Chapter 14

Methylation of NF-κB and its Role in Gene Regulation

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Abstract

The nuclear factor κB (NF-κB) is one of the most pivotal transcription factors in mammalian cells. In many pathologies NF-κB is activated abnormally. This contributes to the development of various disorders such as cancer, acute kidney injury, lung disease, chronic inflammatory diseases, cardiovascular disease, and diabetes. This book chapter focuses on how methylation of NF-κB regulates its target genes differentially. The knowledge from this chapter will provide scientific strategies for innovative therapeutic intervention of NF-κB in a wide range of diseases.

Keywords: arginine, epigenetic enzymes, gene regulation, lysine, methylation, NF-κb, transcription factor

1. Introduction

The nuclear factor κB (NF-κB) is one of the most pivotal transcription factors in mammalian cells. In many pathologies NF-κB is activated abnormally. This contributes to the development of various disorders such as cancer, acute kidney injury, lung disease, chronic inflammatory diseases, cardiovascular disease, and diabetes [1]. NF-κB family is comprised of five family members: p65 (RelA), RelB, c-Rel, p50/p105 (NF-kB1), and p52/p100 (NF-kB2). Among them, the Rel homology domain (RHD) at their N-termini is a commonly share feature (**Figure 1**). It is necessary for protein dimerization, the inhibition of NF-κB (IκB) interaction, nuclear targeting, and DNA binding [2]. Additionally, a carboxy-terminal transactivation domain (TAD) also exists in the Rel proteins, such as p65 (**Figure 1**), RelB, and c-Rel. Among the NF-κB dimers, the p65:p50 heterodimer is the prototype.

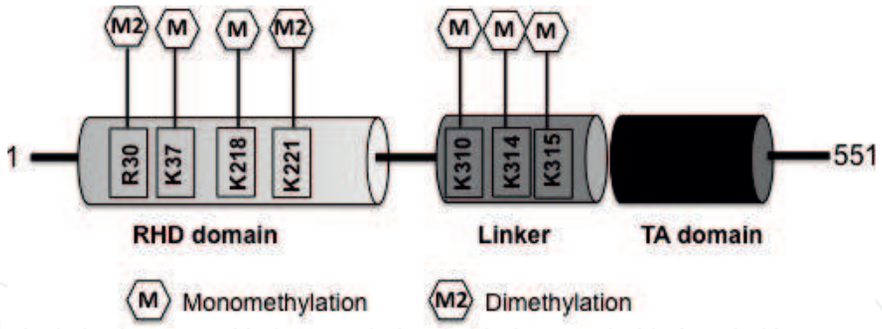

Figure 1. Diagram of the p65 subunit of NF-κB with methylation modifications. In the diagram, the arginine (R) 30 and lysine (K) 37, 218 and 221 are located at the Rel homology domain (RHD) (light gray), other 3 lysine sites K310, 314 and 315 are located in the linker region (gray) between RHD and Transactivation domain (TA) (dark gray).

The activity of NF-κB is frequently regulated by various modifications, namely, post-translational modifications (PTM). Among which, methylation is the newest type of modification that is discovered. The knowledge on NF-κB methylation is still scarce and not popularized among wide range of readers. Thus, in this chapter, we will focus on how methylation of NF-κB regulates its target genes differentially and provide perspectives and future directions in term of the research and application of NF-κB methylation. The knowledge from this chapter will provide scientific strategies for innovative therapeutic intervention of NF-κB in a wide range of diseases.

2. NF-κB signaling pathways

The NF-κB signaling pathways play a very important role in signaling innate and adaptive immune responses and in many cellular processes. NF-κB signaling and subsequent target gene activation can be induced by a variety of factors including cytokines, stress, radiation, and also bacteria and viruses [3]. This signaling can be broken down into two signaling pathways: the canonical and non-canonical branches of the NF-κB pathway (**Figure 2**). In the canonical pathway, activity is regulated by interactions between IκB proteins and the p65:p50 complex. IκB proteins hold NF-κB proteins in inactive conformations by binding in the cytoplasm and preventing nuclear localization. Extracellular signals including cytokines such as interleukin 1 β (IL-1β) and tumor necrosis factor α (TNFα), stress, free radicals, or radiation cause IκB kinase (IKK) activation. IKK is a complex that consists of the IKKα and IKKβ kinases and a third regulatory subunit known as NEMO/IKKγ [4, 5]. In the canonical pathway, IKKβ phosphorylates the N-terminal serine residues 32 and 36 of IκBα, resulting in its polyubiquitination and subsequent rapid proteasomal degradation [3]. This degradation allows the release of p65:p50 into the cytoplasm. The two-unit NF-κB complex then binds to the protein importin and translocates to the nucleus where it further binds to DNA and promotes increased expression of NF-κB target genes [6]. In the noncanonical pathway, the p100 and RelB proteins form an inactive dimer in the cytoplasm. Upon stimulation by a certain group of stimuli, such as B-cell activation factor (BAF) or CD40 ligand (CD40L), IKKα is subsequently activated through NF-κB-inducing kinase (NIK) mediation, leading to the ubiquitin/proteasomal processing of

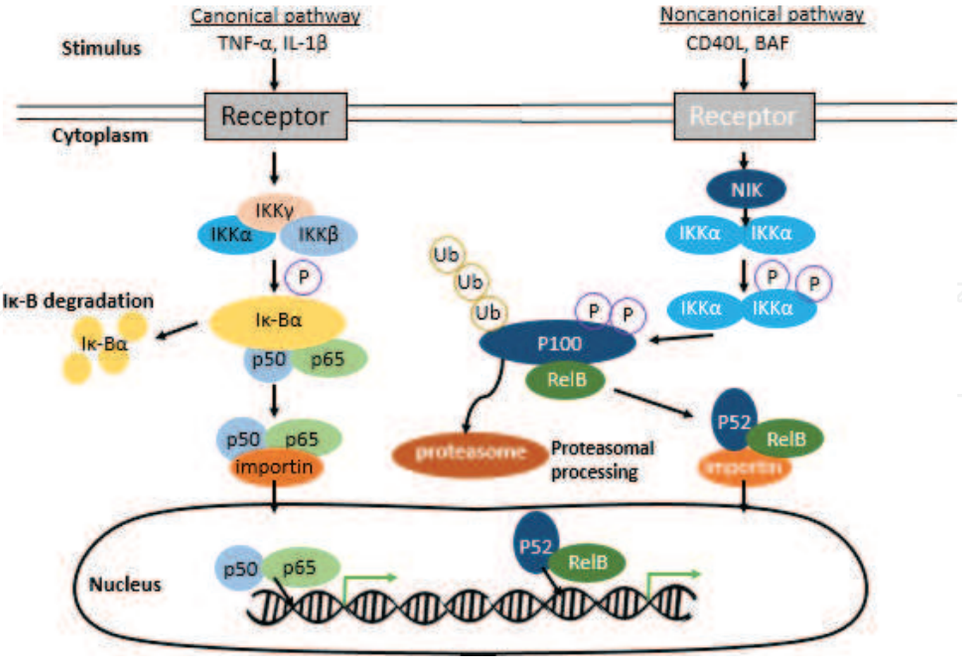

Figure 2. Two important NF-κB pathways. **Left**, in the canonical pathway, NF-κB subunit dimers are bound to inhibitory IκB proteins, which act to hold NF-κB complexes in an inactive state in the cytoplasm. Following stimulus of a receptor, the IκB kinase (IKK) complex becomes phosphorylated. IKK is made of two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO). IKK then phosphorylates IκB proteins which are subsequently ubiquitinated and proteasomally degraded. This releases the bound subunits of NF-κB p65 and p50 into the cytoplasm. Following cytoplasmic release the subunits bind to importin and translocate to the nucleus where they can bind to the promoter and trigger the transcriptional activation of NF-κB target genes. **Right**, in the noncanonical pathway, the p100/RelB dimer remains in an inactive state until stimulated by a signaling cascade triggered by factors including CD40L or BAFF. Following stimulus and subsequent phosphorylation of IKKα by NIK, IKKα phosphorylates p100 associated with RelB, which leads to its ubiquitination and proteasomal processing to p52. The complexed p52/RelB can then translocate to the nucleus and bind to the target gene promoters and further activate the transcription of the NF-κB target genes.

p100 to p52. Once this processing has occurred, the RelB/p52 complex can translocate to the nucleus and bind to DNA to promote increased expression of NF-κB target genes [7].

3. The state of post-translational modifications (PTM) of NF-κB

Given the role of NF-κB in a wide range of important cellular and physiological processes, the potentially disastrous consequences of dysregulated NF-κB necessitates highly complex and finely regulated mechanisms for controlling NF-κB activity. NF-κB signaling can be influenced at multiple levels, many of which converge on various components of the pathway including the IKK complex and the IκB family of proteins [8]. For instance, the IKK complex remains one of the best-studied central regulators of NF-κB activation, and its phosphorylation of IκBα constitutes an essential event for subsequent signal transduction to both the canonical and non-canonical heterodimeric subunits of NF-κB [8, 9] as described above.

In addition to regulation by the IKK complex and the inhibitory IκB proteins, the NF-κB/Rel dimeric proteins are themselves subject to intricate regulation via a host of critical post-translational

modification (PTM) events [10–12]. PTMs on p65, the prototypical subunit of NF-κB, include [13, 14], acetylation [15–19], methylation [20–23], ubiquitination [24], nitrosylation [25], and sumoylation [26]. The consequences of these regulatory modifications are context dependent, and vary based on the nature and abundance of the NF-κB pathway stimulators [11, 22]. Moreover, the sites and/or crosstalk between modifications [16, 27] can yield different outcomes with even the same modifications yielding quite distinct physiological effects [28–31]. Eventually, these PTMs work to dictate the duration and strength of activation and, accordingly, the degree of transcriptional output [10, 32]. Moreover, some of these modifications serve as important means for crosstalk with other signaling pathways [33].

Our laboratory is one of the first few groups to discover that p65 can be methylated on lysine residues upon cytokine stimulation [20]. Subsequently, we pioneered the identification of arginine 30 (R30) methylation of p65 [22]. Below, we will thoroughly discuss the impact of these methylation sites on NF-κB-mediated differential gene regulation.

4. Methylation of the p65 subunit of NF-κB

4.1. Lysine methylation of p65

To date, a total of six lysine methylation sites have been reported: K37, 218, 221, 310, 314, and 315 [18, 20]. By using a novel genetic approach, our lab identified that p65 can be methylated by a lysine methylase, the nuclear receptor-binding SET domain-containing protein 1 (NSD1), and demethylated by a lysine demethylase, the F-box and leucine-rich repeat protein 11 (FBXL11) [20]. This reversible lysine methylation of p65 is targeted at K218/K221 sites and affects NF-κB activity. K218/K221 methylation induces over 80% p65-dependent gene expression in mouse embryonic fibroblast cells (MEFs). The observation indicates that PTMs play an important role in fine-tuning the regulation of NF-κB [20].

Zhang *et al.* provided strong evidence regarding the function of p65 methylation by reporting that in response to TNFα, plant homeodomain finger protein 20 (PHF20) promotes NF-κB transcriptional activity by interacting with methylated p65 at K218/221. The methylation of p65 blocks recruitment of PP2A to p65, thereby leading to the persistent phosphorylation of p65 [34]. By using the immunohistochemistry (IHC) staining method, the authors showed that PHF20 and phosphorylated p65 are localized in the nucleus in glioma tissue specimens. The PHF20 expression levels are also tightly correlated with the clinical tumor grade after univariate analysis with a P value of 0.0018 ($P < 0.05$ is considered to be significant). These findings highlight the interrelated connections between overexpressed PHF20, methylation, and phosphorylation of p65 in human malignant gliomas [34].

In addition to our discovery of the methylation of K218/221, Ea *et al.* revealed that p65 is monomethylated by histone methyltransferase, the Set domain-containing protein 9 (Set9) at K37 in response to both IL-1β and TNFα treatment. The authors showed that TNFα induced p65 monomethylation is essential for the expression of NF-κB regulated genes. Methylated p65 stays in the nucleus, and Set9 might regulate its nuclear function.

Moreover, monomethylation of p65 increases the NF-κB DNA binding ability and recruitment to the promoter of NF-κB target genes [18]. Interestingly, Yang *et al.* described that Set9 may also monomethylate p65 at K314 and K315 in addition to K37, and negatively regulate p65. The authors suggested that methylation of K314/315 inhibits the transcriptional activity of NF-κB through proteasome-mediated degradation, and it downregulates NF-κB target gene expression [19], a phenomenon quite different from that of K37 methylation. Collectively, this evidence suggests the complexity of p65 methylation, and indicates that the same enzyme, such as SET9, may have different functions depending on the lysine residues it modifies. There is also a possibility that K37 modification occurs before K314/K315 methylation. It is likely that K37 methylation is required for gene activation, while K314/315 methylation is required for the termination of NF-κB activity [18].

Besides the methylated lysine residues on p65 discussed above, another SET family member SETD6, was also reported to monomethylate p65 at K310 under basal condition. Levy and colleagues observed that under the unstimulated condition, a proportion of p65 can be monomethylated by SETD6. This methylation event negatively regulates NF-κB target gene expression, including those involved in inflammatory response. The phenotype was proven in various cell lines, such as bone osteosarcoma U2OS, peripheral blood THP-1, and bone marrow-derived macrophages (BMDM), and therefore represents diverse disease models. Interestingly, Levy *et al.* found that K310 monomethylation-mediated NF-κB inhibition is due to the involvement of another protein, the G9A-like protein (GLP). By binding to monomethylated K310, GLP enriches histone H3K9 dimethylation on the p65 target gene promoters, resulting in gene suppression. This SETD6-initiated lysine-methylation repressive pathway can be terminated by p65 phosphorylation at serine 311 (S311) and by the atypical protein kinase PKC-ζ [23]. This study presents a delicate example of how methylation and phosphorylation on p65 may regulate each other and be an integral part of a more sophisticated regulatory system of NF-κB.

An overlook of the biological roles of p65 lysine methylation and their modifying enzymes is shown in **Table 1**. It is evident that under various experimental conditions, p65 lysine methylation may affect NF-κB nucleus localization, transcriptional activity, and NF-κB target gene expression.

4.2. Arginine methylation of p65

Distinct from the methylation of lysine residues, our lab used Mass Spectrometry to discover that p65 can also be symmetrically methylated at arginine 30 residue (R30) [20, 22]. This important modification is carried out by the protein arginine methyltransferase 5 (PRMT5), an enzyme that belongs to the PRMT superfamily, contains 637 amino acids, and catalyzes the formation of symmetrically dimethylated arginine.

We reported that PRMT5 catalyzed p65 dimethylation upon IL-1β treatment. R30 to A mutant (R30A) of p65 decreased NF-κB activity and led to the downregulation of a subgroup of NF-κB inducible genes; among these are cytokine and chemokine genes. Conditional media from cells expressing the R30A mutant of p65 had much less NF-kB-inducing activity than its wild-type cohort. Additionally, through *In Silico* prediction we proposed that dimethylation

Type of methylation	Site modified	Enzymes	Biological function	Reference
Monomethylation	K37	SET9	Stabilizes nuclear localization and enhances p65 binding ability	[18]
Monomethylation	K218	NSD1/FBXL11	Promotes NF-κB transcriptional activity and maintains p65 phosphorylation on S536	[20, 34]
Dimethylation	K221	NSD1/ FBXL11	Promotes NF-κB transcriptional activity and maintains p65 phosphorylation on S536	[20, 34]
Monomethylation	K310	SETD6	Decreases NF-κB target gene expression	[23]
Monomethylation	K314	SET9	Decreases NF-κB activity and target gene expression	[19]
Monomethylation	K315	SET9	Decreases NF-κB activity and target gene expression	[19]
Symmetric dimethylation	R30	PRMT5	Enhances NF-κB DNA binding and transcriptional activities, and increases NF-κB target gene expression	[22, 35]
Asymmetric dimethylation	R30	PRMT1	Reduces NF-κB DNA binding ability and decreases NF-κB target genes expression	[22, 35]

Table 1. Types of NF-κB methylation and its biological roles.

of R30 may mediate *van der Waals* contacts and stabilize domain interactions. The key residues involved are aspartic acid (D) 277, glutamic acid (E) 279, and threonine (T) 191. Since phenylalanine (F) 184 positions closely to R30 and T191, R30 is sandwiched between F184/T191 on one side and D277/E279 on the other. This evidence affirms the importance of R30 methylation in increasing the ability of p65 to bind to DNA, resulting in the changes in its target gene expression [22].

Further demonstrating the complexity of R30 methylation, Reintjes *et al.* reported that PRMT1, another member of the PRMT superfamily, may asymmetrically methylate p65 at the same R30 that is symmetrically methylated by PRMT5 [35]. The information of R30 methylation by both PRMT5 and 1 is also included in **Table 1**. Different from PRMT5, PRMT1 is an enzyme containing 361 amino acids which catalyzes the formation of monomethyl-arginine and asymmetric dimethyl-arginine [36]. Reintjes and colleagues proposed an interesting model suggesting that symmetric dimethylation of R30 by PRMT5 seems to be induced at early time points, however, asymmetric dimethylation of R30 by PRMT1 is enriched at later time points. This idea presents an overall picture of the "meticulously calculated" regulation of NF-κB signaling, through symmetric and/or asymmetric R30 dimethylation that occurs at different stages of NF-κB responses. This model represents a specific on/off switch mechanism for adjusting cytokine-induced NF-κB responses [35].

4.3. Differential gene regulation by lysine and arginine methylation

As we mentioned earlier, a total of six lysine methylation sites can be methylated by different histone lysine methyltransferases in response to activating signals. Among them, K37, K218, and K221 are located in the RHD domain, while K310, K314, and K315 are in the linker region between RHD and the transactivation domain (TA) [36] (**Figure 1**). Using site mutagenesis, we generated the K218/221Q double mutant (DKQ) or the K37Q single mutant of p65. We found that in response to cytokines, such as IL-1β treatment, ~350 genes were rapidly induced within 5 min after treatment, while an additional ~300 genes were significantly upregulated 30 min later. Additionally, 1500 genes were further induced between the time points of 1 and 24 h. We revealed that *early growth response protein 1* (*EGR1*) was upregulated within 30 min and then began to decrease after 2 h. While *C-X-C motif chemokine 10* (*CXCL10*, also known as *IP10*), and *Interleukin 8* (*IL-8*) were upregulated after 1 h or longer treatment. However, their expression is much more stable than the *EGR* gene [21]. To further explore the different effects of DKQ and K37Q on gene expression, we conducted an Illumina array analysis, observing that DKQ is responsible for ~50%, while K37Q is only responsible for ~25% of NF-κB target gene regulation. Among these genes, some were exclusively regulated by either DKQ or K37Q, while others were commonly regulated by both DKQ and K37Q. This is a very interesting phenomenon. Our work showed that a very tiny difference in NF-κB methylation, such as methylation on different lysine residues, could lead to dramatically different gene induction patterns. By using ChIP-seq and bioinformatics approaches, we further uncovered that NF-κB target genes can be classified into multiple subgroups based on the effects of DKQ or K37Q (up- or down-regulation, or lysine site sensitivity) [21]. This data offers a valuable picture of the dynamic complexity of gene regulation by methylation of NF-κB on different lysine residues.

To further determine the difference between K and R methylation of p65 on NF-κB regulation, we conducted similar experiments as described above [21]. R30A and DKA (K218/221 K-A) mutants were generated in HEK293 cells. Illumina microarray experiments were carried out to analyze the gene populations affected by these mutations. We found that ~75% of NF-κB target genes were down-regulated by twofold or more by the R30A mutation, while significantly fewer (~48%) genes were downregulated by the DKA mutation. This data suggests that R30 methylation is in charge of most NF-κB target gene expression, while K218/221 methylation controls a much smaller population of the genes. Not surprisingly, Ingenuity Pathway Analysis **(Figure 3)** revealed that R30A and DKA control different functional networks. For instance, the top network for R30A is regarding the functions of *Cellular Movement, Hematological System Development and Function*, *Immune Cell Trafficking*, while the top network for DKA is related to *Cell-mediated Immune Response*, *Cellular Development*, *Cellular Function and Maintenance*, affirming the quite distinct cellular functions of R30 and K218/221 methylation. **Figure 4** illustrates a representative network from either R30A or DKA regulated genes. Importantly, the NF-κB complex is identified as a critical node in both networks. Two typical NF-κB target genes, IL8 (CXCL8) and IP10 (CXCL10), are also shown as important components in both networks.

Collectively, the evidence described above proves that methylation on different lysine residues or on different types of amino acids (lysine *vs.* arginine) on the p65 subunit of NF-κB,

Rank	Top Networks (Ingenuity Pathway Analysis): Associated Network Functions	
	R30A	DKA
1.	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	Cell-mediated Immune Response, Cellular Development, Cellular Function and Maintenance
2.	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Inflammatory Response	Cell Cycle, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function
3.	Cardiovascular Disease, Organismal Injury and Abnormalities, Glomerular Injury	Cellular Development, Connective Tissue Development and Function, Tissue Development
4.	Respiratory System Development and Function, Cell Morphology, Embryonic Development	Cancer, Cell Death and Survival, Organismal Injury and Abnormalities
5.	Cellular Function and Maintenance, Humoral Immune Response, Protein Synthesis	Nucleic Acid Metabolism, Small Molecule Biochemistry, Gene Expression

Figure 3. Top networks that are affected by either R30A or DKA mutations. Ingenuity pathway analysis (IPA), showing top five different functional networks that are associated with R30A or DKA mutation.

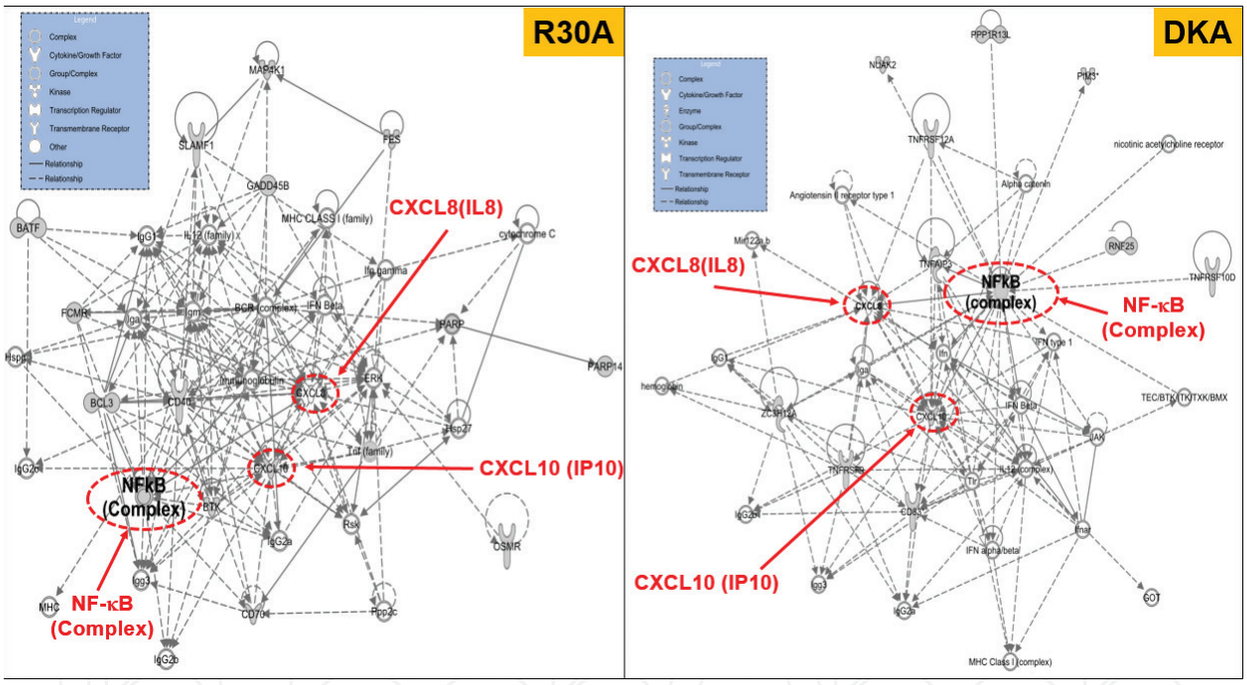

Figure 4. Example networks of R30A and DKA mutations, with NF-κB as a master node. **Left panel,** R30A mutation affects NF-κB orchestrated network. **Right panel,** DKA mutation interferes with NF-κB signaling. However, these two networks show quite distinct topographies and interactions with other signaling components. Note: Both *IL8* and *IP10* are within the networks.

dictates differential gene regulation, leading to complex and distinct outcomes. The work on methylation of NF-κB has offered a unique angle for understanding the mechanisms underlying the extreme plasticity of the biological responses led by the finely tuned regulation of NF-κB. The knowledge gained by this study will enable us to better understand why NF-κB is dysregulated in a variety of disease states, thus providing critical guidance to the design of disease-specific therapeutics.

5. Histone methylases as potential therapeutic targets in cancer

Due to the important role of NF-κB methylation in differential gene regulation, it is logical to recognize the essential roles of the enzymes that catalyze these methylation modifications. These enzymes are frequently histone methylases, and there are quite a few examples. Since the role of histone methylases in cancer has been well reviewed by Albert and Helin [37], below, we will only focus on PRMT5.

PRMT5 has been increasingly recognized as an important tumor promoter. We and others have observed elevated PRMT5 expression in cancers of the colon, pancreas, ovary, kidney, lung, bladder, liver, breast, prostate, cervix, and skin. This suggests that high levels of this enzyme may promote tumorigenesis, at least in part by facilitating NF-κB-induced gene expression [22, 38]. For instance, by conducting colorectal cancer (CRC) tissue microarray (TMA), we found that PRMT5 is overexpressed in polyps, advanced stages of colorectal cancer, and in the metastatic stage [39]. Similarly, PRMT5 is also overexpressed in various stages of pancreatic cancer, especially in the metastatic stage [39]. We proved that overexpression of PRMT5 promotes CRC HT29 cell and pancreatic cancer PANC1 cell proliferation, anchorage-independent growth, and cell migration ability. Knockdown of PRMT5 by shRNA showed the opposite effect, confirming PRMT5 functions as a tumor promoter in these cancers [39].

Additionally, overexpression of PRMT5 has been shown to be associated with poor epithelial ovarian cancer prognosis [40]. In a clinical study with 150 ovarian cancer patient samples, the overexpression of PRMT5 is found to be highly correlated with the Federation of Gynecology and Obstetrics (FIGO) advanced stage, which includes poor cell differentiation, high proliferation activity, and lymph node involvement. The overall survival rate of patients with low PRMT5 expression is 90%. In contrast, only 30% of patients with high PRMT5 expression survived. The progression-free survival rate is 50% for patients with low PRMT5 expression, but in those with high PRMT5 expression the rate is only 10% [40].

Moreover, Kumar and colleagues showed that the expression level of PRMT5 is inversely correlated with oropharyngeal squamous cell carcinoma (OPSCC) patient outcome. For instance, high PRMT5 expression correlated with low overall survival and had over 1.7 times higher death risk than the patient who has low PRMT5 expression [41]. Together, these studies have identified PRMT5 as a promising therapeutic target in cancers.

To date, multiple efforts have been made to develop the small molecule inhibitors of PRMT5. For instance, EPZ015666 was reported [39, 42] to inhibit PRMT5 methyltransferase activity in panels of mantle cell lymphoma (MCL) cell lines (Maver-1, Mino, Granta-519, Jeko-1and Z-138). It also significantly inhibits tumor growth in Z-138 and Maver-1 MCL xenograft mouse model as compare with vehicle control.

Recently, by adapting the AlphaLISA technique into a sensitive high throughput screening platform, our lab identified PR5-LL-CM01 as a potent PRMT5 small molecule inhibitor. PR5-LL-CM01 showed greater potency than EPZ015666 in both PDAC and CRC model [39].

These examples highlight the great potential of using histone methylases, such as PRMT5, as novel therapeutic targets in cancer.

Likewise, other histone methylases (**Table 1**) that methylate NF-κB may also play critical roles in the development and progression of cancer and other hyper NF-κB driven diseases. Therefore, they constitute a group of highly promising future therapeutic targets for these pathological conditions.

6. Conclusion, perspective, and future directions

The implications of methylation of NF-κB are multi-fold and far reaching. Methylation provides a snapshot of the complexity underlying the regulation of this important transcription factor. Even with the studies done to date, researchers have just begun to understand the crosstalk between these different PTMs and their implications in normal cellular function and disease. Two interesting questions remain. First, how does methylation of these residues on the same subunit affect NF-κB function? Second, can we reconcile the effects of other kinds of PTMs coupled with methylation both in normal and diseases states? A deeper understanding of these aspects will shed important light on the overall strategies for the development of new therapeutic approaches to treat the affected diseases.

Cancer is one of the leading causes of morbidity and mortality worldwide. Methylation of NF-κB as described in this review highlights its significance in cancers and other inflammatory diseases. Over the past decade, several transformative discoveries in epigenetics have led to the development of novel therapies that target epigenetic enzymes. However, the inquiries into acetylation and methylation modifications of lysines and arginines have been mainly focused on histone proteins. Important research identifying methylation residues on important non-histone proteins like NF-κB may be crucial to developing therapeutic interventions that target these modifications. For instance, the PRMT5 inhibitor identified in our laboratory has paved the way for future drug development to treat cancers and other disease with hyper PRMT5-driven NF-κB activity [22, 39]. In addition to PRMT5, other histone methylases, such as NSD1, have been reported by us and others as a significant player in cancer development [20, 43–45]. Although researchers have been trying to develop a small NSD1 inhibitor for cancer treatment, no NSD1 specific inhibitor has yet been reported due to the large size of NSD1 enzyme and the lack of sufficiently sensitive assay development. Future effort on this front and other histone methylases are equally as important in developing new medicines that target PRMT5.

Additionally, as mentioned in the Introduction, the prototypical NF-κB is comprised of a heterodimer of p65 and p50 subunits. Though multiple sites of methylation have been discovered on the p65 subunit of NF-κB, the potential methylation of the p50 subunit is quite understudied. With recent advances in proteomics and prediction software, novel methylation site(s) on p50 could arise in the near future. The study on p50 methylation could provide more a complete picture in terms of NF-κB regulation, and may possibly lead to novel discoveries regarding the methylation-mediated regulation of this subunit as well.

Since NF-κB is an important transcription factor that also plays a fundamental role in normal cells, one must consider important factors such as specificity of inhibiting modification only in cancer cells but not in normal cells. Multi-targeted approaches that simultaneously cripple several signaling pathways in cancer cells would be ideal, and a better understanding of the crosstalk between these pathways will advance the drug development process. In the future, a combination of advanced animal models, Cas9/CRISPR system, and more sophisticated bioinformatics approaches will serve as invaluable tools to study the implications of methylation on NF-κB and its interactions with other critical cellular factors that are important in the disease context. This will help to expedite the development of therapeutic tools to combat these deadly diseases.

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Abbreviation

BAF	B-cell activation factor
BMDM	bone marrow-derived macrophages
CD40L	CD40 ligand
ChIP-seq	chromatin immunoprecipitation (ChIP) with DNA sequencing
CRC	colorectal cancer
CXCL10	C-X-C motif chemokine 10 (also known as IP10)
EGR1	early growth response protein
FBXL11	F-box and leucine-rich repeat protein 11
FIGO	Federation of Gynecology and Obstetrics
GLP	G9A-like protein
IHC	immunohistochemistry
IKK	IκB kinase
IL-1β	interleukin 1 β
IL-8	Interleukin 8

MCL	mantle cell lymphoma
MEFs	mouse embryonic fibroblast cells
NF-κB	nuclear factor κB
NIK	NF-κB-inducing kinase
NSD1	nuclear receptor-binding SET domain-containing protein 1
OPSCC	oropharyngeal squamous cell carcinoma
PHF20	plant homeodomain finger protein 20
PKC-ζ	Protein kinase C zeta
PRMT1	protein arginine methyltransferase 1
PRMT5	protein arginine methyltransferase 5
PTM	post-translational modifications
RHD	Rel homology domain
Set9	Set domain-containing protein 9
TAD	transactivation domain
TMA	tissue microarray
TNFα	tumor necrosis factor α

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