

# NF- $\kappa$ B: no longer an island, but a piece of a continent

*Gioacchino Natoli*

**The Keystone meeting on NF- $\kappa$ B in Inflammation and Disease held in January covered several aspects of NF- $\kappa$ B regulation. Many new concepts emerged in signalling, transcriptional control and the involvement of NF- $\kappa$ B in disease, particularly cancer.**

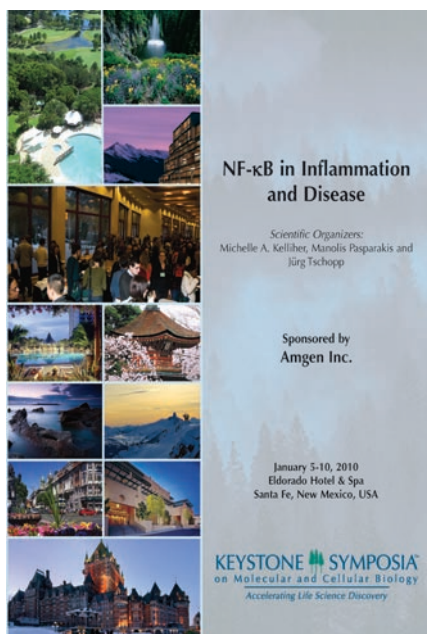
"No man is an island, entire of itself; every man is a piece of the continent, a part of the main."

*Meditation XVII, John Donne (1572–1631)*

When thinking about a suitable title to express the spirit of the last Keystone meeting on NF- $\kappa$ B, held in Santa Fe (New Mexico, USA) between 5 and 10 January 2010, I decided to borrow this citation from John Donne, which was quoted by Yinon Ben-Neriah (Hebrew U., Jerusalem) in his talk. Far from being an isolated field, the study of NF- $\kappa$ B regulation has moved from analysing the properties of individual members of this transcription factor family to several broad areas of interest that provide an extensive surface for scientific interaction. These areas encompass the transduction of signals from cellular receptors to the I $\kappa$ B kinase (Ikk) complex and the subsequent nuclear translocation of NF- $\kappa$ B; the interplay between inducible transcription factors and of these with chromatin and its regulators; and the role of these increasingly defined processes in diseases as diverse as cancer, metabolic disorders and infections. In several of these fields, the study of NF- $\kappa$ B has provided many exportable paradigms, and thus many scientists of the old guard were flanked by numerous new faces at this meeting, making it one of the most exciting and interdisciplinary on this topic in recent years.

## Signalling through ubiquitin and ROS

Several talks discussed the role of K48, K63 and linear ubiquitin (Ub) chains in the transduction of signals from the tumour necrosis factor receptors (TNF-Rs) and Toll-like receptors (TLRs) to the Ikk complex



(Hayden & Ghosh, 2008). The emerging model is that the use of Ub chains differs between the TNF-R and TLR pathways. In TNF-R1 signalling, the ring-finger protein Traf2 is recruited by the adaptor TRADD, thus providing a scaffold for the recruitment of the protein kinase Rip1 and the Ub ligases cIAP1 and cIAP2. Although Traf2 was proposed initially to act as an E3 ligase that generates signalling-competent K63 chains, structural data by Hao Wu (Cornell U.), indicate that it might not be able to interact with E2 enzymes, and thus might not be a functional E3. Conversely, cIAPs could be responsible for the ubiquitination of Rip1, the kinase activity of which is dispensable for signalling—as reported by Michelle

Kelliher (U. Massachusetts Medical School). She also showed that Rip1 has an essential lysine for anchoring Ub chains, which are then bound by the Tak1-binding proteins Tab2/3 and the Ub binding domain of Nemo/Ikk $\gamma$ —known as NUB or UBAN. The selectivity of Tab2 for K63-linked chains is supported by the structure of its Ub binding domain in complex with K63-linked di-Ub and tri-Ub, which was described by Dave Komander (Medical Research Council, Cambridge, UK). This process brings Tak1 and Ikk into close proximity, resulting in Ikk activation. As shown by Vishva Dixit (Genentech, San Francisco), K63 chains are attached to Rip1 by cIAPs in the first 5 min after stimulation, when initial signalling events occur, and are then replaced with K48 chains, which provide a proteasomal targeting signal. This transition is catalysed by A20, an enzyme with both deubiquitinase and E3 ligase activities: a single zinc (Zn) finger in A20 was found to be required for both E3 ligase activity and for the recognition of K63 chains.

In the TLR/interleukin-1 receptor (IL-1R) signalling pathway, the ring-finger protein Traf6 synthesizes K63 chains by using the E2 Ubc13 as a partner. James Chen (U. Texas Southwestern Medical Center) reported the provocative finding that free K63 Ub chains released by Traf6 might be able to activate downstream signalling, acting as second messengers. These chains would be recognized selectively by Tab2, leading to Tak1 activation and stimulation of adjacent Ikk molecules recruited through the NUB/UBAN domain of Nemo. In this model, the selective activation of this pathway by the free Ub chains is guaranteed by the direct interaction

between Traf6 (bound to the receptor) and Tab2 (bound to Tak1).

The relative role of K63-linked and linear Ub chains was a matter of debate, as mice lacking *Ubc13*, a component of the K63-specific E2 complex, surprisingly showed normal NF- $\kappa$ B activation in response to various agonists (Yamamoto *et al*, 2006). Kazu Iwai (Osaka U.) discussed the generation of linear Ub chains—in which the carboxy-terminal glycine of an Ub molecule is conjugated to the amino-terminal methionine of another Ub moiety—by the linear Ub chain assembly complex (LUBAC; Kirisako *et al*, 2006) and their role in NF- $\kappa$ B signalling. Enforced expression of LUBAC is sufficient to activate NF- $\kappa$ B in a manner that depends on direct binding of Nemo, to which LUBAC could attach linear poly-Ub. In turn, the recognition of linear Ub by the NUB/UBAN domain of another Nemo molecule would lead to dimerization and kinase activation. LUBAC activates NF- $\kappa$ B in mouse embryonic fibroblasts lacking *Ubc13*, and the deletion of a LUBAC component reduces the expression of NF- $\kappa$ B target genes, supporting a role for linear Ub chains in Ikk activation. Ivan Dikic (Goethe U., Frankfurt) showed the structure of the NUB/UBAN domain of Nemo in combination with linear di-Ub. This UBAN domain has a 100-fold higher affinity for linear chains than for K63-linked di-Ub. Even when binding to longer chains was analysed, Nemo preferentially bound linear Ub chains over K63-linked ones. The UBAN domain of Nemo forms a coiled-coil homodimer that associates with two linear di-Ub molecules, one for each monomer, and Ub binding results in conformational changes that might be involved in Ikk activation—possibly in a Tak1-independent manner. Furthermore, mutations in Nemo that mediate the association with linear Ub impair NF- $\kappa$ B activation in response to multiple signals. Two additional points were raised in the discussion: the affinity of the UBAN domain for K63-linked Ub is increased strongly by the C-terminal Zn finger of Nemo, which is required for signalling (Laplantine *et al*, 2009); and the lack of phenotype of the *Ubc13* deletion on the TLR/IL-1R signalling pathway (Yamamoto *et al*, 2006) could reflect an incomplete deletion, as a more complete deletion had strong effects on TAK1 ubiquitination and Ikk activation (Yamazaki *et al*, 2009).

Signalling to NF- $\kappa$ B in response to DNA damage involves the sumoylation of nuclear Nemo by the SUMO E3 ligase Piasy, followed

by its ataxia telangiectasia-mutated (ATM)-mediated phosphorylation, ubiquitination and nuclear export (Huang *et al*, 2003). In the cytoplasm, Nemo binds to and activates Ikk1/2, thereby leading to NF- $\kappa$ B activation. Claus Scheidereit (Max Delbrück Center Berlin-Buch) showed a requirement for the generation of poly-ADP ribose (PAR) chains by Parp1 for NF- $\kappa$ B activation in response to DNA damage. Automodified Parp1 assembles a PAR-dependent Nemo–Piasy–ATM complex within 30 min after exposure to a DNA-damaging agent. The formation of this complex requires a PAR-binding motif in Piasy. After complex formation, ATM reaches the cytoplasm and activates TRAF6, leading to the generation of K63-linked Ub chains and eventually activating Ikk. Shigeki Miyamoto (U. Wisconsin–Madison) showed that Nemo knock-in mice harbouring mutations in the sumoylation sites had selective defects in Ikk activation after DNA damage. He showed that Ikk activation in response to DNA damage is terminated through an NF- $\kappa$ B-regulated negative feedback involving a SUMO protease.

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An old-standing controversy in the field is whether reactive oxygen species (ROS)—which are generated rapidly in response to multiple stimuli—have a role in NF- $\kappa$ B activation (Hayakawa *et al*, 2003). Martin Krönke (U. Cologne) reported that riboflavin kinase (RFK) links TNF-R1—and some TLRs—to the ROS-generating NADPH oxidases. RFK is recruited to TNF-R1 after stimulation and acts as a bridge to NADPH oxidases. No direct recruitment to TLRs was found, although RFK was nonetheless required for ROS generation. Depletion experiments in cells and analyses of deficient mice both suggest that the generation of ROS through this pathway could be dispensable for signalling to Ikk but is required for activation of the inflammasome—a multiprotein complex responsible for the cleavage of pro-IL-1 $\beta$  and the production of its mature form (Martinon *et al*, 2009). These data are in agreement with those presented by Jurg Tschöpp (U. Lausanne), who

reported a thioredoxine-regulated mechanism of inflammasome activation. On oxidation, thioredoxin releases Txnip, which binds to and activates the Nlrp3 component of the inflammasome; lack of Txnip impairs Nlrp3 inflammasome activation. Txnip also controls glucose metabolism and is upregulated by high glucose in pancreatic  $\beta$ -cells—the activity of the inflammasome in these cells is induced by glucose, and neutralization of IL-1 $\beta$  improves metabolic control in diabetic patients.

### Transcriptional control

Several talks dealt with the mechanisms of transcriptional control by NF- $\kappa$ B, including the role of oscillations in NF- $\kappa$ B nuclear translocation, the role of alternative binding sites in the control of specific responses and the interplay with chromatin regulators. Sankar Ghosh (Columbia U., New York) reported the complex effects of a S276A mutation in the p65/RelA subunit—which disrupts binding to the acetyltransferase p300—on the induction of NF- $\kappa$ B-regulated genes. His data point to an unexpected complexity in the p300-mediated regulation of NF- $\kappa$ B target genes, with opposite responses at different genes. Gioacchino Natoli (European Institute of Oncology, Milan) presented a new strategy for the identification of inflammatory gene enhancers, which allowed the elucidation of novel organizational principles of these *cis*-regulatory elements. NF- $\kappa$ B, IRF and AP1 sites are combined in these enhancers with binding sites for factors that control tissue-specific expression. Another approach involving computational analyses of  $\kappa$ B sites and expression data in *p50*<sup>-/-</sup> cells allowed Christine Cheng (Alex Hoffmann Lab, U. California, San Diego) to define a dominant mechanism of cross-inhibition of interferon regulatory factor 3 (IRF3) binding exerted by p50 homodimers. These data, together with a comprehensive analysis of the binding specificity of different NF- $\kappa$ B dimers provided by Trevor Siggers (Bulyk Lab, Harvard Medical School), suggest that the information content of alternative  $\kappa$ B sites is much richer than expected, thus urging for more refined approaches to functionally characterize different classes of binding sites.

Oscillations in NF- $\kappa$ B activity—which are due to cycles of degradation and resynthesis of I $\kappa$ B $\alpha$ —were also discussed. Michael White (U. Liverpool) discussed the role of NF- $\kappa$ B oscillations at a single cell level in the control of target gene expression; his data showed that changes in oscillation frequency

alter the pattern of downstream gene expression. However, Alex Hoffmann reported that NF- $\kappa$ B-dependent gene transcription is normally activated in mutant cells without oscillatory behaviour. Hoffmann also reported a role of specific I $\kappa$ Bs in the generation of some NF- $\kappa$ B dimers, the formation of which would be disfavoured owing to the low affinity of the interacting monomers and might rely on the presence of a specific I $\kappa$ B.

The impact of chromatin regulators on the NF- $\kappa$ B transcriptional response was discussed by Ido Amit (Broad Institute, Boston), who described an innovative, systems-level, approach to identify and validate regulators that control specificity in the transcriptional response to pathogens. In turn, pathogens also control NF- $\kappa$ B-dependent gene expression by manipulating chromatin, a topic covered by Laurence Arbibe (Pasteur Institute). She showed that a virulence factor of *Shigella flexneri*, OspF, induces gene-specific inhibitory effects through an alteration of the chromatin state of target genes. OspF is a phosphatase that deactivates multiple MAP kinases, which might be sufficient to cause transcriptional effects; gene specificity could be acquired through the reported phosphothreonine lyase activity of OspF and the presence of a domain capable of interacting directly with histones.

### NF- $\kappa$ B and disease

The emerging links between NF- $\kappa$ B and cancer were covered by several talks in the meeting. David Baltimore (Caltech) discussed the role of two NF- $\kappa$ B-regulated microRNAs, miR-146a and miR-155, in tumorigenesis. miR-146a inhibits the expression of two signal transducers that are upstream of NF- $\kappa$ B: Traf6 and Irak1. *miR-146a*<sup>+</sup> mice overexpress Traf6 and Irak1, and suffer from both myeloid and lymphoid hyper-proliferation, which eventually leads to a seemingly lymphoid leukaemia, suggesting that this miRNA has tumour-suppressor activity. Conversely, miR-155, which is often highly expressed in lymphoid and myeloid leukaemia, does not inhibit the pathway but rather acts as an oncogene, promoting lymphomagenesis when overexpressed in B cells and myeloid proliferation when expressed in haematopoietic stem cells. The role of Traf family signal transducers in tumorigenesis was underscored in two other talks. Laura Pasqualucci (Columbia U., New York) reported that Trafs and additional components of the NF- $\kappa$ B signalling pathway—including A20

and Tak1—are mutated in more than 50% of activated B-cell subtype, diffuse large B-cell lymphomas. Mutations lead to hyperactivation of the NF- $\kappa$ B pathway, although the mechanisms involved are different in each case. Bill Hahn (Dana-Farber Cancer Institute, Boston) also discussed activating mutations and Traf2 amplification. Moreover, he showed the results of a short hairpin RNA-based synthetic lethality screening for the identification of transducers required for survival of cells expressing activated K-Ras. Tbk1, an Ikk-related kinase that is not part of the normal NF- $\kappa$ B signalling pathway, was found to be required for NF- $\kappa$ B activation and survival in response-activated K-Ras, thus suggesting that Tbk1 is co-opted to activate NF- $\kappa$ B in this context. A paralogue of Tbk1, Ikke was previously reported by the Hahn group to be a breast cancer oncogene (Boehm *et al*, 2007). Ikke interacts with Traf2 and might promote its transforming potential. It can substitute for Ikke in transformation assays, which is consistent with a model in which Traf2 acts downstream of Ikke.

### The emerging model is that the use of Ub chains differs between the TNF-R and TLR pathways

Several studies focused on mouse models of cancer with genetic alterations in the NF- $\kappa$ B pathway. Michael Karin (U. California, San Diego School of Medicine) reported a role of Ikk subunits in castration-resistant prostate cancer. His data point to a role of the B-cell infiltrate of the tumour in the release of cytokines—such as lymphotoxin A—that promote Ikk1 activation. Ikk1 in turn promotes the survival and growth of prostate epithelial cells in the presence of androgen receptor blockers. Yinon Ben-Neriah showed a complex interplay between constitutive activation of the Wnt pathway in *Ck1*<sup>+</sup> intestinal crypts, inflammation—probably driven by NF- $\kappa$ B—the DNA-damage response and senescence. Blunting the inflammatory response with a non-steroidal anti-inflammatory drug abolishes senescence, but if p21 (Cdkn1a) is expressed, tumour formation is prevented. Manolis Pasparakis (U. Cologne) discussed genetic mouse models harbouring Ikk-activating or inhibiting mutations in intestinal epithelia, and their consequences on gut inflammation and tumorigenesis. Balanced levels of NF- $\kappa$ B activity seem to be required to prevent

intestinal damage and cancer. Inder Verma (Salk Institute, La Jolla) suggested the use of advanced lentiviral vectors to transduce a small amount of cells in tissues and provide physiological models of tumorigenesis in the lung. By using this approach, he found that silencing p53 in pneumocytes in an *Ikk2*<sup>+</sup> background delays tumorigenesis.

The biological roles of the adaptor protein p62—which is a scaffold for atypical PKCs—in cancer and metabolic control were discussed by Jorge Moscat (U. Cincinnati). p62 is overexpressed in 60% of lung adenocarcinomas and is required for Ras-induced, Traf6-mediated NF- $\kappa$ B activation and lung tumorigenesis. Downstream of p62, the atypical PKCs exert opposite effects on oncogenesis, with PKC $\zeta$  acting as a tumour suppressor, in part through the repression of IL-6. Increased IL-6 production in PKC $\zeta$ <sup>+</sup> mice also promotes obesity and glucose intolerance, possibly owing to the metabolic actions of this cytokine. The link between NF- $\kappa$ B, inflammation and metabolic control was also discussed by Steve Shoelson (Harvard Medical School), who showed that obesity and a high-fat diet activate NF- $\kappa$ B in peripheral tissues and monocytes. This effect is reversed by the anti-inflammatory drug salicylate and its derivatives, which were found to decrease insulin resistance and reduce blood glucose levels, and are now being used in clinical trials to treat diabetes. Surprisingly, the effects of salicylate on insulin sensitivity were first reported in 1876 (!) but never exploited for therapeutic purposes.

As can be distilled from this report, this meeting was extremely lively and broadly covered several topics. The advances reported and the vibrant controversies that arose testify that this mature field is surprisingly vital, stimulating and able to attract scientists from many disciplines.

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*Gioacchino Natoli is at the European Institute of Oncology, Milan, Italy.*

*E-mail: gioacchino.natoli@ifom-ieo-campus.it*

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