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Review

NF-kappaB-inducing kinase in cancer

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ABSTRACT

Dysregulation of the alternative NF-κB signaling has severe developmental consequences that can ultimately lead to oncogenesis. Pivotal for the activation of the alternative NF-κB pathway is the stabilization of the NF-κB-inducing kinase (NIK). The aim of this review is to focus on the emerging role of NIK in cancer. The documented subversion of NIK in cancers highlights NIK as a possible therapeutic target. Recent studies show that the alterations of NIK or the components of its regulatory complex are manifold including regulation on the transcript level, copy number changes, mutations as well as protein modifications. High NIK activity is associated with different human malignancies and has adverse effects on tumor patient survival. We discuss here research focusing on deciphering the contribution of NIK towards cancer development and progression. We also report that it is possible to engineer inhibitors with high specificity for NIK and describe developments in this area.

1. Introduction

The aberrant upregulation of the NF-κB signaling pathway is a hallmark of many cancers. In this context, the contribution of the classical NF-κB pathway is well-documented and reviewed in the literature [1–3]. In contrast, the role of the alternative NF-κB pathway in the development of cancer was notably overlooked. We discuss in this review recent research that highlights not only the important function that the alternative NF-κB pathway plays in tumorigenesis but also creates the basis for future studies in the direction of utilizing NIK as a target in combination therapies.

A key mediator of the alternative NF-κB pathway is the NF-κB-inducing kinase (NIK) which was originally identified as a TNF receptor-associated factor 2 (TRAF2) binding partner in a two-hybrid screen of a human B-cell cDNA library. NIK is a serine/threonine-specific protein kinase belonging to the mitogen activated kinase kinase kinase (MAP3K) family of protein kinases [4]. The human *MAP3K14* gene (Fig. 1A) is located on chromosome 17 (q21.31) spanning approximately 54 kb and encodes the 104 kDa NIK protein (Fig. 1B), which contains various regulatory and binding domains. NIK contains a N-terminal IAP-binding motif (IBM) which binds the baculovirus inhibitor of apoptosis protein repeat 2 (BIR2) domain of cIAP1 ensuring maximal NIK degradation [5]. The adjacent TRAF3-binding motif (aa: 78–84) mediates the repression of NIK under basal, non-stimulated conditions [6]. The kinase domain of NIK is located between amino acids 375–655 [7]. Further, NIK contains a nuclear localization signal (NLS) (aa: 136–149), a nuclear export signal (NES) (aa: 795–805) [8], as well as a C-terminal non-catalytic domain (aa: 675–947) where molecules like TRAFs [9] and p100 [10] or substrates like IκB kinase alpha (IKKα) [11], which is phosphorylated by the kinase activity, are bound.

The functional relevance of the Thr559 phosphorylation site within the activation loop of the kinase domain (Fig. 1C) however, is still being controversially discussed. While some studies indicate a crucial role of T559 phosphorylation to mediate downstream signaling [11,12], other studies suggest a dispensable role of T559 phosphorylation and a phosphorylation-independent constitutively active conformation of NIK [7,9].

NIK is well known for its role in the activation of the alternative NF-κB signaling pathway which has been reviewed extensively elsewhere [13,14]. In addition, NIK has also been implicated in the regulation of the classical NF-κB activation. It has been shown that upon stimulation with CD40L, B-cell-activating factor (BAFF) or CD70, NIK-deficiency represses classical and alternative NF-κB signaling but had no impact on TNF-induced NF-κB signaling, indicating a ligand-specific NIK-dependency [15]. Further, another study provides evidence for an enhancing role of NIK on classical NF-κB signaling upon IL-1β stimulation. These data indicate that TRAF3-deficiency promotes basal and IL-1β-induced canonical NF-κB gene expression *in vivo* and *in vitro* in a NIK-dependent manner [16].

In quiescent cells, low basal abundance of NIK protein is maintained by an interacting regulatory complex (Fig. 2A) consisting of cIAP1/2, TRAF2 and TRAF3, whereby NIK is K48-linked ubiquitinylated by cIAP1/2 and degraded by the proteasome [17]. The functions of cIAP1 and cIAP2 in the regulatory complex are redundant [17] and therefore addressed as cIAP1/2. Upon receptor stimulation (Fig. 2B) by the respective ligand, TRAF2/3 are recruited to the receptor typically followed by cIAP1/2-dependent TRAF3 degradation, leading to the stabilization of NIK [17]. NIK accumulation precedes the processing of

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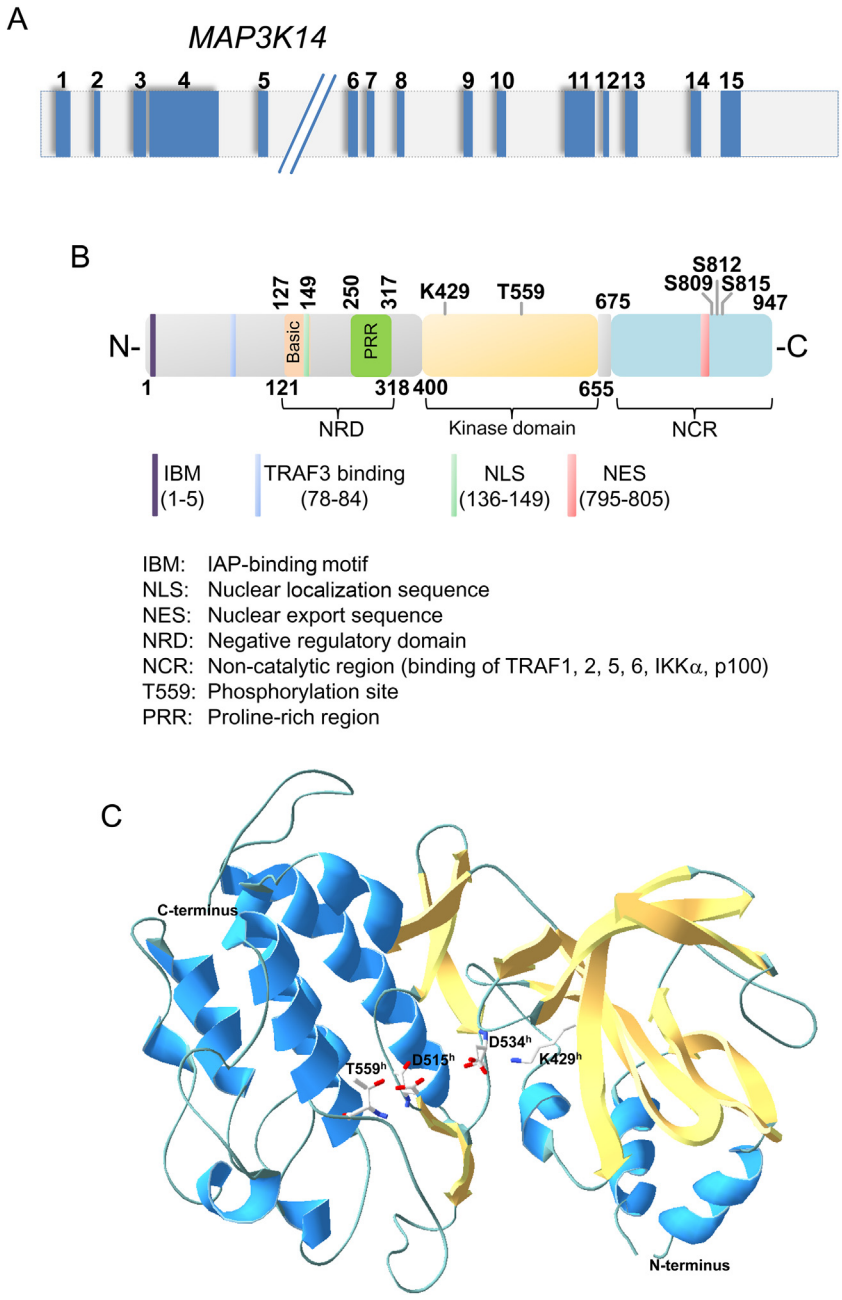


Fig. 1. The *MAP3K14* gene, protein domains and crystal structure of the catalytic domain of human NF-κB-inducing kinase (NIK). (A) The *MAP3K14* gene (Entrez gene: 9020) consists of 15 exons and spans the region from 45,263,119-45,317,064 (GRCh38/hg38) on the minus strand of chromosome 17. (B) The protein domains of NIK are depicted in different colors and modification sites are numbered according to the sequence of the human protein. (C) The crystal structure of the catalytic domain of human NIK determined by X-ray diffraction at 2.9 Å (4G3D) is visualized using DeepView v4.1. The active site amino acids (K429, D515, D534) and the T559 phosphorylation site are depicted and colored as CPK model.

p100 (*NFKB2*) to p52 leading to the translocation of p52/RelB to the nucleus and the regulation of target genes [18]. The activation of NIK is negatively regulated through phosphorylation at S809, S812 and S815 by IKKα (Fig. 2B, [19]). The subsequent turnover of induced NIK is proteasome-dependent, but cIAP1/2-independent [20]. Also, the deubiquitinylase ovarian tumor domain containing 7B (OTUD7B) prevents further proteasomal degradation of TRAF3, therefore providing the basis for the NIK regulatory complex ensuring NIK turnover after induction [21].

The alternative NF-κB pathway and therefore NIK plays a crucial role in B-cell maturation [22], lymphoid organ development [23] and innate and adaptive immunity [24]. In this review, we focus on the alterations of NIK leading to the development or progression of different cancers and the underlying mechanisms. Furthermore, we examine functions of NIK, independent of its role in the induction of the alternative NF-κB pathway. Finally, we comment on recently developed inhibitors of NIK and their therapeutic implications.

2. Dysregulation of NIK-induced signaling supports cancer initiation and progression

Dysregulation of alternative NF-κB signaling has severe developmental consequences that can lead to oncogenesis [25] (Fig. 3). In embryonal hematopoiesis for example, NF-κB signaling is integral to hematopoietic stem cells homeostasis [26]. In addition, studies in recent years have emphasized the importance of NIK and the alternative NF-κB pathway in the self-renewal of hematopoietic stem/progenitor cells (HSPCs) [27], regulatory T-cell homeostasis [28], B-cell maintenance in mice [22] and dendritic cell cross-priming of CD8+ T-cells [29]. Hence, the development and maintenance of hematological cancers like acute myeloid leukemia (AML), multiple myeloma (MM), Hodgkin lymphoma (HL) and many solid cancers display a dysregulation of not only the classical, but also the alternative NF-κB signaling pathway.

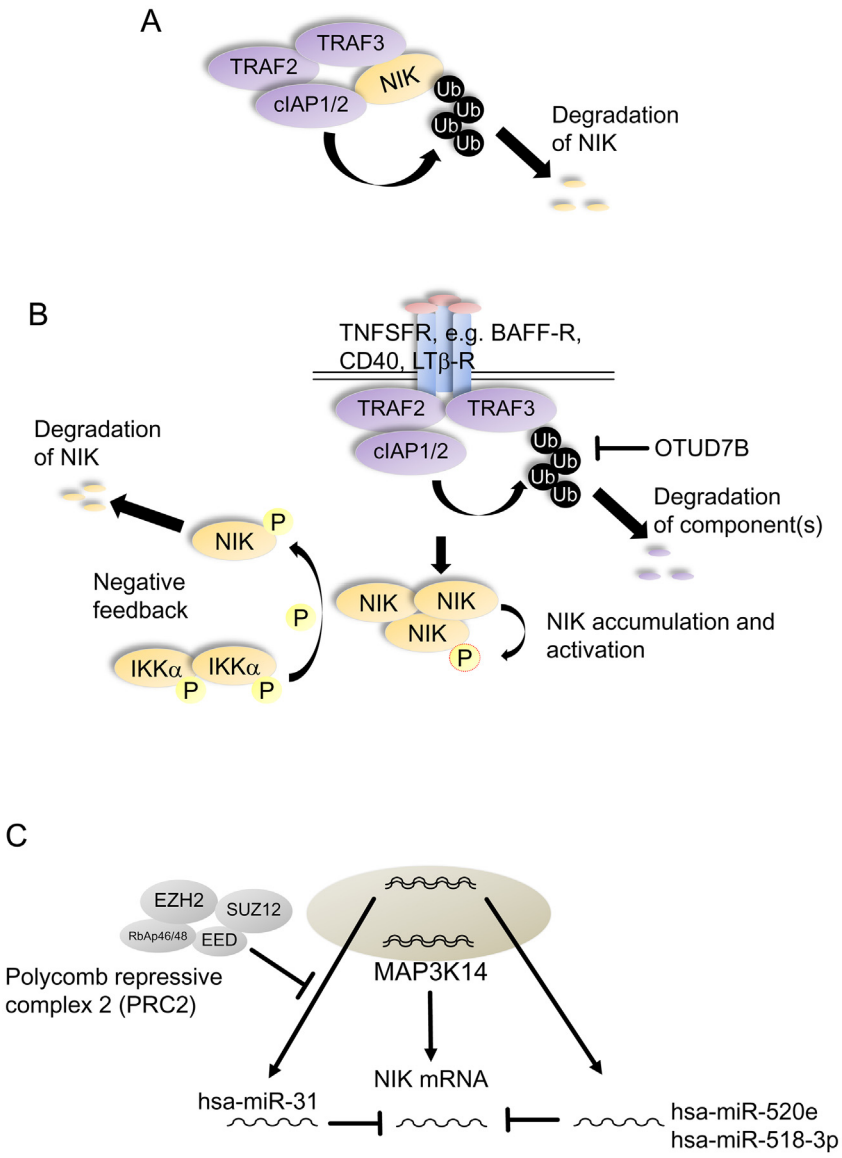


Fig. 2. Regulation of NIK. (A) In quiescent cells, NIK is constantly degraded by the proteasome. The binding of TRAF3 to NIK leads to the recruitment of preassembled TRAF2-cIAP1/2 followed by cIAP1/2-mediated K48-linked ubiquitinylation of NIK. (B) Upon receptor stimulation, TRAF2 and TRAF3 are recruited to the cytosolic domain of the receptor leading to a switch in the target specificity of cIAP1/2 from NIK to TRAF3. TRAF3 is subsequently K48-linked ubiquitinylated and degraded. This liberates NIK followed by its accumulation and activation which may or may not involve the autophosphorylation at Thr559. Stabilized NIK can be negatively regulated by IKKα-dependent phosphorylation. (C) NIK abundance is also regulated by the repression of miRNAs. For instance, the polycomb repressive complex 2 represents a histone methyltransferase activity leading to the trimethylation of histone 3 (H3K27me3) and chromatin silencing repression of miR-31. Two other miRNAs, namely miR-520e and miR-518-3p were found to be repressed in HCC and CRC, respectively leading to excessive NIK stabilization.

2.1. AML and constitutive NIK activity

AML is a cytogenetically heterogeneous clonal disorder of hematopoietic stem cells leading to the inhibition of their differentiation and therefore accumulation of these cells in varying immature stages [30]. Ten percent of all leukemias harbors rearrangements and translocations of the mixed-lineage leukemia 1 (*MLL1,* 11q23; now referred to as *Lysine [K]-specific MethylTransferase 2A* (*KMT2A)*) gene that encodes a protein with histone H3 lysine 4 (H3K4) methyltransferase activity [31]. A common alteration of the *MLL1* gene in myeloid leukemias is a translocation that gives rise to MLL fusion with *ALL1-fused gene from chromosome 9* (AF9). A study by Xiu and colleagues [32] addresses the function of NIK in cells with *MLL-AF9* rearrangement by using mouse models with a hematopoietic system-specific or an inducible, whole body stabilization (NIKERT2) of NIK. Both mouse models utilize the TRAF3 binding-deficient mutant of NIK (NIKDT3^{flSTOP}) for its stabilization. Isolated HSPCs from wildtype (wt) or NIKERT2 mice were transduced with *MLL-AF9* and transplanted into lethally irradiated recipient mice together with rescue cells. Although all recipients developed AML, the recipients which received the *MLL-AF9*-transduced HSPCs with stabilized NIK showed a significant delay in the initiation of *MLL-AF9*-induced AML, suggesting a suppression of AML initiation upon NIK stabilization. Consistently, the repopulation of leukemia stem cells (LSCs), isolated from mice with established AML, was also delayed in recipients treated with tamoxifen. Further analysis uncovered that NIK stabilization suppresses *HOXA9/MEIS2* target genes, likely by regulation of the alternative NF-κB pathway. Another proposed mechanism involves the up- and downregulation of Dnmt3a and Mef2c, respectively by the NIK-regulated transcription factor RelB. Surprisingly, knockdown of Dnmt3a or overexpression of Mef2c did not rescue the NIK-induced delay in leukemogenesis, implying a partial contribution of these factors likewise. Even though NIK stabilization in LSCs decreased proliferation, the apoptosis rate was only slightly increased. This observation is corroborated by the findings of Studencka-Turski et al. [33] who also observed a stabilization of NIK. Here, NIK impairs apoptosis induced by chemotherapeutic drugs. A primary human CD34+ cord blood cell line transduced with *MLL-AF9* was used to show a stabilization of NIK compared to human CD34+ blood stem/progenitor cells, which is due to a K63-linked ubiquitinylation of NIK by the atypical E3 ligase Zinc finger protein 91 homolog (ZFP91). The observed increase in NIK protein, its phosphorylation and processing of p100 to p52 could be explained by the high expression of the lymphotoxin β receptor (LTβR) in the transduced cells. The possible consequence of NIK stabilization was investigated using the classical

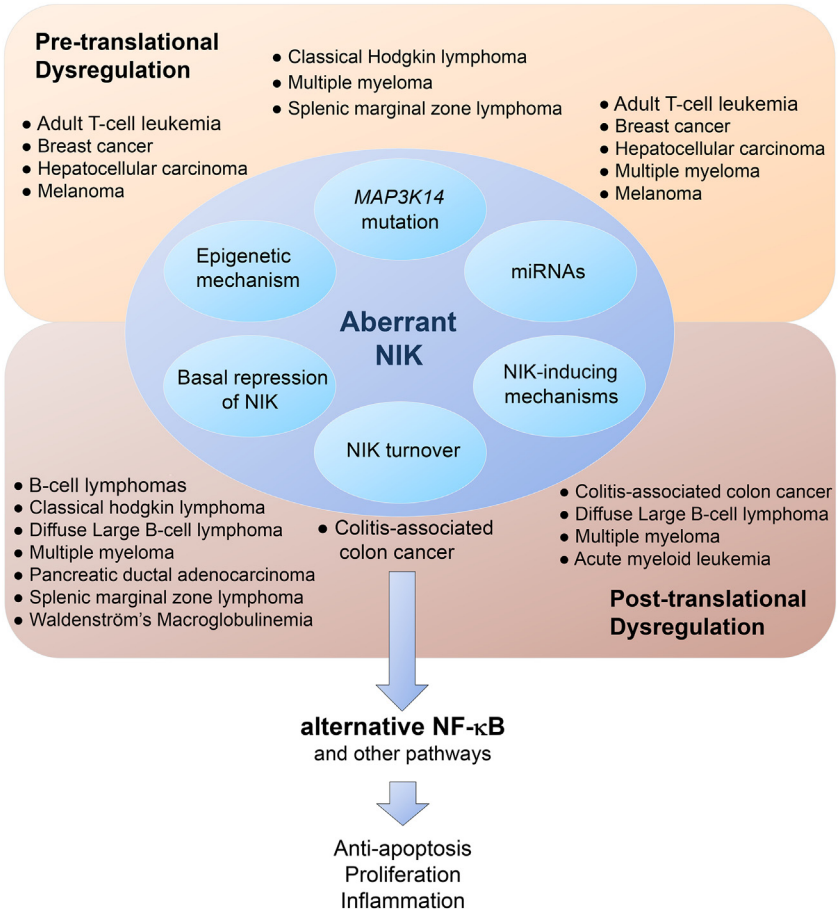


Fig. 3. Contribution of the dysregulation of NIK to various cancers. Different mechanisms achieve a pre- and post-translational dysregulation of NIK. This dysregulation leads to aberrant NIK stability impacting various tumors.

upfront treatment with chemotherapeutic drugs daunorubicin and cytarabine. Results showed that NIK- or ZFP91-deficient CD34+ MLL-AF9 cells are more susceptible to the apoptotic effect of the drug treatment than CD34+ MLL-AF9 cells. These data indicate that NIK and ZFP91 exert an antiapoptotic effect *via* the alternative NF-κB pathway.

2.2. NIK activity in multiple myeloma and Hodgkin lymphoma

An increasing number of publications report the aberrant activation of both NF-κB pathways in MM ([34]). As an approach to scrutinize the involvement of NIK and the alternative NF-κB pathway in MM, Keats and colleagues [35] used high-density oligonucleotide array-based comparative genomic hybridization (CGH) and gene expression profiling data, as well as cytoplasmic immunoglobulin staining-fluorescence *in situ* hybridization (cIg-FISH) for validation, to elucidate underlying mutations leading to high activity of the alternative NF-κB pathway. Starting with 62 patient samples and 46 human myeloma cell lines (HMCLs), they identified biallelic deletions for TRAF2, TRAF3 and cIAP1/2. These initial findings were substantiated by the detection of spiked expression of *MAP3K14*, *LTBR*, and *CD40*, leading to NIK accumulation and increased processing of p100 to p52, as well as a higher transcriptional activity of NF-κB (RelB/p52). Also, the reintroduction of TRAF3 into cell lines with TRAF3 abnormalities restored the function of TRAF3 and decreased NIK protein levels and p100 to p52 processing. In summary, a combination of all deletion events, mutations and gene rearrangements of the aforementioned genes amount to 17% (28/167) in MM patients and 41.3% (19/46) in HMCLs of having abnormalities in NF-κB regulation. These data show direct gain-of-function mutations of NIK and genomic changes in positive and negative regulators of NIK which affect NIK stability. Yet another reason for increased alternative NF-κB activity in MM was found by Morgan et al. [36] using 1421 samples from 958 myeloma patients as well as a dataset from the Clinical Outcomes in Multiple Myeloma to Personal Assessment of Genetic Profile (CoMMpass) study. They identified active fusion genes, overexpression and translocations of *MAP3K14* by sequencing and gene expression analysis. All fusion genes of NIK observed lead to an in-frame tail fusion of NIK starting with exons 4, 5 or 6. The kinase domain starts in exon 5 which suggest that the fusions, except for the one starting with exon 6, should be active, whereas the regulation by TRAF3 is hampered due to the deletion of the IBM and TRAF3 binding site in exon 1 (Fig. 1B). In 2% of the samples of another targeted panel dataset, a translocation t(14;17)(q32.33;q21.31) involving NIK and the *IGH* locus was observed and resulted in overexpression of NIK. Together, these findings highlight the different options to increase the activity of the alternative NF-κB pathway in MM.

Ranuncolo and colleagues [37] showed that almost all biopsies from HL patients (49/50) exhibit a stabilized NIK expression. Therefore, they performed RelB knockdown in HL and non-HL cell lines and found a decrease in the viability of HL cell lines only, which could be rescued by ectopic expression of RelB, confirming the importance of this transcription factor for the survival of HL cell lines. The inhibition or knockdown of NIK mimics the effects of RelB depletion on the viability of HL cell lines. A recent publication sheds light on a possible contribution of genetic alterations to the increased stability of NIK. Otto et al. [38] studied *TRAF3* and *MAP3K14* gene locations using single nucleotide polymorphism (SNP) array data, sequencing, fluorescence immunophenotyping and interphase cytogenetics as a tool for investigation of neoplasms (FICTION) and fluorescent *in situ*

hybridization (FISH) of six classical HL (cHL) cell lines and microdissected Hodgkin and Reed-Sternberg (HRS) cells. The SNP array indicated loss of heterozygosity in three of the six cHL cell lines studied, in which the cell line U-HO1 harbored a biallelic deletion of the *TRAF3* gene. No inactivating mutations were found in *TRAF3*. FICTION further detected *TRAF3* loss in 15% (3/20) and *TRAF3* gain in 35% (7/20) of the primary lymph node sections. The SNP analysis of the *MAP3K14* gene revealed a gain in cHL cell lines L428, HDLM-2 and U-HO1, whereas FICTION analysis detected gains of one to three copies in 31% (5/16) of the primary cases. Loss of *MAP3K14* was slightly less frequent and was observed in 25% (4/16) of the primary cases. These data invariably implicate that genetic alteration of NIK or a component of the NIK regulatory complex is involved in the stabilization of NIK in HL.

2.3. NIK-associated dysregulation in solid tumors

The involvement of NIK is not restricted to hematological cancers but was also observed in solid tumors. Recent findings show that NIK is important for glioma cell invasion [39,40], stem cell phenotype in breast cancer [41], pancreatic cancer progression [42] and tumor-associated angiogenesis [43]. Work from Noort and coworkers [43] that NIK is implicated in pathological angiogenesis showed the comprehensive impact of NIK on the progression of different types of solid tumors. They found the expression of active NIK in endothelial cells under inflammatory condition (inflamed rheumatoid arthritis model) and in a variety of tumor tissues but not in healthy tissue. Furthermore, they observed sprouting of endothelial cells *in vitro* when treated with lymphotoxin, homologous to lymphotoxins, exhibits inducible expression and competes with HSV glycoprotein D for herpesvirus entry mediator, a receptor expressed on T lymphocytes (LIGHT) and CD40L, which could be blocked specifically by silencing NIK, IKKα and to a lesser extent IKKβ, implying a major role of the alternative NF-κB pathway. In $Nik^{-/-}$ mice, tumor-associated angiogenesis was impaired. In the B16 melanoma model, $Nik^{-/-}$ mice exhibited a significantly lower total number of blood vessels (Wt 8.33 ± 6.36 *vs* $Nik^{-/-}$ 0.50 ± 0.34; $p = .05$) and $CXCL12^{+}$ blood vessels (Wt 12.5 ± 1.29 *vs* $Nik^{-/-}$ 2.33 ± 0.33; $p = .005$) within the tumor compared to wildtype mice emphasizing that NIK is an attractive target when it comes to inhibition of tumor-driven neoangiogenesis.

Two recent publications studied the impact of NIK regulation and the resulting alternative NF-κB pathway on the invasiveness of gliomas. In the publication by Cherry et al. [39], they established TNF-like weak inducer of apoptosis (TWEAK) as an inducer of a NIK-driven matrix metallopeptidase 9 (MMP-9) expression that elicits glioma invasiveness. In this respect, NIK functions in its known capacity as activator of the alternative NF-κB pathway because the expression of RelB positively correlated with the invasive behavior of different glioma cell lines tested in a 3D collagen matrix assay. In line with this, depletion of RelB in highly invasive BT25 and U87 cells abated invasiveness. Knockdown of RelA in these cells led to lower expression of RelB resulting in diminished invasiveness. To corroborate this result, the authors showed that overexpression of RelB was able to restore the invasiveness of cells transfected with shRNA against RelA. That MMP-9 is involved in TWEAK-induced cell invasion was shown using MMP-9 inhibitor I in a 3D collagen matrix assay. To strengthen these results with *in vivo* data, the authors studied gliomagenesis *in vivo* using an orthotopic mouse xenograft model. Ectopic expression of NIK in the cell line BT114 and subsequent transplantation into the right cortex revealed that NIK-overexpressing BT114 cells formed larger and more dispersed tumors compared to BT114 control cells. The report by Duran et al. [40] from the same group extends the role of NIK to the posttranscriptional regulation of MT1-MMP (MMP-14), an endopeptidase which degrades various components of the extracellular matrix. In a 3D collagen type I invasion assay, the impact of NIK expression (wildtype and a stabilized NIK(S867A) [44]) on the invasion of different glioma cell lines (BT114, BT116, U87, BT25) was investigated. The results highlighted a critical role of NIK in TWEAK-induced as well as in the constitutive invasion of these cell lines. The mechanistic insight was unraveled by studying pseudopodia formation, localization and enzymatic activity of MT1-MMP. The findings showed that NIK promotes pseudopodia formation. NIK expression does not affect the transcript level of MT1-MMP but changes the localization of MT1-MMP within the pseudopodia as well as its phosphorylation status (pMT1-MMP, Y573) and therefore its enzymatic activity. As NIK is a serine/threonine kinase, the NIK-induced phosphorylation of MT1-MMP is indirect. The increase of pMT1-MMP was also reflected in the immunohistochemical staining for pMT1-MMP in BT114-NIK orthotopic xenograft tumors isolated from mice. A contribution of the classical NF-κB signaling was ruled out because $p65^{-/-}$ and $p65^{-/-}$; $cRel^{-/-}$ MEFs were less invasive than $RelB^{-/-}$ MEFs. Interestingly, MEFs from wildtype and $NIK^{-/-}$ mice showed minimal invasion compared to wildtype $p65^{-/-}$, $p65^{-/-}$; $cRel^{-/-}$ and $RelB^{-/-}$ MEFs, but a robust invasion upon TWEAK stimulation was demonstrated for wildtype MEFs, which was significantly impaired in $NIK^{-/-}$ MEFs. Finally, the ectopic expression of NIK in $p65^{-/-}$; $cRel^{-/-}$ MEFs led to increased invasion and pseudopodial localization of pMT1-MMP. The clinical significance was tested by analyzing NIK and MT1-MMP expression in 'The cancer genome atlas' data sets (glioblastoma and lower grade glioma patients) through the cBioPortal for Cancer Genomics. The results established that in both patient data sets, poor survival of patients correlated with a higher expression of NIK and MT1-MMP. Taken together, these two reports imply that NIK plays a critical role in tumor invasion and is a prognostic marker for gliomas. In yet another solid tumor, NIK is stabilized and contributes to increased NF-κB signaling, proliferation and anchorage-independent growth, hallmarks of pancreatic cancer [42]. Döppler et al. [42] observed that NIK protein and its phosphorylation at the T559 residue but not NIK mRNA level is upregulated in pancreatic ductal adenocarcinoma (PDAC). The authors inspected the components of the NIK regulatory complex and found diminished amounts of TRAF2 in 7 out of 9 PDAC cell lines, due to continuous proteasomal degradation. A luciferase reporter assay on cell lines transfected with NIK shRNA revealed a decrease in NF-κB activity. The reduced NIK level resulted in less anchorage-independent growth and colony formation in soft agar assays. Both phenomena were reverted by ectopic expression of a constitutively active NIK. The proliferation rate of cell lines with NIK shRNA was reduced by about 50%. Most importantly, these *in vitro* findings correlated with clinical data. From 55 human PDAC samples, 69% displayed decreased TRAF2 levels, 18% however showed increased TRAF2 and NIK, and a low level of pT559-NIK. There was a correlation between TRAF2 levels and the tumor grade or better the degree of differentiation of the PDAC. Seventy percent of the moderately differentiated and 80% of the poorly differentiated PDACs had low TRAF2 and high NIK levels. In PDACs, like the genetic ablation of *TRAF3* in HL, loss of a component of the regulatory complex of NIK leads to NIK accumulation and consequently the activation of the alternative NF-κB pathway.

A more hidden role of NIK in cancer development is associated with the fact that NIK is abnormally activated in obesity, leading to hyperglycemia which might be involved in the stimulation of tumorigenesis [45]. Sheng et al. [46] showed an increase in NIK activity in two mouse models of dietary obesity and genetic obesity, respectively. Examination of the metabolic function of NIK *in vivo* led to the observation of hyperglycemia and greater glucose tolerance in NIK knockout mice, whereas hepatic glucose production was reduced. Accordingly, overexpression of kinase inactive NIK in the liver of mice fed with high-fat diet (HFD) or *ob/ob* (mutated leptin) mice ameliorated the induced hyperglycemia. The authors found that the increase in NIK activity in obesity led to an enhanced glucagon stimulation of hepatic glucose production. Inhibition of NIK in the liver decreased the glucagon response. The effect of NIK on glucagon was at least in part attributed to a stabilization of the transcription factor cAMP response element-binding protein (CREB).

2.4. NIK-associated disorders in stem cells

Within a tumor population, there exist cancer stem cells (CSCs) which possess stem cell properties that can initiate and sustain growth of a tumor and enable propagation of metastasis. The study by Vazquez-Santillan and coworkers [41] established that upregulated NIK plays a crucial role in regulating the phenotype of breast cancer stem cells (BCSCs). For the isolation of BCSCs from luminal (MCF7) and triple negative (MDA-MB-231) breast cancer cell lines, specific antibodies against surface markers (CD44, CD24 or Epithelial Cell Adhesion Molecule (ESA)) were used. The stem cell populations were defined as $CD44^+/CD24^{-/low}$ for MCF7 and $CD44^+/ESA^+$ for MDA-MB-231. Both cell lines showed different amounts of BCSCs (MCF7 0.7–1.4%; MDA-MB-231 34%). The tumorigenic potential of BCSCs from these cell lines was tested in immunodeficient *nu/nu* mice. BCSCs from MCF7 and MDA-MB-231 formed tumors within 120 and 90 days, respectively. In both cell lines and the HER2 positive cell line SKBR3, higher levels of NIK mRNA were detected compared to non-stem cells. To study the impact of this change in NIK mRNA on the phenotype, population, clonogenic and tumorigenic potential of BCSCs, shRNA-mediated silencing of NIK or overexpression was performed. NIK knockdown reduced the expression of SOX2, OCT4, NANOG, ALDH1A3 and ALDH8A1 in MCF7, SOX2, OCT4, ALDH1A3 and ALDH8A1 in MDA-MB-231, and SOX2, OCT4 and ALDH1A3 in SKBR3. Concurrently, the BCSC fraction was reduced in MCF7 and MDA-MB-231 cell lines. Limited dilution assays revealed reduced clonogenic potential of MDA-MB-231-shNIK cells compared with MDA-MB-231-siLuc cells. The tumorigenicity of MDA-MB-231-shNIK cells in *nu/nu* mice was also reduced. The forced expression of NIK (MCF7-NIK+ and SKBR3-NIK+) yielded as expected the opposite results, increased expression of some stem cell markers, increase in the number and clonogenicity of BCSCs as well as in the number of tumors formed in *nu/nu* mice. Further studies revealed that NIK also activated the ERK pathway to regulate the stemness of BCSCs. Finally, examination of patient material (191 breast cancer tissue samples; 60.7% luminal, 13.6% triple negative and 9.4% HER2+ subtype) demonstrated that NIK expression was significantly higher in HER2+ breast carcinomas and correlated positively with HER2 expression. The triple negative carcinomas exhibited the lowest expression of NIK. Immunohistochemistry analysis further revealed that NIK was expressed in 79.5% (152 samples) of breast cancer tissues.

Similar data were obtained from surgically resected tissue specimens from 82 patients with breast carcinoma who underwent surgical treatment [47]. Positive NIK expression was significantly higher in cancer tissue (63.4%) compared to tumor-adjacent normal tissue (25.6%). The correlation of the clinical parameter of the breast cancer patients demonstrated that high NIK expression was significantly associated with the clinical stage and prognosis of the patients. The Kaplan-Meier five-year survival analysis showed a median survival time of 35 months and 51.5 months for NIK positive and NIK negative patients, respectively. Poor prognosis was also observed in renal cell carcinoma where high cytoplasmic NIK was associated with lower cancer-specific survival ($p = 0.006$) and a stratified 10-year survival rate from 85% (low) to 65% (high, $p = 0.005$) [48].

2.5. The role of miRNA in the regulation of NIK

The impact of non-coding RNAs on the regulation of gene expression has been extensively studied. NIK is regulated by various microRNAs (miRNAs) which belong to the non-coding RNAs (Fig. 2C). Yamagishi et al. [49] studied the miRNA expression profile of clinical adult T-cell leukemia (ATL) samples and identified miR-31 as one of the most profoundly repressed miRNAs in all ATL individuals. They used gene expression arrays and luciferase 3'-UTR assays to test the functional significance of this downregulation and observed high luciferase activity for the reporter containing NIK 3'-UTR sequence upon anti-miR-31 treatment. Using anti-miR-31, miR-31 precursor and mutations within the miR-31 binding site of NIK, the authors showed convincingly that NIK is regulated by miR-31 and the amount of miR-31 is inversely correlated with the amount of NIK. Most importantly, they also observed that miR-31 negatively regulates the BAFF- and CD40L-stimulated accumulation of NIK. The regulation of NIK by miR-31 affected the proliferation of the tumor and its response to an apoptotic trigger, establishing miR-31 as a tumor suppressor that acts *via* NIK regulation. Mechanistically, the repression of miR-31 arises from genomic deletions (21 ATL cases, 12.5%) or from epigenetic silencing of miR-31. Computational analysis identified an assembly of YY1-binding motifs upstream of miR-31, a recruitor of the Polycomb repressive complex (PRC, Fig. 2C). Chromatin immunoprecipitation analysis of a broad area containing the miR-31 coding region showed higher levels of methylation at H3K9 and H3K27, suggesting repression of miR-31. Microarray data showed a positive correlation between PRC2 components and NIK expression, suggesting that PRC regulates NIK by the epigenetic repression of miR-31. This was confirmed by PRC2 knockdown in ATL cell lines leading to decreased levels of NIK, p52 as well as phospho-IκBα, which were restored using anti-miR-31. Knockdown of PRC2 also sensitizes the tumor cell line TL-Om1 to apoptosis, underlining the importance of NIK for the survival of the tumor. Although it is difficult to envision that NIK is regulated on the mRNA level as its abundance is mainly regulated on the protein level by the cIAP1/2-TRAF2-TRAF3 regulatory complex, these results nevertheless show clearly that miR-31 impacts the trigger-induced abundance of NIK [49].

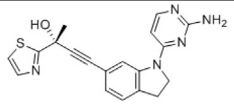
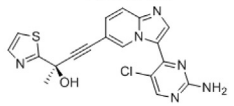
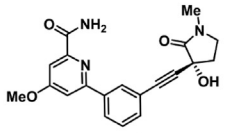
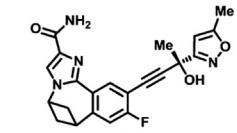
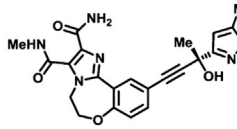
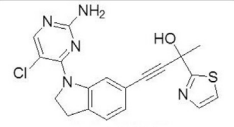
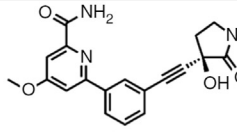
Using the same techniques as above, another study examined hepatoma cell lines and clinical hepatocellular carcinoma (HCC) tissues for the levels of miR-520e [50]. DNA hypermethylation in the upstream region of miR-520e resulted in the downregulation of miR-520e in HCC tissues and hepatoma cell lines. The authors demonstrated that overexpression of miR-520e suppressed the growth of hepatoma cells *in vitro* (HepG2, H7402 cell lines) and *in vivo* (HepG2-miR-520e, intratumoral injection; BALB/c athymic nude mice), whereas silencing the expression of miR-520e by anti-miR-520e resulted in enhanced cell proliferation (Chang liver cells, L-O2 cell lines). Luciferase-3'-UTR reporter assay containing the 3'-UTRs of potential targets established NIK as a direct target of miR-520e. Therefore, the authors tested whether NIK is responsible for the observed effects of miR-520e on tumor proliferation. The transfection of anti-miR-520e promoted proliferation of Chang liver cells. This effect was partially attenuated when siRNA targeting human NIK mRNA was co-transfected with anti-miR-520e. Interestingly, miR-520e and silencing of NIK impacted the phosphorylation of ERK1/2 and nuclear translocation of RelA, implying that the tumor growth is regulated by a NIK-ERK1/2-NF-κB axis.

Another example of NIK regulation by miRNA is published for colorectal cancer (CRC) in which miR-518a-3p is significantly downregulated [51]. The mechanism resulting in the downregulation of miR-518a-3p is unclear but epigenetic silencing could be involved as suggested by the data from Menigatti et al. [52] for HT29 cells. Luciferase-3' UTR reporter assays demonstrated a negative effect on luciferase activity by the NIK 3'-UTR sequence. This result was confirmed by inhibition of miR-518a-3p which led to an increase in luciferase activity, implying that NIK is a direct target of this miRNA. Using the precursor or mimics of miR-518a-3p resulted in a decrease in NIK protein level. The authors found that the suppression of NIK either by miR-518a-3p or shRNA against NIK resulted in the downregulation of a subset of anti-apoptotic genes, including Bcl-xL, XIAP and cFLIP. On the other hand, overexpression of miR-518a-3p enhanced procaspase-3 processing. Therefore, NIK and the downstream NF-κB activity are correlated with resistance to apoptosis and tumor proliferation in CRCs.

3. Functions of NIK independent of alternative NF-κB regulation

The impact of NIK is mostly correlated with the downstream signaling towards the activation of transcription factor complex RelB/p52. Lately, several publications report functions of NIK independent of the

Table 1
NIK Inhibitors discussed in section "Mechanisms of NIK inhibition and therapeutic implications".

Inhibitor	Structure	Ki (nM) vs NIK	Reference
AM-0216/AM-0561	 AM-0216  AM-0561	2.0/0.3 high time fluorescent resolution (HTRF) assay	[57]
Compound 4f	 4f	0.23±0.17 (n=2) ATP consumption assay	[58]
Compounds 32 and 33	 32  33	0.4±0.4 ATP consumption assay 0.47±0.27 ATP consumption assay	[59]
B022	 B022	4.2 ATP consumption assay	[63]
SMI1	 SMI1	0.23±0.17 ATP consumption assay	[64]

alternative NF-κB pathway [53–56]. Zhang et al. [56] demonstrated that NIK supports the expansion of tumor-initiating cells (TICs) co-purified with a $CD24^{med}CD49f^{hi}$ population from premalignant ErbB2-expressing mammary glands. The accumulation of NIK led to increased nuclear localization of IKKα where it phosphorylates the cyclin-dependent kinase (CDK) inhibitor p27/Kip1 at S183 and stimulates its nuclear export or exclusion. In human breast cancer, nuclear IKKα is inversely correlated with nuclear p27 in about 73.7% of metastatic invasive ductal carcinomas (IDCs) but only in 25% of non-metastatic IDCs, suggesting that the pro-metastatic function of IKKα lies in its control of p27 localization. The upstream signal leading to the NIK-IKKα module is unknown but the authors speculate that RANKL/RANK might be responsible.

The study by Boutaffala et al. [53] investigated the role of NIK in TNFR1/RIP1-induced apoptosis. Upon co-stimulation with TWEAK/TNF or LTβR agonist/TNF, $NIK^{-/-}$ MEFs displayed resistance to apoptosis, whereas $NIK^{+/+}$ MEFs showed a substantial decrease in survival. TNFR1 can trigger a TRADD-dependent (RIP1-independent) as well as a RIP1-dependent apoptotic pathway. When the authors triggered the RIP1-dependent apoptosis (TNF/Smac mimetic), they observed that $NIK^{-/-}$ MEFs were resistant. Also, the TWEAK/TNF- or LTβR agonist /TNF-induced caspase activation was equally inhibited in the presence of necrostatin-1 (Nec-1), a potent RIP1 kinase inhibitor. The direct interaction between RIP1 and NIK was shown by using overexpression of different RIP1 constructs and FLAG-NIK MEFs and verified on the endogenous level. As the kinase activity of NIK was important for the pro-apoptotic function of NIK, the authors investigated phosphorylation of RIP1 by NIK. Data derived with recombinant RIP1 in the presence of NIK as well as *in vivo* data displayed a significant increase in RIP1 phosphorylation, which led to an increase in autophosphorylation activity as shown by inhibition with Nec-1. Further results demonstrated that NIK was associated with and required for the formation of the RIP1/FADD/caspase-8 death complex IIb upon TWEAK/TNF- or LTβR agonist/TNF-induced apoptosis. In conclusion, a new role for NIK downstream of LTβR is postulated that involves the induction of TNFR1-driven cortical thymic epithelial cell death and thymus involution, independent of the alternative NF-κB pathway.

Jung and coworkers [54] identified a novel pool of NIK protein in the mitochondria by immunofluorescence staining and colocalization with Mito-RFP in glioma, breast cancer and pancreatic cancer cell lines. Results demonstrated that NIK regulates the phosphorylation and subcellular localization of Drp1 to control mitochondrial dynamics, velocity and directional migration to the periphery of migrating cells. Studies in knockout mice indicated that this unconventional role of NIK

does not require IKKα or IKKβ, meaning it is independent of classical and alternative NF-κB signaling.

Parvatiyar et al. [55] studied the RNA and DNA recognition pathways for the detection of viral infection and uncovered a surprising role for the NIK regulatory complex constituents, TRAF3, TRAF2 and cIAP1/2. Specifically, using the respective knockout mice, TRAF3, TRAF2 and cIAP1/2 were implicated in the positive regulation of type I interferon (IFN) activation in the RNA pathway and negative regulation of type I IFN activation in the DNA pathway, implying that NIK might have a regulatory function. To verify this, cells were transduced with B-DNA causing a decrease in TRAF3 level concomitant with an increase in NIK expression leading to a gain in interferon regulatory factor 3 (IRF3) phosphorylation. Co-immunoprecipitations using epitope-tagged NIK, stimulator of interferon genes (STING) and IRF3 in HEK293T cells revealed that NIK interacted with STING as well as IRF3. NIK also associated with STING endogenously. Interaction of NIK with STING elevated the aggregation of STING and also enhanced STING–IRF3 complex formation. NIK operated in a kinase activity-dependent manner in order to facilitate STING-dependent signaling. On the contrary, IKK$\alpha^{-/-}$ mice showed increased the interferon activation upon B-DNA stimulation. The transcription factor component p100 was also dispensable in eliciting the induction of IFN in the DNA pathway. Collectively, these results led to the conclusion that NIK could also function independently of the alternative NF-κB pathway.

4. Mechanisms of NIK inhibition and therapeutic implications

An association of dysregulated alternative NF-κB pathway with cancer development and progression is substantiated by an ever increasing number of studies. The stabilization of NIK is the upstream event leading to the activation of alternative NF-κB, arguing for NIK as a potential therapeutic target. However, inhibitor studies on NIK were limited by missing structural data until 2012 when the crystal structures for the catalytic domain of murine (4G3E) and human (4G3D, 4DN5) NIK were published [7,9]. Thus far, the inhibitor design revolved around the catalytic ATP-binding site of NIK (Table 1).

Demchenko and colleagues [57] tested two novel NIK inhibitors (AM-0216, AM-0561) together with an isometric control. Selection of these compounds was performed using the KINOMEscan® survey and K_dELECT assays by DiscoveRx (Eurofins). The K_i for AM-0216 and AM-0561 are 2 nM and 0.3 nM, respectively. Both compounds were tested on multiple myeloma cell lines (MMCLs) with mutated components of the alternative NF-κB pathway (NIK, TRAF2, TRAF3, cIAP1/2) leading to NIK activation. As controls, MMCLs with activating mutations for the classical NF-κB or alternative NF-κB pathway by NIK-independent mechanisms and MMCLs with low NF-κB activity were used. To determine the effectiveness of inhibition, the mRNA expression of three NF-κB target genes (*BIRC3*, *TNFAIP3*, *NFKB2*) was used for quantification. Both compounds effectively inhibited only NIK-dependent NF-κB activation in the range of 1–5 μM. In addition, BAFF receptor-induced NF-κB activity was inhibited by AM-0216 in MM-S1 cells. Importantly, the inhibitors induced cytotoxicity and apoptosis in these cell lines and prevented clone formation. Due to the poor pharmacokinetic properties of these inhibitors however, *in vivo* experiments were not feasible. Therefore, modifications of these Amgen inhibitors could lead to candidate compounds useful for future *in vivo* studies.

Two recent papers from the same group demonstrated different approaches towards the discovery and development of potent NIK inhibitors [58,59]. Castanedo et al. [59] used a structure-guided optimization method. Starting from a modestly potent lead, the authors improved the design by exploiting the different binding modes in NIK and phosphoinositide-3-kinase (PI3K) to discover a series of potent inhibitors selective for NIK. Starting with a compound with a K_i of 1.3 ± 0.3 μM which had a novel structure compared to published NIK inhibitors, they used structure-based methods to maintain and improve NIK inhibition while minimizing PI3K inhibition. Two Inhibitors emerged that have strong NIK biochemical potency (K_i: 0.4 ± 0.4 and 0.47 ± 0.27 nM) which clearly inhibited the alternative over classical NF-κB pathway in HeLa cells. Importantly, the pharmacokinetic properties of these inhibitors were suitable for preliminary *in vivo* experiments but had to be refined for more robust *in vivo* evaluation of NIK pharmacology. A scaffold-hopping approach was used by the same group to meliorate the potency, kinase selectivity and *in vivo* pharmacokinetic properties [58]. Besides measuring the inhibition of NIK activity, the authors also performed a luciferase-based NF-κB activity assay to test the efficacy of inhibition in HEK293 cells. The final compound was highly potent and selective for NIK with suitable pharmacokinetic properties for advanced ADME (absorption, distribution, metabolism and excretion) and pharmacology experiments. This inhibitor inhibits besides NIK, only KHS1 with greater than 50% in a panel of 228 kinases tested at 100 nM. It also inhibits BAFF-induced B-cell survival *in vitro* as well as survival of splenic marginal zone B-cells *in vivo*.

The discovery of miRNAs and their vital role in gene regulation and tumor development opens up the possibility for the development of miRNAs as therapeutic agents [60,61]. Ma and coworkers [62] analyzed the therapeutic effect following synthetic miR-520e delivery in lung tumor. The downregulation of miR-520e in non-small cell lung cancer (NSCLCs), similar to hepatomas [50], was confirmed in human tumor samples and in different cell lines. Overexpression of miR-520e in various cell lines resulted in growth inhibition as shown by proliferation and colony formation assays. H460 cells, which form rapidly growing tumor xenografts within 3–4 weeks, were grafted subcutaneously into the lower back of NOD/SCID mice until observable tumors were formed. Twelve days thereafter, intratumoral injection of 200 μg miR-520e in a lipid-based vehicle began and was repeated for four times every second day. Compared to the controls (PBS, vehicle or miR-NC), miR-520e administration led to substantial reduction of the tumor size of the xenograft. Also, immunohistochemistry analysis of sections obtained from the xenograft revealed a strong increase in caspase-3 staining. The experiment was repeated using tail vein injection of 100 μg miR-520e as systemic delivery method, resulting in data consistent with the local delivery of miR-520e. Serum levels of alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen and alkaline phosphatase were examined to exclude detrimental adverse effects of systemic delivery. Based on the earlier described effect of miR-520e on the growth of hepatoma cell lines and HCC [50], it is likely that the downregulation of NIK by miR-520e contributes to the observed effects in NSCLC. Therefore, the miR-520e replacement therapy seems to be an implementable option.

The development of NIK inhibitors for cancer therapy might also benefit from studies of NIK inhibitors in other diseases where NIK is dysregulated. Ren et al. [63] reported that the utilization of NIK inhibitor B022 leads to the suppression of inflammatory gene expression in mouse hepatocyte cell line Hepa 1 and primary mouse hepatocytes. In addition, B022 reversed the mortality rate of *STOP-NIK* mice (Cre-mediated deletion of the STOP sequences activates NIK overexpression) with NIK-induced liver injury caused by liver-specific activation of the *NIK* transgene. The authors showed that B022 reduced the amount of p52, proinflammatory cytokines (IL-6, CCL2, TNF) and iNOS. B022 injection produced the same effects in toxin (CCL_4)-induced liver injury, which is strongly associated with NIK, indicating that B022 ameliorates CCl_4-induced liver injury primarily through the inhibition of p52 generation and ROS formation. Although B022 is effective in protecting the liver, its pharmacokinetic is still suboptimal and awaits further improvements.

Additional support for the discovery of efficient NIK inhibitors comes from a study based on the chronic autoimmune disease systemic lupus erythematosus [64]. The authors have shown in a lupus NZB/W F1 mouse model that inhibition of NIK by the highly efficacious NIK small molecule inhibitor SMI1 (K_i: 0.230 ± 0.170 nM) boosts survival and renal function. SMI1 is highly specific for NIK as shown by a screen

for off-target kinases, where it inhibits only 3 (KHS1, LRRK2, PKD1) out of 222 kinases greater than 75%. In *in vitro* assays, SMI1 effectively inhibits p100 processing upon stimulation with BAFF, OX40L, CD40L and TWEAK. Based on *in vivo* data obtained in a survival study using a mouse model, SMI1 represents a good candidate which could also be used in other settings like tumor growth reduction.

Apart from the sole inhibition of NIK activity, it seems feasible that treatment of hematological malignancies with a combination of NIK-alleviating and chemotherapeutic agents can result in a beneficial reduction of therapeutic resistance. As shown by Studencka-Turski and colleagues [33], stabilization of NIK is supported by the ZFP91-dependent K63-linked ubiquitinylation in cord blood cells with rearranged *MLL-AF9* gene. The authors report that NIK or ZFP91 silencing accompanied by simultaneous treatment with daunorubicin or cytarabine sensitize these CD34+ MLL-AF9 cells to apoptosis.

The aberrant stabilization of NIK observed in different cancers seems to confer a survival advantage for the tumor cells exposed to chemotherapeutic drug treatment. This raises the interesting question whether modulators could be engineered as a therapeutic tool to overcome NIK stabilization. Targets of these modulators could be the components of the NIK regulatory complex as well as NIK itself. Herein, it must be taken into consideration that the components of the NIK regulatory complex have multiple functions in processes independent of cancer development. The TRAFs are adaptor molecules in receptor-mediated signaling and cIAP1/2 are apoptosis inhibitors [65,66]. A feasible strategy could be to make use of the expression of a tumor-specific component (*e.g.* tumor-specific ncRNA) which act as a trigger for the action of the modulator [67].

To date, there are no NIK inhibitors in clinical trials. In 2016, TRACON Pharmaceuticals, Inc. announced a strategic licensing collaboration with Janssen Pharmaceutica N.V. for a novel, potent, orally bioavailable inhibitor TRC694 (formerly JNJ-64290694) of NIK discovered by Janssen. TRC694 is currently undergoing preclinical development. Another interesting development by Epizyme, Inc. is the drug Tazemetostat (EPZ-6438), an EZH2 inhibitor, which is currently in clinical phase II trials (NCT03348631, NCT02601950, NCT02875548, NCT01897571, NCT03456726, NCT03155620) against different tumors. As described previously, EZH2 is a component of PRC2 and negatively regulates miR-31, which in turn leads to an increase in NIK abundance [49].

5. Conclusion

The regulation of NIK abundance by its regulatory complex is pivotal for the timely activation of the alternative NF-κB pathway. Here we reviewed diverse intrinsic alterations of NIK stability in the context of various human malignancies. Many of these studies have focused on the regulation of NIK and components of its regulatory complex, using the alternative NF-κB activity as readout. The results indicate that an increase in NIK stability impacts various aspects of tumor development. In this regard, a therapeutic strategy of NIK inhibition could be very promising. We have highlighted recent advances in the development of efficacious NIK inhibitors. While most of these inhibitors are highly specific for NIK, much work is needed to improve the pharmacokinetic properties for suitable use in *in vivo* studies. Noteworthy is the discovery that independent of alternative NF-κB activation, NIK is also implicated in apoptosis, detection of viral infections and the cell cycle. Future research in these areas can further expand our understanding of this enigmatic molecule and aid the customizing of therapeutic strategies for targeting NIK.

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Conflict of interest statement

The authors declare that there is no conflict of interests.

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