5-2016

THE ROLES OF MALT1 IN NF-κB ACTIVATION AND SOLID TUMOR PROGRESSION

Deng Pan

THE ROLES OF MALT1 IN NF-κB ACTIVATION AND SOLID TUMOR PROGRESSION

by

Deng Pan, B.S.

APPROVED:

_____

Xin Lin, Ph.D. , Advisory Professor

_____

Paul Chiao, Ph.D.

_____

Dos Sarbassov, Ph.D.

_____

M. James You, M.D., Ph.D.

_____

Chengming Zhu, Ph.D.

APPROVED:

_____

Dean, The University of Texas

Graduate School of Biomedical Sciences at Houston

THE ROLES OF MALT1 IN NF-κB ACTIVATION AND SOLID TUMOR PROGRESSION

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Deng Pan, B.S.

Houston, Texas

May, 2016

DEDICATION

This Ph.D thesis work is dedicated to

My parents and my wife,

for their love and support.

ACKNOWLEDGEMENTS

I would like to first acknowledge my mentor, Dr. Xin Lin, for his support and guidance during the course of my doctoral work since I joined his laboratory in 2010. Dr. Lin has spent tremendous amount of time in training his postdoctoral trainee and gradate students. I am very fortunate to have the opportunity to start my scientific career with Dr. Lin. Dr.Lin always emphasizes many key aspects and qualities of scientific researches: creativity, persistence, critical thinking, and enjoyment. As his trainee, I will carry on his philosophy of science throughout my career.

I would like to acknowledge my father, Dr. Weiqing Pan, who is also a professor working on parasites and malaria in the Second Military Medical University in Shanghai, China. During college years, I was very fortunate to have an opportunity to conduct research in a laboratory of his collaborator, Dr. Ming Li, and also in his own lab during summer and winter holidays. Thanks to those valuable lab experience, I have developed strong interests and motivation on science and it finally drives me to purse a doctoral degree after finishing medical school. As a father and a scientist, he always gives me encouragement and valuable suggestions.

I would like also like to thank my past committee members, Dr. Bryant Darnay and Dr. Hui-kuan Lin, and my current committee members, Dr. Paul Chiao, Dr. Dos Sarbassov, Dr. M. James You and Dr. Chengming Zhu, for their supports, suggestions and guidance on my projects.

I would like to thank all current and past lab members in Dr. Xin Lin's lab: Dr.Yun You, Dr. Changying Jiang, Dr.Zhicheng Zhou, Dr. Donghyun Joo, Dr. Marzenna Blonska, Dr.

Yifan Zhu, Dr. Bing Tang, Dr. Sara Gorjestani, Harrison You, Jeffery You, Dr. Zhongliang Ma, Dr.Xueqiang Zhao and Dr.Guoxing Zheng, for their help, support and valuable discussions.

Finally, I would like to thank my family members. My father Weiqing Pan, my mother Ying Ding, and my wife Wenqian Liang, for their endless love and support. I would also like to thank many of my friends, especially Shanshan Gao, Junchen Diao, Lihe Chen, Tianjiao Dai, Yu Huang and Zhenzhen Qu, who made my life in Houston very much enjoyable.

THE ROLES OF MALT1 IN NF-κB ACTIVATION AND SOLID TUMOR PROGRESSION

Deng Pan, B.S.

Supervisory Professor: Xin Lin, Ph.D.

The transcription factor NF-κB plays a central role in many aspects of biological processes and diseases, such as inflammation and cancer. Although it has been suggested that NF-κB is critical in tumorigenesis and tumor progression, the molecular mechanism by which NF-κB is activated in solid tumor remains largely unknown. In the current work, we focus on growth factor receptor-induced NF-κB activation and tumor progression, including epidermal growth factor receptor (EGFR)-induced NF-κB in lung cancer and heregulin receptor (HER2)-induced NF-κB in breast cancer. We found that Mucosa-associated lymphoma translocation protein 1 (MALT1), also known as paracaspase, is required for EGFR-induced NF-κB activation by recruiting E3 ligase TRAF6 to the IKK complex. In addition, MALT1 also contributes to many malignant phenotypes, including tumor cell proliferation, survival, migration and metastasis. Furthermore, by generating transgenic mice in which EGFR-associated lung adenocarcinoma will be developed in the absence of MATL1, we found that MALT1 specifically contributes to the EGFR- but not K-ras-induced lung adenocarcinoma by activating both NF-κB and STAT3 pathway, suggesting a crucial role of MALT1 in EGFR-associated lung cancer progression. In a separate study, we found that MALT1, together with its interaction partners CARMA3 and BCL10 (also known as the CBM complex), is required for HER2-induced NF-κB activation. Consistently, the CBM complex contributes to malignant phenotypes in breast cancer cells, including proliferation,

survival, migration, invasion and metastasis. Furthermore, we showed that the development of mammary tumor was delayed in MALT1-deficient mice (*MMTV-Neu; Malt1*$^{-/-}$), indicating that MALT1 contributes to the onset and progression of breast cancer *in vivo*. To further explore the role of MALT1 in cancer therapy, we found that MALT1 and the CBM complex are involved in resistance of chemotherapy induced apoptosis. MALT1 deficiency promotes doxorubicin-induced apoptosis. Mechanistically, the CBM complex is required for DNA damage-induced NF-κB activation, which antagonizes doxorubicin-induced apoptosis. In summary, our studies have revealed the multi-functional roles of MALT1 and the CBM complex in growth factor-associated solid tumors and cancer therapy.

Table of Contents

APPROVAL PAGE i
TITLE PAGE ii
COPYRIGHT iii
DEDICATION iv
ACKNOWLEDGEMENTS v
ABSTRACT vii
CHAPTER 1: INTRODUCTION 1
1.1 General overview of lung cancer and breast cancer 1
1.1.1.1 Overview of lung cancer 1
1.1.1.2 Molecular origins of lung cancer 2
1.1.1.3 Mouse models in lung cancer 4
1.1.2.1 Overview of breast cancer 5
1.1.2.2 Risk factors of breast cancer 6
1.1.2.3 Subtypes of breast cancer 7
1.1.2.4 Mouse models of breast cancer 10
1.2 The transcription factor NF-κB 11
1.2.1 Discovery of NF-κB 11
1.2.2 Canonical NF-κB pathway 13
1.2.3 Non-canonical NF-κB pathway 15
1.2.4 The role of NF-κB signaling in inflammation 16
1.2.5 The role of NF-κB signaling in hematopoietic malignancies 17
1.2.6 The role of NF-κB signaling in solid tumors 18
1.3 The biochemical properties and functions of MALT1 20
1.3.1 The discovery of MALT1 20
1.3.2 The function of CARMA1-BCL10-MALT1 (CBM) complex in lymphocytes 24
1.3.3 The function of CBM complex in other cell types 27
1.4 The proteolytic function of MALT1 28
1.4.1 MALT1 cleaves BCL10 to regulate T-cell adhesion 28

1.4.2 MALT1 cleaves A20 to regulate NF-κB activation 29
1.4.3 MALT1 cleaves CYLD to regulate JNK activation 29
1.4.4 MALT1 cleaves RelB to regulate NF-κB activation 30
1.4.5 MALT1 cleaves HOIL-1 to regulate NF-κB activation 31
1.4.6 MALT1 cleaves Regnase-1 to regulate mRNA stability 32
1.4.7 MALT1 cleaves Roquin to regulate mRNA stability 33
1.4.8 Phenotypes of MALT1 deficient and mutant mice 34
1.4.9 MALT1 as a therapeutic target in ABC-DLBCL 35
CHAPTER 2: THE ROLE OF MALT1 IN EGFR-INDUCED NF-κB ACTIVATION AND EGFR-ASSOCIATED LUNG CANCER PROGRESSION 38
2.1 Introduction 39
2.1.1 MALT1 is a therapeutic target in lymphoma 39
2.1.2 NF-κB signaling in EGFR associated-cancer 40
2.1.3 Specific aims and hypothesis 41
2.2 Materials and methods 42
2.2.1 Antibodies and reagents 42
2.2.2 Cell cultures 42
2.2.3 Immunoblotting (IB) 43
2.2.4 Immunoprecipiation assay 43
2.2.5 Electrophoretic mobility shift assay (EMSA) 43
2.2.6 Gene silencing and reconstitution 44
2.2.7 MTT Assay 44
2.2.8 Colony formation assay 45
2.2.9 Migration Assay 45
2.2.10 Wound healing assay 45
2.2.11 Lung metastasis model 46
2.2.12 Mouse strains and models 46
2.2.13 Histology and Immunohistochemical staining 47
2.2.14 Real-time PCR 47
2.2.15 Statistical analysis 48
2.3 Results 49

2.3.1 MALT1 is required for EGF-induced NF-κB activation 49
2.3.2 MALT1 is specifically required for EGF-induced NF-κB activation and does not affect other EGFR-activated pathways 51
2.3.3 The protease activity of MALT1 is not required for EGF-induced NF-κB activation 53
2.3.4 MALT1 functions as a scaffold protein to recruit TRAF6 to IKK complex 57
2.3.5 MALT1 contributes cell proliferation 60
2.3.6 MALT1 contributes to cell migration and metastasis 62
2.3.7 MALT1 contributes to EGFR-associated lung adenocarcinoma progression in a mouse model 66
2.3.8 MALT1 is not required for K-ras-induced lung adenocarcinoma progression 69
2.3.9 MALT1 contributes to NF-κB and STAT3 activation in vivo 71
2.3.10 MALT1 controls NF-κB dependent IL-6 production in response to EGFR 73
2.4 Discussion 77
2.4.1 MALT1 only functions as a scaffold protein in EGFR-mediated NF-κB pathway 77
2.4.2 MALT1 in EGFR-associated tumor malignancy 78
2.4.3 The role of MALT1 in STAT3 pathway in lung cancer 80
CHAPTER 3 THE ROLE OF CBM COMPLEX IN HER2-INDUCED NF-κB ACTIVATION AND HER2-ASSOCIATED BREAST CANCER PROGRESSION 81
3.1 Introduction 82
3.1.1 Overview of HER2 and breast cancer 82
3.1.2 The role of NF-κB signaling in HER2+ breast cancer 82
3.1.3 Critical observations and hypothesis 83
3.2 Materials and Methods 85
3.2.1 Antibodies and reagents 85
3.2.2 Cells 85
3.2.3 Immunoblotting and gel shift assay 85
3.2.4 cDNA preparation and Quantitative PCR 86
3.2.5 Cell migration and invasion assay 87
3.2.6 MTT Assay 87
3.2.7 Colony formation assay 87

3.2.8 Anoikis assay 88
3.2.9 Mouse xenograft tumor model 88
3.2.10 Mouse lung metastatic tumor model 89
3.2.11 Mammary tumor mouse model 89
3.2.12 Histology and immunohistochemical staining 89
3.2.13 Statistical analysis 90
3.3 Results 91
3.3.1 PKC but not AKT activity is required for HER2-induced NF-κB activation 91
3.3.2 The CBM complex is required for HER2-induced NF-κB activation 95
3.3.3 The CBM complex contributes to cell proliferation and avoidance of apoptosis 100
3.3.4 The CBM complex contributes to cell migration and invasion 106
3.3.5 CARMA3 contributes to migration and invasion through regulating HER2-induced MMP1 and MMP13 expression 110
3.3.6 MALT1 contributes to breast cancer progression in vivo 114
3.4 Discussion 117
3.4.1 HER2-induced NF-κB is mediated by PKC-CBM-IKK axis instead of AKT 117
3.4.2 The role of NF-κB in HER2-associated breast tumor progression 118
CHAPTER 4. THE ROLE OF CBM COMPLEX IN DNA DAMAGE-INDUCED NF-κB ACTIVATION AND RESISTANCE TO CHEMOTHERAPY-INDUCED CELL DEATH 121
4.1 Introduction 122
4.1.1 General overview DNA repair and DNA damage response 122
4.1.2 Molecular mechanism by which DNA damage-induces NF-κB activation 124
4.2 Materials and Methods 128
4.2.1 Cells 128
4.2.2 Doxorubicin-induced apoptosis 128
4.2.3 Immunoblotting assay 128
4.2.4 EMSA 129
4.2.5 Immunoprecipiation assay 129
4.3 Results 130
4.3.1 MALT1 regulates doxorubicin-induced apoptosis 130

4.3.3 The CBM complex is required for DNA damage-induced NF-κB activation 134
4.3.4 PKC is not required for DNA damage-induced NF-κB activation 136
4.3.5 CARMA3 associates with TRAF6 upon DNA damage induction 138
4.4 Discussion **141**
4.4.1 The CBM complex-mediated NF-κB activation 141
4.4.2 The upstream activator of CARMA3 142
4.4.3 Therapeutic implications 143
CHAPTER 5. CONCLUSION, DISCUSSION AND FUTURE DIRECTIONS **145**
5.1 Conclusion **145**
5.2 The protease activity of MALT1 **147**
5.3 Future directions of studies on MALT1 **149**
5.3.1 MALT1 and autoimmune disease 150
5.3.2 The role of MALT1 in the effector function in Th17 cells 150
5.3.3 BCL10-independent function of MALT1 in macrophages 153
Bibliography **157**
VITA **194**

List of Figures

Figure 1. Structural basis of MALT1, cIAP2 and cIAP-MALT1 fusion protein. 23

Figure 2. The CARMA1-BCL10-MALT1 complex mediated NF-κB activation and structural basis of CARMA protein family members. 25

Figure 3. The substrates of MALT1. 37

Figure 4. MALT1 is required for EGFR- and PKC-induced NF-κB activation. 50

Figure 5. MALT1 is specifically required for EGFR-induced NF-κB activation but not for other EGFR-downstream pathways. 52

Figure 6. Protease-deficient and wild-type MALT1 reconstitution equally rescued EGFR- and PMA/Ionomycin-induced NF-κB activation. 54

Figure 7. MALT1 protease inhibitor does not affect EGF-induced NF-κB activation. 56

Figure 8. MALT1 functions as a scaffold protein to recruit TRAF6 to IKK complex. 58

Figure 9. MALT1 contributes to EGFR-associated cell proliferation. 61

Figure 10. MALT1 is required for cell migration and metastasis. 63

Figure 11. TRAF6 and IKK complex are required for cell migration. 64

Figure 12. MALT1 protease activity is not required for cell migration. 65

Figure 13. MALT1 contributes to EGFR-associated lung adenocarcinoma progression in a mouse model. 68

Figure 14. MALT1 is dispensable for K-ras-induced lung adenocarcinoma progression. 70

Figure 15. MALT1 controls NF-κB and STAT3 activation in EGFR-driven lung cancer model. 72

Figure 16. IL-6 and IKK complex are required for STAT3 activation in vitro. 74

Figure 17. MALT1 is required for EGFR-induced IL-6 production. 75

Figure 18. The working model summarizing the finding in this study. 76

Figure 19. Heregulin specifically activates HER2 receptor. 92

Figure 20. HER2-induced NF-κB activation does not require AKT activity. 93

Figure 21. HER2-induced NF-κB activation requires PKC activity. 94

Figure 22. Knockdown efficiency of CARMA3, BCL10 and MALT1. 96

Figure 23. The CARMA3-BCL10-MALT1 complex is required for HER2-induced NF-κB activation. 97

Figure 24. CARMA3 and BCL10 do not affect HER2-induced AKT activation. 99

Figure 25. CARMA3 and BCL10 are required for SKBR3 cell proliferation. 102

Figure 26. CARMA3 and BCL10 are required for detachment induced apoptosis in SKBR3 cell line. 103

Figure 27. The CBM complex is required for anchorage-independent growth of SKBR3 cells in vitro. 104

Figure 28. CARMA3 is required for tumor progression on a xenograft mouse model. 105

Figure 29. CARMA3 and BCL10 are required for serum and HRG induced cell migration and invasion. 107

Figure 30. MALT1 is required for serum induced cell migration and invasion. 108

Figure 31. CARMA3 is required for lung metastasis in a mouse model. 109

Figure 32. MMP1 and MMP13 are upregulated upon HRG stimulation in SKBR3 cells. 111

Figure 33. Upregulation of MMP1 and MMP13 depends on PKC-IKK-NF-κB pathway. 112

Figure 34. CARMA3 is required for HER2-induced MMP1 and MMP13 upregulation. 113

Figure 35. MALT1 contributes to HER2-induced mammary tumor progression in vivo. 115

Figure 36. The working model summarizing the findings in this study. 116

Figure 37. Schematic overview of DNA damage induced NF-κB activation. 127

Figure 38. MALT1 regulates pro-survival signaling to antagonize doxorubicin-induced apoptosis in MEFs. 131

Figure 39. MALT1 is required for DNA damage-induced NF-κB activation. 133

Figure 40. The CBM complex is required for DNA damage-induced NF-κB activation. 135

Figure 41. PKC is not required DNA damage-induced NF-κB activation. 137

Figure 42. CARMA3 inducibly associates with TRAF6 upon doxorubicin treatment. 139

Figure 43. Proposed working model of DNA damage-induced NF-κB activation. 140

Figure 44. MALT1 deficient mice develop autoimmune phonotypes. 152

Figure 45. MALT1 and its protease activity negatively regulate CXCL-1 expression in macrophages. 155

Figure 46. MALT1 is specifically induced in macrophages and functions independently of BCL10. 156

CHAPTER 1: INTRODUCTION

1.1 General overview of lung cancer and breast cancer

1.1.1.1 Overview of lung cancer

According to the cancer statistics reported in 2008, lung cancer is the leading cause of cancer death in the United States and worldwide (Jemal et al. 2008). About 85% of lung cancers are non-small cell lung cancer (NSCLC), while 15% of lung cancers are small-cell lung cancers. As most NSCLC are diagnosed at the advanced stage, it is associated with a poor prognosis with a 5-year survival rate at 15.9% (Ettinger et al. 2013). The NSCLC can be further divided into three subtypes according to the pathological findings, adenocarcinoma (ADC, around 50%) , squamose-cell carcinoma (SCC, around 40%), and large-cell lung cancer. ADC generally occurs in more distal airways and is the associated with never smokers, while SCC generally occurs in more proximal airways and is closely associated with smoking. Recent studies suggest that the susceptibility and risk of lung cancer could be increased in patients with inherited genetic alternations, including rare mutations in tumor suppression genes such as p53, retinoblastoma (*Rb*), and oncogenes such as *EGFR* (epidermal growth factor receptor) gene (Sanders et al. 1989, Hwang et al. 2003, Bell et al. 2005). Genome wide association study (GWAS) also indicates an association between lung cancer susceptibility and single nucleotide polymorphisms (SNPs) at 15q24-15q25.1 (Amos et al. 2008, Hung et al. 2008, Thorgeirsson et al. 2008). Lung cancers at the early stage are often asymptomatic. Therefore, early diagnosis of lung cancer is extremely important. The diagnosis of lung cancer includes imaging tests (X-ray, CT scan and positron emission tomographyscan), biopsy and sputum cytology. In term of lung cancer treatment, surgery is

a preferred treatment for patients with early stage of lung cancer. However, most patients with advanced or metastatic lung cancer are not suitable for surgery treatment. In addition to surgery, other treatment options includes chemotherapy, radiation therapy, targeted therapy and immunotherapy. For example, lung cancers with activating EGFR mutations can be treated with targeted therapy by using EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as erlotinib and gefitinib (Gazdar 2009). Noticeably, immunotherapy recently becomes an emerging and promising way to treat lung cancer (Massarelli et al. 2014). In March 2015, the FDA approved the PD-1 checkpoint inhibitor nivolumab for treatment of advanced squamous NSCLC that has stopped responding to chemotherapy reagent . This approval is based on the data of a phase III clinical trail showing that patients receiving nivolumab lived an average 3.2 months longer than patients who receiving standard chemotherapy (Brahmer et al. 2015).

1.1.1.2 Molecular origins of lung cancer

Activating K-ras mutation is responsible for about 15%-25% of NSCLC. An amino acid changing mutation at the position 12, 13 or 61 will generate the constitutive activating form of K-ras, which has a strong transformation potential (Bos 1989). The activating K-ras will constantly activates MEK-ERK pathway to send a constitutive proliferation signal to the cell (McCormick 2015). Despite tremendous efforts spent on identifying drugs that specifically inactivate mutant K-ras, targeting K-ras associated tumors is still difficult (McCormick 2015). Given the structural similar of four Ras isoforms-H-Ras, N-Ras, K-Ras4a, and K-Ras4B, it is difficult to achieve high specificity of mutant K-ras alone. However, a recent study reported the development of small molecules irreversibly bind to

oncogenic K-Ras (G12C) and induce apoptosis in K-ras (G12C)-associated lung cancer cells (Ostrem et al. 2013). Therefore, development small molecules that specifically target oncogenic K-ras is still under insensitive investigation.

In comparison, EGFR mutation accounts for nearly 10% of NSCLC in the United States and 35% of NSCLC in Asia (Herbst et al. 2008). Two most frequent (more than 80% in total) mutations of EGFR involve in-frame deletion of exon 19 and L858R mutation in the exon 21. Both EGFR mutants become constitutively active and activates a number of downstream pathways, including PI3K-AKT, RAS-MAPK and NF-κB pathways (Sharma et al. 2007). Therefore, oncogenic EGFR signaling promotes cell proliferation, survival and metastasis (Sharma et al. 2007). Fortunately, lung cancer with EGFR mutations can be treated by target therapy using EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib (Gazdar 2009). Although lung cancers with EGFR mutations usually have an initial response to EGFR TKIs, they quickly develop drug resistance to EGFR TKIs by generating new mutations. For example, NSCLCs with EGFR T790M mutations are not responsive to EGFR TKIs (Kobayashi et al. 2005, Kosaka et al. 2006). Amplification of porto-oncogene MET is another mechanism in which EGFR mutant NSCLCs become resistance to EGFR TKI treatment (Bean et al. 2007, Engelman et al. 2007). While K-ras and EGFR mutations are two major mutations in NSCLC, other mutations also contributes to the tumorigenesis of NSCLC, such as *ALK* (anaplastic lymphoma kinase) rearrangement (Solomon et al. 2009), *BRAF* mutations (Davies et al. 2002), and PTEN (Phosphatase and Tensin homolog) downregulation (Soria et al. 2002).

1.1.1.3 Mouse models in lung cancer

Mouse models are extremely powerful preclinical models to investigate the mechanisms by which human cancers develop *in vivo*. Based on the genetical alternations in human NSCLC, genetically engineered mouse (GEM) models for lung cancers have been generated by introducing mutations in human NSCLC. For example, using the Cre-Lox based expression system (Sauer 1987, Sauer and Henderson 1988), Jackson et al. generated a mouse model in which murine lung adenocarcinoma can be developed *in vivo* by conditional expression of oncogenic K-ras induced by adenoviral Cre recombinase (Jackson et al. 2001). The histology of K-ras-induced murine carcinoma closely resembles that in human NSCLC (Jackson et al. 2001), suggesting that murine models of lung cancers are biologically related to human lung cancer. In order to specifically achieve inducible expression of mutant K-ras in type II alveolar epithelial cells in mice, Fisher et al. generated a conditional expression of activating mutant K-ras-induced lung cancer model by crossing a tetracycline operator-regulated oncogenic K-ras with the clara cell secretory protein-reverse tetracycline activator (CCSP-rtTA) mice (Fisher et al. 2001). In this mouse model, lung adenocarcinoma can be induced by feeding mice with doxycycline (Fisher et al. 2001). Subsequently, through a similar approach, Politi et al. generated a mouse model in which EGFR-mediated lung adenocarcinoma ($EGFR^{L858R}$;CCSP-rtTA and $EGFR^{\Delta L747–S752}$;CCSP-rtTA) can be induced by feeding mice with doxycycline (Politi et al. 2006). In addition to EGFR and K-ras, other genetic mutations/amplification/deletion based murine lung cancer models are also available (Tichelaar et al. 2000, Zhao et al. 2001, Meuwissen et al. 2003). These murine models

provided excellent tools to study the development of human lung cancer in the context of complicate genetic settings.

1.1.2.1 Overview of breast cancer

Breast cancer is the leading cause of cancer death in women, and is the second most common type of cancer worldwide (Warrier et al. 2015). Breast cancer can be categorized into two major categories: noninvasive breast cancer, also known as *in situ* breast cancer, in which tumor cells remains in particular location in the breast and do not spread across surrounding tissues (Webb and Koch 1997); invasive breast cancer, which is also known as infiltrating breast cancer, in which tumor cells great normal tissue and spread across other parts of tissues and distant organs in the body (Weigelt et al. 2005). The invasive breast cancer can be further divided into two types: invasive ductal carcinoma (IDC), in which cancer originates from the mild ducts; invasive lobular carcinoma (ILC), in which cancer originates from lobules. According to the molecular characteristics and pathology of invasive breast cancer, it can be also decided in to several subgroups, including endocrine-sensitive breast cancer, HER-2 positive breast cancer, triple-negative breast cancer, inflammatory breast cancer, medullary carcinoma, metaplastic carcinoma, mutinous carcinoma, papillary carcinoma, tubular carcinoma, Paget's disease and male breast breast cancer. Breast cancer can show no symptom at the early stage and shows symptoms at the late stages, including skin changes, pain, irritation and symptoms of local inflammation. Due to the asymptomatic feature of breast cancer, early diagnosis and prevention screening is critical. According to the american cancer society beast cancer screening guideline, it is

suggested that women with average risk of breast cancer should take annually regular mammography screening starting from age 45 years. Evidence suggested that screening mammography in women aged 40 to 69 years old is associated with reduced breast cancer death (Oeffinger et al. 2015). In terms of therapy, breast cancer can be treated by surgery, radiation therapy, chemotherapy, hormone therapy and targeted therapy. Noticeably, breast cancer is one of the few cancers can be traced with individualized targeted therapy, which significantly improved survival rates (Perez 2011). The prognosis of breast cancer is highly related with the stages of cancer, and the five-year relative survival rate ranges from 100% for stage 0 to 22% for stage IV, according to the SEER database from National Cancer Institute (http://seer.cancer.gov/).

1.1.2.2 Risk factors of breast cancer

Like most types of other cancers, the risk of breast cancer includes gender (mostly women), aging, personal and familial history, ethnicity, dense of breast tissue and genetic factors (Singletary 2003). About 5-10% of breast cancer are though to be inherited breast cancer (Hedenfalk et al. 2001). *BRCA1* (Breast-cancer susceptibility gene 1) and *BRCA2* (Breast-cancer susceptibility gene 2) are two most common causes of hereditary breast cancer . The average breast cancer risk with *BRCA1* mutation is between 55-65% while the rate for *BRCA2* mutations is about 45% (Hedenfalk et al. 2001, Jiang and Greenberg 2015). Both BRCA1 and BRCA2 function as tumor suppressors and play a central role in homology-directed DNA damage repair pathway and cell cycle checkpoints (Jiang and Greenberg 2015). In addition to BRCA genes, changes of many other genes can also lead to

inherited breast cancers, including *ATM, TP53, CHEK2, PTEN,CDH1, STK11, and PALB2*. Noticeably, many of the genes involved in inherited breast cancer have a functions in DNA damage repair (DDR) pathways. According to TCGA database, most frequently mutated genes in breast cancer includes both well known oncogenes and tumor suppressor genes, such as *P53* and *PTEN*.

1.1.2.3 Subtypes of breast cancer

Like all other cancers, breast cancer is a genetic disease. According to the gene expression profiles, breast cancers can be classified into serval major subgroups: luminal breast cancer, human epidermal growth factor receptor 2 + (HER2+) breast cancer , and basal like breast cancer (Perou et al. 2000, Sorlie et al. 2001). Each subtype of breast cancer has different biological and clinical presentations, including risk factor, strategies of treatment, and preferential sites of metastasis. Luminal breast cancer, also known as estrogen receptor (ER)-positive breast cancer, accounts for about two-thirds of breast cancers (Ignatiadis and Sotiriou 2013). According to the expression profiles, luminal breast cancers can be further divided into two categories: luminal A breast cancer, in which cancer cells have higher expression of ER or ER regulated genes; luminal B breast cancer, in which cancer cells have lower level of ER or ER regulated genes. The luminal B breast cancer usually shows lower progesterone expression, higher tumor rate, and being more proliferative (Creighton 2012). The treatment strategy is also highly depends on the subgroup. For example, endocrine therapy alone is recommended for luminal A type of breast cancer with a high expression of

ER, while both endocrine and chemotherapy are recommended to luminal B type of breast cancer ,which is generally more aggressive.

HER2 is a transmembrane receptor belongs to the epidermal growth factor receptor family. Upon binding to its ligand heregulin, HER2 activates NF-κB a number of downstream pathways, including PI3K/Akt and Ras/Raf/MAPK signaling (Yarden and Sliwkowski 2001). It has also been suggested that NF-κB pathways is playing critical role for HER2-asscociated breast cancer progression (Merkhofer et al. 2010). However, the molecular mechanism by which HER2-induced NF-κB activation remains undetermined and controversial. HER2 positive breast cancer cells highly express HER2 due to gene amplification or enhanced mRNA or protein level. As a result, HER2+ breast cancer cells have constitutive activated HER2 downstream signaling and are highly proliferative and resistant to apoptosis. Breast cancers with oncogenic HER2 can be treated by monoclonal antibody trastuzumab, which binds to the extracellular domain of HER2 receptor (Molina et al. 2001). The mechanism includes antibody-dependent cell-mediated cytotoxicity (ADCC), inhibition of the extracellular domain (ECD) of HER2 receptor, inhibition of HER2 dimerization, and internalization of HER2 (Molina et al. 2001, Arteaga et al. 2012). It has been suggested that combination of trastuzumab and chemotherapy produced a benefit in disease-free survival (Yin et al. 2011). Even though trastuzumab is a very efficient and effective way to treat HER2+ breast cancer, like almost all other type of targeted therapy, cancer cells eventually become resistant to trastuzumab. The mechanisms by which breast cancer cells become be resistant to trastuzumab includes (1) structural mutation in HER2, (2) alternative activation of other tyrosine kinase receptors, such as insulin-like growth factor

receptor and (3) alternation of HER2 downstream pathway molecules (Vu and Claret 2012). Therefore, identifying new therapeutic target in HER2+ breast cancer will be an urgent task.

The basal-like breast cancer is typically associated with more aggressive behavior and poor prognosis, and they typically do not express hormone receptors nor HER2 receptor, and is often named as "triple negative" breast cancer (Rakha et al. 2008). Although majority of basal-like cancers are triple negative and major of triple negative cancers are basal-like, they are not interchangeable. The expression profiles and clinical outcome can be very different between a triple negative breast cancer and a basal-like breast cancer. The histological definition of basal-like breast cancer includes: (i) lack of ER, PR and HER2 expression, (ii) expression of one or more of the followings: CD5/6, CK14 and CK17, (iii) lack of ER and HER2 in conjunction with CD5/6 and/or EGFR; and (iv) lack of ER, PR and HER2 in conjunction with expression of CD5/6 and EGFR (Nielsen et al. 2004, Cheang et al. 2008, Badve et al. 2011). The basal-like breast cancer is associated with specific pattern of distal metastasis with a preference in lung and brain and poorer prognosis (Banerjee et al. 2006, Fulford et al. 2007). It has been suggested that basal-like breast cancer is associated with *BRCA1* deficiency, as a large majority of breast cancers with BRCA1 mutations have basal-like cancer phenotype (Foulkes et al. 2003). Although the reason for the underlying association between basal-like breast cancer and loss of BRCA1 is not completely clear, it has been indicated that BRCA1 inactivation plays a role in differentiation of mammary epithelial cells (Furuta et al. 2005). In terms of cancer treatment, basal-like breast cancer now can only be treated by chemotherapy other than surgery, since it does not express hormone receptors nor HER2 receptors.

1.1.2.4 Mouse models of breast cancer

The biology of breast cancer can be studied *in vivo* by using mouse models. Although xenograft model allows investigation on the tumor proliferation and metastasis *in vivo*, there are fundamental limitations of this approach. First, since most xenografts are established in immunodeficient mice such as *SCID* (severe combined immunodeficiency) mice, the interaction between tumor cells and immune cells cannot be studied in such models. In fact, growing number of evidence suggests the immune system is playing a central role in tumorigenesis and tumor progression *in vivo* (Schreiber et al. 2011). Second, xenografts are usually being subcutaneously injected into sides of the body, where the microenvironment is completely different from the *in situ* microenvironment. Third, many established tumor cell lines have already undergone extensive evolution and therefore it is difficult to recapture early events in tumorigenesis.

In comparison, genetically engineered mice (GEM) are powerful preclinical models to study breast cancer progression *in vivo* by manipulating both oncogenes and tumor suppressor genes specifically in the mammary gland. To achieve this, transgenic mice carrying breast cancer-causing oncogenes have been generated since late 1980s, such as *Erbb2*, *PyMT* (polyoma virus middle T) and *Myc*, under transcriptional control of mouse mammary tumor virus (MMTV) promoter (Sinn et al. 1987, Muller et al. 1988, Guy et al. 1992, Guy et al. 1992). These transgenic mice develop mammary tumors similar to human breast cancers and undergo spontaneous metastasis (Sinn et al. 1987, Muller et al. 1988, Guy et al. 1992). In addition, advances in genetic engineering also allow more precise control of

the timing and developmental context of breast cancer. For example, to study the contribution of tyrosine kinase c-Src in the development of PyMT-induced breast cancer, Marcotte et al. crossed the *MMTV-PyMT* mice with a mammary epithelial cell-specific c-Src deletion mouse model (*c-Src$^{lox/lox}$; MMTV-Cre*) (Marcotte et al. 2012). In this model, authors demonstrated that c-Src contributes to PyMT-induced breast cancer tumorigenesis by regulating cell-cycle progression (Marcotte et al. 2012). In summary, transgenic mouse models for breast cancer closely recapitulate human breast cancer and serve as powerful preclinical models for scientific investigation.

1.2 The transcription factor NF-κB

1.2.1 Discovery of NF-κB

In 1986, the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) was first discovered as a protein complex that binds to the κ light chain enhancer specifically in the B cells by David Baltimore's group (Sen and Baltimore 1986, Singh et al. 1986, Staudt et al. 1986, Weinberger et al. 1986, Baltimore 2009). Since NF-κB specifically binds to κ light chain enhancer, it is crucial to the inducibility of κ enhancer (Atchison and Perry 1987, Lenardo et al. 1987). Although NF-κB was initially thought to be specific in B cells, Sen and Baltimore soon discovered that NF-κB also exist in many other cell types, including Jurkat cells and even HeLa cells (Sen and Baltimore 1986). To further prove the concept that NF-κB is the transcription factor that responsible for expression of κ light chain, Sen and Baltimore used the 70Z/3 cells, in which there is no detectable NF-κB and transcription of the κ gene can be induced by LPS (Parslow and Granner 1982, Sen and

Baltimore 1986). The hypothesis was that LPS would induce NF-κB. Consistent with this hypothesis, Sen and Baltimore showed that LPS potently induced NF-κB in 70Z/3 cells, which clearly demonstrates that NF-κB is responsible for the expression of κ gene (Sen and Baltimore 1986). To further investigate the activation mechanism of NF-κB, Sen and Baltimore showed that the induction of NF-κB does not require protein synthesis (Sen and Baltimore 1986). Furthermore, a combination of LPS and cyclohgeximide which blocks new protein systhesis causes a superinduction of NF-κB. Based on this data, Sen and Baltimore suggest that (i) NF-κB pre-exists in the cells and (ii) the induction of NF-κB may be mediated by posttranslational modification of precursor factor of NF-κB (Sen and Baltimore 1986). Impressive as it is, this notion turns out to be very precise and true even after 20 years of intensive investigation of NF-κB.

To further investigate the molecular mechanism by which NF-κB is activated, Baeuerle and Baltimore found that although NF-κB is virtually undetectable in 70Z/3 cells, it becomes detectable only in the cytosolic fraction after denaturation and renaturation (Baeuerle and Baltimore 1988). In addition, the authors showed that NF-κB becomes detectable in the nuclear fraction upon treatment with phorbol esters (Baeuerle and Baltimore 1988). This data indicates that NF-κB pre-exists in an inactive form in the cytosol and is released to the nucleus upon stimulation. Subsequently, Baeuerle and Baltimore identified a 68kD protein that specifically inhibits NF-κB DNA binding capability, and therefore it is named as inhibor-κB (IκB) (Baeuerle and Baltimore 1988). Based on this observation, it was further hypothesized that the activation of NF-κB requires posttranslational modification on

IκB protein (Baeuerle and Baltimore 1988). It turned out to be exact the case. Together, these early studies have established most fundamental framework of the NF-κB pathway.

1.2.2 Overview of NF-κB pathway

NF-κB family consists five members, including p65 (RelA), p50, p52, c-Rel and RelB (Hayden and Ghosh 2012). They share an N-terminal Rel homology domain (RHD), which is responsible for binding DNA and homo- or heterodimerization with other members (Hayden and Ghosh 2012). The NF-κB dimers bind to the κB sites of the promoters or enhancers of the target genes and thereby regulate transcription by recruiting coactivators and repressors. RelA, c-Rel and RelB positively regulate gene expression through the transcription activation domain (TAD), while p50 and p52 do not have TAD and therefore they may repress gene transcription if they are not associated with a TAD-containing NF-κB subunit. However p50 and p52 can still positively regulate transcription if they forms a heterodimer with a TAD containing NF-κB subunit (Hayden and Ghosh 2012). The NF-κB signaling can be categorized into two categories according to the activation mechanisms: canonical NF-κB pathway and noncanonical NF-κB pathway.

1.2.2 Canonical NF-κB pathway

The canonical NF-κB signaling can be triggered by the activation of many kinds of receptors on immune cells, including antigen receptors, pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), TNF receptor (TNFR) and IL-1 receptor (IL-1R) (Hayden and Ghosh 2012). In the quiescent state, the NF-κB family members are kept in cytosol and

remains inactive state by associating with IκB proteins. The IκB protein family contains IκBα, IκBβ, IκBε, IκBζ, B-cell lymphoma 3 (BCL-3), IκBns, NF-κB1 (also known as p100) and NF-κB2 (also knowns as p105) (Beg and Baldwin 1993). These IκB proteins are characterized by containing ankyrin repeat domains (ANK) (Beg and Baldwin 1993). Upon stimulation, IκB proteins will be rapidly phosphorylated by IκB kinase (IKK) complex. Once phosphorylated, IκB proteins will be K48-linked ubiquitinated and processed by proteasome for rapid degradation. The degradation of IκB proteins allows NF-κB subunits to translocate into the nucleus and initiate transcription. The IKK complex is composed by three different proteins, IKKβ (also known as IKK2), IKKα (also known as IKK1) and NF-κB essential modulator (NEMO), which is also known as IKKγ. In canonical NF-κB pathway, the IKK complex is activated through several distinct mechanism depending on the type of stimuli.

For example, the TNFR-induced NF-κB activation requires the recruitment and assembly of several death domain (DD)- and caspase recruitment domain (CARD)-containing proteins, including TRADD (TNFRSF1A-associated via death domain), TRAF2 (TNF receptor-associated factor 2), RIP1 (receptor interacting protein 1), cIAP1 (cellular inhibitor of apoptosis protein 1) and cIAP2 (cellular inhibitor of apoptosis protein 2) (Hayden and Ghosh 2014). Mechanistically, cIAP1 and cIAP2 function as E3 ligase to trigger ubiquitination of IKK complex (Mahoney et al. 2008). In addition, the linear ubiquitin chain assembly complex (LUBAC) is also required for TNF-induced IKK ubiquitination (Haas et al. 2009, Rahighi et al. 2009, Gerlach et al. 2011, Ikeda et al. 2011, Tokunaga et al. 2011). At the mean time, the ubiquitination of RIP1 recruits TAK1 to

phosphorylate IKK complex (Takaesu et al. 2003, Sato et al. 2005, Shim et al. 2005, Ea et al. 2006, Li et al. 2006). Both ubiquitination and phosphorylation are required for a complete activation of IKK (Hayden and Ghosh 2014). In comparison, the toll-like receptors activate IKK complex through a different mechanism, in which different scaffold proteins are involved (Kawai and Akira 2007). Upon binding to its ligand, the Toll IL-1R (TIR) domain of TLR4 recruits other TIR-containing adaptor proteins, such as MyD88 (myeloid differentiation primary response gene 88) and TICAM1 (TIR domain-containing adaptor molecule 1) (Medzhitov et al. 1998, Kawai et al. 1999, Yamamoto et al. 2002). Upon activation TLR4 activation, MyD88 recruits IRAK-4 (IL1-R-associated kinases-4), which further recruits IL-1R-associated kinases-1 to form IRAK-4:IRAK-1 complex (Kanakaraj et al. 1998, Li et al. 2002, Suzuki et al. 2002). The IRAK-4:IRAK-1 complex recruits TNF receptor-associated factor 6 (TRAF6), which further recruits TAK1 to phosphorylate IKK complex and NF-κB activation (Adhikari et al. 2007).

1.2.3 Non-canonical NF-κB pathway

The discovery of Non-canonical NF-κB pathway originates from the finding that NIK (NF-κB-inducing kinase) mediates the proteolytic processing of p100 (NF-κB2) to p52 (Xiao et al. 2001). P100 (NF-κB2) is an IκB-like protein and functions to inhibit RelB translocation (Solan et al. 2002). After processing to p52, it can induced noncanonical NF-κB activation by forming heterodimer with RelB (RelB:p52 dimer) (Coope et al. 2002). The noncanonical NF-κB signaling is induced by several specific cytokines in the TNF cytokine family, such as CD40 ligand, B-cell activating factor (BAFF) and lymphotoxin-β (Sun 2011). The hallmark

of noncanonical is the involvement of IKKα and NIK rather than phosphorylation of IκB proteins. For example, upon CD40 binds to its ligand CD40L, TRAF2 is recruited to the receptor and target TRAF3 for proteasome degradation. Since TRAF3 functions to destabilize NIK by inducing NIK ubiquitination and degradation, TRAF3 degradation leads to a stabilization of NIK, which phosphorylates and activates IKKα. The activated IKKα will further phosphorylate p100, which binds and keeps NF-κB subunit p52 in the cytosol. Once phosphorylated, p100 will be targeted by $SCF^{\beta TRCP}$ complex and release p52 into the nucleus to initiate transcription by forming heterdimer with RelB (Sun 2011).

1.2.4 The role of NF-κB signaling in inflammation

NF-κB signaling plays a central role in inflammation as it controls a variety of pro inflammatory cytokines, chemokines and adhesion molecules. Both activation of antigen receptors and pattern recognition receptors (PPR) lead to NF-κB activation and trigger a series of immune responses. In addition, many inflammatory cytokines, such as TNFα and IL-1, are potent activator of NF-κB (Hayden and Ghosh 2012). Given the importance of NF-κB in inflammation, many chronicle inflammatory diseases are associated with aberrant NF-κB, such as rheumatoid arthritis, atherosclerosis, chronic obstructive pulmonary disease (COPD), asthma, multiple sclerosis, inflammatory bowel disease (IBD), and ulcerative colitis (Tak and Firestein 2001). In addition to production of proinflammatory cytokines, NF-κB also mediates the expression of anti-apoptotic genes, including cIAP-1 and c-IAP2, which functions to suppress the activation of caspase-8 (Wang et al. 1998). Therefore, NF-κB plays

a central role in mediating inflammatory responses by coordinately control immune cells activation and survival.

1.2.5 The role of NF-κB signaling in hematopoietic malignancies

NF-κB has both direct and indirect impact on human cancers. Mutations affecting NF-κB pathway are causes for many types of lymphomas. For example, a truncated p100 (NF-κB2) protein was found in rearranged B-cell lymphomas (Neri et al. 1991). The MALT B cell lymphoma with t(1;14)(p22;q32) translocation involves a recurrent breakpoint upstream of the promoter of *Bcl10*, which encodes a protein BCL10 (Willis et al. 1999). While wild-type BCL10 leads to both NF-κB activation and apoptosis, the truncated form of BCL10 expressed in MALT lymphoma constitutively activates NF-κB without causing apoptosis. MALT1 lymphoma with a t(11;18)(q21;q21) genetic translocation also has constitutive NF-κB activation, which is activated by cIAP2-MALT1 fusion protein created by t(11;18)(q21;q21) translocation (Levine et al. 1989). Oncogenic NF-κB signaling also plays a pathogenic role in activated B cell (ABC) type of diffuse large B cell lymphoma (DLBCL), but not in germinal center B-cell-like (GCB) type of DLBCL (Davis et al. 2001). About 10% of ABC-DLBCL have mutated *CARD11* gene, which encodes for protein CARMA1 (Lenz et al. 2008). CARMA1 is required for TCR and BCR induced NF-κB activation in lymphocytes (Wang et al. 2002). Most mutations in CARMA1 in ACB-DLBCL occurs in the coiled-coil domain and makes CARMA1 a constitutive activator of NF-κB (Lenz et al. 2008).

Oncogenic NF-κB also plays a fundamental role in the oncogenesis of multiple myeloma (MM). Annuziata et al. and Keats et al. showed that many MM cell lines and primary cells from MM patients have mutations in signaling components in NF-κB pathway (Annunziata et al. 2007, Keats et al. 2007). Several mutations involved in positive regulators in both canonical and noncanonical pathway have been identified, including CD40, LTβR, TAC1, NIK, NF-κB1 and NF-κB2 (Annunziata et al. 2007, Keats et al. 2007). In addition, authors also identified loss of function mutations in negative regulators of NF-κB pathway, including TRAF2, TRAF3, cIAP-1, cIAP-2 and CYLD (Annunziata et al. 2007, Keats et al. 2007). Mutations in genes encoding those proteins leads to impaired negative regulation of NF-κB and results enhanced NF-κB activation in MM patients.

1.2.6 The role of NF-κB signaling in solid tumors

NF-κB has also been implicated of playing a positive roles during the onset and progression of many types of solid tumors. Myelin et al. showed that NF-κB signaling is required for the progression for oncogenic K-ras (K-ras^{G12D}) induced lung adenocarcinoma in a genetically engineered mouse model (Meylan et al. 2009). Consistently, Ling et al. showed that IKKβ (IKK2) is required for oncogenic K-ras (K-ras^{G12D}) induced pancreatic ductal adenocarcinoma in a transgenic mouse model (Ling et al. 2012). In addition, by using a systematic RNA interference screening to detect synthetic lethal molecules in K-ras mutant cell lines, Barbie et al. identified TBK1 as a crucial protein for cell survival in K-ras mutant cells (Barbie et al. 2009). Mechanistically, TBK1 functions to activate NF-κB signaling in K-ras mutant cell lines and suppression of TBK1 results apoptosis in those cell lines (Barbie et

al. 2009). Together, these lines of investigations proved a critical role of NF-κB signaling in oncogenic K-ras-associated solid tumor progression.

In addition to K-ras, epidermal growth factor receptor (EGFR) also induces NF-κB activation in many types of solid tumors. For example, Jiang et al. demonstrated that CARMA3 is involved in EGFR induced NF-κB activation and contributes to cell proliferation in A431 cell line (Jiang et al. 2011). In addition, Yang et al. demonstrated that EGFR-induced NF-κB activation is required for EGFR-associated tumor progression via upregulation of the expression of pyruvate kinase M2 (PKM2), which plays a central role in tumor metabolism (Mazurek et al. 2005, Yang et al. 2012). In addition to a direct involvement of NF-κB signaling in EGFR-associated tumor malignancy, NF-κB activation is also responsible for drug resistance of EGFR tyrosine kinase inhibitors (TKIs) (Bivona et al. 2011). Through a pooled RNA interference screening, Bivona et al. showed that knockdown of several component of NF-κB pathway specifically enhanced apoptosis induced by TKI erlotinib in EGFR-mutant lung cancer cells (Bivona et al. 2011). In addition, overexpression of several positive regulators in NF-κB pathway rescued EGFR-mutant lung cancer cells from TKI treatment (Bivona et al. 2011). Therefore, NF-κB can be served as a potential drug target in combination with EGFR TKIs. In summary, NF-κB is crucial for the tumorigenesis and development of EGFR-associated cancer. However, the molecular mechanism by which EGFR activates NF-κB signaling remains to be fully established.

In addition to lung cancer, NF-κB is also playing a crucial role in the tumorigenesis of breast cancer. Bissau et al. found that activated NF-κB was predominantly found in estrogen receptor (ER)-negative and heregulin (HRG) receptor HER2-positive primary breast cancer

samples (Biswas et al. 2004). The authors further demonstrated that HRG activates NF-κB through HER2 and IKK complex, as inhibition of HER2 and IKK complex blocked HRG-induced NF-κB activation and induced cell apoptosis (Biswas et al. 2004). In addition, Kendellen et al. showed that both canonical and noncanonical NF-κB signaling promote breast cancer tumor-initiating cells (TICs) (Kendellen et al. 2014). The authors demonstrated that both canonical and noncanonical NF-κB signaling are activated in TICs and are required for their self-renew potential (Kendellen et al. 2014). Consistently, through generating a transgenic mouse model (MMTV-ErbB2/IκBαSR) in which mammary tumor will be developed in the absence of NF-κB activation, Liu et al. showed that NF-κB pathway governs mammary tumorigenesis and stem cell expansion in this mouse model (Liu et al. 2010). Furthermore, NF-κB is also directly involved in mammary tumor migration and metastasis. Huber et al. showed that IKK-NF-κB signaling axis is required for induction of epithelial-mesenchymal transition (EMT), which is a fundamental process for cell migration and metastasis (Huber et al. 2004, Kalluri and Weinberg 2009). In summary, NF-κB signaling plays a multifunctional roles in the tumorigenesis and development of mammary tumors, especially in HER2-positive breast cancers. However, the molecular mechanism by which HER2 activates NF-κB remains to be investigated.

1.3 The biochemical properties and functions of MALT1

1.3.1 The discovery of MALT1

MALT1, also known as paracaspase, was initially identified by Vishva Dixit and his colleagues in the year of 2000 (Uren et al. 2000). Based on the sequence comparison of

caspase-like domains from several species, Uren et al. identified two homologs of caspase-like proteins, paracaspase and metacaspase (Uren et al. 2000). To determine the binding protein of paracaspase, the authors performed a two hybrid screen using the prodomain of paracaspase as a bait, and they identified BCL10 (B cell lymphoma 10) as a strong interaction protein with human paracaspase (Uren et al. 2000). This result has been verified by the authors through a series of biochemical experiments, including *in vitro* co-immunoprecipitation (co-IP) and colocalization revealed by immunofluorescence experiment (Uren et al. 2000).

With a further search of paracaspase homologs, Uren et al. further identified that human paracaspase matched to the sequence of MALT1 (Mucosa Associated Lymphoid Tissue lymphoma translocation protein 1), which was involved in a t(11;18)(q21;q21) genetic translocation. The t(11;18)(q21;q21) was initially described in 1989 in two cases of non-Hodgkin lymphomas (Levine et al. 1989). Subsequently, this translocation was also reported in other types of MALT lymphomas by other groups (Griffin et al. 1992, Horsman et al. 1992, Leroux et al. 1993, Auer et al. 1997, Ott et al. 1997). Mechanistically, the t(11;18)(q21;q21) translocation generates a chimeric protein with a fusion of the N-terminus of cIAP2 with the C-terminus of paracaspase. The cIAP2-MALT1 transcript contains three baculovirus IAP repeats (BIRs) from cIAP2 and the caspase-like domain from MALT1. The fusion protein cIAP2-MALT1 is a potent activator of NF-κB, as overexpression of cIAP2-MALT1 resulted an 80-fold to 300-fold of NF-κB induction (Uren et al. 2000).

The full length MALT1 contains a death domain (DD) at its N-terminus, following by two immunoglobulin domains, and a caspase-like domain at its C-terminus (Figure 1) (Uren

et al. 2000). The major function of MALT1 involves in antigen receptor signaling in lymphocytes and lymphocytes activation. In 2003, MALT1 deficient transgenic mice were independently generate by two groups (Ruefli-Brasse et al. 2003, Ruland et al. 2003) . Both groups showed a clear defect on TCR-induced NF-κB activation in T cells isolated from MALT1 deficient mice (Ruefli-Brasse et al. 2003, Ruland et al. 2003). In addition, both groups showed a defect of JNK activation and IL-2 production in MALT1 deficient T cells.

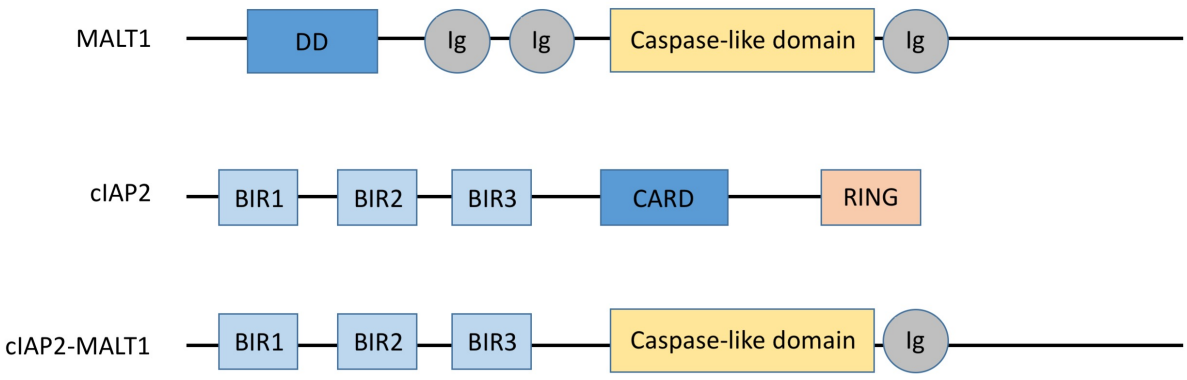

Figure 1. Structural basis of MALT1, cIAP2 and cIAP-MALT1 fusion protein.

MALT1 contains one death domain (DD), two immunoglobulin (Ig) domains and one caspase-like domain at its C-terminus followed by another Ig-like domain. cIAP2 consists of three baculovirus IAP repeats (BIRs), a caspase associated recruitment domain (CARD) and a really interesting new gene (RING) domain. The fusion protein cIAP2-MALT1 is composed of three BIR domains from the N-terminal of cIAP2 and the caspase-like domain from the C-terminus of MALT1.

1.3.2 The function of CARMA1-BCL10-MALT1 (CBM) complex in lymphocytes

In response to TCR activation, MALT1 forms a complex with two scaffold protein, CARD11 (Caspase recruitment domain-containing protein 11), also known as CARMA1 (CARD-containing MAGUK protein 1), and BCL10 (B-cell lymphoma/leukemia 10) (Blonska and Lin 2009). In 2002, several groups independently identified that CARMA1 is required for TCR-induced NF-κB activation and it is an interacting partner with BCL10 upon TCR stimulation (Gaide et al. 2002, Pomerantz et al. 2002, Wang et al. 2002). CARMA1 is phosphorylated by PKC (protein kinase C) upon TCR stimulation (Matsumoto et al. 2005, Sommer et al. 2005, Shambharkar et al. 2007). Phosphorylation of CARMA1 triggers its conformational change and exposes its CARD domain, which rapidly recruits BCL10 and MALT1 to form the CARMA1-BCL10-MALT1 complex, also known as the CBM complex (Matsumoto et al. 2005, Sommer et al. 2005, Shambharkar et al. 2007). The activated CBM complex presents in a highly polymerized structure and is able to recruit downstream factors such as E3 ubiquitin ligase TRAF6 to activate the IKK complex (Sun et al. 2004, Qiao et al. 2013). It is proposed that both TRAF6-mediated ubiquitination and TAK1-mediated phosphorylation are required to a full activation of the IKK complex (Figure 2A) (Sun et al. 2004).

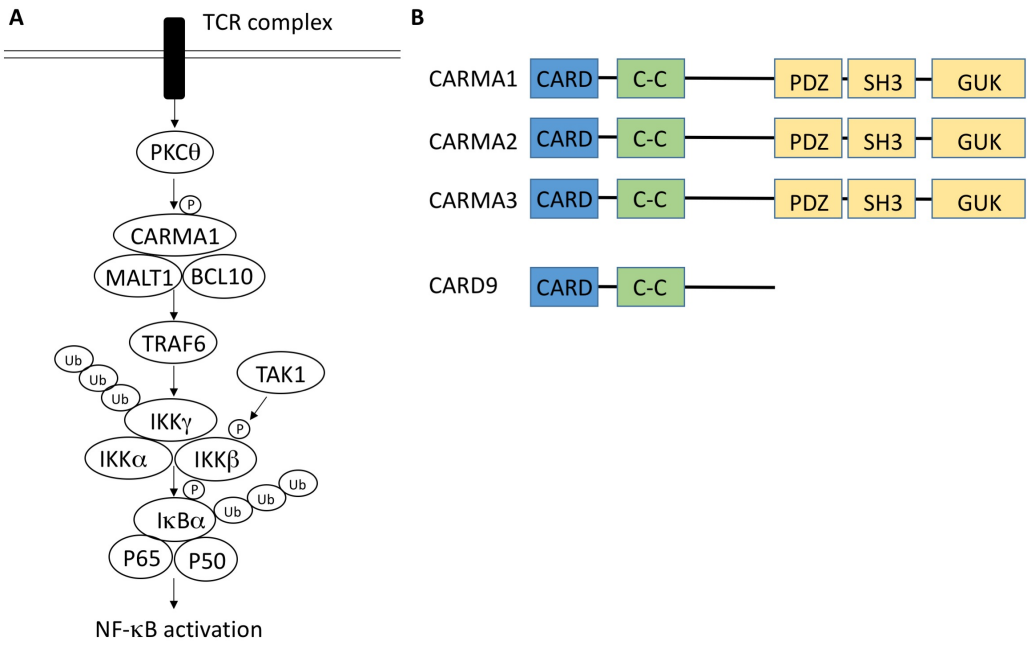

Figure 2. The CARMA1-BCL10-MALT1 complex mediated NF-κB activation and structural basis of CARMA protein family members.

(A) Molecular mechanism by which TCR induces NF-κB activation. TCR activates CARMA1 through PKCθ-dependent phosphorylation in the linker region in CARMA1. The activated CARMA1 recruits MALT1 and BCL10 to form the CARMA1-BCL10-MALT1 complex, which further recruits E3 ligase such as TRAF6 to mediate IKKγ ubiquitination. At the mean time, TAK1 phosphorylates IKKβ to fully activates IKK complex. The activated IKK complex phosphorylates IκBα, which will undergo ubiquitination and proteasome degradation. (B) The structural basis of CARMA protein family members. CARMA1, CARMA2 and CARMA3 are composed by a CARD domain and a coil-coiled (C-C) domain at the N-terminus, and a membrane associated guanylate kinase (MAGUK) domain at this C-

terminus. The MAGUK domain includes a PDZ domain, a SH3 domain and a GUK domain. In comparison, CARD9 only contains the N-terminal CARD and C-C domain.

1.3.3 The function of CBM complex in other cell types

In addition to TCR and BCR mediated NF-κB, the CBM complex is also involved in NF-κB activation in response to many types of stimuli in a variety of cells (Blonska and Lin 2011). Based on the similarity of sequences and structure, the CARMA protein family consists CARMA1, CARMA2, CARMA3 and CARD9 (Figure 2B), which lacks the MAGUK domain. Among them, CARMA1 is primarily expressed in hematopoietic tissue; CARMA2 is expressed in placenta; CARMA3 is expressed in most non-hematopoietic cells; CARD9 is only expressed in myeloid cells. It has been shown CARMA1, CARMA3 and CARD9 functions similarly in terms of forming the CBM complex to mediate NF-κB activation. In addition to lymphocytes, CARMA1-BCL10-MALT1 complex also mediates the immunoreceptor tyrosine-based activation motif (ITAM)-coupled natural killer (NK) cells receptors-induced NF-κB activation (Gross et al. 2008). The CARD9-BCL10-MALT1 complex is required for c-type lectin receptors, including Dectin-1, Dectin-2 and Dectin-3, induced NF-κB activation in myeloid cells, such as macrophages and dendritic cells (Bertin et al. 2000, Gross et al. 2006, Hara et al. 2007, Hsu et al. 2007, Bi et al. 2010, Zhao et al. 2014). It has been also reported that CARD9 is essential for the activation of myeloid cells through ITAM associated receptors (Hara et al. 2007). In non-hematopoietic cells, CARMA3-BCL10-MALT1 complex is required for G protein-coupled receptors (GPCR)-induced NF-κB activation, as CARMA3- and BCL10-deficient cells are defective for lysophosphatidic acid (LPA)-mediated NF-κB activation (Grabiner et al. 2007). Similar observations suggesting a requirement of CBM complex in GPCR-induced NF-κB activation have been reported independently from other groups (Klemm et al. 2007, Wang et al. 2007,

McAllister-Lucas et al. 2010). In addition to GPCR, recently Jiang et al. showed that CARMA3 is required for epidermal growth factor receptor (EGFR)-mediated NF-κB activation, as CARMA3-deficient cells are defective to epidermal growth factor (EGF)-induced NF-κB activation (Jiang et al. 2011). Mechanistically, it is believed that CARMA3-BCL10-MALT1 complex in non-hematopoietic cells functions similar to CARMA1-BCL10-MALT1 in lymphocytes in a PKC dependent manner, as both GPCR and EGFR activation cause the activation of PKC. Yet there is no evidence suggesting that PKC directly activates CARMA3.

1.4 The proteolytic function of MALT1

1.4.1 MALT1 cleaves BCL10 to regulate T-cell adhesion

Although MALT1 is thought to operate as a protease-like caspase based on their sequence similarity, it was not until 2008 that MALT1 has been demonstrated to contain protease activity in mammalian cells for the first time. Initially, Lucas et al. showed that a mutation on a critical cystine residue (C453→A) on the putative catalytic domain only resulted a modest reduction in NF-κB activity, indicating that the protease activity of MALT1 is dispensable for the NF-κB activation, which is thought to be the major function of MALT1 at that time (Lucas et al. 2001). Two milestone studies published in 2008 by Rebeaud et al. and Coornaert et al. clearly demonstrated that MALT1 contains protease activity and the protease activity contributes to T cell activation and NF-κB activation (Coornaert et al. 2008, Rebeaud et al. 2008). Rebeaud et al. found that MALT1 cleaves its binding partner BCL10 at the Arg288 site upon stimulation with either PMA/Ionomycin or CD3/CD28 in a number of

lymphoma and leukemia cell lines (Rebeaud et al. 2008). In addition, the authors proved a protease activity of MALT1 *in vitro* by incubating of purified MALT1 with a Bcl-10-derived fluorogenic peptide substrate (Leu-Arg-Ser-Arg) mimicking the Bcl-10 cleavage site (Rebeaud et al. 2008). The authors further demonstrated that MALT1 dependent BCL10 cleavage regulates adhesion of T cells to fibronectin but not NF-κB activation (Rebeaud et al. 2008). However, the mechanism by which the processed BCL10 regulates cell adhesion remains unclear.

1.4.2 MALT1 cleaves A20 to regulate NF-κB activation

Coornaert et al. showed that TCR stimulation results in cleavage of A20 (also known as *Tnfaip3*) at the Arg228 site in a MALT1 dependent manner in Jurkat cells and primary human T cells (Coornaert et al. 2008). A20 is a zinc finger protein which is involved in negatively regulates inflammatory genes by down regulating NF-κB activity in response to a variety of stimuli in immune cells (Lee et al. 2000). Through proteolytic cleavage, MALT1 enhances NF-κB activity by generating two inactive A20 fragments in the Jurkat cells (Coornaert et al. 2008). By generating mutations on A20 and performing *in vitro* cleavage assay, the authors further demonstrated that MALT1 is an arginine specific protease (Rebeaud et al. 2008). This is consistent with the cleavage site on both human and mouse BCL10 (Rebeaud et al. 2008). In fact, arginine specific cleavage applies to all subsequently identified substrates for MALT1.

1.4.3 MALT1 cleaves CYLD to regulate JNK activation

Similar to A20, the ubiquitin-specific-processing protease CYLD is also involved in negative regulation of a variety of signaling pathways, including NF-κB (Kovalenko et al. 2003, Trompouki et al. 2003), Jun amino-terminal kinases (JNK) (Reiley et al. 2007), and transforming growth factor-β (TGF-β) signaling (Lim et al. 2012). Staal et al. showed that CYLD is cleaved into two fragments upon TCR stimulation in Jurkat cells, and upon BCR stimulation in SSK41 and Raji cells, respectively (Staal et al. 2011). In addition, MALT1 proteolytic activity is required for such cleavage, as demonstrated by treating cells with small peptide inhibitor z-VRPR-Fmk, which can specifically block the proteolytic activity of MALT1 (Rebeaud et al. 2008, Staal et al. 2011). Consistent with previous notion that MALT1 is a arginine specific protease, MALT1 processes both human and mouse CYLD at the Arg324 site, as a (R324→A) CYLD mutant is not cleavable by MALT1 (Staal et al. 2011). Interestingly, MALT1 dependent CYLD cleavage regulates JNK pathways instead of NF-κB. Steal et al. shows that MALT1 enhances JNK activation by cleaving and inactivating CYLD (Staal et al. 2011). Therefore, MALT1 proteolytic activity is a positive regulator in multiple pathways downstream of TCR signaling.

1.4.4 MALT1 cleaves RelB to regulate NF-κB activation

RelB is a member of the NF-κB transcription factors. It has been suggested that RelB plays a negative roles in T cell activation and inflammation, as RelB-deficient mice display a multiorgan inflammation phenotype (Weih et al. 1995). Hailfinger et al. showed that MALT1 cleaves RelB at Arg85 upon either PMA/Ionomycin stimulation or CD3/CD28 stimulation in Jurkat cells and murine primary T cells (Hailfinger et al. 2011). RelB cleavage induces its

proteasomal degradation and enhances the DNA binding activity of RelA or c-Rel containing NF-κB heterodimers (Hailfinger et al. 2011). Hailfinger et al. shows that the RelB mutant (Arg85→G) is resistant to MALT1 dependent cleavage and inhibit the expression of NF-κB target gene *Il2* (Hailfinger et al. 2011). The authors further demonstrated that RelB is constantly processed by MALT1 in activated B-cell (ABC) type of diffuse large B cell lymphoma (DLBCL) cells, in which MALT1 is constitutively activated (Ferch et al. 2009, Hailfinger et al. 2009). RelB processing optimizes the proliferation and survival potential of ABC-DLBCL as RelB overexpression impairs the survival of ABC-DLBCL cell lines (Hailfinger et al. 2011). An independent study also suggests that there is excessively higher level of RelB in MALT1-deficient T cells compared with wild-type controls (Brustle et al. 2012). In addition, the physiological role of MALT1 dependent RelB cleavage is also linked to the differentiation of functional Th17 cells, although the exact mechanism is not clear yet (Brustle et al. 2012). Together, these studies demonstrate that MALT1 regulates NF-κB activation by proteolytically process negative regulator RelB.

1.4.5 MALT1 cleaves HOIL-1 to regulate NF-κB activation

The linear ubiquitin assembly complex (LUBAC) includes HOIL-1 (heme-oxidized IRP2 ubiquitin ligase 1), HOIP (HOIL-1-interacting protein) and SHARPIN (SHANK associated RH domain interactor) (Kirisako et al. 2006, Gerlach et al. 2011). LUBAC is the only E3 ligase complex that generates a linear form of ubiquitination, in which C-terminal glycine is directly linked to the N-terminal methionine of another ubiquitin (Kirisako et al. 2006). The linear form of ubiquitination plays a critical role in NEMO ubiquitination and

subsequent NF-κB activation (Tokunaga et al. 2009). Using a 10-plex tandem mass tag TAILS N-terminal peptide proteomic approaches, Klein et al. attempted to systematically identify substrates of MALT1 upon T cell activation (Klein et al. 2015). The authors demonstrate that MATL1 cleaves and inactivates HOIL-1 in the LUBAC complex to reduce linear ubiquitination and NF-κB activation (Klein et al. 2015). MALT1 processes HOIL-1 at the site Gly166 upon PMA/Ionomycin stimulation *in vitro* and *in vivo* (Klein et al. 2015). This study provides first evidence that MALT1 is involved in negative regulation of NF-κB activation (Klein et al. 2015).

1.4.6 MALT1 cleaves Regnase-1 to regulate mRNA stability

Regnase-1, also known as zinc finger CCCH-type containing 12A (*Zc3h12a*) or MCP-induced protein 1 (MCPIP1), is a novel ribonuclease (RNase) that is involving in regulating cytosine stabilities during TLR activation (Matsushita et al. 2009). Generation of *Zc3h12a* deficient mice resulted an embryonic lethality at day 12 with an early onset of fetal autoimmune disease, suggesting that regnase-1 plays a critical role in negative regulation of inflammatory responses (Matsushita et al. 2009). Mice with a conditional depletion of regnase-1 in CD4-positive T cells (CD4-Cre; $Reg1^{fl/fl}$) show autoimmune inflammatory disease, suggesting regnase-1 also plays a negative role in regulation of T cell activation (Uehata et al. 2013). Indeed, upon TCR stimulation, a set of cytokine RNA is stabilized in regnase-1-deficient T cells compared with wild-type controls, suggesting regnase-1 negatively regulates RNA stability in response to stimulation in T cells (Uehata et al. 2013). Interestingly, TCR stimulation leads to MALT1 dependent cleavage regnase-1 at the site

Arg111 (Uehata et al. 2013). The cleavage of regnase-1 leads to its inactivation, and thereby stabilizing its target RNAs, including c-Rel, Ox40, Il2 and IκB*α* (Uehata et al. 2013). In summary, Uehata et al. provides the first evidence suggesting that MALT1 protease activity controls T cell activation by regulating inflammatory genes at the RNA level (Uehata et al. 2013).

1.4.7 MALT1 cleaves Roquin to regulate mRNA stability

Roquin is a CCCH-type zinc finger protein that regulates target mRNA level via binding to the constitutive decay element (CDE) region in the 3' UTR to trigger mRNA decay (Leppek et al. 2013). Roquin regulates a variety of coding mRNA, including *Icos* mRNA, *Tnfrsf4* mRNA, and *Tnf* mRNA (Yu et al. 2007, Glasmacher et al. 2010, Leppek et al. 2013, Vogel et al. 2013). Jeltsch et al. shows an inflammatory phenotypes in lungs with enhanced T helper cell 17 (Th17) differentiation in mice lacking roquin expression in CD4+ T cells (Jeltsch et al. 2014). Furthermore, the authors shows that both roquin-1 and roquin-2 were cleaved by MALT1 upon either PMA/Ionomycin or CD3/CD28 stimulation, indicating Roquin is processed upon T cell activation (Jeltsch et al. 2014). MALT1 preferentially cleaves both human and mouse roquin-1 at the site Arg510 and roquin-2 at the site Arg509. The cleavage of roquin-1 and roquin-2 leads to an impaired activity in destabilizing targeting mRNA involved in Th17 differentiation, including IL-6, ICOS, c-Rel, IRF4, IκBNS and IκBζ (Jeltsch et al. 2014). Thus, MALT1 promotes Th17 differentiation by negatively regulating roquin-1 and roquin-2 through proteolytic inactivation.

1.4.8 Phenotypes of MALT1 deficient and mutant mice

In 2003, two group independently generated MALT1 deficient transgenic mice by disruption *Malt1* exons through homologous recombination in embryonic stem cells (Ruefli-Brasse et al. 2003, Ruland et al. 2003). Ruefli-Brasse et al. showed that MALT1 is essential to both T cell and B cell activation through regulating NF-κB activation (Ruefli-Brasse et al. 2003). However, Ruland et al. demonstrated that while MALT1 is absolutely required for T cell activation and NF-κB activation in response to CD3/CD28 stimulation in T cells, MALT1 is not required for B cell activation nor NF-κB activation in B cells (Ruland et al. 2003). The underlying reason for this discrepancy remains to be determined.

In 2014, three independent groups reported the generation of protease defective mutant form of MALT1 knock-in mice (Gewies et al. 2014, Jaworski et al. 2014, Bornancin et al. 2015). Consistent with all previous findings, the activation of T cells from MALT1 protease deficient mutant mice are significantly impaired compared with controls (Gewies et al. 2014, Jaworski et al. 2014, Bornancin et al. 2015). This observation confirmed an essential role of MALT1 protease activity in T cell activation *in vivo*. Surprisingly, all three groups consistently show that MALT1 protease deficient knock-in mice display severe autoimmune phenotypes with multiorgan inflammation (Gewies et al. 2014, Jaworski et al. 2014, Bornancin et al. 2015). Jaworski et al. and Bornancin et al. suggest that this autoimmune phenotypes are linked to an impairment of regulatory T helper cell (Treg) development in MALT1 protease deficient knock-in mice, as reconstitution of wild-type Treg partially rescued the autoimmune phenotypes (Jaworski et al. 2014, Bornancin et al. 2015). However, the molecular mechanism by which MALT1 regulates the development of natural

Treg (nTreg) remains to be determined. On the other hand, Gweies et al. suggest that the autoimmune-like diseases in MALT1 protease-deficient mice are linked to an impairment of regnase-1 and roquin dependent down-regulation of interferon γ (INFγ), which was found excessively higher in MALT1 protease-deficient mutant T cells compared with controls (Gewies et al. 2014). In summary, the underlying causes for the autoimmune phenotypes in MALT1 protease deficient mice could be complicated and remain to be further investigated.

1.4.9 MALT1 as a therapeutic target in ABC-DLBCL

The protease activity of MALT1 plays a critical role in lymphocytes activation by cleaving a number of substrates (Figure 3). Consequently, the protease activity also contributes to certain types of lymphoma. Lymphoma can be categorized into Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL). Diffuse large B cell lymphoma (DLBCL) is the most common type of NHL. According to the gene expression profiles, DLBCL can be classified into two subgroups, activated B cell-like (ABC) type of DLBCL and germinal center B cell-like (GCB) type of DLBCL (Alizadeh et al. 2000). In 2006, through a shRNA based screening approach, Ngo et al. identifies that CARD11 and MALT1 shRNAs are selective toxic to ABC type of DLBCL, suggesting a requirement of CARMA1 and MALT1 for ABC-DLBCL survival (Ngo et al. 2006). Subsequently, Hailfinger et al. show that the protease activity of MALT1 is constitutively activated in ABC-DLBCL but not GCB-DLBCL cell lines (Hailfinger et al. 2009). Based on this finding, the authors further treat ACB-DLBCL with MALT1 peptide inhibitor, z-VRPR-Fmk, which specifically blocks the protease activity *in vitro*. Treatment with z-VRPR-Fmk significantly inhibits NF-κB

activation and induced cell apoptosis specifically in ABC-DLBCL (Hailfinger et al. 2009). Together, Halfinger et al. has provided the first evidence suggesting MALT1 protease activity is a promising drug target to treat ABC-DLBCL. In addition, Ferch et al. verifies the finding by Halfinger et al. and suggests that inhibition of MALT1 protease activity is selectively toxic to ABC-DLBCL (Ferch et al. 2009). Although inhibition MALT1 with v-VRPR-Fmk induces cell apoptosis in ABC-DLBCL, the efficacy of small peptide inhibition is relatively low. In 2012, two groups independently identify small molecules inhibitors, MI-2 and derivatives of active phenothiazines, which work specifically to inhibit the protease activity of MALT1 *in vitro* and *in vivo*. Both inhibitors work potently in inhibiting NF-κB activation and induce cell apoptosis in ABC-DLBCL (Fontan et al. 2012, Nagel et al. 2012). These studies provide opportunities to treat ABC-DLBCL by using MALT1 inhibitors.

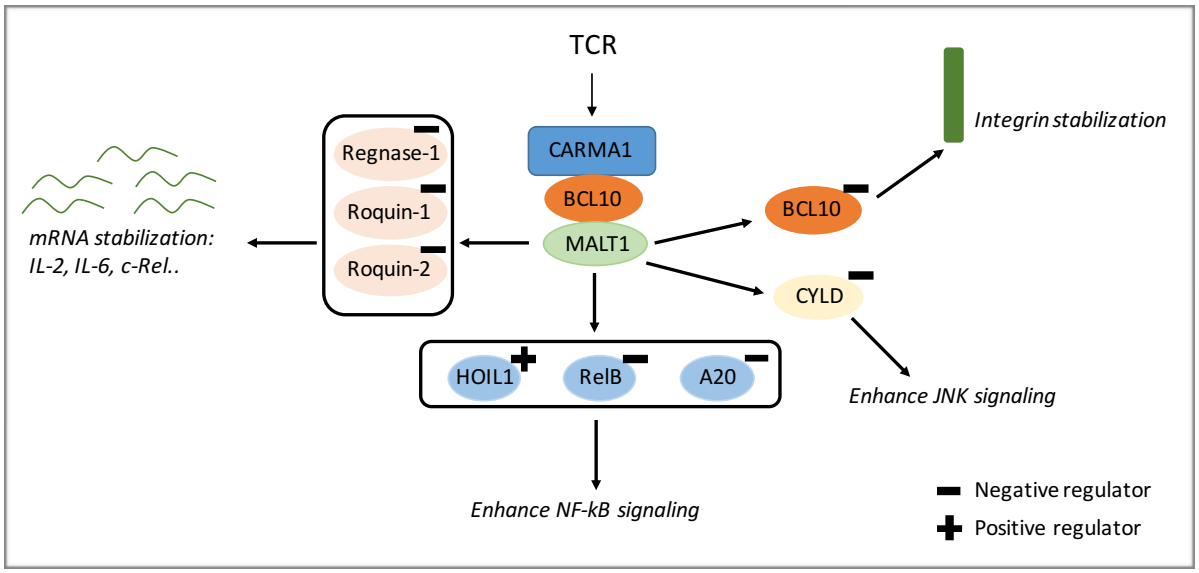

Figure 3. The substrates of MALT1.

MALT1 cleaves a number of substrates involving several biological pathways. MALT1 cleaves BCL10 to regulate the stability of intergrin and T cell adhesion. MALT1 cleaves and inactivates CYLD to upregulate JNK signaling. MALT1 also cleaves HOIL1 (positive regulator of NF-κB pathway), RelB (negative regulator of NF-κB pathway) and A20 (negative regulator of NF-κB pathway) to enhance NF-κB activation. MALT1 cleaves ribonuclease Regnase-1, Roquin-1 and Roquin-2 to enhance the stability of many cytokines at the RNA level to optimize lymphocytes activation and inflammation response.

CHAPTER 2: THE ROLE OF MALT1 IN EGFR-INDUCED NF-κB ACTIVATION AND EGFR-ASSOCIATED LUNG CANCER PROGRESSION

This chapter is based upon " Deng Pan, Changying Jiang, Zhongliang Ma, Marzenna Blonska, M. James You and Xin Lin. MALT1 is required for EGFR-induced NF-κB activation and contributes to EGFR-driven lung cancer progression. Oncogene advance online publication 18 May 2015; doi: 10.1038/onc.2015.146"

2.1 Introduction

2.1.1 MALT1 is a therapeutic target in lymphoma

MALT1 is an upstream signaling component of NF-κB with high therapeutic potential. MALT1 has been shown to be a key mediator in NF-κB activation in lymphocytes, and is required for lymphocyte activation and survival through TCR and BCR signaling (Thome 2008). Activation of these receptors leads to the activation of protein kinase C, which phosphorylates the Caspase recruitment domain and membrane-associated guanylate kinase-like domain (CARMA) family proteins such as CARMA1 in lymphocytes (Sun et al. 2000, Wang et al. 2002). Phosphorylation of the CARMA protein triggers a conformational change and further recruits MALT1 and B-cell lymphoma protein 10 (BCL10), resulting in the assembly of the CARMA–BCL10–MALT1 (CBM) complex (Matsumoto et al. 2005, Sommer et al. 2005). The CBM complex activates the IκB kinase to trigger NF-κB activation (Blonska and Lin 2011). MALT1 is considered a critical component in constitutive NF-κB activation in certain types of lymphoma. In MALT lymphoma-associated with the genetic translocation t(11;18)(q21;21), a cIAP2 (cellular inhibitor of apoptosis 2)-MALT1 fusion protein gives constitutive NF-κB signals (Akagi et al. 1999, Dierlamm et al. 1999). In activated B-cell-like (ABC) diffuse large B-cell lymphoma (DLBCL), MALT1 also has a tumor-promotive role by bridging the constitutive BCR signaling to a dysregulated NF-κB activity (Ngo et al. 2006).

The therapeutic value of MALT1 is associated with the protein's caspase-like domain, which contains an arginine-specific protease activity. The protease activity of MALT1 facilitates optimal NF-κB and AP-1 activation by cleaving negative regulators, such as A20,

CYLD and RelB (Coornaert et al. 2008, Hailfinger et al. 2011, Staal et al. 2011). In MALT1 lymphoma, the protease activity of the cIAP2–MALT1 fusion protein cleaves and stabilizes NF-κB-inducing kinase (NIK), resulting in a constitutive NF-κB activity, enhanced adhesion and resistance to apoptosis (Rosebeck et al. 2011). In addition, several independent studies of therapeutic applications of MALT1 protease inhibitors in ABC-DLBCL indicated that MALT1 inhibitors are selectively toxic to this type of lymphoma (Ferch et al. 2009, Hailfinger et al. 2009, Nagel et al. 2012). These results suggest that MALT1 inhibitors can potentially serve as therapeutic reagents for certain types of lymphoma. However, the tumor-promotive role of MALT1 has been confirmed only for the lymphoid system. As NF-κB contributes to tumor malignancy in a wide range of cell types, the oncogenic role and therapeutic potential of MALT1 need to be investigated in a nonhematopoietic system, such as solid tumors of epithelial origin. To date, no study has demonstrated a functional role of MALT1 in solid-tumor progression.

2.1.2 NF-κB signaling in EGFR associated-cancer

One of the most frequently mutated and overexpressed genes in solid tumors is the epidermal growth factor receptor (EGFR). EGFR overexpression and gain-of-function mutations are observed in nearly 30% of solid tumors, such as breast cancer, head-and-neck cancer and non-small-cell lung cancer (NSCLC) (Sharma et al. 2007). EGFR-mediated signaling contributes to many important malignant properties of tumors, such as cell growth, proliferation and metabolism. Recently, several groups including ours showed that in addition to its role in the phosphatidylinositide 3-kinase/Akt and MAPK/ERK (mitogen-

activated protein kinase–extracellular signal-regulated kinase) pathways, EGFR-induced NF-κB activation has an essential role in malignant properties such as proliferation, survival, migration and metabolism (Jiang et al. 2011, Yang et al. 2012, Pan and Lin 2013). However, the exact molecular mechanism by which EGFR activates NF-κB remains unclear. In addition, a physiological role of NF-κB signaling in EGFR-associated tumor has not been demonstrated so far.

2.1.3 Specific aims and hypothesis

The goal of this study aims to investigate the roles of MALT1 and its protease activity in solid tumor. Given CARMA3 is required for EGFR-induced NF-κB activation and MALT1 is an inducible binding partner with CARMA1 in lymphocytes (Che et al. 2004, Jiang et al. 2011), we hypothesize that MALT1 is required for EGFR-induced NF-κB activation and involved in EGFR-associated cancer progression. To address this hypothesis, we propose to examine the followings. First, we would like to investigate whether MALT1 mediates EGFR-induced NF-κB activation in human cancer cell lines. Second, we would like to examine whether the protease activity of MALT1 is required for EGFR-induced NF-κB activation in human cancer cell lines. Third, we would like to determine whether MALT1 contributes to EGFR-associated malignant phenotypes, such as proliferation, survival and migration. Finally, we would like to establish a mouse model in which we will be able to investigate a role of MALT1 in EGFR-associated lung cancer progression *in vivo*.

2.2 Materials and methods

2.2.1 Antibodies and reagents

Phosphorylation-specific antibodies to ERK1/2 (9101) and IκBα (9246) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phosphorylated EGFR (sc-12351), IκBα (sc-371), lamin B (sc-6216), ERK (sc-154), IKKγ (FL-419) and actin (sc-8432) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Monoclonal antibodies against the C terminus of MALT1 were generated in the Genentech (South San Francisco, CA, USA) central production facility. DNA-oligo probes for NF-κB (E3291) and Oct-1 (E3241) were purchased from Promega (Madison, WI, USA). Recombinant human EGF was purchased from Sigma-Aldrich (St Louis, MO, USA). PMA (16561-29-8) and ionomycin (56092-82-1) were purchased from Fisher Scientific (Pittsburgh, PA, USA). IL-6-neutralizing antibodies were purchased from Abcam, Cambridge, MA, USA (ab6672) and used at 1:400 dilution. TNFα was purchased from Thermo Fisher Scientific (Rockford, IL, USA). MALT1 protease inhibitor z-VRPR-Fmk was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA).

2.2.2 Cell cultures

Human A431 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). HCC827 cells were cultured with RPMI medium containing 10% FBS. Primary MEFs were isolated at E13.5 and cultured with DMEM–10% FBS. All cells were maintained at 37 °C with 5% CO_2.

2.2.3 Immunoblotting (IB)

The procedure for immunoblotting has been described elsewhere (Grabiner et al. 2007). Briefly, one million of cells were lysed in 100μl of lysis buffer (150 mM NaCl, 50 mM HEPES at pH 7.4, 1 mM EDTA, 1% NP-40, protease inhibitors) for 10 minutes. The cell lysates will be purified by spinning 13,000 r.p.m. for 10 minutes and resulting supernatants will be saved for western blot analysis. 10-12μl of the resulting supernatant were loaded to 10% of SDS-PAGE and probed with antibodies.

2.2.4 Immunoprecipiation assay

Generally five million of cells were lysed in 300μl of lysis buffer (150 mM NaCl, 50 mM HEPES at pH 7.4, 1 mM EDTA, 1% NP-40, protease inhibitors) for 10 minutes. The cell lysates will be purified by spinning 13,000 r.p.m. for 10 minutes and resulting supernatants will be saved for Immunoprecipiation assay. 30μl of the protein lysis will be saved for western blot analysis as input. The rest lysis will be incubated together with indicated antibodies and protein A or protein G agarose beads for overnight at 4 degree. After incubation, the beads will be washed three times with lysis buffer and will be suspended in 30μl of lysis buffer containing SDS for western blot analysis.

2.2.5 Electrophoretic mobility shift assay (EMSA)

For the electrophoretic mobility shift assay, two million cells were starved overnight in DMEM containing 0.5% serum and stimulated with reagents, and nuclear extracts were

isolated. Nuclear extracts (5 μg) were incubated with 1×10^5 c.p.m. of ^{32}P-labeled probes at room temperature for 15 min. The samples were separated on a native Tris-borate-EDTA polyacrylamide gel and analyzed by autoradiography.

2.2.6 Gene silencing and reconstitution

Lentivirus vectors were generated by cotransfection of HEK293T cells with plasmids encoding short hairpin RNA (target sequence for MALT1: 5′-CCTCACTACCAGTGGTTCAAA-3′; TRAF6: 5'-GCCACGGGAAATATGTAATATCT-3'): pCMV-VSV-G (Addgene #8454) and pCMV-dR8.2 (Addgene #8455). The complementary DNA of human MALT1 was amplified by using pcDNA3-MALT1 as a template and cloned into the pBabe-hygro (Addgene #1765) vector. The C464A mutation was introduced via site-directed mutagenesis. To reconstitute MALT1 in A431 cells with a MALT1 knockdown, six synonymous mutations in the short hairpin RNA-targeting sequence were introduced by PCR (5′-CCTCACTACCAGTGGTTCAAA-3′ to 5′-CCGCATTATCAATGGTTTAAG-3′), so that the short hairpin RNA targeted only endogenous MALT1. To produce retrovirus vectors for infecting MEF, pBabe-human MALT1 was cotransfected with pCL-Eco (Addgene #12371).

2.2.7 MTT Assay

HCC827 cells were seeded in wells of a 96-well plate in 100μl of complete DMEM media.

At the indicated time, 10μl of MTT reagent (5mg/ml) was added to the cells. After 3 hours of

incubation, the media containing MTT was removed and metabolized MTT will be dissolved in 100μl of DMSO. The absorbance was measured at 570nm and was recorded accordingly.

2.2.8 Colony formation assay

Cells were mixed with agarose to a final concentration of 0.6% in complete medium, with or without EGF (1 ng/ml). Three weeks after culturing, the numbers and sizes of colonies were determined by inverted microscopy. Nine to ten fields were randomly selected and the size of each colony visualized was determined.

2.2.9 Migration Assay

A431 and HCC827 cells are subjected to transwell migration assay. 1×10^5 cells were suspended in 200μl of serum-free DMEM and seeded to BD Faclon cell culture inserts. The inserts will be placed in a well on a 6-well plate filled with 500μl of complete DMEM to let the cell migrate to the bottom part of insert. 20 hours after seeding, the insert will be washed with PBS for twice and will be fixed with 4% paraformaldehyde for 30 minutes. After fixing, the top layer of inserts (unmigrated cells) will be removed and the inserts will be stained with crystal violet cell staining solution (0.05% weight/volume) for 5 minutes. The resulting stained inserts with be photographed and migrated cell will be quantified by reverse microscopy.

2.2.10 Wound healing assay

Confluent A431 cells were cultured in serum-free DMEM, and a uniform wound was made with a p200 pipette tip. Wounded monolayer cells were washed two or three times to remove detached cells. The initial size of the wound was determined by inverted microscopy immediately after the wash. After 16–20 h of incubation in serum-free medium, the size of the wound was analyzed again. Wound closure was calculated as the percentage of the initial wound area remaining.

2.2.11 Lung metastasis model

To establish a lung metastasis model, three million A431 cells were washed with phosphate-buffered saline, resuspended in 300 μl of serum-free DMEM, and intravenously injected into the tail veins of SCID mice. Three weeks after injection, mice were killed and the lungs were fixed by Bouin's solution (Sigma-Aldrich, #HT10132), so that a metastasis site could be visualized as a white spot on a yellow background. Metastasis sites on each lung lobe were counted. Student's t-test was used to determine statistical significance.

2.2.12 Mouse strains and models

To establish EGFR-induced lung cancer model, tetO-EGFRL858R mice,40 CCSP-rTTA mice41 and Malt1$^{-/-}$ mice (Ruefli-Brasse et al. 2003) were intercrossed to generate control mice (tetO-EGFRL858R; CCSP-rTTA; Malt1$^{+/-}$) and experimental mice (tetO-EGFR^{L858R}; CCSP-rTTA; Malt1$^{-/-}$). After weaning, mice were administrated with doxycycline (Alfa Aesar, Ward Hill, MA, USA) containing water (2 mg/ml) for 2 months. For activating K-ras-induced lung caner model, we crossed LSL-K-ras^{G12D} (Jackson et al. 2001); CCSP-Cre (Li et

al. 2008) mice and Malt1$^{-/-}$ mice to generate control mice (LSL-K-ras^{G12D}; CCSP-Cre; Malt1$^{+/-}$) and experimental mice (LSL-K-ras^{G12D}; CCSP-Cre; Malt1$^{-/-}$). These mouse strains were genotyped by PCR as previously described (Fisher et al. 2001, Jackson et al. 2001, Politi et al. 2006, Li et al. 2008). All animal experiments and procedures were conducted under the protocol and was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas MD Anderson Cancer Center.

2.2.13 Histology and Immunohistochemical staining

Mouse tissues were washed in PBS and fixed in 4% paraformaldehyde solution for overnight and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin by the histology core facility at the University of Texas MD Anderson Cancer Center and examined by a pathologist (MJY). For immunohistochemistry (IHC) staining, standard procedures were carried out according to the manual (DAKO, #K0673, USA). The stained sections were automatically processed by ACIS III system (DAKO, USA) and quantified based on 10–15 randomly chosen fields. Quantification of IHC staining was repeated three times by using at least three different mice per genotype. The following antibodies were used in IHC staining: P-S6 Ribosomal Protein (#4858, Cell Signaling), P-AKT (#3787, Cell Signaling), P-ERK1/2 (#4376, Cell Signaling), P-P65 (#3037, Cell Signaling), P-STAT3 (#9145, Cell Signaling) and STAT3 (#9139, Cell Signaling).

2.2.14 Real-time PCR

Total RNA was isolated using TRIzol RNA isolation reagent (Invitrogen, Grand Island, NY, USA) and reverse transcribed using SuperScriptIII (Invitrogen). Quantitative PCR was performed in triplicates using Power SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA).

2.2.15 Statistical analysis

GraphPad Prism software was used for all statistical analyses. The Student's t-test (two-tailed t-test) was used to evaluate the difference of two groups of data.

2.3 Results

2.3.1 MALT1 is required for EGF-induced NF-κB activation

Our previous results revealed that CARMA3, a MALT1-interacting protein, is involved in EGFR-mediated NF-κB signaling (Jiang et al. 2011). To test whether MALT1 is involved in EGF-induced NF-κB activation, we knocked down MALT1 expression in A431 cells in which EGFR is highly expressed and examined NF-κB activation by gel shift assay upon EGF stimulation. We starved control (shCtl) and MALT1-knockdown (shMALT1) A431 cells with 0.5% FBS containing DMEM overnight (~16 hours) and stimulated with either EGF (100 ng/ml) or PMA (50 ng/ml)/Ionomycin (100ng/ml) or TNFα (10ng/ml) as controls. We found that the suppression of MALT1 expression significantly impaired EGF- and PMA/ Ionomycin-induced NF-κB activation, respectively, but not tumor necrosis factor-α (TNFα)-induced NF-κB activation, indicating that MALT1 is specifically involved in mediating EGF-induced NF-κB activation (Figure 4A). To further confirm the role of MALT1 in EGF-induced NF-κB activation in primary cells, we prepared MALT1-heterozygous ($Malt1^{+/-}$) and -deficient ($Malt1^{-/-}$) primary mouse embryonic fibroblasts (MEFs). Because EGFR expression in primary MEFs is low, we used PMA (50ng/ml) /Ionomycin (100 ng/ml) to activate protein kinase C, a downstream component in EGFR signaling, which can induce NF-κB activation (Stewart and O'Brian 2005, Fan et al. 2009). Consistently, PMA/ Ionomycin-induced NF-κB was completely abolished in MALT1-deficient MEFs (Figure 4b). These data indicates that MALT1 is required for EGFR- and PKC- mediated NF-κB activation.

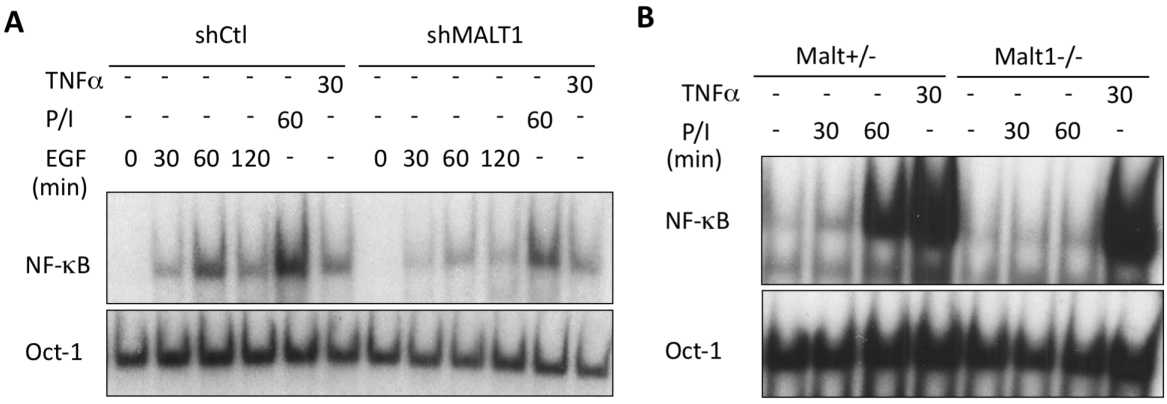

Figure 4. MALT1 is required for EGFR- and PKC-induced NF-κB activation.

(A) A431 cells with a MALT1 knockdown (shMALT1) and control cells (shCtl) were stimulated with EGF (100ng/ml), PMA and Ionomycin (50ng/ml; 100ng/ml) or TNF (10ng/ml) for indicated periods. NF-κB activation and Oct-1 (loading control) levels were determined by the gel shift assay. (B) MEFs from Malt1$^{+/-}$ and Malt1$^{-/-}$ embryos were isolated. Early-passage (P1) MEFs were stimulated with PMA and Ionomycin (50ng/ml; 100ng/ml) or TNF (10ng/ml) for indicated periods. NF-κB activation and Oct-1 levels were determined by the gel shift assay.

2.3.2 MALT1 is specifically required for EGF-induced NF-κB activation and does not affect other EGFR-activated pathways

To access whether MALT1 is also involved in other EGFR-mediated signaling transduction, such as PI3K/AKT/mTOR, MAPK/ERK and JNK pathways, we starved control (shCtl) and MALT1-silenced (shMALT1) A431 cells overnight and stimulated cells with 100ng/ml of human EGF. We lysed cells at 7.5, 15, 30 and 60 minutes after EGF stimulation and analyzed the activation of downstream pathways of EGFR. We found MALT1 suppression specifically abolished EGF-induced p65 nuclear localization (Figure 5A) upon EGF stimulation, but it had no impact on other pathways downstream of EGFR, as shown by the level of p-S6 Kinase, p-ERK and p-JNK (Figure 5B). These data collectively suggest that MALT1 is specifically involved in EGFR-mediated NF-κB activation.

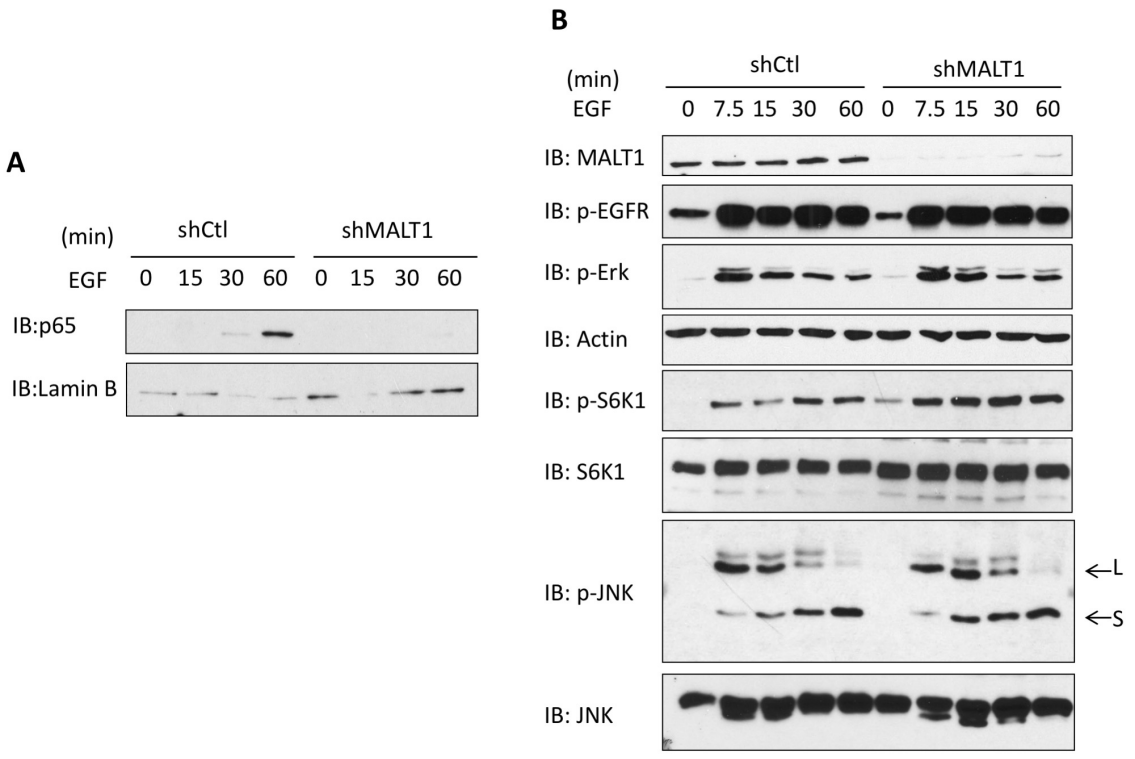

Figure 5. MALT1 is specifically required for EGFR-induced NF-κB activation but not for other EGFR-downstream pathways.

(A) A431 cells were starved with 0.5% FBS containing DMEM and stimulated with 100ng/ml of human EGF for indicated time points. Nuclear lysates from control (shCtl) and MALT1-silenced (shMALT1) cells were analyzed by immunoblotting using indicated antibodies. (B) Control (shCtl) and MALT1-silenced (shMALT1) A431 cells were stimulated with EGF (100ng/ml) for indicated periods. Cell lysates were analyzed by immunoblotting using indicated antibodies. L: Long form of JNK; S: Short form of JNK.

2.3.3 The protease activity of MALT1 is not required for EGF-induced NF-κB activation

It has been reported that MALT1 contains protease activity, which is required for optimal TCR- and BCR-induced NF-κB activity by inhibiting negative regulators in NF-κB pathway. We therefore sought to determine whether MALT1 protease activity contributes to EGF-induced NF-κB activation. To this end, we reconstituted MALT1-silenced cells with either wild-type MALT1 or protease-deficient mutant MALT1 ($MALT1^{C464A}$) in either MALT1-silenced A431 cells or MALT1-deficient MEFs (Figure 6A). We found there was a slight reduction of NF-κB in mutant MALT1 constituted MEFs compared with wild-type MALT1 reconstituted MEFs upon PMA/Ionomycin stimulation (Figure 6B), indicating MALT1 protease activity is involved in PKC mediated NF-κB activation. However, both the wild type and the protease-deficient MALT1 mutant rescued EGF-induced NF-κB in A431 cells (Figure 6C), suggesting the protease activity of MALT1 is largely dispensable for EGFR-induced NF-κB activation.

As an alternative approach, we examined EGF induced NF-κB activation in the presence or absence of MALT1 specific inhibitor (z-VRPR-Fmk). While MALT1 inhibitor completely blocked its protease activity as shown by cleaved BCL10 (Figure 7A), it does not affect NF-κB activation in response to EGF stimulation in A431 cells (Figure 7B). Taken together, these results demonstrate that MALT1 mainly functions as a scaffold protein and is selectively involved in the regulation of EGF-induced NF-κB activation.

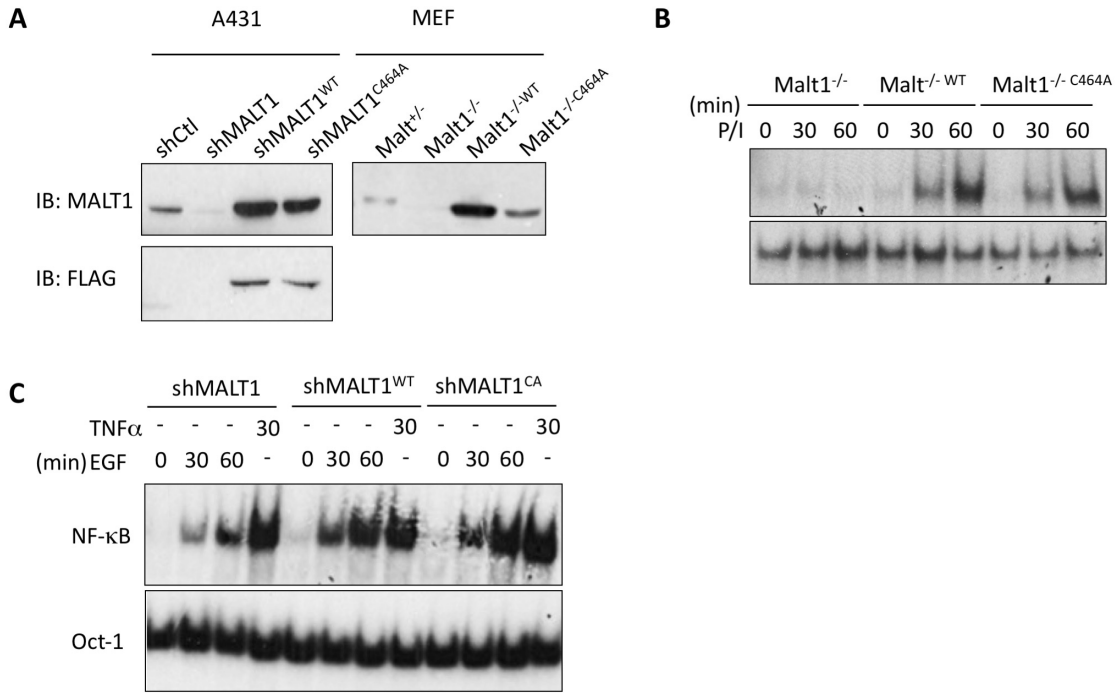

Figure 6. Protease-deficient and wild-type MALT1 reconstitution equally rescued EGFR- and PMA/Ionomycin-induced NF-κB activation.

(A) MALT1-silenced A431 cells and MALT1-deficient MEFs were reconstituted with wild-type MALT1 (shMALT1^{WT} or Malt1$^{-/-WT}$) or a protease-deficient C464A mutant (shMALT1^{C464A} or Malt1$^{-/-C464A}$). Lysates of these cells were analyzed by immunoblotting with anti-MALT1 or anti-Flag antibodies. (B) MALT1-deficient cells (Malt1$^{-/-}$), wild-type MALT1-reconstituted cells (Malt1$^{-/-WT}$) and protease-deficient mutant reconstituted cells (Malt1$^{-/-C464A}$) were stimulated with PMA and Ionomycin (50ng/ml; 100ng/ml) for indicated periods, respectively. Nuclear lysates were isolated and subjected to gel shift analysis for NF-κB activation. (C) A431 cells with a MALT1 knockdown (shMALT1), wild-type MALT1-reconstituted cells (shMALT1^{WT}) and protease-deficient mutant reconstituted cells (shMALT1^{C464A}) were stimulated with EGF (100ng/ml) or TNF (10ng/ml) for indicated

periods. Nuclear lysates were isolated and subjected to gel shift analysis for NF-κB activation.

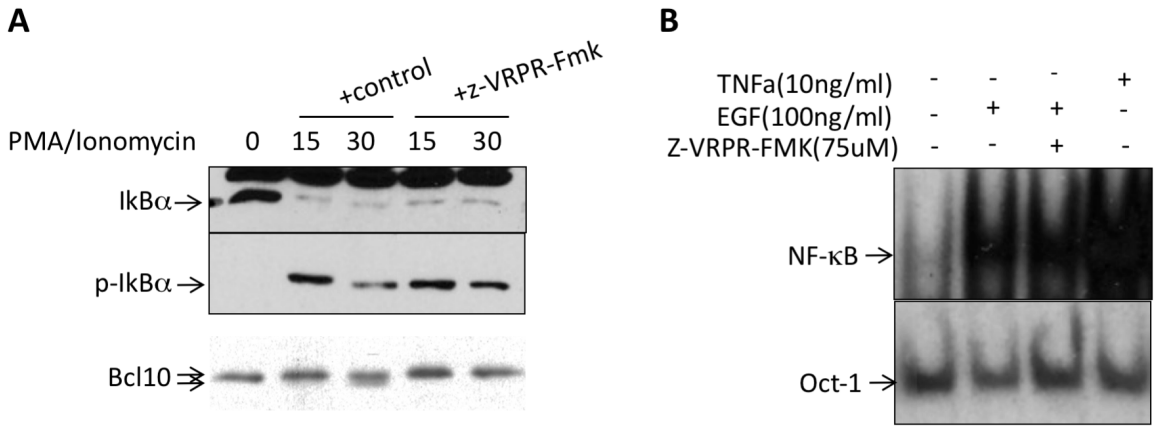

Figure 7. MALT1 protease inhibitor does not affect EGF-induced NF-κB activation.

(A) Jurkat cells were stimulated with PMA and ionomycin (20ng/ml; 100ng/ml) for the indicated periods with or without 75μM MALT1 inhibitor z-VRPR-Fmk. Whole cell lysates were analyzed by immunoblotting with the indicated antibodies. (B) A431 cells were stimulated with EGF (100ng/ml) or TNFα (10ng/ml) for 60 minutes, with or without 75 μM MALT1 inhibitor z-VRPR-Fmk. Nuclear lysates were isolated and subjected to gel shift analysis for NF-κB activation.

2.3.4 MALT1 functions as a scaffold protein to recruit TRAF6 to IKK complex

To further delineate the molecular mechanism by which MALT1 activates NF-κB in response to EGF stimulation, we examined MALT1-interacting protein upon EGF stimulation. Upon EGF stimulation, MALT1 was inducibly associated with TRAF6, an E3 ligase that activates IKK complex (Figure 8A). Consistently, we also observed a notable, but weak association between MALT1 and IKK at a later time point upon EGF stimulation (Figure 8A), suggesting that MALT1 bridges TRAF6 to IKK complex in response to EGFR activation. We further found that the association of MALT1 and TRAF6 was specifically induced by EGF but not TNF, indicating that MALT1 functions specifically downstream of EGFR (Figure 8B). In addition, MALT1 silencing abolished the association between IKKγ and TRAF6 upon EGF stimulation (Figure 8C), suggesting that MALT1 is functionally required for recruiting TRAF6 to IKK complex upon EGFR activation. To further confirm whether TRAF6 is functionally required for EGF-induced NF-κB activation, we knocked down TRAF6 expression by shRNA (Figure 8D) and examined NF-κB activation by gel shift assay. Consistently, TRAF6 inhibition significantly abolished EGF- and PMA/Ionomycin-induced NF-κB activation, respectively, suggesting that TRAF6 is also required for EGF-induced NF-κB activation (Figure 8E). Collectively, these results indicate that MALT1 serves as a scaffold protein recruiting TRAF6 to activate IKK complex in response to EGFR stimulation.

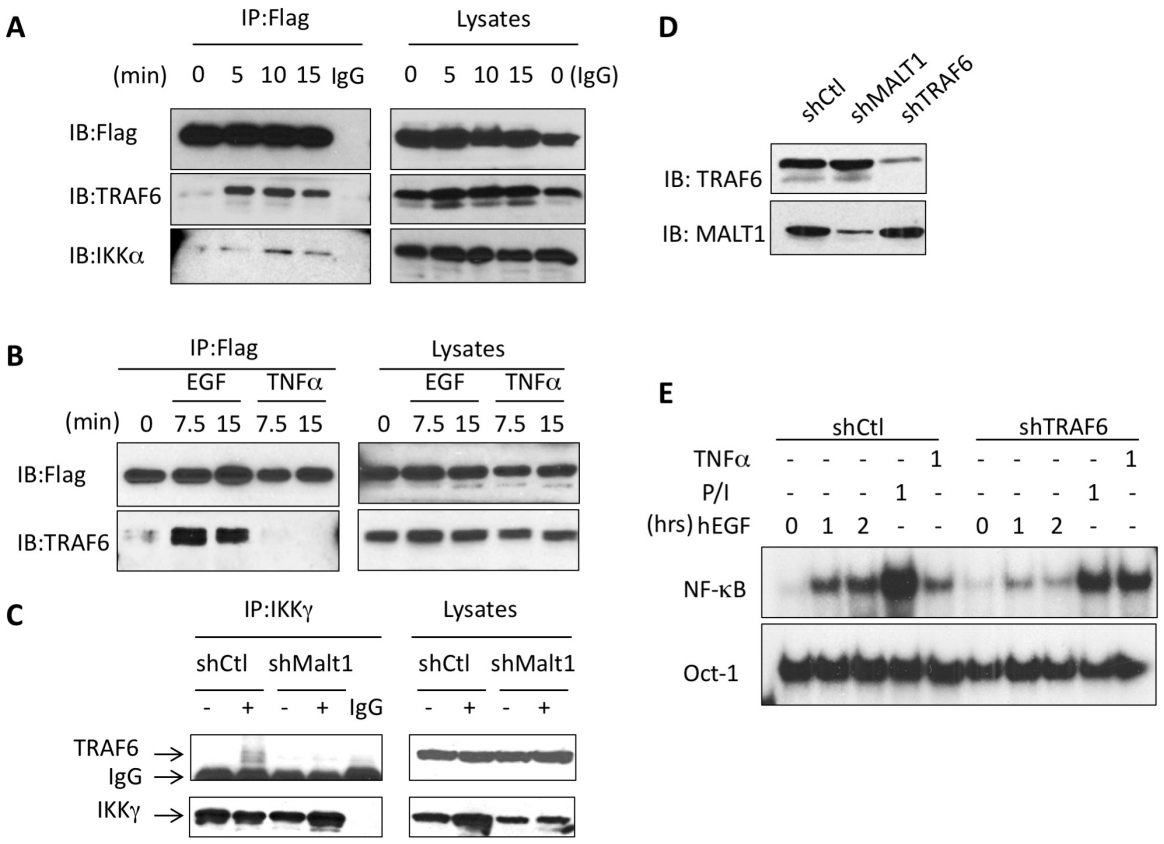

Figure 8. MALT1 functions as a scaffold protein to recruit TRAF6 to IKK complex.

(A) MALT1-reconstituted cells were stimulated with EGF (100ng/ml) for indicated time and MALT1-Flag was immunoprecipitated (IP) by anti-Flag conjugated beads. The IP samples and lysates were analyzed by immunoblotting using the indicated antibodies. (B) MALT1-reconstituted cells were stimulated with EGF (100ng/ml) and TNFα, respectively. MALT1-Flag was immunoprecipitated (IP) by anti-Flag conjugated beads. The IP samples and lysates were analyzed by immunoblotting using the indicated antibodies. (C) Control (shCtl) or MALT1-silenced (shMALT1) A431 cells were either unstimulated or stimulated with EGF (100ng/ml) for 15 minutes and IKKγ was immunoprecipitated (IP). The IP samples and lysates were analyzed by immunoblotting using the indicated antibodies. (D) The lysates of

A431 cells with control knockdown (shCtl), MALT1 knockdown (shMALT1) and TRAF6 knockdown (shTRAF6) were analyzed by immunoblotting using indicated antibodies. (E) A431 cells with a TRAF6 knockdown (shTRAF6) and control cells (shCtl) were stimulated with EGF (100ng/ml), PMA and Ionomycin (50ng/ml; 100ng/ml) or TNF (10ng/ml) for indicated time points, respectively. NF-κB activation and Oct-1 (loading control) levels were determined by the gel shift assay.

2.3.5 MALT1 contributes cell proliferation

Based on the observation that MATL1 is specifically required for EGF-induced NF-κB activation, we asked whether EGFR-MALT1-NF-κB signaling contributes to EGFR-associated tumor malignancy. To this end, we used both A431 and HCC 827 cell line, which is a lung cancer cell lines with homozygous constitutive activating EGFR mutation. First, we examined the role of MALT1 in cell proliferation. We observed a modest suppression of cell proliferation in MALT1-silenced HCC827 cells compared to controls as determined by MTT assay (Figure 9A). By using the soft agar colony formation assay to compare anchorage-independent proliferation, we found that MALT1 suppression significantly reduced the size of colonies formed in the agarose compared with controls in both A431 cells and HCC827 cells (Figure 9B-C), suggesting MALT1 plays a more profound role in 3D proliferation. In addition, while the size of colonies from control cells increased with the supplementation with EGF (2ng/ml), MALT1-silenced A431 cells did not increase with the supplementation with EGF (2ng/ml) (Figure 9 B-C). This result suggests that MALT1-silenced cells do not respond to EGF stimulation in terms of proliferation. Collectively, these data suggest that MALT1-dependent NF-κB activity is required for EGFR-dependent cell proliferation *in vitro*.

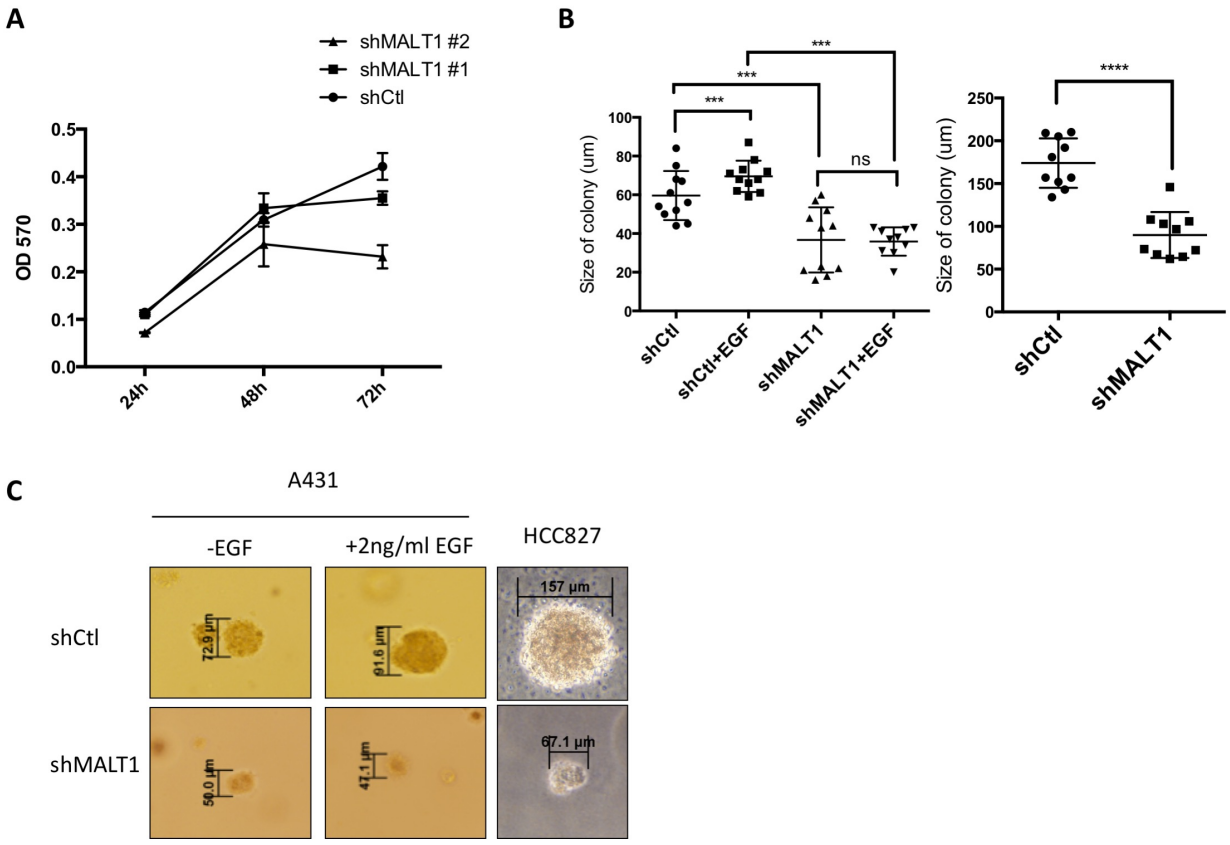

Figure 9. MALT1 contributes to EGFR-associated cell proliferation.

(A) HCC827 cells with MALT1 knockdown (shMALT1) and control cells (shCtl) were subjected to the MTT assay to determine their proliferation rates at 24, 48, 72 hours. OD, optical density. (B) A431 (left) and HCC827 (right) cells with a MALT1 knockdown (shMALT1) and control cells (shCtl) were subjected to soft agar colony formation analysis with or without EGF (2ng/ml). The sizes of cell colonies were calculated and analyzed. (C) Representative pictures showing the size of shCtl and shMalt1 colonies of A431 and HCC827 cells.

2.3.6 MALT1 contributes to cell migration and metastasis

It has been suggested that NF-κB singling is involved in cell invasiveness by regulating epithelia-mesenchymal transaction (EMT), a key process in tumor metastasis (Huber et al. 2004). Therefore, we performed *in vitro* and *in vivo* assays in A431 cells, and a human lung cancer cell line HCC827. First, we found MALT1-silenced cells showed a dramatical defect in cell migration and motility compared with control cells (shCtl) transwell migration assay (Figure 10A-B). Consistently, shMALT1 also showed a defect in wound healing compared with control (Figure 10C). To validate this finding *in vivo*, We compared cell metastasis in a lung metastasis model and found that the number of lung metastatic spots was significantly reduced in MALT1-silenced cells compared to controls (Figure 10D).

To access whether this effect is NF-κB-dependent, we treated cells with IKK inhibitor, and found that IKK inhibition similarly blocked cell migration *in vitro* (Figure 11). In addition, TRAF6-silenced cells showed a consistent defect of cell migration (Figure 11), which indicates MALT1-TRAF6-IKK signaling controls cell migration. In addition, we found that treating MALT1 inhibitor does not affect cell migration in either A431 or HCC827 cell lines (Figure 12), suggesting MALT1 protease activity does not contribute to tumor migration. Taken together, these data suggest that MALT1-mediated NF-κB activity regulates cell migration *in vitro* and metastasis *in vivo*.

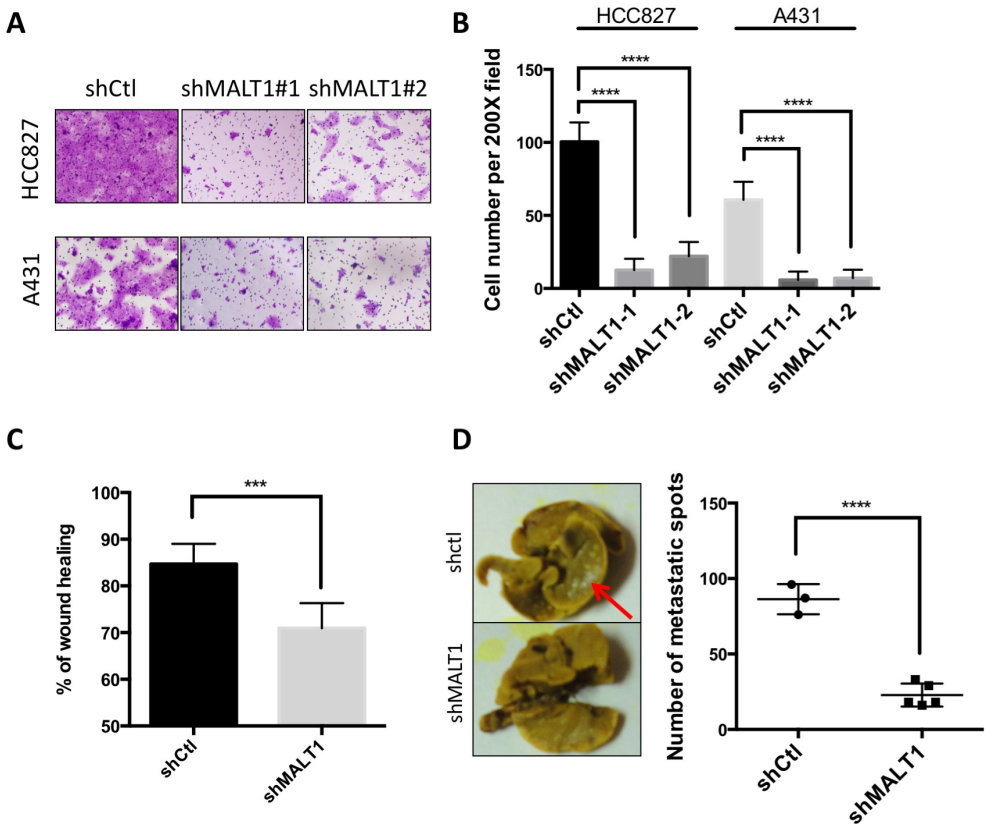

Figure 10. MALT1 is required for cell migration and metastasis.

(A) A431 and HCC827 cells with a MALT1 knockdown (shMALT1#1 and shMALT1#2) or a control knockdown (shCtl) were analyzed by transwell migration assays. Cells were fixed and stained 20 hours after seeding. (B) Cell numbers in five random fields were calculated and compared. (C) MALT1 silenced A431 cells and controls were analyzed by a wound-healing assay in the presence of EGF (1 ng/ml). The percentage of wound closure were calculated and analyzed. (D) MALT1 silenced A431 cells and controls were intravenously injected into SCID mice. Four weeks after injection, mice were sacrificed and the lungs were washed, fixed and stained with Bouin solution. Metastasis sites were visualized as white spots (left panel) and quantitated (right panel).

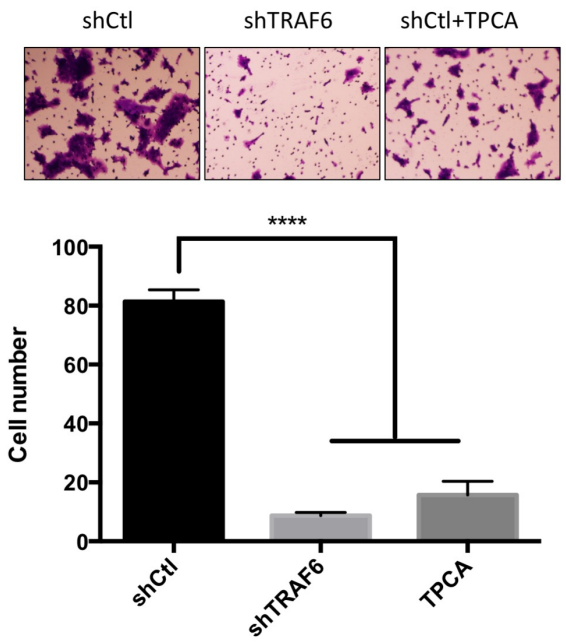

Figure 11. TRAF6 and IKK complex are required for cell migration.

Transwell migration assays were performed using shCtl A431 cells, shTRAF6 A431 cells and shCtl A431 cells with IKK inhibitor TPCA (1μM) treatment, respectively. **** $p<0.0001$.

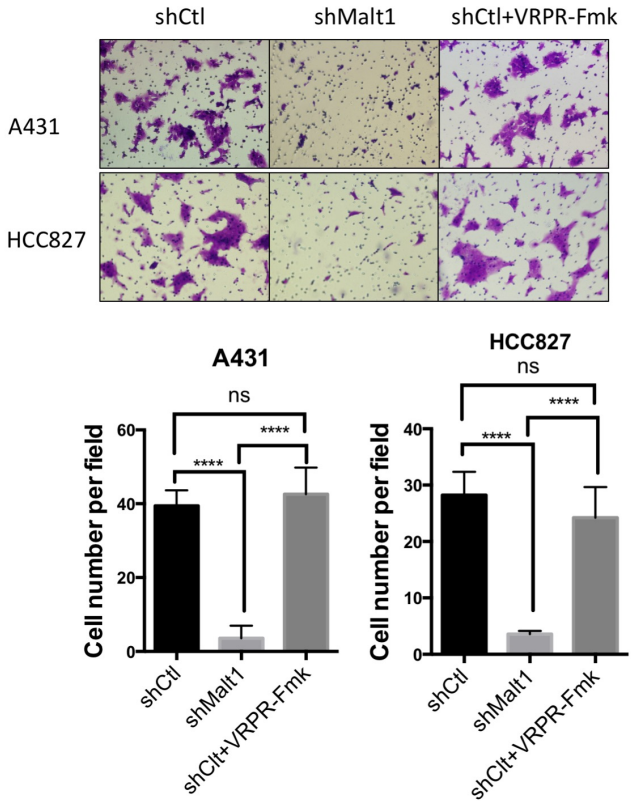

Figure 12. MALT1 protease activity is not required for cell migration.

Transwell migration assays were performed using shCtl (A431 and HCC827) cells, shMALT1 cells and shCtl cells treated with MALT1 inhibitor z-VRPR-Fmk (75μM), respectively. **** $p<0.0001$; ns, no significance.

2.3.7 MALT1 contributes to EGFR-associated lung adenocarcinoma progression in a mouse model

We sought to establish a physiological model to further investigate whether and how MALT1-mediated NF-κB activation affects EGFR-driven tumor progression *in vivo*. Since EGFR mutation and overexpression have been frequently found in non-small cell lung cancer (NSCLC), we crossed MALT1-knockout mice to a lung cancer mouse model (tetO-$EGFR^{L858R}$; CCSP-rtTA; $Malt1^{+/-}$ or tetO-$EGFR^{L858R}$; CCSP-rtTA; $Malt1^{-/-}$), in which mutant EGFR-driven lung cancer will be developed in the presence or absence of MALT1 expression (Figure 13A). After we successfully got $EGFR^{L858R}$/CCSP-rtTA/$Malt1^{+/-}$- and $EGFR^{L858R}$/CCSP-rtTA/$Malt1^{-/-}$-triple transgenic mice, we found both Malt1 heterozygous and knockout mice express human EGFR mRNA in a similar level, indicating that MALT1 does not affect the expression and induction of mutant EGFR transgene (Figure 13B). To access lung tumor burden, we compared the ratio of lung weight to body weight. We found that Malt1 knockout mice showed significant lower ratio of lung to body weight compared with heterozygous controls (0.0350 ± 0.001570, n=8 vs. 0.02771 ± 0.001672, n=7 p=0.0073) (Figure 13C), suggesting a reduced lung tumor burden in Malt1 knockout mice. We reviewed the pathological slides of lung tissue and found that mice with homozygous Malt1 knockout showed a reduced level of cellularity and the frequency of atypical cells with irregular nuclei in lungs in comparison with Malt1 heterozygous controls, suggesting a less malignant phenotype in Malt1 homozygous knockout mice (Figure 13D). In addition, Malt1 heterozygous lungs also had less alveolar space and more frequent focal glandular neoplasms compared with Malt1 knockout littermates (Figure 13D). Collectively, these data suggests

that Malt1 homozygous knockout mice had a reduced lung cancer burden and malignancy compared with its heterozygous controls.

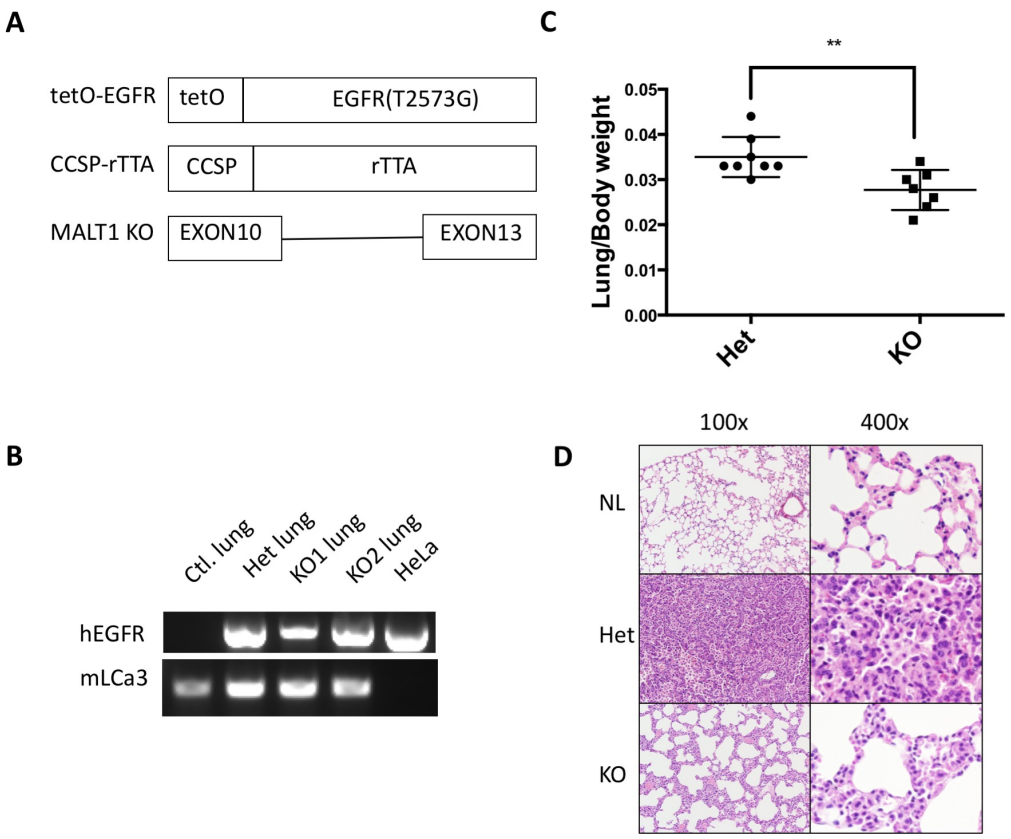

Figure 13. MALT1 contributes to EGFR-associated lung adenocarcinoma progression in a mouse model.

(A) Graphic presentation of the generation of tetO-EGFR^{L858R} ; CCSP-rtTA; MALT1$^{-/-}$ triple-transgenic mice. (B) Total RNA were extracted from the lungs of tetO-EGFR^{L858R}; CCSP-rtTA; Malt1$^{+/-}$ mice (Het), tetO-EGFR^{L858R}; CCSP-rtTA; Malt1$^{-/-}$ mice (KO), and HeLa cells, respectively. Reverse transcription PCR was performed using primers to amplify human EGFR and mouse LCa3, respectively. (C) The ratio of lung to body weight was compared between tetO-EGFR^{L858R}; CCSP-rtTA; MALT1$^{+/-}$ (Het) (n=8) and tetO-EGFR^{L858R} ; CCSP-rtTA; MALT1$^{-/-}$ (KO) mice (n=7). **, $p<0.01$. (D) H&E staining of the normal lung (NL), lung from tetO-EGFR^{L858R} ; CCSP-rTTA; MALT1$^{+/-}$ mice (Het) and lung from tetO-EGFR^{L858R} ; CCSP-rTTA; MALT1$^{-/-}$ mice (KO).

2.3.8 MALT1 is not required for K-ras-induced lung adenocarcinoma progression

Since MALT1 is specifically involved in EGFR but not K-ras induced NF-κB activation, we would like to examine whether MALT1 is specifically required for EGFR-induced NF-κB activation. To this end, we also generated another mouse model in which lung tumor is driven by mutant K-ras expression with or without MALT1 expression (LSL-K-ras^{G12D}; CCSP-Cre; MALT1$^{+/-}$ or LSL-K-ras^{G12D}; CCSP-Cre; MALT1$^{-/-}$) (Figure 14A). However, we observed similar tumor burdens (0.01573 ± 0.001575, n=5 vs. 0.01548 ± 0.001560, n=5) between control mice (LSL-K-ras^{G12D}; CCSP-Cre; MALT1$^{+/-}$) and MALT1 deficient mice (LSL-K-ras^{G12D}; CCSP-Cre; MALT1$^{-/-}$) (Figure 14B). The number of visible tumors on the surface of lungs were also similar between control mice and MALT1 deficient mice (Figure 14C). In terms of histology, both control mice and MALT1 deficient mice develops similar size and pathology of lung adenocarcinoma (Figure 14D). Therefore, these data indicate that MALT1 contributes to EGFR-induced but not K-ras-induced lung tumor progression *in vivo*.

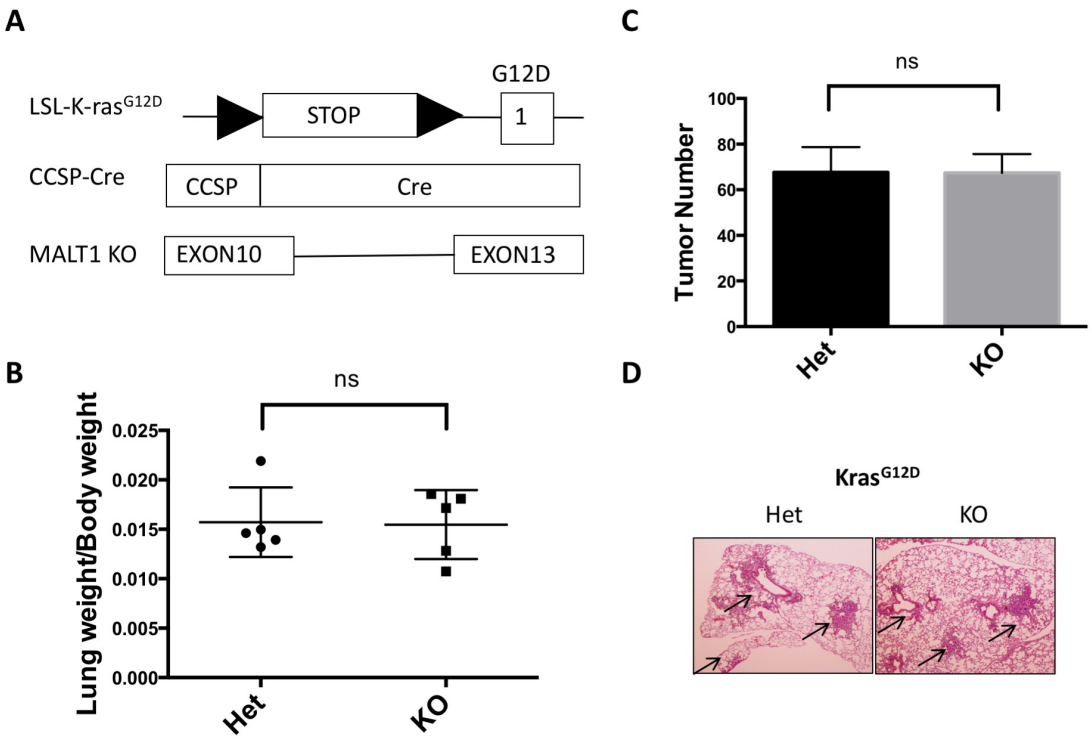

Figure 14. MALT1 is dispensable for K-ras-induced lung adenocarcinoma progression.

(A) Graphic presentation of the generation of LSL-K-ras^{G12D}; CCSP-Cre; MALT1$^{-/-}$ triple-transgenic mice. (B) The ratio of lung to body weight was compared between LSL-K-ras^{G12D}; CCSP-Cre; MALT1$^{+/-}$ (Het) (n=5) and LSL-K-ras^{G12D}; CCSP-Cre; MALT1$^{-/-}$ (KO) mice (n=5). ns, no statistically significance. (C) The number of tumors on the lung in LSL-K-ras^{G12D}; CCSP-Cre; MALT1$^{+/-}$ (Het) (n=3) and LSL-K-ras^{G12D}; CCSP-Cre; MALT1$^{-/-}$ (KO) mice (n=3). ns, no statistically significance. (D) H&E staining of the lung from LSL-K-ras^{G12D} ; CCSP-Cre; MALT1$^{+/-}$ mice (Het) and lung from LSL-K-ras^{G12D} ; CCSP-Cre; MALT1$^{-/-}$ mice (KO). Arrow shows tumor cells.

2.3.9 MALT1 contributes to NF-κB and STAT3 activation *in vivo*

To further dissect how MALT1 deficiency inhibits EGFR-dependent tumor growth, we examined the activation of downstream pathways of EGFR. Consistent with our *in vitro* data, we found a lower NF-κB activity in Malt1-knockout tumor bearing lungs compared with controls as showed by a reduced level of phosphorylated p65, while the level of phosphorylated-S6 ribosomal protein, phosphorylated AKT and phosphorylated ERK remained similar between Malt1 heterozygous and knockout mice (Figure 15). These results were consistent with our observation in *vitro* and suggest that MALT1 affects NF-κB activation *in vivo*.

It has been shown that STAT3 is also one of the downstream effectors of EGFR and is activated in EGFR-associated lung tumor in both mouse models and human samples (Gao et al. 2007). To our surprise, we found a significant defect of phosphorylated STAT3 level in Malt1-knockout mice compared with its heterozygous control, while the level of total STAT3 is comparable between Malt1 heterozygous and knockout mice (Figure 15). Thus, our data also suggest that MALT1 contributes to the EGFR-dependent activation of STAT3 *in vivo*.

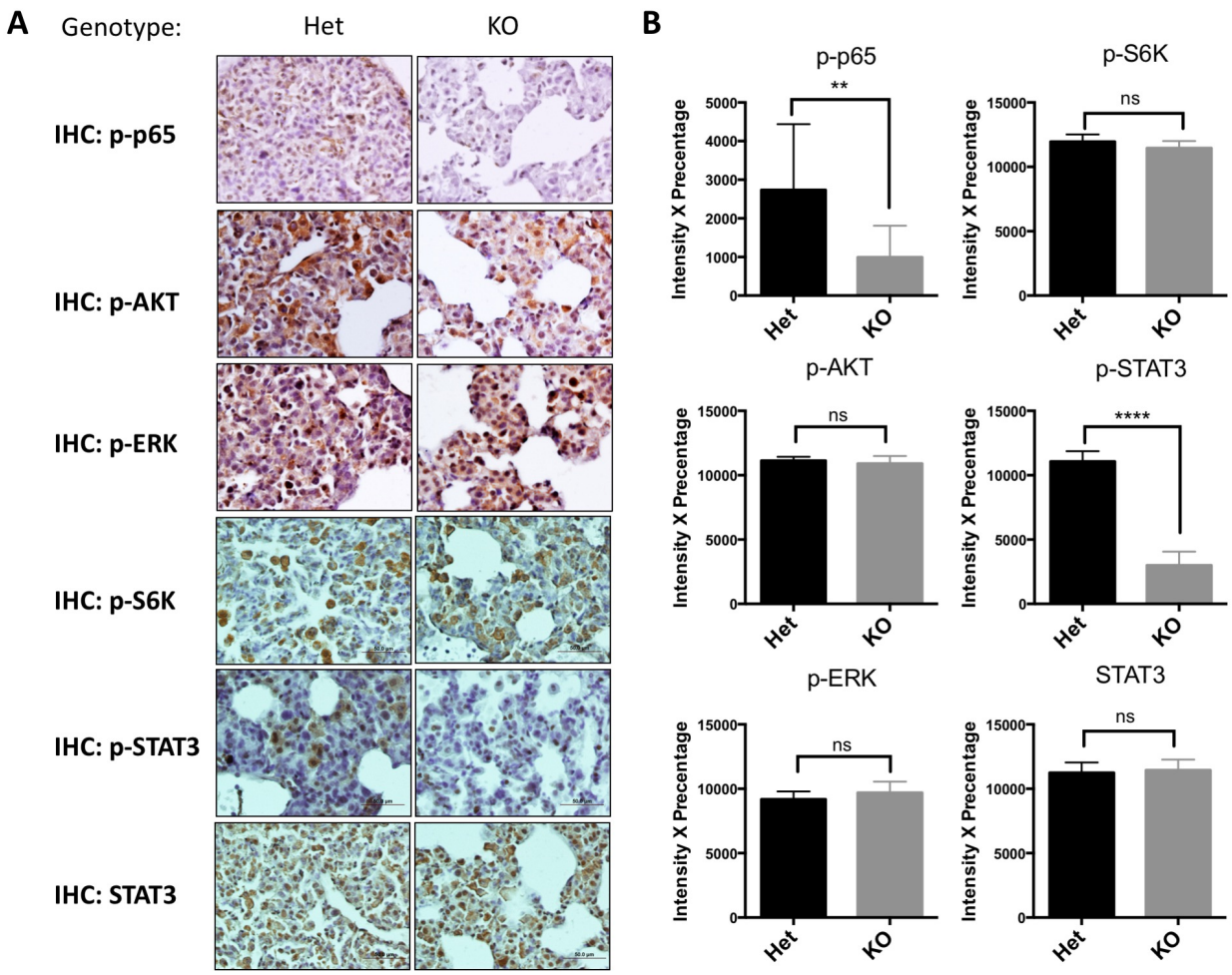

Figure 15. MALT1 controls NF-κB and STAT3 activation in EGFR-driven lung cancer model.

(A) Representative IHC staining of lung sections from tetO-EGFR^{L858R}; CCSP-rtTA; MALT1$^{+/-}$ and tetO-EGFR^{L858R}; CCSP-rtTA; MALT1$^{-/-}$ mice by using indicated antibodies. (B) The intensity × percentage of positive signals in (A) were compared for each staining. 10-15 random fields were chosen for each genotype, and were repeated three times using different mice (n=3) for each genotype.

2.3.10 MALT1 controls NF-κB dependent IL-6 production in response to EGFR

It has been suggested that NF-κB regulates IL-6 production. We found both IL-6 neutralization and IKK inhibition significantly reduced p-STAT3 level in HCC827 cells, suggesting that both NF-κB and IL-6 are required for p-STAT3 activation in HCC827 cells (Figure 16). In addition, MALT1-silenced HCC827 cells showed a lower level of p-STAT3 compared with control cells. This result is consistent with a lower p-STAT3 level in the lung tumor from MALT1 deficient mice compared with the control mice *in vivo*. To further determine whether MALT1 controls IL-6 production upon EGFR activation, we took A431 cells and examined IL-6 production upon EGF stimulation. We found MALT1-silenced cells produced significant less IL-6 compared to controls, while cells treated with MALT1 inhibitor produced similar amount IL-6 production as control (Figure 17A). Consistently, MALT1-silenced HCC827, but not cells treated with MALT1 inhibitor, showed a similar defect in IL-6 production (Figure 17B). In our mouse model, we found that IL-6 mRNA level is much lower in the tumor bearing lungs of Malt1 knockout mice compared to its heterozygous controls (Figure 17C). Taken together, these data indicate that MALT1 controls EGFR-driven IL-6 production *in vitro* and *in vivo*. In summary, our data suggests that MALT1 contributes EGFR-associated lung cancer progression by coordinately controlling both NF-κB and STAT3 activation through IL-6-mediated crosstalk between these two pathways (Figure 18).

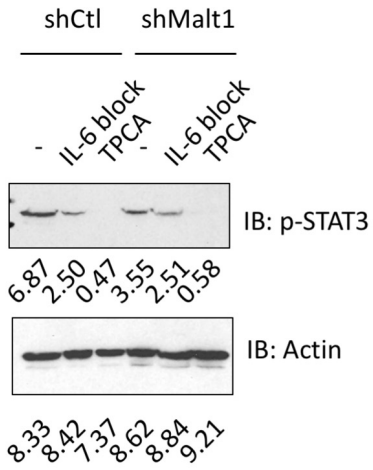

Figure 16. IL-6 and IKK complex are required for STAT3 activation *in vitro*.

Control (shCtl) and MALT1-silenced (shMalt1) HCC827 cells were treated with IL-6 neutralizing antibodies and IKK inhibitor TPCA (1μM) for 12 hours, respectively. Lysates of these cells were analyzed by immunoblotting with indicated antibodies. Band intensity was quantified by using Image J.

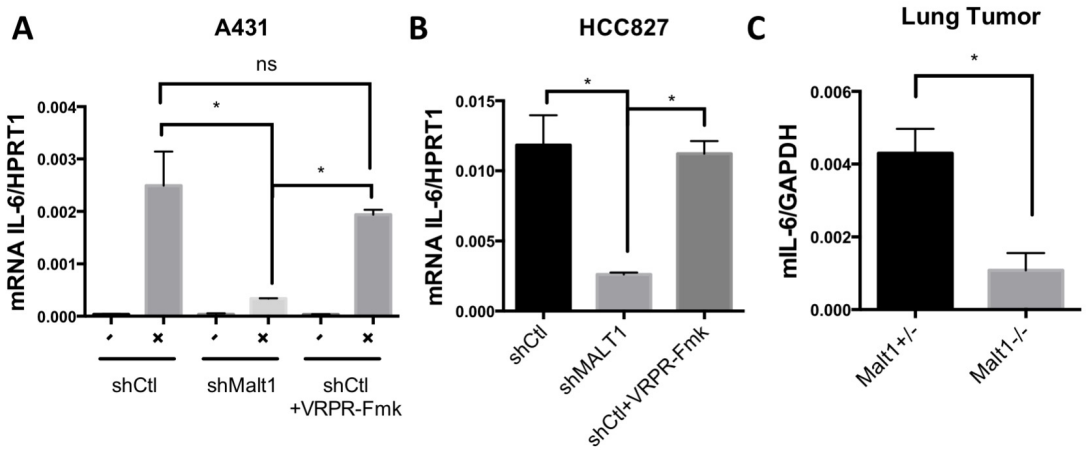

Figure 17. MALT1 is required for EGFR-induced IL-6 production.

(A) Control A431 cells (shCtl), MALT1 knockdown cells (shMALT1), and control cells treated with MALT1 inhibitor z-VRPR-Fmk (75μM) were stimulated with EGF (100ng/ml), respectively. Total RNA was extracted for quantitative PCR analysis. The ratio of mRNA of human IL-6 to human HPRT1 were calculated and compared in the graph. *, $p<0.05$. (B) Total RNA was extracted from control (shCtl) HCC827 cells, MALT1 knockdown cells (shMALT1), and control cells treated with MALT1 inhibitor z-VRPR-Fmk (75μM), respectively. The ratio of mRNA of human IL-6 to human HPRT1 were calculated and compared in the graph. *, $p<0.05$. (C) Total RNA was extracted from the lung of tetO-$EGFR^{L858R}$; CCSP-rtTA; $MALT1^{+/-}$ and tetO-$EGFR^{L858R}$; CCSP-rtTA; $MALT1^{-/-}$ mice. The ratio of IL-6 to GAPDH was presented in the graph. *, $p<0.05$.

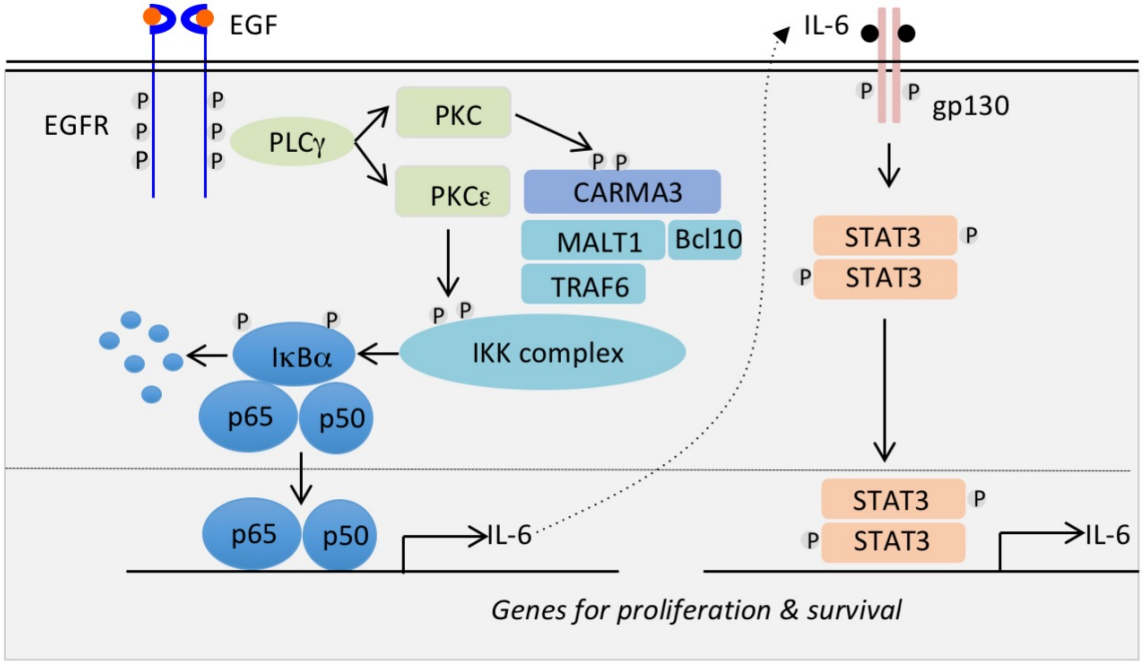

Figure 18. The working model summarizing the finding in this study.

MALT1 is required for EGFR-induced NF-κB activation by recruiting E3 ligase TRAF6 to induce the activation of the IKK complex. The EGFR-activated NF-κB is responsible to transcribe IL-6, which in turns to activate STAT3 pathway. Together, NF-κB and STAT3 signaling coordinately promote EGFR-associated lung cancer progression.

2.4 Discussion

2.4.1 MALT1 only functions as a scaffold protein in EGFR-mediated NF-κB pathway

NF-κB is one transcription factor that has been shown directly activated by EGFR. Although previously it has been shown that EGFR activates NF-κB in a CARMA3- and BCL10-dependent manner, the exact molecular mechanism by which EGFR activates NF-κB is still not clear. In this study, we have provided biochemical and genetic evidence suggesting that EGFR-induced NF-κB is mediated by MALT1. We found that MALT1 functions downstream of BCL10 and recruits E3 ligase TRAF6 to IKK complex upon EGFR stimulation. In lymphocytes, the activation of NF-κB requires two parallel signals to induce IKK ubiquitination and phosphorylation in response to TCR and BCR stimulation (Shambharkar et al. 2007). A previous report suggests that EGFR activation induce IKK phosphorylation through PKC (Yang et al. 2012). Since we found MALT1 deficiency does not alter the level of phosphorylated IKK in response to EGF stimulation, we propose that MALT1-TRAF6 complex is responsible for IKK ubiquitination in response to EGF stimulation. This activation mechanism is similar to TCR- and BCR-induced NF-κB. However, unlike TCR- and BCR-induced NF-κB, the protease activity of MALT1 is not required for EGFR-induced NF-κB activation, although we found there was a slight reduction of PMA/Ionomycin induced NF-κB in MEFs. This result is in part due to lack of MALT1's substrates in non-hematoploitic cells, such as A20 and RelB. It may also due to EGFR does not activate NF-κB as strong as CD3/CD28 or PMA and Ionomycin does.

However, even the cells express CYLD, which is cleaved by MALT1 in response to TCR stimulation (Staal et al. 2011), we cannot detect proteolysis events upon EGF stimulation. Therefore, our results suggest a differential requirement of MALT1 protease activity depending different types of stimuli.

2.4.2 MALT1 in EGFR-associated tumor malignancy

EGFR is playing an oncogenic role in many types of human cancer. While it has been well established that EGFR downstream effector pathways such as AKT and MAPK are playing pivotal roles in cancer initiation and progression, less is known about how other signaling such as NF-κB contributes to EGFR-associated tumor progression. Here we show MALT1 contributes to several important oncogenic phenotypes in EGFR-associated tumors. Our study found that MALT1 plays an essential role in tumor migration and invasion *in vitro*, as well as in a lung metastasis model *in vivo*. We propose such defect is through NF-κB-dependent gene expression based on the data that MALT1 deficiency specifically blocks NF-κB activation but not other signaling pathways downstream of EGFR. In line with this notion, inhibition of IKK kinase activity and TRAF6-silencing phenocopy MALT1-silencing in terms of cell migration, suggesting that MALT1 controls cell migration through NF-κB pathway. Indeed, our microarray data revealed that the expression of several matrix metalloproteinases (MMPs) is significantly down regulated in response to EGF stimulation in CARMA3-silenced cells (data not shown). This is consistent with the notion that NF-κB regulates the expression of many genes involved in migration and invasion (Huber et al.

2004). However, MALT1 may also contribute to migration and invasion in an NF-κB-independent manner. For example, a recent study showed that BCL10 regulates actin dynamics and polymerization (Rebeaud et al. 2008). Therefore, it is possible that MALT1 also affects tumor cell migration and invasion by regulating these processes.

Although study from our group and others suggests that NF-κB is an important transcription factor for EGFR-associated malignancy (Jiang et al. 2011), this notion has not been tested in physiological tumor models. In this study, we used an EGFR-driven lung cancer mouse model to test how MALT1 is involved in the progression of EGFR-induced lung tumor progression. Our model shows that MALT1 dependent NF-κB contributes to the progression of EGFR-induced adenocarcinoma, but is not required for the tumor onset. This result highly correlates with our in *vitro* data that MALT1 inhibition suppresses tumor growth but does not completely suppress tumor cells. Interestingly, although NF-κB activity has been shown to be important to K-ras dependent lung cancer progression (Meylan et al. 2009), MALT1 is dispensable for both onset and progression of K-ras-induced lung cancer. This finding is consistent with the hypothesis that MALT1 is specifically involved in EGFR-induced NF-κB activation but not K-ras-mediated NF-κB, which is likely mediated by TBK1 (Barbie et al. 2009). Therefore, our result has provided the genetic evidence supporting a rationale of targeting MALT1 or other components in NF-κB signaling in EGFR-associated lung cancer.

2.4.3 The role of MALT1 in STAT3 pathway in lung cancer

Another interesting finding in this study is that MALT1 deficiency abolishes the activation STAT3 *in vivo*. Previous studies suggest constitutively activated STAT3 is found in 50% of human NSCLC samples (Gao et al. 2007). In addition, a blockage of STAT3 induces growth arrest of tumor, suggesting the functional importance of STAT3 in EGFR-associated lung cancer (Gao et al. 2007). However, how STAT3 is aberrantly activated in NSCLC is not clear. Here, we provide genetic evidence suggesting that STAT3 activation in NSCLC is likely controlled by MALT1-dependent NF-κB activation. This result suggests that NF-κB not only controls cell proliferation and survival by itself, but also indirectly activate STAT3. Therefore, a blockage of the crosstalk between NF-κB and STAT3 can be used as a potential therapeutic strategy for the treatment of EGFR-associated lung cancer. In the current study, we showed that IL-6 is one of the effectors that bridging NF-κB to STAT3 in response to EGFR activation. This result supports an idea to block IL-6 in EGFR-dependent lung cancer to interrupt the crosstalk between NF-κB and STAT3. In fact, elevated IL-6 level has been connected to the survival of patients with advanced NSCLC (Songur et al. 2004). As anti-IL-6 and anti-IL-6 receptor antibodies are currently tested in several clinical studies including some types of cancers (Guo et al. 2012), targeting IL-6 in EGFR-dependent NSCLC is worth to be tested in preclinical models in the future.

CHAPTER 3 THE ROLE OF CBM COMPLEX IN HER2-INDUCED NF-κB ACTIVATION AND HER2-ASSOCIATED BREAST CANCER PROGRESSION

This chapter is based upon " Deng Pan, Yifan Zhu, Zhicheng Zhou, Tingting Wang, Harrison You, Changying Jiang and Xin Lin. The CBM complex Underwrites NF-kappaB Activation to Promote HER2-associated Tumor Malignancy. Mol Cancer Res. 2015 Sep 21. ePub ahead of print."

3.1 Introduction

3.1.1 Overview of HER2 and breast cancer

The human epidermal growth factor receptor 2 (Her2)/Neu protein is one of the members of the epidermal growth factor receptor (EGFR) family. Amplification and overexpression of HER2/Neu have been found in approximately 20-30% of breast cancers (Mitri et al. 2012). In addition, HER2 amplification and overexpression are associated with poor prognosis in patients with breast cancer. Although targeted therapy against HER2, such as Trastuzumab, has been shown to work well against HER2-positive breast cancer, most patients were found to develop acquired resistance within 1 year of treatment (Rexer and Arteaga 2013). Therefore, seeking new therapeutic targets downstream of HER2 signaling may provide alternative therapies for HER2-positive breast cancers.

3.1.2 The role of NF-κB signaling in HER2+ breast cancer

NF-κB is part of a family of transcription factors that regulate the expression of a wide variety of genes, including those involved in cell survival and inflammation (Hayden and Ghosh 2012). It has been proposed that NF-κB plays a crucial role in the progression of many types of tumors, including HER2-positive breast cancer (Bailey et al. 2014). For example, it has been shown that NF-κB is crucial for cell proliferation and invasion in HER2-positive breast cancer cells (Merkhofer et al. 2010). In addition, the activation of NF-κB has been functionally linked to drug resistance in both HER2-positive and negative breast cancer cells (Bailey et al. 2014, Oida et al. 2014). Although it has been suggested that NF-κB

regulates many aspects of HER2-positive breast cancer progression, little is known about the mechanistic relationship between HER2 activation and NF-κB activation.

NF-κB transcription factors include five subunits, RelA, RelB, c-REL, p105/p50 and p100/p52. Canonical NF-B activation requires an upstream signal that activates the IB kinase (IKK) complex, which phosphorylates IκB and triggers its degradation via the ubiquitination/ proteasome pathway. The degradation of IκB releases NF-B subunits to the nucleus and initiates transcription. It has been previously suggested that HER2-induced NF-κB activation is mediated by AKT activation (Pianetti et al. 2001). However, a later study showed that AKT is not required for the HER2-induced NF-κB activation (Merkhofer et al. 2010). The same study further suggested that HER2 activates NF-κB through a canonical pathway that depends on IKKγ, a component of IKK complex (Merkhofer et al. 2010). However, the molecular link between HER2 and the IKK complex still remains to be determined.

3.1.3 Critical observations and hypothesis

The caspase recruitment domain (CARD) and membrane-associated guanylate kinase-like domain protein (CARMA) family of proteins includes CARMA1, CARMA2 and CARMA3 (Blonska and Lin 2011). CARMA1 is expressed predominantly in the lymphoid cells and is required for T cell receptor (TCR)- and B cell receptor (BCR)-induced NF-κB activation (Gaide et al. 2002, Wang et al. 2002). The activation of CARMA1 triggers the association of CARMA1 with B-cell lymphoma 10 (BCL10) and mucosa associated

lymphoid tissue lymphoma translocation protein 1 (MALT1), and forms CARMA1-BCL10-MALT1 (CBM) complex. The CBM complex recruits E3 ligases such as TRAF6 to activate IKK complex via ubiquitination in response to T cell receptor stimulation (Sun et al. 2004). Similarly, CARMA3 is mainly expressed in the non-hematopoietic cells and is required for protein kinase C (PKC)-induced NF-κB activation, in which CARMA3 forms the CBM complex through a similar mechanism as that of CARMA1 (Jiang and Lin 2012). In our previous studies, we found that the CBM complex is required for G protein coupled receptor (GPCR)- and EGFR-induced NF-κB activation (Grabiner et al. 2007, Jiang et al. 2011, Pan et al. 2015). Therefore, we hypothesized that the CBM complex mediates HER2-induced NF-κB activation and contributes to HER2-associated tumor malignancy. In the current study, we used biochemical and genetic approaches to investigate the role of the CBM complex in HER2-mediated NF-κB activation and HER2-positive breast cancer.

3.2 Materials and Methods

3.2.1 Antibodies and reagents

Antibodies against phospho-EGFR (sc-12351), EGFR (sc-03), BCL10 (sc-5611), ERK (sc-154), Neu (sc-284), and Actin (sc-8432), were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against p-AKT (Ser473; 9271S), p-AKT (Thr308) (9275), AKT (4685), p-ERK1/2 (9101), p-IkBa (9246) and p-HER2 (2241) were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal antibodies against MALT1 were generated by Genentech (San Francisco, CA). Human heregulin-b-1 (Cat. #AF-100-03) was purchased from Pepro Tech (Rocky Hill, NJ). Recombinant human EGF was purchased from Sigma-Aldrich (St. Louis, MO). Phorbol 12 myristate 13 acetate (PMA; 16561-29-8) and ionomycin (56092-82-1) were purchased from Fisher BioReagents (Pittsburgh, PA). TNFα was purchased from Thermo Fisher Scientific (Rockford, IL). Akt1/2 kinase inhibitor (A6730) was purchased from Sigma-Aldrich (MO, USA).

3.2.2 Cells

SKBR3 cells were grown in McCoy's medium containing 10% fetal bovine serum at 37ºC with 5% CO_2. Mouse embryonic fibroblasts were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37ºC with 8% CO_2.

3.2.3 Immunoblotting and gel shift assay

These experiments were performed as described (Pan et al. 2015). For immunoblotting assay, one million of cells were lysed in 100μl of lysis buffer (150 mM NaCl, 50 mM HEPES

at pH 7.4, 1 mM EDTA, 1% NP-40, protease inhibitors) for 10 minutes. The cell lysates will be purified by spinning 13,000 r.p.m. for 10 minutes and resulting supernatants will be saved for western blot analysis. 10-12μl of the resulting supernatant were loaded to 10% of SDS-PAGE and probed with antibodies. For the electrophoretic mobility shift assay, two million cells were starved overnight in DMEM containing 0.5% serum and stimulated with reagents, and nuclear extracts were isolated. Nuclear extracts (5 μg) were incubated with 1×10^5 c.p.m. of ^{32}P-labeled probes at room temperature for 15 min. The samples were separated on a native Tris-borate-EDTA polyacrylamide gel and analyzed by autoradiography.

3.2.4 cDNA preparation and Quantitative PCR

Total RNA was extracted using TRIzol Reagent (Life Technologies, Grand Island, NY) according to manufactory protocol. cDNA was synthesized by using iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA). Quantitative polymerase chain reaction (PCR) was performed by using the Fast SYBR green master Kit (Life Technologies, Grand Island, NY) according to the protocol. Primers for quantitative PCR were described as follows. CARD10-F: 5' CCCGCACCCCCTAAGAGA 3'; CARD10-R: 5' TGTCAGAGGATGAGGACGAAGA3'; MMP1-F: 5'GAGCTCAACTTCCGGGTAGA3'; MMP1-R: 5' CCCAAAAGCGTGTGACAGTA3'; MMP3-F: 5' CAAAGCTTCAGTGTTGGCTG3'; MMP3-R: 5' GGCCAGGGATTAATGGAGAT3'; MMP7-F: 5' GGCCAAAGAATTTTTGCATC3'; MMP7-R: 5'GAGCTACAGTGGGAACAGGC3'; MMP13-F: 5' ACTGAGAGGCTCCGAGAAATG3'; MMP13-R: 5'

GAACCCCGCATCTTGGCTT3’; MMP14-F: 5’ GGCTACAGCAATATGGCTACC3’; MMP14-R: 5’ GATGGCCGCTGAGAGTGAC3’.

3.2.5 Cell migration and invasion assay

We added $1x10^5$ SKBR3 cells to a cell culture insert (353097, Franklin Lakes, NY) and the lower chambers were placed with 0.7 ml of serum-free medium containing 50 ng/ml heregulin (HRG) or complete medium containing 10% fetal bovine serum (FBS). After 16 hours of incubation, migrated cells were fixed, stained and quantified. The cell invasion assay was performed in a similar manner to the migration assay with matrigel on the lower chamber. After 48 hours of incubation, migrated cells were fixed, stained and quantified.

3.2.6 MTT Assay

SKBR3 cells were seeded in wells of a 96-well plate in 100μl of complete or serum-free (0.5% FBS) of MyCoy’s media. At the indicated time, 10μl of MTT reagent (5mg/ml) was added to the cells. After 3 hours of incubation, the media containing MTT was removed and metabolized MTT will be dissolved in 100μl of DMSO. The absorbance was measured at 570nm and was recorded accordingly.

3.2.7 Colony formation assay

These experiment were performed as described previously (Pan et al. 2015). For colony formation assay, SKBR3 cells were mixed with agarose to a final concentration of 0.6% in

complete medium, with or without HRG (1 ng/ml). Three weeks after culturing, the numbers and sizes of colonies were determined by inverted microscopy. Nine to ten fields were randomly selected and the size of each colony visualized was determined.

3.2.8 Anoikis assay

We mixed $1x10^6$ cells with complete medium containing 1% methylcellulose in the presence or absence of 10ng/ml HRG and seeded this mixture in 6-well plates coated with 2 ml of poly 2-hydroxyethyl methacrylate (10 g/L in 100% ethanol; 25249-16-5, Sigma-Aldrich). The cells were incubated at 37ºC for 72 hours and stained with Annexin V and propidium iodide according to manufactory protocol (556547, BD Biosciences, San Jose, CA). The stained cells were analyzed using a flow cytometer (Becton Dickinson FACS Canto II).

3.2.9 Mouse xenograft tumor model

Six-week-old female SCID mice were used for this experiment. We suspended $1x10^6$ cells in a 1:3 mixture of appropriate medium versus matrigel and injected subcutaneously into the fat pad of SCID mice. Six weeks after inoculation, mice were euthanized and the tumor size was measured. All animal experiments and procedures were conduced under the protocol and were approved by the Institutional Animal Care and Use Committee (IACUC) at the The University of Texas MD Anderson Cancer Center.

3.2.10 Mouse lung metastatic tumor model

Six-week-old female SCID mice were used for the lung metastatic animal model. We suspended $1x10^7$ cells in serum free medium and injected the cells into tail vein. Six weeks after injection, the animals were euthanized and the lungs were washed, fixed and stained in Bouin's solution. Metastatic lesions were photographed and counted.

3.2.11 Mammary tumor mouse model

Malt1 knockout mice (Ruefli-Brasse et al. 2003) were backcrossed to 6 generations with the FVB/N background and then crossed with MMTV-Neu-NDL mice (Siegel et al. 1999), which are maintained with a pure FVB/N background. After 12 weeks, female mice were palpated weekly to determine whether the tumors have developed. Mice were euthanized when the tumor size reached 100mm in diameter. Statistical analysis of mouse survival was performed using survival curve parameters (Log-rank test) in Prism GraphPad 6 (La Jolla, CA).

3.2.12 Histology and immunohistochemical staining

Mammary tumors isolated from *MMTV-Neu* mice were washed in phosphate-buffered saline and fixed in 4% paraformaldehyde solution for overnight and embedded in paraffin. The paraffin sections were stained in hematoxylin and eosin by the histology core facility at MD Anderson. For immunohistochemical (IHC) staining, standard procedures were carried out according to the manual (DAKO, #K0673, Carpinteria, CA).

3.2.13 Statistical analysis

All groups were compared using the Student's *t* test (2-tailed unpaired *t* test) in Prism Graphpad 6 unless otherwise indicated. $p<0.05$ was considered statistically significant.

3.3 Results

3.3.1 PKC but not AKT activity is required for HER2-induced NF-κB activation

The role of PI3K-AKT in HER2-induced NF-B activation remains controversial (Pianetti et al. 2001). To determine whether PI3K-AKT signaling is required for HER2-induced NF-κB activation in breast cancer cell lines, we tested the effect of an AKT inhibitor on NF-κB activation in response to stimulation of HRG in SKBR3 cells, in which HER2 is highly expressed. To ensure the specificity of HRG to HER2 activation, we first confirmed that HRG only activates only HER2 and not EGFR in SKBR3 cells (Figure 19). We found that although the AKT inhibitor specifically and efficiently blocked the activation of AKT in response to HRG stimulation (Figure 20A), the activation of NF-kB was intact in the presence of the AKT inhibitor compared with controls, as determined by the gel shift assay (Figure 20B). This result suggests that AKT activity is dispensable for HER2-induced NF-B activation. In contrast, we found that HER2-induced NF-B activation was completely blocked in SKBR3 cells treated with PKC inhibitor (GF209103X) (Figure 21A). The PKC inhibitor specifically affected PMA- and ionomycin- but not TNF-induced NF-B (Figure 21B). In addition, the PKC inhibitor had no effect on HER2 activation or its other downstream targets such as ERK activation (Figure 21C). Taken together, these data indicate that HER2-induced NF-κB activation requires PKC rather than AKT activity.

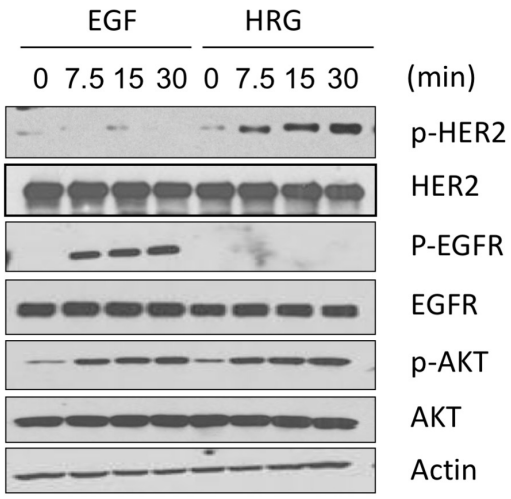

Figure 19. Heregulin specifically activates HER2 receptor.

SKBR3 cells were stimulated with EGF (100ng/ml) or HRG (50ng/ml) for 7.5, 15 or 30 minutes. Whole cell lysates were subjected to immunoblotting with the indicated antibodies.

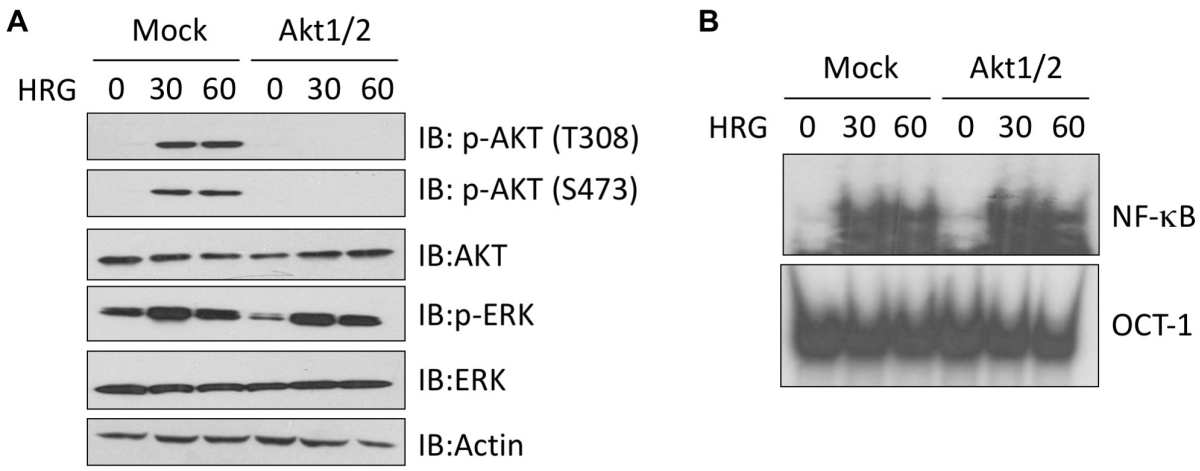

Figure 20. HER2-induced NF-κB activation does not require AKT activity.

(A) SKBR3 cells were treated with AKT1/2 kinase inhibitor (20μM) or vehicle control and stimulated with heregulin (HRG; 50ng/ml) for 30 or 60 minutes. Whole cell lysates were subjected to immunoblotting with indicated antibodies. (B) Nuclear extracts from the experiment shown in panel A were isolated and subjected to the gel shift assay using probes binding to NF-B or Oct-1 (loading control).

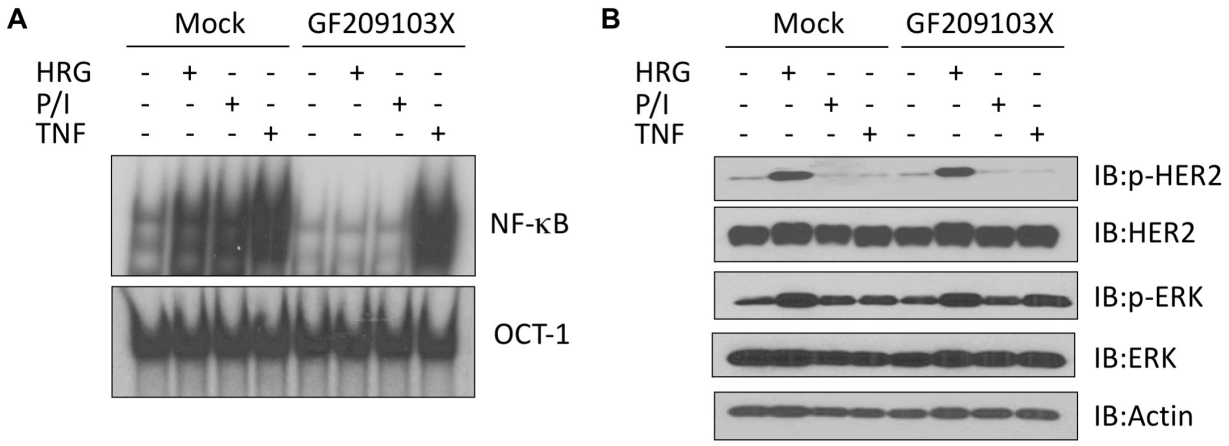

Figure 21. HER2-induced NF-κB activation requires PKC activity.

(A) SKBR3 cells were treated with a PKC inhibitor (GF209103X; 1uM) or vehicle control and were stimulated with HRG (50ng/ml),phorbol 12 myristate 13 acetate (50ng/ml) and ionomycin (100ng/ml), or TNF (10ng/ml) for 1 hour. Whole cell lysates were subjected to immunoblotting with the indicated antibodies. (B) Nuclear extracts from same experiment shown in panel C were isolated and subjected to the gel shift assay using probes binding to NF-κB or Oct-1 (loading control). Experiments have been independently repeated 2-3 times.

3.3.2 The CBM complex is required for HER2-induced NF-κB activation

It has been shown that the CBM complex is positioned immediately downstream of PKC in response to several types of stimuli, such as T cell and B cell receptor and GPCR stimulation (Blonska and Lin 2009). Therefore, we sought to determine whether the CBM complex also links PKC to NF-κB activation in response to HER2 stimulation. We silenced the expression of CARMA3, BCL10 and MALT1, respectively, in SKBR3 cells by infecting cells with shRNA (Figure 22) and examined NF-κB activation after HRG stimulation using the gel shift assay. We found that HER2-induced NF-κB activation was significantly reduced in CARMA3, BCL10 and MALT1-silenced SKBR cells, respectively (Figure 23A-B). Consistently, HER2-induced NF-κB activation was also abolished in CARMA3 deficient (*Card10*$^{-/-}$) mouse embryonic fibroblasts (MEFs) compared with wild-type (*Card10*$^{+/+}$) controls (Figure 23C). In addition, CARMA3 or BCL10 silencing specifically inhibited the activation of NF-κB, but not the AKT pathway, as determined by the level of both phosphorylated AKT (Thr 308) and phosphorylated AKT (Ser 473) after HRG stimulation (Figure 24). Taken together, these results indicate that the CBM complex is specifically required for HER2-induced NF-κB activation.

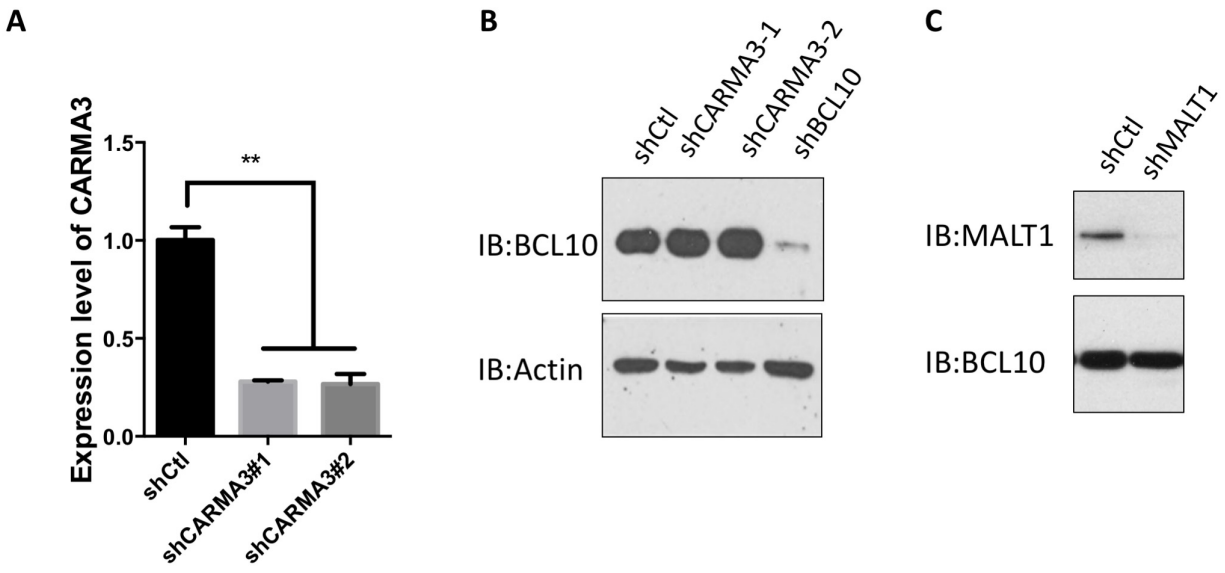

Figure 22. Knockdown efficiency of CARMA3, BCL10 and MALT1.

(A**)** Total RNA from SKBR3 cells with control knockdown (shCtl) or CARMA3 knockdown by 1 of 2 different shRNA (shCARMA3-1 and shCARMA3-2) were extracted for quantitative PCR analysis to determine the relative expression level of CARMA3 to GAPDH. (B-C) Whole cell lysates from SKBR3 cells with control knockdown (shCtl), BCL10 knockdown, or MALT1 knockdown were subjected to immunoblotting with the indicated antibodies.

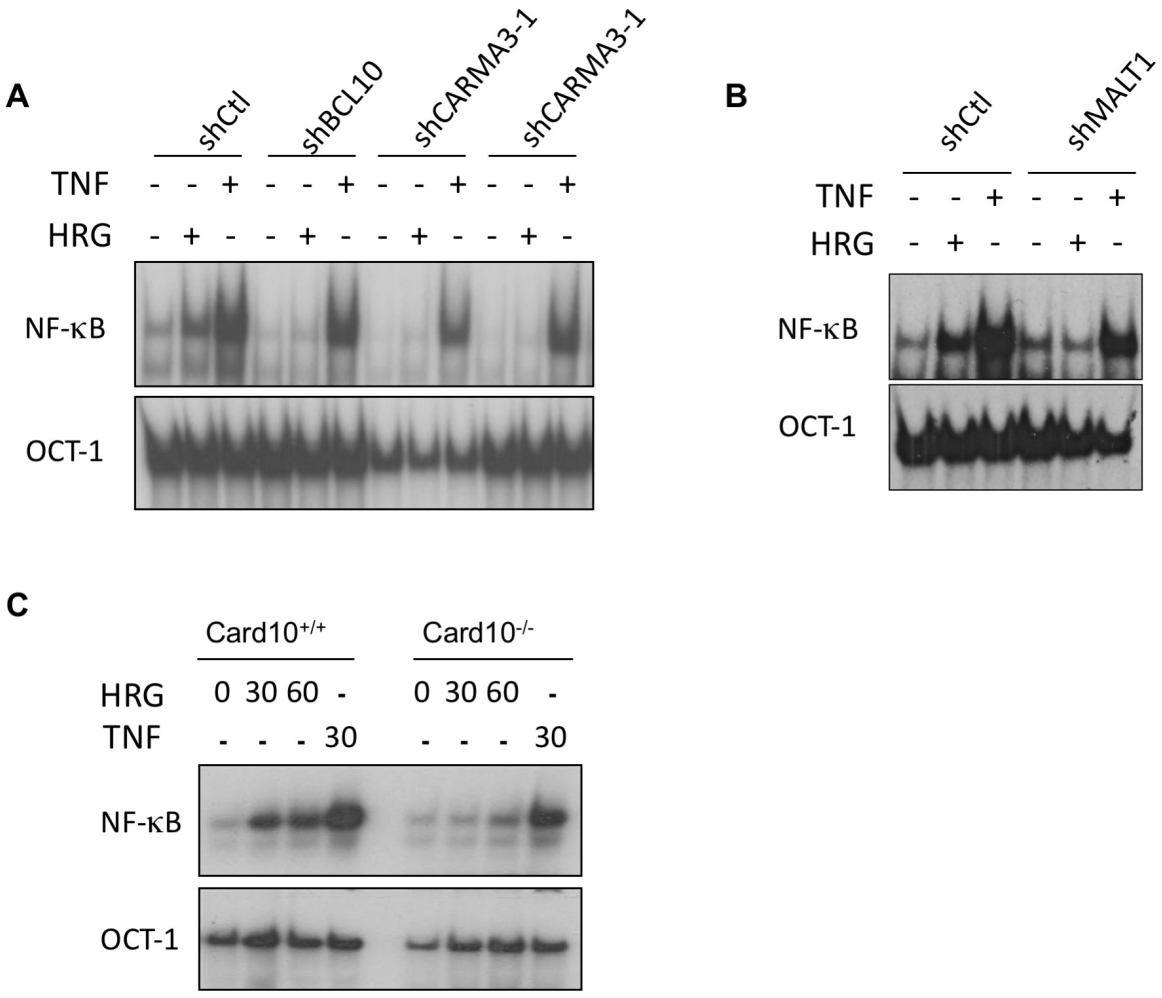

Figure 23. The CARMA3-BCL10-MALT1 complex is required for HER2-induced NF-κB activation.

(A) SKBR3 cells with control knockdown (shCtl), BCL10 knockdown (shBCL10), or CARMA3 knockdown by 1 of 2 different shRNAs (shCARMA3-1 and shCARMA3-2) were stimulated with heregulin (HRG; 50ng/ml) or TNF (10ng/ml) for 1 hour. Nuclear extracts were isolated and subjected to the gel shift assay by using probes binding to NF-κB or Oct-1 (loading control). (B) SKBR3 cells with control knockdown (shCtl), or MALT1 knockdown (shMALT1) were stimulated with HRG (50ng/ml) or TNF (10ng/ml) for 1 hour. Nuclear

extracts were isolated and subjected to the gel shift assay by using probes binding to NF-κB or Oct-1 (loading control). (C) Wild-type (*Card10*$^{+/+}$) and CARMA3-deficient (*Card10*$^{-/-}$) mouse embryonic fibroblasts were stimulated with HRG (50ng/ml) for 30 and 60 minutes or with TNF (10ng/ml) for 30 minutes. Nuclear extracts were isolated and subjected to the gel shift assay by using probes binding to NF-κB or Oct-1 (loading control). Experiments have been independently repeated 2-3 times.

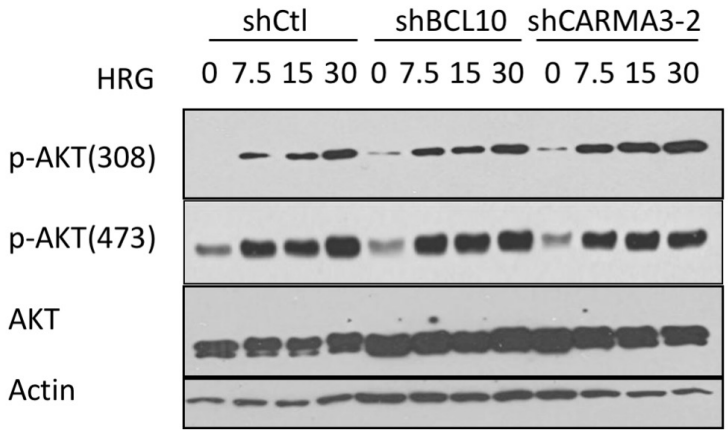

Figure 24. CARMA3 and BCL10 do not affect HER2-induced AKT activation.

SKBR3 cells with control knockdown (shCtl), BCL10 knockdown (shBCL10), or CARMA3 knockdown (shCARMA3-2) were stimulated with HRG (50ng/ml) for 7.5, 15 or 30 minutes. Whole cell lysates were subjected to immunoblotting with the indicated antibodies. Experiments have been independently repeated 2-3 times.

3.3.3 The CBM complex contributes to cell proliferation and avoidance of apoptosis

Because NF-κB plays a critical role in cell survival, we examined the contribution of CARMA3 and BCL10 to cell proliferation and survival in SKBR3 cells. We found that cells with silenced CARMA3 or BCL10 proliferated at a significantly slower rate than control cells (Figure 25A). The difference was more dramatic in the presence of HRG, suggesting that CARMA3 and BCL10 are required for HRG-stimulated cell proliferation (Figure 25B). We also found that cells with silenced CARMA3, BCL10 or MALT1 become more sensitive to detachment induced cell apoptosis, a phenomenon called "anoikis," compared with control cells (Figure 26). Taken together, these data suggest that CARMA3 and BCL10 are required for HRG-stimulated cell proliferation and cell survival *in vitro*.

To examine the role of the CBM complex in terms of tumor growth, we used the colony formation assay to determine whether the CBM complex contributes to tumor formation *in vitro*. We found that SKBR3 cells with silenced CARMA3 or BCL10 formed fewer colonies on the soft agar than did control cells (Figure 27A-B). Similarly, cells with silenced MALT1 also formed much smaller colonies than did control cells (Figure 27C). These results suggest that the CBM complex is required for tumor formation *in vitro*.

To further investigate the role of CARMA3 in tumor formation *in vivo*, we performed xenograft experiment by injecting CARMA3-silenced or control SKBR3 cells into SCID mice. Consistent with the results of the colony formation assays, we found that CARMA3-silenced SKBR3 cells formed significantly smaller tumors than control cells (Figure 28), suggesting that CARMA3 also contributes to the tumor growth *in vivo*. Collectively, these

results indicate that the CBM complex contributes to the proliferation and survival of SKBR3 breast cancer cells *in vitro* and *in vivo*.

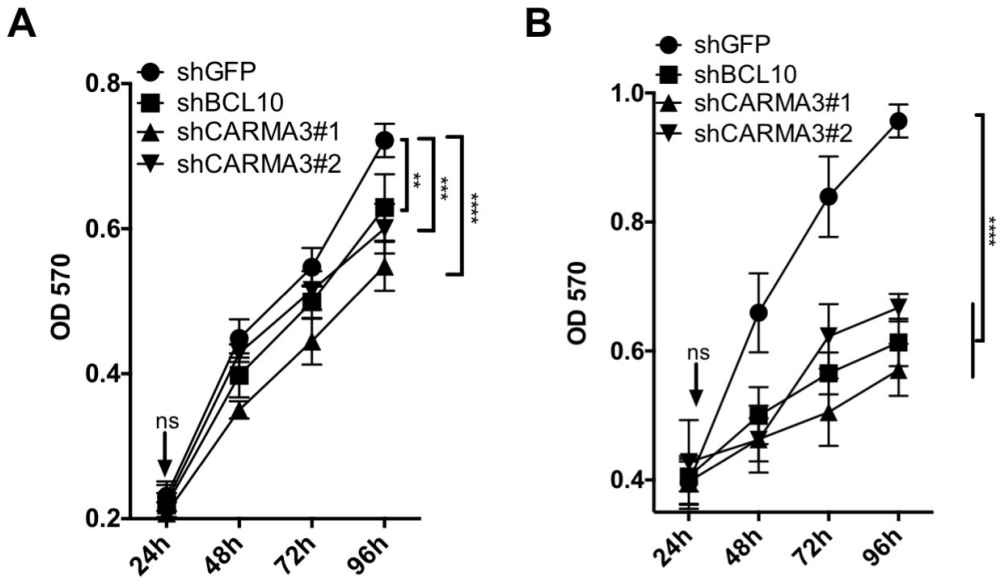

Figure 25. CARMA3 and BCL10 are required for SKBR3 cell proliferation.

(A) SKBR3 cells with control knockdown (shCtl), BCL10 knockdown (shBCL10), or CARMA3 knockdown by 1 of 2 different shRNAs (shCARMA3-1 and shCARMA3-2) were subjected to the colony formation assay in the presence or absence of 10 ng/mL heregulin for 2 weeks. Five random fields were photographed; one was presented here. (B) Quantification of colonies in panel A in the presence or absence of HRG. Experiments have been independently repeated 2-3 times.

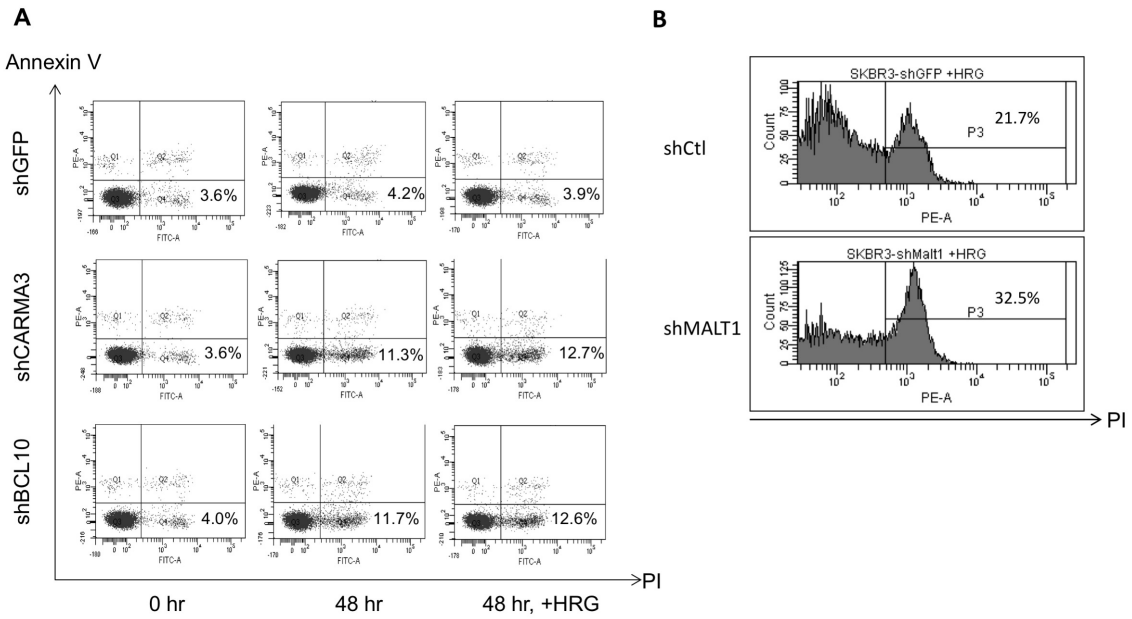

Figure 26. CARMA3 and BCL10 are required for detachment induced apoptosis in SKBR3 cell line.

SKBR3 cells with control knockdown (shCtl), BCL10 knockdown (shBCL10), or CARMA3 knockdown (shCARMA3) were mixed with complete medium containing 1% methylcellulose in the presence or absence of 10ng/ml heregulin. After 48 hours, apoptotic cells were examined by staining with Annexin V and propidium iodide. (B) SKBR3 cells with control knockdown (shCtl) or MALT1 knockdown (shMALT1) were mixed with complete medium containing 1% methylcellulose in the presence or absence of 10ng/ml heregulin. After 48 hours, apoptotic cells were examined by staining with propidium iodide. Experiments have been independently repeated 2-3 times.

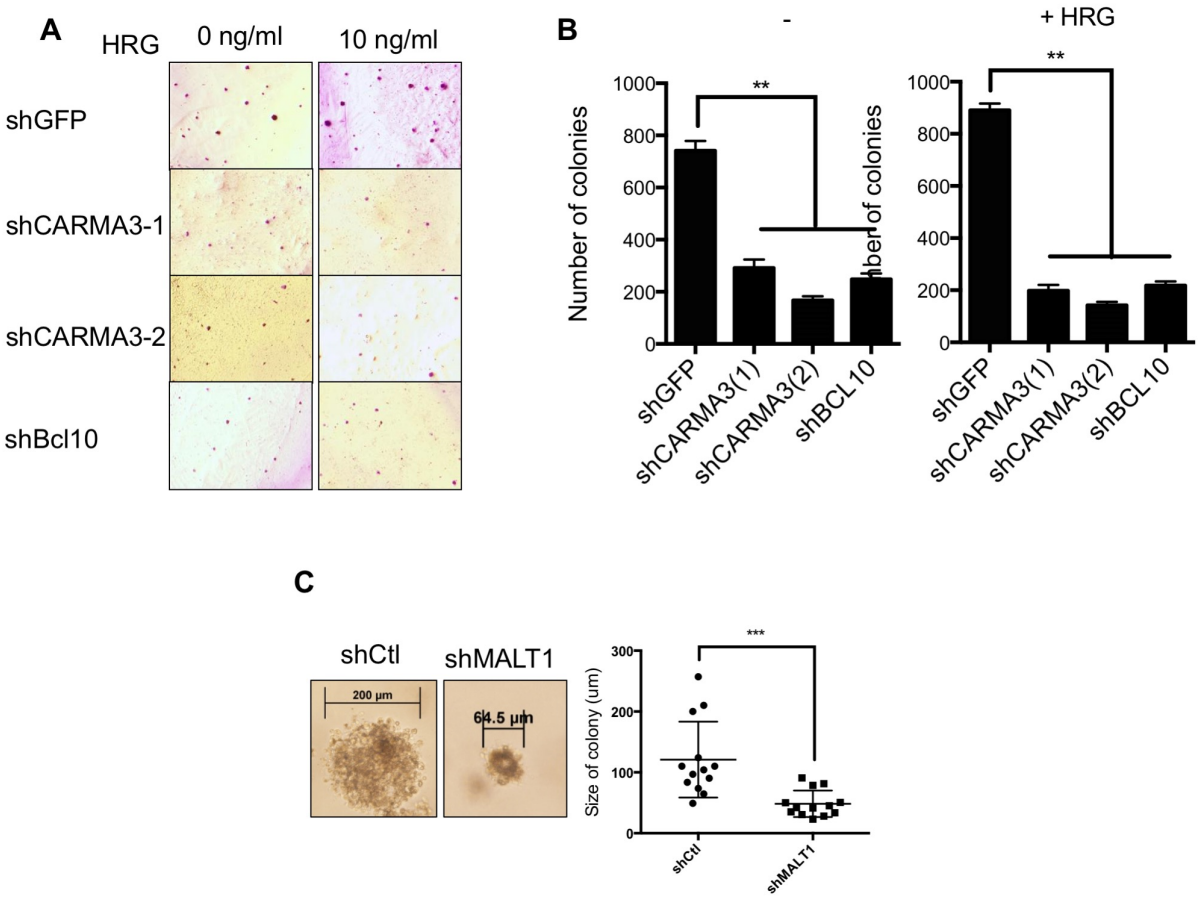

Figure 27. The CBM complex is required for anchorage-independent growth of SKBR3 cells *in vitro*.

(A) SKBR3 cells with control knockdown (shCtl), BCL10 knockdown (shBCL10), or CARMA3 knockdown by 1 of 2 different shRNAs (shCARMA3-1 and shCARMA3-2) were subjected to the colony formation assay in the presence or absence of 10 ng/mL heregulin for 2 weeks. Five random fields were photographed; one was presented here. (B) Quantification of colonies in panel A in the presence or absence of HRG. (C) SKBR3 cells with control knockdown (shCtl) or MALT1 knockdown (shMALT1) were subjected to the colony formation assay in the presence of HRG (10 ng/ml). The sizes of the colonies were quantified and analyzed (right).

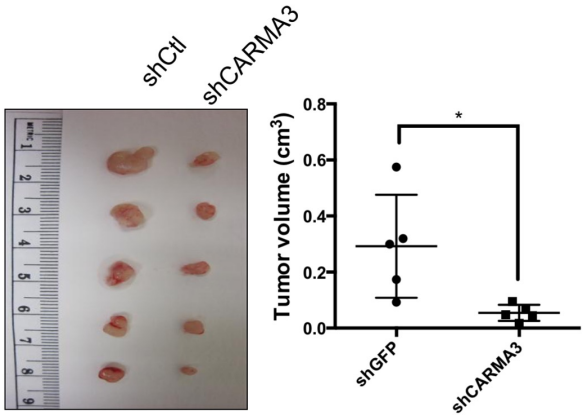

Figure 28. CARMA3 is required for tumor progression on a xenograft mouse model. SKBR3 cells with control knockdown (shCtl) or CARMA3 knockdown (shCARMA3-2) were subcutaneously injected on each side of 5 SCID mice to develop xenograft tumors. The mice were euthanized 6 weeks after injection and size of the tumors was determined.

3.3.4 The CBM complex contributes to cell migration and invasion

It has been suggested that NF-κB signaling plays a crucial role in cell migration and invasion (Huber et al. 2004). Therefore, we examined the involvement of the CBM complex in HRG-induced cell migration and invasion *in vitro* using the transwell migration assay. We found that silencing CARMA3 or BCL10 significantly inhibited cell migration (Figure 29A) and invasion (Figure 29B) in response to either serum or HRG stimulation. Similar results were also found in SKBR3 cells with silenced MALT1 (Figure 30). These results suggest that the CBM complex is required for HRG-induced cell migration and invasion *in vitro*.

To further validate this finding *in vivo*, we generated a spontaneous lung metastasis model by injecting CARMA3-silenced and control SKBR3 cells into SCID mice. We found significantly fewer lung metastatic sites in mice injected with CARMA3-silenced cells than in mice injected with control cells (Figure 31), suggesting that CARMA3 contributes to breast cancer metastasis *in vivo*. Taken together, these results indicate that the CBM complex contributes to cell migration and invasion *in vitro* and *in vivo*.

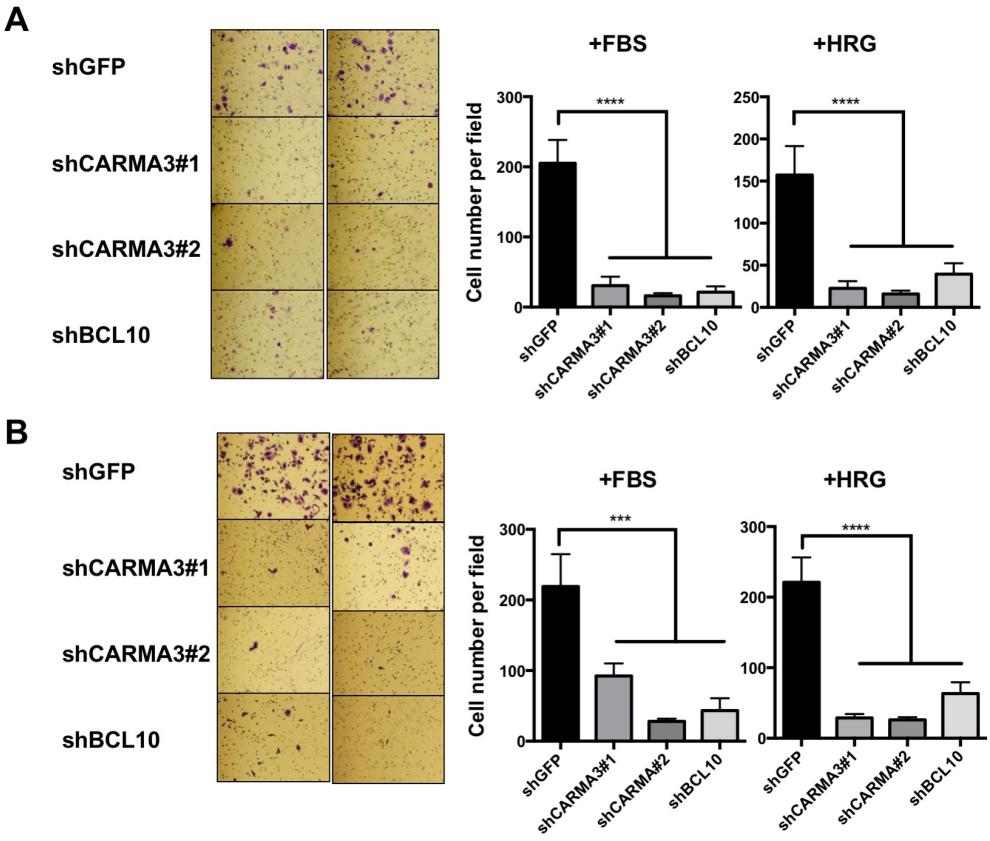

Figure 29. CARMA3 and BCL10 are required for serum and HRG induced cell migration and invasion.

(A) SKBR3 cells with control knockdown (shCtl), BCL10 knockdown (shBCL10), or CARMA3 knockdown by 1 of 2 different shRNAs (shCARMA3-1 and shCARMA3-2) were subjected to the transwell migration assay in the presence of either fetal bovine serum or heregulin (10ng/ml). After 16 hours, cells were fixed, stained, and photographed using inverted microscope. The number of migrated cells was quantified (right panel). (B) SKBR3 cells with shRNA knockdown as in A were subjected to the invasion assay for 48 hours to penetrate matrigel. Following incubation, the cells were fixed, stained, and quantified (right panel).

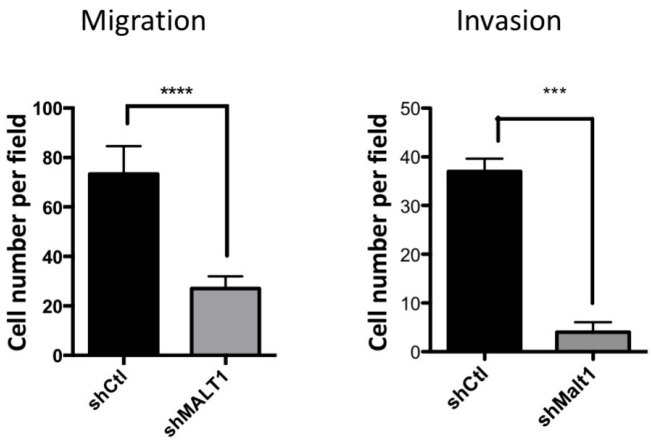

Figure 30. MALT1 is required for serum induced cell migration and invasion.

SKBR3 cells with control knockdown (shCtl) or MALT1 knockdown (shMALT1) were subjected to the transwell migration and invasion assays in the presence of fetal bovine serum. After 16 hours (for migration) and 24 hours (for invasion), cells were fixed, stained, and photographed using inverted microscope. The number of migrated cells were quantified.

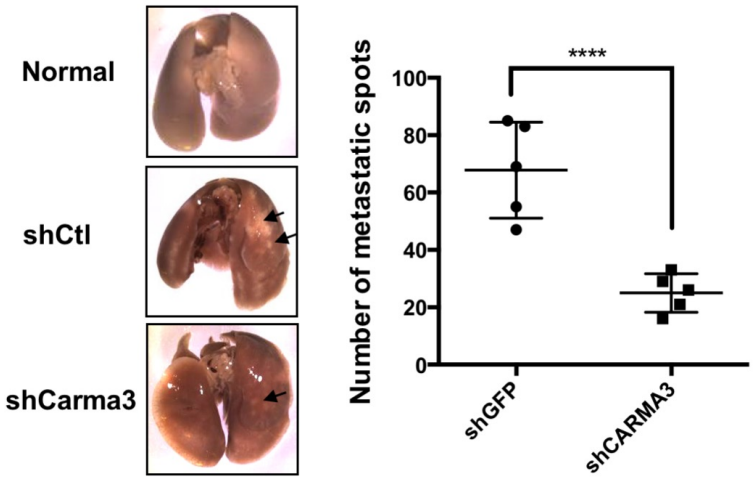

Figure 31. CARMA3 is required for lung metastasis in a mouse model.

SKBR3 cells with a control knockdown (shCtl) or CARMA3 knockdown (shCARMA3) were intravenously injected into 6-week-old female SCID mice. After 6 weeks, mice were euthanized and lungs were washed, fixed, and stained with Bouin's solution. The number of metastatic tumors was quantified and analyzed.

3.3.5 CARMA3 contributes to migration and invasion through regulating HER2-induced MMP1 and MMP13 expression

To further examine the mechanism by which the CBM complex controls cell migration and invasion, we examined a set of matrix metalloproteinases (MMPs) and found that the expression levels of MMP1 and MMP13 were significantly upregulated after HRG stimulation in SKBR3 cells (Figure 32 A-B). In comparison, MMP3 and MMP7 were not responsive to HRG stimulation whereas MMP14 was downregulated by HRG stimulation (Figure 32C-E). Furthermore, the induction of MMP1 and MMP13 was significantly blocked by treatment with an IKK inhibitor (TPCA) or NF-κB translocation inhibitor (NF-κBi), or PKC inhibitor (GF109203X), respectively (Figure 33). These data indicate that the induction of MMP1 and MMP13 depends on PKC- and IKK-mediated canonical NF-B activation in response to HRG stimulation.

Because the CBM complex is required for NF-κB activation in response to HRG stimulation, we next determined whether the CBM complex regulates HER2-induced MMP induction. We found that the induction of MMP1 and MMP13 was significantly blocked in CARMA3-silenced cells compared with control cells, indicating that CARMA3 is required for HER2-induced MMP1 and MMP13 expression (Figure 34). Collectively, our results indicate that the CBM complex-dependent NF-κB pathway regulates MMP1 and MMP13 expression in response to HER2 activation.

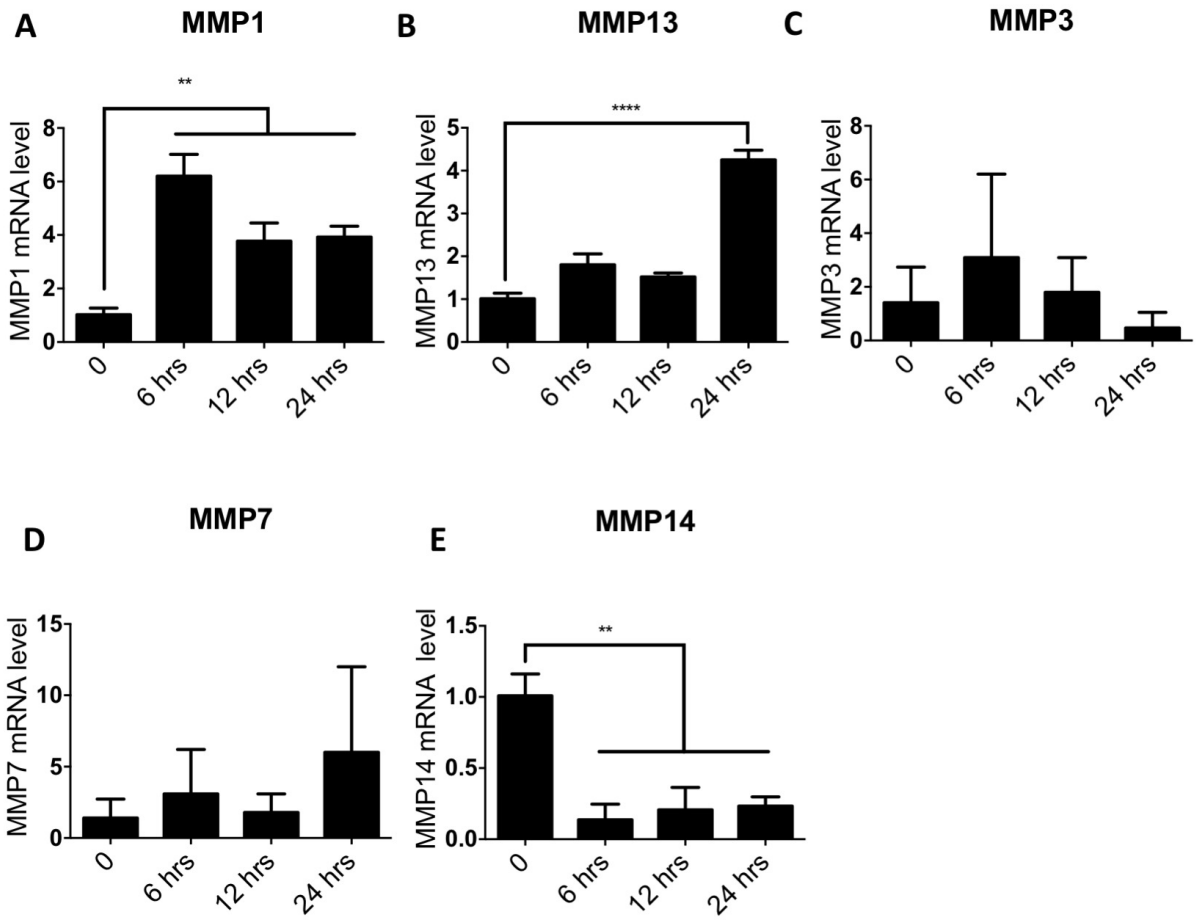

Figure 32. MMP1 and MMP13 are upregulated upon HRG stimulation in SKBR3 cells. SKBR3 cells were stimulated with (HRG; 50ng/ml) for 6, 12, or 24 hours, respectively. Total RNA was extracted for quantitative polymerase chain reaction (PCR) analysis. Levels of the indicated mRNAs were normalized to GAPDH.

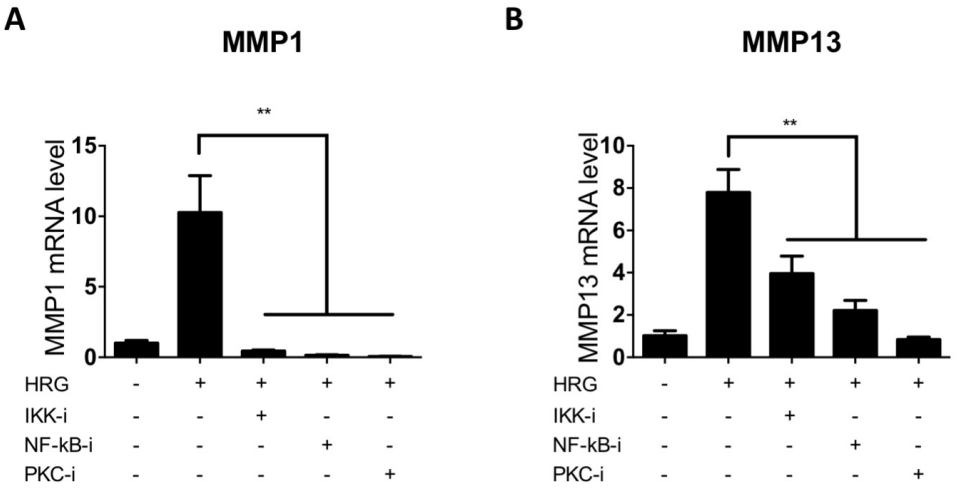

Figure 33. Upregulation of MMP1 and MMP13 depends on PKC-IKK-NF-κB pathway.

SKBR3 cells were stimulated with HRG (50ng/ml) for 24 hours in the presence of the IKK inhibitor TPCA (IKKi), NF-κB translocation inhibitor SN50 (NF-Bi), or protein kinase C inhibitor GF109203X (PKCi). Total RNA was extracted for quantitative PCR analysis. Level of MMP1 and MMP13 were normalized to GAPDH.

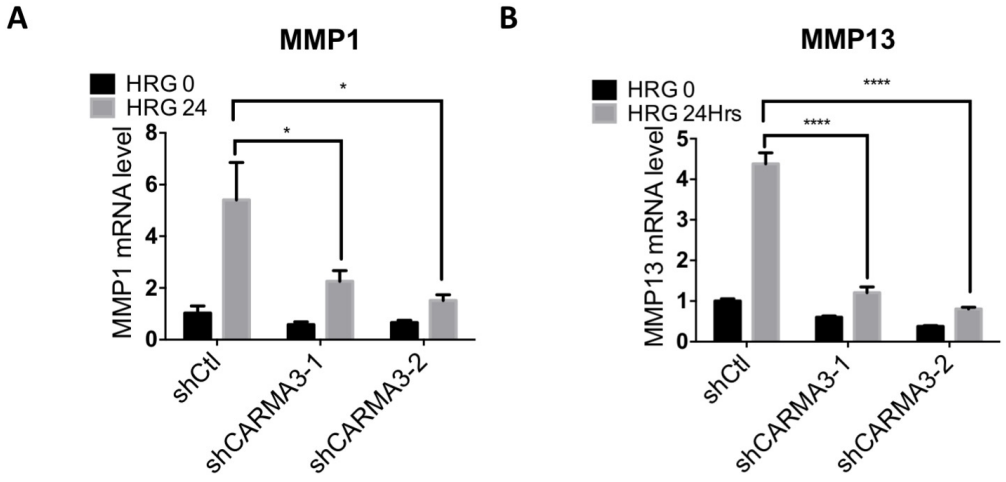

Figure 34. CARMA3 is required for HER2-induced MMP1 and MMP13 upregulation. SKBR3 cells with control knockdown (shCtl), or CARMA3 knockdown by 1 of 2 different shRNA (shCARMA3-1 and shCARMA3-2) were stimulated with HRG (50ng/ml) for 24 hours. Total RNA was extracted for quantitative PCR analysis. Levels of MMP1 and MMP13 were normalized to GAPDH.

3.3.6 MALT1 contributes to breast cancer progression *in vivo*

To investigate the contribution of the CBM complex contributes to HER2-induced breast cancer progression, we backcrossed Malt1 knockout mice (Malt1$^{-/-}$) from a C57BL/6 background to an FVB/N background and then crossed these mice with *MMTV-Neu* mice to generate *MMTV-Neu; Malt1*$^{-/-}$ mice, in which mammary tumors develop in the absence of Malt1 expression. Both control mice (*MMTV-Neu; Malt1*$^{+/+}$ and *MMTV-Neu; Malt1*$^{+/-}$) and Malt1 knockout mice (*MMTV-Neu; Malt1*$^{-/-}$) developed mammary tumors owing HER2 overexpression (Figure 35A). However, we found that Malt1 knockout mice (*MMTV-Neu; Malt1*$^{-/-}$) showed longer tumor latency than controls (Figure 35B). In addition, the overall survival durations in Malt1 knockout mice (*MMTV-Neu; Malt1*$^{-/-}$) were significant longer than in control mice (Figure 35C), indicating that the mammary tumors progressed at a slower rate in Malt1 knockout mice than in control mice. Together, these data indicates that MALT1 contributes to HER2-induced mammary tumor progression *in vivo*.

In summary, this study demonstrated that the CBM complex plays a crucial role in HER-associated beast tumor by regulating HER-induced NF-κB pathway (Figure 36).

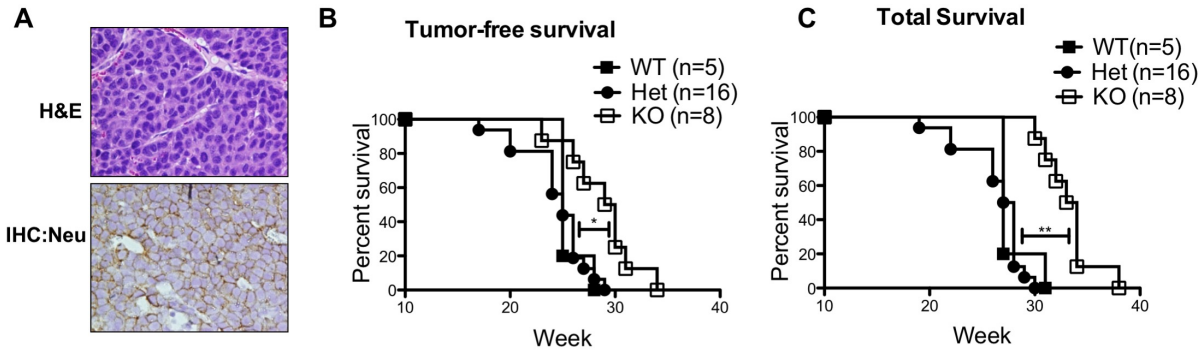

Figure 35. MALT1 contributes to HER2-induced mammary tumor progression *in vivo*. (A) Hematoxylin and eosin staining of mammary tumors developed in *MMTV-Neu* mice (upper panel) and immunohistochemistry staining with anti-Neu antibodies of mammary tumors developed in *MMTV-Neu* mice (lower panel). (B) Mammary tumor latency was compared among wild-type mice (*Malt1*$^{+/+}$*; MMTV-Neu*; n=5), Malt1 heterozygous mice (*Malt1*$^{+/-}$*; MMTV-Neu*; n=16) and Malt1 knockout mice (*Malt1*$^{-/-}$*; MMTV-Neu*; n=9). Log-rank test; P=0.0423 (C) The overall survival time of the mice in panel B was calculated and analyzed. Log-rank test; P=0.0040

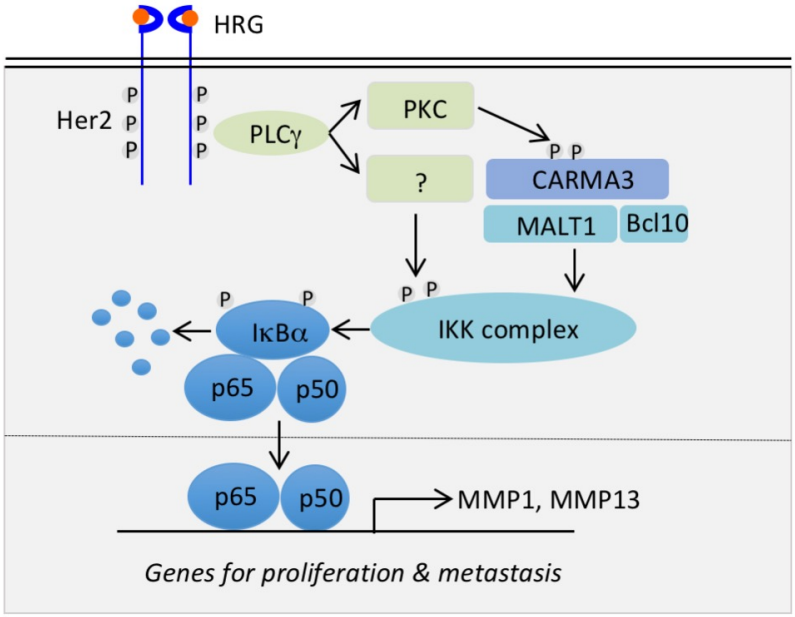

Figure 36. The working model summarizing the findings in this study.

The CARMA3-BCL10-MALT1 complex is required for HER2-mediated NF-κB activation in breast cancer cells. The HER2-induced NF-κB contributes to many malignant phenotypes of breast cancers, including proliferation, survival, migration and metastasis. Mechanistically, NF-κB transcribes MMP1 and MMP13 to facilitate migration and invasion.

3.4 Discussion

3.4.1 HER2-induced NF-κB is mediated by PKC-CBM-IKK axis instead of AKT

Although NF-κB plays a central role in many aspects of malignancy in HER2-positive breast cancer, the molecular mechanism by which HER2 activates NF-κB remains to be determined. Our previous studies identified the CBM complex as a central mediator of T cell receptor- and B cell receptor-induced NF-κB activation in lymphoid cells (Wang et al. 2002). In addition, our group and others reported that both GPCR and EGFR utilize the CBM complex to activate NF-κB in nonhematopoietic cells (Grabiner et al. 2007, Klemm et al. 2007, McAllister-Lucas et al. 2010, Jiang et al. 2011). In the current study, using biochemical and genetic approaches, we showed that HER2-induced NF-κB activation is mediated by the CBM complex. In addition, we found that PKC activity is required for the activation of NF-κB in response to HRG stimulation. This result is consistent with the idea that PKC is the upstream activator of CARMA1 or CARMA3 (Blonska and Lin 2011). Thus, we propose that the PKC-CBM axis is the major pathway that links HER2 to NF-κB activation.

The PKC-CBM axis also mediates EGFR-induced NF-κB activation, as we have previously shown (Jiang et al. 2011, Pan et al. 2015). Thus, our study reveals a shared mechanism by which the CBM complex mediates NF-κB activation in response to activation of the receptors in the EGFR family, and possibly activation of other types of tyrosine kinase receptors such as fibroblast growth factor receptor, which also activates PKC (Jiang and Lin 2012). Further investigation is needed to determine whether the CBM complex mediates

NF-κB activation when other types of tyrosine kinase receptors outside of the EGFR family are activated.

3.4.2 The role of NF-κB in HER2-associated breast tumor progression

A major treatment strategy for HER2-positive breast cancer is to block the HER2 receptor using monoclonal antibodies such as trastuzumab (Mitri et al. 2012). However, the inevitable obstacle for such therapy is that patients quickly develop drug resistance. Several preclinical studies showed that a combination of trastuzumab with a PI3K inhibitor overcomes trastuzumab resistance (Eichhorn et al. 2008, Serra et al. 2008, Junttila et al. 2009), indicating that blocking the major downstream effector pathways is an alternative way to prevent trastuzumab resistance. Although a previous study suggested that AKT activation is responsible for NF-κB activity in HER2-overexpressed breast cancer cell lines (Pianetti et al. 2001), we showed that NF-κB and AKT are indeed 2 independent pathways downstream of HER2, because the AKT inhibitor did not affect NF-κB activation and the PKC inhibitor did not affect AKT activation. These findings provide a molecular basis and rationale for targeting the PKC-CBM complex-NF-κB pathway in HER2-positive breast cancer. It is also worth examining in the future studies whether combining an NF-κB inhibitor with trastuzumab and a PI3K inhibitor leads to synergistic effects.

Using functional assays and mouse models, we also showed that the CBM complex is required for tumor cell proliferation, survival, and migration. We observed that the CBM complex is played the most significant role in terms of cell migration and invasion. This observation is consistent with a previous report that silencing IKKα abolished NF-κB activation and decreased the invasiveness of SKBR3 cells (Merkhofer et al. 2010). However, the mechanism by which NF-κB controls tumor cell migration and invasion remains largely unknown. In the current study, we sought to determine whether CBM complex-mediated NF-κB activity affected the expression of MMP family, which is known to play a crucial role in tumor metastasis. Indeed, we found that MMP1 and MMP13 were highly induced after HRG stimulation in a PKC-, CARMA3-, IKK complex- and NF-κB-dependent manner, which strongly suggests that HER2-induced NF-κB is crucial for the induction of these MMPs. It has been reported that MMP1 and MMP13 are involved in breast cancer metastasis (Pivetta et al. 2011, Liu et al. 2012)(24, 25). Thus, our data indicate that NF-κB signaling contributes to cell invasiveness at least in part through MMP1 and MMP13 in HER2-positive breast cancer cells.

In summary, our data reveal the molecular mechanism by which HER2 activates NF-κB through PKC and the CBM complex. In addition, our results also highlighted the contribution of the CBM complex and NF-κB signaling to the characteristics of malignancy in HER2-positive breast cancer cells and provide a molecular basis for targeting NF-κB signaling in HER2-positive breast cancer.

CHAPTER 4. THE ROLE OF CBM COMPLEX IN DNA DAMAGE-INDUCED NF-κB ACTIVATION AND RESISTANCE TO CHEMOTHERAPY-INDUCED CELL DEATH

4.1 Introduction

4.1.1 General overview DNA repair and DNA damage response

It is estimated that there are about 10,000 of DNA lesions occur within each cells in our human body each day (Pivetta et al. 2011). As the central hereditary materials in the cell nuclei, the integrity of DNA is extremely important. If not being repaired properly, DNA damage can lead to genetic instability and eventually accelerate the onset of cancer. The causes of DNA damage come from a variety of sources, such as ionizing radiation, chemotherapeutic drugs, ultraviolet exposure, reactive oxygen species and mechanical stress on chromosomes. The types of DNA damage includes base modifications, such as methylation and oxidation, mispairs, which come from mistakes in DNA synthesis, cross-liked nucleotides, such as intrastrand and interstrand links, and double-stranded DNA breaks. Depending on the types of DNA damage, cell utilizes different mechanisms to repair DNA damage.

Base excision repair (BER) corrects small base DNA lesion most commonly caused by oxidation, deamination or alkylation. The common steps of BER includes excision, incision, end processing repair synthesis and ligation (Krokan and Bjoras 2013). Nucleotide excision repair (NER) is an important mechanism the removes DNA damage induced by ultraviolet. The NER pathway can be categorized into global genome nucleotide excision repair (GG-NER) and transcription-coupled nucleotide excision repair (TC-NER) (Marteijn et al. 2014). DNA mismatch repair (MMR) is also a highly conserved biological pathway to maintain genomic stability from *E.coli* to the mammalian cells. The general role of MMR is to recognize and correct base substitution mismatches during DNA replication. In *E.coli*,

MMR is initiated by MutS, MutL and MutH. MutS is the mismatch recognition protein which recognizes base-base mismatches or small nucleotide insertion or deletion. After recognition, MutL, which constantly interacts with MutS, recruits and activates the endonuclease MutH, which nicks the newly synthesized DNA and corrects the strand. After nicking, helices II is loaded and generates a single-strand DNA, which binds to SSB (single stranded DNA-binding protein) and is protected from nuclease attack (Ramilo et al. 2002). Afterwards, endonuclease such as Exon and RecJ will be recruited to excise the nicked strand from the nick site and resulting single-stranded gap will be repaired by DNA polymerase III, SSB and DNA ligase to finish the process. The human MMR pathway is very similar to that in *E.coli*, and a defect of MMR pathway is tightly linked to the development of cancer. For example, it has been shown that germ-line mutations in hMSH2 is associated with hereditary non-polyposis colorectal cancer (HNPCC) (Fishel et al. 1993, Leach et al. 1993).

In case of double stranded DNA break, cells either utilize homologous recombination (HR) or non homologous end joining (NHEJ), which are important repair processes to repair double stranded DNA break. For example, the initial process of NHEJ involves the binding of the protein complex Ku70/80 heterodimer with both ends of DNA double stranded break. Subsequently, the Ku70/80 complex will attract DNA-dependent protein kinase catalytic subunit (DNA-PKCS) to bring the DNA ends together. Meanwhile, nucleases and polymerases will be recruited to the double stranded break to remove or fill-in single stranded overhangs. Finally, the ligase/XRCC4 complex will catalyze the ligation process (Weterings and Chen 2008). The NHEJ plays a particular important role in the V(D)J recombination, a process in which lymphocytes diversity is generated in the vertebrate

immune system. The error-prone feature of NHEJ is beneficial and maximize the diversity of immune receptors (Jung and Alt 2004). By contrast, HR is commonly considered as error free as it ensure accurate repair by using the other undamaged sister chromatid as a temple.

In addition to DDR, DNA damage can also trigger a variety of cellular processes, including replisome stability, transcription, cell cycle regulation, metabolic changes and autophagy, chromatin remodeling, RNA processing and apoptosis. Two key signaling components in the DDR pathway are protein kinases ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia And Rad3-Related Protein). ATM/ATR phosphorylates a number of substrates to regulate many cellular processes. For example, upon DNA damage, ATM/ATR phosphorylate CHK1 (Checkpoint kinase 1) and CHK2 (Checkpoint kinase 2) to induce cell cycle arrest and apoptosis by inhibition of the activity of cyclin-dependent kinase (CDK) and stabilization of P53 (Jackson and Bartek 2009).

4.1.2 Molecular mechanism by which DNA damage-induces NF-κB activation

It has been suggested that DNA damage also triggers the activation of NF-κB, which provides a pro-survival signals to the cell to analogize apoptosis induced by DNA damage (Janssens and Tschopp 2006). Unlike traditional NF-κB activation induced by extracellular stimuli, DNA damage-induced NF-κB is activated from a signal from the nuclei. Therefore, DNA damage-induced NF-κB is also called as “inside out” NF-κB activation (McCool and Miyamoto 2012). As the central inducer of DDR, ATM is also required for DNA damage-induced NF-κB activation. By using ATM-deficient cells, Piret et al. showed that ATM is

required for CPT (camptothecin)-induced NF-κB activation (Piret et al. 1999). This study established a molecular link between ATM and DNA damage-induced NF-κB activation (Piret et al. 1999). Subsequently, Wu et al. showed that ATM phosphorylates NEMO (IKKγ) at the site (Ser85) to promote its ubiquitin-dependent nuclear export, which also bridges the nuclear export of ATM (Wu et al. 2006). Since NEMO is an essential component in NF-κB pathway, this study further established the ATM-NEMO axis in DNA damage-induced NF-κB activation (Wu et al. 2006).

Huang et al. showed that the C-terminal zing finger (ZF) of NEMO is required for ultraviolet- and etoposide-induced NF-κB activation, while ZF domain is largely dispensable for LPS- and TNF-induced NF-κB activation. This data is one of the earliest mechanistic insights suggesting DNA damage utilizes key signaling component in the canonical NF-κB pathway to activate NF-κB. Subsequently, Huang et al. further showed that both sumoylation and ubiquitination of NEMO by SUMO-1 (Small Ubiquitin-Like Modifier 1) is required for DNA damage-induced NF-κB activation. Furthermore, the authors showed that ATM-independent SUMO-1-mediated sumoylation of NEMO triggers the nuclear localization of NEMO. In addition ATM-dependent ubiquitylation of NEMO mediates the activation of the IKK complex in the cytosol (Huang et al. 2003). Taken together, both studies indicate an essential role of NEMO in the DNA damage-induced NF-κB pathway.

Subsequently, a number of studies also indicate the involvement of other signaling molecules in DNA-damage induced NF-κB pathway. These molecules involve TAK1 (Transforming Growth Factor-Beta-Activated Kinase 1), RIP1 (Receptor-Interacting Protein 1), ELKS (essential regulatory subunit of the IKK complex), PIASy (protein inhibitor of activated STATy), PIDD (p53-inducible death-domain-containing protein) and TRAF6 (Hinz et al. 2010, Wu et al. 2010, Yang et al. 2011). However, it still remains unclear that how NEMO is activated upon DNA damage induction (Figure 37). As a central inducer of mediating NEMO ubiquitination and activation, here we propose to test a potential role of the CBM complex in DNA damage-induced NF-κB activation.

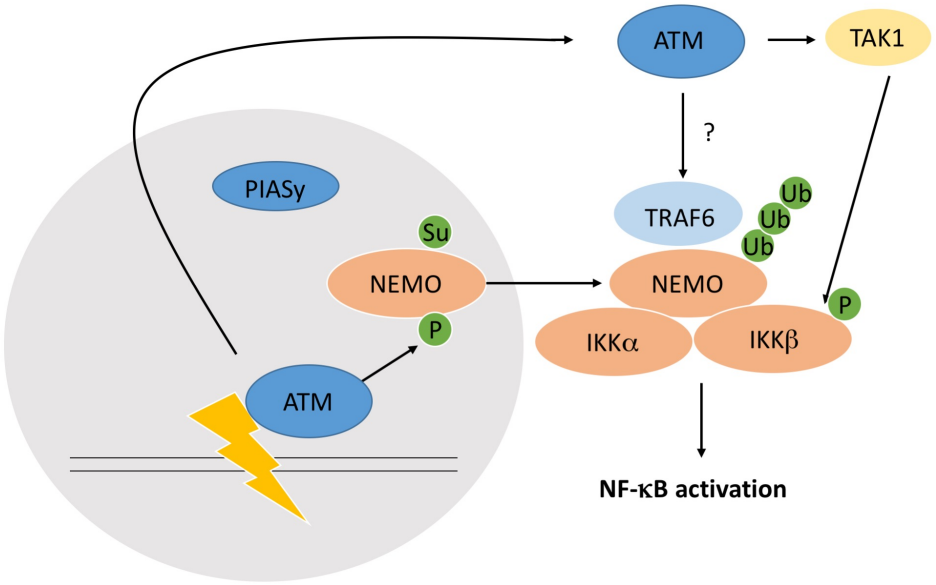

Figure 37. Schematic overview of DNA damage induced NF-κB activation.

DNA damage-induced NF-κB activation requires the activation of ATM, which phosphorylates NEMO in the nucleus to trigger the nuclear export of NEMO. In addition, TAK1 and TRAF6 are also believed to mediate the activation of IKK complex. In this model, the molecular mechanism by which TRAF6 is activated remains unclear.

4.2 Materials and Methods

4.2.1 Cells

Primary MEFs were generated from E13.5 from *Malt1*$^{+/-}$ mice. MEFs were grown in DMEM medium containing 10% fetal bovine serum at 37ºC with 8% CO_2.

4.2.2 Doxorubicin-induced apoptosis

To access Doxorubicin-induced cell apoptosis, Annexin V and propidium iodide (PI) staining was performed. Briefly, doxorubicin (1.5ug/ml) will be introduced into confluent MEFs in 6-well plate. After 6 hours and 12 hours, respectively, MEFs were digested by 0.05% tripsin and cells will be washed by PBS and suspended in 100μl of 1X binding buffer provided by Annexin V staining kit (eBioscience, #88-8007). 5μl of Annexin V-FITC will be added to the cell suspension and will be incubated for 15 minutes at 4 degree. 3μl of PI-APC will be added to the suspension and will be incubated for 5 minutes at 4 degree. The suspension will be washed 1 time will 1 X binding buffer and will be suspended in 200μl of 1 X binding buffer. The pro-apoptotic and apoptotic cells will be access by flow cytometry.

4.2.3 Immunoblotting assay

For immunoblotting assay was performed as described (Pan et al. 2015). One million of cells were lysed in 100μl of lysis buffer (150 mM NaCl, 50 mM HEPES at pH 7.4, 1 mM EDTA, 1% NP-40, protease inhibitors) for 10 minutes. The cell lysates will be purified by spinning 13,000 r.p.m. for 10 minutes and resulting supernatants will be saved for western

blot analysis. 10-12μl of the resulting supernatant were loaded to 10% of SDS-PAGE and probed with antibodies.

4.2.4 EMSA

EMSA (electrophoretic mobility shift assay) was performed as described (Pan et al. 2015). Two million cells were starved overnight in DMEM containing 0.5% serum and stimulated with genotoxic reagents, including doxorubicin, VP16, and campthothecin. Nuclear extracts were isolated and were incubated with 1×10^5 c.p.m. of ^{32}P-labeled probes at room temperature for 15 minutes. The samples were separated on a native Tris-borate-EDTA polyacrylamide gel and analyzed by autoradiography.

4.2.5 Immunoprecipiation assay

Generally, five million of cells were lysed in 300μl of lysis buffer (150 mM NaCl, 50 mM HEPES at pH 7.4, 1 mM EDTA, 1% NP-40, protease inhibitors) for 10 minutes. The cell lysates will be purified by spinning 13,000 r.p.m. for 10 minutes and resulting supernatants will be saved for Immunoprecipiation assay. 30μl of the protein lysis will be saved for western blot analysis as input. The rest lysis will be incubated together with indicated antibodies and protein A or protein G agarose beads for overnight at 4 degree. After incubation, the beads will be washed three times with lysis buffer and will be suspended in 30μl of lysis buffer containing SDS for western blot analysis.

4.3 Results

4.3.1 MALT1 regulates doxorubicin-induced apoptosis

Since MALT1 contributes to cell survival by regulating growth factor-induced NF-κB pathway in tumor cells (Pan et al. 2015, Pan et al. 2015), we propose to test whether MALT1 also affects cell death induced by other agents, such as doxorubicin, which is a drug frequently used in chemotherapy (Lao et al. 2013). To this end, we treated primary *Malt1*-heterozygous MEFs (*Malt1*$^{+/-}$) and *Malt1*-knockout MEFs (*Malt1*$^{-/-}$) with 1.5μg/ml of doxorubicin for 6 hours and 12 hours, respectively. We observed that in MALT1-deficient MEFs there were more floating cells than controls, indicating that there were more apoptotic cells in MALT1-deficient MEFs than control cells. We further quantified the dead cells and apoptotic cells by AnnexinV and PI staining. Before treatment, there was no difference of combined apoptotic and dead cell population between controls MEFs and MALT1-deficient MEFs (2.26%+3.18% vs. 2.73%+2.76%) (Figure 38). However, we observed that there were about 2 fold of combined dead cells and apoptotic cells in MALT1-deficient MEFs compared with controls for both 6 hours (1.94%+3.33% vs. 4.27%+5.97%) and 8.5 hours (1.68% +2.95% vs 4.72%+5.07%) after doxorubicin treatment (Figure 38). This data indicates that MALT1 regulates pro-survival signaling to antagonize doxorubicin-induced apoptosis in MEFs.

0hr

6hrs

8.5hrs

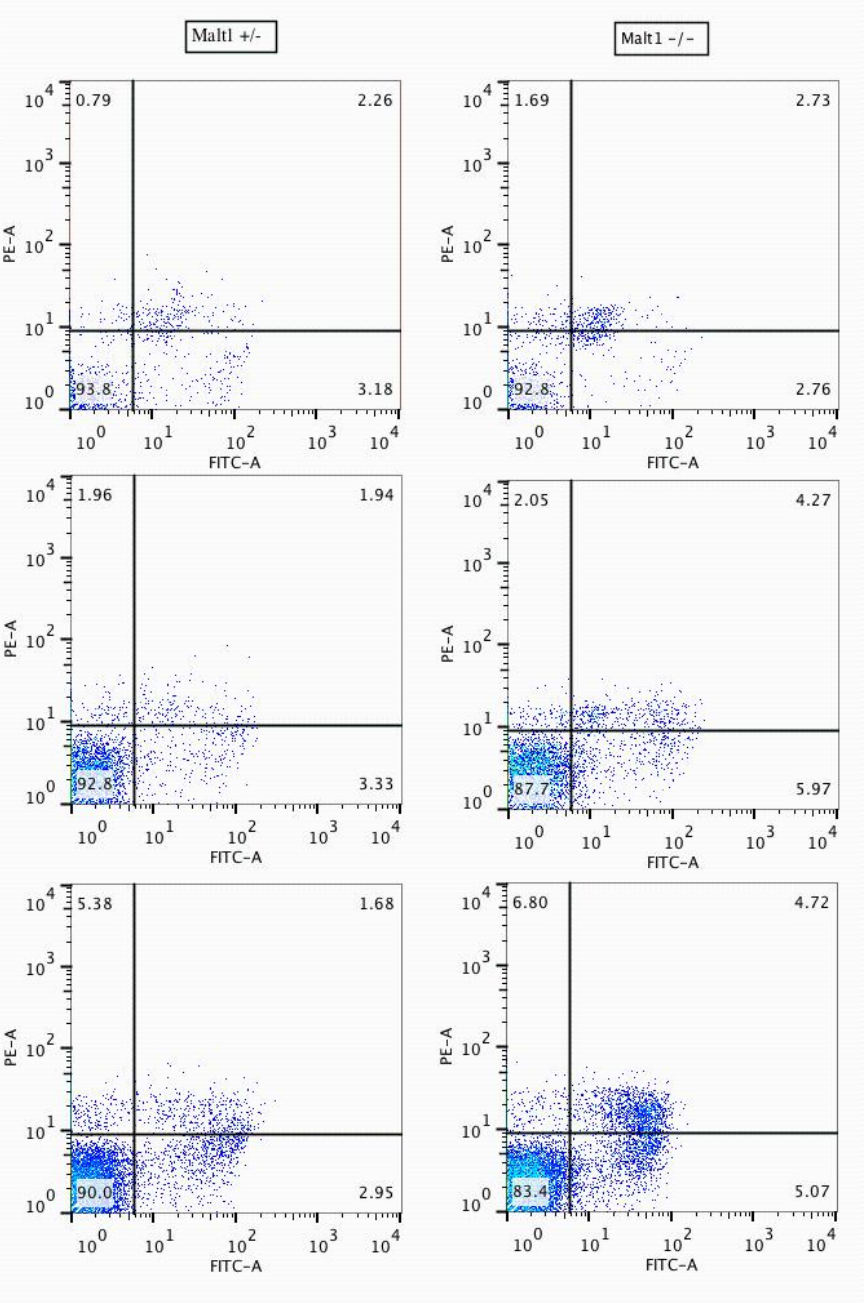

Figure 38. MALT1 regulates pro-survival signaling to antagonize doxorubicin-induced apoptosis in MEFs.

Malt1$^{+/-}$ and *Malt1*$^{-/-}$ MEFs were treated with doxorubicin (1.5μg/ml) for 6 and 8 hours, respectively. Dead cell and apoptotic cell population were accessed by Annexin V and PI staining.

4.3.2 MALT1 is required for DNA damage induced NF-κB activation

Since MALT1 is involved in NF-κB activation in response to many kinds of stimuli, including antigen receptors, c-type lectin receptors GPCR, EGFR and HER2 (Blonska and Lin 2011, Pan and Lin 2013), we reasoned that MALT1 may also affects apoptosis by regulating NF-κB activity in response to DNA damage, as NF-κB provides pro-survival signals. To this end, we treated control MEFs (*Malt1*$^{+/-}$) and MALT-deficient MEFs (*Malt1*$^{-/-}$) with doxorubicin (1.5μg/ml) and examined NF-κB activation by EMSA. We found that doxorubicin potently induced NF-κB activation in control cells, which is consistent with previous findings (Janssens and Tschopp 2006). However, doxorubicin-induced NF-κB activation was completely defective in MALT1-deficient MEFs (Figure 39A), indicating that MALT1 is required for doxorubicin-induced NF-κB activation.

Doxorubicin is a genotoxic reagent and induces DNA damage by inhibiting the activity of topoisomerase II, which relaxes the supercoils in DNA for transcription (Pommier et al. 2010). Therefore, to generalize our finding, we hypothesize that MALT1 is required for DNA damage induced NF-κB activation. To this end, we treated control MEFs (*Malt1*$^{+/-}$) and MALT-deficient MEFs (*Malt1*$^{-/-}$) with two other genotoxic reagent VP16 (etoposide) (10μM) and CPT (2μM), respectively. Consistent with our hypothesis, both VP16- and CPT-induced NF-κB activation were defective in MALT1-deficient cells compared with controls (Figure 39B), indicating that MALT1 is required for DNA damage-induced NF-κB activation.

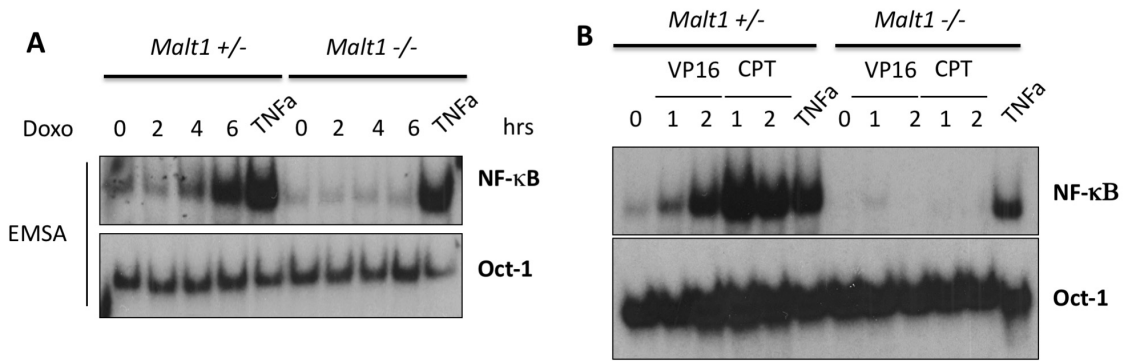

Figure 39. MALT1 is required for DNA damage-induced NF-κB activation.

(A) MEFs from *Malt1*$^{+/-}$ and *Malt1*$^{-/-}$ embryos were isolated. Early-passage (P1) MEFs were stimulated with doxorubicin (1.5μg/ml) or TNF (10ng/ml) for indicated periods. NF-κB activation and Oct-1 levels were determined by the gel shift assay. (B) *Malt1*$^{+/-}$ and *Malt1*$^{-/-}$ MEFs were stimulated with VP16 (10μM), CPT (2μM) or TNF (10ng/ml) for indicated periods. NF-κB activation and Oct-1 levels were determined by the gel shift assay.

4.3.3 The CBM complex is required for DNA damage-induced NF-κB activation

MALT1 forms a complex with CARMA3 and BCL10 in non-hematopoietic cells to activate IKK complex and NF-κB (Blonska and Lin 2009). Therefore, we would like to test that whether CARMA3 and BCL10 are also involved in DNA damage-induced NF-κB activation. We isolated CARMA3-deficient (*Card10-/-*) and BCL10-deficient (*Bcl10-/-*) MEFs together with wild-type controls. We treated these cells with doxorubicin and found that, both CARMA3-deficient and BCL10-deficient cells were completely defective for NF-κB activation upon doxorubicin treatment (Figure 40A). This data indicates that CARMA3 and BCL10 are also involved in DNA damage-induced NF-κB activation.

To further test whether the same phenomenon can be observe in other cell types, we tested Jurkat cells and its derivatives JPM50.6 cells, in which the expression of CARMA1 is disrupted (Wang et al. 2002). We treated JPM50.6 cells and wild-type Jurkat cells as control with different types of genotoxic reagents, including doxorubicin (1.5μg/ml), VP16 (10μM), CPT (2μM), or TNF (10ng/ml) as control. Consistent with the finding in MEFs, we found that genotoxic reagents-induced NF-κB activation was significantly reduced in JPM50.6 cells compared with control (Figure 40B), indicating that the CARMA1-BCL10-MALT1 complex in the hematopoietic cells is also required for DNA damage-induced NF-κB activation. In addition, all cells showed similar level of P53 stabilization (Figure 40B), indicating the general DNA damage response is not affected by the CBM complex. Together, these results indicate that CBM complex specifically mediates DNA damage-induced NF-κB activation.

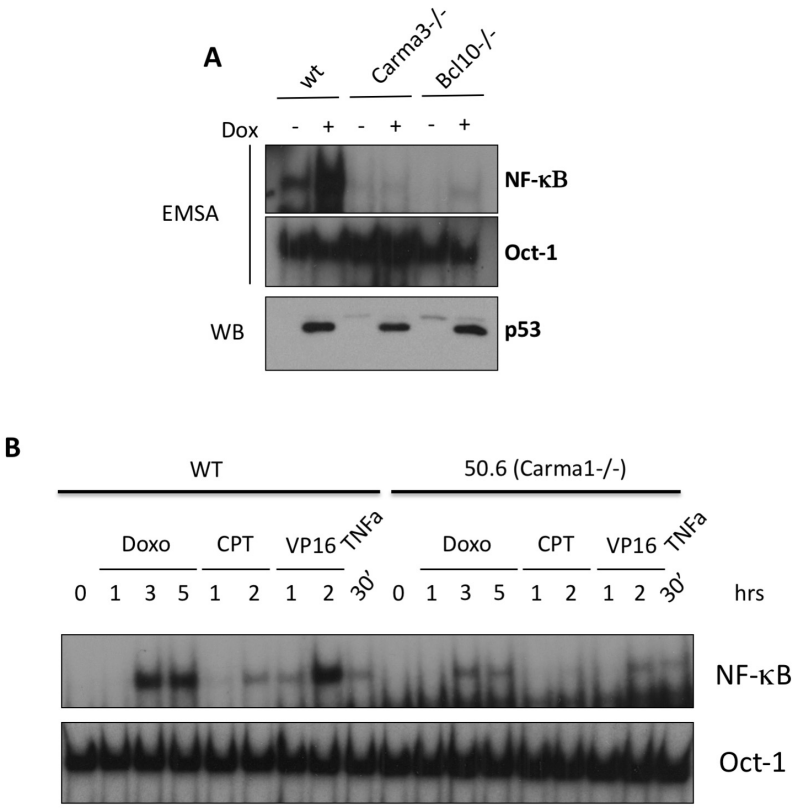

Figure 40. The CBM complex is required for DNA damage-induced NF-κB activation.

(A) MEFs from *Card10*$^{+/+}$, *Card10*$^{-/-}$, *Bcl10*$^{+/+}$ *and Bcl10*$^{-/-}$ embryos were isolated. Early-passage (P1) MEFs were stimulated with doxorubicin (1.5μg/ml) for indicated periods. NF-κB activation and Oct-1 levels were determined by the gel shift assay. (B) Wild-type Jurkat and 50.6 cells were treated with doxorubicin (1.5μg/ml), VP16 (10μM), CPT (2μM), or TNF (10ng/ml) for indicated periods. NF-κB activation and Oct-1 levels were determined by the gel shift assay.

4.3.4 PKC is not required for DNA damage-induced NF-κB activation

It has been suggested that ATM is required for DNA damage-induced NF-κB activation (Hinz et al. 2010). In order to verify this finding, we treated primary MEFs with ATM inhibitor KU55933 (5μM) and examined NF-κB activation upon doxorubicin (1.5μg/ml) treatment. Consistent with previous findings, inhibition of ATM activity reduced NF-κB activation induced by genotoxic stress. This result indicates that ATM is required for DNA damage-indued NF-κB activation.

It has been suggested that CBM complex is activated by PKC, which phosphorylates CARMA1 at the linker domain upon T-cell activation (Matsumoto et al. 2005). In addition, it has been reported that doxorubicin can activate PKC delta (PKCδ) (Diaz Bessone et al. 2011). Therefore, we hypothesize that the CBM complex is activated by PKC isoform upon DNA damage induction. To address this hypothesis, we pre-treated primary MEFs with PKC inhibitor (GF109203X) and induce NF-κB activation by either doxorubicin (1.5μg/ml) or PMA (100ng/ml). Surprisingly, although PKC inhibitor efficiently blocked PMA-induced NF-κB activation, it did not affect doxorubicin-induced NF-κB activation (Figure 41). This data indicates that PKC is not involved in DNA damage-induced NF-κB activation, and CBM complex is activated through other undefined mechanisms.

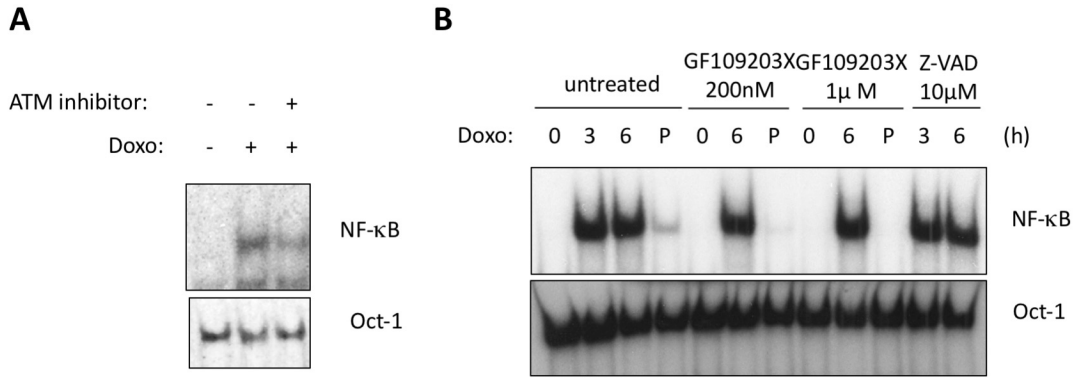

Figure 41. PKC is not required DNA damage-induced NF-κB activation.

(A) Primary MEFs were either left untreated or pretreated with ATM inhibitor KU55933 (5μM), following by treating with doxorubicin (1.5μg/ml). NF-κB activation and Oct-1 levels were determined by the gel shift assay. (B) Primary MEFs were either left untreated or pretreated with PKC inhibitor GF109203X or caspase inhibitor Z-VAD with indicated doses , following by treating with either doxorubicin (1.5μg/ml) or PMA (100ng/ml). NF-κB activation and Oct-1 levels were determined by the gel shift assay.

4.3.5 CARMA3 associates with TRAF6 upon DNA damage induction

Our previous study indicates that TRAF6 is inducible associated with MALT1 upon EGF treatment in A431 cells (Pan et al. 2015). It has also been reported that TRAF6 is required for DNA damage-induced NF-κB activation (Hinz et al. 2010). However, how TRAF6 is activated upon DNA damage is not clear. Based on our current data, we hypothesize that the CBM complex functions upstream of TRAF6 and is required for TRAF6 recruitment. To this end, we generated immortalized MEFs with stable expression of CARMA3-HA similar at an endogenous level. After treating the CARMA3-HA reconstituted MEFs with doxorubicin, we immunoprecipitated CARMA3-HA and examined the interacting protein with CARMA3. Interestingly, we observed inducible association of TRAF6 with CARMA3 upon doxorubicin treatment (1.5μg/ml) (Figure 42). This data indicate that CARMA3 recruits TRAF6 upon DNA damage induction. We therefore propose that the CBM complex functions upstream of TRAF6 to mediate DNA damage-induced NF-κB activation (Figure 43). The upstream mechanism that activates the CBM complex remains to be determined in the future.

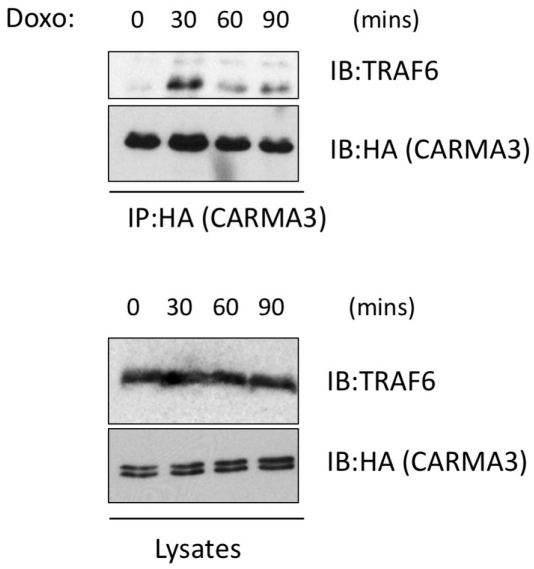

Figure 42. CARMA3 inducibly associates with TRAF6 upon doxorubicin treatment. CARMA3-HA-reconstituted MEFs were treated with doxorubicin (1.5μg/ml) for indicated time points and CARMA3-HA were immunoprecipitated (IP) with anti-HA antibodies. The IP samples and lysates were analyzed by immunoblotting using the indicated antibodies.

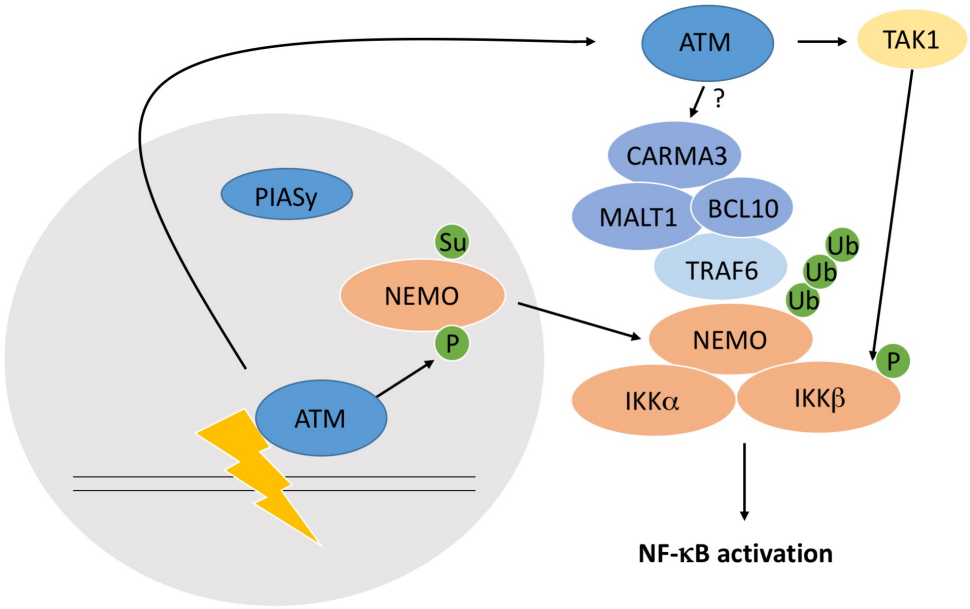

Figure 43. Proposed working model of DNA damage-induced NF-κB activation.

DNA damage-induced NF-κB activation requires the activation of ATM, which phosphorylates NEMO in the nucleus to trigger the nuclear export of NEMO. Upon DNA damage induction, the CARMA3-BCL10-MALT1 (CBM) complex is activated though a PKC-independent mechanism. The CBM complex may function to recruit TRAF6 to activate IKK upon DNA damage induction.

4.4 Discussion

4.4.1 The CBM complex-mediated NF-κB activation

In this study, we showed that the CBM complex is required for DNA damage-induced NF-κB activation. This is a particularly interesting finding as the CBM complex is generally considered only involved in receptors-activated NF-κB, such as antigen receptors (Wang et al. 2002), c-type lectin receptors (Bertin et al. 2000, Bi et al. 2010, Zhao et al. 2014), GPCR (Grabiner et al. 2007), EGFR (Jiang et al. 2011, Pan et al. 2015) and HER2 (Pan et al. 2015). Since the signals come from the nuclei in DNA damage response, our study indicates that the CBM complex has a broader impact in NF-κB activation in response to stimuli from both outside and inside the cells.

It has been suggested that TRAF6 is required for DNA damage-induced NF-κB activation (Hinz et al. 2010). However, the molecular mechanism by which TRAF6-mediates NF-κB activation upon genotoxic stress induction has not been identified. Generally, TRAF6 functions to activate IKK complex by ubiquitination (Sun et al. 2004), and it is recruited by an upstream scaffold protein, such as the CBM complex (Sun et al. 2004, Pan et al. 2015). We have previously shown that MALT1 is required for bridging TRAF6 to IKK complex in response to EGF stimulation in A431 cells (Pan et al. 2015). Here, we showed that DNA damage stimulation induced the association between CARMA3 and TRAF6, indicating that the CBM complex may functions as the upstream component of TRAF6 in response to DNA damage. Since the CBM complex can induce TRAF6 oligomerization and activation in the T

cells (Sun et al. 2004), we hypothesize that CBM complex activates TRAF6 upon genotoxic stress induction.

As the activation of IKK complex requires two parallel signals, including ubiquitination induced by E3 ligases such as TRAF6 and phosphorylation by kinases such as TAK1 (Blonska and Lin 2011, Pan and Lin 2013). It has been shown that TAK1 is also required for DNA damage-induced NF-κB activation, suggesting that TAK1 is likely responsible for phosphorylation of the IKK complex. It still remains to be determined that whether the CBM complex is also required for the activation of TAK1. Therefore, future studies is needed to further characterize the roles of CBM complex in DNA damage signaling.

4.4.2 The upstream activator of CARMA3

The results of this study revealed a role of CBM complex in DNA damage-induced NF-κB activation. However, it remains unclear that what is the upstream molecules of the CBM complex in response to DNA damage. Generally, CARMA1 or CARMA3 is activated by PKC through phosphorylation in the linker region of CARMA protein. For example, it has been shown that CARMA1 is activated by PKCβ at the site Ser552 (Matsumoto et al. 2005). Functionally, phosphorylation of CARMA1 triggers a conformational change and exposes the CARD domain to recruit BCL10 and MALT1 (Matsumoto et al. 2005, Qiao et al. 2013). The activated CBM complex forms a oligomerized structure and is able to recruit downstream molecules such as TRAF6 to activate IKK complex (Qiao et al. 2013).

However, PKC activity is dispensable for DNA damage-induced NF-κB activation, indicating either other kinases phosphorylate CARMA3 in response to DNA damage, or CARMA3 is activated through a different mechanism upon DNA damage. For example, Moreno-Garcia et al. showed that ubiquitination of CARMA1 regulates NF-κB activation in lymphocytes (Moreno-Garcia et al. 2010). Therefore, the activation mechanism of CARMA3 need to be further investigated in the future.

4.4.3 Therapeutic implications

NF-κB provides a pro-survival signal to the cell to antagonize apoptosis signal upon DNA damage. Therefore, cancer cell may utilize NF-κB signaling as a mechanism to avoid chemotherapy- and radiotherapy-induced apoptosis. Consistent with this notion, we showed that doxorubicin-induced cell apoptosis became more efficient in MALT1-deficient cells compared with control, indicating that NF-κB contributes to the resistance of chemotherapy-induced NF-κB activation. In addition, since NF-κB also plays a role in tumor migration and invasion (Huber et al. 2004, Pan and Lin 2013, Pan et al. 2015, Pan et al. 2015), DNA damage response may also potentially promote tumor progression. For instance, Niu et al. showed that DNA damage induced up-regulation of microRNA-21 to promote cancer cell migration and invasion in an NF-κB and STAT3 dependent manner (Niu et al. 2012). DNA damage-induced NF-κB also unregulated the expression of IL-6, which also promote cancer development by activation of STAT3 signaling (Guo et al. 2012). Therefore, targeting DNA

damage-induced NF-κB pathway is potentially beneficial when it is combined with radiotherapy or chemotherapy. The biological outcome of combination with targeting NF-κB and radiotherapy/chemotherapy need to be tested in the future. Therefore, our study also provides a molecular basis for targeting the CBM complex to block DNA damage-induced NF-κB pathway.

CHAPTER 5. CONCLUSION, DISCUSSION AND FUTURE DIRECTIONS

5.1 Conclusion

The CBM complex is best known for a role in mediating antigen receptor-induced NF-κB activation in lymphocytes (Blonska and Lin 2011). As NF-κB is absolutely critical for the activation of lymphocytes, the CBM complex is also crucial for lymphocytes activation. For the same reason, constitutive activation of CBM complex-mediated NF-κB pathway leads to lymphoma (Davis et al. 2001). However, the role of CBM complex in solid tumor has not been explored previously. As increasing numbers of literatures demonstrated a crucial role of NF-κB signaling in different types of solid tumors, such as lung cancer (Meylan et al. 2009) and breast cancer (Biswas et al. 2004), we propose to investigate a role of the CBM complex in solid tumor progression.

We found that the CBM complex is required for growth factor-induced NF-κB activation, including EGFR-induced NF-κB and HER-2-induced NF-κB (Jiang et al. 2011, Pan et al. 2015, Pan et al. 2015). In the EGFR-induced NF-κB pathway, the CBM complex functions as a scaffold protein complex to mediate IKK activation by recruiting E3 ligase TRAF6. In addition, MALT1 is required for many tumor malignant phenotypes *in vitro*, including cell proliferation, survival and migration. To further investigate a role of MALT1 in EGFR-associated malignancy *in vivo*, we generated a lung cancer mouse model in which lung adenocarcinoma will be developed in the absence of MALT1. In this model, we further confirmed that MALT1 is specifically involved in EGFR- but not K-ras-associated lung

cancer progression. Furthermore, EGFR-MALT-NF-κB signaling axis also contributes to lung cancer progression *in vivo* by activating STAT3 pathway though IL-6 expression. Therefore, our study revealed multi-functional roles of MALT1 in the tumorigenesis and progression of EGFR-assocaited lung cancer (Pan et al. 2015).

In addition, we also generated a breast cancer mouse model in which HER2-associated mammary tumor will be developed in the absence of MALT1 expression. Consistent with our *in vitro* finding suggesting a role of MALT1 in HER2-induced NF-κB activation and HER2-associated malignancy, we found that MALT1 also promotes HER2-associated mammary tumor progression *in vivo*. In addition, MALT1 contributes to HER2-associated tumor metastasis by regulating HER2-dependent expression of MMP1 and MMP13 (Pan et al. 2015). In conclusion, our studies demonstrate the role of CBM complex and MALT1 in growth-factor-related solid tumor progression, and provide molecular basis of targeting CBM complex and NF-κB pathway in EGFR- and HER2-associated tumor.

In addition to a role of growth factor receptor-induced NF-κB activation, we also investigated a role of CBM complex in DNA damage-induced NF-κB activation. DNA damage-induced NF-κB activation provides a pro-survival signal to the cells to antagonize cell apoptosis induced by DNA damage response. Therefore, understanding the molecular mechanism by which DNA damage induces NF-κB activation is particularly important. Here, we showed that DNA damage-induced NF-κB activation requires the CBM complex. In addition, CARMA3 is inducibly associated with TRAF6 in response to DNA damage

induction, suggesting a possible role of CBM complex in recruiting TRAF6 to IKK complex. However, how CBM complex is activated remains to be investigated. We hypothesize that either CARMA1/CARMA3 is directly activated by kinases other than PKC, or CARMA1/ CARMA3 is activated by other unknown mechanism, such as ubiquitination. Since DNA damage-induced NF-κB contributes to DNA damage induced tumor malignancy (Niu et al. 2012), our study provides evidence suggesting CBM complex and NF-κB may serve as potential therapeutic target in combination with radiotherapy and chemotherapy.

5.2 The protease activity of MALT1

The protease activity is the key to lymphocytes activation. Currently, the substrates of MALT1 includes A20 (Coornaert et al. 2008), BCL10 (Rebeaud et al. 2008), CYLD (Staal et al. 2011), RelB (Hailfinger et al. 2011), HOIL-1 (Klein et al. 2015), Regnase-1 (Uehata et al. 2013), Roquin-1 and Roquin-2 (Jeltsch et al. 2014). Most of the substrates are negative regulators of a variety of cellular activates, including NF-κB activation, JNK activation and regulation of RNA stability. This notion has been further validated by generation of the MALT1 protease-deficient mice by three different groups (Gewies et al. 2014, Jaworski et al. 2014, Bornancin et al. 2015). All of these studies have provided genetic evidence suggesting a critical role of MALT1 protease activity in lymphocytes activation. However, to date, the protease activity of MALT1 only functions in the lymphocytes and lymphomas.

In our current study, we determine to analyze a potential role of MALT1 protease activity in non-hematopoietic cells. However, we found that the protease activity of MALT1

is completely dispensable for both EGFR-induced NF-κB activation and HER2-induced NF-κB activation. Our data suggests that the protease activity of MALT1 does not contributes to growth factor-induced NF-κB activation. Consistent with our biochemical data, the protease activity of MALT1 is also dispensable for tumor malignant phenotypes in EGFR-associated tumor cell lines. Therefore, our data suggests that MALT1 plays a differential role in hematopoietic and non-hematopoietic cells.

The underlying reasons for a differential involvement of MALT1 protease activity remain to be determined. Here we propose several possibilities. First, as a matter of fact, many of the substrates of MALT1 are preferentially expressed in T-cells, such as A20 and RelB. Therefore, the dispensability of MALT1 in growth factor-induced NF-κB activation may due to a lack of essential substrates in non-hematopoietic cells. Indeed, we are not able to detect A20 and RelB expression in A431 cells. If this is the case, it also suggest a differential regulatory mechanism of NF-κB in different cell types. Second, even if substrates expressed in non-hematopoietic cells, MALT1 may not associate with the substrates upon EGFR or HER2 activation. For example, it has been reported that MALT1 cleaves CYLD in lymphocytes upon CD3/CD38 stimulation (Staal et al. 2011). However, we cannot detect the cleavage of CYLD in A431 cells, suggesting that CYLD may not associate with MALT1 upon EGFR stimulation. Third, although studies suggest that MALT1 becomes activated by oligomerization upon the formation of CBM complex (Qiao et al. 2013), there is no direct evidence demonstrating that MALT1 becomes activated upon other types of stimulation, such as EGFR or HER2 stimulation. It is possible that the activation of MALT1 protease activity

requires additional signals, which are limited in the hematopoietic cells or even only in the T-cells. For example, Pelzer et al. showed that monoubiquitination of MALT1 is required for the activation of the protease activity of MALT1 (Pelzer et al. 2013). However, it is unclear that where is the signal comes from and which E3 ligases are required to induce the monoubiquitination of MALT1. It also remains unknown that whether monoubiquitination of MALT1 is specific in T-cells. Therefore, the molecular requirements of activation of the protease activity of MALT1 remain to be further investigated in both hematopoietic cells and non-hematopoietic cells.

5.3 Future directions of studies on MALT1

MALT1 has been extensively investigated for 15 years since the discovery of MALT1 as a caspase-like protein in the year of 2000 (Uren et al. 2000). It is now well established that MALT1 functions as a part the CBM complex and mediates NF-κB activation in response to TCR or BCR stimulation in the lymphocytes (Ruefli-Brasse et al. 2003). Our current studies and others have also shown that MALT1 has a similar function in response to different types of stimuli, such as c-type lectin rector-induced NF-κB activation in myeloid cells (Bertin et al. 2000, Bi et al. 2010, Zhao et al. 2014), GPCR-induced NF-κB activation (Grabiner et al. 2007), EGFR-induced NF-κB activation (Jiang et al. 2011, Pan et al. 2015) in lung cancer cells and HER2-induced NF-κB (Pan et al. 2015) activation in breast cancer cells. In 2008, a milestone discovery demonstrated that MALT1 does have protease activity and it is critical to T-cell activation (Coornaert et al. 2008). Subsequently, a number of studies

also identified many other substrates of MALT1, and they play different roles to either positively or negatively regulate T-cell activation. Although it is clear that MALT1 and its protease activity contributes to NF-κB activation and lymphocytes activation, several main questions regarding to MALT1 need to be addressed in the future.

5.3.1 MALT1 and autoimmune disease

First, three different groups have reported that the protease-deficient MALT1 knock-in mice develop spontaneous autoimmune disease (Gewies et al. 2014, Jaworski et al. 2014, Bornancin et al. 2015). Consistently, we also observed that MALT1-deficient mice developed autoimmune-like diseases when mice were maintained in FVB/N background (Figure 44). It has been hypothesized that a deficiency of naive regulatory T-cells (Treg) development in MALT1 protease-deficient mice contributes the autoimmune disease (Jaworski et al. 2014). In comparison, Gewies et al. proposed that an excessive IFNγ production causes the autoimmune disease in MALT1 protease-deficient mice (Gewies et al. 2014). Therefore, the exact causes for autoimmune diseases in MALT1 and MALT1-protease-deficient mice remains to be further investigated.

5.3.2 The role of MALT1 in the effector function in Th17 cells

Two groups independently showed that MALT1-deficient mice were resistant to the induction of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (Brustle et al. 2012, Mc Guire et al. 2013). This result suggests that MALT1-deficiency affects the effector functions of inflammatory Th17 cells. Brustle et al.

further showed that while MALT1 is not required for the differentiation of Th17 cells, MALT1 is required for the effector function of Th17 cells by affecting IL-17 expression (Brustle et al. 2012). Since there is only one nonfunctional sequence of putative NF-κB elements in the mouse *Il17a* locus (Ruan et al. 2011), it is not likely that MALT1 regulates IL-17 expression directly through NF-κB. Therefore, the exact mechanism in which MALT1 regulates the effector function of Th17 cells need to be further investigated.

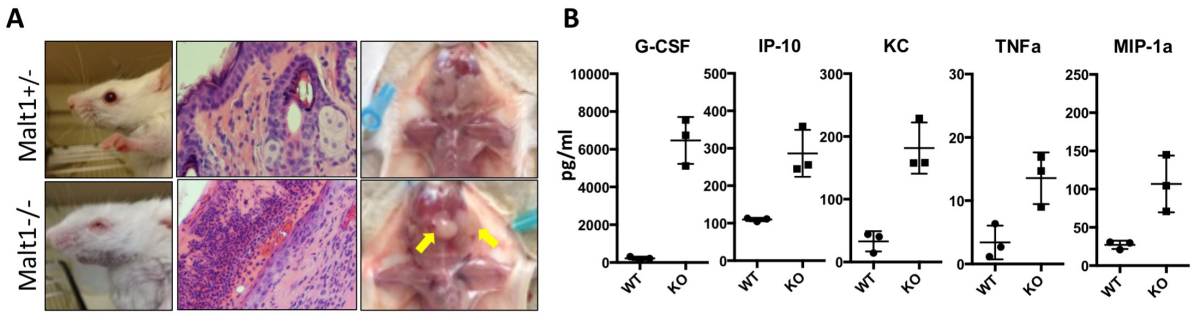

Figure 44. MALT1 deficient mice develop autoimmune phonotypes.

(A) MALT1 deficient mice develops spontaneous conjunctivitis (left panel). H&E staining showed a significant neutrophils infiltration at conjunctiva in MALT1 deficient mice compared with MALT1 heterozygous control (middle panel). Enlarged cervical lymph nodes in MALT1 deficient mice (right panel). (B) Cytokine level in the serum in MALT1 wild-type mice (WT) and Malt1$^{-/-}$ (KO) mice, respectively.

5.3.3 BCL10-independent function of MALT1 in macrophages

Since the activation of MALT1 depends on BCL10 and CARMA1/CARMA3, to date, all of functions of MALT1 is BCL10-dependent. However, if BCL10 and MALT1 are functionally overlapping, it raises a general question that why two protein are doing the exact same function? By using multi-plex cytokines array, we found that MALT1-deficient macrophages, but not WT or BCL10-deficient macrophages, produced significantly more CXCL-1 upon LPS stimulation, while the production of other cytokines were not changed or mildly affected (Figure 45A). We have further confirmed that the expression level of CXCL-1 is much higher in MALT1-deficient macrophages than WT or BCL10-deficient macrophages (Figure 45B). These results indicate that MALT1 negatively regulates CXCL-1 production upon macrophage activation, and this function is BCL10-independent. Importantly, inhibition of MALT1 protease activity produces a similar level of CXCL-1 as MALT1 -eficient macrophages do (Figure 45C), suggesting MALT1 protease activity is required to regulate this process. Together, these results indicate that MALT1 and its protease activity has a novel function in negatively regulating CXCL-1 expression independently of BCL10 in macrophages. Since the function is BCL10-independent, it also raises an interesting question: How does protease activity of MALT1 being activated independently of BCL10? To address this question, we examined the expression level of MALT1 and found that the expression level MALT1 is significantly induced in macrophages upon LPS and polyI:C stimulation (Figure 46 A-B). However, MALT1 induction was only found in macrophages but not in MEFs upon LPS stimulation, suggesting TLR-induced MALT1 expression is a macrophage-specific phenomenon (Figure 46C). This data suggest that

MALT1 upregulation may serve as an alternative activation mechanism of the protease activity of MALT1. Efforts have been undertaken by our group to further elucidate a new exciting role of MALT1 in macrophages and TLR signaling.

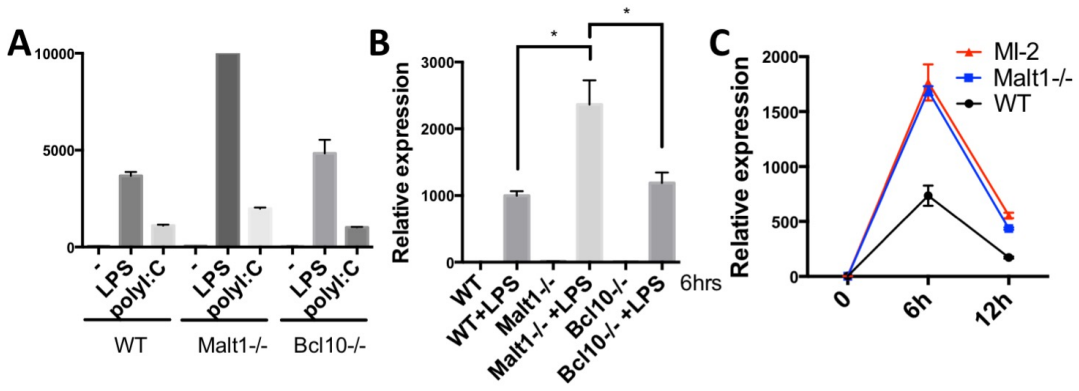

Figure 45. MALT1 and its protease activity negatively regulate CXCL-1 expression in macrophages.

(A) CXCL-1 level (pg/ml) after LPS or poly I:C stimulation (24hrs) in WT, MALT1 deficient and BCL10 deficient BMDM, respectively. (B) Relative mRNA level of CXCL-1 upon LPS stimulation in WT, MALT1 deficient and BCL10 deficient BMDM, respectively. (C) Relative mRNA level of CXCL-1 upon 6 or 12 hours of LPS stimulation in WT, MALT1 deficient macrophages and WT macrophages treated with MALT1 inhibitor MI-2 (2μM) (MI-2), respectively.

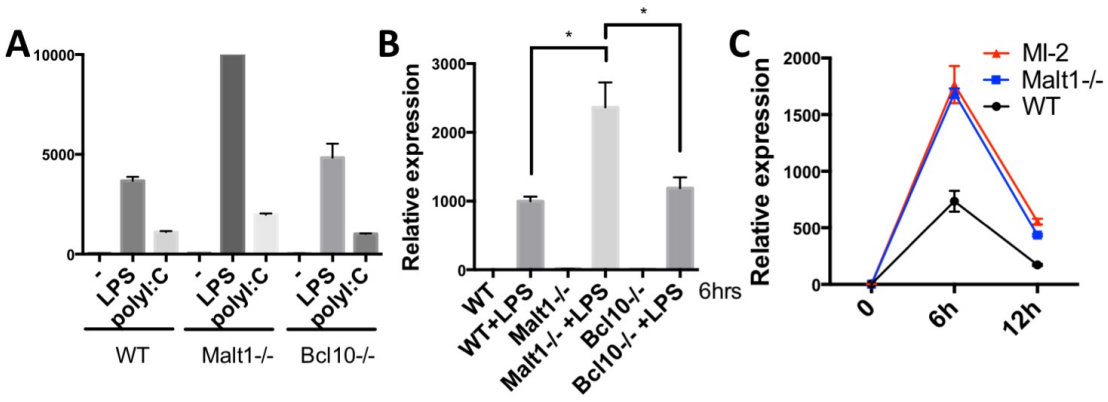

Figure 46. MALT1 is specifically induced in macrophages and functions independently of BCL10.

(A) The protein level of MALT1 as determined by western blot in BMDM. (B) Relative mRNA level of MALT1 upon LPS stimulation in BMDM upon 6 hours of LPS and polyI:C stimulation, respectively. (C) Relative mRNA level of MALT1 6 hours of LPS stimulation in primary mouse embryonic fibroblast (MEFs).

Bibliography

Adhikari, A., M. Xu and Z. J. Chen (2007). "Ubiquitin-mediated activation of TAK1 and IKK." Oncogene **26**(22): 3214-3226.

Akagi, T., M. Motegi, A. Tamura, R. Suzuki, Y. Hosokawa, H. Suzuki, H. Ota, S. Nakamura, Y. Morishima, M. Taniwaki and M. Seto (1999). "A novel gene, MALT1 at 18q21, is involved in t(11;18) (q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue." Oncogene **18**(42): 5785-5794.

Alizadeh, A. A., M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. Hudson, Jr., L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, R. Levy, W. Wilson, M. R. Grever, J. C. Byrd, D. Botstein, P. O. Brown and L. M. Staudt (2000). "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling." Nature **403**(6769): 503-511.

Amos, C. I., X. Wu, P. Broderick, I. P. Gorlov, J. Gu, T. Eisen, Q. Dong, Q. Zhang, X. Gu, J. Vijayakrishnan, K. Sullivan, A. Matakidou, Y. Wang, G. Mills, K. Doheny, Y. Y. Tsai, W. V. Chen, S. Shete, M. R. Spitz and R. S. Houlston (2008). "Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1." Nat Genet **40**(5): 616-622.

Annunziata, C. M., R. E. Davis, Y. Demchenko, W. Bellamy, A. Gabrea, F. Zhan, G. Lenz, I. Hanamura, G. Wright, W. Xiao, S. Dave, E. M. Hurt, B. Tan, H. Zhao, O. Stephens, M. Santra, D. R. Williams, L. Dang, B. Barlogie, J. D. Shaughnessy, Jr., W. M. Kuehl and L. M.

Staudt (2007). "Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma." Cancer Cell **12**(2): 115-130.

Arteaga, C. L., M. X. Sliwkowski, C. K. Osborne, E. A. Perez, F. Puglisi and L. Gianni (2012). "Treatment of HER2-positive breast cancer: current status and future perspectives." Nat Rev Clin Oncol **9**(1): 16-32.

Atchison, M. L. and R. P. Perry (1987). "The role of the kappa enhancer and its binding factor NF-kappa B in the developmental regulation of kappa gene transcription." Cell **48**(1): 121-128.

Auer, I. A., R. D. Gascoyne, J. M. Connors, F. E. Cotter, T. C. Greiner, W. G. Sanger and D. E. Horsman (1997). "t(11;18)(q21;q21) is the most common translocation in MALT lymphomas." Ann Oncol **8**(10): 979-985.

Badve, S., D. J. Dabbs, S. J. Schnitt, F. L. Baehner, T. Decker, V. Eusebi, S. B. Fox, S. Ichihara, J. Jacquemier, S. R. Lakhani, J. Palacios, E. A. Rakha, A. L. Richardson, F. C. Schmitt, P. H. Tan, G. M. Tse, B. Weigelt, I. O. Ellis and J. S. Reis-Filho (2011). "Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists." Mod Pathol **24**(2): 157-167.

Baeuerle, P. A. and D. Baltimore (1988). "Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor." Cell **53**(2): 211-217.

Baeuerle, P. A. and D. Baltimore (1988). "I kappa B: a specific inhibitor of the NF-kappa B transcription factor." Science **242**(4878): 540-546.

Bailey, S. T., P. L. Miron, Y. J. Choi, B. Kochupurakkal, G. Maulik, S. J. Rodig, R. Tian, K. M. Foley, T. Bowman, A. Miron, M. Brown, J. D. Iglehart and D. K. Biswas (2014). "NF-kappaB activation-induced anti-apoptosis renders HER2-positive cells drug resistant and accelerates tumor growth." Mol Cancer Res **12**(3): 408-420.

Baltimore, D. (2009). "Discovering NF-kappaB." Cold Spring Harb Perspect Biol **1**(1): a000026.

Banerjee, S., J. S. Reis-Filho, S. Ashley, D. Steele, A. Ashworth, S. R. Lakhani and I. E. Smith (2006). "Basal-like breast carcinomas: clinical outcome and response to chemotherapy." J Clin Pathol **59**(7): 729-735.

Barbie, D. A., P. Tamayo, J. S. Boehm, S. Y. Kim, S. E. Moody, I. F. Dunn, A. C. Schinzel, P. Sandy, E. Meylan, C. Scholl, S. Frohling, E. M. Chan, M. L. Sos, K. Michel, C. Mermel, S. J. Silver, B. A. Weir, J. H. Reiling, Q. Sheng, P. B. Gupta, R. C. Wadlow, H. Le, S. Hoersch, B. S. Wittner, S. Ramaswamy, D. M. Livingston, D. M. Sabatini, M. Meyerson, R. K. Thomas, E. S. Lander, J. P. Mesirov, D. E. Root, D. G. Gilliland, T. Jacks and W. C. Hahn (2009). "Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1." Nature **462**(7269): 108-112.

Bean, J., C. Brennan, J. Y. Shih, G. Riely, A. Viale, L. Wang, D. Chitale, N. Motoi, J. Szoke, S. Broderick, M. Balak, W. C. Chang, C. J. Yu, A. Gazdar, H. Pass, V. Rusch, W. Gerald, S. F. Huang, P. C. Yang, V. Miller, M. Ladanyi, C. H. Yang and W. Pao (2007). "MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib." Proc Natl Acad Sci U S A **104**(52): 20932-20937.

Beg, A. A. and A. S. Baldwin, Jr. (1993). "The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors." Genes Dev **7**(11): 2064-2070.

Bell, D. W., I. Gore, R. A. Okimoto, N. Godin-Heymann, R. Sordella, R. Mulloy, S. V. Sharma, B. W. Brannigan, G. Mohapatra, J. Settleman and D. A. Haber (2005). "Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR." Nat Genet **37**(12): 1315-1316.

Bertin, J., Y. Guo, L. Wang, S. M. Srinivasula, M. D. Jacobson, J. L. Poyet, S. Merriam, M. Q. Du, M. J. Dyer, K. E. Robison, P. S. DiStefano and E. S. Alnemri (2000). "CARD9 is a novel caspase recruitment domain-containing protein that interacts with BCL10/CLAP and activates NF-kappa B." J Biol Chem **275**(52): 41082-41086.

Bi, L., S. Gojestani, W. Wu, Y. M. Hsu, J. Zhu, K. Ariizumi and X. Lin (2010). "CARD9 mediates dectin-2-induced IkappaBalpha kinase ubiquitination leading to activation of NF-kappaB in response to stimulation by the hyphal form of Candida albicans." J Biol Chem **285**(34): 25969-25977.

Biswas, D. K., Q. Shi, S. Baily, I. Strickland, S. Ghosh, A. B. Pardee and J. D. Iglehart (2004). "NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis." Proc Natl Acad Sci U S A **101**(27): 10137-10142.

Bivona, T. G., H. Hieronymus, J. Parker, K. Chang, M. Taron, R. Rosell, P. Moonsamy, K. Dahlman, V. A. Miller, C. Costa, G. Hannon and C. L. Sawyers (2011). "FAS and NF-kappaB signalling modulate dependence of lung cancers on mutant EGFR." Nature **471**(7339): 523-526.

Blonska, M. and X. Lin (2009). "CARMA1-mediated NF-kappaB and JNK activation in lymphocytes." Immunol Rev **228**(1): 199-211.

Blonska, M. and X. Lin (2011). "NF-kappaB signaling pathways regulated by CARMA family of scaffold proteins." Cell Res **21**(1): 55-70.

Bornancin, F., F. Renner, R. Touil, H. Sic, Y. Kolb, I. Touil-Allaoui, J. S. Rush, P. A. Smith, M. Bigaud, U. Junker-Walker, C. Burkhart, J. Dawson, S. Niwa, A. Katopodis, B. Nuesslein-Hildesheim, G. Weckbecker, G. Zenke, B. Kinzel, E. Traggiai, D. Brenner, A. Brustle, M. St Paul, N. Zamurovic, K. D. McCoy, A. Rolink, C. H. Regnier, T. W. Mak, P. S. Ohashi, D. D. Patel and T. Calzascia (2015). "Deficiency of MALT1 paracaspase activity results in unbalanced regulatory and effector T and B cell responses leading to multiorgan inflammation." J Immunol **194**(8): 3723-3734.

Bos, J. L. (1989). "ras oncogenes in human cancer: a review." Cancer Res **49**(17): 4682-4689.

Brahmer, J., K. L. Reckamp, P. Baas, L. Crino, W. E. Eberhardt, E. Poddubskaya, S. Antonia, A. Pluzanski, E. E. Vokes, E. Holgado, D. Waterhouse, N. Ready, J. Gainor, O. Aren Frontera, L. Havel, M. Steins, M. C. Garassino, J. G. Aerts, M. Domine, L. Paz-Ares, M. Reck, C. Baudelet, C. T. Harbison, B. Lestini and D. R. Spigel (2015). "Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer." N Engl J Med **373**(2): 123-135.

Brustle, A., D. Brenner, C. B. Knobbe, P. A. Lang, C. Virtanen, B. M. Hershenfield, C. Reardon, S. M. Lacher, J. Ruland, P. S. Ohashi and T. W. Mak (2012). "The NF-kappaB

regulator MALT1 determines the encephalitogenic potential of Th17 cells." J Clin Invest **122**(12): 4698-4709.

Che, T., Y. You, D. Wang, M. J. Tanner, V. M. Dixit and X. Lin (2004). "MALT1/paracaspase is a signaling component downstream of CARMA1 and mediates T cell receptor-induced NF-kappaB activation." J Biol Chem **279**(16): 15870-15876.

Cheang, M. C., D. Voduc, C. Bajdik, S. Leung, S. McKinney, S. K. Chia, C. M. Perou and T. O. Nielsen (2008). "Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype." Clin Cancer Res **14**(5): 1368-1376.

Coope, H. J., P. G. Atkinson, B. Huhse, M. Belich, J. Janzen, M. J. Holman, G. G. Klaus, L. H. Johnston and S. C. Ley (2002). "CD40 regulates the processing of NF-kappaB2 p100 to p52." EMBO J **21**(20): 5375-5385.

Coornaert, B., M. Baens, K. Heyninck, T. Bekaert, M. Haegman, J. Staal, L. Sun, Z. J. Chen, P. Marynen and R. Beyaert (2008). "T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20." Nat Immunol **9**(3): 263-271.

Creighton, C. J. (2012). "The molecular profile of luminal B breast cancer." Biologics **6**: 289-297.

Davies, H., G. R. Bignell, C. Cox, P. Stephens, S. Edkins, S. Clegg, J. Teague, H. Woffendin, M. J. Garnett, W. Bottomley, N. Davis, E. Dicks, R. Ewing, Y. Floyd, K. Gray, S. Hall, R. Hawes, J. Hughes, V. Kosmidou, A. Menzies, C. Mould, A. Parker, C. Stevens, S. Watt, S. Hooper, R. Wilson, H. Jayatilake, B. A. Gusterson, C. Cooper, J. Shipley, D. Hargrave, K. Pritchard-Jones, N. Maitland, G. Chenevix-Trench, G. J. Riggins, D. D. Bigner, G. Palmieri,

A. Cossu, A. Flanagan, A. Nicholson, J. W. Ho, S. Y. Leung, S. T. Yuen, B. L. Weber, H. F. Seigler, T. L. Darrow, H. Paterson, R. Marais, C. J. Marshall, R. Wooster, M. R. Stratton and P. A. Futreal (2002). "Mutations of the BRAF gene in human cancer." Nature **417**(6892): 949-954.

Davis, R. E., K. D. Brown, U. Siebenlist and L. M. Staudt (2001). "Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells." J Exp Med **194**(12): 1861-1874.

Diaz Bessone, M. I., D. E. Berardi, P. B. Campodonico, L. B. Todaro, L. Lothstein, E. D. Bal de Kier Joffe and A. J. Urtreger (2011). "Involvement of PKC delta (PKCdelta) in the resistance against different doxorubicin analogs." Breast Cancer Res Treat **126**(3): 577-587.

Dierlamm, J., M. Baens, I. Wlodarska, M. Stefanova-Ouzounova, J. M. Hernandez, D. K. Hossfeld, C. De Wolf-Peeters, A. Hagemeijer, H. Van den Berghe and P. Marynen (1999). "The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas." Blood **93**(11): 3601-3609.

Ea, C. K., L. Deng, Z. P. Xia, G. Pineda and Z. J. Chen (2006). "Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO." Mol Cell **22**(2): 245-257.

Eichhorn, P. J., M. Gili, M. Scaltriti, V. Serra, M. Guzman, W. Nijkamp, R. L. Beijersbergen, V. Valero, J. Seoane, R. Bernards and J. Baselga (2008). "Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235." Cancer Res **68**(22): 9221-9230.

Engelman, J. A., K. Zejnullahu, T. Mitsudomi, Y. Song, C. Hyland, J. O. Park, N. Lindeman, C. M. Gale, X. Zhao, J. Christensen, T. Kosaka, A. J. Holmes, A. M. Rogers, F. Cappuzzo, T. Mok, C. Lee, B. E. Johnson, L. C. Cantley and P. A. Janne (2007). "MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling." Science **316**(5827): 1039-1043.

Ettinger, D. S., W. Akerley, H. Borghaei, A. C. Chang, R. T. Cheney, L. R. Chirieac, T. A. D'Amico, T. L. Demmy, R. Govindan, F. W. Grannis, Jr., S. C. Grant, L. Horn, T. M. Jahan, R. Komaki, F. M. Kong, M. G. Kris, L. M. Krug, R. P. Lackner, I. T. Lennes, B. W. Loo, Jr., R. Martins, G. A. Otterson, J. D. Patel, M. C. Pinder-Schenck, K. M. Pisters, K. Reckamp, G. J. Riely, E. Rohren, T. A. Shapiro, S. J. Swanson, K. Tauer, D. E. Wood, S. C. Yang, K. Gregory, M. Hughes and n. National comprehensive cancer (2013). "Non-small cell lung cancer, version 2.2013." J Natl Compr Canc Netw **11**(6): 645-653; quiz 653.

Fan, Q. W., C. Cheng, Z. A. Knight, D. Haas-Kogan, D. Stokoe, C. D. James, F. McCormick, K. M. Shokat and W. A. Weiss (2009). "EGFR signals to mTOR through PKC and independently of Akt in glioma." Sci Signal **2**(55): ra4.

Ferch, U., B. Kloo, A. Gewies, V. Pfander, M. Duwel, C. Peschel, D. Krappmann and J. Ruland (2009). "Inhibition of MALT1 protease activity is selectively toxic for activated B cell-like diffuse large B cell lymphoma cells." J Exp Med **206**(11): 2313-2320.

Fishel, R., M. K. Lescoe, M. R. Rao, N. G. Copeland, N. A. Jenkins, J. Garber, M. Kane and R. Kolodner (1993). "The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer." Cell **75**(5): 1027-1038.

Fisher, G. H., S. L. Wellen, D. Klimstra, J. M. Lenczowski, J. W. Tichelaar, M. J. Lizak, J. A. Whitsett, A. Koretsky and H. E. Varmus (2001). "Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes." Genes Dev **15**(24): 3249-3262.

Fontan, L., C. Yang, V. Kabaleeswaran, L. Volpon, M. J. Osborne, E. Beltran, M. Garcia, L. Cerchietti, R. Shaknovich, S. N. Yang, F. Fang, R. D. Gascoyne, J. A. Martinez-Climent, J. F. Glickman, K. Borden, H. Wu and A. Melnick (2012). "MALT1 small molecule inhibitors specifically suppress ABC-DLBCL in vitro and in vivo." Cancer Cell **22**(6): 812-824.

Foulkes, W. D., I. M. Stefansson, P. O. Chappuis, L. R. Begin, J. R. Goffin, N. Wong, M. Trudel and L. A. Akslen (2003). "Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer." J Natl Cancer Inst **95**(19): 1482-1485.

Fulford, L. G., J. S. Reis-Filho, K. Ryder, C. Jones, C. E. Gillett, A. Hanby, D. Easton and S. R. Lakhani (2007). "Basal-like grade III invasive ductal carcinoma of the breast: patterns of metastasis and long-term survival." Breast Cancer Res **9**(1): R4.

Furuta, S., X. Jiang, B. Gu, E. Cheng, P. L. Chen and W. H. Lee (2005). "Depletion of BRCA1 impairs differentiation but enhances proliferation of mammary epithelial cells." Proc Natl Acad Sci U S A **102**(26): 9176-9181.

Gaide, O., B. Favier, D. F. Legler, D. Bonnet, B. Brissoni, S. Valitutti, C. Bron, J. Tschopp and M. Thome (2002). "CARMA1 is a critical lipid raft-associated regulator of TCR-induced NF-kappa B activation." Nat Immunol **3**(9): 836-843.

Gao, S. P., K. G. Mark, K. Leslie, W. Pao, N. Motoi, W. L. Gerald, W. D. Travis, W. Bornmann, D. Veach, B. Clarkson and J. F. Bromberg (2007). "Mutations in the EGFR kinase

domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas." J Clin Invest **117**(12): 3846-3856.

Gazdar, A. F. (2009). "Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors." Oncogene **28 Suppl 1**: S24-31.

Gerlach, B., S. M. Cordier, A. C. Schmukle, C. H. Emmerich, E. Rieser, T. L. Haas, A. I. Webb, J. A. Rickard, H. Anderton, W. W. Wong, U. Nachbur, L. Gangoda, U. Warnken, A. W. Purcell, J. Silke and H. Walczak (2011). "Linear ubiquitination prevents inflammation and regulates immune signalling." Nature **471**(7340): 591-596.

Gewies, A., O. Gorka, H. Bergmann, K. Pechloff, F. Petermann, K. M. Jeltsch, M. Rudelius, M. Kriegsmann, W. Weichert, M. Horsch, J. Beckers, W. Wurst, M. Heikenwalder, T. Korn, V. Heissmeyer and J. Ruland (2014). "Uncoupling Malt1 threshold function from paracaspase activity results in destructive autoimmune inflammation." Cell Rep **9**(4): 1292-1305.

Glasmacher, E., K. P. Hoefig, K. U. Vogel, N. Rath, L. Du, C. Wolf, E. Kremmer, X. Wang and V. Heissmeyer (2010). "Roquin binds inducible costimulator mRNA and effectors of mRNA decay to induce microRNA-independent post-transcriptional repression." Nat Immunol **11**(8): 725-733.

Grabiner, B. C., M. Blonska, P. C. Lin, Y. You, D. Wang, J. Sun, B. G. Darnay, C. Dong and X. Lin (2007). "CARMA3 deficiency abrogates G protein-coupled receptor-induced NF-{kappa}B activation." Genes Dev **21**(8): 984-996.

Griffin, C. A., B. A. Zehnbauer, W. E. Beschorner, R. Ambinder and R. Mann (1992). "t(11;18)(q21;q21) is a recurrent chromosome abnormality in small lymphocytic lymphoma." Genes Chromosomes Cancer **4**(2): 153-157.

Gross, O., A. Gewies, K. Finger, M. Schafer, T. Sparwasser, C. Peschel, I. Forster and J. Ruland (2006). "Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity." Nature **442**(7103): 651-656.

Gross, O., C. Grupp, C. Steinberg, S. Zimmermann, D. Strasser, N. Hannesschlager, W. Reindl, H. Jonsson, H. Huo, D. R. Littman, C. Peschel, W. M. Yokoyama, A. Krug and J. Ruland (2008). "Multiple ITAM-coupled NK-cell receptors engage the Bcl10/Malt1 complex via Carma1 for NF-kappaB and MAPK activation to selectively control cytokine production." Blood **112**(6): 2421-2428.

Guo, Y., F. Xu, T. Lu, Z. Duan and Z. Zhang (2012). "Interleukin-6 signaling pathway in targeted therapy for cancer." Cancer Treat Rev **38**(7): 904-910.

Guy, C. T., R. D. Cardiff and W. J. Muller (1992). "Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease." Mol Cell Biol **12**(3): 954-961.

Guy, C. T., M. A. Webster, M. Schaller, T. J. Parsons, R. D. Cardiff and W. J. Muller (1992). "Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease." Proc Natl Acad Sci U S A **89**(22): 10578-10582.

Haas, T. L., C. H. Emmerich, B. Gerlach, A. C. Schmukle, S. M. Cordier, E. Rieser, R. Feltham, J. Vince, U. Warnken, T. Wenger, R. Koschny, D. Komander, J. Silke and H. Walczak (2009). "Recruitment of the linear ubiquitin chain assembly complex stabilizes the

TNF-R1 signaling complex and is required for TNF-mediated gene induction." Mol Cell **36**(5): 831-844.

Hailfinger, S., G. Lenz, V. Ngo, A. Posvitz-Fejfar, F. Rebeaud, M. Guzzardi, E. M. Penas, J. Dierlamm, W. C. Chan, L. M. Staudt and M. Thome (2009). "Essential role of MALT1 protease activity in activated B cell-like diffuse large B-cell lymphoma." Proc Natl Acad Sci U S A **106**(47): 19946-19951.

Hailfinger, S., H. Nogai, C. Pelzer, M. Jaworski, K. Cabalzar, J. E. Charton, M. Guzzardi, C. Decaillet, M. Grau, B. Dorken, P. Lenz, G. Lenz and M. Thome (2011). "Malt1-dependent RelB cleavage promotes canonical NF-kappaB activation in lymphocytes and lymphoma cell lines." Proc Natl Acad Sci U S A **108**(35): 14596-14601.

Hara, H., C. Ishihara, A. Takeuchi, T. Imanishi, L. Xue, S. W. Morris, M. Inui, T. Takai, A. Shibuya, S. Saijo, Y. Iwakura, N. Ohno, H. Koseki, H. Yoshida, J. M. Penninger and T. Saito (2007). "The adaptor protein CARD9 is essential for the activation of myeloid cells through ITAM-associated and Toll-like receptors." Nat Immunol **8**(6): 619-629.

Hayden, M. S. and S. Ghosh (2012). "NF-kappaB, the first quarter-century: remarkable progress and outstanding questions." Genes Dev **26**(3): 203-234.

Hayden, M. S. and S. Ghosh (2014). "Regulation of NF-kappaB by TNF family cytokines." Semin Immunol **26**(3): 253-266.

Hedenfalk, I., D. Duggan, Y. Chen, M. Radmacher, M. Bittner, R. Simon, P. Meltzer, B. Gusterson, M. Esteller, O. P. Kallioniemi, B. Wilfond, A. Borg, J. Trent, M. Raffeld, Z. Yakhini, A. Ben-Dor, E. Dougherty, J. Kononen, L. Bubendorf, W. Fehrle, S. Pittaluga, S.

Gruvberger, N. Loman, O. Johannsson, H. Olsson and G. Sauter (2001). "Gene-expression profiles in hereditary breast cancer." N Engl J Med **344**(8): 539-548.

Herbst, R. S., J. V. Heymach and S. M. Lippman (2008). "Lung cancer." N Engl J Med **359**(13): 1367-1380.

Hinz, M., M. Stilmann, S. C. Arslan, K. K. Khanna, G. Dittmar and C. Scheidereit (2010). "A cytoplasmic ATM-TRAF6-cIAP1 module links nuclear DNA damage signaling to ubiquitin-mediated NF-kappaB activation." Mol Cell **40**(1): 63-74.

Horsman, D., R. Gascoyne, R. Klasa and R. Coupland (1992). "t(11;18)(q21;q21.1): a recurring translocation in lymphomas of mucosa-associated lymphoid tissue (MALT)?" Genes Chromosomes Cancer **4**(2): 183-187.

Hsu, Y. M., Y. Zhang, Y. You, D. Wang, H. Li, O. Duramad, X. F. Qin, C. Dong and X. Lin (2007). "The adaptor protein CARD9 is required for innate immune responses to intracellular pathogens." Nat Immunol **8**(2): 198-205.

Huang, T. T., S. M. Wuerzberger-Davis, Z. H. Wu and S. Miyamoto (2003). "Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress." Cell **115**(5): 565-576.

Huber, M. A., N. Azoitei, B. Baumann, S. Grunert, A. Sommer, H. Pehamberger, N. Kraut, H. Beug and T. Wirth (2004). "NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression." J Clin Invest **114**(4): 569-581.

Hung, R. J., J. D. McKay, V. Gaborieau, P. Boffetta, M. Hashibe, D. Zaridze, A. Mukeria, N. Szeszenia-Dabrowska, J. Lissowska, P. Rudnai, E. Fabianova, D. Mates, V. Bencko, L. Foretova, V. Janout, C. Chen, G. Goodman, J. K. Field, T. Liloglou, G. Xinarianos, A.

Cassidy, J. McLaughlin, G. Liu, S. Narod, H. E. Krokan, F. Skorpen, M. B. Elvestad, K. Hveem, L. Vatten, J. Linseisen, F. Clavel-Chapelon, P. Vineis, H. B. Bueno-de-Mesquita, E. Lund, C. Martinez, S. Bingham, T. Rasmuson, P. Hainaut, E. Riboli, W. Ahrens, S. Benhamou, P. Lagiou, D. Trichopoulos, I. Holcatova, F. Merletti, K. Kjaerheim, A. Agudo, G. Macfarlane, R. Talamini, L. Simonato, R. Lowry, D. I. Conway, A. Znaor, C. Healy, D. Zelenika, A. Boland, M. Delepine, M. Foglio, D. Lechner, F. Matsuda, H. Blanche, I. Gut, S. Heath, M. Lathrop and P. Brennan (2008). "A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25." Nature **452**(7187): 633-637.

Hwang, S. J., L. S. Cheng, G. Lozano, C. I. Amos, X. Gu and L. C. Strong (2003). "Lung cancer risk in germline p53 mutation carriers: association between an inherited cancer predisposition, cigarette smoking, and cancer risk." Hum Genet **113**(3): 238-243.

Ignatiadis, M. and C. Sotiriou (2013). "Luminal breast cancer: from biology to treatment." Nat Rev Clin Oncol **10**(9): 494-506.

Ikeda, F., Y. L. Deribe, S. S. Skanland, B. Stieglitz, C. Grabbe, M. Franz-Wachtel, S. J. van Wijk, P. Goswami, V. Nagy, J. Terzic, F. Tokunaga, A. Androulidaki, T. Nakagawa, M. Pasparakis, K. Iwai, J. P. Sundberg, L. Schaefer, K. Rittinger, B. Macek and I. Dikic (2011). "SHARPIN forms a linear ubiquitin ligase complex regulating NF-kappaB activity and apoptosis." Nature **471**(7340): 637-641.

Jackson, E. L., N. Willis, K. Mercer, R. T. Bronson, D. Crowley, R. Montoya, T. Jacks and D. A. Tuveson (2001). "Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras." Genes Dev **15**(24): 3243-3248.

Jackson, S. P. and J. Bartek (2009). "The DNA-damage response in human biology and disease." Nature **461**(7267): 1071-1078.

Janssens, S. and J. Tschopp (2006). "Signals from within: the DNA-damage-induced NF-kappaB response." Cell Death Differ **13**(5): 773-784.

Jaworski, M., B. J. Marsland, J. Gehrig, W. Held, S. Favre, S. A. Luther, M. Perroud, D. Golshayan, O. Gaide and M. Thome (2014). "Malt1 protease inactivation efficiently dampens immune responses but causes spontaneous autoimmunity." EMBO J **33**(23): 2765-2781.

Jeltsch, K. M., D. Hu, S. Brenner, J. Zoller, G. A. Heinz, D. Nagel, K. U. Vogel, N. Rehage, S. C. Warth, S. L. Edelmann, R. Gloury, N. Martin, C. Lohs, M. Lech, J. E. Stehklein, A. Geerlof, E. Kremmer, A. Weber, H. J. Anders, I. Schmitz, M. Schmidt-Supprian, M. Fu, H. Holtmann, D. Krappmann, J. Ruland, A. Kallies, M. Heikenwalder and V. Heissmeyer (2014). "Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote T(H)17 differentiation." Nat Immunol **15**(11): 1079-1089.

Jemal, A., R. Siegel, E. Ward, Y. Hao, J. Xu, T. Murray and M. J. Thun (2008). "Cancer statistics, 2008." CA Cancer J Clin **58**(2): 71-96.

Jiang, C. and X. Lin (2012). "Regulation of NF-kappaB by the CARD proteins." Immunol Rev **246**(1): 141-153.

Jiang, Q. and R. A. Greenberg (2015). "Deciphering the BRCA1 Tumor Suppressor Network." J Biol Chem **290**(29): 17724-17732.

Jiang, T., B. Grabiner, Y. Zhu, C. Jiang, H. Li, Y. You, J. Lang, M. C. Hung and X. Lin (2011). "CARMA3 is crucial for EGFR-Induced activation of NF-kappaB and tumor progression." Cancer Res **71**(6): 2183-2192.

Jung, D. and F. W. Alt (2004). "Unraveling V(D)J recombination; insights into gene regulation." Cell **116**(2): 299-311.

Junttila, T. T., R. W. Akita, K. Parsons, C. Fields, G. D. Lewis Phillips, L. S. Friedman, D. Sampath and M. X. Sliwkowski (2009). "Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941." Cancer Cell **15**(5): 429-440.

Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." J Clin Invest **119**(6): 1420-1428.

Kanakaraj, P., P. H. Schafer, D. E. Cavender, Y. Wu, K. Ngo, P. F. Grealish, S. A. Wadsworth, P. A. Peterson, J. J. Siekierka, C. A. Harris and W. P. Fung-Leung (1998). "Interleukin (IL)-1 receptor-associated kinase (IRAK) requirement for optimal induction of multiple IL-1 signaling pathways and IL-6 production." J Exp Med **187**(12): 2073-2079.

Kawai, T., O. Adachi, T. Ogawa, K. Takeda and S. Akira (1999). "Unresponsiveness of MyD88-deficient mice to endotoxin." Immunity **11**(1): 115-122.

Kawai, T. and S. Akira (2007). "Signaling to NF-kappaB by Toll-like receptors." Trends Mol Med **13**(11): 460-469.

Keats, J. J., R. Fonseca, M. Chesi, R. Schop, A. Baker, W. J. Chng, S. Van Wier, R. Tiedemann, C. X. Shi, M. Sebag, E. Braggio, T. Henry, Y. X. Zhu, H. Fogle, T. Price-Troska, G. Ahmann, C. Mancini, L. A. Brents, S. Kumar, P. Greipp, A. Dispenzieri, B. Bryant, G.

Mulligan, L. Bruhn, M. Barrett, R. Valdez, J. Trent, A. K. Stewart, J. Carpten and P. L. Bergsagel (2007). "Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma." Cancer Cell **12**(2): 131-144.

Kendellen, M. F., J. W. Bradford, C. L. Lawrence, K. S. Clark and A. S. Baldwin (2014). "Canonical and non-canonical NF-kappaB signaling promotes breast cancer tumor-initiating cells." Oncogene **33**(10): 1297-1305.

Kirisako, T., K. Kamei, S. Murata, M. Kato, H. Fukumoto, M. Kanie, S. Sano, F. Tokunaga, K. Tanaka and K. Iwai (2006). "A ubiquitin ligase complex assembles linear polyubiquitin chains." EMBO J **25**(20): 4877-4887.

Klein, T., S. Y. Fung, F. Renner, M. A. Blank, A. Dufour, S. Kang, M. Bolger-Munro, J. M. Scurll, J. J. Priatel, P. Schweigler, S. Melkko, M. R. Gold, R. I. Viner, C. H. Regnier, S. E. Turvey and C. M. Overall (2015). "The paracaspase MALT1 cleaves HOIL1 reducing linear ubiquitination by LUBAC to dampen lymphocyte NF-kappaB signalling." Nat Commun **6**: 8777.

Klemm, S., S. Zimmermann, C. Peschel, T. W. Mak and J. Ruland (2007). "Bcl10 and Malt1 control lysophosphatidic acid-induced NF-kappaB activation and cytokine production." Proc Natl Acad Sci U S A **104**(1): 134-138.

Kobayashi, S., T. J. Boggon, T. Dayaram, P. A. Janne, O. Kocher, M. Meyerson, B. E. Johnson, M. J. Eck, D. G. Tenen and B. Halmos (2005). "EGFR mutation and resistance of non-small-cell lung cancer to gefitinib." N Engl J Med **352**(8): 786-792.

Kosaka, T., Y. Yatabe, H. Endoh, K. Yoshida, T. Hida, M. Tsuboi, H. Tada, H. Kuwano and T. Mitsudomi (2006). "Analysis of epidermal growth factor receptor gene mutation in patients

with non-small cell lung cancer and acquired resistance to gefitinib." Clin Cancer Res **12**(19): 5764-5769.

Kovalenko, A., C. Chable-Bessia, G. Cantarella, A. Israel, D. Wallach and G. Courtois (2003). "The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination." Nature **424**(6950): 801-805.

Krokan, H. E. and M. Bjoras (2013). "Base excision repair." Cold Spring Harb Perspect Biol **5**(4): a012583.

Lao, J., J. Madani, T. Puertolas, M. Alvarez, A. Hernandez, R. Pazo-Cid, A. Artal and A. Anton Torres (2013). "Liposomal Doxorubicin in the treatment of breast cancer patients: a review." J Drug Deliv **2013**: 456409.

Leach, F. S., N. C. Nicolaides, N. Papadopoulos, B. Liu, J. Jen, R. Parsons, P. Peltomaki, P. Sistonen, L. A. Aaltonen, M. Nystrom-Lahti and et al. (1993). "Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer." Cell **75**(6): 1215-1225.

Lee, E. G., D. L. Boone, S. Chai, S. L. Libby, M. Chien, J. P. Lodolce and A. Ma (2000). "Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice." Science **289**(5488): 2350-2354.

Lenardo, M., J. W. Pierce and D. Baltimore (1987). "Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility." Science **236**(4808): 1573-1577.

Lenz, G., R. E. Davis, V. N. Ngo, L. Lam, T. C. George, G. W. Wright, S. S. Dave, H. Zhao, W. Xu, A. Rosenwald, G. Ott, H. K. Muller-Hermelink, R. D. Gascoyne, J. M. Connors, L. M. Rimsza, E. Campo, E. S. Jaffe, J. Delabie, E. B. Smeland, R. I. Fisher, W. C. Chan and L.

M. Staudt (2008). "Oncogenic CARD11 mutations in human diffuse large B cell lymphoma." Science **319**(5870): 1676-1679.

Leppek, K., J. Schott, S. Reitter, F. Poetz, M. C. Hammond and G. Stoecklin (2013). "Roquin promotes constitutive mRNA decay via a conserved class of stem-loop recognition motifs." Cell **153**(4): 869-881.

Leroux, D., P. Seite, J. Hillion, F. Le Marc'hadour, B. Pegourie-Bandelier, M. C. Jacob, C. J. Larsen and J. J. Sotto (1993). "t(11;18)(q21;q21) may delineate a spectrum of diffuse small B-cell lymphoma with extranodal involvement." Genes Chromosomes Cancer **7**(1): 54-56.

Levine, E. G., D. C. Arthur, J. Machnicki, G. Frizzera, D. Hurd, B. Peterson, K. J. Gajl-Peczalska and C. D. Bloomfield (1989). "Four new recurring translocations in non-Hodgkin lymphoma." Blood **74**(5): 1796-1800.

Li, H., S. N. Cho, C. M. Evans, B. F. Dickey, J. W. Jeong and F. J. DeMayo (2008). "Cre-mediated recombination in mouse Clara cells." Genesis **46**(6): 300-307.

Li, H., M. Kobayashi, M. Blonska, Y. You and X. Lin (2006). "Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation." J Biol Chem **281**(19): 13636-13643.

Li, S., A. Strelow, E. J. Fontana and H. Wesche (2002). "IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase." Proc Natl Acad Sci U S A **99**(8): 5567-5572.

Lim, J. H., H. Jono, K. Komatsu, C. H. Woo, J. Lee, M. Miyata, T. Matsuno, X. Xu, Y. Huang, W. Zhang, S. H. Park, Y. I. Kim, Y. D. Choi, H. Shen, K. S. Heo, H. Xu, P. Bourne, T. Koga, H. Xu, C. Yan, B. Wang, L. F. Chen, X. H. Feng and J. D. Li (2012). "CYLD

negatively regulates transforming growth factor-beta-signalling via deubiquitinating Akt." Nat Commun **3**: 771.

Ling, J., Y. Kang, R. Zhao, Q. Xia, D. F. Lee, Z. Chang, J. Li, B. Peng, J. B. Fleming, H. Wang, J. Liu, I. R. Lemischka, M. C. Hung and P. J. Chiao (2012). "KrasG12D-induced IKK2/beta/NF-kappaB activation by IL-1alpha and p62 feedforward loops is required for development of pancreatic ductal adenocarcinoma." Cancer Cell **21**(1): 105-120.

Liu, H., Y. Kato, S. A. Erzinger, G. M. Kiriakova, Y. Qian, D. Palmieri, P. S. Steeg and J. E. Price (2012). "The role of MMP-1 in breast cancer growth and metastasis to the brain in a xenograft model." BMC Cancer **12**: 583.

Liu, M., T. Sakamaki, M. C. Casimiro, N. E. Willmarth, A. A. Quong, X. Ju, J. Ojeifo, X. Jiao, W. S. Yeow, S. Katiyar, L. A. Shirley, D. Joyce, M. P. Lisanti, C. Albanese and R. G. Pestell (2010). "The canonical NF-kappaB pathway governs mammary tumorigenesis in transgenic mice and tumor stem cell expansion." Cancer Res **70**(24): 10464-10473.

Lucas, P. C., M. Yonezumi, N. Inohara, L. M. McAllister-Lucas, M. E. Abazeed, F. F. Chen, S. Yamaoka, M. Seto and G. Nunez (2001). "Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway." J Biol Chem **276**(22): 19012-19019.

Mahoney, D. J., H. H. Cheung, R. L. Mrad, S. Plenchette, C. Simard, E. Enwere, V. Arora, T. W. Mak, E. C. Lacasse, J. Waring and R. G. Korneluk (2008). "Both cIAP1 and cIAP2 regulate TNFalpha-mediated NF-kappaB activation." Proc Natl Acad Sci U S A **105**(33): 11778-11783.

Marcotte, R., H. W. Smith, V. Sanguin-Gendreau, R. V. McDonough and W. J. Muller (2012). "Mammary epithelial-specific disruption of c-Src impairs cell cycle progression and tumorigenesis." Proc Natl Acad Sci U S A **109**(8): 2808-2813.

Marteijn, J. A., H. Lans, W. Vermeulen and J. H. Hoeijmakers (2014). "Understanding nucleotide excision repair and its roles in cancer and ageing." Nat Rev Mol Cell Biol **15**(7): 465-481.

Massarelli, E., V. Papadimitrakopoulou, J. Welsh, C. Tang and A. S. Tsao (2014). "Immunotherapy in lung cancer." Transl Lung Cancer Res **3**(1): 53-63.

Matsumoto, R., D. Wang, M. Blonska, H. Li, M. Kobayashi, B. Pappu, Y. Chen, D. Wang and X. Lin (2005). "Phosphorylation of CARMA1 plays a critical role in T Cell receptor-mediated NF-kappaB activation." Immunity **23**(6): 575-585.

Matsushita, K., O. Takeuchi, D. M. Standley, Y. Kumagai, T. Kawagoe, T. Miyake, T. Satoh, H. Kato, T. Tsujimura, H. Nakamura and S. Akira (2009). "Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay." Nature **458**(7242): 1185-1190.

Mazurek, S., C. B. Boschek, F. Hugo and E. Eigenbrodt (2005). "Pyruvate kinase type M2 and its role in tumor growth and spreading." Semin Cancer Biol **15**(4): 300-308.

Mc Guire, C., P. Wieghofer, L. Elton, D. Muylaert, M. Prinz, R. Beyaert and G. van Loo (2013). "Paracaspase MALT1 deficiency protects mice from autoimmune-mediated demyelination." J Immunol **190**(6): 2896-2903.

McAllister-Lucas, L. M., X. Jin, S. Gu, K. Siu, S. McDonnell, J. Ruland, P. C. Delekta, M. Van Beek and P. C. Lucas (2010). "The CARMA3-Bcl10-MALT1 signalosome promotes

angiotensin II-dependent vascular inflammation and atherogenesis." J Biol Chem **285**(34): 25880-25884.

McCool, K. W. and S. Miyamoto (2012). "DNA damage-dependent NF-kappaB activation: NEMO turns nuclear signaling inside out." Immunol Rev **246**(1): 311-326.

McCormick, F. (2015). "KRAS as a Therapeutic Target." Clin Cancer Res **21**(8): 1797-1801.

Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh and C. A. Janeway, Jr. (1998). "MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways." Mol Cell **2**(2): 253-258.

Merkhofer, E. C., P. Cogswell and A. S. Baldwin (2010). "Her2 activates NF-kappaB and induces invasion through the canonical pathway involving IKKalpha." Oncogene **29**(8): 1238-1248.

Meuwissen, R., S. C. Linn, R. I. Linnoila, J. Zevenhoven, W. J. Mooi and A. Berns (2003). "Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model." Cancer Cell **4**(3): 181-189.

Meylan, E., A. L. Dooley, D. M. Feldser, L. Shen, E. Turk, C. Ouyang and T. Jacks (2009). "Requirement for NF-kappaB signalling in a mouse model of lung adenocarcinoma." Nature **462**(7269): 104-107.

Mitri, Z., T. Constantine and R. O'Regan (2012). "The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in Therapy." Chemother Res Pract **2012**: 743193.

Molina, M. A., J. Codony-Servat, J. Albanell, F. Rojo, J. Arribas and J. Baselga (2001). "Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits

basal and activated Her2 ectodomain cleavage in breast cancer cells." Cancer Res **61**(12): 4744-4749.

Moreno-Garcia, M. E., K. Sommer, H. Shinohara, A. D. Bandaranayake, T. Kurosaki and D. J. Rawlings (2010). "MAGUK-controlled ubiquitination of CARMA1 modulates lymphocyte NF-kappaB activity." Mol Cell Biol **30**(4): 922-934.

Muller, W. J., E. Sinn, P. K. Pattengale, R. Wallace and P. Leder (1988). "Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene." Cell **54**(1): 105-115.

Nagel, D., S. Spranger, M. Vincendeau, M. Grau, S. Raffegerst, B. Kloo, D. Hlahla, M. Neuenschwander, J. Peter von Kries, K. Hadian, B. Dorken, P. Lenz, G. Lenz, D. J. Schendel and D. Krappmann (2012). "Pharmacologic inhibition of MALT1 protease by phenothiazines as a therapeutic approach for the treatment of aggressive ABC-DLBCL." Cancer Cell **22**(6): 825-837.

Neri, A., C. C. Chang, L. Lombardi, M. Salina, P. Corradini, A. T. Maiolo, R. S. Chaganti and R. Dalla-Favera (1991). "B cell lymphoma-associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-kappa B p50." Cell **67**(6): 1075-1087.

Ngo, V. N., R. E. Davis, L. Lamy, X. Yu, H. Zhao, G. Lenz, L. T. Lam, S. Dave, L. Yang, J. Powell and L. M. Staudt (2006). "A loss-of-function RNA interference screen for molecular targets in cancer." Nature **441**(7089): 106-110.

Nielsen, T. O., F. D. Hsu, K. Jensen, M. Cheang, G. Karaca, Z. Hu, T. Hernandez-Boussard, C. Livasy, D. Cowan, L. Dressler, L. A. Akslen, J. Ragaz, A. M. Gown, C. B. Gilks, M. van

de Rijn and C. M. Perou (2004). "Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma." Clin Cancer Res **10**(16): 5367-5374.

Niu, J., Y. Shi, G. Tan, C. H. Yang, M. Fan, L. M. Pfeffer and Z. H. Wu (2012). "DNA damage induces NF-kappaB-dependent microRNA-21 up-regulation and promotes breast cancer cell invasion." J Biol Chem **287**(26): 21783-21795.

Oeffinger, K. C., E. T. Fontham, R. Etzioni, A. Herzig, J. S. Michaelson, Y. C. Shih, L. C. Walter, T. R. Church, C. R. Flowers, S. J. LaMonte, A. M. Wolf, C. DeSantis, J. Lortet-Tieulent, K. Andrews, D. Manassaram-Baptiste, D. Saslow, R. A. Smith, O. W. Brawley, R. Wender and S. American Cancer (2015). "Breast Cancer Screening for Women at Average Risk: 2015 Guideline Update From the American Cancer Society." JAMA **314**(15): 1599-1614.

Oida, K., A. Matsuda, K. Jung, Y. Xia, H. Jang, Y. Amagai, G. Ahn, S. Nishikawa, S. Ishizaka, E. Jensen-Jarolim, H. Matsuda and A. Tanaka (2014). "Nuclear factor-kB plays a critical role in both intrinsic and acquired resistance against endocrine therapy in human breast cancer cells." Sci Rep **4**: 4057.

Ostrem, J. M., U. Peters, M. L. Sos, J. A. Wells and K. M. Shokat (2013). "K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions." Nature **503**(7477): 548-551.

Ott, G., T. Katzenberger, A. Greiner, J. Kalla, A. Rosenwald, U. Heinrich, M. M. Ott and H. K. Muller-Hermelink (1997). "The t(11;18)(q21;q21) chromosome translocation is a frequent and specific aberration in low-grade but not high-grade malignant non-Hodgkin's lymphomas of the mucosa-associated lymphoid tissue (MALT-) type." Cancer Res **57**(18): 3944-3948.

Pan, D., C. Jiang, Z. Ma, M. Blonska, M. J. You and X. Lin (2015). "MALT1 is required for EGFR-induced NF-kappaB activation and contributes to EGFR-driven lung cancer progression." Oncogene.

Pan, D. and X. Lin (2013). "Epithelial growth factor receptor-activated nuclear factor kappaB signaling and its role in epithelial growth factor receptor-associated tumors." Cancer J **19**(6): 461-467.

Pan, D., Y. Zhu, Z. Zhou, T. Wang, H. You, C. Jiang and X. Lin (2015). "The CBM Complex Underwrites NF-kappaB Activation to Promote HER2-associated Tumor Malignancy." Mol Cancer Res.

Parslow, T. G. and D. K. Granner (1982). "Chromatin changes accompany immunoglobulin kappa gene activation: a potential control region within the gene." Nature **299**(5882): 449-451.

Pelzer, C., K. Cabalzar, A. Wolf, M. Gonzalez, G. Lenz and M. Thome (2013). "The protease activity of the paracaspase MALT1 is controlled by monoubiquitination." Nat Immunol **14**(4): 337-345.

Perez, E. A. (2011). "Breast cancer management: opportunities and barriers to an individualized approach." Oncologist **16 Suppl 1**: 20-22.

Perou, C. M., T. Sorlie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, C. A. Rees, J. R. Pollack, D. T. Ross, H. Johnsen, L. A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S. X. Zhu, P. E. Lonning, A. L. Borresen-Dale, P. O. Brown and D. Botstein (2000). "Molecular portraits of human breast tumours." Nature **406**(6797): 747-752.

Pianetti, S., M. Arsura, R. Romieu-Mourez, R. J. Coffey and G. E. Sonenshein (2001). "Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN." Oncogene **20**(11): 1287-1299.

Piret, B., S. Schoonbroodt and J. Piette (1999). "The ATM protein is required for sustained activation of NF-kappaB following DNA damage." Oncogene **18**(13): 2261-2271.

Pivetta, E., M. Scapolan, M. Pecolo, B. Wassermann, I. Abu-Rumeileh, L. Balestreri, E. Borsatti, C. Tripodo, A. Colombatti and P. Spessotto (2011). "MMP-13 stimulates osteoclast differentiation and activation in tumour breast bone metastases." Breast Cancer Res **13**(5): R105.

Politi, K., M. F. Zakowski, P. D. Fan, E. A. Schonfeld, W. Pao and H. E. Varmus (2006). "Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors." Genes Dev **20**(11): 1496-1510.

Pomerantz, J. L., E. M. Denny and D. Baltimore (2002). "CARD11 mediates factor-specific activation of NF-kappaB by the T cell receptor complex." EMBO J **21**(19): 5184-5194.

Pommier, Y., E. Leo, H. Zhang and C. Marchand (2010). "DNA topoisomerases and their poisoning by anticancer and antibacterial drugs." Chem Biol **17**(5): 421-433.

Qiao, Q., C. Yang, C. Zheng, L. Fontan, L. David, X. Yu, C. Bracken, M. Rosen, A. Melnick, E. H. Egelman and H. Wu (2013). "Structural architecture of the CARMA1/Bcl10/MALT1 signalosome: nucleation-induced filamentous assembly." Mol Cell **51**(6): 766-779.

Rahighi, S., F. Ikeda, M. Kawasaki, M. Akutsu, N. Suzuki, R. Kato, T. Kensche, T. Uejima, S. Bloor, D. Komander, F. Randow, S. Wakatsuki and I. Dikic (2009). "Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation." Cell **136**(6): 1098-1109.

Rakha, E. A., J. S. Reis-Filho and I. O. Ellis (2008). "Basal-like breast cancer: a critical review." J Clin Oncol **26**(15): 2568-2581.

Ramilo, C., L. Gu, S. Guo, X. Zhang, S. M. Patrick, J. J. Turchi and G. M. Li (2002). "Partial reconstitution of human DNA mismatch repair in vitro: characterization of the role of human replication protein A." Mol Cell Biol **22**(7): 2037-2046.

Rebeaud, F., S. Hailfinger, A. Posevitz-Fejfar, M. Tapernoux, R. Moser, D. Rueda, O. Gaide, M. Guzzardi, E. M. Iancu, N. Rufer, N. Fasel and M. Thome (2008). "The proteolytic activity of the paracaspase MALT1 is key in T cell activation." Nat Immunol **9**(3): 272-281.

Reiley, W. W., W. Jin, A. J. Lee, A. Wright, X. Wu, E. F. Tewalt, T. O. Leonard, C. C. Norbury, L. Fitzpatrick, M. Zhang and S. C. Sun (2007). "Deubiquitinating enzyme CYLD negatively regulates the ubiquitin-dependent kinase Tak1 and prevents abnormal T cell responses." J Exp Med **204**(6): 1475-1485.

Rexer, B. N. and C. L. Arteaga (2013). "Optimal targeting of HER2-PI3K signaling in breast cancer: mechanistic insights and clinical implications." Cancer Res **73**(13): 3817-3820.

Rosebeck, S., L. Madden, X. Jin, S. Gu, I. J. Apel, A. Appert, R. A. Hamoudi, H. Noels, X. Sagaert, P. Van Loo, M. Baens, M. Q. Du, P. C. Lucas and L. M. McAllister-Lucas (2011). "Cleavage of NIK by the API2-MALT1 fusion oncoprotein leads to noncanonical NF-kappaB activation." Science **331**(6016): 468-472.

Ruan, Q., V. Kameswaran, Y. Zhang, S. Zheng, J. Sun, J. Wang, J. DeVirgiliis, H. C. Liou, A. A. Beg and Y. H. Chen (2011). "The Th17 immune response is controlled by the Rel-RORgamma-RORgamma T transcriptional axis." J Exp Med **208**(11): 2321-2333.

Ruefli-Brasse, A. A., D. M. French and V. M. Dixit (2003). "Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase." Science **302**(5650): 1581-1584.

Ruland, J., G. S. Duncan, A. Wakeham and T. W. Mak (2003). "Differential requirement for Malt1 in T and B cell antigen receptor signaling." Immunity **19**(5): 749-758.

Sanders, B. M., M. Jay, G. J. Draper and E. M. Roberts (1989). "Non-ocular cancer in relatives of retinoblastoma patients." Br J Cancer **60**(3): 358-365.

Sato, S., H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi and S. Akira (2005). "Essential function for the kinase TAK1 in innate and adaptive immune responses." Nat Immunol **6**(11): 1087-1095.

Sauer, B. (1987). "Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae." Mol Cell Biol **7**(6): 2087-2096.

Sauer, B. and N. Henderson (1988). "Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1." Proc Natl Acad Sci U S A **85**(14): 5166-5170.

Schreiber, R. D., L. J. Old and M. J. Smyth (2011). "Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion." Science **331**(6024): 1565-1570.

Sen, R. and D. Baltimore (1986). "Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism." Cell **47**(6): 921-928.

Sen, R. and D. Baltimore (1986). "Multiple nuclear factors interact with the immunoglobulin enhancer sequences." Cell **46**(5): 705-716.

Serra, V., B. Markman, M. Scaltriti, P. J. Eichhorn, V. Valero, M. Guzman, M. L. Botero, E. Llonch, F. Atzori, S. Di Cosimo, M. Maira, C. Garcia-Echeverria, J. L. Parra, J. Arribas and J. Baselga (2008). "NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations." Cancer Res **68**(19): 8022-8030.

Shambharkar, P. B., M. Blonska, B. P. Pappu, H. Li, Y. You, H. Sakurai, B. G. Darnay, H. Hara, J. Penninger and X. Lin (2007). "Phosphorylation and ubiquitination of the IkappaB kinase complex by two distinct signaling pathways." EMBO J **26**(7): 1794-1805.

Sharma, S. V., D. W. Bell, J. Settleman and D. A. Haber (2007). "Epidermal growth factor receptor mutations in lung cancer." Nat Rev Cancer **7**(3): 169-181.

Shim, J. H., C. Xiao, A. E. Paschal, S. T. Bailey, P. Rao, M. S. Hayden, K. Y. Lee, C. Bussey, M. Steckel, N. Tanaka, G. Yamada, S. Akira, K. Matsumoto and S. Ghosh (2005). "TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo." Genes Dev **19**(22): 2668-2681.

Siegel, P. M., E. D. Ryan, R. D. Cardiff and W. J. Muller (1999). "Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer." EMBO J **18**(8): 2149-2164.

Singh, H., R. Sen, D. Baltimore and P. A. Sharp (1986). "A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes." Nature **319**(6049): 154-158.

Singletary, S. E. (2003). "Rating the risk factors for breast cancer." Ann Surg **237**(4): 474-482.

Sinn, E., W. Muller, P. Pattengale, I. Tepler, R. Wallace and P. Leder (1987). "Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo." Cell **49**(4): 465-475.

Solan, N. J., H. Miyoshi, E. M. Carmona, G. D. Bren and C. V. Paya (2002). "RelB cellular regulation and transcriptional activity are regulated by p100." J Biol Chem **277**(2): 1405-1418.

Solomon, B., M. Varella-Garcia and D. R. Camidge (2009). "ALK gene rearrangements: a new therapeutic target in a molecularly defined subset of non-small cell lung cancer." J Thorac Oncol **4**(12): 1450-1454.

Sommer, K., B. Guo, J. L. Pomerantz, A. D. Bandaranayake, M. E. Moreno-Garcia, Y. L. Ovechkina and D. J. Rawlings (2005). "Phosphorylation of the CARMA1 linker controls NF-kappaB activation." Immunity **23**(6): 561-574.

Songur, N., B. Kuru, F. Kalkan, C. Ozdilekcan, H. Cakmak and N. Hizel (2004). "Serum interleukin-6 levels correlate with malnutrition and survival in patients with advanced non-small cell lung cancer." Tumori **90**(2): 196-200.

Soria, J. C., H. Y. Lee, J. I. Lee, L. Wang, J. P. Issa, B. L. Kemp, D. D. Liu, J. M. Kurie, L. Mao and F. R. Khuri (2002). "Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation." Clin Cancer Res **8**(5): 1178-1184.

Sorlie, T., C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, T. Thorsen, H. Quist, J. C. Matese, P. O. Brown, D. Botstein, P.

E. Lonning and A. L. Borresen-Dale (2001). "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications." Proc Natl Acad Sci U S A **98**(19): 10869-10874.

Staal, J., Y. Driege, T. Bekaert, A. Demeyer, D. Muyllaert, P. Van Damme, K. Gevaert and R. Beyaert (2011). "T-cell receptor-induced JNK activation requires proteolytic inactivation of CYLD by MALT1." EMBO J **30**(9): 1742-1752.

Staudt, L. M., H. Singh, R. Sen, T. Wirth, P. A. Sharp and D. Baltimore (1986). "A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes." Nature **323**(6089): 640-643.

Stewart, J. R. and C. A. O'Brian (2005). "Protein kinase C-{alpha} mediates epidermal growth factor receptor transactivation in human prostate cancer cells." Mol Cancer Ther **4**(5): 726-732.

Sun, L., L. Deng, C. K. Ea, Z. P. Xia and Z. J. Chen (2004). "The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes." Mol Cell **14**(3): 289-301.

Sun, S. C. (2011). "Non-canonical NF-kappaB signaling pathway." Cell Res **21**(1): 71-85.

Sun, Z., C. W. Arendt, W. Ellmeier, E. M. Schaeffer, M. J. Sunshine, L. Gandhi, J. Annes, D. Petrzilka, A. Kupfer, P. L. Schwartzberg and D. R. Littman (2000). "PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes." Nature **404**(6776): 402-407.

Suzuki, N., S. Suzuki, G. S. Duncan, D. G. Millar, T. Wada, C. Mirtsos, H. Takada, A. Wakeham, A. Itie, S. Li, J. M. Penninger, H. Wesche, P. S. Ohashi, T. W. Mak and W. C. Yeh

(2002). "Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4." Nature **416**(6882): 750-756.

Tak, P. P. and G. S. Firestein (2001). "NF-kappaB: a key role in inflammatory diseases." J Clin Invest **107**(1): 7-11.

Takaesu, G., R. M. Surabhi, K. J. Park, J. Ninomiya-Tsuji, K. Matsumoto and R. B. Gaynor (2003). "TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway." J Mol Biol **326**(1): 105-115.

Thome, M. (2008). "Multifunctional roles for MALT1 in T-cell activation." Nat Rev Immunol **8**(7): 495-500.

Thorgeirsson, T. E., F. Geller, P. Sulem, T. Rafnar, A. Wiste, K. P. Magnusson, A. Manolescu, G. Thorleifsson, H. Stefansson, A. Ingason, S. N. Stacey, J. T. Bergthorsson, S. Thorlacius, J. Gudmundsson, T. Jonsson, M. Jakobsdottir, J. Saemundsdottir, O. Olafsdottir, L. J. Gudmundsson, G. Bjornsdottir, K. Kristjansson, H. Skuladottir, H. J. Isaksson, T. Gudbjartsson, G. T. Jones, T. Mueller, A. Gottsater, A. Flex, K. K. Aben, F. de Vegt, P. F. Mulders, D. Isla, M. J. Vidal, L. Asin, B. Saez, L. Murillo, T. Blondal, H. Kolbeinsson, J. G. Stefansson, I. Hansdottir, V. Runarsdottir, R. Pola, B. Lindblad, A. M. van Rij, B. Dieplinger, M. Haltmayer, J. I. Mayordomo, L. A. Kiemeney, S. E. Matthiasson, H. Oskarsson, T. Tyrfingsson, D. F. Gudbjartsson, J. R. Gulcher, S. Jonsson, U. Thorsteinsdottir, A. Kong and K. Stefansson (2008). "A variant associated with nicotine dependence, lung cancer and peripheral arterial disease." Nature **452**(7187): 638-642.

Tichelaar, J. W., W. Lu and J. A. Whitsett (2000). "Conditional expression of fibroblast growth factor-7 in the developing and mature lung." J Biol Chem **275**(16): 11858-11864.

Tokunaga, F., T. Nakagawa, M. Nakahara, Y. Saeki, M. Taniguchi, S. Sakata, K. Tanaka, H. Nakano and K. Iwai (2011). "SHARPIN is a component of the NF-kappaB-activating linear ubiquitin chain assembly complex." Nature **471**(7340): 633-636.

Tokunaga, F., S. Sakata, Y. Saeki, Y. Satomi, T. Kirisako, K. Kamei, T. Nakagawa, M. Kato, S. Murata, S. Yamaoka, M. Yamamoto, S. Akira, T. Takao, K. Tanaka and K. Iwai (2009). "Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation." Nat Cell Biol **11**(2): 123-132.

Trompouki, E., E. Hatzivassiliou, T. Tsichritzis, H. Farmer, A. Ashworth and G. Mosialos (2003). "CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members." Nature **424**(6950): 793-796.

Uehata, T., H. Iwasaki, A. Vandenbon, K. Matsushita, E. Hernandez-Cuellar, K. Kuniyoshi, T. Satoh, T. Mino, Y. Suzuki, D. M. Standley, T. Tsujimura, H. Rakugi, Y. Isaka, O. Takeuchi and S. Akira (2013). "Malt1-induced cleavage of regnase-1 in CD4(+) helper T cells regulates immune activation." Cell **153**(5): 1036-1049.

Uren, A. G., K. O'Rourke, L. A. Aravind, M. T. Pisabarro, S. Seshagiri, E. V. Koonin and V. M. Dixit (2000). "Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma." Mol Cell **6**(4): 961-967.

Vogel, K. U., S. L. Edelmann, K. M. Jeltsch, A. Bertossi, K. Heger, G. A. Heinz, J. Zoller, S. C. Warth, K. P. Hoefig, C. Lohs, F. Neff, E. Kremmer, J. Schick, D. Repsilber, A. Geerlof, H. Blum, W. Wurst, M. Heikenwalder, M. Schmidt-Supprian and V. Heissmeyer (2013).

"Roquin paralogs 1 and 2 redundantly repress the Icos and Ox40 costimulator mRNAs and control follicular helper T cell differentiation." Immunity **38**(4): 655-668.

Vu, T. and F. X. Claret (2012). "Trastuzumab: updated mechanisms of action and resistance in breast cancer." Front Oncol **2**: 62.

Wang, C. Y., M. W. Mayo, R. G. Korneluk, D. V. Goeddel and A. S. Baldwin, Jr. (1998). "NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation." Science **281**(5383): 1680-1683.

Wang, D., Y. You, S. M. Case, L. M. McAllister-Lucas, L. Wang, P. S. DiStefano, G. Nunez, J. Bertin and X. Lin (2002). "A requirement for CARMA1 in TCR-induced NF-kappa B activation." Nat Immunol **3**(9): 830-835.

Wang, D., Y. You, P. C. Lin, L. Xue, S. W. Morris, H. Zeng, R. Wen and X. Lin (2007). "Bcl10 plays a critical role in NF-kappaB activation induced by G protein-coupled receptors." Proc Natl Acad Sci U S A **104**(1): 145-150.

Warrier, S., G. Tapia, D. Goltsman and J. Beith (2015). "An update in breast cancer screening and management." Womens Health (Lond Engl).

Webb, C. and T. Koch (1997). "Women's experiences of non-invasive breast cancer: literature review and study report." J Adv Nurs **25**(3): 514-525.

Weigelt, B., J. L. Peterse and L. J. van 't Veer (2005). "Breast cancer metastasis: markers and models." Nat Rev Cancer **5**(8): 591-602.

Weih, F., D. Carrasco, S. K. Durham, D. S. Barton, C. A. Rizzo, R. P. Ryseck, S. A. Lira and R. Bravo (1995). "Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family." Cell **80**(2): 331-340.

Weinberger, J., D. Baltimore and P. A. Sharp (1986). "Distinct factors bind to apparently homologous sequences in the immunoglobulin heavy-chain enhancer." Nature **322**(6082): 846-848.

Weterings, E. and D. J. Chen (2008). "The endless tale of non-homologous end-joining." Cell Res **18**(1): 114-124.

Willis, T. G., D. M. Jadayel, M. Q. Du, H. Peng, A. R. Perry, M. Abdul-Rauf, H. Price, L. Karran, O. Majekodunmi, I. Wlodarska, L. Pan, T. Crook, R. Hamoudi, P. G. Isaacson and M. J. Dyer (1999). "Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types." Cell **96**(1): 35-45.

Wu, Z. H., Y. Shi, R. S. Tibbetts and S. Miyamoto (2006). "Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli." Science **311**(5764): 1141-1146.

Wu, Z. H., E. T. Wong, Y. Shi, J. Niu, Z. Chen, S. Miyamoto and V. Tergaonkar (2010). "ATM- and NEMO-dependent ELKS ubiquitination coordinates TAK1-mediated IKK activation in response to genotoxic stress." Mol Cell **40**(1): 75-86.

Xiao, G., E. W. Harhaj and S. C. Sun (2001). "NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100." Mol Cell **7**(2): 401-409.

Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda and S. Akira (2002). "Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling." J Immunol **169**(12): 6668-6672.

Yang, W., Y. Xia, Y. Cao, Y. Zheng, W. Bu, L. Zhang, M. J. You, M. Y. Koh, G. Cote, K. Aldape, Y. Li, I. M. Verma, P. J. Chiao and Z. Lu (2012). "EGFR-induced and PKCepsilon monoubiquitylation-dependent NF-kappaB activation upregulates PKM2 expression and promotes tumorigenesis." Mol Cell **48**(5): 771-784.

Yang, Y., F. Xia, N. Hermance, A. Mabb, S. Simonson, S. Morrissey, P. Gandhi, M. Munson, S. Miyamoto and M. A. Kelliher (2011). "A cytosolic ATM/NEMO/RIP1 complex recruits TAK1 to mediate the NF-kappaB and p38 mitogen-activated protein kinase (MAPK)/MAPK-activated protein 2 responses to DNA damage." Mol Cell Biol **31**(14): 2774-2786.

Yarden, Y. and M. X. Sliwkowski (2001). "Untangling the ErbB signalling network." Nat Rev Mol Cell Biol **2**(2): 127-137.

Yin, W., Y. Jiang, Z. Shen, Z. Shao and J. Lu (2011). "Trastuzumab in the adjuvant treatment of HER2-positive early breast cancer patients: a meta-analysis of published randomized controlled trials." PLoS One **6**(6): e21030.

Yu, D., A. H. Tan, X. Hu, V. Athanasopoulos, N. Simpson, D. G. Silva, A. Hutloff, K. M. Giles, P. J. Leedman, K. P. Lam, C. C. Goodnow and C. G. Vinuesa (2007). "Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA." Nature **450**(7167): 299-303.

Zhao, B., S. S. Chua, M. M. Burcin, S. D. Reynolds, B. R. Stripp, R. A. Edwards, M. J. Finegold, S. Y. Tsai and F. J. DeMayo (2001). "Phenotypic consequences of lung-specific inducible expression of FGF-3." Proc Natl Acad Sci U S A **98**(10): 5898-5903.

Zhao, X. Q., L. L. Zhu, Q. Chang, C. Jiang, Y. You, T. Luo, X. M. Jia and X. Lin (2014). "C-type lectin receptor dectin-3 mediates trehalose 6,6'-dimycolate (TDM)-induced Mincle

expression through CARD9/Bcl10/MALT1-dependent nuclear factor (NF)-kappaB activation." J Biol Chem **289**(43): 30052-30062.

VITA

Deng Pan was born in Shanghai, China on November 8, 1986, the son of Weiqing Pan and Ying Ding. After completing his work at Kongjiang high school, Shanghai, China in 2005, he entered Southern Medical University in Guangzhou, China. He received the degree of Bachelor of Medicine, with a major in diagnostic imaging from Southern Medical University in June, 2010. In September of 2010 he entered The University of Texas Graduate School of Biomedical Sciences at Houston.

Permanent address:

Zhengtong Rd. Lane 90, BLDG #2, 1401

Shanghai, China 200433