

# Crosstalk of reactive oxygen species and NF- $\kappa$ B signaling

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**NF- $\kappa$ B proteins are a family of transcription factors that are of central importance in inflammation and immunity. NF- $\kappa$ B also plays important roles in other processes, including development, cell growth and survival, and proliferation, and is involved in many pathological conditions. Reactive Oxygen Species (ROS) are created by a variety of cellular processes as part of cellular signaling events. While certain NF- $\kappa$ B-regulated genes play a major role in regulating the amount of ROS in the cell, ROS have various inhibitory or stimulatory roles in NF- $\kappa$ B signaling. Here we review the regulation of ROS levels by NF- $\kappa$ B targets and various ways in which ROS have been proposed to impact NF- $\kappa$ B signaling pathways.**

**Keywords:** reactive oxygen species (ROS); NF- $\kappa$ B; oxidative stress; antioxidants

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## NF- $\kappa$ B

NF- $\kappa$ B proteins are a family of transcription factors and are of central importance in inflammation and immunity [1, 2]. NF- $\kappa$ B transcription factors also regulate the expression of hundreds of genes that are involved in regulating cell growth, differentiation, development, and apoptosis. The mammalian NF- $\kappa$ B proteins consist of five different related family members that bind as homodimers or heterodimers to 10-base pair  $\kappa$ B sites. All of these family members have a Rel-homology (RHD) domain that is essential for DNA binding and dimerization. The three Rel members of the family, RelA (also known as p65), RelB, and cRel, have a C-terminal transcription activation domain (TAD) that serves to positively regulate gene expression. The two other mammalian NF- $\kappa$ B proteins are synthesized as larger p105 and p100 precursor proteins, which have C-terminal ankyrin repeats that inhibit DNA binding until partially processed by proteasome to the smaller p50 and p52 products, respectively [1-3]. In addition, these proteins lack a TAD and therefore do not generally activate transcription unless paired as a heterodimer with one of the Rel proteins. All NF- $\kappa$ B proteins are capable of homodimerization or heterodimerization with the other NF- $\kappa$ B proteins with the excep-

tion of RelB, which can only form heterodimers. Though most NF- $\kappa$ B dimer combinations result in the regulation of similar sets of genes, the ability to interact in various homo- and hetero- dimer configurations contributes to their ability to bind with varying affinities to  $\kappa$ B sites in distinct DNA sequences, and they thus regulate unique, as well as overlapping, gene sets.

Given this diversity, it is therefore not surprising that a substantial variety of mechanisms have arisen to regulate the DNA binding activity of various NF- $\kappa$ B homo- and hetero- dimers. In general, NF- $\kappa$ B activity is principally regulated by the I $\kappa$ B proteins, which, like p100 and p105, possess ankyrin repeats and are generally inhibitory of DNA binding. Three of these proteins are considered “typical” I $\kappa$ Bs, namely I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ . These proteins bind to NF- $\kappa$ B proteins and mask their DNA binding domains. They also possess potent nuclear export signals (NES) and generally remove NF- $\kappa$ B proteins from the nucleus, and are thus inhibitory in multiple ways. Two other I $\kappa$ Bs, I $\kappa$ B $\zeta$  and Bcl-3, are considered “atypical”. They are found in the nucleus, are inducibly expressed, bind only to p50 and p52 homodimers, and under certain circumstances may act to repress or to activate these homodimers. The activity of the typical I $\kappa$ Bs is controlled through phosphorylation by upstream I $\kappa$ B kinases (IKKs).

Although there are many ways of activating NF- $\kappa$ B, two main signaling pathways have been described that lead to the activation of NF- $\kappa$ B target genes. These are

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referred to as the canonical (or classical) and noncanonical (or alternative) pathways [1, 2, 4, 5]. These two pathways may usually be distinguished by whether the p50 product of p105 (canonical) or p52 product of p100 (non-canonical) is involved. Since p50 is frequently associated with RelA and p52 is frequently associated with RelB, the regulation of these two NF- $\kappa$ B heterodimers has been the most studied and they are considered the prototypical heterodimers for the canonical and noncanonical pathways, respectively.

The canonical NF- $\kappa$ B pathway is activated mostly by the stimulation of proinflammatory receptors, such as the TNF Receptor superfamily, the Toll-Like receptor family (TLRs), and by cytokine receptors for the Interleukins. It is also activated by genotoxic agents as well. Upstream receptors that activate the canonical signaling pathway typically activate an IKK complex consisting of IKK $\alpha$  and IKK $\beta$ , which are the catalytic kinases, and IKK $\gamma$  (also known as NEMO), which acts as a regulatory subunit. Recruitment and activation of this IKK complex is usually dependent on various TNF Receptor Associated Factor (TRAF) family proteins and sometimes on RIP kinases. The p105 (*nfkb1* gene product) is constitutively processed by the proteasome into p50, which is held inactive as a heterodimer with RelA (or c-Rel) by its interaction with the inhibitory I $\kappa$ B proteins. (I $\kappa$ B $\alpha$  has been the most studied). Phosphorylation of I $\kappa$ B $\alpha$  on serines 32 and 36 by the IKK complex (primarily IKK $\beta$ ) targets it for ubiquitination. Subsequently the ubiquitinated I $\kappa$ B $\alpha$  is degraded by the proteasome and this unmasks the DNA binding activity of the p50/RelA heterodimer and also allows it to translocate to the nucleus where it can bind to  $\kappa$ B sites and activate gene transcription [1, 2, 4, 5].

Noncanonical NF- $\kappa$ B activation is stimulated by specific TNF receptor family members that signal through the recruitment of TRAF2 and TRAF3. These include LT $\beta$ R, CD40, CD27, CD30, BAFF-R, RANK, and others [6-8]. The upstream kinase in the noncanonical pathway is the NF- $\kappa$ B-inducing kinase (NIK). Continual degradation of NIK in resting cells prevents constitutive activation of the noncanonical NF- $\kappa$ B pathway [9]. Degradation of NIK is mediated by a complex between TRAF3, TRAF2, and cIAPs 1 and 2, which ubiquitinates NIK, targeting it for proteosomal degradation, and the degradation of TRAF2 or TRAF3 by receptor-stimulated processes prevents NIK degradation by this complex and results in NIK stability [10, 11]. Stabilization of NIK results in the stimulation of downstream noncanonical signaling events.

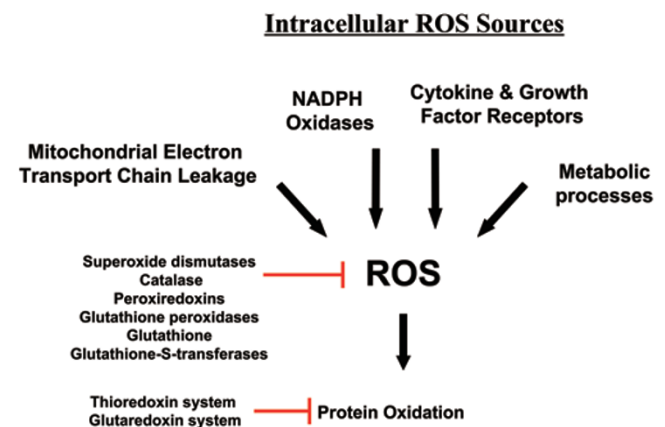
In contrast to the canonical pathway, p100 is processed to p52 only after the noncanonical pathway is stimulated. The ankyrin-repeat inhibitory portion of unprocessed

p100 therefore acts like a negative regulator by inhibiting DNA binding and nuclear localization of the NF- $\kappa$ B heterodimer. Thus p100 processing is a critical step in the noncanonical NF- $\kappa$ B signal pathway. Processing of p100 is triggered by its phosphorylation, which, unlike in canonical signaling, is dependent on an IKK complex made of homodimeric IKK $\alpha$ . IKK $\alpha$  is in turn activated by its phosphorylation by the recently stabilized NIK. The phosphorylation of p100 by IKK $\alpha$  on its C-terminus targets it for ubiquitination and partial degradation by the proteasome [6, 7, 12], thus freeing p52 and its hetero- or homo-dimeric partner to bind to DNA in the nucleus and affect transcription.

## Reactive oxygen species (ROS)

There are many cellular sources of reactive oxygen species (ROS) within a cell (Figure 1). These sources can be broadly divided into two main categories. Firstly, there are those biological processes that release ROS as a byproduct, or a waste product, of various other necessary reactions and secondly, there are those processes that generate ROS intentionally, either in molecular synthesis or breakdown, as part of a signal transduction pathway, or as part of a cell defense mechanism.

In the first category, the mitochondria are in a large measure the greatest source of ROS, since the reactions that occur during oxidative phosphorylation processes frequently lose electrons during their transfer between



**Figure 1** Intracellular Sources of ROS. The mitochondria are a major source of ROS, especially through electron leakage from Complexes I and III. ROS are also produced by NAD(P)H oxidases, sometimes in response to cytokines and other growth factor receptors, which may also utilize other pathways to produce ROS for use in their signaling pathways. Lastly, metabolic enzymes often create ROS as side products or through nonspecific reactions.

electron transport chain complexes. These electrons react with molecular oxygen to produce ROS. In consequence of this, the toxic buildup of ROS and cellular oxidation is usually alleviated by enzymes such as the superoxide dismutases, catalase, and peroxiredoxins, as well as systems of antioxidants and their associated enzymes, such as the thioredoxin and glutathione systems (Figure 1) [13-15]. These systems not only serve to repair oxidative damage, but also contribute to the overall response of the cell to ROS by acting as oxidative sensors in signal transduction pathways. For instance, thioredoxin-1 oxidation has been proposed to serve in translating information on the redox state of the cell into ASK1 kinase activity through various mechanisms [16-20].

In the second category of ROS sources are many enzymes that generate ROS for diverse purposes, although none more robustly than the phagocytic NADPH oxidase, NOX2 (gp91), which uses NADPH to reduce molecular oxygen, thus producing superoxide [21, 22]. This superoxide is typically used as a defense tool against infectious pathogens, and is converted in phagosomal compartments by superoxide dismutase and myeloperoxidase to hypochlorous acid (HOCl), which is a potent microbicidal compound [23]. However, during this process, some leakage of ROS from the phagosome occurs; and they enter the cytosol, contributing to the oxidative stress of the cell. In addition to NOX2, other NADPH oxidases of this family have been characterized in many cell types [21, 22], though the family members typically produce ROS less robustly. In addition to their cell defense function, local recruitment of NADPH oxidases, including non-phagocytic oxidases, has more recently been implicated in the production of ROS by growth factors and cytokines, though some of these may produce ROS by other means [24]. Consequently, these ROS are generated intentionally by the cell to function specifically within signaling pathways. Likewise, other ROS-generating enzymes, such as lipoxygenases and cyclooxygenases, create ROS to function within specific catabolic or anabolic processes, and typically produce substantially less ROS than NADPH oxidases. Thus, there are many different potential intracellular sources of ROS (Figure 1), much of which is capable of influencing, or being influenced by, NF- $\kappa$ B activity.

### NF- $\kappa$ B: Protecting from ROS

As mentioned previously, ROS are toxic in cells at certain levels, due to the oxidative stress they exert by their reaction with proteins, lipids, and nucleic acids. The correct cellular response to ROS production is consequently critical in order to prevent further oxidative damage, and

to maintain cell survival. However, when too much cellular damage has occurred, it is to the advantage of a multicellular organism to remove the cell for the benefit of the surrounding cells. Reactive oxygen species can therefore trigger both apoptotic and necrotic cell death depending on the severity of the oxidative stress [25-27]. Although there are a few exceptions where NF- $\kappa$ B contributes to cell death [28], in most cases the expression of NF- $\kappa$ B target genes typically promotes cellular survival. Therefore it is not surprising that ROS would modulate an NF- $\kappa$ B response and that NF- $\kappa$ B target genes would attenuate ROS to promote survival. One of the main signaling pathways that intersects with NF- $\kappa$ B with regard to ROS and cell death is the crosstalk that occurs between NF- $\kappa$ B and JNK. Crosstalk from NF- $\kappa$ B to JNK is known to prevent sustained JNK activation and thus prevents cell death through both apoptosis and necrosis [29-31]. This extensive crosstalk occurs in multiple ways, and has been reviewed elsewhere [32-36].

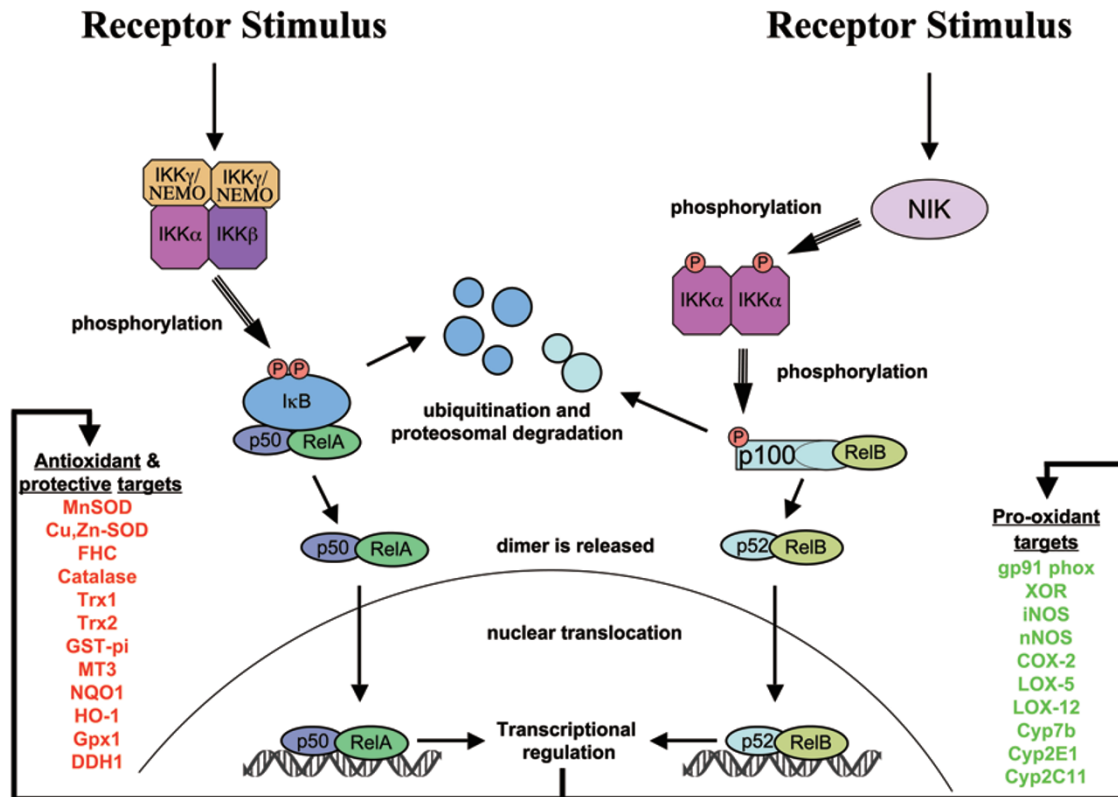
### Antioxidant NF- $\kappa$ B targets

One of the most important ways in which NF- $\kappa$ B activity influences ROS levels is via increased expression of antioxidant proteins (Figure 2). Here we discuss a few of the known as well as proposed NF- $\kappa$ B targets that may contribute to protection from ROS.

**Manganese Superoxide Dismutase (MnSOD, or SOD2)** is perhaps the most famous of NF- $\kappa$ B targets with antioxidant activity, due to numerous studies [37-40]. MnSOD is a mitochondrial enzyme that protects cells from oxidative stress by converting  $\cdot\text{O}_2^-$  into  $\text{H}_2\text{O}_2$ . Mice lacking MnSOD die perinatally after birth due to massive oxidative stress [41]. It is down-regulated in many oxidative diseases [42], and may be up-regulated in some cancers [43].

Likewise, its cytoplasmic relative, **Copper-Zinc Superoxide Dismutase (Cu,Zn-SOD, or SOD1)** has been shown to be an NF- $\kappa$ B target in at least one study [44]. It catalyzes a similar reaction, causing the dismutation of  $\cdot\text{O}_2^-$  into  $\text{H}_2\text{O}_2$ . SOD1-deficient mice have a shortened life span, have persistent oxidative damage and develop hepatocellular carcinoma [45].

**Ferritin Heavy Chain (FHC)** is the second-most well-known NF- $\kappa$ B target that protects from oxidative damage [46]. An iron storage protein, FHC does not directly scavenge ROS, but protects the cell from oxidative damage by preventing iron-mediated generation of highly reactive  $\cdot\text{OH}$  radicals from  $\text{H}_2\text{O}_2$  (Fenton reaction). Thus FHC may synergize with MnSOD to rid the cell of ROS by preventing the generation of more highly reactive species ( $\cdot\text{O}_2^-$  and  $\cdot\text{OH}$ ) and promoting the breakdown of



**Figure 2** Activation of NF-κB and regulation of downstream transcriptional antioxidant and pro-oxidant targets. NF-κB is activated primarily by two pathways: the canonical pathway (shown on left) and the noncanonical pathway (shown on right). Downstream binding of the NF-κB proteins to DNA regulates downstream transcriptional targets. Shown are many potential antioxidant and pro-oxidant targets that have been proposed in the literature.

H<sub>2</sub>O<sub>2</sub> into water by peroxidases and catalases [47].

Little evidence exists for the regulation of *Catalase* by NF-κB. However, one report suggests that this is the case [48], and one other study suggests that catalase could be the target of inhibitory p50 homodimers, since its promoter is bound by p50 in unstimulated cells and catalase is down-regulated when canonical NF-κB activation occurs [49].

While the evidence is not yet overwhelming, both thioredoxins, *Thioredoxin-1 (Trx1)* and *Thioredoxin-2 (Trx2)*, two of the most important cellular antioxidants in the cell, have been reported to be regulated by NF-κB [38, 39]. Thioredoxins protect from oxidative stress by means of their 2-cysteine active site that reacts with ROS and is also able to reduce oxidized proteins. They also serve as hydrogen donors to the thioredoxin-dependent peroxide reductases. Trx1 is expressed in the cytoplasm and nucleus. Inactivation of Trx1 in mice also results in early embryonic lethality [50]. Trx2 is localized within the mitochondria and is also indispensable for cell survival [51, 52]. Deletion of Trx2 causes massive apoptosis due to the accumulation of intracellular ROS, resulting in early

embryonic lethality in homozygous mice [52].

*Glutathione S-transferase pi (GST-pi)* is up-regulated by oxidative stress through NF-κB [53]. GST-pi is a phase II enzyme that catalyzes the reaction of the GSH thiolate to toxic electrophilic compounds, thus allowing highly reactive carcinogens or radicals to be eliminated by excretion machinery [54, 55]. It also is proposed to contribute to the repair of damage from oxidative stress. Disruption of the gene encoding GST-Pi in HCT116 cells showed that GSTP1 protects HCT116 cells from oxidative stress and resultant apoptosis under growth-limiting conditions [56].

*Metallothionein-3 (MT3)* has been shown to be an NF-κB target in keratinocytes and fibroblasts [57]. Metallothioneins are low-molecular-weight, cysteine-rich proteins which bind to many different metals [58]. In addition to regulating metal toxicity, the cysteine residues in metallothioneins can scavenge O<sub>2</sub><sup>-</sup> and OH radicals [59].

*NAD(P)H dehydrogenase [quinone] 1 (NQO1)* is an NF-κB target that is activated in response to the DNA crosslinking agent mitomycin C [60]. This FAD-binding

protein is a cytoplasmic 2-electron reductase that reduces quinones to hydroquinones. Since it is a 2-electron reductase, its enzymatic activity prevents the one electron reduction of quinones that produces radical species [61]. Interestingly, NQO1 deletion also prevents the activation of NF- $\kappa$ B [62].

**HO-1** is a heme oxygenase that is up-regulated by NF- $\kappa$ B [63-65] and other transcription factors in response to oxidative stress and hypoxia. Heme oxygenase-1 catalyzes heme degradation, resulting in the formation of carbon monoxide and biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase [66]. Since bilirubin is a potent antioxidant, it is thought that HO-1 is therefore protective from oxidative stress.

**Glutathione peroxidase-1 (Gpx1)** is an abundant cytoplasmic enzyme that catalyzes the conversion of H<sub>2</sub>O<sub>2</sub> into water using glutathione as a substrate [67]. It is one of the most important members of antioxidant proteins. Although it prefers H<sub>2</sub>O<sub>2</sub> as a substrate, it also can reduce lipid peroxides [67], as well as peroxynitrite [68]. In skeletal muscles cells, glutathione peroxidase is up-regulated by NF- $\kappa$ B in response to oxidative stress. The glutathione peroxidase promoter is bound by all five of NF- $\kappa$ B subunits in U937 cells in response to LPS, signifying an important NF- $\kappa$ B target [49].

**Dihydrodiol dehydrogenase (DDH1 or AKR1C1)** is one of many dehydrogenase enzymes regulated by NF- $\kappa$ B [69]. DDH1 is a phase-2 aldoketo reductase and oxidizes transdihydrodiols of polycyclic aromatic hydrocarbons. Like many phase-2 enzymes that activate toxic compounds to eliminate them from the body, the reactive products downstream of its reaction have been associated with induction of ROS [70]. However, ectopic expression of DDH1 has been shown to lower the basal levels of ROS in some cell types, suggesting that DDH1 can act as a protective enzyme [71].

### Pro-ROS NF- $\kappa$ B targets

Since NF- $\kappa$ B is important in inflammation, some enzymes that promote the production of ROS are also regulated as its targets, especially in cells of the immune system. Below, we discuss a few involved in the generation of ROS.

During the inflammatory process, expression of the phagocytic **NADPH oxidase NOX2 (gp91 phox)** is dependent on, and induced by, NF- $\kappa$ B [72]. As mentioned previously, NADPH oxidase enzymes are specifically devoted to the production of ROS. NADPH oxidases use NADPH to produce superoxide, which is used in immune defenses, and also is used for cell signaling.

An enzyme that exists in two intraconvertible forms

that catalyze either reduction or oxidation reactions, **Xanthine Oxidase/Dehydrogenase (XOR, or Xanthine Oxidoreductase)** is regulated by NF- $\kappa$ B [73]. The dehydrogenase form is the most dominant form *in vivo*, however it may be converted to the oxidase form through the oxidation of its protein sulphhydryl groups [74]. XOR typically catalyzes the interconversion of Xanthine and Urate with NAD<sup>+</sup> and water as cofactors. However, the enzyme has very low specificity, and transfer of electrons to O<sub>2</sub> instead of NAD<sup>+</sup> results in the generation of superoxide and hydrogen peroxide [75]. ROS production from XOR is implicated in several pathological conditions, including heart failure [75].

The **Inducible Nitric Oxide Synthase, or iNOS (NOS2)** is heavily upregulated by NF- $\kappa$ B [76-79] and **Neuronal Nitric Oxide Synthase, or nNOS (NOS1)** is also an NF- $\kappa$ B target [80, 81]. While technically nitric oxide synthases actually produce a reactive nitrogen species (i.e., nitric oxide, or NO) and not reactive oxygen species, we mention them here because NO is often produced where it can react with superoxide leading to formation of the highly reactive peroxynitrite. While peroxynitrite itself is highly reactive as both an oxidant and nitrating agent, it also reacts with CO<sub>2</sub> to form Nitrosoperoxycarbonate (ONOOCO<sub>2</sub><sup>-</sup>), which then homolyzes to form carbonate (CO<sub>3</sub><sup>·-</sup>) and nitrogen dioxide radicals (NO<sub>2</sub><sup>·</sup>). Peroxynitrite can cause various kinds of cellular damage, including damage to DNA, and can activate cell death pathways [82]. At low levels, peroxynitrite, and its resultant radicals may participate in signal transduction pathways, in a large part by tyrosine nitration [83]. Thus the expression of nitric oxide synthases can potentiate ROS damage as well as signaling.

**Cyclooxygenase-2 (COX-2, also known as Prostaglandin G/H synthase 2)** is a well-known NF- $\kappa$ B target involved in inflammation [84, 85] that converts arachidonic acid into prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) by a free radical mechanism involving a protein tyrosyl radical generated by cooperation from a heme prosthetic group [86]. During the second step of the reaction that produces PGH<sub>2</sub>, superoxide is also generated [86]. Thus, superoxide is a side product of this reaction, and may contribute to oxidative stress as well as signaling.

Other enzymes, in addition to COX-2, that generate ROS during arachidonic acid metabolism have also been reported to be NF- $\kappa$ B targets. Among these are **arachidonate 12-lipoxygenase (LOX-12, or ALOX12)** [87] and **arachidonate 5-lipoxygenase (LOX-5, or ALOX5)** [88]. As with COX-2-mediated reactions, oxidized metabolites and byproducts of these enzymatic reactions contribute to ROS within the cell [89, 90]. In addition, the metabolic products of LOX-12 and LOX-5, 12(S)-hydroxyeicosa-

tetranic acid and leukotriene B<sub>4</sub>, respectively, have been shown to activate and induce NADPH oxidases [91].

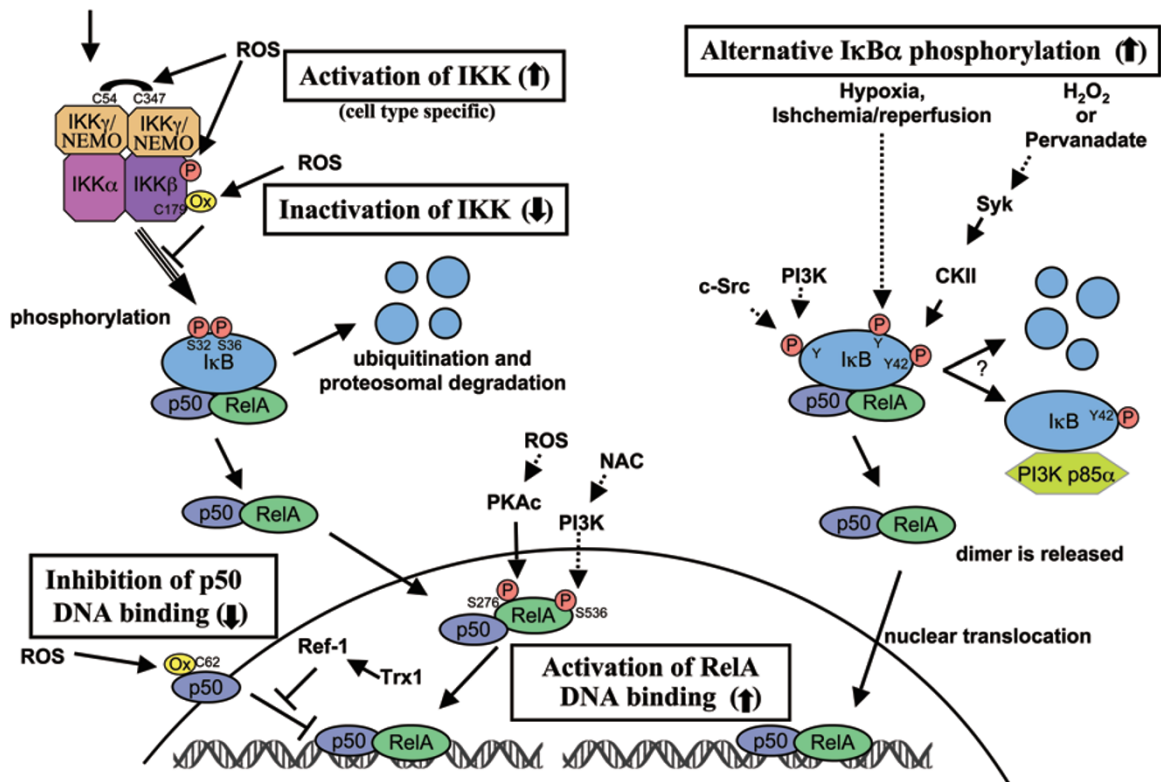
**Cytochrome p450 enzymes**, which are phase I enzymes that detoxify toxic compounds, have long been known to produce ROS when uncoupled, particularly H<sub>2</sub>O<sub>2</sub> and hydroxyl radicals [92-95]. *Cyp2E1*, *Cyp2C11*, and *Cyp7b* are all known to have NF- $\kappa$ B promoter elements. *Cyp2E1* and *Cyp2C11* are both down-regulated by pro-inflammatory cytokines [96, 97], suggesting a negative regulation by NF- $\kappa$ B, while *Cyp7b* is up-regulated [98]. Both *Cyp2E1* and *Cyp2C11* are known to be able to produce ROS through uncoupled reactions [95, 99-101].

### Influence of ROS on NF- $\kappa$ B activation

Having examined some of the transcriptional targets of NF- $\kappa$ B that affect ROS amounts within the cells, let us

now turn to the ways in which ROS affect the activity of NF- $\kappa$ B (Figure 3). A difficulty in defining ROS contributions to signaling is that ROS can often function in multiple places (i.e. upstream or downstream) within a given pathway, and sometimes in opposing ways (i.e. inhibitory or stimulatory). Such seems to be the case with regards to ROS functions in the NF- $\kappa$ B pathway. For instance ROS often stimulates the NF- $\kappa$ B pathway in the cytoplasm, but inhibits NF- $\kappa$ B activity in the nucleus [102]. Thus, overexpression of the antioxidant protein TRX1 was shown to diminish NF- $\kappa$ B activation by inhibiting I $\kappa$ B degradation [103], but others have shown that TRX1 translocation to the nucleus during TNF or PMA stimulation serves to enhance NF- $\kappa$ B DNA binding [104, 105]. ROS have been reported to both activate and to repress NF- $\kappa$ B signaling. While many of the differences noted in the literature are probably due to the use of different methodology, many of the differences are also attributed

### Receptor Stimulus



**Figure 3** Crosstalk of ROS with NF- $\kappa$ B signaling pathways. ROS interacts with NF- $\kappa$ B at various places within the signaling pathway. Many of these interactions occur in a cell type-specific manner. ROS has been proposed to both activate and inactivate the IKK complex leading to an effect on the downstream targets. Often ROS has been shown to activate NF- $\kappa$ B through alternative I $\kappa$ B $\alpha$  phosphorylation, which may or may not result in the degradation of I $\kappa$ B $\alpha$ . Lastly, ROS may influence the DNA binding properties of the NF- $\kappa$ B proteins themselves. Oxidation of p50 on its DNA binding domain has been shown to prevent its DNA binding, and must be reversed in the nucleus by a Trx1-dependent process involving Ref-1. On the other hand, the phosphorylation of RelA that is influenced by ROS-dependent processes leads to greater NF- $\kappa$ B activation.

to the study of different upstream pathways and cell-specific differences.

What are the targets of ROS in the NF- $\kappa$ B pathway? The best characterized way in which ROS affects signaling is through its reaction with cysteine, especially at an enzyme's catalytic sites, where the cysteine has a low pKa and exists in the thiolate form [106]. A primary example is that of protein tyrosine phosphatases, which have been shown to be inactivated by ROS through oxidation of catalytic cysteines [107-109]. ROS has also been shown to inactivate dual specificity phosphatases [110], which dephosphorylate phospholipids, in addition to both phospho-tyrosine and phospho-serine/threonine residues. In the absence of phosphatase activity, prolonged phosphorylation stimulates the activity of kinases and other enzymes within the cell.

Initial oxidation of cysteines, resulting in sulfenic acid is usually reversible by the cellular antioxidant machinery, but further oxidation to sulfinic and then to sulfonic acids results in irreversible inactivation of the phosphatases by ROS [107, 111]. Sulfenic acid is usually unstable and may react with cellular glutathione to form a disulfide bond resulting in an S-glutathionated protein. While a S-glutathionated enzyme is often still inactive, it may then be reduced to its normal state by glutaredoxin in the cytoplasm. If another cysteine instead of glutathione is in close proximity to a recently oxidized cysteine in the form of sulfenic acid, an intramolecular disulfide bond can form, possibly leading to a change in protein conformation and thus preventing or initiating protein activity.

### Direct regulation of NF- $\kappa$ B heterodimers by ROS

Direct oxidation of NF- $\kappa$ B by ROS inhibits its DNA binding ability [112]. A specific cysteine of p50 is especially sensitive to oxidation. This cysteine, Cys-62 is in the RHD and therefore its oxidation inhibits DNA binding [113-115]. This oxidation is probably followed by an S-glutathionylation event since glutathionated NF- $\kappa$ B has been shown to have less transcriptional activity [116, 117]. This oxidation may be selectively reduced and the p50 DNA binding restored by a nuclear enzyme that is associated with base excision repair called Ref-1 (also called APE1) [118], apparently in part through its direct interaction with TRX1 [104, 119]. This same cysteine may likewise be S-nitrosylated by NO [120], which is produced by the iNOS protein that is up-regulated as an NF- $\kappa$ B target, thus acting as a negative feedback loop [121].

There are other more indirect ways that ROS influences DNA binding of NF- $\kappa$ B proteins. The phosphory-

lation of RelA on Ser-276 is required for expression of a subset of NF- $\kappa$ B-dependent genes [122]. Phosphorylated Ser-276 is necessary for the interaction of RelA with CBP/300 [123], as well as the positive transcription elongation factor b [122]. PKAc mediates phosphorylation of Ser-276 [123, 124], and this event is thought to be dependent on ROS based on a variety of reasons [125], among which is the finding that anti-oxidant treatment inhibits Ser-276 phosphorylation and CBP/300 binding [126].

Aside from the regulation of Ser-276 phosphorylation by ROS, Ser-536 phosphorylation of RelA is induced by NAC through a PI3-kinase-mediated mechanism in a variety of cell lines and this event contributes to its DNA-binding activity [127].

### ROS regulation of upstream NF- $\kappa$ B activating pathways.

Exogenously added H<sub>2</sub>O<sub>2</sub> regulates NF- $\kappa$ B activation, and it does so in part through alternative phosphorylation of I $\kappa$ B $\alpha$  (Figure 3). While typically I $\kappa$ B $\alpha$  is usually phosphorylated on serines 32 and 36, which leads to its ubiquitination and degradation, H<sub>2</sub>O<sub>2</sub> affects the phosphorylation of I $\kappa$ B $\alpha$  on Tyr42 or other tyrosine residues, and I $\kappa$ B $\alpha$  may or may not be degraded as part of the process [128-131]. Although IKK is phosphorylated, IKK is not required in this case, and I $\kappa$ B $\alpha$  phosphorylation may be mediated by casein kinase II, possibly downstream of Syk [129, 130]. Although this event is observed in response to exogenous H<sub>2</sub>O<sub>2</sub>, there is reason to believe that alternative phosphorylation and inactivation of I $\kappa$ B $\alpha$  occurs under physiological conditions because it is also observed under conditions of pervanadate treatment, indicating a possible role for ROS in inhibiting phosphatases, and has also been observed during hypoxia, reoxygenation following hypoxia and in ischemia/reperfusion injury *in vivo* [132-138]. Degradation of I $\kappa$ B $\alpha$  may not be necessary in this case because Tyr42-phosphorylated I $\kappa$ B $\alpha$  is bound by the SH2 domains of p85 $\alpha$  regulatory subunit of PI3K, thus unmasking NF- $\kappa$ B and allowing it to translocate to the nucleus [136]. PI3K as well as c-Src has been implicated in alternative tyrosine phosphorylation of I $\kappa$ B $\alpha$  [132, 134, 136].

ROS modification of I $\kappa$ B $\alpha$  has also been shown to lead to inhibition of NF- $\kappa$ B activation. Glutathionylation of I $\kappa$ B $\alpha$  has been detected at cysteine 189, thus preventing phosphorylation events and subsequent degradation [139]. However, most of the inhibitory action of ROS on NF- $\kappa$ B with respect to I $\kappa$ B $\alpha$  has been tied to I $\kappa$ B $\alpha$  stability due to the inhibition of the proteasome [140].

IKK is another primary target for ROS in influencing NF- $\kappa$ B signaling. H<sub>2</sub>O<sub>2</sub> has been shown in some cells to

inactivate IKK [141-144]. An inhibitory effect may be mediated by ROS oxidation of IKK $\beta$  on cysteine 179, since it is found to be S-glutathionated upon exposure to ROS, thus inactivating its kinase activity [142] and leading to a reduction in NF- $\kappa$ B signaling. Deficiency of glutaredoxin 1, which repairs S-glutathionation, leads to reduced nuclear translocation of RelA and substantial loss of NF- $\kappa$ B binding [142]. IKK inactivation through oxidation of Cys-179 of IKK $\beta$  has also been shown upon arsenite treatment [145] and this cysteine residue is also a target of S-nitrosylation by nitric oxide [146]. More importantly, Cys-179 is the oxidation target of anti-inflammatory cyclopentone prostaglandins, PGA and 15d-PGJ<sub>2</sub> [147], suggesting that oxidation of this residue regulates IKK in physiological settings.

Conversely, some studies have shown that ROS, in particular, H<sub>2</sub>O<sub>2</sub>, can activate IKKs in some cell types. Dimerization of IKK $\gamma$ /NEMO was potentiated by H<sub>2</sub>O<sub>2</sub> through formation of disulfide bonds between Cys54 and Cys 347, thus implicating the NEMO subunit in positive regulation by ROS [148], though the other IKK subunits were likely inhibited. Another study reported activation of IKK by H<sub>2</sub>O<sub>2</sub>, but in this report NF- $\kappa$ B activation was shown to be inhibited due to lack of I $\kappa$ B $\alpha$  degradation despite its ubiquitination, suggesting that the proteasome was also inhibited [149]. One study showed that phosphorylation of both catalytic IKK subunits was potentiated by H<sub>2</sub>O<sub>2</sub> [150], which may suggest inhibition of an IKK phosphatase.

Kinases upstream of IKK could be potentially regulated by ROS. MEKK1 was originally suggested to play a role in NF- $\kappa$ B activation. Since it is a redox-sensitive kinase that is inactivated by glutathionylation at C1238, this could potentially be a link to ROS inactivation of IKK in some cells [151]. However, an essential role for MEKK1 in IKK activation has been dismissed based on findings in MEKK1-deficient cells [152], and MEKK3, which may be required instead of MEKK1 [153, 154], lacks the cysteine that makes MEKK1 a redox-sensitive kinase [155]. To our knowledge, TAK1, which has also been suggested to be required for canonical NF- $\kappa$ B activation [154, 156-159], is not known to be redox-regulated.

NIK, the upstream kinase in the noncanonical pathway is believed to be activated by ROS through inhibition of phosphatases [160]. NIK phosphorylation of IKK $\alpha$  is increased upon treatment of H<sub>2</sub>O<sub>2</sub> within a narrow range. However, given that ROS can sometimes inactivate the proteasome, the increased activity of NIK may be in part due to its increased stability as well since it is constitutively degraded by the proteasome.

One potential redox-regulated kinase that affects

IKK activation is Akt. Akt positively influences IKK $\beta$ -mediated NF- $\kappa$ B activation through the downstream activation of mTOR in association with Raptor [161, 162]. Akt has a kinase domain that is subject to oxidation events that inactivate the kinase activity by forming a disulfide bond between Cys-297 and Cys-311 [163], and thus ROS could prevent IKK activation by inhibiting Akt. However, not only is Akt itself regulated by ROS, but PTEN, an upstream inhibitor of Akt activation, has a catalytic cysteine that is oxidized by ROS, thus inactivating its phosphatase activity [164]. Thus, Akt can be regulated both positively and negatively by ROS.

## Conclusion

In summary, ROS interacts with NF- $\kappa$ B signaling pathways in many ways. The transcription of NF- $\kappa$ B-dependent genes influences the levels of ROS in the cell, and in turn, the levels of NF- $\kappa$ B activity are also regulated by the levels of ROS. Depending on the context, ROS can both activate and inhibit NF- $\kappa$ B signaling. A high degree of complexity characterizes ROS interactions with NF- $\kappa$ B pathways owing to the capability for ROS to act in many ways and at numerous places simultaneously. Another complication is that many ROS effects and interactions appear to be cell type-specific. Though we have learned much about the way that ROS influences signaling there is still doubtless a great deal that is yet to be elucidated.

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