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PATHOBIOLOGY IN FOCUS

Off the beaten pathway: the complex cross talk between Notch and NF-κB

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The canonical Notch pathway that has been well characterized over the past 25 years is relatively simple compared to the plethora of recently published data suggesting non-canonical signaling mechanisms and cross talk with other pathways. The manner in which other pathways cross talk with Notch signaling appears to be extraordinarily complex and, not surprisingly, context-dependent. While the physiological relevance of many of these interactions remains to be established, there is little doubt that Notch signaling is integrated with numerous other pathways in ways that appear increasingly complex. Among the most intricate cross talks described for Notch is its interaction with the NF-κB pathway, another major cell fate regulatory network involved in development, immunity, and cancer. Numerous reports over the last 11 years have described multiple cross talk mechanisms between Notch and NF-κB in diverse experimental models. This article will provide a brief overview of the published evidence for Notch–NF-κB cross talk, focusing on vertebrate systems.

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CANONICAL NOTCH SIGNALING

Canonical Notch signaling has been recently reviewed by several authors,[1–6] and the reader is referred to these reviews for detailed information and additional references. Briefly, there are four mammalian Notch receptors (Notch-1, -2, -3, and -4) and five ligands (Jagged-1/-2, Delta-like 1, 3, and 4).[7] Notch receptors and ligands are heterodimeric type I membrane proteins that require cell–cell contact for activation to regulate cell fate decisions. Notch signaling plays context-dependent roles in differentiation, proliferation, and apoptosis, and has been implicated in cancer progression both as an oncogene and a tumor suppressor. Notch receptors heterodimers include an extracellular subunit (N^{EC}) containing multiple EGF-like repeats and three LIN-12-like repeats, and a transmembrane subunit (N^{TM}).[1] The 103-amino-acid C-terminal hydrophobic region of N^{EC}, together with the 65 amino acids at the N terminus of the N^{TM} subunit, forms the 'heterodimerization domain' (HD), which mediates the interaction between N^{EC} and N^{TM}.[8] The LNR repeats prevent ligand-independent dissociation of the two subunits by 'folding over' the HD.[9] Upon ligand binding to N^{EC}, N^{TM} and N^{EC} dissociate and the ligand-bound N^{EC} is endocytosed into the ligand-expressing cell.[10] This unmasks the HD and triggers an extracellular cleavage in it by ADAM (a disintegrin and metalloproteinase) 10 or 17,[1,2] followed by an intramembranous cleavage by the γ-secretase complex.[11] The cleavage of N^{TM} by γ-secretase releases an intracellular domain of Notch of approximately 97 kDa (N^{IC}) that translocates to the nucleus to act as a switch for transcription factor CSL (CBF-1/suppressor of hairless/Lag-1, also known as RBP-Jκ in mammals). CSL binds to responsive elements whose canonical sequence is CGTGGGAA and represses target genes by recruiting a multi-protein corepressor complex.[1,2] Upon binding of N^{IC} to CSL, the corepressor complex is replaced with a coactivator complex which includes MAML1 (Mastermind in *Drosophila*), SKIP, and histone acetyltransferases PCAF, GCN5, or p300. This converts CSL into a transcriptional activator, driving the transcription of CSL target genes. These include among others the HES and HEY families, homologs of *Drosophila* 'Enhancer of Split' bHLH transcription factors.[12] Other Notch target genes have been shown to play critical roles in carcinogenesis and cancer progression. These include the following: $p21^{Cip/Waf}$,[13] cyclin D1,[14] cyclin A,[15] several subunits of nuclear factor-κB

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(NF-κB),[16] PPAR family nuclear receptors,[17,18] E3 ubiquitin ligase SKP2, which mediates degradation of $p27^{kip1}$,[19] c-Myc,[20,21] and many others.

NF-κB SIGNALING

A comprehensive review of NF-κB biology is beyond the scope of this manuscript, and the reader is referred to recent articles for further information.[22–30] NF-κB/Rel transcription factors control the expression of a multitude of genes with multiple functions in immunity, inflammation, and cancer.[29,30] The NF-κB family include homo- or heterodimers formed by p50, p65 (RelA), c-Rel, p52, and RelB.[29,30] All five proteins contain a Rel homology domain (RHD), which participates in dimerization and DNA binding. The RHD contains a nuclear localization sequence (NLS), which in non-stimulated cells is masked through binding of IκB family inhibitory proteins. These include IκB-α, IκB-β, IκB-ε, Bcl-3, as well as NF-κB precursor proteins p100 (p52 precursor) and p105 (p50 precursor).[30] IκB-α, IκB-β, and IκB-ε physically interact with and sequester NF-κB in the cytosol.[31,32] IκBα also contains a nuclear export signal, which allows it to sequester NF-κB in the nucleus and export it back to the cytoplasm.[30] Both p100 and p105 have IκB activity. Bcl-3, conversely, is a nuclear protein that functions primarily as a coactivator. A common feature of these proteins is the presence of five to seven ankyrin repeats, which are required for the interaction with NF-κB.[33] The ankyrin region binds the RHD, masking the NLS and preventing nuclear transport of NF-κB.

There are at least three mechanisms for activation of NF-κB:[24,30] (1) The canonical IKK pathway involves phosphorylation of IκB proteins by a specific kinase (IKK) 'signalosome' multiprotein complex, consisting of at least three subunits, IKKα, IKKβ, and multiple copies of regulatory subunit IKKγ/NEMO. The IKK complex is activated by stimuli such as T-cell receptor (TCR) ligation, TNF-α, IL-1, or LPS. The IKK signalosome, acting primarily through the catalytic activity of IKK-β, phosphorylates IκBα Ser32 and Ser36 or equivalent residues in IκBβ and ε.[30] This triggers polyubiquitination at Lys21 and Lys22 of IκBα (or equivalent residues in IκBβ and ε), and proteasomal degradation of IκBs, allowing nuclear transport of NF-κB dimers. Activation of the IKK signalosome is mediated, at least in some cases, by monoubiquitination of IKKγ/NEMO, which helps recruit ubiquitin-binding proteins to the complex, such as kinase TAK1,[34] which ultimately activate IKKβ. In the case of genotoxic effects, IKK activation can involve nuclear SUMOylation of IKKγ/NEMO, its phosphorylation by atangia–telangiectasia (ATM) kinase, replacement of SUMO with ubiquitin, nuclear export of an ATM/NEMO complex and recruitment of this complex to the IKK signalosome.[30] (2) The non-canonical pathway is activated by ligands such as CD40L and lymphotoxin. These activate NIK kinase, which in turn activates and phosphorylates IKKα homodimers. IKKα phosphorylates the p100 precursor of p52, causing its partial degradation by the proteasome to generate p52/RelB heterodimers, or 'NF-κB2'. These have affinity for a subset of κB responsive elements and generate a distinctive gene expression pattern.[30] (3) Atypical or IKK-independent pathways, activated by stimuli such as hypoxia, oxidizing radicals, or UV radiation, which involve either Tyr phosphorylation of IκBα on Tyr 42 or Ser/Thr phosphorylation in its C-terminal PEST domain, leading to IκBα ubiquitination and degradation.[30]

ROLES OF IKK KINASES

IKKα and β, although structurally very similar, have different roles in the NF-κB pathway, and different NF-κB-independent functions. IKKβ, as a component of the IKK signalosome, appears predominant in the canonical pathway activation cascade and IκBα phosphorylation, while IKKα is predominant in the activation of the alternative, p52/RelB pathway (see above). Additionally, IKKα has multiple nuclear functions within and outside the NF-κB pathway. These include chromatin remodeling at NF-κB-dependent promoters by phosphorylation of Histone H3 (Ser10),[35] phosphorylation of p65/RelA,[36] and phosphorylation of co-repressor SMRT, leading to NF-κB-dependent transcriptional activation.[37] IKKα can increase cyclin D1 expression by phosphorylating and activating the estrogen receptor-α (ERα) and it coactivator SRC-3 at the cyclin D1 promoter,[38,39] as well as by phosphorylating β-catenin at the same promoter[40] and stabilizing cytoplasmic β-catenin.[41] Both these activities may contribute to oncogenic effects, especially in breast cancer. In a possible feedback loop, IKKα can also directly phosphorylate cyclin D1, leading to its degradation.[42]

IKKβ, for its part, participates in regulating the TCR signal in a complex feedback loop. First, it functions downstream of the TCR in a T-cell specific complex including CARMA, BCL-10, and MALT1 (CBM), which leads to canonical NF-κB activation.[30] Subsequently, it phosphorylates BCL-10, leading to its dissociation from the CBM complex and attenuating the signal.[43] IKKβ phosphorylates p65/RelA at Ser536, stimulating its nuclear migration.[44] IKKβ also phosphorylates and inactivates proapoptotic transcription factor FOXO3a.[45] By phosphorylating a 14-3-3 protein that binds AU-rich binding tristetraprolin (TPP), IKKβ inhibits the binding of TPP to multiple growth factor and cytokine mRNAs, stabilizing these transcripts.[46] IKKβ can also activate, and in some cases attenuate, the MEK–ERK pathway through phosphorylation of p105, which releases ERK pathway kinase TPL2/COT, and conversely, through degradation of scaffold protein DOK1.[47,48]

This list of functions far from exhaustive, but it illustrates the complexity of effects through which the IKK kinases can mediate cross talk between NF-κB-activating stimuli and multiple other pathways regulating proliferation, apoptosis, and inflammation.

CROSS TALK BETWEEN NOTCH AND NF-κB

Over the past 11 years, numerous reports have described regulation of NF-κB by Notch and vice versa through different, context-dependent mechanisms. We will briefly summarize the mechanisms of cross talk that have been described so far (Table 1).

Transcriptional Regulation of NF-κB Pathway Members by Notch

Oswald *et al*[49] originally described transcriptional activation of the p100 promoter by CSL-responsive element that overlaps with a κB site. Through this effect, Notch would increase expression of the p52 precursor (Figure 1a). The overall effect would then depend on cellular context. Since p100 has IκB activity, the end result could be inhibition of the non-canonical pathway (see above). However, in the presence of active IKKα, this could also result in the rapid generation of p52/RelB heterodimers. Subsequently, Cheng *et al*[16] showed that Notch-1 upregulates the expression of p50, p65, RelB, and c-Rel NF-κB subunits in murine bone marrow hematopoietic precursors (Figure 1a). Through this effect, Notch-1 controlled the LPS-induced proliferation of B cells and GM-CSF-induced differentiation of dendritic cells. Oakley *et al*[50] reported that in stellate liver cells, CSL in the absence of Notch activation acts as a repressor of IκBα expression. Notch-1^{IC} caused de-repression of IκBα expression, and thus lower NF-κB activity.

Transcriptional Regulation of Notch Pathway Members by NF-κB

This cross talk mechanism has been described primarily in the context of B cells (Figure 1a). Bash *et al*[51] reported transcriptional upregulation of Notch ligand Jagged-1 by NF-κB in B cells. More recently, Notch-2 and NF-κB have been reported to cooperate in marginal zone (MZ) B-cell development. In this model, NF-κB stimulates the expression of two known Notch targets, HES-5 and Deltex-1.[52]

Physical Interaction between Notch and NF-κB

In 1996, Guan *et al*[53] reported that ectopically overexpressed Notch-1^{IC} has an IκB-like activity in Jurkat cells, specifically associating with the p50 subunit of p50–p65 heterodimers and preventing κB-dependent transactivation. Interestingly, in that report, the authors showed the effect to be dose-dependent in co-transfection experiments (Figure 1b). Low amounts of Notch-1 stimulated NF-κB transcriptional activity, while higher amounts inhibited it. Subsequently, the same group mapped the interaction between Notch-1^{IC} and p50 to a 109 amino-acid stretch including residues 1773–1881.[54] This is at the very N terminus of Notch-1^{IC} and overlaps with the RAM23 domain, which participates in Notch interaction with CSL. This would imply that these two interactions of Notch-1 may compete with one another. Under the conditions studied in that report, where Notch-1^{IC} was overexpressed by transfection, the interaction was

Table 1 Evidence of Notch-NF-κB cross talk

Model	Effect	Mechanism	Reference
Jurkat T-ALL cells	Notch-1 induces p100/p52 expression	Transcriptional, CSL mediated	49
Mouse hematopoietic precursors	Notch-1 induces p50, p65, RelB and c-Rel	Transcriptional, CSL mediated	16
Stellate liver cells	Notch-1 induces IκBα	Transcriptional, CSL mediated	50
B cells	NF-κB induces Jagged-1	Transcriptional, canonical NF-κB	51
Marginal zone B cells	NF-κB induces HES-5 and Deltex-1	Transcriptional, canonical NF-κB	52
Jurkat T-ALL cells	Notch-1 inhibits NF-κB (dose-dependent)	Physical interaction between p50 and Notch-1^{IC}	53,54
Murine T cells	Notch-1 stimulates NF-κB	Physical interaction between p50/c-Rel and Notch-1^{IC}	56
Human primary keratinocytes	Notch ligand peptide stimulates NF-κB	Unknown, short-term (IKK activation?)	18
Murine erythroleukemia cells	Cell-associated Jagged-2 stimulates NF-κB	Unknown, short-term (IKK activation?)	57
Murine T-ALL (Notch-3^{IC} transgenic)	Notch-3^{IC} stimulates canonical NF-κB	IKK signalosome dependent	58
Murine T-ALL (Notch-3^{IC} transgenic)	Notch-3^{IC} stimulates alternative NF-κB2	Physical interaction between Notch-3^{IC} and IKKα homodimers	59
Murine T-ALL (Notch-1^{IC} transgenic)	Notch-1^{IC} stimulates canonical NF-κB	Physical interaction between Notch-1^{IC} and IKK signalosome. Also, CSL-dependent effects	61
CEM T-ALL cells	Notch-1^{IC} stimulates canonical NF-κB	Physical interaction between Notch-1^{IC} and IKK signalosome	61

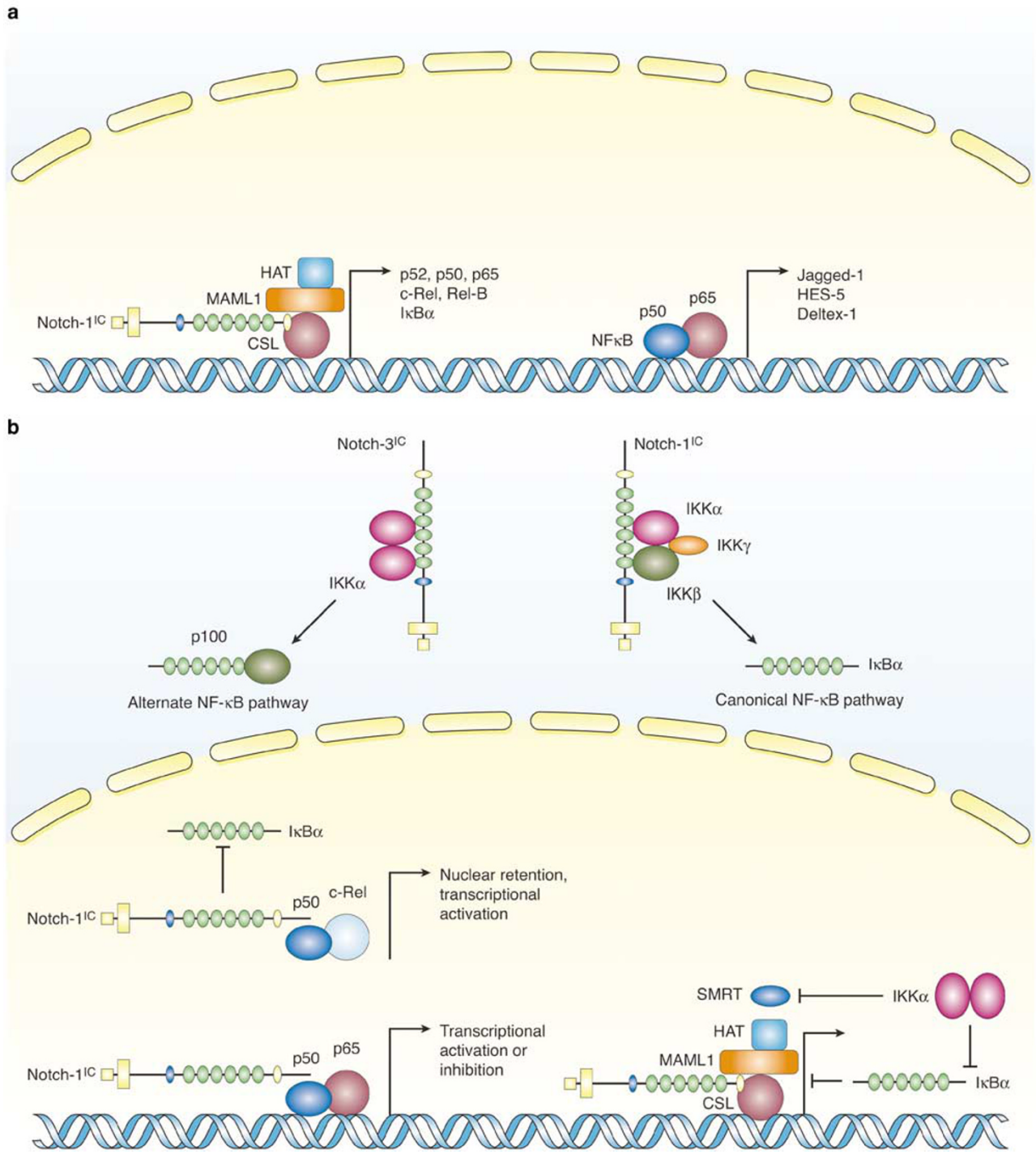

Figure 1 (**a**) Reciprocal transcriptional regulation between Notch and NF-κB. Notch has been reported to increase transcription of p52, p50, p65/RelA, RelB, and c-Rel, as well as IκBα, thus potentially stimulating or inhibiting NF-κB activity. Conversely, NF-κB has been reported to increase transcription of Notch ligand Jagged-1, as well as Notch targets Deltex-1 and HES-5. (**b**) Non-transcriptional cross talk mechanisms. Notch-1^{IC} binds to p50, causing either inhibition or stimulation of NF-κB p50/p65 depending on relative amounts. Also, Notch-1^{IC} by binding p50 blocks the binding of IκBα to p50/c-Rel complexes, thus preventing IκBα from causing nuclear export of p50/c-Rel. This results in increased permanence of p50/c-Rel in the nucleus and increased transcriptional activity. Notch-3^{IC} binds and activates IKKα homodimers, resulting in activation of the alternate 'NF-κB2' pathway. Notch-1^{IC} binds and activates the IKK signalosome, resulting in activation of the canonical NF-κB pathway. The distinct nuclear consequences of the canonical NF-κB and alternate 'NF-κB2' pathway are not depicted for clarity. In the nucleus, IKKα can de-repress Notch transcriptional activity by phosphorylating SMRT and/or by phosphorylating nuclear IκBα, which appears to function as a nuclear repressor of Notch activity.

predominantly nuclear and had inhibitory effects on NF-κB.[54] More recent data from the Osborne group, while confirming the physical interaction between Notch-1 and p50, have the opposite functional implications. First, Palaga *et al*[55] showed that Notch-1 activates NF-κB downstream of TCR activation in murine T cells. The mechanism of this effect was later clarified by the same group. Shin *et al*[56] recently showed that Notch-1 is responsible for the late but not the early wave of NF-κB activation that follows TCR activation. This effect is mediated by p50-mediated physical interaction of Notch-1^{IC} with p50/c-Rel complexes. Through this interaction, Notch-1 competes with IκBα and prevents it from exporting NF-κB from the nucleus (Figure 1b). This increased nuclear retention results in activation of NF-κB-dependent transcription. So far, only Notch-1 has been reported to interact with NF-κB. Whether other Notch homologs have similar activities remains unclear.

Notch and the IKKs

Nickoloff *et al*[12] showed that in normal human keratinocytes, Notch activation by a soluble ligand could trigger terminal differentiation and cornification. In that study, the authors showed that Notch activation was associated with rapid activation of NF-κB. This lasted approximately 2 h and was suppressed by a late increase in PPARα. The authors also showed that the soluble Notch ligand triggered rapid phosphorylation of IκBα, suggesting that the canonical, IKK signalosome-mediated pathway was somehow activated by the Notch ligand. Similar effects were observed by the same group in murine erythroleukemia (MEL) cells undergoing erythroid differentiation.[57] In this model, Notch-1 expression is required to maintain survival during differentiation, but must be downregulated for terminal differentiation to occur. Antisense-mediated knockdown of Notch-1 caused differentiating cells to undergo apoptosis, while constitutive overexpression of Notch-1^{IC} prevented differentiation. In an effort to dissect some of the pathways responsible for the survival effects of Notch-1 in this context, the authors showed that exposure of MEL cells to stromal cells expressing Notch ligand Jagged-2 caused rapid and short-lived activation of NF-κB, which was reversed by 4 h.[57]

Recent observations have shed some light on the possible mechanisms for rapid activation of NF-κB by Notch. Bellavia *et al*[58] showed that Notch-3 activates multiple NF-κB pathways during T-cell differentiation. Notch-3^{IC} overexpression causes a T-cell leukemia/lymphoma phenotype in transgenic mice. Transgenic premalignant thymocytes and T lymphoma cells overexpress pTα/pre-TCR and have constitutive NF-κB activation providing survival signals for immature thymocytes. Notch-3 can activate the canonical p50/p65 pathway in a way that is dependent on an IKKβ/IKKα/NIK. NF-κB p50/p65 complexes are recruited onto the cyclin D1, Bcl_2-A1, and IL7-receptor-α promoters, resulting in T-cell survival and proliferation.[58,59] In the same cells but in the absence of pTα, Notch-3^{IC} activates the non-canonical NF-κB pathway by physically binding to IKKα homodimers in an NIK-independent manner (Figure 1b). The resulting NF-κB2/p100 processing allows p52/RelB nuclear translocation triggering transcription of Bcl_2-A1 and IL7-receptor-α genes.[60] These observations raise the question of whether the canonical and/or the alternative NF-κB pathways contribute to the transforming effect of Notch-3 in this model. It is still unclear, however, whether endogenously expressed Notch-3 participates in activation of NF-κB through either of these mechanisms.

Vilimas *et al*,[61] examining a murine model of T-cell acute lymphoblastic leukemia induced by overexpression of Notch-1^{IC}, determined that NF-κB target genes are upregulated in expression profiles of Notch-1^{IC}-transformed cells. In these cells, and in human cell lines derived from spontaneous T-cell acute lymphoblastic leukemia, Notch-1^{IC} interacts with the IKK signalosome, increasing its IκBα kinase activity (Figure 1b). Thus, it appears that at least two Notch homologs have the ability to bind IKK kinases, increasing their activity. It remains unclear whether IKK regulation is a physiological function of Notch-1 and/or -3 or a consequence of high-level expression, either in transgenic models or as a consequence of Notch mutations in human T-ALL. Another side of the cross talk between Notch and IKKs has been uncovered by the Bigas group. This group has recently discovered that in colorectal carcinoma cells, nuclear IKKα phosphorylates SMRT not only in association with NF-κB but also in association with CSL.[62] This leads to uncontrolled activation of Notch-mediated signaling (Figure 1b). Interestingly, the same group had described earlier a nuclear function of IκBα as an inhibitor of Notch-dependent transcription.[63] In this case as well, IKKα could de-repress Notch-dependent transcription, presumably by phosphorylating IκBα (Figure 1b). Thus, it appears that at least in some contexts, stimuli that activate NF-κB also lead to Notch activation and inhibitors of NF-κB also inhibit Notch-dependent transcription.

Undefined Mechanisms

Knockdown of Notch-1 in Notch-1 antisense transgenic mice leads to impairment in long-term potentiation in hippocampal neurons, the neurobiological mechanism for long-term memory. NF-κB DNA-binding activity was found to be defective in Notch-1-deficient neurons.[64] The mechanism(s) through which Notch-1 stimulates NF-κB activity in hippocampal neurons remain undetermined. Similarly, in pancreatic carcinoma cells, inhibition of Notch signaling is accompanied by decreased NF-κB activity, through mechanisms that are still to be clarified.[65,66]

CONCLUSIONS AND FUTURE DIRECTIONS

The observations reported in the literature so far offer a complex and incomplete picture of the interactions between these two key cell fate determining pathways. As it is becoming increasingly clear in the case of other pathways, these interactions can be cooperative or antagonistic, and multiple

levels of feedback are possible depending on the context. The physiological relevance of these interactions will need to be thoroughly investigated. However, it can be safely stated that those planning to manipulate the Notch-signaling pathway for experimental or therapeutic purposes would do well to examine the possible effects on NF-κB and vice versa. This could have implications in several fields.

In cancer biology, both Notch and NF-κB are prominent therapeutic targets. If murine and *in vitro* data are confirmed in human disease, the treatment of cancers dependent on Notch activity may benefit from combinations of agents targeting both pathways, for example, inhibiting Notch and IKK activities, or Notch and the proteasome. Evidence in support of this notion is offered by the fact that some cancers in which Notch plays a clearly oncogenic role, such as breast and pancreatic carcinomas, are also often characterized by high NF-κB activity. Ramdass *et al*[67] showed that in a large series of cervical cancer specimens, the NF-κB and Notch pathways were commonly coactivated, as judged by expression of Notch target and NF-κB target genes, and nuclear localization of NF-κB immunoreactivity.

In immunology and inflammation, where the role of NF-κB is paramount, Notch-1 and Notch-3 have been suggested to regulate NF-κB activity during mature T-cell activation (Notch-1) and during thymocyte development (Notch-3). It is conceivable that combined manipulations of the two pathways may be useful in a number of indications, from graft versus host disease to transplant rejection.

In neurology, the possible role of Notch upstream of NF-κB in hippocampal neurons[64] needs to be further investigated as a possible basis for neuronal plasticity in long-term memory. These studies may suggest possible toxic effects of long-term Notch inhibition in the brain, and contribute to our understanding of one of the most fundamental processes in the human mind.

The cross talk between Notch and NF-κB is a biological Pandora's box that has just been opened. Sorting out the mechanisms, biological implications, physiological significance and possible therapeutic applications of the multiple interactions between these two pathways will require considerable effort over the next several years, but is likely to generate invaluable information in a number of biological and medical fields.

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