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Meeting Report

Getting away from it all in Capri: the 2008 EMBO workshop on NF-κB

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The 2008 EMBO Workshop on NF-κB Capri Oct 17-21

As if seeking refuge from the economic storm circling the globe, approximately 100 researchers gathered amidst the winding paths, massive cliffs, and azure sea on the Isle of Capri, Italy, for an EMBO Workshop on 'The NF-κB Network in Development and Disease'. Fittingly, the NF-κB signaling pathway is one of the primary regulators of stress and immunity. The meeting featured talks by leading researchers from Europe and the US, two poster sessions and many opportunities for informal conversations and extended questioning.

NF-κB Signaling

Signaling from receptors to IκB kinase (IKK). Most NF-κB-activating signals induce the recruitment of distinct cytoplasmic factors to the intracellular domain of a given stimulated cell-surface receptor, leading to activation of the IKK complex. IKK then phosphorylates the NF-κB inhibitor IκB, thus releasing NF-κB to affect nuclear target gene expression. The IKK complex contains two catalytic subunits – IKKα and IKKβ – and a non-catalytic regulatory subunit, NEMO. NEMO's primary function in NF-κB signaling seems to be to guide the IKK complex to its proper destination during cell signaling.

Keynote speaker Michael Karin discussed the multiple signaling complexes at the CD40 receptor that participate in activation of both IKK and the MAP kinase MEKK1 (which induces p38/JNK signaling). Karin showed that CD40-mediated activation of both kinases requires NEMO; however, whereas IKK activation uses a NEMO complex that remains bound to CD40, MEKK1 activation uses a NEMO complex that is released from the receptor. Karin also reported that in response to CD40L or BAFF stimulation, TRAF2 mediates K63-linked ubiquitination of c-IAP1/2. This modification enhances the K48 ubiquitin (Ub) ligase activity of c-IAP1/2 towards TRAF3, which results in TRAF3 degradation with consequent NIK stabilization and ultimately alternative NF-κB pathway activation. TRAF3 proteolysis is also required for cytoplasmic translocation of the NEMO complex that mediates MEKK1 activation and JNK/p38 signaling.

James Chen described a role for K63 polyubiquitination in activation of NF-κB in antiviral innate immunity pathways that are initiated by membrane-bound toll-like receptors (TLRs) or the cytosolic RIG-I-like receptor. He showed that IKK activation in these MyD88- (i.e., TLR) and MAVS- (i.e., RIG-I) dependent antiviral pathways requires the catalytic activity of Ubc13 as well as K63 ubiquitination. Chen also reported that the deubiquitinating enzyme CYLD specifically cleaves K63-, but not K48-linked poly-Ub chains.

Rudi Beyaert provided evidence that the MALT1 paracaspase cleaves target proteins at sites distinct from those recognized by classical caspases. In T-cell receptor (TCR)-induced activation of NF-κB, MALT1 cleaves the A20 adapter protein at Arg439, thereby preventing A20-mediated inhibition of NF-κB signaling, and thus enabling NF-κB-dependent activation of *IL-2* gene expression.

The IKK complex. Fabrice Agou and Alain Israël reported that the NEMO dimer consists of an elongated, largely coiled-coil structure that is stabilized by binding to specific proteins. That is, NEMO contains one N-terminal IKKα/β-binding domain and two Ub-binding motifs – one (called NOA) within its central coiled-coil leucine-zipper domain (CC2-LZ) and another within its C-terminal zinc finger – that cooperate to provide specificity for NEMO's binding to K63-linked poly-Ub chains. Israël also reported that NEMO might be regulated negatively by monoubiquitination, an event that interferes with NEMO's poly-Ub-binding activity. Results from Gilles Courtois' lab suggest that the adapter protein TRAF6 interacts with NEMO through a two-step process, with ubiquitinated TRAF6 first binding to the NOA domain as a means of facilitating the docking of TRAF6 to an N-terminal sequence of NEMO.

Shigeki Miyamoto reported that DNA-damaging agents (e.g., etoposide) and other stress stimuli (e.g., hydroxyurea)

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cause NEMO to be SUMOylated by SUMO ligase PIASγ and phosphorylated by the checkpoint kinase, ataxia telangiectasia mutated (ATM). He also presented evidence that SENP2, a SUMO-specific protease, interacts with and de-SUMOylates NEMO. Moreover, the *SENP2* gene is a target of NF-κB, which provides another route of negative feedback regulation of NF-κB signaling.

Adaoha Ihekwaba described combined computational and experimental approaches to analyze the temporal behavior of the NF-κB pathway. Ihekwaba first characterized the kinetics of IκBα phosphorylation by IKK, and then used an IKK inhibitor to experimentally show the effect of decreased IκBα phosphorylation on NF-κB activity oscillations. The importance of RelA oscillations between the cytoplasm and the nucleus was also discussed in posters by Louise Ashall, Kate Sillitoe (Michael White's group) and Alessandra Agresti. Agresti and Ashall proposed that the classical IκBα-mediated negative feedback loop, which controls the dynamics of these oscillations, allows for repeated sampling of the cellular microenvironment, thus controlling the 'late' induction of NF-κB target genes.

NF-κB-mediated gene regulation. Activation of NF-κB ultimately results in signal- and cell type-specific regulation of target genes. Sankar Ghosh presented data showing that IκBβ knockout mice display increased resistance to septic shock induced by lipopolysaccharide (LPS). This resistance is a consequence of the loss of the sustained production of TNFα that occurs in wild-type mice due to the association of IκBβ with a RelA/c-Rel dimer bound to the *TNF*α promoter. Véronique Baud described a negative role for RelA in NF-κB signaling, namely the ability of RelA to form dimers with RelB in the nucleus, which blocks the binding of RelB complexes to DNA in response to TNFα.

Gioacchino Natoli discussed the transcriptional co-regulator Jmjd3, a histone H3K27-me demethylase. The *Jmjd3* gene is a direct NF-κB target and is induced in macrophages in response to LPS. Natoli reported that Jmjd3 has over 4000 genomic DNA-binding sites, many located at the promoter/enhancer regions of LPS-inducible genes. The binding of Jmjd3 to these sites enables RNA polymerase II to continue transcribing DNA, and thereby cooperates with NF-κB in a feed-forward loop required for sustained expression of anti-microbial and pro-inflammatory genes after stimulation with LPS.

Neil Perkins described how NF-κB transcriptional activity, post-translational modifications and promoter binding can vary between the G1, S and G2/M phases of the cell cycle, with consequent regulation of specific cell-cycle target genes, such as cyclin D1. Perkins also showed that ATR (ataxia telangiectasia related)-dependent activation of Chk1 can both inhibit constitutive IKKα activity and phosphorylate RelA at Thr505, ultimately modulating RelA's transcriptional activity.

Alan Chariot reported that NF-κB p100 undergoes increased processing to p52 in the Hut-78 T-lymphoma line, which expresses a truncated form of p100 (called p85). Matrix metalloprotease (MMP9), which plays important roles in metastasis and tissue invasion, is one of the primary gene targets of p52 in these cells. At the MMP9 promoter, p52 interacts with Ash2, a component of the MLL1/MLL2, H3K4-me methylase complex.

Disease

Cancer. In many human cancers enhanced NF-κB activity provides proliferative and survival signals. Using a mouse model of prostate cancer, Michael Karin showed that knocking out IKKα in prostate epithelial cells reduces metastases formation without affecting primary prostatic tumor development. This pro-metastatic activity is attributable to a nuclear function of IKKα, which represses the expression of metastasis inhibitor maspin. Karin also described a role for both IKKα and IKKβ in the switch from androgen-dependence to androgen-independence in prostate cancer. IKKα acts in prostate cancer cells in an NF-κB-independent pathway, whereas IKKβ acts in the hematopoietic compartment in an NF-κB-dependent manner to produce cytokines that cause IKKα activation.

Manolis Pasparakis showed that liver-specific deletion of NEMO results in extensive spontaneous hepatocyte apoptosis, followed by hepatocyte proliferation and carcinogenesis. Pasparakis reported that liver-specific expression of a constitutively active IKKβ mutant or administration of the anti-oxidant BHA to mice can block cancer in liver-specific NEMO-deficient mice, suggesting that defective NF-κB signaling is responsible for the development of hepatocellular carcinoma in these NEMO-deficient mice. Using other mouse knockouts, Pasparakis reported that reduced NF-κB signaling in the heart endothelium is protective against atherosclerosis – an effect due, in part, to the downregulation of adhesion molecules such as VCAM1.

Guido Franzoso discussed the basis for NF-κB-JNK crosstalk (controlling cell fate) in the liver. He showed that the NF-κB target *gadd45*β (a direct inhibitor of the JNK kinase, MKK7), is induced by TNF-R1 during liver regeneration after partial hepatectomy, and that this upregulation of *gadd45*β is required for hepatocyte survival and proliferation during liver regeneration. He also showed that this hepatic function of Gadd45β is mediated through a suppression of sustained TNF-R1-induced MKK7/JNK signaling.

Macrophages can play a role in promoting inflammation-associated carcinogenesis. Toby Lawrence reported that mice harboring IKKβ-deficient macrophages show an enhanced ability to clear infection, indicating that IKKβ inhibits the development of M1-polarized macrophages during microbial infection. Lawrence also presented evidence that IKKβ is involved in the maintenance of tumor-associated macrophages, and that inhibition of IKKβ in these cells enhances their cytotoxic activity against tumor cells, suggesting that macrophage-specific inactivation of IKKβ has a potential application in anti-cancer therapy.

Antonio Leonardi reported that NGAL, a secreted acute-phase protein that binds to iron, is the product of an NF-κB target gene and a mediator of the pro-tumorigenic activity of NF-κB in thyroid cancer. NGAL is required for malignant transformation and survival of thyroid cancer cells *in vitro*, and it promotes the growth of thyroid tumor xenografts in mice.

Crosstalk between the Wnt and NF-κB signaling pathways in colon cancer was discussed by Yinon Ben-Neriah. Using mice with gut-specific deletion of CKIα (causing constitutive activation of Wnt/β-catenin signaling), Ben-Neriah showed a causative link between Wnt activation, cellular senescence,

induction of NF-κB target genes and activation of the p53 tumor suppressor. Furthermore, he reported that gut-specific, p53 and CKIα double knockout mice show increased epithelial cell proliferation and carcinogenesis. He proposed that gut homeostasis and senescence after CKIα ablation involves both a cell-autonomous and a non-cell-autonomous pathway, dependent on Wnt signaling and NF-κB/TNFα-elicited inflammation, respectively.

CYLD is a tumor suppressor protein with deubiquitinating activity that can block activation of NF-κB in response to various stimuli. Jessica Hutti presented evidence that IKKε can phosphorylate CYLD at Ser418, and that CYLD phosphorylation is required for IKKε-induced transformation of cells in culture.

Tom Gilmore discussed the ability of an overexpressed REL mutant to convert the gene expression profile of the human BJAB B-lymphoma cell line from that of a relatively 'benign' lymphoma to one of a more aggressive subtype of B-cell lymphoma. He also described a splice variant of REL with enhanced DNA-binding and transactivating properties that is overexpressed in some B-cell lymphomas.

HTLV-1 can cause adult T-cell leukemia in humans, and HTLV-1's primary transforming protein is Tax, which promotes NF-κB signaling. Françoise Bex described how Tax assembles into nuclear dense bodies, which contain p50, RelA, and other transcription and splicing factors. Bex reported that Tax is SUMOylated and ubiquitinated at K280/284. She reported that ubiqutinated Tax is found in the cytoplasm, whereas SUMOylation promotes the formation of Tax nuclear bodies. Both of these modifications require Tax phosphorylation at Ser 300/301 and are important for Tax's ability to induce NF-κB.

Evidence was also presented for new links between the IKK/NF-κB pathway and growth inhibitory pathways in certain cancers. Véronique Baud presented data showing that knockdown of RelB in mouse embryo fibroblasts leads to increased cell proliferation, and that RelB inhibition enhances xenograft tumor growth *in vivo*. RelB-mediated inhibition of cell proliferation is apparently caused by upregulation of p53. Neil Perkins also linked the alternative NF-κB pathway to p53 function, reporting that NF-κB/p52 and p53 co-operatively regulate autophagy in response to the chemotherapeutic drug cisplatin. Elad Horwitz (Ben-Neriah lab) presented data showing that inhibition of NF-κB in the early stages of a chemically induced liver cancer can enhance tumor growth. Similarly, Antonio Costanzo showed that IKKα nuclear localization inhibits squamous cell carcinoma (SCC) progression *in vitro* and *in vivo*, and this IKKα-mediated negative regulation of SCC growth involves crosstalk with the TGFβ pathway.

Immunodeficiency diseases. Mutations in *NEMO* cause an array of human disorders primarily affecting the skin and the immune system, including incontinentia pigmenti and ectodermal dysplasia with immunodeficiency (EDA-ID). Matilde Valeria Ursini's lab reported that 60–90% of the patients affected by these disorders have mutations deleting exons 4–10 of the *NEMO* locus, which severely disable NEMO protein activity; many other single amino acid substitutions or small gene deletions can result in hypomorphic NEMO proteins. Fabrice Agou and Alain Israël showed that most disease-associated point mutants of NEMO interfere with either NEMO dimerization or poly-Ub binding. However, Ashish Jain described two patients with EDA-ID who presented with an 80% reduction in NEMO protein levels due to a chromosomal rearrangement affecting an intronic region of *NEMO* that contains a transcriptional enhancer. Cells from these patients exhibit impaired transcription of the *IL-12-p35* gene, which is associated with an inability of both RelA/c-Rel and IKKα to co-localize to the *IL-12-p35* promoter after LPS stimulation.

Anne Puel described the range of gene mutations that occur in a variety of human immunodeficiencies. Namely, some patients have mutations that convert the IκBα protein into a constitutive NF-κB inhibitor, whereas others harbor partially inactivating mutations in *NEMO*, or completely inactivating mutations in the TLR adapter Myd88 and IRAK4. In most cases, cells from these patients display defects in NF-κB (and JNK/p38) activation in response to IL-1β and LPS.

NF-κB in Development

Ruth Schmidt-Ullrich used mouse models to investigate the reciprocal crosstalk between the EdaR-NF-κB and Wnt/β-catenin pathways during hair follicle (HF) development. She reported that Wnt/β-catenin controls the induction of HF development and directly regulates components of the Eda-A1/EdaR/NF-κB signaling pathway, whereas NF-κB activity is required for proper hair placode growth. Furthermore, the genes encoding the Wnt inhibitor Dkk4 and the Wnt-family protein Wnt-10B seem to be transcriptional targets of NF-κB, both playing a role in HF development. Ingo Haase reported that mice with skin-specific knockout of either IKKβ or IκBα show phenotypes that resemble human psoriasis. Inactivation of factors involved in either NF-κB activation (i.e., IKKβ) or NF-κB inhibition (i.e., IκBα) in epidermal keratinocytes results in inflammation with increased keratinocyte proliferation, suggesting that tight control of NF-κB signaling is required for the prevention of chronic skin inflammation. Using IKK mutant mouse models, Marc Schmidt-Supprian showed that IKK-mediated NF-κB activation mediates a pro-survival signal in λ light chain-, but not in κ light chain-expressing B cells during their development in the bone marrow.

Brain and Behavior

NF-κB subunits are expressed in specific regions of the brain including the hippocampus and cortex, suggesting a role for NF-κB in memory and behavior. Using transgenic mice expressing the IκBα-super-repressor (SR) in neurons of these regions of the brain, Sylvie Mémet reported that inhibition of brain NF-κB results in defects in both memory formation in a water maze test and in synaptic plasticity. Barbara Kaltschmidt showed that overexpression of p50 or the IκBα-SR decreases axon length in hippocampal neurons. Similarly, in a transgenic model, expression of IκBα-SR reduces axon length of mossy fibers. Kaltschmidt and Mémet also showed that the gene encoding the α catalytic subunit of PKA, involved in synaptic plasticity and long-term memory, is a neuronal NF-κB target. Mariagrazia Grilli reported that p50 knockout

mice show hippocampal neurogenesis defects, which correlate with impairment of short-term spatial memory performance.

Evolution

NF-κB signaling is also important in invertebrates, including insects in which it controls developmental processes and immune responses to microbial pathogens. Irina Udalova reported that many NF-κB-binding sites in the promoters of developmental and immune genes of *Drosophila melanogaster* occur at analogous locations in six other fly species, suggesting that these κB sites have retained their regulatory roles in gene expression. Dominique Ferrandon described genetic results, indicating that the fly TAK1 adapter protein TAB2 is involved in activation of the Imd pathway – mediating host defense against gram-negative bacteria – through a mechanism that depends on IKK/NF-κB, but not on JNK. Tom Gilmore described a naturally occurring variant allele of NF-κB in the sea anemone *Nematostella vectensis*, which alters NF-κB's DNA-binding and transactivation properties and may represent an adaptation to selective pressure exerted by environmental stress.

Concluding Remarks

At this meeting, much attention focused on the various roles of NF-κB in cell signaling, physiology, development and human disease. The elucidation of key protein structures and protein–protein interactions, combined with the use of tissue-specific mouse knockouts and the characterization of human disease-associated mutations in NF-κB signaling, are generating profound insights into the function of this important signaling pathway. All participants look forward to returning to Capri in future years to discuss further developments in this rapidly moving field.

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