AD_____

Award Number: W81XWH-08-1-0763

TITLE: Characterization of IKBKE as a Breast Cancer Oncogene

PRINCIPAL INVESTIGATOR: Alicia Zhou

CONTRACTING ORGANIZATION: Dana-Barber Cancer Institute
Boston, MA 02115

REPORT DATE: October 2011

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE *(DD-MM-YYYY)* | 2. REPORT TYPE | 3. DATES COVERED *(From - To)* |
|---|---|---|
| 01-10-2011 | Annual Summary | 1 Oct 2008 - 30 Sep 2011 |

**4. TITLE AND SUBTITLE**
Characterization of IKBKE as a Breast Cancer Oncogene

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-08-1-0763

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Alicia Zhou

E-Mail: aliciay_zhou@dfci.harvard.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Dana-Barber Cancer Institute
Boston, MA 02115

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

A summary is presented of research performed during three years of a project to further characterize the breast cancer oncogene IKKε. Two specific aims were pursued. The first was to determine the role of ubiquitination in the upstream regulation of IKKε. The second aim created a mammary-specific constitutive IKKε transgenic mouse model to study the role of IKKε in breast cancer initiation and maintenance. The long term goals of this research were to elucidate the mechanism of IKKε upstream regulation in order to better understand how it is dysregulated and overexpressed in human breast cancer and also to create a genetic animal model of IKKε breast cancer initiation for further study.

**15. SUBJECT TERMS**
IKKepsilon, breast cancer, ubiquitin, oncogenic transformation, mammary mouse tumor model

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 11 | 19b. TELEPHONE NUMBER *(include area code)* |

**Table of Contents**

**Page**

## Introduction

Pathological specimens derived from breast cancer patients and mammary carcinoma cell lines display elevated or constitutive nuclear-factor-κB (NF-κB) activity[1], underscoring the importance of understanding this pathway for the development of future targeted therapeutics. The NF-κB family of proteins plays critical roles in many different biological processes including inflammation, innate and adaptive immunity and apoptosis. Because of their diverse functions, deregulation of NF-κB transcription factors is also linked to oncogenesis. Recently, NF-κB signaling has been shown to be activated in solid tumors, including human breast cancers[1, 2]. Other studies have also established a connection between chronic inflammation, NF-κB activity, and epithelial cancers[3]. These observations, taken together, have implicated many members of the NF-κB family as potential oncogenes.

Previous work in the Hahn Lab has identified *IKBKE*, the gene that encodes the non-canonical NF-κB pathway kinase IKKε, as a breast cancer oncogene that mediates mammalian cell transformation[4]. Expression of IKKε in human mammary epithelial cells (HMEC) facilitates anchorage-independent growth in soft agar and promotes tumorigenesis in mice. However, this transforming potential is abolished upon introduction of the NF-κB super-repressor, a degradation-resistant form of IκBα, indicating that IKKε-mediated transformation requires NF-κB signaling. In addition, IKKε is amplified and overexpressed in a considerable portion of human breast cancer cell lines and primary tumor samples.

Although these studies allowed us to identify *IKBKE* as a breast cancer oncogene, the mechanisms and signals by which IKKε is activated were not well studied. Furthermore, we had never investigated the role of IKKε in tumor initiation and tumor maintenance in the setting of an intact animal. Thus, over the course of the past three years, I have pursued the following specific aims to elucidate the regulation and function of IKKε in the development of breast cancer.

## Specific Aims

1. Investigate the role of ubiquitination in IKKε-mediated cell transformation
    a. Confirm and characterize IKKε ubiquitination
    b. Identification of the IKKε ubiquitin-accepting residues
    c. Determine the functional relevance of IKKε ubiquitination in mammary cell transformation

2. Investigate the role of IKKε in breast cancer initiation and maintenance
    a. Investigate the role of *IKBKE* in breast cancer initiation
    b. Investigate the role of *IKBKE* in breast cancer maintenance

**Body**

***Specific Aim 1a:*** I have demonstrated that IKKε is ubiquitinated in several cell contexts. First, Flag-tagged IKKε (F-IKKε) and HA-tagged ubiquitin were transiently expressed in HEK293T cells and an immunoprecipitation for either FLAG or HA was performed. When the precipitate was analyzed for IKKε by immunoblot, an ubiquitin ladder was observed in cells expressing IKKε (Fig 1A). In transformed kidney and mammary epithelial cell lines, HA1EM and HMLEM, that express F-IKKε and MF-IKKε, an immunoprecipitation for IKKε followed by immunoblot for IKKε reveals the endogenously polyubiquitinated species of IKKε (Fig. 1B and IC respectively). Finally, in MCF-7 breast cancer cells, which are known to harbor an *IKBKE* amplification and overexpress IKKε protein, an immunoprecipitation for IKKε was performed and subsequent analysis by immunoblot for both IKKε and ubiquitin revealed endogenous ubiquitinated species of IKKε (Figure 1D).

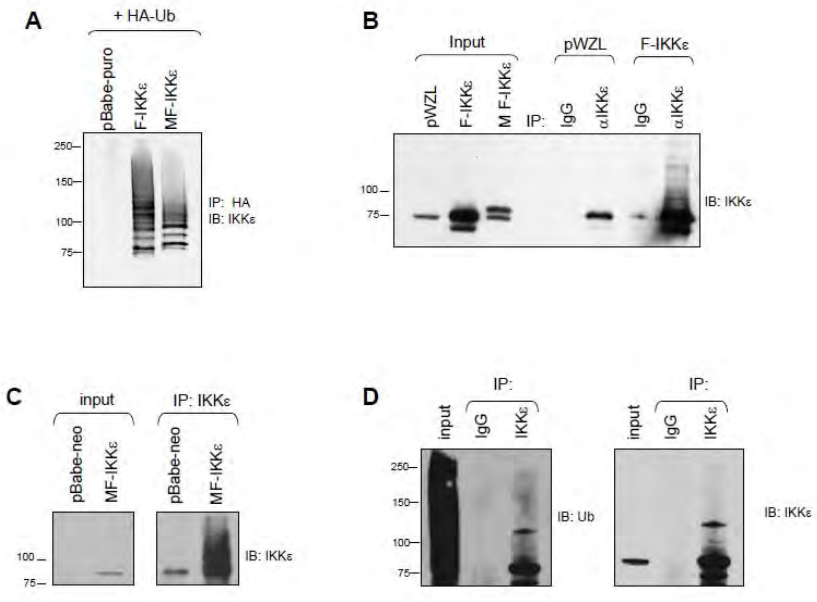
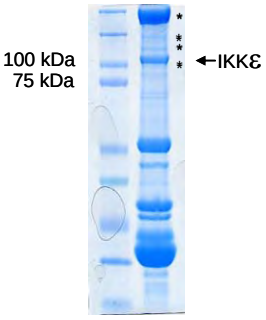



**Figure 1. IKKε is ubiquitinated**.
(A) HEK293T cells were cotransfected with HA-ubiquitin and either pBabe-puro empty vector, F-IKKε or, MF-IKKε. Immunoprecipitation (IP) was performed with an HA antibody followed by immunoblot analysis (WB) with an IKKε antibody
(B) IP with an IKKε antibody from HA1EM cells stably expressing either pWZL, F- IKKε or MF-IKKε. Immunoblot analysis by ubiquitin and IKKε antibody.
(C) IP with IKKε antibody from HMLE-MEK$^{DD}$ cells stably expressing either pWZL or MF-IKKε. Immunoblot analysis by IKKε antibody.
(D) IP with an IKKε antibody from MCF-7 breast cancer cells. Immunoblot analysis by ubiquitin and IKKε antibody.

***Specific Aim 1b:*** To determine the residues on which IKKε is being modified by ubiquitination, a mass spectrometry approach was taken. I transiently cotransfected GST-tagged IKKε and HA-tagged ubiquitin in HEK293T cells and performed a GST immunoprecipitation. The




| | | | | | |
|---|---|---|---|---|---|
| 1 | MQSTANYLWH | TDDLLGQGAT | ASVYKARNKK | SGELVAVKVF | NTTSYLRPRE |
| 51 | VQVREFEVLR | KLNHQNIVKL | FAVEETGGSR | QKVLVMEYCS | SGSLLSVLES |
| 101 | PENAFGLPED | EFLVVLRCVV | AGMNHLRENG | IVHRDIKPGN | IMRLVGEEGQ |
| 151 | SIYKLTDFGA | ARELDDDEKF | VSVYGTEEYL | HPDMYERAVL | RKPQQKAFGV |
| 201 | TVDLWSIGVT | LYHAATGSLP | FIPFGGPRRN | KEIMYRITTE | KPAGAIAGAQ |
| 251 | RRENGPLEWS | YTLPITCQLS | LGLQSQLVPI | LANILEVEQA | KCWGFDQFFA |
| 301 | ETSDILQRVV | VHVFSLSQAV | LHHIYIHAHN | TIAIFQEAVH | KQTSVAPRHQ |
| 351 | EYLFEGHLCV | LEPSVSAQHI | AHTTASSPLT | LFSTAIPKGL | AFRDPALDVP |
| 401 | KFVPKVDLQA | DYNTAKGVLG | AGYQALRLAR | ALLDGQELMF | RGLHWVMEVL |
| 451 | QATCRRTLEV | ARTSLLYLSS | SLGTERFSSV | AGTPEIQELK | AAAELRSRLR |
| 501 | TLAEVLSRCS | QNITETQESL | SSLNRELVKS | RDQVHEDRSI | QQIQCCLDKM |
| 551 | NFIYKQFKKS | RMRPGLGYNE | EQIHKLDKVN | FSHLAKRLLQ | VFQEECVQKY |
| 601 | QASLVTHGKR | MRVVHETRNH | LRLVGCSVAA | CNTEAQGVQE | SLSKLLEELS |
| 651 | HQLLQDRAKG | AQASPPPIAP | YPSPTRKDLL | LHMQELCEGM | KLLASDLLDN |
| 701 | NRIIERLNRV | PAPPDV | | | |

**Figure 2. Mass spectrometry of IKKε**.
(Left) GST-IKKε and HA-ubiquitin (HA-Ub) were coexpressed in HEK293T cells and lysates from 45 x 10 cm plates were loaded on SDS-PAGE and stained with Coomassie Blue. The bands noted by asterisks were submitted for mass spectrometry (Right) Summary of mass spectrometry data. Underlined amino acids were identified by mass spectrometry. Lysines (K) are bolded and the three lysines in red were found to be ubiquitinated.

immunoprecipitates were then subjected to SDS-PAGE followed by Coomassie blue staining. Four bands of interest were identified and submitted for mass spectrometry analysis (Figure 2). We obtained 58.2% coverage of the IKKε protein and 64.7% (22 out of 34) coverage of the internal lysines. From this analysis, three lysine residues were identified as modified by ubiquitin: K30, K401, and K416.

***Specific Aim 1c:*** To determine the functional relevance of the ubiquitination of IKKε in the context of cell transformation and cancer, I generated site-specific lysine-to-arginine IKKε mutants for the three lysine residues that were identified in Aim 1b. These IKKε mutants have been retrovirally introduced into HA1EM cell to create stable cell lines that express these mutant constructs (Figure 3A). These mutants were assessed for transformation capacity by soft agar analysis (Figure 3B). This transformation analysis indicates that the K30R and K401R mutants exhibit an impaired transformation capacity as compared to that of WT and the K416R mutant.

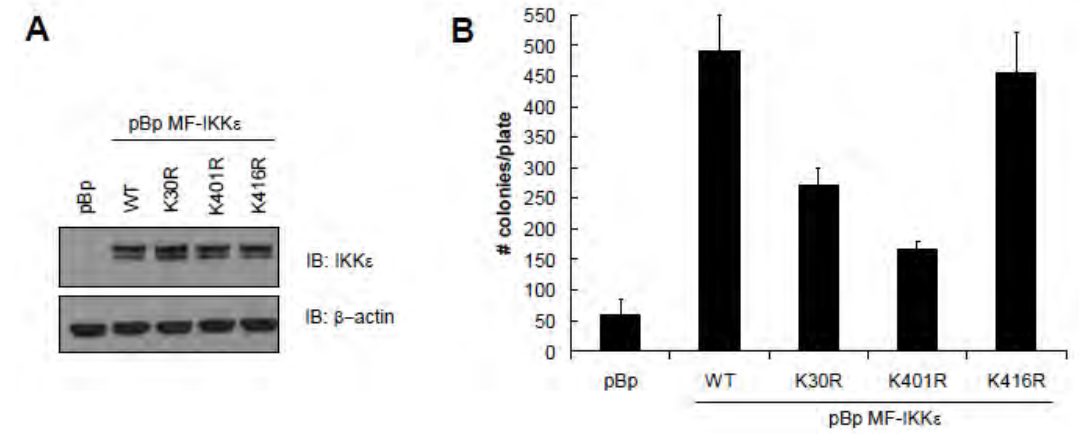



**Figure 3. Transformation phenotype of IKKε ubiquitination mutants**.
(A) HA1EM cells stably expressing MF-IKKε WT and mutants were made. IKKε levels were assessed by immunoblot.
(B) Soft agar analysis of HA1EM cells expressing WT and mutants of MF-IKKε. Colonies were counted 3 weeks after cells were plated.

The K30R and K401R IKKε mutants showed an impaired transformation capacity in Figure 3. I, thus, further examined what the effects of the K30R and K401R mutants were on other IKKε functions including IKKε kinase

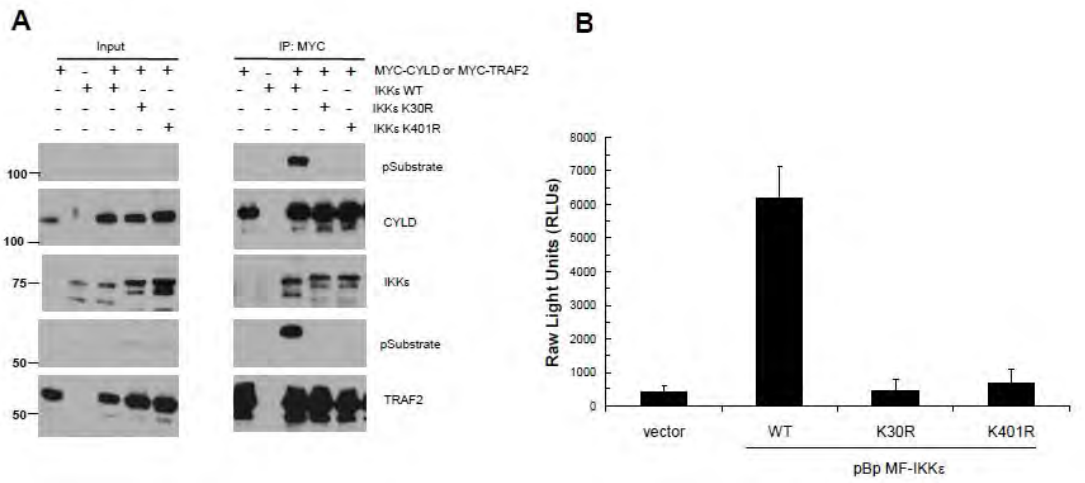



**Figure 4. Functional consequences of IKKε mutants on kinase and NF-κB activity**.
(A) 293T cells were cotransfected with MYC-CYLD or MYC-TRAF2 and IKKε WT or mutant. Immunoprecipitation for MY and immunoblot for pSubstrate, IKKε, CYLD and TRAF2 was performed.
(B) ONE-GLO Luciferase Assay performed on 293T cells stably expressing a NF-κB luciferase reporter. These cells were transfected with either eGFP control or IKKε WT and mutants.

activity and subsequent NF-κB activation. Other studies in the Hahn Lab have identified CYLD (cylindromatosis) and TRAF2 (TNF receptor-associated factor 2) as kinase targets of IKKε[5]. As a result from our previous studies, an IKKε phospho-substrate antibody was generated and has been used to identify CYLD and TRAF2 as IKKε kinase targets. I cotransfected wildtype, K30R and K401R mutant IKKε with either Myc-tagged CYLD or Myc-tagged TRAF2. After immunoprecipitation for Myc, an immunoblot using phospho-substrate (pSubstrate) antibody showed that wildtype IKKε phosphorylates both TRAF2 and CYLD, but neither of the mutant forms of IKKε was capable of this phosphorylation activity (Figure 4A). A NF-κB luciferase reporter assay was used to determine the ability of these IKKε mutants to activate the NF-κB pathway (Figure 4B). Consistent with the kinase activity data, these results indicated that WT but not mutant IKKε was able to induce the NF-κB luciferase reporter activity.

***Further Studies:*** Having completed the three aims I had originally set out to achieve, my next goal was to characterize what type of ubiquitination was occurring on IKKε. It is know that the NF-κB pathway is regulated by various forms of ubiquitination. Whereas canonical K48-linked ubiquitination serves as a protein degradation signal on the IκB proteins, non-canonical K63-linked ubiquitination has been observed to be an activating modification on IKKγ[6,7]. In MCF-7 cells that were treated with a cocktail of proteasome inhibitors (MG132 + Velcade), I was able to show that although the overall ubiquitin load of the cell is increased, the levels of IKKε protein remain the same (Figure 5A). This indicates that IKKε is subject to proteasome-independent ubiquitin modification. Next, I cotransfected 293T cells with Myc-IKKε and mutant a HA-ubiquitin construct in which all other lysines except K63 are replaced with Arg. I immunoprecipitated for Myc and immunoblotted for HA (Figure 5B) and was able to show that although wildtype IKKε and each of the single mutant K30R and K401R IKKε

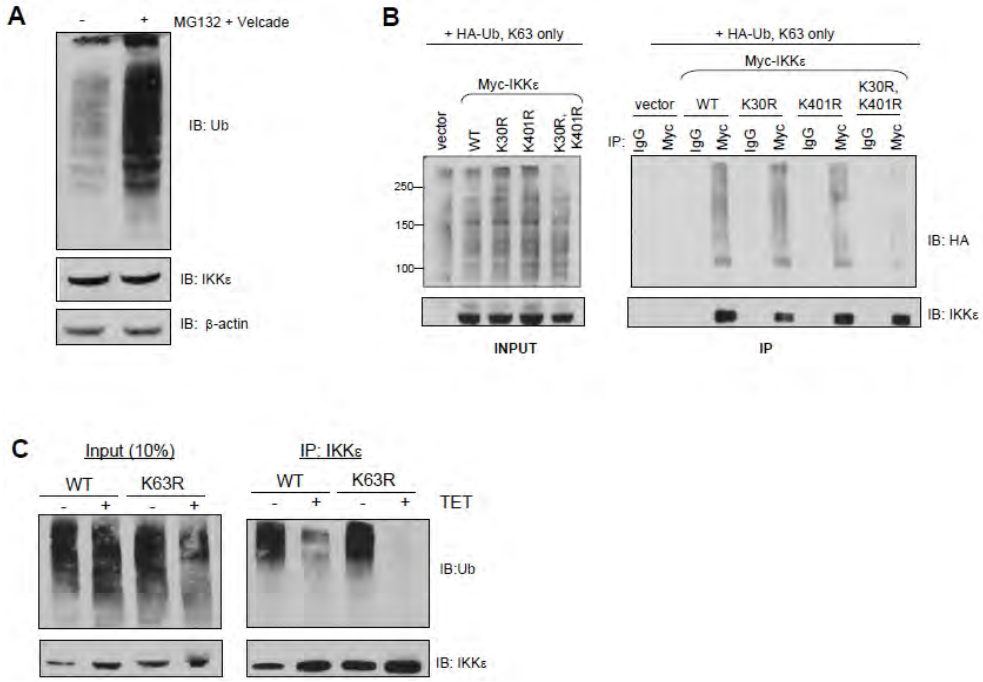



**Figure 5. Characterization of IKKε ubiquitination**.
(A) MCF-7 cells were treated with a cocktail of MG132 and Velcade for 4 hours. After treatment, cell lysates were collected and immunoblots for ubiquitin, IKKε and β-actin protein were performed.
(B) 293T cells were cotransfected K63 only HA-Ubiquitin along with Myc-IKKε. Immunoprecipitation for Myc was preformed followed by immunoblot for HA.
(C) U2OS shUb-Ub(WT) or shUb-Ub(K63R) cells were treated with tetracycline (TET) for 96 hours, immunoprecipitated for IKKε and immunoblotted for ubiquitin.

mutants were being modified by the K63-only ubiquitin, the K30R, K401R double mutant was no longer undergoing this modification. This result indicates that IKKε is being modified by K63-linked ubiquitin chains on both K30 and K401. Mutation at only one of these residues is not sufficient to eliminate the ubiquitination of IKKε by K63 chains, but the mutation of both residues results in the disappearance of the higher mobility polyubiquitination ladder, thus indicating that IKKε is being modified by K63 chains only on these two residues. Through collaboration with the James Chen Lab (UT Southwestern), we were able to obtain a more physiologically relevant system to address this issue as well. The Chen Lab provided us with a set of engineered U2OS cells in which the cell's endogenous ubiquitin could be inducibly suppressed by tetracycline treatment. Since the ubiquitin protein is essential for cell survival, a tetracycline-inducible exogenous ubiquitin construct was introduced in parallel to these cells. In the U2OS shUb-Ub(WT) cells, the exogenous construct expressed a wild-type ubiquitin. In the U2OS shUb-Ub(K63R) cells, the exogenous construct expressed a K63R mutant form of ubiquitin – this mutant harbors a lysine-to-arginine mutation at the K63 residue, rendering this ubiquitin incapable of forming K63-linked chains. In this system, immunoprecipitation for IKKε followed by immunoblot for ubiquitin showed that IKKε is capable of undergoing modification by the WT ubiquitin chains but is no longer able to be modified by the K63R chains (Figure 5C). Taken together, these data are evidence that IKKε normally undergoes K63-linked ubiquitination at K30 and K401 under physiological conditions.

My most recent work on this project has been to determine the E3 ubiquitin ligase that is responsible for IKKε modification. Through a thorough literature search, I was able to determine that the TRAF family of proteins was a relevant family of E3 ubiquitin ligase proteins which are known to associate with various NF-κB activation pathways. In addition, the TRAF family of proteins are known to contain a RING domain and have been characterized to show specificity towards catalyzing K63-chain ubiquitination. As a result, this family of proteins was determined to be good candidates for catalyzing IKKε ubiquitination. In HA1EM cells expressing F-IKKε and MF-IKKε, I was able to demonstrate that IKKε forms a complex with TRAF2 and the scaffolding protein TANK (Figure 6A). To do this, I immunoprecipitated TANK from these cell lysates and subsequently immunoblotted for IKKε and TRAF2. My results indicate that all three of the proteins do indeed form a complex. Next, I

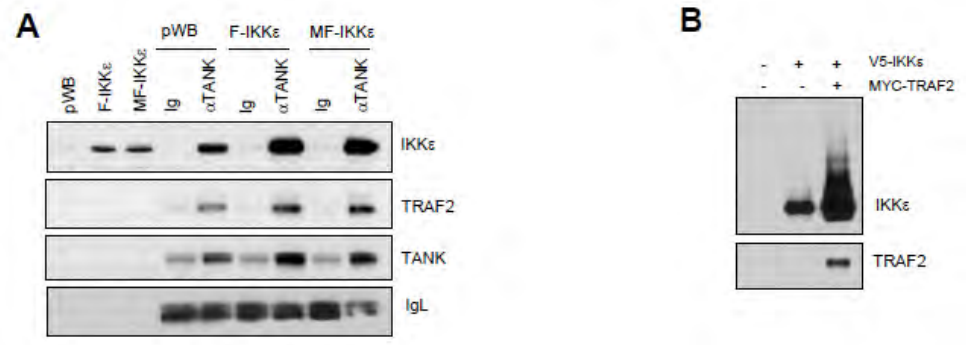



**Figure 6. TRAF2 complexes with and ubiquitinates IKKε.**

(A) HA1EM cell lysates expressing vector, F-IKKε and MF-IKKε were immunoprecipitated for TANK and immunoblotted for IKKε, TRAF2 and TANK.

(B) 293T cells were co-transfected with V5-IKKε and MYC-TRAF2. Cell lysates were collected and immunoblotted for IKKε and TRAF2.

co-transfected 293T cells with V5-tagged IKKε and Myc-TRAF2. After a subsequent immunoblot for IKKε, I was able to show that IKKε is robustly ubiquitinated in the presence of TRAF2. Taken together, these data indicate that IKKε physically associates with TRAF2 in a complex with TANK and that TRAF2 is able to catalyze the ubiquitination of IKKε.

***Specific Aim 2:*** In my previous progress reports, I have reported that we had successfully generated a MMTV-IKKε mouse model. However, unfortunately, recent analysis of our cohort revealed that despite successful transgene integration, after several rounds of breeding over the duration of 1.5 years, the transgene was no longer being expressed in the tissues of the mice. As a result, we were forced to begin anew on this project in early 2010. Since then, I have generated a completely new MMTV-IKKε construct (Figure 7A). The new construct was submitted for injection and we have now identified three successful founder mice by PCR genotyping (Figure 7B). We are currently working to expand these three founder lines.

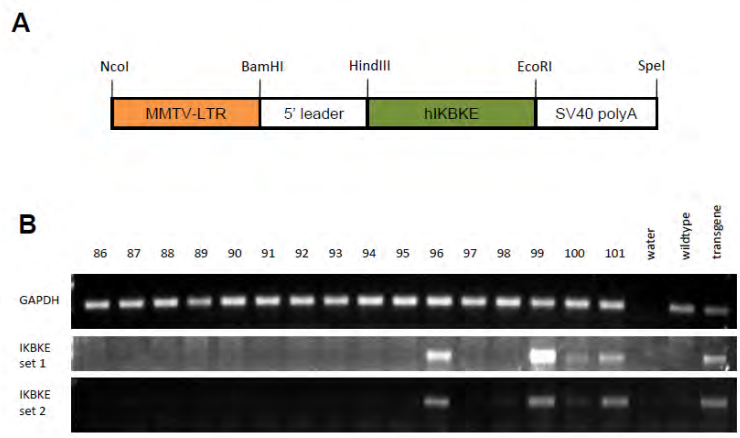



**Figure 7. MMTV-IKKε transgenic mouse model**
(A) The constitutive MMTV-IKKε construct. The construct was created by subcloning the cDNA of the human *IKBKE* gene, with HindIII and EcoRI, downstream of the MMTV promoter
(B) Genotyping PCR results. PCR was performed on DNA isolated from tails of putative MMTV-IKKε founder mice. Two distinct primer sets specific for the human *IKBKE* cDNA were used along with a GAPDH housekeeping gene control.

We will shortly move ahead with our original intended experiments to determine if IKKε expression in the mammary gland of the mouse is sufficient to cause tumor formation. We have also generated a large cohort of WAP-Cre/p53 floxed mice, these mammary-specific p53 null mice show a mammary tumor phenotype at an average onset of 9 months. Should the MMTV-IKKε mice not develop a significant mammary tumor phenotype, we are prepared to cross these mice into the p53 null background in order to determine if mammary-specific expression of IKKε causes tumor growth acceleration in these mice.

**Key Research/Training Accomplishments**

- Confirmed IKKε ubiquitination in the context of mammary cell transformation
- Identified three lysine residues within IKKε that are subject to ubiquitination by mass spectrometry
- Generated IKKε point mutants in which the identified lysine residues are mutated and generated cell lines that stably express the IKKε mutant constructs
- Demonstrated that IKKε lysine mutants show a distinct change in transformation phenotype as well as in IKKε kinase function and NF-κB activation

- Demonstrated that IKKε is being modified by K63-linked ubiquitination
- Identified TRAF2 as a putative E3 ligase that catalyzes the ubiquitination of IKKε
- Generated founder mice for the constitutive MMTV-IKKε transgenic mouse model
- Generated compound transgenic WAP-Cre/p53$^{fl/fl}$ mouse model

**Reportable Outcomes**

- Established expression constructs for Flag-tagged and Myr-Flag-tagged IKKε point mutants: K30R, K401R, K41R and K30R K401R
- Established HA1EM cell lines that constitutively express F- and MF-IKKε point mutants
- Established two new animal models: MMTV-IKKε and WAP-Cre/p53$^{fl/fl}$

**Conclusion**

Taken altogether, the data that I have summarized in this report offer significant evidence that IKKε undergoes modification and regulation by K63-linked ubiquitination on residues K30 and K401. Further, I have shown that the mutation of either of these two residues results in the abrogation of IKKε kinase function and subsequent ability to activate NF-κB. Most importantly, I have shown that these two residues are essential for the ability of IKKε to cause cell transformation. Finally, I have been able to identify TRAF2 as a putative E3 ligase that associates with and catalyzes the ubiquitination of IKKε. These results offers new insights into the regulation of IKKε, a known breast cancer oncogene, allowing for the future development of more targeted therapeutics against IKKε.

I have also successfully generated both a constitutive transgenic mouse model of mammary-specific IKKε expression. This genetic model of IKKε transformation will allow us to better understand the *in vivo* role of IKKε in breast tumor initiation and maintenance for future studies.

**References**

1. Karin, M., Cao, Y., Greten, F.R. & Li, Z.W. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2, 301-10 (2002).

2. Biswas, D.K. et al. 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, 10137-42 (2004).

3. Karin, M. & Greten, F.R. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 5, 749-59 (2005).

4. Boehm, J.S. et al. Integrative genomic approaches identify IKBKE as a breast cancer oncogene. *Cell* 129, 1065-79 (2007).

5. Hutti et al. Phosphorylation of the tumor suppressor CYLD by the breast cancer oncogene IKKepsilon promotes cell transformation. *Mol Cell.* 34, 461-72 (2009).

6. Chen, F., Bhatia, D., Chang, Q. & Castranova, V. Finding NEMO by K63-linked polyubiquitin chain. *Cell Death Differ* 13, 1835-8 (2006).

7. Sebban, H., Yamaoka, S. & Courtois, G. Posttranslational modifications of NEMO and its partners in NF-kappaB signaling. *Trends Cell Biol* 16, 569-77 (2006).