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Review

Signals from within: the DNA-damage-induced NF-κB response

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Abstract

An appropriate response to genotoxic stress is essential for maintenance of genome stability and avoiding the passage to neoplasia. Nuclear factor κB (NF-κB) is activated as part of the DNA damage response and is thought to orchestrate a cell survival pathway, which, together with the activation of cell cycle checkpoints and DNA repair, allows the cell in cases of limited damage to restore a normal life cycle, unharmed. In this respect, NF-κB is one of the main factors accounting for chemotherapy resistance and as such impedes effective cancer treatment, representing an important drug target. Despite this high clinical relevance, signalling cascades leading to DNA damage-induced NF-κB activation are poorly understood and the use of highly divergent experimental set-ups in the past led to many controversies in the field. Therefore, in this review, we will try to summarize the current knowledge of distinct DNA damage-induced NF-κB signalling pathways.

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Keywords: NF-κB; DNA damage; signalling

Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ATM-related kinase; BAFF, B-cell-activating factor of the TNF family; CK2, casein kinase 2; CPT, camptothecin; DD, death domain; DNA-PK, DNA-protein kinase; DSB, double-strand break; HDAC, histone deacetylase; HED-ID, hypohydrotic ectodermal dysplasia with severe immunodeficiency; FAT, Frap, ATM and Trapp; IκB, inhibitor of κB; IKK, IκB kinase; IR, γ-irradiation; LRR, leucine-rich repeat; LT, lymphotoxin; NEMO, NF-κB essential modifier; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; NLS, nuclear localization signal; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; RHD, Rel homology domain; RSK1, ribosomal S6 kinase 1; TAD, transcriptional activation domain; TNF, tumour necrosis factor; UV, ultraviolet

Canonical, Alternative and Atypical Pathways: The Roads to NF-κB

Active nuclear factor κB (NF-κB) transcription factors are composed of dimeric combinations of members of the Rel transcription factor family (for reviews, see Rothwarf and Karin[1] and Hayden and Ghosh[2]). In mammals, five different Rel family members have been identified: RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100), which can form hetero- or homodimers. Heterodimers of RelA/p65 and p50 are the most abundant in most cells, and the term NF-κB is often used to refer to this complex. All Rel family members are characterized by the presence of a 300-amino-acid long N-terminal stretch, called the Rel homology domain (RHD), which is responsible for DNA binding, dimerization and interaction with inhibitor of κB (IκB) proteins. Interaction with IκB masks the nuclear localization signal (NLS) present in the RHD and sequesters NF-κB in an inactive form in the cytoplasm. In addition, RelA/p65, RelB and c-Rel also possess a transcriptional activation domain (TAD), rendering NF-κB a potent transcription factor. p50 and p52 do not have similar domains and therefore homodimers of these proteins can repress transcription of NF-κB-dependent target genes.

The NF-κB/Rel family of transcription factors is involved in diverse processes such as immunity, inflammation, cell proliferation, apoptosis or embryonal development (for details, see elsewhere in this journal). Therefore, it is not surprising that the list of known NF-κB activators is long and comprises both cytokines or bacteria and viruses as well as oxidative, genotoxic, physiological or chemical stress factors.[3]

Research from the past 20 years revealed that three major NF-κB activating pathways can be distinguished.[2,4,5]

In the first canonical pathway (Figure 1), the prototypical NF-κB dimer RelA/p50 is activated upon release from its inhibitor IκBα through action of the classical IκB kinase (IKK) complex. Signal-induced activation of the IKK signalosome, consisting of two kinases (IKKα and IKKβ) and a regulatory scaffolding subunit NF-κB essential modifier (NEMO), results in phosphorylation, ubiquitination and degradation of IκBα, exposing the NLS of NF-κB, leading to its nuclear accumulation. This pathway is typically activated by proinflammatory cytokines or pathogen-associated molecular patterns (PAMPs), inducing a rapid and strong NF-κB-activating signal, needed for a fast response in acute stress situations, such as viral infection.

A second pathway, described more recently, is called the alternative pathway and relies on the activation of an alternative IKK signalosome complex consisting exclusively of IKKα homodimers (although additional compounds might be identified in the future) (Figure 1). Intriguingly, in this case, IKKα functions completely independently of IKKβ and NEMO.

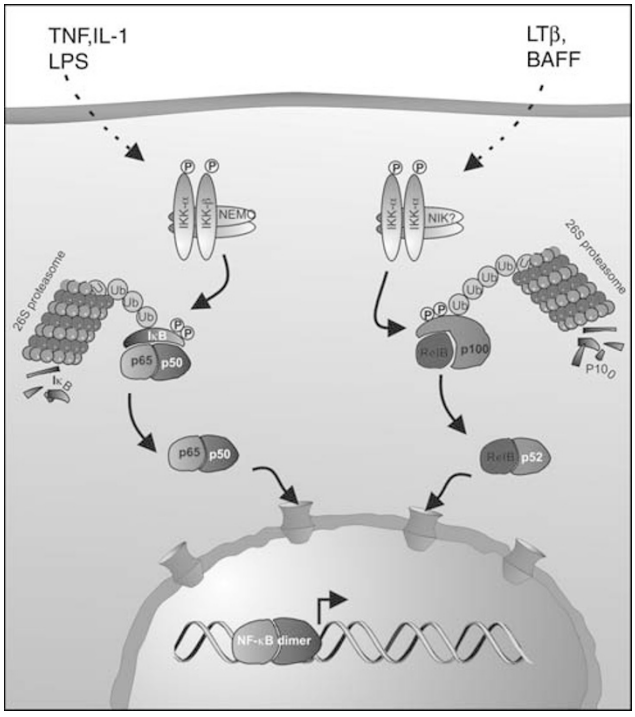

Figure 1 Overview of canonical and alternative NF-κB-inducing signalling pathways. Left: the classical pathway, triggered by cytokines such as IL-1 or TNF and products of bacterial or viral origin, requires the presence of IKKβ and NEMO and leads to phosphorylation of IκBα, liberating an active p65 : p50 heterodimer. Right: the alternative pathway, triggered by particular members of the TNFR superfamily, such as LT β or BAFF, involves IKKα and NIK and results in phosphorylation and processing of p100, generating a functional RelB : p52 heterodimer

The alternative pathway is predominantly active in B-cells and is induced by a particular subset of TNFR family ligands, such as B-cell-activating factor of the tumour necrosis factor (TNF) family (BAFF), CD40 or lymphotoxin (LT) β. Upon receptor triggering, adaptor proteins from the TRAF family enable the recruitment of NF-κB-inducing kinase (NIK), a kinase implicated in the phosphorylation of IKKα. IKKα then targets p100, an additional NF-κB/Rel family member, for phosphorylation and ubiquitination, leading to the limited proteolysis of its ankyrin-like C-terminus, liberating a p52 subunit that forms a functional heterodimer with RelB, driving transcription of a specific subset of target genes.

Finally, the third so-called 'atypical pathways' collectively refer to those pathways that do not fall in the abovementioned categories. Originally, all signalling cascades activated by atypical stimuli, such as DNA damage or oxygen stress, were classified as 'atypical' NF-κB activators, as they all induce a slow and weak NF-κB signal (with peak activities reached after 2–4 h). Later studies revealed that these stimuli cannot be categorized in one class, but induce completely unrelated pathways. Thus, ultraviolet (UV)-induced NF-κB signalling appears to occur in an IKK-independent way, while most other genotoxic stress agents activate a pathway that follows more or less the classical NF-κB activation scheme. No consensus has been reached as to how oxygen radicals induce NF-κB activation, but most likely this involves tyrosine phosphorylation of IκBα. In the following part, these atypical pathways will be dissected into more detail. For oxidative stress-induced NF-κB activation, we refer to another review.[6]

More than One Way to Induce Damage by DNA Damage-Inducing Agents: Which Signal Leads to NF-κB Activation?

The cellular perturbations induced by genotoxic stress are manifold and therefore different stresses could lie at the basis of the subsequent NF-κB response. For example, the reported effects of doxorubicin among others include free radical formation, lipid peroxidation, intercalation into DNA with consequent inhibition of macromolecular synthesis or initiation of DNA damage via the inhibition of topoisomerase II.[7]

Several authors argued that mainly cytoplasmic effects such as the production of oxygen radicals or sphingomyelin breakdown products like ceramide cause DNA damage-induced NF-κB activation, while the induction of DNA breaks would be an unrelated event. These studies are mainly based on the use of antioxidants such as PDTC or NAC as inhibitors of the DNA damage-induced NF-κB response (e.g., Simon *et al.*,[8] Mohan and Meltz,[9] Kaltschmidt *et al.*,[10] Boland *et al.*,[11] Bian *et al.*[12] and Wang *et al.*[13]) and on the argument that NF-κB has been described as an oxygen stress-responsive factor (although the latter has been questioned lately[14]). However, there are at least as many reports showing the opposite result (e.g., Li *et al.*[15] and Hellin *et al.*[16]), and it is becoming more and more apparent that the inhibitory effects of PDTC or NAC are not always related to their antioxidant properties.[14,17] Furthermore, camptothecin (CPT) induces NF-κB activation without being able to generate free radicals in a cell,[18] strongly suggesting that other mechanisms must be operational as well.

In the case of UV irradiation, enucleation experiments showed that the nucleus is not needed for NF-κB activation,[19] but that the presence of cellular membranes is required, leading to the hypothesis of a plasma membrane-mediated effect.[8] Two mechanisms have been proposed: UV would lead to clustering of membrane receptors such as TNF-R or IL-1R, which would trigger their ligand-independent activation,[20] or UV would inhibit receptor-inactivating tyrosine phosphatases, leading to constitutive activation of tyrosine kinases of the Scr family.[21] However, these studies are inconsistent with the notion that far lower doses of UV are needed to activate NF-κB in XPA patients, suffering from the DNA repair disease xeroderma pigmentosum group A.[22] Furthermore, Yarosh *et al.*[23] demonstrated that the administration of an UV-irradiated plasmid but not nonirradiated DNA through lipofection is sufficient to induce an NF-κB response, strongly arguing against a membrane-mediated effect. Bender *et al.*[24] solved these apparently contradicting results, by showing that the UV response can be subdivided in an early DNA damage-independent and a late DNA damage-dependent response.

More consensus has been reached concerning the action mechanism of other genotoxic stress agents, such as γ-irradiation (IR)- or topoisomerase-targeting drugs. In these conditions, the activation of NF-κB is strictly dependent on the

presence of an intact nucleus and a drug-sensitive version of topoisomerase I or II enzyme, showing the intimate link between the formation of double-strand breaks (DSBs) and NF-κB activation.[25–27] Topoisomerases are enzymes that control the degree of DNA supercoiling. Type I topoisomerases (targeted by CPT) create transient single-strand breaks in DNA, necessary for the induction of transcription, while topoisomerase II enzymes (inhibited by etoposide-like and doxorubicin-like drugs) are more involved in DNA replication and cell division and therefore create transient DSBs.[28] Chemotherapeutic drugs are not merely inhibiting the action of these enzymes, but interfere with the religation step, thereby causing permanent DNA breaks.[29] Huang *et al*.[26] demonstrated that the single-strand breaks induced by CPT are not sufficient for NF-κB activation, but need to be converted to DSBs during S-phase, when the replication fork collides with DNA–Topo I complexes. This makes DSBs the most likely candidate causing NF-κB activation among all possible DNA lesions generated by DNA damage.

How are DNA DSBs Sensed in a Cell: The Role of ATM, ATR and DNA-PK

Sequence alterations in the DNA are at all times present and can arise from either spontaneous mutations, DNA replication errors, endogenous metabolites such as oxygen radicals, or environmental (exogenous) DNA-damaging agents such as irradiation or chemical mutagens (for reviews, see Zhou and Elledge[30] and Shiloh[31]). Maintenance of genomic stability is a prerequisite for cellular homeostasis and essential for survival of the organism. Therefore, the cell possesses strict safeguarding mechanisms to protect from any unwanted DNA lesion or base pair mismatch.

DNA damage initiates a meticulously orchestrated response coordinated by sensor, transducer and effector proteins that detect the damage and direct the activation of either a cell survival response (consisting of a cell cycle arrest, DNA repair and stress response branch) in cases when the type of damage can be handled, or an apoptotic programme to eliminate the harmed cell in cases of overwhelming damage. It is clear that, when both responses fail, the genetic alteration becomes fixed and the road to neoplasia is open.

The sensors that detect DNA DSBs and subsequently mount the global DNA damage response remain as yet unknown.[30,31] DNA-protein kinase (DNA-PK), a kinase of the phosphoinositide-3-like (PI3-like) protein kinase family, was long considered as an excellent candidate.[32] The holoenzyme consists of a catalytic subunit DNA-protein kinase C (PKC) and two small regulatory accessory proteins Ku70 and Ku80. Ku70/80 heterodimer has a high affinity for DSBs and recruits the catalytic part of DNA-PK to the damaged site, where it participates in DNA repair. However, there is no genetic evidence for an essential role for DNA-PK as activator of the global DNA response.[33] Another likely candidate is ataxia telangiectasia mutated (ATM), another member of the PI3-like protein kinase family that is absent in patients with the genomic instability disorder ataxia telangiectasia (AT).[31,34] Hallmarks of this disease are neurodegeneration, immunodeficiency, radiation sensitivity and cancer predisposition, all reflecting the inability of these patients to mount an appropriate DNA damage response upon irradiation. ATM's kinase activity is activated immediately upon IR induction, suggesting that it is acting at an early stage in the DNA damage response.[35] However, ATM is not activated by free double DNA strands,[36] suggesting that it acts as a primary transducer of DSBs rather than as a real sensor. Work by Bakkenist *et al*.[35] well demonstrated that, in unstimulated cells, ATM resides in the nucleus in a dimer or higher-order multimer with its kinase domain, blocked by interaction with the Frap, ATM and Trapp (FAT) domain of an adjacent ATM molecule. IR induces a transphosphorylation reaction within the FAT domain, liberating the two monomers and turning them into active kinases. They further showed that about 18 DSBs are sufficient to activate over 50% of the ATM molecules in less than 5 min upon IR exposition, making it extremely unlikely that ATM needs to bind DSBs to become activated. Rather, they suggest that ATM might sense higher-order chromatin alterations evoked by the DSBs, and in this way might act as a sensor after all.[35]

ATM has several known substrates, most of them implicated in cell cycle checkpoints (like, e.g., p53, Mdm2, CHK1 and 2), but also proteins involved in DNA repair, such as Nbs1 or BRCA1.[31,34] Recent evidence demonstrates that ATM is also required for the activation of NF-κB in response to various genotoxic stress insults, expanding the known ATM-dependent network.[27,37–39]

Although the NF-κB response to IR is still controversial – many authors utilize IR doses that are high above clinically relevant doses ($\leq$2 Gy)[40] and clear differences in sensitivity to IR are observed between primary and transformed cell types[41] – different groups independently demonstrated that the NF-κB response to IR in ATM-deficient cells is abrogated, both in transformed cell types[37,38] as well as in primary mouse tissues.[38] Furthermore, also CPT and doxorubicin appear to induce NF-κB activation via an ATM-dependent pathway.[27,39,42] Importantly, anthracyclin-like molecules that do not cause DNA breaks but activate NF-κB via unrelated pathways (presumably through the activation of PKC-δ) do not need ATM.[42,43] Also, TNF and LPS activate NF-κB in an ATM-independent way,[37–39] demonstrating that ATM is particularly required in those NF-κB pathways activated by the presence of DNA lesions.

In some cell lines obtained from AT patients, an early and short NF-κB induction upon CPT can still be observed, suggesting possible redundancy with other damage transducers.[39] These authors also observed an intact daunomycin-induced NF-κB response, which is unexpected as this molecule also targets topoisomerase II. Other candidate molecules acting in a parallel pathway to ATM comprise the protein kinases DNA-PK and ATM-related kinase (ATR). DNA-PK was mentioned above and is implicated in non-homologous end joining.[32] A paper by Basu *et al*.[44] showed that MO59J cells, deficient in DNA-PK, no longer activate NF-κB in response to IR. However, later work demonstrated that these cells contain very low numbers of ATM, which could also explain the observed defect.[38] In line with this, several other groups failed to detect a role for DNA-PK in IR, mithoxanthrone, CPT or daunomycin-induced NF-κB activation.[27,38,39] Finally, ATR is a third member of PI3-like protein kinase family

clearly implicated in the DNA damage response.[31] Knockouts of this gene are lethal and no human disease is known associated with mutations in Atr, unambiguously demonstrating the essential role of this kinase.[30] Furthermore, it is very likely that dominant-negative versions of this kinase might also affect ATM-mediated pathways, limiting the available tools to analyse the function of this kinase. At present, there are no data available demonstrating a role for this kinase in NF-κB activation upon genotoxic stress, although one report shows a role for ATR in p14ARF-mediated repression of RelA transcriptional activity (see further).[45]

How does ATM Relay a Signal to the IKK Complex?

With the notable exception of UV light and a specific p53-induced pathway to NF-κB (which are described in the following paragraph), all DNA damage pathways require a functional IKK complex for NF-κB activation. Indeed, the absence of NEMO, IKKα and IKKβ completely abrogates the response to doxorubicin, CPT, etoposide or IR.[15,26,38,42,46–48] Activation of the IKK complex in an ATM-dependent way leads to the phosphorylation of IκBα on serines 32 and 36, and its subsequent ubiquitination, similar to the classical NF-κB pathway (Figure 2).

It has long remained enigmatic how ATM relays a nuclear DNA damage signal to a cytoplasmic IKK complex. Previous authors suggested that ATM might phosphorylate IκBα in a direct way, but this has never been proven *in vivo*.[49] Huang *et al*.[47] realized that all DNA-damaging agents share one peculiarity: they all need an intact zinc-finger region of NEMO to signal to NF-κB. Hypohydrotic ectodermal dysplasia with severe immunodeficiency (HED-ID) is one of the two identified human genetic disorders that are caused by mutations in the X-linked gene coding for Ikkγ (NEMO)[50–52] (see also elsewhere in this issue). The majority of the mutations causing HED-ID occur in exon 10, affecting the C-terminal zinc-finger region of NEMO.[53] Interestingly, stable transfection of a NEMO construct containing two zinc-finger point mutations occurring in HED-ID or of a deletion construct removing the zinc-finger was able to restore NF-κB signalling in NEMO-deficient 1.3E2 cells upon LPS, but not CPT or UV stimulation, suggesting a differential requirement for this region by

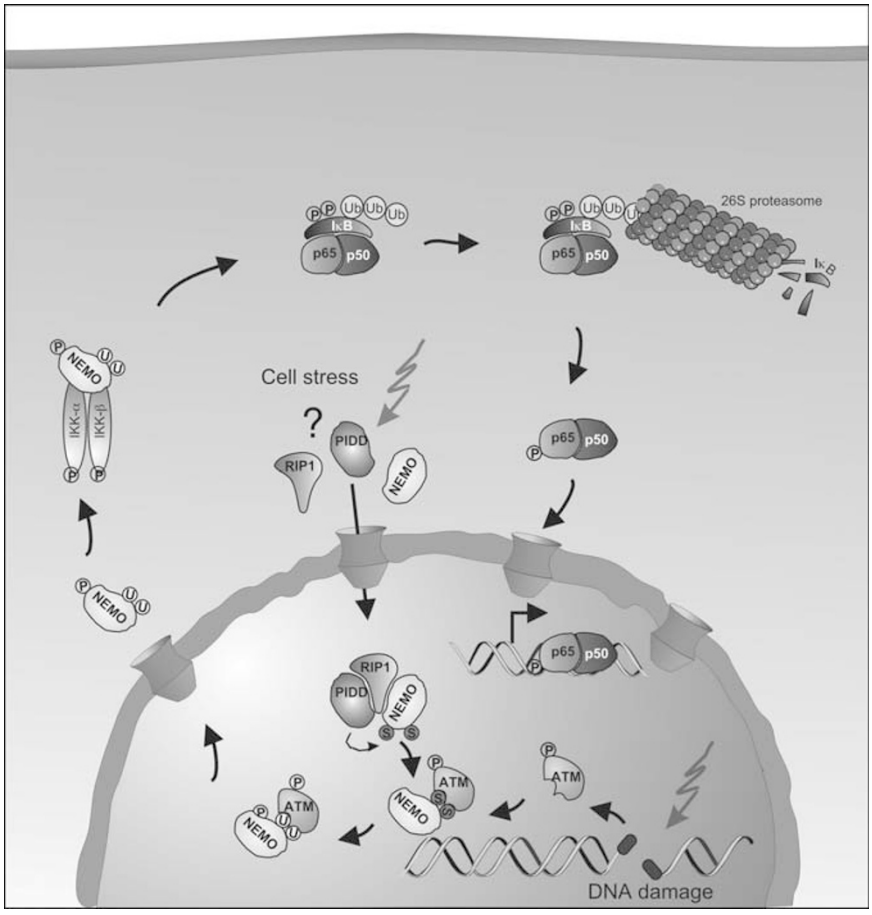

Figure 2 Overview of the IKK-dependent genotoxic stress-induced NF-κB pathway. Upon genotoxic stress two independent parallel pathways are activated, both needed for subsequent NF-κB activation. The first one leads to activation and nuclear translocation of the DD protein PIDD, followed by its recruitment of RIP1 and NEMO. It is as yet unclear if this inducible complex is formed in the cytoplasm or in the nucleus. In any case, once in this complex, NEMO becomes rapidly sumoylated in a PIDD-dependent manner. A second pathway triggered upon DNA damage leads to the phosphorylation and activation of the kinase ATM. Both pathways are integrated when sumoylated NEMO is recognized as a substrate for kinase-active ATM, leading to its phosphorylation and ubiquitination in an ATM-dependent way. Ubiquitinated NEMO then translocates back to the cytosol, activating the IKK complex and triggering NF-κB activation

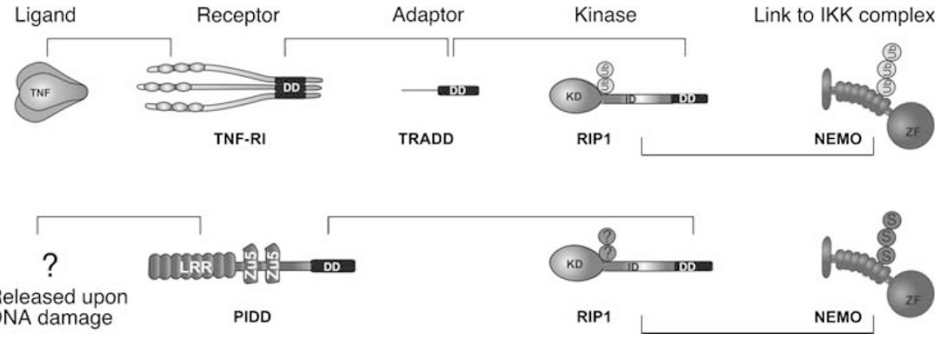

Figure 3 Comparison of TNF and DNA damage-induced signalling cascades. TNFRI and PIDD activate similar signalling cascades, in which homotypic DD–DD interactions, an essential role of RIP1 as recruiter of NEMO and regulation through specific post-translational modifications are recurrent themes. Upon ligand binding, TNFRI recruits the adaptor protein TRADD and the kinase RIP1 via its intracellular DD, leading to the extensive ubiquitination of RIP1. RIP1 enables recruitment of NEMO, which also becomes ubiquitinated, presumed to be critical for subsequent IKK activation. PIDD can be viewed as an intracellular sensor of DNA damage, detecting a signal of unknown origin via its LRR. This leads to a DD-dependent recruitment of RIP1, inducing an unknown nuclear modification of the latter. RIP1 is essential for recruitment of NEMO into the complex, leading to its sumoylation. Later, both pathways deviate, as sumoylated NEMO is further recognized by ATM, leading to an altered NEMO post-translational modification, and subsequently IKK activation. DD: death domain; KD: kinase domain; ID: intermediate domain; ZF: zinc-finger; LRR: leucine-rich repeats; ZU5: zonula occludens (ZO)-1 and unc5-like netrin receptor domain

different NF-κB-inducing stimuli.[47] Huang *et al*.[54] further demonstrated that the zinc-finger region of NEMO is needed for DNA damage-induced sumoylation of NEMO and most likely mediates interaction with a protein needed in the sumoylation pathway, while the actual sumo-sites lie outside the zinc-finger region (Figure 2). Although absolutely essential, sumoylation of NEMO is not sufficient to allow DNA damage-induced NF-κB activation. In a second pathway, sumo-NEMO is recognized and phosphorylated by ATM and tagged by ubiquitination at the same lysine residues, showing a central role for different post-translational modifications of NEMO in genotoxic stress-induced NF-κB activation. It has been proposed that the different modifications target the molecule to different subcellular compartments, that is, sumoylation would lead to NEMO nuclear accumulation and thus recognition by ATM, while ubiquitination most likely releases the molecule from the ATM scaffold and allows its cytoplasmic translocation.[54,55] As has been shown before for other signalling cascades,[56–59] ubiquitination of NEMO is a prerequisite for IKK activation. As such, NEMO could act as the factor mediating the link between DNA damage and ATM activation in the nucleus and activation of the IKK complex in the cytosol.

Previously, RIP1 had been shown to play a role in ATM-dependent NF-κB signalling pathways,[48] but it was unclear how these data could be reconciled with the present data on NEMO sumoylation. Also, the initial factor activated upon DNA damage that drives NEMO sumoylation remained enigmatic. These questions became more clear with the identification of PIDD as a factor linking DNA damage and NEMO sumoylation[89] (Figure 2).

PIDD is a leucine-rich repeat (LRR)- and death domain (DD)-containing protein previously implicated in the activation of caspase-2 through the formation of a so-called 'PIDDosome', consisting of the adaptor protein RAIDD, PIDD and caspase-2.[60] Upon genotoxic stress, PIDD translocates to the nucleus, establishes a complex with RIP1 and NEMO (either preformed in the cytosol or formed once inside the nucleus), and enables DNA damage-induced NEMO sumoylation in this complex.[89] While PIDD and RIP1 interact in a direct way via their DD, the interaction between NEMO and PIDD is mediated via RIP1, explaining the absolute requirement for RIP1 in genotoxic stress-induced NF-κB activation.[48] Interestingly, we observed that the RIP1/NEMO and RAIDD/caspase-2 pathways induced by PIDD appear to be mutually exclusive, suggesting that PIDD could act as a switchboard either activating a cell survival pathway in cases where the amount and type of DNA damage could be handled, or activating an apoptotic pathway in cases of overwhelming damage.[89] Our data further revealed the remarkable similarity between the TNF and DNA damage-induced NF-κB pathway (Figure 3). In the latter pathway, PIDD could act as a kind of intracellular receptor, detecting intracellular stress signals via its LRR motifs. Via their DD, both receptors then recruit RIP1 (in the case of TNFRI via the adaptor protein TRADD), which subsequently becomes modified. While in the case of TNF RIP1 becomes clearly ubiquitinated, the type of modification in the case of DNA damage is as yet unknown, but appears to be exclusively limited to the nuclear pool of RIP1. In a subsequent step, RIP1 recruits NEMO, which also becomes post-translationaly modified, although again the type of modification appears to be strictly stimulus-specific. Most likely, stimulus-specific post-translational modifications allow NEMO and RIP1 to integrate various signalling pathways via the recruitment of specific ubiquitin- or sumo-binding proteins. Similarly, in the IL-1 pathway, TAB2 proteins have been demonstrated to function as K63-polyubiquitin receptors, representing a new class of signal transducers, activating kinases in a ubiquitin-dependent, nonproteolytic mechanism.[61]

CK2 and Friends: the IKK-Independent Pathways

Although the IKK complex is clearly involved in the NF-κB activating pathways induced by IR or chemotherapeutic drugs, the role of this kinase in response to UV is more controversial (Figure 4). Thus, Li *et al*.[15] and Bender *et al*.[24] showed that a dominant-negative IκBα construct in which

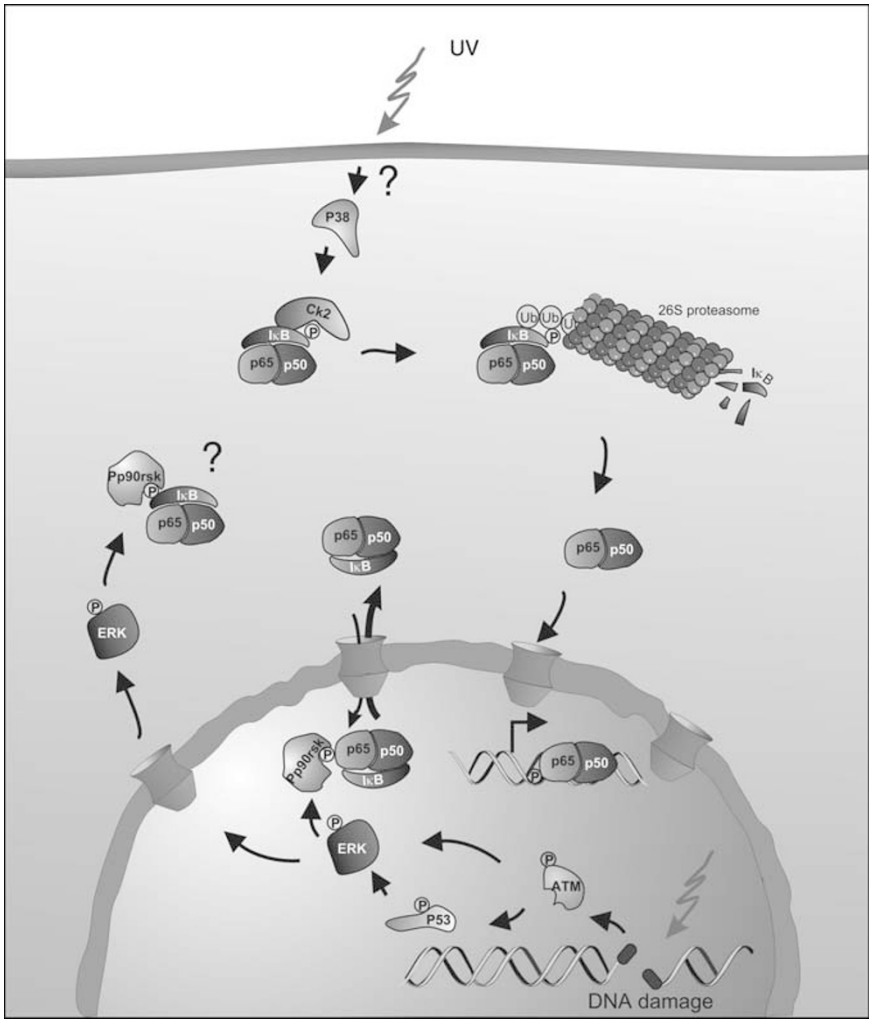

Figure 4 IKK-independent pathways of genotoxic stress-induced NF-κB activation. Genotoxic stress leads to activation of two IKK-unrelated kinases, implicated in NF-κB activation. Activated ATM and/or phospho-p53 induce ERK-dependent phosphorylation of ribosomal kinase 6 (pp90rsk). pp90rsk has been suggested to be implicated in phosphorylation of IκBα (either alone or in conjunction with the IKK complex), or alternatively could be implicated in a nuclear pathway leading to phosphorylation of p65 present in inactive NF-κB/IκB complexes constitutively shuttling between the cytosol and nucleus. Normally, nuclear NF-κB/IκB complexes are immediately transported back to the cytosol. Phosphorylation of p65 at Ser536 weakens its affinity for IκBα, leading to a slow accumulation of active p65/p50 heterodimers and resulting in weak NF-κB transcriptional activity. Stimulation with UV triggers a nucleus-independent pathway, culminating in the activation of CK2 via an as yet unknown, but p38-dependent mechanism. CK2 induces phosphorylation of C-terminal residues on IκBα, leading to its degradation in a ubiquitin- and proteasome-dependent manner, and inducing NF-κB activation

Ser32 and 36 were mutated to Ala was still degraded upon UV treatment, while this construct resisted TNF-dependent degradation. In addition, no activation of the IKK complex could be measured in an *in vitro* kinase assay in response to UV.[15] Huang *et al*.[47] could confirm these latter results, but did detect a complete abrogation of the UV-induced NF-κB response in IKKα/β double KO cells. Through the use of a specifically designed IκBα-phosphospecies 'trap', they further demonstrated the presence of small amounts of serine phosphorylated IκBα in response to UV treatment. Thus, although the knockout data of IKKα/β still obscure the discussion on the role of the IKK complex in UV, the absence of IKK kinase activity in response to UV shows that its function is clearly distinct from those in other pathways, and suggests that other kinases might be implicated as well. Neither the group of Karin[15] nor the group of Herrlich[24] could detect any evidence for tyrosine phoshorylation of IκBα in response to UV, but they did show that IκBα is degraded in a ubiquitin and proteasome-dependent manner. To identify the UV-activated IκBα kinase, Kato *et al*.[62] used a solid-phase kinase assay and in this way spotted casein kinase 2 (CK2) as a likely candidate. Indeed, it had been shown before that UV induced phosphorylation of C-terminal residues in IκBα in a region known to contain six CK2 phosphorylation sites.[24] Reconstitution of IκBα$^{-/-}$ cells with an IκB construct in which these sites were mutated did not restore UV-induced NF-κB activation, while the TNF-dependent signalling cascade was re-established. Furthermore, downregulation of CK2 through siRNA treatment or immunodepletion with a CK2-specific antibody abrogated the UV response to NF-κB. These data are also consistent with the notion that cells derived from a patient suffering from xeroderma pigmentosum, a genetic disorder

characterized by an increased sensitivity to UV-induced damage, can be rendered UV resistant by transfection of CK2β, presumably through the induction of a CK2-dependent antiapoptotic NF-κB response.[63] Although it remains to be identified how CK2 is activated upon UV stimulation, the pathway appears to involve a p38-dependent step, as shown by the absence of UV-induced NF-κB activation in p38$^{-/-}$ cells.[62]

Another NF-κB pathway clearly distinct from the classical dogma comprises activation of the kinase pp90rsk in an ERK- and possibly p53-dependent way. The link between NF-κB and p53 upon genotoxic stress is one of the most controversial fields in DNA damage literature (see also further). Two schools exist, one claiming that p53 is a prerequisite for NF-κB induction and that p53-induced NF-κB activation plays a merely apoptotic role,[64,65] the other claiming that genotoxic stress-induced p53 and NF-κB activation represent two independent parallel pathways, both activated by ATM, which might however interfere with each other's action through distinct mechanisms.[42,66,67] The first argument is based on the fact that some authors showed that the p53-null tumour cell line Saos-2 is resistant to doxorubicin or etoposide-induced NF-κB activation, while doxocycline-induced expression of p53 restores NF-κB activation.[65] The other school claims that p53-null cells such as Saos-2 or 10(1) murine embryo fibroblasts do activate NF-κB as efficiently as p53 wt cells in response to genotoxic stress.[42] The reason underlying these opposite results is at present not understood, but the numerous reports showing efficient NF-κB activation upon genotoxic stress in cell lines transformed with SV40 large T antigen (resulting in inactivation of p53) or in cell lines harbouring mutations in p53 make the absolute requirement for p53 in DNA damage-induced NF-κB activation rather unlikely (e.g., Panta *et al*.[42] and Arlt *et al*.[68]). Furthermore, an *in vivo* liver model of doxorubicin-treated mice did not reveal any functional link between p53 and NF-κB, but rather showed that NF-κB activation precedes p53 activation, making it unlikely to be a consequence of p53 activity.[66]

Nevertheless, p53 induction *in se* (by doxoxyclin-mediated expression or transient overexpression) leads to a slow activation of NF-κB. Bohuslav *et al*.[65] showed that this pathway occurs in an IκBα phosphorylation and IKK-independent way, involving activation of the ERK kinase and ribosomal S6 kinase pp90rsk (as judged by treatment with ERK inhibitors or siRNA for pp90rsk). These latter observations have been troubled by a report showing that DNA damage indeed leads to activation of ERK and pp90rsk, but in a p53-independent way.[69] pp90rsk has been proposed as a kinase of IκBα in the TPA pathway, although it has to act in conjunction with the IKK complex to induce complete degradation of IκBα.[70] Bohuslav *et al*.[65] proposed an alternative, IKK-independent pathway, in which pp90rsk forms a complex with p65 and induces its phosphorylation on Ser 536. This reduces its affinity for IκBα, leading to a steady accumulation of free p50/p65 heterodimers in the nucleus and transcriptional activation of NF-κB. The importance of these latter pathways in DNA damage-dependent NF-κB activation remains to be established.

Finally, mechanisms involving tyrosine phosphorylation of IκBα have been described,[71] but these are mainly associated with oxidative stress- or hypoxic injury-induced NF-κB activation, and are beyond the scope of the present review.

Is DNA Damage-Induced NF-κB Transcriptionally Active? RelA as a Repressor of Gene Activation

The NF-κB response can be divided into two phases that occur in distinct subcellular compartments (for reviews, see Hayden and Ghosh[2] and Chen and Greene[72]) (Figure 5). A first cytoplasmic phase comprises the IKK-dependent phosphorylation and degradation of either IκBα in the canonical pathway or p100 in the alternative pathway, and as such leads to the nuclear translocation of a NF-κB heterodimer. Experimentally, activation of this first phase can be demonstrated by gelshift assays, in which an increased NF-κB DNA binding can be observed. The second, mainly nuclear phase involves post-translational modifications of NF-κB subunits, leading to the modulation of their transcriptional potential. Thus, degradation of IκB and nuclear translocation of NF-κB are not sufficient to induce maximal NF-κB transcriptional gene activation, but an additional level of regulation involving phosphorylation and acetylation of key residues in NF-κB subunits is required. This 'transactivation phase' is thought to

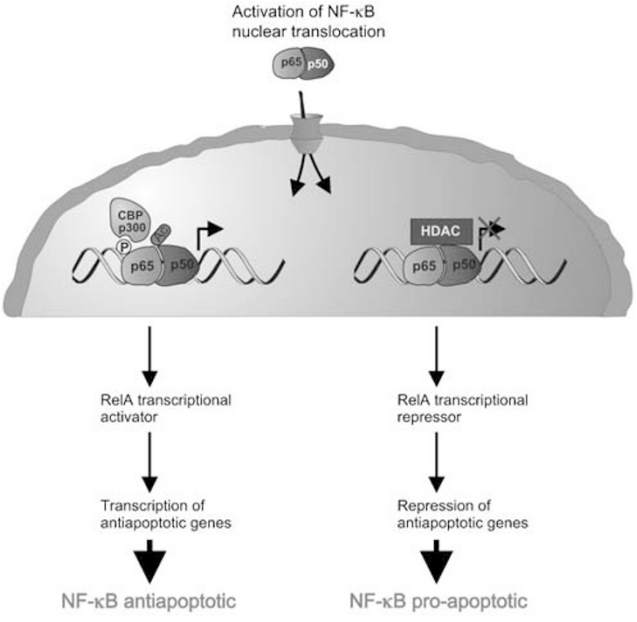

Figure 5 RelA as repressor or activator of gene expression? Most pathways triggering NF-κB nuclear translocation also induce phosphorylation of its subunits, enhancing its transcriptional potential through different mechanisms, one of them being recruitment of coactivator proteins such as CBP/p300 and subsequent RelA acetylation. Through transcriptional activation of antiapoptotic gene expression, NF-κB mediates resistance against programmed cell death. Recently, it has become clear that under particular circumstances (in the presence of tumour suppressor genes or upon induction by UV or doxorubicin in certain cell types) NF-κB can repress expression of certain target genes. The conversion of RelA from activator to repressor is associated with an altered pattern of post-translational modifications of the RelA subunit, resulting in its interaction with members of the HDAC family rather than with coactivator proteins. Repression of antiapoptotic target genes converts NF-κB in a proapoptotic and tumour suppressor factor, rather than being antiapoptotic and tumour promoting

determine the strength and duration of the transcriptional response and can be evaluated experimentally by measuring NF-κB-dependent gene transcription.[72]

Interestingly, although NF-κB (in this context referring to the prototypical RelA/p50 heterodimer) is typically regarded as a potent activator of gene expression, this viewpoint has been challenged lately as it has been demonstrated that under some circumstances RelA can actually repress expression of certain target genes.[73–75]

Campbell *et al*.[73,74] noticed that while UV, doxorubicin and daunorubicin clearly induce nuclear translocation of NF-κB, they did not lead to increased NF-κB-dependent gene expression. In the first report, they correlated the absence of NF-κB transcriptional activity with an increase in p53 transcriptional activity.[74] Indeed, it had been shown before that p53 counteracts the nuclear actions of NF-κB through competition for the same pool of coactivators such as CBP, and thus the observed defects in NF-κB gene expression upon UV or doxorubicin treatment could be merely the result of concomitant p53 activation.[67,76] However, they later observed that the absence of NF-κB-dependent gene transcription in response to UV or doxorubicin treatment also occurred in p53-negative cells.[73] Furthermore, upon treatment with these stimuli, NF-κB not merely lost its ability to induce transcription, but rather acquired a dominant transcriptional repressor function, an effect that was particularly clear when UV or doxorubicin treatment was combined with TNF treatment. Importantly, not all DNA-damaging agents lead to repression of RelA-mediated gene transcription, as etoposide did stimulate NF-κB-induced gene expression. Therefore, conversion of RelA from an activator to a repressor of gene transcription appears to remain limited to a specific subset of DNA damage stimuli, and is most likely also restricted to a subset of target genes and a subset of cell types. Indeed, although other groups confirmed the repressor effects of doxorubicin-induced RelA,[75] many authors did measure doxo- or daunorubicin-induced NF-κB transcriptional activity and synergistic effects upon combined TNF and daunorubicin treatments, with their observations being made in other cell types.[11,46,66,68] Furthermore, Campbell *et al*.[73] demonstrated that while some target genes are downregulated upon UV treatment, other NF-κB target genes, such as IκBα, were upregulated, showing clearly additional regulatory effects at the promotor level.

An important question remains as to how RelA is converted from gene activator to gene repressor. Saccani *et al*.[77] previously described the exchange of NF-κB dimers over time at certain promotors as a way to finetune *NF-κB* gene expression. Thus, rapidly activated dimers (such as p50/RelA) are later replaced by slowly activated dimers (e.g. RelB/p52) during dendritic cell maturation. As only a subset of Rel family members contain TAD (RelA, RelB and c-Rel), different dimers contain different transcriptional potential (e.g. homodimers of p50 and p52 repress gene expression) and therefore it is plausible that repression of NF-κB activity is achieved by activation of a different subset of Rel members by UV. However, Campbell *et al*.[73] could demonstrate that up to 8 h upon treatment, UV-induced NF-κB complexes could be shifted by a RelA-specific antibody in gel shift assays, excluding the latter possibility. Furthermore, genetic evidence showed that the observed transcriptional repression was strictly dependent on the presence of RelA, as the defect was absent in RelA$^{-/-}$ MEF cells or in cells treated with siRNA against RelA.

Several post-translational modifications of RelA have been described that alter its transcriptional properties, the most important ones being phosphorylation and acetylation.[2,72] RelA is phosphorylated at key residues both in its RHD and TAD, which all lead to an enhanced transcriptional response. In addition, phosphorylation events also modulate DNA binding and oligomerization of NF-κB or weaken the affinity for IκBα, all resulting in a more persistent NF-κB response. Several kinases have been implicated in phosphorylation of RelA, with the exception of PKA all acting in the nucleus. Interestingly, one of the proposed kinases is the IKK complex itself, targeting Ser536 and thereby enhancing NF-κB's transcriptional activity.[2] This reveals that in most cases NF-κB nuclear translocation and transactivation are activated by one and the same pathway, and thus, that it is no surprise that NF-κB is generally regarded as a potent activator of gene transcription.

Phosphorylation of RelA/p65 is thought to be critical to create a docking site for CBP/p300, a coactivator protein that bridges transcription factors and the components of the cellular transcriptional machinery.[78] In addition, CBP/p300 contains intrinsic histone acetyltransferase activity and also mediates transfer of acetyl groups to nonhistone proteins such as the RelA transcription factor.[79] Acetylation of RelA/p65 modulates its DNA-binding capacity,[79] its transcriptional activity,[80] its interaction with IκB proteins and its subcellular localization.[79] Although still controversial,[81] a model was proposed in which acetylated p65 is protected from inhibition by IκBα, but, following the recruitment of histone deacetylases (HDACs), more particularly HDAC3, the deacetylated p65 interacts with IκBα and is exported to the cytoplasm, providing a mechanism for NF-κB downregulation.[79]

It is plausible that different stimuli induce different post-translational modifications of RelA, determining the outcome of RelA transcriptional events. Rocha *et al*.[45] reported that the tumour suppressor ARF represses RelA transcriptional events through phosphorylation of Thr505 in an ATR-dependent manner. This subsequently led to an interaction of RelA with HDAC1, resulting in a transcriptionally inactive complex. UV- or doxorubicin-mediated effects on RelA appeared to occur independently of Thr505 phosphorylation, although also in this case an increased association of RelA with several HDAC's could be observed.[73] Thus, the authors proposed a mechanism in which a transcriptional activator complex consisting of RelA and coactivator proteins is converted to an active repressor complex, consisting of RelA and HDACs, which still can associate with target promotors and as such downregulate their expression. This is in slight disagreement with the findings of other authors, which could not detect stable association of nonmodified RelA on endogenous loci, and proposed that the absence of Ser536 phosphorylation and acetylation in response to doxorubicin treatment blocks the ability of RelA to bind promotors of target genes.[75]

Whatever the exact mechanism may be, it is clear that the altered transcriptional properties of UV or

doxorubicin-induced RelA clearly influence the outcome of NF-κB-mediated effects and change our current dogma of NF-κB as an activator of antiapoptotic gene expression.

Functional Consequences of DNA Damage-Induced NF-κB Activation: Pro- or Antiapoptotic Factor?

As has been mentioned earlier, most interest for the role of NF-κB in DNA damage pathways comes from the field of cancer therapy, where resistance of tumours to chemotherapy is a major problem. Inducible chemoresistance can be acquired in many ways.[82,83] Apart from the overexpression of MDR1, a transmembrane protein implicated in export of chemotherapeutic drugs from cells, another important mechanism involves resistance to chemotherapy-induced apoptosis. The landmark publications of the group of Baldwin showed a key role for chemotherapy-induced NF-κB activation in preventing cell death,[84,85] and since then numerous papers showing the potential to enhance tumour therapy by inhibition of NF-κB activation contributed to the dogma of NF-κB as antiapoptotic factor and tumour promotor (for a review, see Nakanishi and Toi[83]). However, several papers that appeared during the last 5 years[10,12,13,64,73,74] showed that under some circumstances NF-κB can contribute to genotoxic stress-induced apoptosis, and forced the scientific field to re-evaluate this simple model. Owing to their investigations on the interplay between tumour suppressor genes and NF-κB function, the group of Perkins clarified the apparent dual role of NF-κB and showed that the tumour suppressor status of a cell plays a decisive role in the functional outcome of NF-κB.[45,67,86] They reasoned that as NF-κB is a cell survival factor and inducer of cellular proliferation, and p53 induces cell cycle arrest or apoptosis, mechanisms that integrate the activities of both pathways in conditions when they are activated at the same time must exist (such as genotoxic stress treatment). Indeed, they and others[76,87] could show that p53 counteracts NF-κB function by blocking its transcriptional potential through competition for coactivators or through conversion of Rel members from activators to repressors. For example, p53 induces a switch from p52–Bcl-3 activator complexes to p52–HDAC1 complexes.[86] Similarly, another tumour suppressor gene *ARF* induces phosphorylation of Thr505 on RelA, leading to its association with HDAC1, suppressing its transcriptional activity.[45] Both effects could explain the results of Ryan *et al.*,[64] who showed that p53-induced NF-κB activation is not transcriptionally active and contributes to apoptosis, presumably through p53-mediated repression of NF-κB induced antiapoptotic gene expression. In addition, other p53-independent mechanisms must exist, as well that contribute to NF-κB's proapoptotic functions, as shown by the paper of Campbell *et al.*[73](see before).

A model is emerging in which NF-κB tumorigenic properties are kept carefully in balance by tumour suppressor genes that convert the molecule from an activator to a repressor of antiapoptotic gene expression.[88] Therefore, in early tumours still expressing wild-type p53 and/or ARF, NF-κB could actually inhibit tumour development.[64] Later, when mutations in tumour suppressor genes are accumulating and mechanisms to keep NF-κB in check are no longer operational, NF-κB's antiapoptotic and cell proliferation-inducing capacities can freely contribute to tumour progression and inhibition of NF-κB activity will be a prerequisite for successful cancer therapy.

Perspectives

Although the signalling cascades initiated upon DNA damage and inducing NF-κB activation remained for a long-time obscure, significant progress during the last few years allows the emergence of a general picture. With the exception of UV light, which induces an entirely unrelated pathway based on the activation of CK2, all DNA-damaging agents require the presence of ATM, as a sensor of DNA DSBs and the IKK complex for NF-κB activation. A genotoxic stress-inducible complex consisting of PIDD, RIP1 and NEMO provides the long-sought link between nuclear DNA damage and cytoplasmic IKK activation. Upon DNA damage, NEMO becomes sumoylated in a PIDD- and RIP1-dependent manner. Sumoylated NEMO is a substrate for ATM, which phosphorylates the protein, tagging it for subsequent ubiquitination. This presumably allows NEMO to translocate to the cytoplasm and trigger the activation of the classical IKK complex.

Many questions remain, however, such as how PIDD becomes activated by genotoxic stress and assists in sumoylation of NEMO, where the inducible PIDD/RIP1/NEMO complex is formed, what is the exact role of NEMO post-translational modifications and why different modifications of NEMO are needed to activate the (apparently) same IKK complex in response to different signals.

Interestingly, the functional consequences of genotoxic stress-induced NF-κB activation can be quite different from TNF-induced NF-κB activation, and appear to depend on the cytotoxic drug used. In particular cell types, UV- and doxorubicin-induced NF-κB leads to transcriptional repression of selected antiapoptotic target genes and therefore performs a mainly proapoptotic function, while etoposide or IR, just like TNF, rather activate NF-κB-induced gene transcription and thus the antiapoptotic properties of NF-κB. This is particularly intriguingly as etoposide and doxorubicin appear to induce similar pathways, dependent on PIDD, RIP1 and NEMO, although one cannot exclude the possibility that additional distinct IKK-independent pathways are activated as well. So, what determines whether the activated NF-κB is transcriptionally active or repressing and which subset of target genes is repressed/activated? While we know already a lot about the pathways culminating in IκBα degradation and NF-κB nuclear translocation, this proves that much remains to be learned on the second pathway, modulating NF-κB transcriptional potential through post-translational modifications that alter its capacity to interact with various coactivator/corepressor complexes or other transcription factors. It is likely that different stimuli trigger different modifications of NF-κB and that this underlies the observed differences in transcriptional responses. In addition, the apparently conflicting literature data on the dual role of NF-κB as an antiapoptotic *versus* proapoptotic factor in response to DNA damage can be explained in a similar way

and appear to be linked to the tumour suppressor status in a cell. Wild-type p53 and/or ARF, also activated by genotoxic stress but only present early during tumour development, assist in recruiting RelA subunits to tumour suppressor pathways through modification of the Rel subunits, inducing their association with corepressors rather than coactivators. As such, in cells or tumours retaining p53, NF-κB has often been showed to play a merely apoptotic role.

Whatever the cause may be, the ability of certain stimuli to switch Rel subunits into transcriptional repressors rather than transcriptional activators offers interesting possibilities for the clinic. New drugs aimed at exploiting the transcriptional repressor capacities of NF-κB could have more potential than those merely aimed at inhibiting its function. In this way, one could make profit of the potential beneficial aspects that NF-κB could have in assisting chemotherapy-based strategies designed to induce cell death and tumour regression.

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