conditions (2, 3). The alternative pathway is mainly important for the survival of premature B cells and the development of secondary lymphoid organ (7). The anti-apoptotic activity of the IKK β -driven classical pathway is of particular importance in the case of various immunoreceptors of cytokines, including T- and B-cell receptors (TCR and BCR, respectively), Toll-like receptor 4 (TLR4) and type 1 TNF α receptor (TNFR1), all of which generate pro-survival and pro-death signals upon ligation (8, 9). Under most circumstances the survival signals dominate, but under some conditions where IKK β or NF- κ B activities have been compromised, receptor activation results in cell death (10–12).

NF- κ B AND CELL DEATH PATHWAYS

The decision between life and death is of primary importance in embryonic development, organogenesis, and immune responses. PCD is classified into two major types, apoptosis and necrosis, based on morphological and

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biochemical characteristics and the mechanism of cell killing. Apoptosis is characterized by membrane blebbing, shrinking and condensation of the cell and its organelles, including the nucleus, accompanied by the characteristic 'DNA ladder' generated by internucleosomal DNA degradation (13, 14). There are two well-established pathways that lead to apoptosis; the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (15). Apoptosis is generally considered a process that depends on members of a family of cysteine proteases called caspases (15, 16). However, recent evidence reveals diversification of the apoptosis program with respect to the role of caspases, since apoptosis-like PCD can sometime proceed without caspase activation (17, 18). Furthermore, it was shown that caspase activation does not always lead to cell death (19, 20), and recently clear pro-survival functions were found for caspase-8 (21, 22). Necrosis is characterized by swelling of the cell and its organelles and results in disruption of the cell membrane and cell lysis, often accompanied by inflammation due to release of cellular contents. Catastrophic failure of energy metabolism or massive generation of reactive oxygen species (ROS) are thought to cause necrosis (23). In contrast to the extensive genetic and biochemical analysis of apoptosis, the mechanistic understanding of necrosis is at its infancy, but necrosis, just like apoptosis, can be a programmed response that is specifically elicited by extracellular stimuli acting through defined receptors, including TNFR1.

NF- κ B suppresses both types of PCD, although initially it was thought to antagonize only apoptotic cell death. The first evidence for NF- κ B as an inhibitor of PCD came from the analysis of RelA-knockout mice that die at mid-gestation by massive liver apoptosis (24). The role of NF- κ B in embryonic liver survival, which is brought about by inhibition of TNFR1-mediated apoptosis (25), was underscored by the very similar phenotypes of mice that lack IKK β (4) or IKK γ (26). A protective role for NF- κ B in adult liver has been confirmed in various mouse models of liver damage (10, 27, 28) and was recently shown to be due to inhibition of both apoptosis and necrosis (9). The pro-survival activity of NF- κ B is not limited to the liver and was seen in a variety of biological processes and other cell types, including thymocyte development (29–31), survival of naive *T* cells (32), B-cell development (33), epidermal homeostasis (34), hair follicle development (35), and the central nervous system (36). NF- κ B inhibits apoptosis by DNA damaging anti-cancer drugs and therapeutic radiation, which is mainly mediated by the intrinsic pathway (37), but its most prominent survival promoting activity is manifested by inhibition of apoptosis or necrosis triggered

by death receptors (DRs), including Fas and TNFR1 (2, 9, 38, 39).

MACHINERY AND MECHANISMS BY WHICH NF- κ B SUPPRESSES PCD

Caspases

DRs are characterized by a unique cytoplasmic region termed the death domain (DD), that recruits a number of molecules that lead to activation of various signaling cascades. So far, DR family members include: TNFR1 (aka DR1), Fas/CD95 (aka DR2), DR3 (aka APO-3/LARD/TRAMP/WSL1), TRAILR1 (aka DR4/APO-2), TRAILR2 (aka DR5/KILLER/TRICK2), DR6, ectodysplasin A receptor (EDAR) and p75 nerve growth factor receptor (NGFR) (40, 41). These DRs are grouped into two types based on the signaling complexes they form. The first group comprises Fas, DR4 and DR5, which directly recruit the DD-containing adaptor molecule FADD (Fas associated death domain), procaspase-8, procaspase-10 and the cellular FLICE-inhibitory protein (FLIP) to form death-inducing signaling complexes (DISCs) (42). DISC formation results in activation of caspase-8, which plays a central role in transduction of the apoptotic signal generated by these receptors. Targeted disruptions of the mouse caspase-8 or the FADD genes prevent DR-induced PCD (43, 44). The second group comprises TNFR1, DR3, DR6, and EDAR. In contrast to DISC forming receptors, TNFR1 does not directly interact with FADD (45, 46). Instead, TNFR1 forms a signaling complex (complex I) at the plasma membrane by recruiting the adaptor protein TRADD (TNFR1 associated death domain protein) and the signaling proteins TRAF2 (TNFR associated factor 2), TRAF5 and RIP1 (receptor interacting protein 1). Complex I leads to activation of IKK, through phosphorylation and activation of IKK β (47, 48), c-Jun N-terminal kinase (JNK) and p38 MAP kinase (MAPK) (49). In a second step, complex I dissociates from TNFR1, which can then recruit FADD and caspase-8, and trigger an apoptotic response (46). The dissociation of complex I from the receptor is likely to depend on FLIP degradation.

NF- κ B as a transcription factor leads to induction of genes whose products prevent PCD. One of the best elicitors of NF- κ B activation is TNF α , which is a rather poor inducer of PCD. TNF α triggers PCD only when new protein or RNA synthesis are inhibited or in NF- κ B-deficient cells. NF- κ B exerts its pro-survival activity by inducing expression of several anti-apoptotic genes, including c-FLIP, Bcl-X_L, A1/Bfl-1, cellular inhibitor of apoptosis (c-IAPs), X-chromosome-linked IAP (XIAP), TRAF1,

and TRAF2 (2, 39). cFLIP inhibits apoptosis by interfering with activation of caspase-8 (46). c-IAPs and XIAP directly bind and inhibit effector caspases which act downstream of initiator caspases in both the extrinsic and intrinsic pathways to block progression of the apoptotic signal.

Bcl-2 Family Members

NF- κ B induces expression of several members of the Bcl-2 family, most notably Bcl-X_L and A1/Bfl-1, which prevent apoptosis by inhibiting mitochondrial permeability transition, mitochondrial depolarization, and the release of cytochrome *c* (2, 39). In general Bcl-2 family members are mainly involved in regulation of the intrinsic apoptotic pathway. However, it was described that DRs can trigger apoptosis through two different pathways (50). In Type I cells, activated caspase-8 directly leads to activation of downstream effector caspases, but in type II cells, which exhibit poor DISC formation, death signaling requires an additional amplification loop. This loop is based on the ability of caspase-8 to cleave Bid, a BH3-only pro-apoptotic Bcl-2-family member, to generate truncated tBid which induces release of cytochrome *c* from mitochondria (51, 52). The released cytochrome *c* triggers apoptosome formation, followed by activation of caspase-9, which in turn activates the downstream effector caspase-3 (15). Type II DR signaling can be blocked by anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-X_L (15). Although the mechanism by which TNFR1 triggers PCD differs from that used by Fas, the fact that Bcl-X_L expression prevents TNFR1-induced PCD in NF- κ B-deficient cells indicates a role for mitochondrial amplification (53). NF- κ B-induced expression of Bcl-X_L and A1/Bfl-1 can therefore suppress mainly the intrinsic death pathway but in some cases will also inhibit the extrinsic pathway.

JNK

Ligation of TNFR1 and other immunoreceptors also results in JNK and p38 MAPK activation (38). The role of JNK in PCD has been quite controversial, because it has both survival and death enhancing effects. Three JNK isoforms, JNK1-3, are activated through specialized MAPK cascades, which also include the MAPK kinases (MKKs) MKK4 and 7 and numerous MKK kinases (MAP3Ks) (54, 55). The clearest evidence for JNK as regulator of PCD comes from analysis of knockout mice. Crossing a JNK1-deficiency into an IKK β -deficient strain delays the onset of liver apoptosis by 4 days (L. Chang and M.K., unpublished). Furthermore, JNK1- or JNK2-

deficient mice are relatively resistant to induction of fulminant hepatitis in response to concanavalin A (ConA), a pathology that depends on activation of TNFR1 and other DRs (10).

JNK phosphorylates anti-apoptotic Bcl-2 family members, including Bcl-2 and Bcl-X_L (56–58), as well as pro-apoptotic members, including Bim and Bmf (59). JNK also phosphorylates 14-3-3 proteins which associate with Bax, thereby releasing Bax from 14-3-3 mediated inhibition (60). The released Bax translocates to mitochondria and induces apoptosis. Recently, a novel role for JNK in TNFR1-mediated apoptosis was reported: JNK induces caspase-8-independent cleavage of Bid to generate jBID, another pro-apoptotic cleavage product which induces the release of the apoptotic mediator Smac/DIABLO from mitochondria (53). Smac/DIABLO binds to cIAP1, and releases caspase-8 from inhibition by the TRAF2-cIAP complex (61). This pathway is distinct from classical DR-mediated apoptosis pathways, in which caspase-8 acts upstream to mitochondria through generation of tBid in Type II cells (51, 52).

As mentioned above, JNK also possesses anti-apoptotic functions, and JNK1/JNK2 double knockout fibroblasts were reported to display increased sensitivity to TNF α -induced PCD (62). It was suggested, JunD, whose regulation by JNK is not well established (63), binds to the promoter of c-IAP2 gene and promotes its expression (62). In addition, JNK phosphorylates and inactivates Bad, thereby promoting survival of growth factor-deprived cells (21). Furthermore, mice lacking MKK4 die due to liver apoptosis, suggesting a survival role for JNK signaling (64). However, it remains to be established whether liver apoptosis in these mice is TNFR1 dependent and a more recent report indicates that JNK activity is required for hepatoblast proliferation rather than survival (65). To make things more complicated, it was found that JNK1 and JNK2 have distinct roles in the control of cell proliferation and PCD (66, 67). One way to explain these apparently paradoxical observations is that the JNK system does not function like a simple ON/OFF switch. Instead it acts as a contextual modulator and a rheostat, and that the final outcome depends on the magnitude and duration of JNK activation in relations to other signaling pathways. Thus, it is not surprising that initially JNK activation was found not to be required for TNFR1-mediated PCD (38) and was even suggested to have a minor survival role (68). Clearly a JNK-independent pathway is required to transduce the major TNFR1-generated death signal, but concomitant JNK activation enhances this signal.

Recent results indicate that it is not JNK alone that modulates the strength of the TNFR1-generated death signal,

but it is the ratio between JNK and NF- κ B activities that controls cell survival or death, not only in response to TNFR1 but also in response to other death stimuli (69, 70). Most importantly, it is turned out that NF- κ B is a negative regulator of JNK activation by TNF α and other death stimuli (71). It was observed that whereas TNF α leads to transient JNK activation in WT cells, it leads to highly prolonged JNK activation in cells that lack IKK β , TRAF2 and 5 or RelA and therefore are unable to activate NF- κ B (9, 72–74). Furthermore, the pro-survival activity of NF- κ B was shown to depend on its ability to prevent prolonged JNK activation (9, 72–74). Prolonged JNK activation was also seen in mice lacking IKK β in liver cells (hepatocytes) following ConA administration, which as mentioned above causes massive TNFR1-dependent hepatocytes death (10). In the liver, however, TNFR1 and JNK signaling are also required for regeneration or compensatory hepatocyte proliferation following partial hepatectomy or chemically-induced injury (75, 76). Thus, NF- κ B may be a critical regulator of cell life and death through its ability to control JNK activation (Fig. 1). Interestingly, such a crosstalk seems to function also in *Drosophila*, in which JNK activation is prolonged in cells lacking Relish, a *Drosophilal* homolog of NF- κ B (77). However, the exact function of prolonged JNK signaling in the fly system remains obscure.

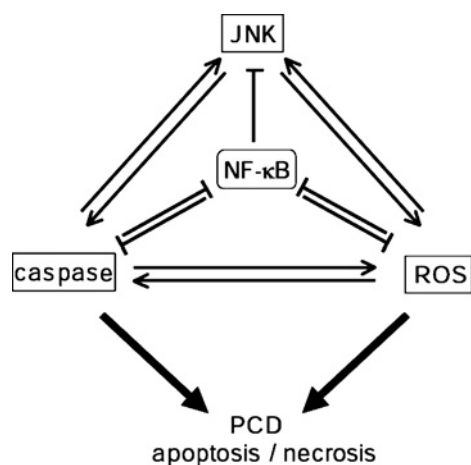


Fig. 1. Control of cell survival and death through the NF- κ B-JNK crosstalk. Positive feedback loops exist between ROS and caspases, caspases and JNK, and JNK and ROS. Negative feedback loops exist between NF- κ B and caspases, and NF- κ B and ROS. NF- κ B functions as a pro-survival transcription factor by inducing the expression of anti-apoptotic genes, such as the Bcl-2 family members and caspase inhibitors, and anti-oxidant genes such as MnSOD and FHC. Activation of NF- κ B also results in inhibition of prolonged JNK activation, mostly through inhibition of ROS accumulation. Inhibition of NF- κ B enhances PCD, that can be either apoptotic or necrotic in nature by removing the negative feedback loops.

Reactive Oxygen Species (ROS)

Prolonged JNK activation in NF- κ B-deficient cells implies that NF- κ B induces expression of JNK inhibitors or attenuates JNK activation signals. Such a function was proposed for GADD45 β , whose gene is activated by NF- κ B (72). GADD45 β was suggested to bind to MKK7 and inhibit its kinase activity (78). Another study concluded that the NF- κ B-induced JNK inhibitor is the caspase inhibitor XIAP (79). However, analysis of GADD45 β - or XIAP-deficient fibroblasts failed to reveal changes in the kinetics of JNK activation (39, 80), suggesting that NF- κ B regulates JNK activation through a different mediator.

This mediator has turned out to be ROS, most likely H₂O₂ (74). ROS, including H₂O₂, O₂⁻ and HO \cdot radicals, are constantly generated in cells through many enzymatic pathways (81), but their major source is leakage of electrons from the mitochondrial respiratory chain. ROS accumulation, referred to as oxidative stress, results in cytotoxicity and hence cells possess diverse antioxidant systems for ROS detoxification. For instance, O₂⁻ anions are rapidly converted to H₂O₂ by the action of superoxide dismutase (SOD), and then H₂O₂ is eliminated by several peroxidases including peroxiredoxins (Prx), glutathione peroxidase (Gpx), and catalase. Although ROS are rapidly eliminated and kept at very low levels, certain extracellular stimuli can enhance ROS production and cells use ROS as second messengers (81). Defects in ROS elimination result in oxidative stress and irreversible damage to many cellular components.

ROS can modulate the activity of various signaling systems, but there is no evidence that they can directly activate protein kinases. Instead, ROS activate kinases through oxidation of kinase interacting molecules. For instance, ROS oxidize thioredoxin bound to ASK1, thereby causing ASK1 activation (82). ROS also activate many tyrosine kinases by inactivating protein tyrosine phosphatases (PTPs), through oxidation of the highly reactive cysteine residue at their catalytic site (83–87). Importantly, ROS act as the critical mediators of the NF- κ B-JNK crosstalk through their ability to inhibit the activity of various MAPK phosphatases (MKPs) involved in JNK inactivation (81).

TNF α induces ROS accumulation in a variety of cell types and these ROS are important mediators of PCD (23). TNF α -induced ROS accumulation is easily seen in NF- κ B-deficient cells, but not in NF- κ B competent cells (9, 74). Treatment of cells with the antioxidant butylated hydroxyanisole (BHA) has no effect on transient activation of JNK triggered by TNF α in NF- κ B competent cells, but it effectively suppresses prolonged JNK activation in

TNF α -NF- κ B-deficient cells (9, 74). Importantly, BHA markedly suppresses TNF α -mediated PCD in NF- κ B deficient cells or mice (9, 74). This protective effect of BHA is due to its ability to prevent the oxidation of MKPs, thus ensuring transient JNK activation (9). Expression of dominant negative mutants of MKPs leads to prolonged JNK activation allows the killing by TNF α of NF- κ B competent cells, which otherwise are TNF α -resistant (9). However, prolongation of JNK activation by expression of dominant negative MKPs does not convert IL-1 to a death cytokine, indicating that prolonged JNK activation alone is not sufficient for cell killing and that it needs to act in conjunction with another TNFR1-generated death-promoting signal (H.K. and M.K., unpublished results).

Why does the loss of NF- κ B activity result in ROS accumulation? It has turned out that NF- κ B induces expression of several anti-oxidant genes such as MnSOD, Ferritin heavy chain (FHC), glutathione S-transferase, and metallothionein (88). Overexpression of MnSOD, the mitochondrial isozyme protects cells from TNF α -induced cytotoxicity (9, 89). MnSOD-deficient mice exhibit neonatal lethality due to increased oxidative stress, whereas mice deficient in CuZnSOD, a cytosolic SOD, develop normally (90), indicating an important role for the mitochondrial antioxidant system. Overexpression of FHC also suppresses TNF α -induced PCD along with attenuation of prolonged JNK activation (91). FHC seems to act by accelerating the conversion of H₂O₂ to H₂O. Another interesting observation is that TNF α induces expression of a number of the cytochrome p450 family members, such as Cyb1 that enhances ROS production in NF- κ B-deficient fibroblasts (71). Taken together, NF- κ B protects cells from oxidative stress by activating expression of various antioxidant systems, whose failure enhances TNF α -induced PCD.

The mechanism of TNF α -induced ROS production is still unclear. One possible source of ROS could be the cytosolic phospholipase A2 (cPLA2) (92). However, several lines of evidence suggest that mitochondria are the main source of ROS during TNF α -induced PCD (23). First, treatment with TNF α results in damage and dysfunction of the mitochondrial electron transport chain thereby leading to ROS production (93–95). Second, overexpression of mitochondrial anti-oxidant enzymes, such as MnSOD inhibits TNF α -induced PCD (9, 89). Furthermore, BHA, an antioxidant which accumulates in mitochondria, protects cells from TNF α -induced PCD (9, 74, 94). Third, inhibition of peroxiredoxin III (PrxIII) increases susceptibility to TNF α -mediated cytotoxicity (96). Although the mechanisms of mitochondrial involvement in PCD are diverse and controversial, the involvement of mitochondrial ROS generation in cell death is likely to be part of them (97).

Compared to our understanding of DR-induced caspase activation, the mechanism of DR-induced ROS production is obscure. Furthermore, recent studies suggest that DR induces necrosis by a different manner from apoptosis. Fas activation fails to disrupt mitochondrial membrane potential or induce necrosis in FADD-deficient *T* cells (98). TNF α also fails to induce ROS accumulation and necrosis in FADD-deficient fibroblasts (99). Such results indicate an essential role of FADD in ROS production and programmed necrosis. Another protein involved in ROS production is RIP1. Fas and TNF α -induce ROS accumulation or PCD in RIP1-deficient cells (98, 99). In contrast to the established function of RIP1 as an adaptor molecule in NF- κ B activation by TNFR1, its kinase activity is necessary for Fas-induced necrosis, which mostly occurs in caspase-8-deficient cells (98). Interestingly, inhibition of caspases results in severe damage to mitochondria, increased ROS accumulation and cell death (92, 100, 101). These observations are consistent with results showing that enforced FADD oligomerization results in PCD in the absence of caspase activation (102). However, although DR can induce ROS accumulation without caspase activation in certain cell types, caspase activation can induce mitochondrial damage and ROS accumulation (51, 52, 103–106). Translocation of tBID, which is generated by caspase-8, to mitochondria results in cytochrome *c* release and loss of mitochondrial membrane potential (51, 52). In addition, caspase activation is necessary for rapid loss of mitochondrial membrane potential following cytochrome *c* release (103–105). Recent study identified NDUFS1, the 75 kDa subunit of respiratory complex I, is a critical caspase substrate whose cleavage results in ROS accumulation (106). Thus, caspase-dependent and independent mechanisms might be involved in ROS accumulation.

JNK activation may also enhance ROS accumulation, thereby potentiating TNF α -stimulated necrosis (107). Although the mechanism by which JNK potentiates ROS accumulation is still unclear, a positive feedback loop between ROS accumulation and JNK activation may exist (Fig. 1). Such a loop may also involve caspase activation. Although caspases are not involved in TNF α -induced prolonged JNK activation in NF- κ B-deficient cells (74), caspase-mediated cleavage of upstream MAP3Ks was shown to result in constitutive JNK activation (108). JNK activation also contributes to caspase activation, an effect mediated through enhanced cytochrome *c* release, during UV-induced apoptosis (109). Alternatively, JNK causes caspase activation through jBID formation during TNFR1 signaling (53). Importantly, NF- κ B suppresses all of these amplification loops by induction of different anti-apoptotic proteins including caspase inhibitors and Bcl-2

family members, as well as anti-oxidants (Fig. 1). Interestingly, negative feedback loops exist between NF- κ B and various death promoting proteins. Caspases-mediated cleavage of RelA and IKK β can prevent NF- κ B activation (79, 110). Caspases can also cleave I κ B generating a degradation-resistant NF- κ B inhibitor (111). Oxidation of a cysteine residue in the RHD of RelA prevents its binding to DNA (112), whereas oxidation of another cysteine residue within the cativation loop of IKK β interferes its activation (113). It is unlikely, however, that all of these regulatory loops and modifications take place at the same time and a major challenge for the future is to sort out the events that do take place during different physiological and pathophysiological forms of cell death. As discussed below, it should be possible to take advantage of some of these regulatory loops for designing drugs and therapeutic strategies that enhance the killing of cancer cells. Importantly, there is more than one way to die or to be killed. Fas and TNF α can induce both apoptosis and necrosis and so do anti-cancer drugs. In L929 cells, for instance, TNF α triggers mostly necrosis, whereas Fas can induce necrosis only when apoptotic pathway is suppressed (114). FADD and RIP play central role in controlling the choice between the two death pathways (115). Also of importance is the realization that NF- κ B activation also inhibits programmed necrosis, in addition to its well-established role in the prevention of apoptosis.

NF- κ B INHIBITION AND CANCER THERAPY

The pivotal role of the IKK β -driven NF- κ B pathway in inhibition of PCD, tumor promotion and tumor progression, together with the observation of constitutively activated NF- κ B in a large number of solid and hematopoietic malignancies, strongly suggest that IKK β and/or NF- κ B inhibitors would be useful in cancer therapy. In fact, many non-specific IKK β /NF- κ B inhibitors have already been shown promising results in cancer therapy conducted both in animal models and human tumors. Specific IKK β /NF- κ B inhibitors are also being developed at a rapid pace and some reports have documented their efficacy in triggering apoptosis in cancer cell lines in combination with either death-inducing cytokines or chemotherapeutic drugs (116, 117). An ideal IKK β /NF- κ B inhibitor should be specific, being capable of preventing NF- κ B activation without an effect on other signaling systems. Most likely, such inhibitors will not exhibit cell type specificity, which may be an added bonus, as NF- κ B contributes to cancer development and the neoplastic/invasive phenotype not only within the malignant cell but also in tumor-infiltrating inflammatory cells (118). Although robust inhibition of

NF- κ B is a highly desired property, the ideal inhibitor should be reversible, such that when drug administration is terminated, NF- κ B activity would be restored thereby preventing long-term immune deficiency and susceptibility to infections. Although it is difficult to envision IKK β /NF- κ B inhibitors that will be more active in malignant cells than in normal cells, it should be possible to take advantage of the fact that under normal conditions most cells do not contain activated NF- κ B and only cancer cells exhibit constitutively active NF- κ B. Thus, administration of reversible IKK β /NF- κ B inhibitors may temporarily enhance the death sensitivity of the malignant cell without exerting such an effect in most normal cells.

To avoid severe side effects which IKK β /NF- κ B-specific inhibitors may cause, alternative approaches, based on selective inhibition of anti-PCD targets of NF- κ B which does not affect the expression of genes required for immune responses, should be considered. For example, an important pro-survival factor regulated by NF- κ B is the anti-oxidant enzyme MnSOD or SOD2 (119). Thus, SOD2 inhibitors may compromise one particular function of NF- κ B: the suppression of PCD, while leaving other functions intact. In fact, it has been reported that inhibition of SOD in human leukemia cells caused accumulation of O $_2^-$, which was followed by ROS-mediated damage to mitochondria, the release of cytochrome and apoptosis (120). Given its regulation by NF- κ B, whose activity is elevated in most types of cancer, it is likely that MnSOD expression is higher in malignant cells than in normal cells and therefore the former may be more sensitive to SOD inhibitors, in fact, it was reported that certain oestrogen derivatives, acting as SOD inhibitors, selectively kill human leukaemia cells but not normal lymphocytes (120). In case they are not too potent on their own, the SOD inhibitors could be used as adjuvants with conventional anti-cancer therapies.

CONCLUDING REMARKS

IKK/NF- κ B signaling pathways promote cell survival through inhibiting various components of apoptotic machinery or induction of genes encoding anti-oxidant proteins. Regardless of mechanism, many cancer cells, of either epithelial or hematopoietic origin, use NF- κ B to achieve resistance to anticancer drugs, radiation and death cytokines. Hence, inhibition of IKK-driven NF- κ B activation offers an attractive strategy for treatment of many different malignancies. Certain anti-cancer drugs may work much better with IKK β /NF- κ B inhibitors than others. For instance the combined application of TRAIL or TRAIL-inducers, such as IFNs, with anti-inflammatory

or anti-TNF(therapy alongside with IKK β /NF- κ B inhibitors may result in selective killing of malignant cells not achieved by either agent alone (121). It should be noted, however, that due to the critical role of NF- κ B in the activation of innate and adaptive immune responses there may be a certain amount of risk due to induced immunodeficiency that is likely to be associated with long term use of IKK β /NF- κ B inhibitors. Hence, alternative approaches that selectively inhibit NF- κ B-regulated anti-apoptotic targets, but spare other genes required for immune responses are particularly attractive.

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IKK/NF- κ B signaling: balancing life and death — a new approach to cancer therapy

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IKK kinase/NF- κ B (IKK/NF- κ B) signaling pathways play critical roles in a variety of physiological and pathological processes. One function of NF- κ B is promotion of cell survival through induction of target genes, whose products inhibit components of the apoptotic machinery in normal and cancerous cells. NF- κ B can also prevent programmed necrosis by inducing genes encoding antioxidant proteins. Regardless of mechanism, many cancer cells, of either epithelial or hematopoietic origin, use NF- κ B to achieve resistance to anticancer drugs, radiation, and death cytokines. Hence, inhibition of IKK-driven NF- κ B activation offers a strategy for treatment of different malignancies and can convert inflammation-induced tumor growth to inflammation-induced tumor regression.

NF- κ B proteins and I κ B kinase signaling pathways

The mammalian NF- κ B family contains 5 members: NF- κ B1 (p105 and p50), NF- κ B2 (p100 and p52), c-Rel, RelB, and RelA (p65). These proteins share a Rel homology domain (RHD), which mediates DNA binding, dimerization, and interactions with specific inhibitory factors, the I κ Bs, which retain NF- κ B dimers in the cytoplasm. Many stimuli activate NF- κ B, mostly through I κ B kinase-dependent (IKK-dependent) phosphorylation and subsequent degradation of I κ B proteins. The liberated NF- κ B dimers enter the nucleus, where they regulate transcription of diverse genes encoding cytokines, growth factors, cell adhesion molecules, and pro- and antiapoptotic proteins (1, 2). The IKK complex consists of 2 highly homologous kinase subunits, IKK α and IKK β , and a nonenzymatic regulatory component, IKK γ /NEMO (3).

Two NF- κ B activation pathways exist (Figure 1). The first, the classical pathway, is normally triggered in response to microbial and viral infections or exposure to proinflammatory cytokines that activate the tripartite IKK complex, leading to phosphorylation-induced I κ B degradation. This pathway, which mostly targets p50:RelA and p50:c-Rel dimers, depends mainly on IKK β activity (4). The other pathway, the alternative pathway, leads to selective activation of p52:RelB dimers by inducing processing of the NF- κ B2/p100 precursor protein, which mostly occurs as a heterodimer with RelB in the cytoplasm. This pathway is triggered by certain members of the TNF cytokine family, through selective activation of IKK α homodimers by the upstream kinase NIK (5). Both pathways regulate cell survival and death (6); the classical pathway is responsible for inhibition of programmed cell death (PCD) under most conditions (2, 3). The alternative pathway is important for survival of premature B cells and development of secondary lymphoid organs (7). The antiapoptotic activity of the IKK β -driven classical pathway is important for various immu-

noreceptors, including T and B cell receptors, TLR4, and type 1 TNF- α receptor (TNFR1), all of which generate pro-survival and pro-death signals upon ligation (8, 9). Under most circumstances, the survival signals dominate, but under conditions where IKK β or NF- κ B activities have been compromised, receptor activation results in cell death (10–12).

The survival function of NF- κ B: mechanisms and mediators

Pathways of cell death. PCD can be either apoptotic or necrotic. Apoptosis is characterized by membrane blebbing, shrinking, and condensation of the cell and its organelles (13, 14). Two well-established pathways lead to apoptosis: the death receptor (DR) (extrinsic) pathway and the mitochondrial (intrinsic) pathway (15). Both pathways depend on cysteine proteases called caspases (15, 16). However, apoptosis-like PCD can sometimes proceed without caspase activation (17, 18). Furthermore, caspase activation does not always lead to cell death (19), and caspase-8 also has pro-survival functions (20, 21). Necrosis is characterized by swelling of the cell and its organelles, culminating in membrane disruption and cell lysis, often accompanied by inflammation. Failure of energy metabolism and massive generation of ROS are each thought to cause necrosis (22).

NF- κ B suppresses both PCD types, although initially it was thought to antagonize only apoptosis. The first clear evidence for NF- κ B as a PCD inhibitor was provided by RelA knockout mice that die mid-gestation by massive liver apoptosis (23). The role of NF- κ B in embryonic liver survival, brought about by inhibition of TNFR1-mediated apoptosis (24), is underscored by the very similar phenotypes of mice lacking IKK β (4, 25) or IKK γ (26). A protective role for NF- κ B in adult liver was confirmed in mouse models of liver damage (10, 27, 28) and involves inhibition of both apoptosis and necrosis (9). We will discuss the various mechanisms by which NF- κ B suppresses PCD (Figure 2).

NF- κ B and caspases. There are 2 groups of DRs, based on their signaling complexes. The first group comprises Fas, DR4, and DR5, which directly recruit the death domain-containing (DD-containing) adaptor Fas-associated death domain (FADD), procaspase-8, procaspase-10, and the cellular FLICE-inhibitory protein (FLIP) to form death-inducing signaling complexes (DISCs) (29). The second group comprises TNFR1, DR3, DR6, and ectodysplasin A receptor (EDAR). TNFR1 forms a signaling complex

Nonstandard abbreviations used: ATO, arsenic trioxide; DD, death domain; DISC, death-inducing signaling complex; DR, death receptor; FADD, Fas-associated death domain; FHC, ferritin heavy chain; FLIP, FLICE-inhibitory protein; IKK, I κ B kinase; MKP, MAPK phosphatase; MnSOD, manganese superoxide dismutase; PCD, programmed cell death; RHD, Rel homology domain; RIP, receptor-interacting protein; SOD, superoxide dismutase; TNFR1, type 1 TNF- α receptor; TRAF, TNFR-associated factor; XIAP, X chromosome-linked inhibitor of apoptosis.

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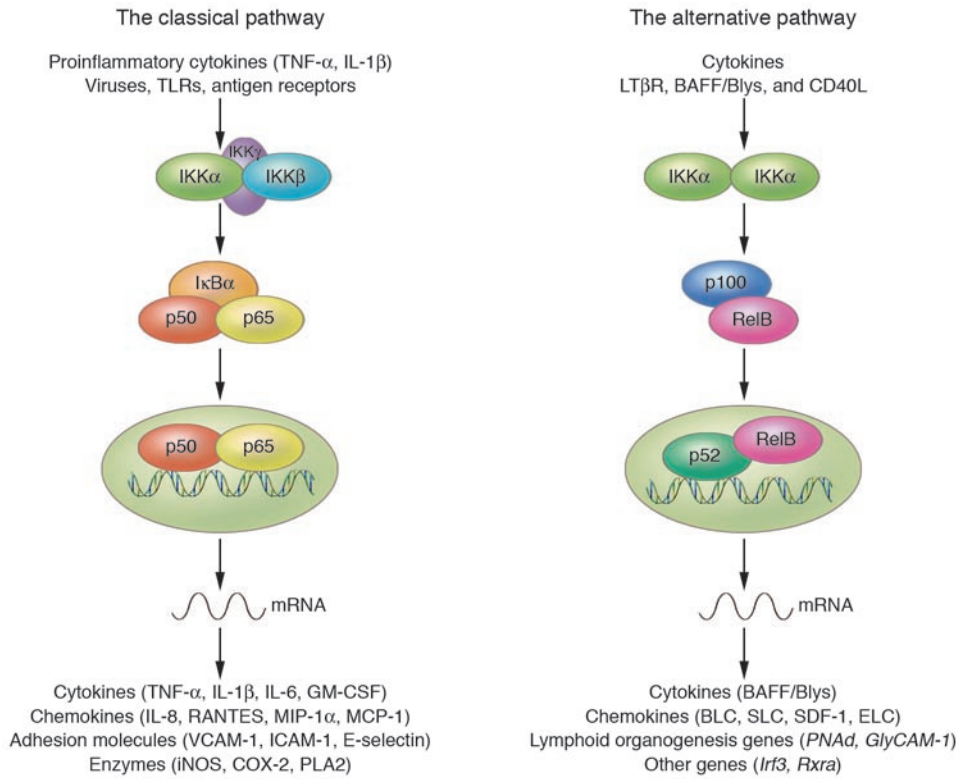


Figure 1

IKK/NF- κ B signaling pathways. The classical pathway is activated by a variety of inflammatory signals, resulting in coordinate expression of multiple inflammatory and innate immune genes. The alternative pathway is strictly dependent on IKK α homodimers and is activated by lymphotoxin β receptor (LT β R), B cell-activating factor belonging to the TNF family (BAFF), and CD40 ligand (CD40L). The alternative pathway plays a central role in the expression of genes involved in development and maintenance of secondary lymphoid organs. BLC, B lymphocyte chemoattractant; ELC, Epstein-Barr virus-induced molecule 1 ligand CC chemokine; MCP-1, monocyte chemoattractant protein-1; MIP-1 α , macrophage inflammatory protein-1 α ; PLA2, phospholipase A2; SDF-1, stromal cell-derived factor-1 α ; SLC, secondary lymphoid tissue chemokine.

(complex I) at the plasma membrane by recruiting the adaptor TNFR1-associated DD protein (TRADD) and the signaling proteins TNFR-associated factor 2 (TRAF2), TRAF5, and receptor-interacting protein 1 (RIP1). After assembly, complex I dissociates from TNFR1, which can then recruit FADD and caspase-8 and trigger an apoptotic response (30).

NF- κ B as a transcription factor induces genes whose products prevent PCD. An elicitor of NF- κ B activation is TNF- α , which is a rather poor inducer of PCD. TNF- α triggers PCD only when new protein or RNA synthesis is inhibited or in NF- κ B-deficient cells. NF- κ B exerts its pro-survival activity through several anti-apoptotic proteins, including FLIP, Bcl-X_L, A1/Bfl-1, cellular inhibitor of apoptosis (c-IAP), X chromosome-linked inhibitor of apoptosis (XIAP), TRAF1, and TRAF2 (2, 31). FLIP inhibits apoptosis by interfering with caspase-8 activation (30). c-IAP and XIAP directly bind and inhibit effector caspases, acting downstream of initiator caspases.

NF- κ B and Bcl-2 family members. NF- κ B induces expression of several Bcl-2 family members, most notably Bcl-X_L and A1/Bfl-1, which prevent apoptosis by inhibiting permeability transition and depolarization of mitochondria, and cytochrome *c* release (2, 31). DRs can trigger apoptosis through different pathways (32). In certain cells, activated caspase-8 directly activates effector caspases, while in cells with poor DISC formation, death signaling requires an additional amplification loop, based on caspase-8-mediated Bid cleavage and generation of truncated tBid that triggers cytochrome *c* release (33, 34) and activation of caspase-9 and caspase-3 (15). This type of DR signaling can be blocked by antiapoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L (15).

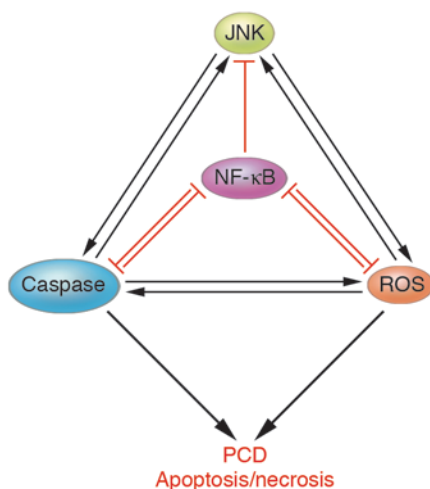
ROS and the NF- κ B-JNK cross-talk. The role of JNK in PCD has been controversial, because it has both survival and death-enhancing effects. The clearest evidence for JNK as regulator of PCD

comes from analysis of knockout mice: JNK1- or JNK2-deficient mice are relatively resistant to induction of fulminant hepatitis in response to concanavalin A, a pathology that depends on activation of TNFR1 and other DRs (10).

The ratio between JNK and NF- κ B activities controls cell survival or death, not only in response to TNFR1 but also in response to other death stimuli (35–37). Whereas TNF- α leads to transient JNK activation in WT cells, it leads to prolonged JNK activation in cells that cannot activate NF- κ B (9, 38–40). The pro-survival activity of NF- κ B depends on this ability to prevent prolonged JNK activation (9, 38–40). Prolonged JNK activation following concanavalin A administration was also seen in mice lacking IKK β in liver cells, resulting in massive TNFR1-dependent hepatocyte death (10). In the liver, however, TNFR1 and JNK signaling is also required for regeneration or compensatory hepatocyte proliferation following partial hepatectomy or chemically induced injury (41, 42). Thus, NF- κ B may be a critical regulator of cell survival and death through its ability to control the duration of JNK activation (Figure 2).

Prolonged JNK activation in NF- κ B-deficient cells implies that NF- κ B induces expression of JNK inhibitors. Such a function was proposed for GADD45 β (43) and XIAP (39). However, analysis of GADD45 β - or XIAP-deficient fibroblasts failed to reveal changes in the kinetics of JNK activation (31, 44), suggesting that NF- κ B regulates JNK activation through a different mediator.

ROS are likely the mediators (40). ROS, including H₂O₂, O₂⁻, and HO[•] radicals, are generated through many enzymatic pathways, but their major source is leakage of electrons from the mitochondrial respiratory chain (45). ROS activate kinases through oxidation of kinase-interacting molecules. For instance, ROS activate tyrosine kinases by inactivating protein tyrosine phosphatases through oxidation of a highly reactive cysteine residue at their

**Figure 2**

Control of cell survival and death through NF- κ B–JNK cross-talk. Positive feedback loops exist between ROS and caspases, caspases and JNK, and JNK and ROS. Negative feedback loops exist between NF- κ B and caspases, and NF- κ B and ROS. NF- κ B functions as a pro-survival transcription factor by inducing the expression of antiapoptotic genes, such as the Bcl-2 family members and caspase inhibitors, and antioxidant genes, such as MnSOD and FHC. Activation of NF- κ B also results in inhibition of prolonged JNK activation, mostly through inhibition of ROS accumulation. Inhibition of NF- κ B enhances PCD, which can be either apoptotic or necrotic, by removing the negative feedback loops.

catalytic site (46–49). Similarly, ROS mediate the NF- κ B–JNK cross-talk through their ability to inactivate various MAPK phosphatases (MKPs) involved in JNK inactivation (45).

TNF- α induces ROS accumulation in many cell types, and these ROS are important mediators of PCD (22). TNF- α -induced ROS accumulation is seen in NF- κ B-deficient cells, but not in NF- κ B-competent cells (9, 40). Treatment of cells with the antioxidant butylated hydroxyanisole (BHA) has no effect on transient JNK activation triggered by TNF- α , but it suppresses prolonged JNK activation and PCD in TNF- α -treated NF- κ B-deficient cells (9, 40). This protective effect is due to BHA's ability to prevent oxidation of MKPs, ensuring transient JNK activation (9). Expression of dominant-negative mutants of MKPs leads to prolonged JNK activation and allows killing by TNF- α of NF- κ B-competent cells, which otherwise are TNF- α -resistant (9).

The loss of NF- κ B activity results in ROS accumulation because NF- κ B induces expression of several antioxidant genes such as manganese superoxide dismutase (MnSOD), ferritin heavy chain (FHC), glutathione-S-transferase, and metallothionein (50). Overexpression of mitochondrial MnSOD protects cells from TNF- α -induced cytotoxicity (9, 51). Overexpression of FHC also suppresses TNF- α -induced PCD along with attenuation of prolonged JNK activation (52). Another interesting observation is that TNF- α induces expression of a number of cytochrome p450 family members, such as CYP1B1, that enhance ROS production in NF- κ B-deficient fibroblasts (37). Taken together, these findings show that NF- κ B protects cells from oxidative stress by activating expression of various antioxidant systems, whose failure enhances TNF- α -induced PCD.

The mechanism of TNF- α -induced ROS production is unclear. One possible source of ROS is the cytosolic phospholipase A2 (53). However, several lines of evidence suggest that mitochon-

dria are the main source of ROS during TNF- α -induced PCD (22). Compared with our understanding of DR-induced caspase activation, the mechanism of DR-induced ROS production is obscure. TNF- α does not induce ROS accumulation and programmed necrosis in FADD- or RIP1-deficient cells, indicating essential roles for FADD and RIP1 (54). In contrast to the established function of RIP1 as an adaptor molecule in NF- κ B activation, its kinase activity is necessary for Fas-induced necrosis, which mostly occurs in caspase-8-deficient cells (55). Interestingly, inhibition of caspases potentiates ROS accumulation and cell death (53, 56). Although DRs can induce ROS accumulation without caspase activation in certain cell types, caspase activation can also lead to mitochondrial damage and ROS accumulation (33, 34, 57–59). Thus, caspase-dependent and -independent mechanisms might be involved in ROS accumulation.

JNK activation may also enhance ROS accumulation, potentiating TNF- α -stimulated necrosis (60). Although the mechanism by which JNK potentiates ROS accumulation is unclear, a positive feedback loop between ROS accumulation and JNK activation may exist (Figure 2). Such a loop may also involve caspase activation. Although caspases are not involved in TNF- α -induced prolonged JNK activation in NF- κ B-deficient cells (40), caspase-mediated cleavage of upstream MAP3Ks may cause constitutive JNK activation (61). JNK activation also contributes to caspase activation, an effect mediated through enhanced cytochrome *c* release, during UV-induced apoptosis (62). Alternatively, JNK causes caspase activation through jBid formation during TNFR1 signaling (63). Importantly, NF- κ B suppresses all of these amplification loops by inducing expression of caspase inhibitors, Bcl-2 family members, and antioxidants (Figure 2). Interestingly, negative feedback loops exist between NF- κ B and various death-promoting proteins. Caspase-mediated cleavage of RelA and IKK β can prevent NF- κ B activation (64, 65). Caspases can also cleave I κ B to generate a degradation-resistant NF- κ B inhibitor (66). Oxidation of a cysteine residue in the RHD of RelA prevents its binding to DNA (67), whereas oxidation of another cysteine within the activation loop of IKK β interferes with its activation (68). It is unlikely, however, that all of these regulatory loops and modifications take place simultaneously, and a major challenge for the future is to sort out the events that do take place during different physiological and pathophysiological conditions. It is possible to use some of these regulatory loops in designing drugs and therapeutic strategies to kill cancer cells. Fas and TNF- α can induce both apoptosis and necrosis, and so do anticancer drugs. In L929 cells, for instance, TNF- α triggers mostly necrosis, whereas Fas can induce necrosis only when the apoptotic pathway is suppressed (69). FADD and RIP play central roles in controlling the choice between the 2 death pathways (70). NF- κ B activation also inhibits programmed necrosis, in addition to its role in prevention of apoptosis.

Proapoptotic functions of NF- κ B?

NF- κ B may induce apoptosis in a cell type- and stimulus-dependent manner. Most commonly, NF- κ B activation inhibits PCD, as evidenced by several knockout mouse models (4, 23, 26, 71). However, under certain circumstances activation of NF- κ B may promote cell death. For instance, NF- κ B may mediate doxorubicin-induced cell death in N-type neuroblastoma cells (72). NF- κ B is also required for anti-CD3-induced apoptosis of double-positive thymocytes (73). Apoptosis in HL-60 cells induced by etoposide or



1- β -D-arabinofuranosylcytosine correlates with NF- κ B activation (74). Human melanoma cells were protected from UV-induced apoptosis by NF- κ B downregulation (75). More recently, it was reported that NF- κ B induced by UV light or daunorubicin/doxorubicin is functionally distinct from the response elicited by TNF- α , and under such conditions NF- κ B may become a repressor of antiapoptotic genes (76). Furthermore, UV light and daunorubicin inhibit TNF- α -induced NF- κ B transcriptional activity, which is antiapoptotic, by enhancing association of RelA with histone deacetylases (76). These results suggest that NF- κ B may mediate apoptosis under certain conditions. However, the pathophysiological relevance of these observations is not clear, and it remains to be demonstrated that NF- κ B has proapoptotic functions *in vivo*. It appears that those agents or stimuli that were reported to induce apoptosis by activating NF- κ B are neither strong nor typical NF- κ B activators, as opposed to TNF- α , IL-1, or LPS, and that they also activate another signaling pathway(s), which may be more relevant to cell killing than NF- κ B.

Tumor suppressors interact with NF- κ B pathway. Suppression of cell proliferation, induction of premature senescence, and/or induction of apoptosis are some mechanisms through which tumor suppressors inhibit cancer development. In general, NF- κ B acts antagonistically to tumor suppressors, based on its ability to promote cell survival, inhibit PCD, and enhance cell proliferation (77). However, in some cases NF- κ B may collaborate with, rather than antagonize, certain tumor suppressors.

Although p53 stabilization decreases upon NF- κ B activation (78), under special circumstances apoptosis induced by p53 may involve activation of NF- κ B (79). Similar to situations in which NF- κ B activation promotes apoptosis, NF- κ B induction by p53 does not involve classical IKK activation and I κ B degradation. Instead, p53 may stimulate the serine/threonine kinase ribosomal S6 kinase 1 (RSK1), which in turn phosphorylates RelA (80). The lower affinity of RSK1-phosphorylated RelA for I κ B α decreases I κ B α -mediated nuclear export, prolonging RelA nuclear residence (80). NF- κ B also plays an essential role in activation of p53 to initiate proapoptotic signaling in response to ROS accumulation. Consequently, NF- κ B-dependent p53 activity induces p53-regulated genes, such as Puma and p21^{waf1} (81). However, a more common observation, seen *in vivo*, is that NF- κ B activation counteracts p53-induced apoptosis by destabilizing p53, perhaps through enhanced Mdm2 expression (78, 82).

Another tumor suppressor, BRCA1, can bind RelA to serve as a coactivator (83). Treatment of 293T cells with TNF- α induces an interaction between endogenous RelA and BRCA1, mediated by the RHD of RelA and the N-terminal region of BRCA1. Forced BRCA1 expression significantly enhances the ability of TNF- α or IL-1 β to induce NF- κ B target genes, and inhibition of NF- κ B by the chemical inhibitor SN-50 blocks this effect (83). Nonetheless, it remains to be seen whether any of these responses documented *in vitro* occurs *in vivo*.

NF- κ B and proapoptotic genes. NF- κ B has been implicated as a transcriptional activator of some proapoptotic genes, such as Fas/CD95 (84), FasL (85), DR4, and DR5 (86). FasL is expressed in activated T cells and represents a major cytotoxic effector through which T cells kill their targets. FasL expression is under the stringent control of various transcription factors, including NF- κ B (85). Recently, it was reported that certain types of cancer cells also express FasL, which may contribute to their ability to escape immune surveillance and resist immunotherapy. Overexpression

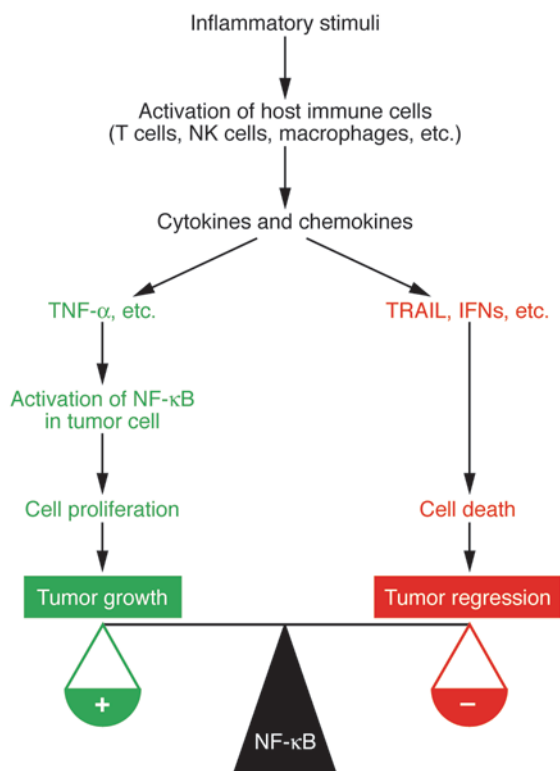
of the Myc family member Max in non-small cell lung cancer cell lines markedly increases basal FasL promoter activity and enhances NF- κ B-mediated FasL induction. Thus, high levels of Max and stress-induced NF- κ B activation may elevate FasL expression in human lung cancer cells (85). TNF- α combined with IFN- α accelerates NF- κ B-mediated apoptosis by enhancing Fas expression in human colon adenocarcinoma RPMI4788 cells (84). However, there may be another explanation for these results, as type I IFNs and related cytokines, such as IL-10, may actually function as NF- κ B inhibitors (87). Another TNF family member, TRAIL, triggers apoptosis through engagement of DR4 and DR5. The c-Rel subunit of NF- κ B induces expression of both receptors, while a degradation-resistant mutant of I κ B α (I κ B super-repressor) or a transactivation-deficient mutant of c-Rel reduces DR expression (86). However, NF- κ B was shown to be a major impediment to TRAIL-mediated tumor killing (88).

IKK/NF- κ B and cancer

IKK/NF- κ B links inflammation to cancer. Based on many functions of NF- κ B target genes, a close relationship between NF- κ B and cancer was proposed (89) and recently reviewed (89–94). The association of NF- κ B activation with inflammation-associated tumor promotion, progression, and metastasis is well documented and was demonstrated in several mouse models (88, 95, 96). The IKK β -dependent NF- κ B activation pathway is a critical molecular link between inflammation and colon cancer in a mouse model (95). Activation of IKK β in enterocytes, which give rise to the malignant component of this tumor, suppresses apoptosis of preneoplastic cells, whereas its activation in myeloid cells promotes production of various cytokines that serve as growth factors for the transformed enterocytes. Inhibition of 1 of these factors, IL-6, interferes with tumor growth but has no effect on tumor cell survival (97). Conversely, inactivation of IKK β in enterocytes results in a dramatic decrease in tumor number due to increased apoptosis but has no effect on proliferation of transformed enterocytes or tumor growth (95).

The role of NF- κ B in inflammation-associated cancer was also demonstrated in Mdr2-deficient mice, which develop cholestatic hepatitis followed by hepatocellular carcinoma (96). In this model, the inflammatory process triggered chronic activation of NF- κ B in hepatocytes, most likely through enhanced production of TNF- α by adjacent endothelial and inflammatory cells. Switching NF- κ B off in Mdr2^{-/-} mice from birth to 7 months of age had no effect on the course of hepatitis or early phases of tumorigenesis (96). By contrast, suppressing chronic NF- κ B activation at later stages resulted in the apoptotic death of transformed hepatocytes and failure to progress to hepatocellular carcinoma (96).

NF- κ B activation also plays a critical role in inflammation-driven tumor progression as demonstrated in a syngeneic colon and mammary cancer xenograft mouse model (88). Cancer cells in this model were introduced into syngeneic immunocompetent mice to form metastatic growths in the lungs. Once the metastases were established, the mice were given a sublethal dose of LPS to elicit systemic inflammation, which stimulated tumor growth. Remarkably, inhibition of NF- κ B in cancer cells converted LPS-induced tumor growth to LPS-induced tumor regression without affecting the ability of the cancer cells to migrate to the lung and establish metastatic growths (88). Further investigation revealed that inflammation-induced tumor growth in this model was mediated by TNF- α produced by host immune cells, whereas LPS-induced



regression of NF- κ B-deficient tumors was mediated by TRAIL, whose synthesis is induced by IFNs in host inflammatory cells in response to LPS-mediated activation of TLR4 (Figure 3). These results indicate that NF- κ B is a major mediator of inflammation-induced tumor progression through overcoming the potential tumor-killing by TRAIL induction (88). Given that NF- κ B activation in cancer cells may be a major hindrance to TRAIL-induced apoptosis, NF- κ B or IKK inhibitors may potentiate the activity of either recombinant TRAIL or TRAIL inducers, such as type I and type II IFNs, to achieve enhanced tumor killing (Figure 3).

Whereas the role of NF- κ B in inflammation-induced tumor promotion, growth, and progression is becoming clear (88, 95, 96), its role in tumor initiation is still ambiguous (98–101). As NF- κ B regulates a large group of genes that have different functions, some of which display cell-type specificity, NF- κ B may have distinct roles in different cell types. For instance, in normal epidermal keratinocytes, NF- κ B proteins are present in the cytoplasm of basal cells but are nuclear in more differentiated suprabasal cells, suggesting that NF- κ B activation is linked to growth arrest (98, 102). Indeed, inhibition of NF- κ B signaling in the murine epidermis results in an increased apoptosis, hyperproliferation of surviving cells, and spontaneous development of squamous cell carcinomas (99, 103). Correspondingly, application of a pharmacological NF- κ B inhibitor to mouse skin induced epidermal hyperplasia (98). In contrast, overexpression of active NF- κ B subunits in transgenic epithelium produced hypoplasia and growth inhibition (98). Contrary to the requirement of an intact IKK/NF- κ B pathway for H-ras-mediated fibroblast transformation (104), NF- κ B inhibition synergized with oncogenic H-ras to induce transformation of primary human keratinocytes (100). Congruously, activation of NF- κ B in normal human epidermal keratinocytes triggered cell-cycle arrest (100). These results suggest that the IKK β -dependent NF- κ B pathway

Figure 3

Inhibition of NF- κ B in cancer cells converts inflammation-induced tumor growth to tumor regression. Activation of the innate and adaptive immune system can have profound influence on tumor growth and development. In addition to its role in activation of immune cells, NF- κ B within the malignant cell is a major modulator of the tumor response to inflammation. Activation of NF- κ B promotes tumor growth and confers resistance to death cytokines, such as TRAIL. Conversely, inhibition of NF- κ B prevents inflammation-stimulated tumor growth and enhances inflammation-induced tumor regression mediated by TRAIL.

in epidermal keratinocytes promotes keratinocyte growth arrest and differentiation to maintain the barrier function of the epidermis, whose perturbation may result in severe inflammation (105). Interestingly, formation of mouse squamous cell carcinomas in response to a chemical carcinogen (106) and following inhibition of NF- κ B (107) is dependent on TNF- α . Similar observations were recently made in a mouse model of chemically induced hepatocellular carcinoma, where deletion of IKK β in hepatocytes promoted tumor development by enhancing compensatory proliferation, whereas an additional deletion of IKK β in liver myeloid cells prevented tumor development by depriving the transformed hepatocytes of essential growth factors (108).

NF- κ B inhibitors in cancer therapy. The pivotal role of the IKK β /NF- κ B signaling pathway in inhibition of PCD, tumor promotion, and tumor progression, together with the occurrence of constitutively activated NF- κ B in various solid and hematopoietic malignancies, strongly suggests that IKK β and/or NF- κ B inhibitors would be useful in cancer therapy. In fact, much effort is currently invested in developing various IKK β and/or NF- κ B inhibitors and testing their efficacy in both animal models and human cancer (109, 110). Many inhibitors currently available are not specific for either IKK β or NF- κ B. These include antiinflammatory agents such as sulfasalazine and trans-resveratrol, NSAIDs such as aspirin and sulindac sulfide, cyclopentenone prostaglandins, proteasome inhibitors, and glucocorticoids (90, 109–112). However, specific IKK β inhibitors are being developed, and a few publications have documented their efficacy in triggering apoptosis in cancer cell lines in combination with either death-inducing cytokines or chemotherapeutic drugs (113–115).

Even nonspecific IKK β /NF- κ B inhibitors may be effective when used as adjuvants with conventional anticancer treatments. As many signaling pathways may be simultaneously activated and/or inactivated in a given malignant cell, collectively contributing to its neoplastic phenotype, nonspecific IKK β /NF- κ B inhibitors may affect several signaling pathways at once and lead to much more effective killing of such cells. The anticancer drug arsenic trioxide (ATO), which is useful for treating promyelocytic leukemia (116) and possibly multiple myeloma (117), is a noteworthy example. ATO is not a specific inhibitor for IKK β or NF- κ B and may have several molecular targets, since it was found that trivalent arsenicals, a chemical class to which ATO belongs, are potent JNK activators (118) as well as IKK β inhibitors (68). JNK activation in this case is mostly due to the ability of trivalent arsenicals to directly interact with the catalytic cysteine of JNK phosphatases, whereas in the case of IKK β the target is the aforementioned reactive cysteine within the activation loop. An additional effect of ATO on JNK activity may be due to NF- κ B inhibition and accumulation of ROS (35). Thus, by inhibiting IKK and activating JNK, ATO may trigger apoptosis in many different types of cancers. Since NF- κ B inhibi-



tion usually does not result in spontaneous apoptosis, it is unlikely that even specific IKK β /NF- κ B inhibitors would be functional as monotherapeutic agents in most cancers. Indeed, using Jurkat cells as a model, the IKK β inhibitor AS602868 was not cytotoxic on its own but strongly potentiated killing by TNF- α (113). Based on our analysis of knockout mice and tumor models, we predict that IKK β /NF- κ B inhibitors will be useful adjuvants for conventional chemotherapeutic drugs, ionizing radiation, or tumoricidal cytokines, such as IFNs or TRAIL (Figure 3).

NF- κ B regulates PCD through a cross-talk with JNK, ROS, and caspases, and an important pro-survival factor regulated by NF- κ B is the antioxidant enzyme MnSOD (9, 51). Thus, MnSOD2 inhibitors may target a particular NF- κ B function, the suppression of ROS production and PCD, while leaving other functions, such as innate immunity, intact. In fact, inhibition of superoxide dismutase (SOD) in human leukemia cells caused accumulation of O $_2^-$, which was followed by ROS-mediated mitochondrial damage, cytochrome c release, and apoptosis (119). Given its regulation by NF- κ B, whose activity is elevated in most types of cancer, it is likely that MnSOD expression is higher in malignant cells than in normal cells, and therefore the former may be more sensitive to SOD inhibitors. In fact, certain estrogen derivatives, acting as SOD inhibitors, selectively kill human leukemia cells but not normal lymphocytes (119). In case such compounds are not sufficiently potent on their own, they need to be tested as adjuvants for more conventional chemotherapeutic and radiotherapeutic approaches.

Concluding remarks

The inhibition of IKK β /NF- κ B appears to be a promising strategy for cancer therapy when combined with established cytotoxic drugs, death cytokines, or therapeutic radiation. Certain anti-

cancer drugs may work much better with IKK β /NF- κ B inhibitors than others. For instance, the combined application of TRAIL or TRAIL inducers, such as IFNs (Figure 3), with anti-inflammatory or anti-TNF- α therapy alongside IKK β /NF- κ B inhibitors may result in selective killing of malignant cells not achieved by either agent alone (88). An important advantage of IKK β /NF- κ B inhibitors over conventional therapeutics is their ability to block NF- κ B activation also in infiltrating inflammatory cells, which are an important source of tumor growth and survival factors. It should be noted, however, that, given the critical role of NF- κ B in innate and adaptive immune responses, there may be a certain amount of risk due to induced immunodeficiency caused by long-term use of IKK β /NF- κ B inhibitors. Hence, alternative approaches should be considered. For instance, an approach based on selective inhibition of antiapoptotic targets of NF- κ B, without affecting target genes required for immune responses, would be particularly attractive.

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