Preface

Activation of the transcription factor nuclear factor-kappa B (NF-κB) is essential for normal immune and inflammatory responses, cell survival and proliferation, and the maintenance of cellular and tissue homeostasis. In addition to these crucial physiological functions, aberrant NF-κB activation occurs in a wide range of chronic inflammatory and autoimmune diseases, solid tumors, leukemia, and lymphomas. Due to this central pathophysiological role in a diverse array of diseases, understanding the precise mechanisms underlying normal and deregulated NF-κB signaling has become a major field of biomedical research. An overarching goal of this extraordinary research effort is the identification of targets in the many emerging NF-κB signaling pathways that are amenable to pharmacological blockade or manipulation. The reward for these endeavors will be the emergence of novel classes of highly specific anti-inflammatory, immunomodulatory, or anticancer drugs.

Almost 30 years of basic research has resulted in the NF-κB activation pathway becoming the paradigm for inducible receptor to nuclear signal transduction. Indeed, efforts to unravel NF-κB signaling mechanisms have been the breeding ground for seminal discoveries with far-reaching impact in the signal transduction field. These contributions include defining the role of ubiquitination-mediated protein degradation in signal transduction and the more recent revelations of the many nondegradative ubiquitination mechanisms as key posttranslational modifications controlling signaling cascades. As the number of original papers focusing on the mechanisms and functions of normal and pathophysiological NF-κB signaling increases, it is clear that the field of NF-κB research remains highly active, continues to expand, and still exists at the forefront of driving the techniques employed by the wider signal transduction community.

In this volume of Methods in Molecular Biology, prominent researchers in the NF-κB field have contributed essential insight into the methods and techniques required to dissect the complex mechanisms of NF-κB activation, regulation, and function. This will provide a timely and invaluable resource for researchers seeking to perform experiments aimed at understanding the role of NF-κB signaling in health and disease.

Part I (Standard Approaches to Detect NF-κB Pathway Activation) contains three chapters describing the now "classic" methods to assay NF-κB pathway activation in cultured cells or tissues. Fittingly the book begins with a chapter by Ramiswami and Hayden describing the electrophoretic mobility shift assay (EMSA) that was the technique initially employed to identify regulators of kappa light chain expression in B cells that led to the first description of NF-κB. Following this is a chapter by Starokadomskyy and Burstein that describes the methods used to detect IκB degradation; then Collins and colleagues provide the approaches required to measure NF-κB transcriptional activity using luciferase reporter assays. These three powerful methodologies have been and remain the mainstay techniques used by researchers studying the activation status of NF-κB signaling in a multitude of experimental systems.

The chapters in Part II (Detection and Analysis of NF-κB Signaling) expand the range of methods used to study NF-κB activation and introduce the techniques used to specifically analyze classical and alternative NF-κB signaling pathways. Following the transcriptional regulation theme of Chapter 3, this section begins with a description by Colleran

et al. of chromatin immunoprecipitation (ChIP) as it is used to detect DNA binding of classical NF-κB dimers. Next are two chapters highlighting the methods employed to study classical NF-κB activation in the context of specific cell types and activation mechanisms. In the first of these, Mihalas and Meffert describe techniques to assess the levels of IKK activity in neurons following excitatory neurotransmission-induced signaling. Jiang and Lin then provide insight into the multiple approaches that can be employed to study epidermal growth factor (EGF)-induced NF-κB activation. The next four chapters by Remouchamps and Dejardin, Qu and Xiao, McCorkell and May, and Gray and May introduce the noncanonical NF-κB pathway and provide detailed descriptions of the methods and protocols used to examine this alternative mechanism in various cell types induced by separate stimuli. Part II concludes with a chapter by Jackson and Miyamoto outlining genetic complementation of NEMO-deficient cells and examining how this approach can be used to study nuclear to cytoplasmic NF-κB signaling in response to genotoxic stress.

Part III (Methods to Study the Control of NF-κB Signaling) details a series of methods to explore the mechanisms that control separate NF-κB signaling pathways. The first two chapters dissect the mechanisms of T cell receptor (TCR)-induced NF-κB activation beginning with Paul and Schaefer (Chapter 12) who describe elegant imaging techniques used to study the formation of POLKADOTS that are signaling platforms necessary for TCR-induced IKK activation. Nagel and Krappmann then demonstrate an innovative fluorogenic cleavage assay used to measure the TCR-induced paracaspase activity of MALT1, thus allowing assessment of this key signaling intermediate as a potential therapeutic target for the treatment of inflammatory and autoimmune diseases and lymphomagenesis. Analysis of signal transduction proteins involved in NF-κB activation continues in Chapters 14 and 15 in which Reichardt and colleagues and Varfolomeev et al. discuss the methodologies used to study the roles of the TRAF and c-IAP proteins, respectively, in NF-κB activation. Again, these techniques provide the methodological foundation for studies addressing the potential therapeutic effects of targeting these crucial signaling intermediates.

In the remainder of Part III, procedures to assess the mechanisms and function of post-translational modifications of critical NF-κB pathway signaling proteins and the NF-κB proteins themselves are presented. This section begins with chapters by Shembade and Harhaj, and Sasaki and colleagues, each describing separate approaches to study nondegradative ubiquitination in NF-κB signaling including analysis of key protein-protein interactions and linear ubiquitination of NEMO. The next two chapters describe the techniques of DELFIA and microscale thermophoresis (Vincendeau et al.) and a sophisticated fluorescence spectroscopy approach (Dubosclard et al.), each of which is employed to specifically study NEMO-ubiquitin binding interactions. Methods to examine ubiquitin-dependent degradation of IκBα are then discussed by Chong et al. who describe an in vitro biochemical approach to reconstitute the ubiquitination system. The topic of ubiquitination controlling NF-κB signaling continues in Chapters 21 and 22 in which Collins et al. and Li and colleagues describe protocols to examine the direct ubiquitination of NF-κB proteins. Both chapters describe separate approaches to assess the ubiquitin-dependent degradation of active NF-κB that is a crucial negative regulatory mechanism preventing deregulated sustained NF-κB activity. In the remaining chapters in Part III, techniques are described to dissect the mechanisms of methylation and acetylation of NF-κB proteins. In Chapter 23, Lu and Stark demonstrate how to perform immunoprecipitations followed by mass spectrometry to identify methylation sites on p65, and then Chen and Chen conclude this section by describing experimental methods to monitor the in vitro and in vivo functions of acetylated or methylated forms of NF-κB.

Approaches to study the role of aberrant NF-κB signaling in diseases including cancer and inflammation and the effects of blocking this activity are explored in depth in Part IV (Analyzing and Targeting NF-κB Activity in Disease). The section begins with a chapter by Wessel and Hanson describing how to quantitate active NF-κB in dermal fibroblast from human skin biopsies obtained from immunodeficiency patients. The next four chapters address the role of dysregulated NF-κB signaling in cancer starting with a comprehensive description by Gilmore and Gélinas of methods to qualitatively evaluate the in vitro transforming activity of Rel proteins. This is followed by a discussion by Bassères and Baldwin of how to apply an RNA interference strategy to target NF-κB signaling in lung cancer cells; then Allen and Van Waes provide a detailed description of methods to immunohistochemically analyze NF-κB in human tumor tissue samples. In Chapter 29, Gaurnier-Hausser and Mason describe approaches to obtain, process, and analyze NF-κB activity in canine malignant lymphoid tissue including techniques to determine the effects of inhibiting classical NF-κB signaling in canine lymphoma using the NEMO-binding domain (NBD) peptide. Importantly, this chapter emphasizes the exciting potential of using outbred animal patient populations as highly representative, clinically relevant models of human diseases.

Methodologies to determine the effects of exogenously inhibiting classical NF-κB in vitro and in vivo in disease models are described in detail in Chapters 30–34. In each of these chapters, the authors provide approaches targeting the NBD; however, the techniques discussed can be considered a blueprint of methods that can be modified to assess other inhibitors of NF-κB signaling in these disease models. This section begins with a protocol from McCorkell and May to analyze the effects of the NBD peptide on vascular inflammation in vivo. Application of the NBD is explored further by Swarnker and Abu-Amer who describe in detail how to synthesize and purify the peptide and apply this approach to study osteoclast differentiation. In the next chapter, Zhao et al. describe methods employed to study NF-κB signaling in mouse models of aging, and they demonstrate how to assess the effects of blocking NF-κB activation in these mice using a novel NBD-protein transduction domain (PTD) fusion peptide. In Chapter 33, Sehnert and colleagues outline the development of an exciting new "sneaking ligand" approach that allows specific in vivo delivery of the NBD to only activated vascular endothelial cells at sites of inflammation. This strategy is expanded in the next chapter in which Sehnert et al. describe how to test the effects of this novel endothelial cell-targeting approach on the development of arthritis in mice. Part IV concludes with a chapter by Shaked and colleagues who describe detailed in vitro and in vivo approaches to analyze NF-κB activation in mouse intestinal epithelial cells using an innovative genetic model of intestinal inflammation.

Part V (Bioinformatics and Modeling Approaches to Study NF-κB) contains three chapters that focus on applying powerful bioinformatics and mathematical methodologies to NF-κB biology. In the first of these chapters, Siggers and colleagues describe the use and computational bioinformatics analysis of protein binding microarrays that provide a high-throughput method to measure proteins binding to distinct DNA sequences. In Chapter 37, Finnerty and Gilmore describe a combination of bioinformatics and phylogenetic approaches to study NF-κB evolution based on menu-driven, open-source computer programs that are readily accessible and can be used without training in phylogenetic methods. The volume then concludes with a chapter by Mitchell et al. describing the state-of-the-art in silico and mathematical modeling approaches that are leading to the development of a highly sophisticated, expansive and ultimately predictive "wiring diagram" of NF-κB activation.

In light of the continuously expanding fields of basic, applied, and translational biomedical research in which NF-κB signaling plays a crucial role, it was not possible to generate

a methods volume encompassing all of the techniques, approaches, and protocols used in the wide arena of NF-κB biology. However, the 38 highly detailed protocols ranging from basic but powerful biochemical approaches, to complex computational and mathematical modeling, provide the insight necessary for researchers to expertly analyze NF-κB signaling in their own fields of biomedical or biological investigation. The promise of identifying powerful new drugs targeting aberrant NF-κB signaling pathways in many debilitating and destructive diseases remains the overarching goal driving much of the research in this field. It is certain that in the pursuit of this goal, the techniques described here will be central to the discovery effort. It is therefore my hope that this volume provides the essential "go-to" resource for current and future workers pursuing this goal, and that the book finds its home on the many laboratory benches at which the boundaries of understanding of NF-κB biology are being relentlessly pushed forward.

In closing, I would like to extend my most sincere thanks and appreciation to all of the authors who contributed to this volume for their extraordinary willingness to share their expertise and for their patience as this book came to life.

Philadelphia, PA, USA ***Michael J. May, Ph.D.***

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