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ARTICLE *in* CANCER CAUSES AND CONTROL · FEBRUARY 2011

Impact Factor: 2.74 · DOI: 10.1007/s10552-011-9745-4 · Source: PubMed

Published in final edited form as:
Cancer Causes Control. 2011 May ; 22(5): 785–801. doi:10.1007/s10552-011-9745-4.

Genomics of the NF-κB signaling pathway: hypothesized role in ovarian cancer

Kristin L. White,
Department of Health Sciences Research, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, USA

David N. Rider,
Department of Health Sciences Research, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, USA

Kimberly R. Kalli,
Department of Oncology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, USA

Keith L. Knutson,
Department of Immunology, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, USA

Gail P. Jarvik, and
University of Washington, Seattle, WA, USA

Ellen L. Goode
Department of Health Sciences Research, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905, USA

Ellen L. Goode: egoode@mayo.edu

Abstract

Objective—We sought to review evidence linking nuclear factor-kappa B (NF-κB) to ovarian cancer and to identify genetic variants involved in NF-κB signaling.

Methods—PubMed was reviewed to inform on ovarian cancer biology and NF-κB signaling and to identify key genes. Public linkage disequilibrium (LD) data were analyzed to identify informative inherited variants (tagSNPs) using ldSelect.

Results—We identified 319 key NF-κB genes including five NF-κB subunits, 167 activating genes, and 55 inhibiting genes. We found that the 1000 Genomes Project was the most informative LD source for most genes (92.8%), and we identified 13,027 LD bins ($r^2 \geq 0.9$, minor allele frequency ≥0.05) and 1,018 putative-functional variants worthy of investigation. We also report that reliance on a commonly used genome-wide SNP array and genotype imputation with HapMap Phase II data provides data on only 74% of the common inherited NF-κB SNPs of interest.

Conclusions—Compelling evidence suggests that NF-κB plays a critical role in ovarian cancer, yet inherited variation in these genes has not been thoroughly assessed in relation to disease risk or

Correspondence to: Ellen L. Goode, egoode@mayo.edu.

Electronic supplementary material The online version of this article (doi:10.1007/s10552-011-9745-4) contains supplementary material, which is available to authorized users.

outcome. We present a collection of variants in key genes and suggest creation of a custom genotyping array as an optimal approach.

Keywords

Association studies; Genetic variation; NF-kappaB; Etiology; tagSNPs; Single nucleotide polymorphisms

Introduction

Ovarian cancer is the fifth leading cause for cancer death among women in the United States [1]. Due to a lack of effective screening strategies and the non-specific nature of early signs and symptoms associated with this disease, most patients are diagnosed with advanced disease. For the approximate 25% of women who are diagnosed with disease confined to the ovary or ovaries, 5-year survival rates are high (75–90%); however, for the 75% of women diagnosed with stage III and IV disease, the likelihood of long-term disease-free survival is low (15–20%) [2–4]. Identification of factors associated with risk and clinical outcome is essential to understand the pathogenesis of ovarian cancer, enabling preventative and therapeutic advances to be made in this rare but deadly disease.

Recent studies indicate that there are two distinct types of ovarian cancer, Type I and Type II [5], and that a proportion of cases of both types likely originate in pelvic tissues outside the ovary. Ovarian cancer is originally thought to develop de novo from the ovarian surface epithelium that is derived from mesodermal epithelium and contains no müllerian-type tissue. However, serous, endometrioid, and clear cell ovarian carcinomas are all derived from müllerian-type tissue indicating that ovarian tumors may not develop de novo in the ovary, but instead develop in müllerian-derived tissues outside ovary (such as the cervix, endometrium, and fallopian tubes) and involve the ovary secondarily [6–8]. Type I ovarian cancers (clear cell, mucinous carcinomas, and low-grade serous and endometrioid) are usually confined to the ovaries at diagnosis, develop from borderline tumor precursors, and are relatively genetically stable with typical somatic mutations, including *KRAS*, *BRAF*, *ERBB2*, and *PIK3CA* but not *TP53* [9]. Type II ovarian cancers (high-grade serous, high-grade endometrioid, malignant mixed mesodermal tumors, and undifferentiated carcinomas) are more aggressive, are believed to develop from intraepithelial carcinomas in the fallopian tube, and are genetically unstable with characteristic somatic *TP53* mutations but rarely mutations characteristic of Type I tumors [7].

Previous data also suggest that ovarian cancer pathogenesis may be dependent on the inflammatory response within the ovarian surface epithelium. This hypothesis is supported by an etiological hypothesis of “incessant ovulation,” which postulates that increased lifetime ovulations are associated with increased risk of ovarian cancer [10]. Ovulation causes repeated trauma to the ovarian surface epithelium that requires repair mediated by the inflammatory response through the recruitment of leukocytes, cytokines, and chemokines, release of nitrous oxide, DNA repair, and tissue restructuring [10–14]. This restructuring can result in epithelial cells trapped internally in inclusion cysts, where the local microenvironment appears to induce them to gain quasi-neoplastic characteristics such as increased p53 accumulation and a ninefold higher proliferation index than surface epithelial cells [15].

Nuclear factor-kappaB (NF-κB) refers to a family of transcription factors (also called the Rel family) that regulate the expression of over 400 genes. These regulated genes are critical to numerous cellular processes and include a multitude of cytokines and chemokines, pro-inflammatory genes, adhesion molecules, acute phase response proteins, complement

factors, stress response genes, oxidative enzymes, apoptosis genes, growth factors, and other transcription factors [16–18]. Due to this regulatory role, NF-κB has been implicated in several diseases including cardiovascular disease and various cancers [19, 20]. Evidence indicates that NF-κB is constitutively activated in many cancer cell lines, influencing cancer progression and response to chemotherapy [21, 22]. Therefore, we set out to review the hypothesis that NF-κB may play a role in ovarian cancer specifically and to identify the optimal approach for a large-scale study of inherited variation in this pathway and ovarian cancer risk.

A role for NF-κB in ovarian cancer

The NF-κB family of transcription factors has received much attention for its role in cancer since the discovery that c-rel is the human homologue of the avian retroviral oncogene v-rel [23]. Subsequent work has demonstrated roles for NF-κB-mediated signaling in promoting survival from pro-apoptotic stimuli, angiogenesis, proliferation, and inflammation—all characteristics of malignancy [24]. All of these NF-κB-influenced functions appear to play a role in ovarian cancer, underlying the importance of further understanding NF-kB-mediated events in order to better identify women at risk of developing ovarian cancer and to facilitate development of more effective therapeutic strategies.

NF-κB has been shown to be important directly in tumor cells. For example, the mitogen lysophosphatidic acid that is found in ascites fluid may induce proliferation by increasing levels of cyclin D1, an important cell cycle control protein [25]. LPA-induced increases in activity of a cyclin D1 promoter-driven reporter gene required the NF-κB site. Ovarian tumors can acquire enhanced NF-κB functions that allow them to circumvent apoptotic pathways and afford protection against environmental insults such as anti-tumor immune effectors or chemotherapy. Inhibition of NF-κB activity in vitro or in human cell line xenografts increases the sensitivity of the cells to apoptosis induced by both cisplatin [26] and paclitaxel [27], drugs representing the platinum/taxane combination most commonly used as first-line treatment for ovarian cancer. Ovarian cancers are resistant to tumor necrosis factor alpha (TNFα)-induced apoptosis because tumor cells can upregulate pathways leading to the activation of NF-κB and induction of apoptosis inhibitory proteins [28]. In addition, using a dominant-negative IkBα construct to block NF-κB activity in the SKOV3 ovarian cancer cell line resulted in reduced expression of VEGF and IL-8, leading to reduced tumorigenicity, invasiveness, and angiogenesis in athymic mice [29]. Similar reductions were seen in cell line invasiveness, IL-8 production, and anchorage-independent growth after treatment with an IKKβ inhibitor in experiments performed after determining that elevated IKKβ levels and a 9-gene “IKKβ signature” were associated with worse outcome in ovarian cancer patients [30].

In addition to studies that assess all ovarian cancer cells as one population, cells phenotypically and functionally characterized as potential ovarian cancer stem cells have been shown to have constitutively activated NF-κB [31]. The group characterizing these cells further determined that expression of transcripts from the microRNA cluster miRNA199A2/214 are lower in the CD44-positive stem cell-like population than in CD44-negative, “mature” ovarian cancer cells, and that decreases in expression of this specific miRNA cluster are associated with decreased expression of Twist1, a transcription factor that controls IKKβ levels [32]. Because Twist1 has an antagonistic function in NF-κB-mediated cytokine expression, it appears that the result of reduced expression of this miRNA cluster and decreased Twist 1 levels is increased NF-κB activity. Due to the strong potential for chemoresistant stem cells to contribute to ovarian cancer recurrence after initially effective chemotherapy, it is clear that understanding the potential role of NF-κB in driving stem cell activity is a critical area of study.

In addition to direct actions in tumor cells, NF-κB-mediated events also contribute to the microenvironment and peritoneal inflammation associated with ovarian cancer. For example, activated thrombin, found in the inflammatory environment of the peritoneum in ovarian cancer patients, appears to induce monocytes to develop a tumor-associated macrophage phenotype. Monocytes treated with activated thrombin have elevated levels of activated NF-κB; supernatants from these cells increase ovarian cancer cell invasion in vitro. This increased invasiveness can be blocked with a neutralizing antibody targeting IL-8, an NF-κB-driven cytokine [33]. The central role of NF-κB in inflammatory responses has developed increased interest in the context of ovarian cancer over the last decade because of the clear association of anti-tumor immune responses with improved outcome [34]. Ovarian tumors are infiltrated with a variety of immune effectors, including anti-tumor T-cells [35–38]. The type of immune infiltrate influences the disease course, with elevated levels of suppressive T regulatory cells associated with poor outcome [38] and higher CD8/Treg ratios associated with improved survival [39]. The type of anti-tumor immune response that develops is controlled by chemokines and cytokines produced by infiltrating tumor macrophages, dendritic cells, and the tumors themselves to control the mobilization of the various T-cell subsets. For example, one mechanism by which ovarian cancer modulates immunity is through the production of interleukin-10 (IL-10). Synthesized directly by the tumor and infiltrating immune effectors, IL-10 blocks NF-κB activation in T-cells and dendritic cells and prevents activation of effective ovarian cancer anti-tumor immunity [40–42].

Because of the vast body of evidence supporting the importance of NF-κB-regulated events to ovarian cancer cells and the anti-tumor immune response, we hypothesize that much of the unexplained risk of this inflammation-related cancer is due to genetic variations in the NF-κB pathway [43].

Nuclear factor-kappaB subunits and primary signaling pathways

NF-κB is present in cells in an uninduced state, requiring activation to initiate nuclear translocation and the ability to influence gene expression. It consists of five members, p50, p52, p65, Rel-B, and c-Rel [44], that can form multiple homo- and hetero-dimers (p65/p50, p65/p65, p50/c-Rel, p50/p50, c-Rel/c-Rel, and p52/p52), the most common of which are p65/p50 and p52/Rel-B [45, 46]. The NF-κB dimers have specific non-redundant functions in various tissue and cell types [22, 46]. p50, p65, and p52 are found in most cell types and at the highest concentration in the thymus, whereas Rel-B and c-Rel are primarily found in lymphoid tissues [46].

The primary NF-κB signaling pathway is referred to as the canonical pathway (Fig. 1). Stimulation by TNFα and other ligands initiates a signaling cascade that assembles the IκB kinase (IKK) complex and promotes the degradation of the inhibitors of κB (IκB), resulting in the activation and nuclear translocation of NF-κB dimers [16]. The canonical pathway is particularly important for the ability of the myeloid (innate) cells of the immune system, including monocytes, macrophages, and dendritic cells, to communicate with the adaptive immune system [47]. NF-κB is also important for the innate immune cells to sense “danger” through a family of molecules known as the toll-like receptors (TLRs) [48].

Much less is understood about the non-canonical NF-κB pathway. This signaling cascade involves ligation by CD40, B-cell activating factor (BAFF), and lymphotoxin beta (LTβ), subsequent IKKα activation, and poly-ubiquitination and partial degradation of p100, which yields activated p52 and Rel-B dimers (Fig. 1) [45, 46, 49, 50]. In contrast to the canonical pathway, the non-canonical NF-κB receptor-mediated pathway is important for lymphoid organ development and the adaptive immune response [47]. Targeted disruptions in the individual NF-κB subunits can lead to abnormal splenic architecture. Due to the spleen’s

important role in both human and mouse immunity, it is likely that these splenic abnormalities play a role in some forms of immunodeficiency. NF-κB is also known to play key roles in the architecture of other immune organs such as the lymph nodes, which are important for the eradication of both infection and malignancy. One of the best-characterized roles of the non-canonical pathway is in the development of an adaptive immune response. For example, targeted disruption of IKKα expression blocks maturation of B-cells in the germinal centers of lymph nodes, indicating the important role of IKKα in the acquisition of humoral immunity [51].

A role for polymorphisms

Inherited variation in key genes may account for some portion of ovarian cancer susceptibility. Familial aggregation of ovarian and breast cancer has long been established, and account for an estimated 5–10% of epithelial ovarian cancer cases [52, 53]. Highly penetrant mutations in *BRCA1*, *BRCA2*, and DNA mismatch repair genes explain less than 40% of familial epithelial ovarian cancer risk [54]; however, approximately two-thirds of families with three or more cases of familial breast or ovarian cancer had *BRCA1* or *BRCA2* mutations [55, 56]. The remaining inherited risk is most likely due to combinations of common, less-penetrant alleles [57–61]. The most powerful method for identification of moderate risk alleles is a genetic association study examining frequencies of variants such as single-nucleotide polymorphisms (SNPs) in candidate genes of key biological pathways, which has been successful in identifying new risk alleles for a variety of cancers [62–64], or via genome-wide association studies (GWAS). Thus far, variants in the 2q31, 3q25, 8q24, 9p22, 17q21, and 19p13 chromosomal regions [65–67] and in genes involved in cell cycle control [68], steroid hormone metabolism [69], DNA repair [70], and one-carbon metabolism [71] have been associated with ovarian cancer susceptibility. Additional evidence is accumulating that inherited factors, including those in inflammation, relate to clinical outcome in ovarian cancer [72–75].

The identification of polymorphisms worthy of study in given genes can draw on putative-functional or location data (reviewed by Bhatti et al. [76]) or can rely on informative tagSNPs selected on the basis of linkage disequilibrium (LD, the correlation of alleles along a chromosome). The structure of LD for common variants has been mapped by the International HapMap Project using genome-wide genotyping [77], and the LD structure of both common and rarer variants is improved by the 1000 Genomes Project [78] using genome-wide sequencing. These projects allow investigators to harness the power of LD for common disease prediction [79–83]. Due to the strong LD within a region, only a few SNPs need to be assayed to successfully predict all of the common genetic variation once SNP correlations are estimated for a core of individuals. If LD structure within a particular gene is known, careful selection of a reduced set of SNPs that identify or “tag” a particular haplotype may provide as much information as genotyping all available SNPs [84–86]. These tagSNPs then theoretically represent all genetic variation exceeding a specified minor allele frequency (MAF). Thus, by genotyping the LD-selected subset of common variants, it is possible to perform a comprehensive analysis of all common variants in a candidate gene.

The purpose of the current investigation is to identify and classify the highest priority genes within the NF-κB signaling pathway, to evaluate publicly available LD data in the encoding genes, and to select a panel of SNPs representing genetic variation within these genes. The resulting set of annotated genes and variants is now available to the scientific community.

Methods

Classification of genes

Using the PubMed1 search engine in December 2009, we identified genes involved in NF-κB signaling through a literature search including the phrases “NF-kappaB,” “pathway,” and “gene.” Genes were categorized into NF-κB subpathways (canonical, non-canonical, or both canonical and non-canonical) and biological function within the subpathways. Biological function definitions were as follows: subunits (NF-κB proteins), activation (role in signaling cascade leading to NF-κB subunit activation), inhibition (inhibitory role), degradation (involved in proteasomal degradation), and nuclear function (nuclear proteins involved in transcription).

Evaluation of genotype data sources

We assessed three types of large-scale LD characterization projects: (1) genome-wide sequencing efforts by the 1000 Genomes Project2,3; (2) genome-wide SNP genotyping projects, including those of the International HapMap Project4 and Perlegen Sciences5; (3) gene-centric resequencing projects, such as several NHLBI Program for Genomic Applications (PGAs),6,7,8 SeattleSNPs,9 the National Institute of Environmental Health Sciences Environmental Genome Project SNPs Program (NIEHS SNPs),10 Pharmacogenetics and Risk of Cardiovascular Disease (PARC),11 and Molecular Diversity and Epidemiology of Common Disease (MDECODE).12 Based on European or European-American data availability for NF-κB genes, we identified, from among these sources, those sources which were appropriate for LD-based SNP selection and applied the tagSNP selection approach described below.

LD binning and choice of genotype data source

Within each genotyped data source and for each gene, we binned SNPs with MAF ≥ 0.05 that were pairwise correlated at $r^2 \geq 0.9$ using the algorithm implemented in ldSelect [87]. For genome-wide projects (the 1000 Genomes Project, the International HapMap Project, and Perlegen Sciences), gene regions were defined as 5 kb upstream and downstream using genomic coordinates based on RefSeq release 29 (NCBI build 36). For genes with data available in multiple LD sources, we then identified the “best source” of data as the source with the highest number of LD bins after tagSNP selection. It is conservative to include a greater number of tagSNPs rather than a smaller number of tagSNPs in the event that one source overestimated LD For example, even with an equal number of underlying SNPs, if Source A indicated a larger number of LD bins than Source B, we proceed with Source A’s tagSNPs as analysis of data from Source A samples suggested lower LD within the gene and thus the need for more LD bins than Source B. More often, however, the source with the greatest number of LD bins was also the source with the greatest number of underlying SNPs, thus clearly capturing the greatest amount of genetic variation.

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[1] http://www.ncbi.nlm.nih.gov/pubmed/.
[2] http://www.1000genomes.org/; accessed April 2010.
[3] ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/pilot_data/release/2010_03/pilot1/CEU.SRP000031.2010_03.genotypes.vcf.gz.
[4] http://www.hapmap.org; accessed November 2008.
[5] http://genome.perlegen.com/; accessed November 2008.
[6] http://pga.swmed.edu/; accessed November 2008.
[7] http://cardiogenomics.med.harvard.edu/home; accessed November 2008.
[8] http://innateimmunity.net/; accessed November 2008.
[9] http://pga.mbt.washington.edu/; accessed November 2008.
[10] http://egp.gs.washington.edu; accessed November 2008.
[11] http://droog.gs.washington.edu/parc/; accessed November 2008.
[12] http://droog.gs.washington.edu/mdecode/; accessed November 2008.

SNP selection

After LD binning using the best data source for each gene, we identified particular tagSNPs to assay within each bin. Using Illumina iSelect HD Custom Genotyping annotation (San Diego, CA), we prioritized tagSNPs first based on expected assay performance. One tagSNP was selected in bins with less than ten SNPs, two tagSNPs were selected in bins with 10–29 SNPs, and three tagSNPs were selected in bins with 30 or more SNPs. Within each gene, additional putative-functional SNPs with MAF ≥ 0.05 (that were not tagSNPs) were also identified as non-synonymous, 5′ UTR, 3′ UTR, 1 kb 5′ upstream, residing in a splice site (dbSNP version 129), or residing in an miRNA-binding site.13,14

Comparison to genome-wide panels

In order to assess the utility of creating a custom NF-κB SNP panel versus use of a commercially available platform, we compared our prioritized SNPs to: (1) SNPs available on the Illumina Infinium HD Human610-Quad BeadChip genome-wide SNP panel (San Diego, CA) that has been used in prior ovarian cancer GWAS [65] and (2) SNPs in the International HapMap Project Phase II which prior ovarian cancer GWAS have used as the basis for imputation of up to 2.5 million SNPs [65]. We estimated "coverage" of SNPs within NF-κB genes versus those available in two ways: (1) as the proportion of NF-κB LD bins with at least one tagSNP included on these panels and (2) as the proportion of NF-κB SNPs either tagged or directly assessed in bins included on these panels.

Results

Identification of the key components of NF-κB signaling

Our review identified 319 key genes in the NF-κB signaling pathway; these are classified in Table 1 by subpathway (canonical, non-canonical, or both) and biological function (subunits, activation, inhibition, degradation, and nuclear function). We excluded the 26S proteasome (degratory role) and RNA polymerase II (nuclear role) from consideration due to a lack of specificity to NF-κB signaling and an excessive number of genes encoding the many subunits required for the functional protein complexes. Supplemental Table 1 provides detailed information for each gene including protein descriptions, location, size, and references attesting to each gene's relevance in NF-κB signaling. The major components of the NF-κB signaling pathway are summarized below.

Activation of NF-κB subunits—Components of NF-κB signaling cascades include ligands, receptors, and intracellular target molecules that lead to IKK complex activation, IκB degradation, and activation of NF-κB dimers. The primary canonical NF-κB signaling pathway is initiated by binding of TNFα, a proinflammatory cytokine produced mainly by macrophages, but also by mast cells, lymphoid cells, endothelial cells, and fibroblasts (Fig. 2) [88, 89]. Interleukin-1 (IL-1), B-cell activating factor belonging to the TNF family BAFF [90], CD40 ligand [18], lymphotoxin beta (LTβ) [91], tumor necrosis factor receptor superfamily, member 11a, NF-κB activator (called RANK) [92], and T-cell receptor (TCR) [18] and lipopolysaccharide (LPS) ligands [88] binding also initiate NF-κB signaling (Figs. 2, 3, 4; Table 1, Supplemental Table 1). After ligation, specific receptors recruit and activate intracellular target molecules, eventually leading to activation of the IKK complex [88, 93, 94]. In the canonical pathway, the IKK complex contains three kinases, IKKα, IKKβ, and NF-κB essential modulator (NEMO or IKKγ), with various roles in the activation of NF-κB. IKKβ is responsible for phosphorylating IκB, marking it for proteolysis [95]. NEMO is a

13http://www.microrna.org/microrna/home.do.
14http://www.targetscan.org/.

non-enzymatic regulatory subunit that modulates and aids in IKKα and IKKβ activation. In the non-canonical pathway, IKKα initiates processing of inhibitory p100 to functional p52 [46]. Activation of the IKK complex leads to processing of p100 and p105 and degradation of IκB, freeing NF-κB to enter the nucleus [46, 95]. For more details, see Fig. 2 for TNFα [88, 96–103] and TCR ligand signaling pathways [18, 104–114], Fig. 3 for IL-1 [88, 96–98, 115–125] and LPS signaling pathways [18, 88, 126–129], and Fig. 4 for the non-canonical signaling pathway [50, 130, 131].

Inhibition—Several molecules play inhibitory roles by seizing key molecules, preventing apoptosis, or interfering with proteasomal degradation (Table 1, Supplemental Table 1) [93]. The primary NF-κB inhibitors are the seven members of the IκB family, IκBα, IκBβ, IκBε, IκBγ, p100, p105, and BCL-3 [44]. The IKK complex phosphorylates IκBs at two serine residues, marking the inhibitors for poly-ubiquitination by the $SCF^{\beta\text{-}TrCP}$ complex and subsequent degradation by the 26S proteasome. Without ubiquitination and proteolysis of IκB, NF-κB complexes remain inactive in the cytoplasm [44, 46]. Preliminary evidence indicates that each IκB family member has a unique and specific role in the NF-κB pathway [132–135]. IκBα, IκBβ, IκBε, and IκBγ mask NF-κB's nuclear localization signal (NLS), which sequesters NF-κB in the cytoplasm, preventing nuclear translocation and transcription [132]. IκBα and IκBβ have a similar binding affinity for NF-κB; however, IκBα also contains a nuclear export signal (NES) that enables IκBα to shuttle NF-κB complexes out of the nucleus. IκBβ does not contain a NES, but is more effective than IκBα at masking NF-κB's NLS and preventing p65 and p50 translocation [46, 132–135]. p100 and p105 are inhibitory precursors of p52 and p50, respectively, that are partially degraded, yielding functional p52 and p50. Although evidence is unclear, BCL-3 does not appear to have an inhibitory role, but instead may facilitate activation of p50 and p52 in the nucleus [44].

Degradation—Proteolysis of inhibitory molecules is critical in regulating activation of the NF-κB signaling cascade [93]. The $SCF^{\beta\text{-}TrCP}$ complex marks the majority of inhibitors in the NF-κB pathway for degradation through poly-ubiquitination. The main components of this complex are beta-transducin repeat containing (β-TrCP) [136–138], S-phase kinase-associated protein 1 (Skp1), cullin 1 (Cul1), ring-box 1 (RBX1 or Roc1), and ubiquitin-conjugating enzyme E2D 1 (UBE2D1 or HbcH5; Table 1, Supplemental Table 1) [139]. This complex recognizes phosphorylated inhibitors, and, through a series of events, it ubiquitinates these inhibitors, thus marking them for partial or complete degradation by the 26S proteasome [93]. Figure 5 provides more detail on the $SCF^{\beta\text{-}TrCP}$ complex partial degradation of p105/p100 and poly-ubiquitination of IκB [18, 93, 139–143].

Nuclear function—After nuclear translocation, DNA-bound, transcription-promoting NF-κB complexes associate with the RNA polymerase II transcription machinery to initiate gene expression. RNA polymerase II can only initiate transcription after recruitment and assembly of the replication complex components. The general transcription factors, transcription factor for RNA polymerase II (TFII)-A, -B, -E, -F, and -H, contain either a TATA box region, a binding site for nuclear proteins, or an Inr element with a downstream promoter region that functions in a similar manner as the TATA box [93, 144, 145]. Other critical components for transcription of NF-κB-regulated genes include TATA-binding protein (TBP), 14 TBP-associated factors (TAFs) [93, 145], K(lysine) acetyltransferase 2B (called PCAF) [146], cAMP-responsive element-binding protein 1 (CREB) [147], CREB-binding protein (CBP), and E1A-binding protein p300 (p300) [146, 148]. Figure 6 provides more detail on the components of the RNA polymerase II transcription complex [146–148].

SNP selection

Evaluation of LD data sources—Among the LD sources listed in "Methods", we found that data from the following were optimal at the time of analysis (Table 2): the genome-wide sequencing 1000 Genomes Project Low-coverage Pilot on 60 unrelated individuals of European descent,15,16 the two genome-wide genotyping projects the International Hapmap Project Phase II Release 2417 and Perlegen Sciences,18 and the three gene resequencing programs SeattleSNPs,19 NIEHS SNPs,20 and Innate Immunity SNPs.21 For 319 NF-κB identified candidate genes, 1000 Genomes Project data were available for all 302 autosomal genes (and none of the 17 chromosome X genes), International HapMap Project data were available for all but one gene (*GTF2H2*), and Perlegen Sciences data were available for 297 candidate genes; SeattleSNPs had resequenced 42 genes, Innate Immunity SNPs had resequenced 21 genes, and NIEHS SNPs had resequenced nine genes. Using data from each source, tagSNP selection was performed for all genes to bin SNPs with MAF ≥ 0.05 and r^2 ≥ 0.9. The 1000 Genomes Project was found to be the most informative source for 296 genes (92.8%), the International HapMap Project Phase II for 16 genes (5.0%, including 15 on chromosome X), Innate Immunity SNPs for three genes (0.9%, including one on chromosome X), SeattleSNPs for three genes (0.9%), and NIEHS SNPs for one gene (0.3%, including one on chromosome X); Perlegen Sciences data was not the most informative source for any gene. Supplemental Table 2 provides the most informative source identified for each gene.

TagSNPs and putative-functional SNPs—Summarizing across all genes and using the optimal LD source, 13,027 LD bins covering 36,181 common SNPs (mean 81 bins [range 0–905 bins] per gene, mean 226 SNPs [range 0–3,069 SNPs] per gene) were identified. Putative-functional SNPs, some of which were also tagSNPs, were identified; putative-functional SNPs were identified in 260 genes including 173 non-synonymous SNPs, 81 5′ UTR SNPs, 389 3′ UTR SNPs, 304 SNPs 1 kb 5′ upstream, and 165 SNPs residing in an miRNA-binding site. No splice site SNPs were identified. Supplemental Table 2 provides detailed information about the number of bins within each gene as well as the number of SNPs tagged by tagSNPs. In total, a collection of 14,231 SNPs of interest to genotype in 319 NF-κB genes has been assembled representing common underlying genetic variation among populations of European descent. These SNPs do not attempt to cover rare variants (MAF < 5%).

Coverage on genome-wide arrays—We evaluated coverage of our 13,027 identified NF-κB tagSNPs on the Illumina Infinium HD Human610-Quad BeadChip and in HapMap Phase II data (often used for imputation). Details per gene are provided in Supplemental Table 2, and Fig. 7 summarizes coverage across genes. Generally, use of the Illumina 610-Quad without additional imputation would directly assess 22% of the identified NF-κB bins and provide data on 50% of the total SNPs with MAF > 0.05 in each gene. Thus, the NF-κB tagSNPs included on the Illumina 610-Quad tend to tag larger LD bins, reflecting a preference in Illumina's SNP selection for the most informative tagSNPs and placement of lower priority on singleton bins. The approach of dropping smaller bins is reasonable due to cost constraints, because smaller bins convey information on a minimal set of underlying SNPs. However, this coverage is not optimal for many applications. With additional

[15] http://www.1000genomes.org/; accessed April 2010.
[16] ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/pilot_data/release/2010_03/pilot1/CEU.SRP000031.2010_03.genotypes.vcf.gz.
[17] http://www.hapmap.org; accessed November 2008.
[18] http://genome.perlegen.com/; accessed November 2008.
[19] http://pga.mbt.washington.edu/; accessed November 2008.
[20] http://egp.gs.washington.edu; accessed November 2008.
[21] http://innateimmunity.net/; accessed November 2008.

imputation using HapMap Phase II data, coverage increases; on average, 43% of NF-κB bins would be assessed, providing data on 74% of the included SNPs. Again, larger bins have been identified by HapMap Phase II; however, the current availability of 1000 Genomes Project data indicates that less than optimal NF-κB coverage is provided by the use of HapMap Phase II imputation. We note that this is not due to the discovery of novel rare variants in 1000 Genomes Project data, as NF-κB LD binning was limited to SNPs with MAF > 0.05. Coverage and statistical power is further reduced with use of HapMap Phase II imputation due to the use of imputation rather than directly genotyping SNPs of interest. To address these issues, GWAS panels are currently under commercial development, which are based on 1000 Genomes Project data, and these will fill a critical void in commercial arrays. In the interim, current ovarian cancer GWAS are not expected to have optimal inclusion of NF-κB tagSNPs. Custom SNP arrays based on the NF-κB variants identified here will provide maximal information about these key genes of interest to ovarian cancer and other phenotypes.

Discussion

NF-κB is a key regulator of expression of hundreds of genes involved in crucial cellular processes related to normal cellular function and carcinogenesis. Its signaling pathway involves a complex cascade of events initiated by immune and other signaling proteins, regulated by several inhibitory molecules, and that eventually leads to NF-κB-mediated regulation of gene expression. There is an abundance of evidence linking a prolonged, aberrant inflammatory response with ovarian cancer. As the most lethal gynecologic malignancy, the quest for a better understanding of ovarian cancer genomics is critical. Due to NF-κB's vital role in responding to inflammatory signals, initiating expression of pro-inflammatory genes, and proven links between ovarian carcinogenesis and inflammation, a study examining variations in genes involved in the NF-κB pathway and ovarian cancer risk is warranted.

Here, we summarized the relevance of the NF-κB signaling pathway in ovarian cancer and identified 319 genes encoding critical components categorized by subpathway and biological function. Additionally, we identified 14,231 tagSNPs and putative-functional SNPs within each of these genes. Of LD data sources available in April 2010, we found that the data from the 1000 Genomes Project was more informative for the vast majority of genes than that from the International HapMap Project and other projects. We also found that coverage of variation in these genes on the commonly used [65] commercial panel Illumina Infinium HD Human610-Quad BeadChip was suboptimal, even considering imputation of missing genotypes using the International HapMap Project Phase II data. Thus, in order to comprehensively assess inherited variation in the NF-κB signaling pathway, a customized SNP panel, such as an Illumina iSelect HD Custom Genotyping, is preferable. As new versions of data from the 1000 Genomes Project are unveiled, it will be critical to re-evaluate genomic coverage of these candidate genes. The collection of SNPs we have currently identified is available to the scientific community studying association between variation in NF-κB signaling and any phenotype, including ovarian and other cancers [149–152] as well as autoimmune [153], vascular [154], and muscular disorders [155].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was made possible by NCI funding R01-CA122443, Mayo Clinic SPORE in Ovarian Cancer (P50 CA136393), and support from the Mayo Foundation.

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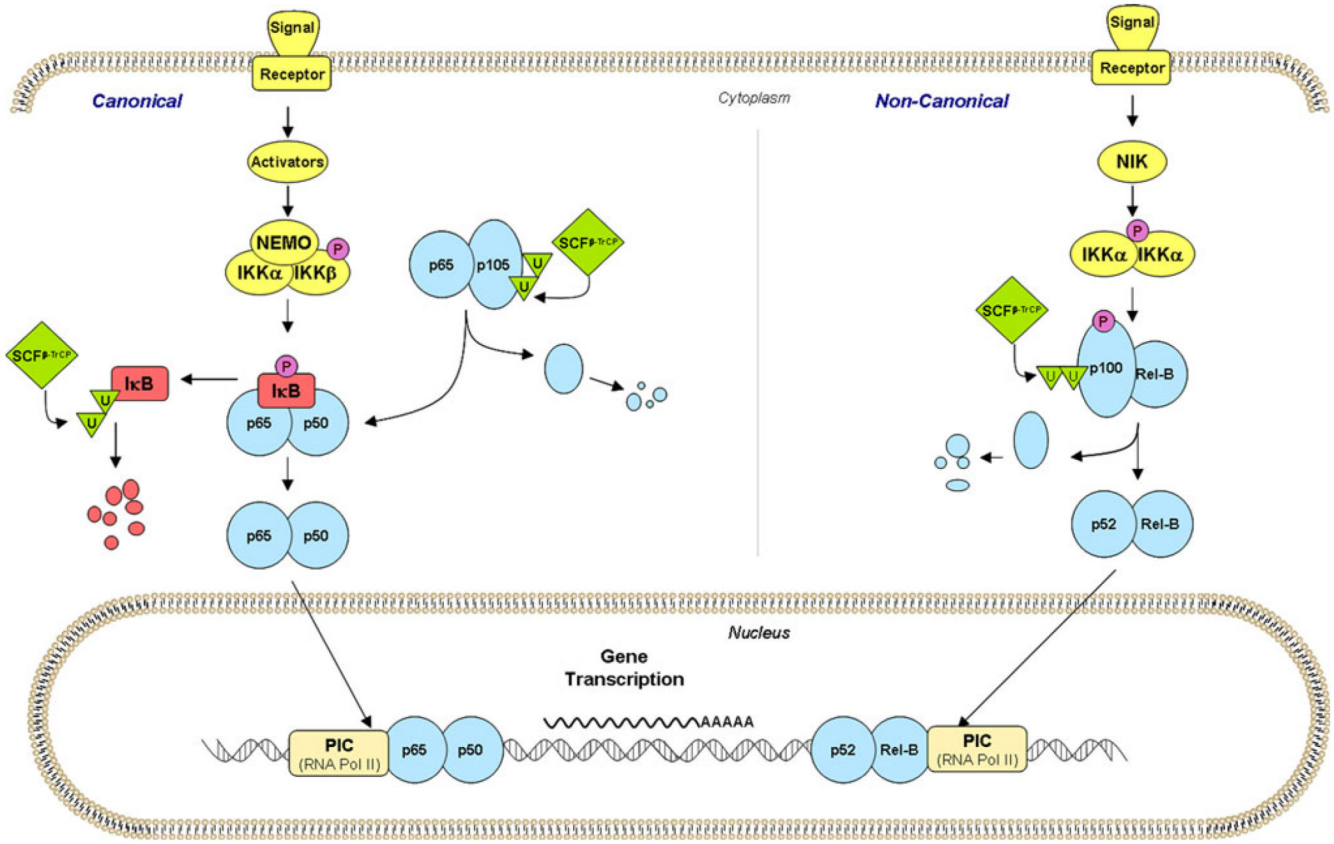

Fig. 1.

Key components of NF-κB signaling pathways. Color-coding of molecules: activators = *yellow*, NF-κB subunits = *blue*, inhibitors = *red*, degradators = *green*, nuclear function = *cream*. Canonical pathway: Binding of extracellular signaling molecules to receptors leads to recruitment of activation proteins, phosphorylation of IKKβ, activation of the IKK complex (IKKα, IKKβ, and NEMO), and processing of p105 and p100 into functional p50 and p52 by the ubiquitin ligase $SCF^{\beta\text{-}TrCP}$ complex. Activated IKK complex phosphorylates IκB, marking it for ubiquitination by the $SCF^{\beta\text{-}TrCP}$ complex and degradation. The main canonical NF-κB dimer, p65/p50, is activated and translocates to the nucleus to initiate gene transcription with the RNA polymerase II preinitiation complex (PIC). Non-canonical pathway: Ligase and receptor coupling leads to NIK and IKKα activation, which initiates processing of p100 into functional p52 by the $SCF^{\beta\text{-}TrCP}$ complex. The p52/Rel-B dimer translocates to the nucleus to initiate gene expression through the RNA polymerase II PIC

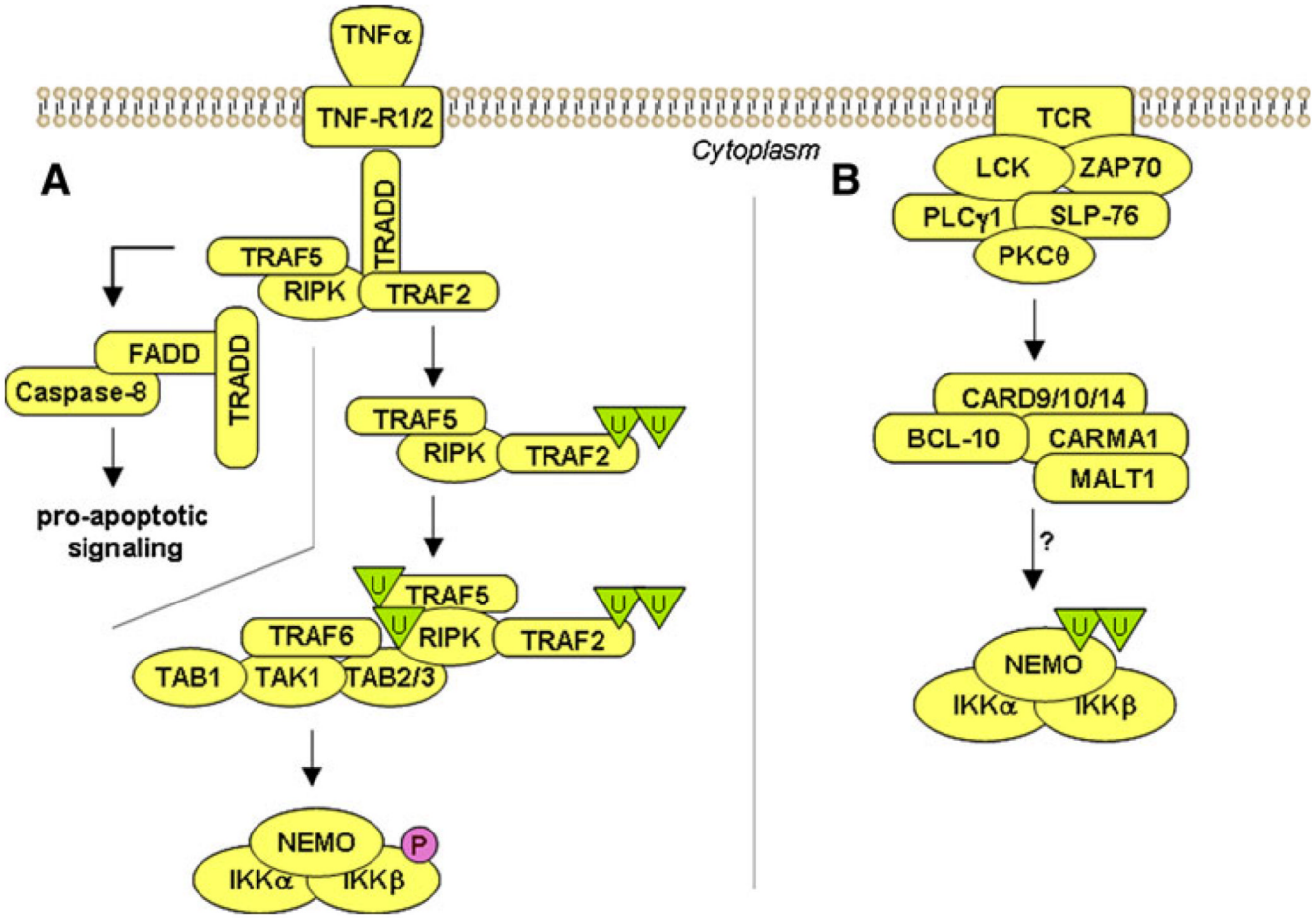

Fig. 2.

TNFα and TCR activation pathways. **A** TNFα binding to its receptors TNF-R1 and TNF-R2 triggers a coupling between TNF-R1/2 and TRADD via their death domains (*DD*). TRADD recruits TRAF2, RIPK1, RIPK2, and RIPK3, and TRAF5. Following TNF-R1 endocytosis, TRADD dissociates from the receptor complex and associates with FADD and caspase-8, initiating pro-apoptotic signaling pathways. After TRAF2 auto-poly-ubiquitination, RIPK1 is poly-ubiquitinated, which recruits TAB 1, TAB 2, TAB 3, TAK1, and TRAF6 to the complex. TAB 2 forms a bridge between TRAF6 and TAK1, allowing TAK1 to activate the IKK complex by phosphorylating IKKβ. **B** Activation of the TCR-CD3 complex induces phosphorylation of ITAMs on TCRζ and CD3. ZAP70 and LCK are recruited, LCK phosphorylates ZAP70, PLCγ1, and the scaffold protein SLP-76, and recruits PKCθ to the complex. PKCθ recruits CARMA1, CARD9, CARD10, CARD14, BCL-10, and MALT1, leading to activation of the IKK complex

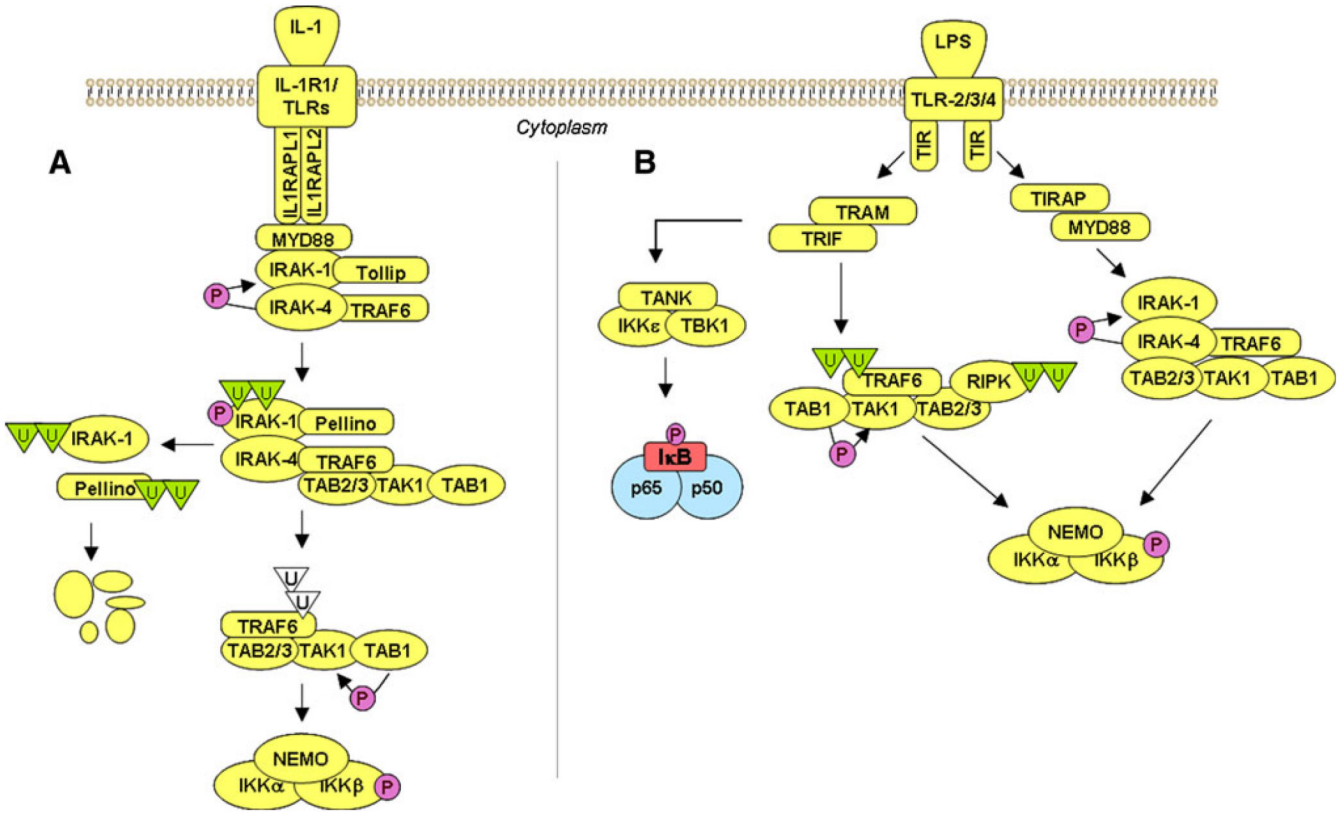

Fig. 3.

IL-1 and TLR activation pathways. **A** Ligand binding to IL-1R1 leads to coupling of IL-1RAPcP1, IL-1RAPcP2, and MYD88 with receptors. MYD88 recruits IRAK-1 and the adaptor proteins Tollip, TRAF6, and IRAK-4 to the receptor complex. After IRAK-1 phosphorylation by IRAK-4, IRAK-1, and TRAF6 dissociate from the receptor complex and associate with TAB 1, TAB 2, TAB 3, and TAK1. Pellino proteins phosphorylate IRAK-1, initiating IRAK-1 and Pellino poly-ubiquitination and degradation, freeing the TRAF6/TAB 1/TAB 2/TAB 3/TAK1 complex. TAB 2 phosphorylates TAK1, which phosphorylates and activates IKKβ. **B** Toll-like receptors (*TLRs*) are critical components of the innate immune response as they are conserved pattern recognition receptors (*PRRs*) that detect conserved pathogen-associated microbial patterns (*PAMPs*) present on many antigens. There are over ten TLRs that have been identified, all of which recognize a variety of different PAMPs and lead to NF-#x003BA;B activation. TLRs and IL-1R1 have a shared Toll-IL-1R (*TIR*) domain that mediates interactions with downstream molecules. Due to this shared TIR domain, TLRs initiate a similar signaling cascade as IL-1 involving MYD88, IRAK-1, IRAK-4, TRAF6, TAB 2, and TAK1, but also involve other cascades that include TIRAP, TRIF, TANK, and TBK1

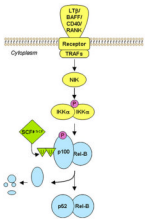

Fig. 4.

Non-canonical activation pathways. Receptor binding of LTβ, BAFF, CD40, and RANK initiate the non-canonical signaling pathway. TRAF proteins and other intracellular target molecules are recruited, eventually leading to the activation of NIK through an unknown mechanism. NIK contains binding domains that interact with TRAF2, TRAF3, TRAF5, and TRAF6, but the specific roles of TRAF and other proteins in the non-canonical pathway is still controversial. After NIK phosphorylation, IKKα phosphorylates p100, marking it for poly-ubiquitination and partial degradation by the $SCF^{\beta\text{-}TrCP}$ complex and 26S proteasome. Partial degradation of p100 yields functional p52, which most commonly forms heterodimers with Rel-B and translocates to the nucleus to initiate gene transcription

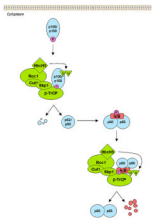

Fig. 5.

Ubiquitination and proteasomal degradation of IκBs. The E3 protein-ubiquitin ligase $SCF^{\beta\text{-}TrCP}$ complex consists of β-TrCP, an F-box protein with a WD repeat region that serves as the docking subunit for the $SCF^{\beta\text{-}TrCP}$ complex, the adaptor proteins Skp1 and Cul1, and the RING protein Roc1 that recruits the E2 ubiquitin-conjugating enzyme UbcH5. The precursor proteins p100 and p105 are partially degraded to yield functional p52 and p50, respectively. p105 contains a glucine-rich region (*GRR*), which separates the functional from inhibitory portions of the protein and is essential for its processing. After being marked for ubiquitination by the $SCF^{\beta\text{-}TrCP}$ complex, the proteasome degrades p105 and p100 until it hits the GRR region signaling the completion of processing and the functional subunit is released, or the proteasome is unable to properly unfold the GRR region, forcing it to complete processing prematurely and release p50/p65. Additionally, the $SCF^{\beta\text{-}TrCP}$ complex processes IκB after IKKβ phosphorylates IκBα, IκBβ, and IκBε. β-TrCP recognizes the degradation signal and UbcH5 and the $SCF^{\beta\text{-}TrCP}$ complex polyubiquitinate lysine residues on IκBα, which marks it for degradation by the 26S proteasome

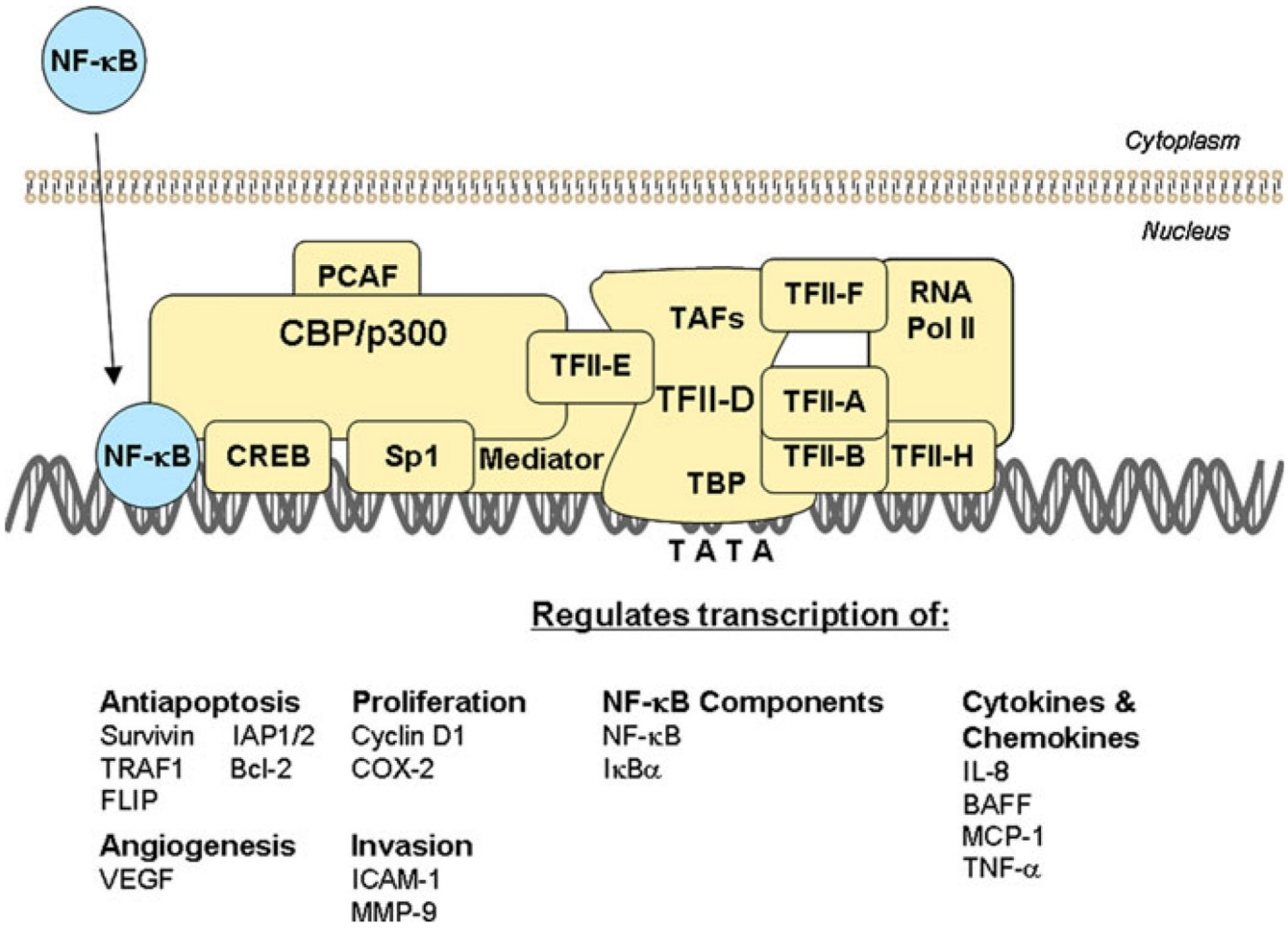

Fig. 6.

RNA polymerase II transcription complex. Formation of the transcription pre-initiation complex (*PIC*) begins with the general transcription factor TFII-D, which is composed of the TATA-binding protein TBP and 14 TBP-associated factors (*TAFs*). The transcription factor, TBP binds to the TATA box in the promoter region and is necessary for completion of the PIC complex. TAF1 and TAF5 appear to be the “organizers” of the complex with TAF1 serving as the scaffold for TAF2, TAF4, TAF5, TAF6, TAF9, TAF11, and TAF12 and branching across two of three lobes in TFII-D. TAF5 binds to TAF1, TAF7, TAF11, TAF12, TAF13, and TBP and contains connections to all three of the TFII-D lobes. Creation of the TFII-D complex recruits TFII-A to bind to both the DNA and TFII-D, stabilizing their interaction. TFII-B binds to TBP and the DNA, serving as a scaffold for RNA polymerase II. TFII-F enters the complex with RNA polymerase II, both of which bind to the TFII-B/TBP complex. After RNA polymerase II is bound, TFII-E and TFII-H enter, completing the PIC and initiating transcription

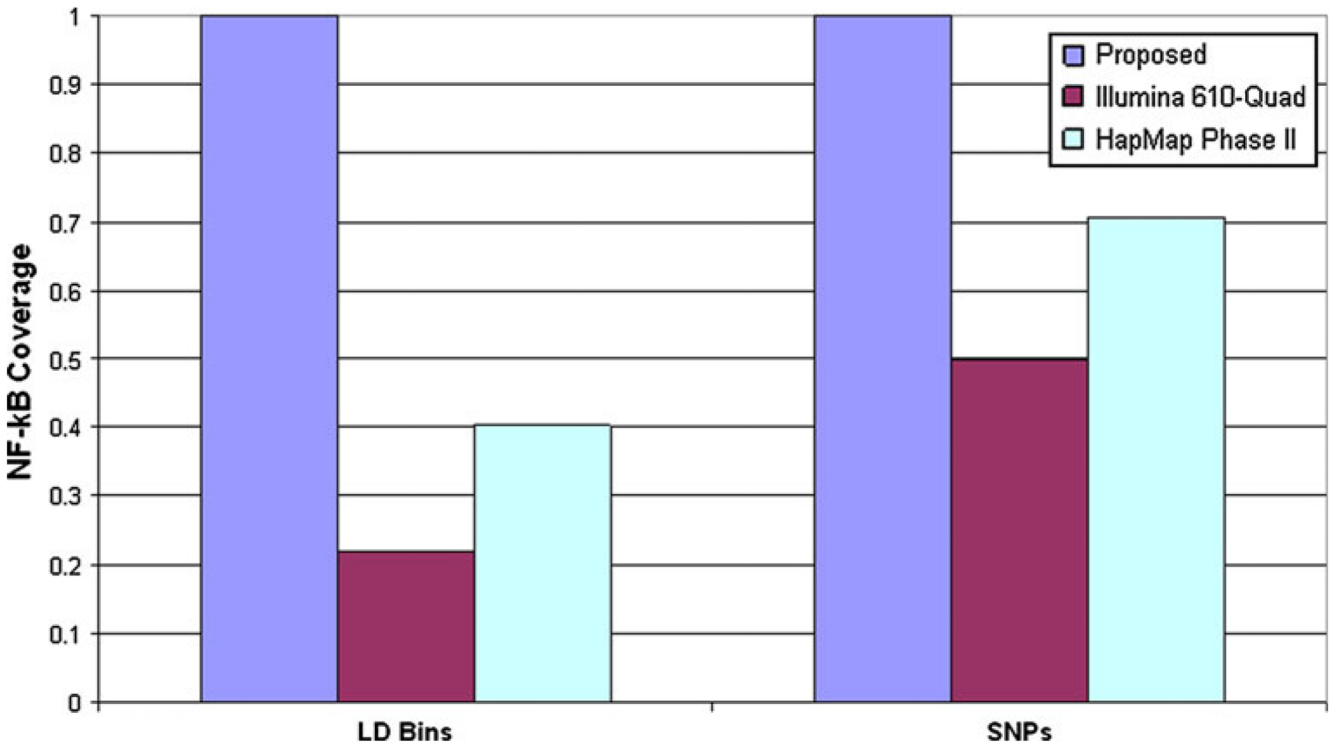

Fig. 7.

Coverage of inherited variation in 319 NF-κB signaling genes. Coverage calculations are based on tagSNPs in NF-κB genes ($r^2 \geq 0.9$ and MAF ≥ 0.05) using the most informative source available, including 1000 Genomes Project Low-coverage Pilot data for 92.8% of genes (see "Methods"). LD Bins reflects the proportion of LD bins with at least one tagSNP on the Infinium HD Human610-Quad BeadChip genome-wide SNP panel (Illumina 610-Quad) or imputable based on HapMap Phase II data. SNPs reflect the proportion of SNPs interrogated, either directly or indirectly, via genotyping the included tagSNPs on the Illumina 610-Quad or imputing based on HapMap Phase II data (representing SNPs directly assessed or in the covered LD bins). Results for the Illumina 610-Quad and HapMap Phase II are presented due to use in current ovarian cancer genome-wide association studies (e.g., Song et al. [65])

Table 1

NF-κB genes classified by function and subpathway

Function	Subpathway			
	Canonical	**Non-Canonical**	**Both**	**Unknown**
Subunits	*NFKB1, REL, RELA*	*NFKB2, RELB*	–	–
Activation	*AHR, AKT1, AKT2, ATM, AZI2, BCL10, BIRC3, BRCA1, CARD10, CARD11, CARD14, CARD4, CARD9, CASP1, CASP2, CASP8, CC2D1A, CD247, CD28, CD3D, CD3E, CD3G, CDC37, CDK4, CFLAR, CRADD, CRP, CSNK2A2, CSNK2B, ECSIT, EGF, EGFR, EIF2AK2, ERBB2, FADD, FRAP1, HRAS, HSP90AA1, HSP90AA2, HSP90AB1, IKBKB, IKBKE, IKBKG, IL12A, IL15, IL17A, IL18, IL1A, IL1B, IL1R1, IL1R2, IL2, IL6, IL8, IL1RAPL1, IL1RAPL2, ILK, IRAK1, IRAK2, IRAK4, KRAS, LAT, LCK, LCP2, LBP, MALT1, MAP2K1, MAP2K2, MAP2K3, MAP2K4, MAP2K5, MAP2K6, MAP2K7, MAP3K1, MAP3K11, MAP3K2, MAP3K3, MAP3K7, MAP3K7IP1, MAP3K7IP2, MAP3K7IP3, MAP3K8, MAP4K1, MAPK14, MAPK8, MTDH, MYD88, NCAM1, NOD2, NRK, PDPK1, PELI1, PELI2, PELI3, PIK3CA, PIK3CB, PIK3CG, PIK3R1, PLCG1, PRKACA, PRKACB, PRKCA, PRKCQ, PRKDC, PTPRC, RAF1, RHOA, RIPK1, RIPK2, RIPK3, SPAG9, STAT1, SYK, TBK1, TBKBP1, TICAM1, TICAM2, TIFA, TIRAP, TNF, TNFRSF10A, TNFRSF10B, TNFRSF10D, TNFRSF1A, TNFSF10, TOLLIP, TP53, TRA@, TRADD, TRAF2, TRAF7, TRB@, TRD@, TRG@, TRIP6, VISA, ZAP70*	*CD40, CD40LG, DYNLL1, LTA, LTB, LTBR, MAP3K14, TNFRSF11A, TNFRSF13B, TNFRSF13C, TNFSF11*	*CHUK, FKBP5, IKBKAP, TANK, TLR1, TLR10, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TNFRSF1B, TRAF5, TRAF6, TRAPPC9, XIAP*	–
Inhibition	*ARRB1, ARRB2, ATR, BAG4, BIRC2, CARD6, CARD8, CDKN2A, CHEK1, CLU, CYLD, EGR1, ESR1, ESR2, G3BP2, HEXIM1, HMOX1, IL10, IL1RN, IL3, IRAK3, ITGA3, MCC, MDM2, NALP12, NFKBIA, NFKBIB, NFKBIE, NFKBIZ, NKIRAS1, NKIRAS2, NR1D1, OTUD7B, PGR, PKN1, PPARA, PPARG, PPP1R13L, PPP6C, PTPN13, PYCARD, RNF216, SOD1, TAL1, TNFAIP3, TNIP1, TNIP2, TNIP3, TRAF1, TRIB3*	*IFNG, TRAF3, TRAF31P2*	*TTRAP*	*NKRF*
Degradation	*CSNK2A1, GSK3A, GSK3B, HNRNPAB, RPS6KA1, VCP*	–	*BTRC, CAPN1, CUL1, FBXW11, FBXW7, RBX1, SKP1, SUMO4, UBE2D1, UBE2D2, UBE2 N, UBE2V1, USP11*	–
Nuclear function	*BCL3, DHX9, F2R, KPNA3, NCOA3, NKAP, PRKCZ, RNF25, RPS6KA4, RPS6KA5, STAT3*	*KPNA2, KPNA6*	*ATF1, CREB1, CREBBP, EP300, FUS, GTF2A1, GTF2A2, GTF2B, GTF2E1, GTF2E2, GTF2F1, GTF2F2, GTF2H1, GTF2H2, GTF2H3, GTF2H4, GTF2H5, HDAC1, HDAC3, HDAC6, JUN, KAT2B, MAPK1, MAPK3, MCM5, MCM7, POLR2A, POLR2B, POLR2C, POLR2D, POLR2E, POLR2F, POLR2G, POLR2H,POLR2I, POLR2 J, POLR2 K, POLR2L, RPS6KA3, SP1,*	–

Function	Subpathway			
	Canonical	Non-Canonical	Both	Unknown
			TAF1, *TAF10*, *TAF11*, *TAF12*, *TAF13*, *TAF15*, *TAF2*, *TAF3*, *TAF4*, *TAF4B*, *TAF5*, *TAF6*, *TAF7*, *TAF8*, *TAF9*, *TBP*	
Unknown	–	–	–	*NFKBIL1*, *NFKBIL2*, *NFRKB*, *TCEAL7*

Columns represent a NF-κB subpathway categorization (canonical, non-canonical, both, or unknown); rows represent the biological function of the gene (subunit, activation, inhibition, degradation, nuclear function, or unknown)

Table 2

Characteristics of linkage disequilibrium data sources

Data source	Samples	*n* Total SNPs/genes	N NF-κB genes	Ref.
Genome-wide sequencing				
1000 Genomes Project Low-coverage Pilot	60 unrelated CEU	7.7 million SNPs	302	1
Genome-wide genotyping				
International HapMap Project Phase II	60 unrelated CEU	4.0 million SNPs	318	2
Perlegen Sciences	24 European-American	1.6 million SNPs	297	3
Gene-specific sequencing				
SeattleSNPs PGA: Panel 1	23 unrelated CEPH	203 genes	39	4
SeattleSNPs PGA: Panel 2	23 unrelated CEU	105 genes	3	4
Innate Immunity SNPs PGA	23 unrelated European	83 genes	21	5
NIEHS SNPs Panel 1	22 unrelated European	201 genes	9	6

N genes/SNPs based on human coding genes (release 29 in genome build 36.3); N NF-κB from among $n = 319$ listed in Supplemental Table 1; CEU, samples collected in 1980 from U.S. residents with northern and western European ancestry by the Centre d'Etude du Polymorphisme Humain (CEPH); References: 1. http://www.1000genomes.org; accessed April 2010; 2. http://www.hapmap.org; accessed November 2008; 3. http://genome.perlegen.com/; accessed November 2008; 4. http://pga.mbt.washington.edu; accessed November 2008; 5. http://www.innateimmunity.net; accessed November 2008; 6. http://egp.gs.washington.edu; accessed November 2008