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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.					
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Introduction

Pathological specimens derived from breast cancer patients and mammary carcinoma cell lines display elevated or constitutive nuclear-factor- κ B (NF- κ B) activity¹, underscoring the importance of understanding this pathway for the development of future targeted therapeutics. The NF- κ B 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- κ B transcription factors is also linked to oncogenesis. Recently, NF- κ B 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- κ B activity, and epithelial cancers³. These observations, taken together, have implicated many members of the NF- κ B family as potential oncogenes.

Previous work in the Hahn Lab has identified *IKBKE*, the gene that encodes the non-canonical NF- κ B pathway kinase IKK ϵ , as a breast cancer oncogene that mediates mammalian cell transformation⁴. Expression of IKK ϵ 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- κ B super-repressor, a degradation-resistant form of I κ B α , indicating that IKK ϵ -mediated transformation requires NF- κ B signaling. In addition, IKK ϵ 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 IKK ϵ is activated were not well studied. Furthermore, we had never investigated the role of IKK ϵ 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 IKK ϵ in the development of breast cancer.

Specific Aims

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

2. Investigate the role of IKK ϵ 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 IKK ϵ is ubiquitinated in several cell contexts. First, Flag-tagged IKK ϵ (F-IKK ϵ) 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 IKK ϵ by immunoblot, an ubiquitin ladder was observed in cells expressing IKK ϵ (Fig 1A). In transformed kidney and mammary epithelial cell lines, HA1EM and HMLEM, that express F-IKK ϵ and MF-IKK ϵ , an immunoprecipitation for IKK ϵ followed by immunoblot for IKK ϵ reveals the endogenously polyubiquitinated species of IKK ϵ (Fig. 1B and 1C respectively). Finally, in MCF-7 breast cancer cells, which are known to harbor an

IKBKE amplification and overexpress IKK ϵ protein, an immunoprecipitation for IKK ϵ was performed and subsequent analysis by immunoblot for both IKK ϵ and ubiquitin revealed endogenous ubiquitinated species of IKK ϵ (Figure 1D).

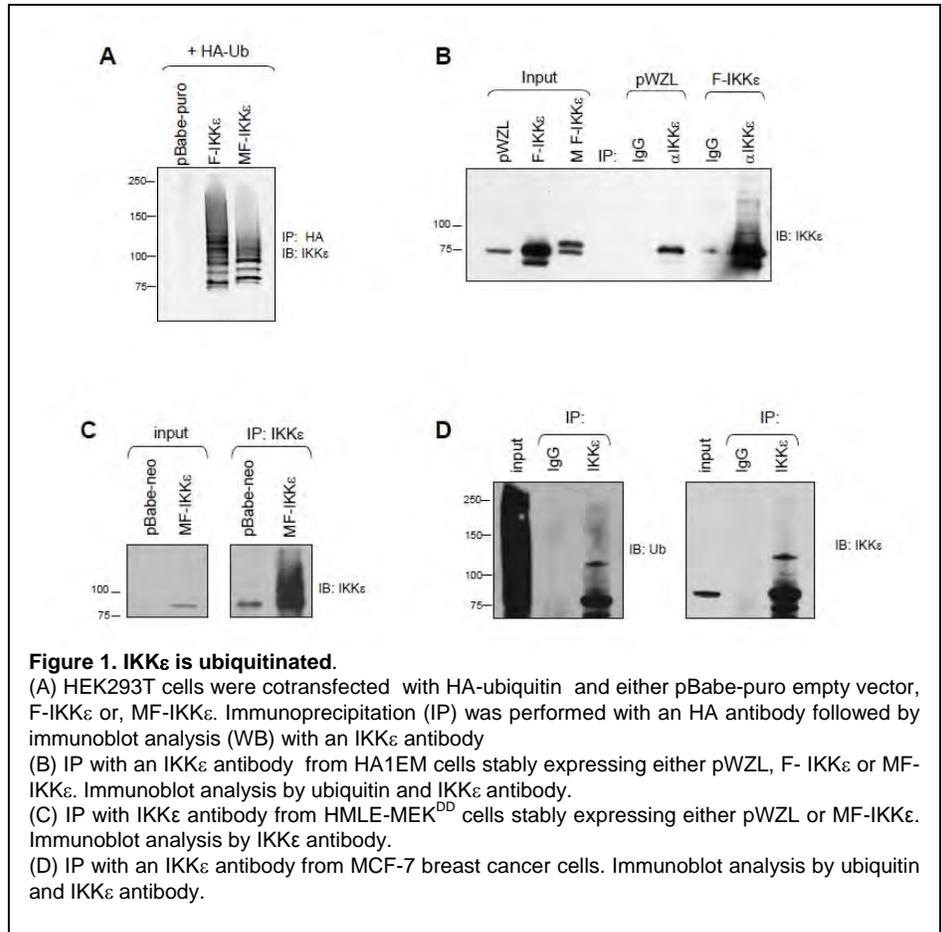


Figure 1. IKK ϵ is ubiquitinated.

(A) HEK293T cells were cotransfected with HA-ubiquitin and either pBabe-puro empty vector, F-IKK ϵ or, MF-IKK ϵ . Immunoprecipitation (IP) was performed with an HA antibody followed by immunoblot analysis (WB) with an IKK ϵ antibody

(B) IP with an IKK ϵ antibody from HA1EM cells stably expressing either pWZL, F-IKK ϵ or MF-IKK ϵ . Immunoblot analysis by ubiquitin and IKK ϵ antibody.

(C) IP with IKK ϵ antibody from HMLE-MEK^{DD} cells stably expressing either pWZL or MF-IKK ϵ . Immunoblot analysis by IKK ϵ antibody.

(D) IP with an IKK ϵ antibody from MCF-7 breast cancer cells. Immunoblot analysis by ubiquitin and IKK ϵ antibody.

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

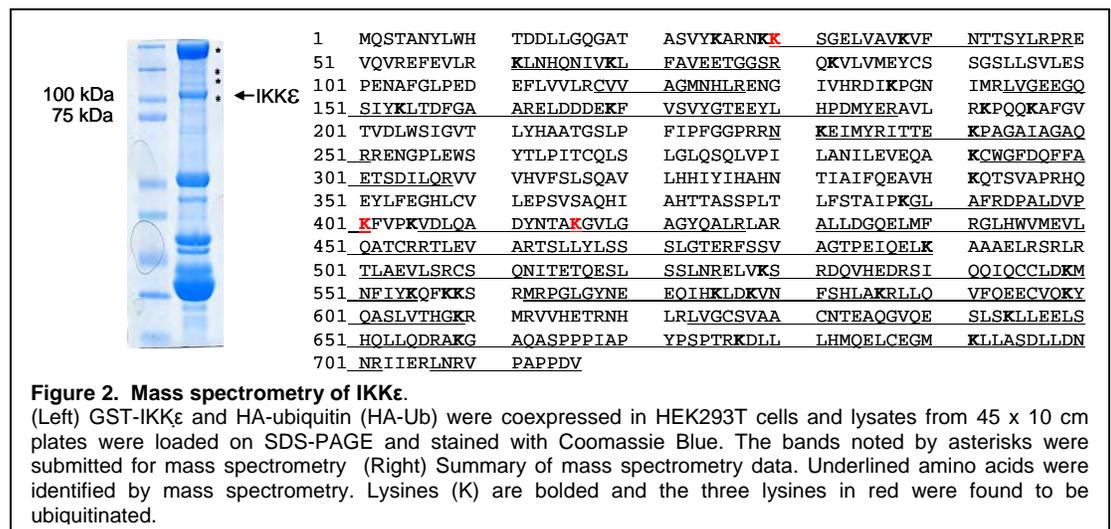
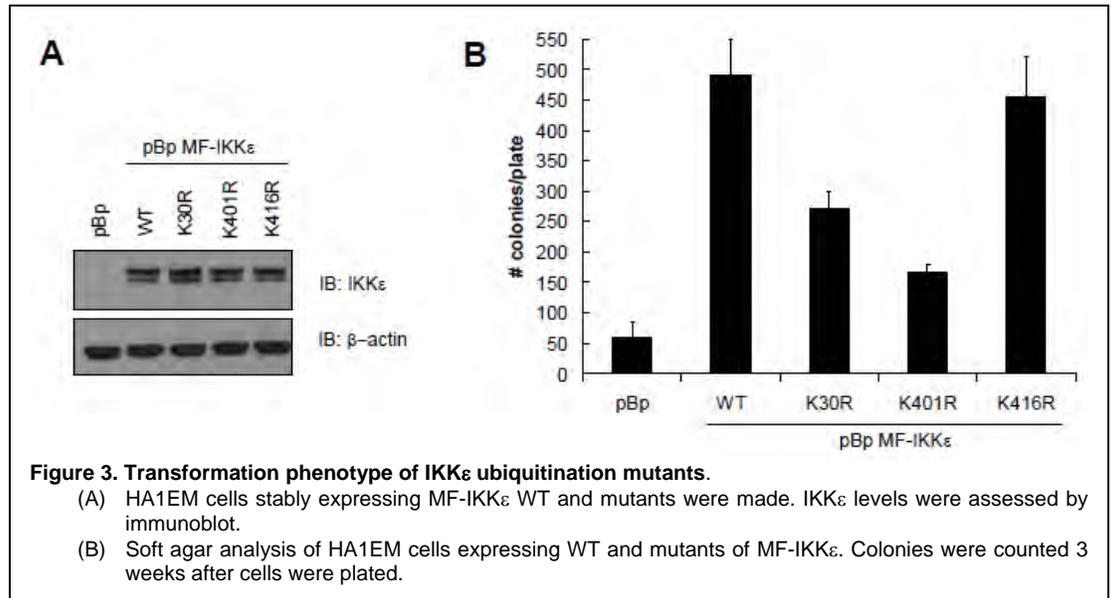


Figure 2. Mass spectrometry of IKK ϵ .

(Left) GST-IKK ϵ 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.

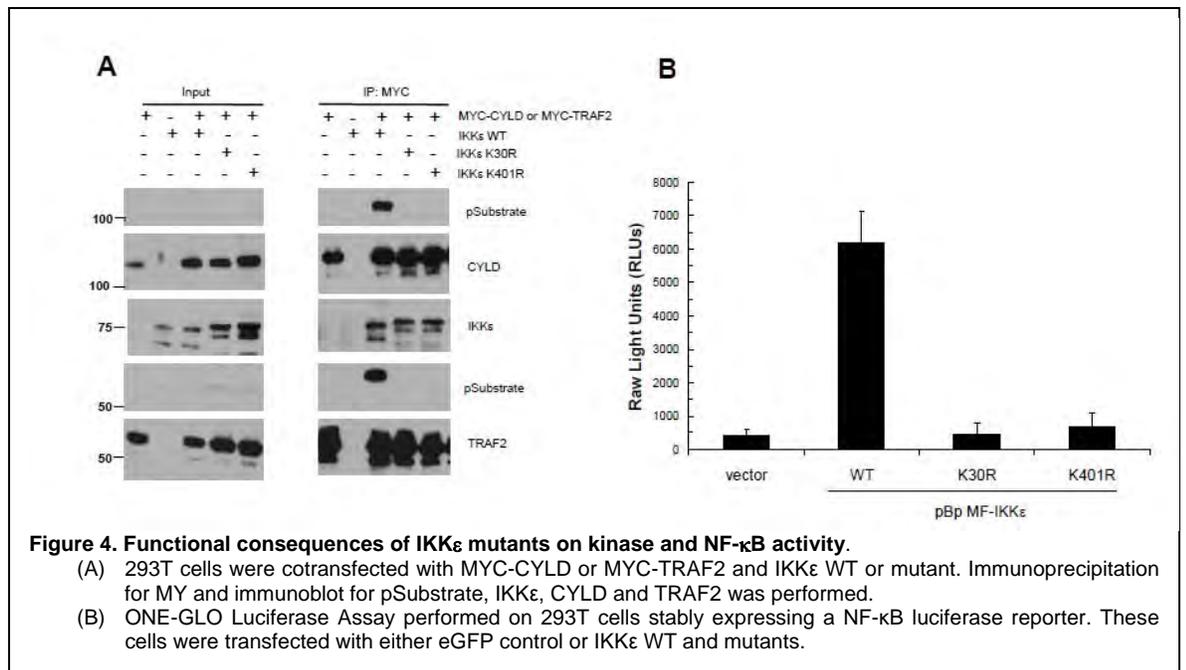
immunoprecipitates were then subjected to SDS-PAGE followed by Coomassie blue staining. Four bands of interest were identified and submitted for mass spectrometry analysis (Figure 2). We obtained 58.2% coverage of the IKK ϵ protein and 64.7% (22 out of 34) coverage of the internal lysines. From this 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 IKK ϵ in the context of cell transformation and cancer, I generated site-specific lysine-to-arginine IKK ϵ mutants for the three lysine residues that were identified in Aim 1b. These IKK ϵ mutants have been



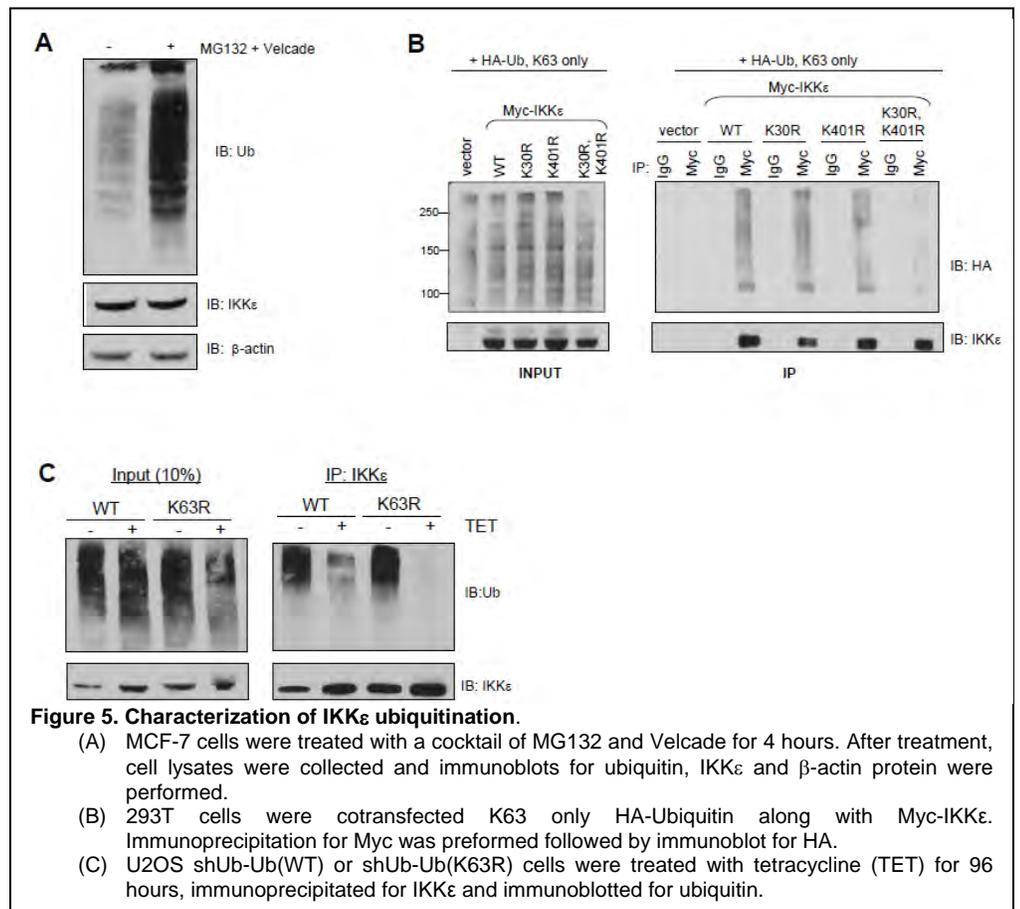
retrovirally introduced into HA1EM cell to create stable cell lines that express these mutant constructs (Figure 3A). These mutants were assessed for transformation capacity by soft agar analysis (Figure 3B). This transformation analysis indicates that the K30R and K401R mutants exhibit an impaired transformation capacity as compared to that of WT and the K416R mutant.

The K30R and K401R IKK ϵ 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 IKK ϵ functions including IKK ϵ kinase



activity and subsequent NF- κ B activation. Other studies in the Hahn Lab have identified CYLD (cylindromatosis) and TRAF2 (TNF receptor-associated factor 2) as kinase targets of IKK ϵ ⁵. As a result from our previous studies, an IKK ϵ phospho-substrate antibody was generated and has been used to identify CYLD and TRAF2 as IKK ϵ kinase targets. I cotransfected wildtype, K30R and K401R mutant IKK ϵ with either Myc-tagged CYLD or Myc-tagged TRAF2. After immunoprecipitation for Myc, an immunoblot using phospho-substrate (pSubstrate) antibody showed that wildtype IKK ϵ phosphorylates both TRAF2 and CYLD, but neither of the mutant forms of IKK ϵ was capable of this phosphorylation activity (Figure 4A). A NF- κ B luciferase reporter assay was used to determine the ability of these IKK ϵ mutants to activate the NF- κ B pathway (Figure 4B). Consistent with the kinase activity data, these results indicated that WT but not mutant IKK ϵ was able to induce the NF- κ B 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 IKK ϵ . It is known that the NF- κ B pathway is regulated by various forms of ubiquitination. Whereas canonical K48-linked ubiquitination serves as a protein degradation signal on the I κ B proteins, non-canonical K63-linked ubiquitination has been observed to be an activating modification on IKK γ ^{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

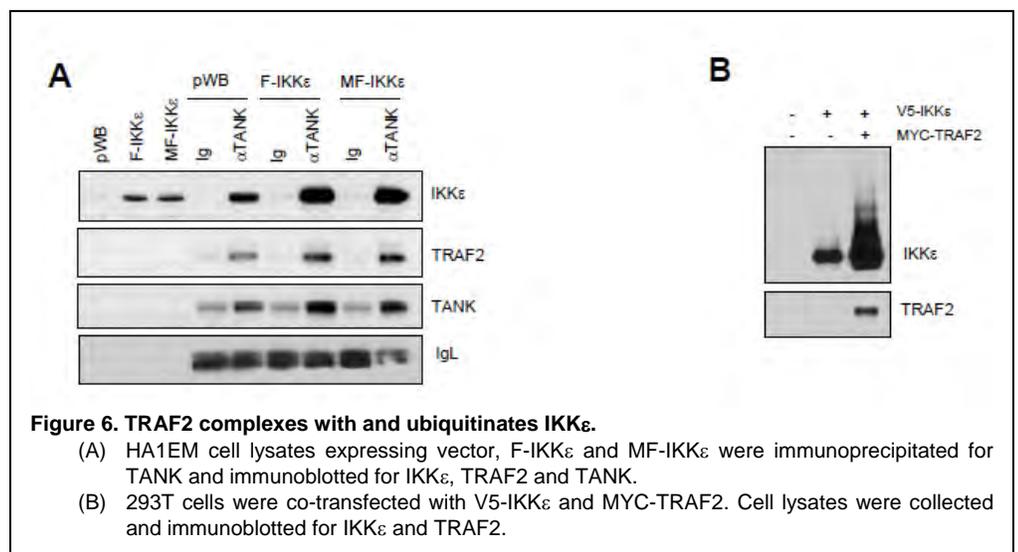


increased, the levels of IKK ϵ protein remain the same (Figure 5A). This indicates that IKK ϵ is subject to proteasome-independent ubiquitin modification. Next, I cotransfected 293T cells with Myc-IKK ϵ and mutant a HA-ubiquitin construct in which all other lysines except K63 are replaced with Arg. I immunoprecipitated for Myc and immunoblotted for HA (Figure 5B) and was able to show that although wildtype IKK ϵ and each of the single mutant K30R and K401R IKK ϵ

mutants were being modified by the K63-only ubiquitin, the K30R, K401R double mutant was no longer undergoing this modification. This result indicates that IKK ϵ 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 IKK ϵ by K63 chains, but the mutation of both residues results in the disappearance of the higher mobility polyubiquitination ladder, thus indicating that IKK ϵ 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 IKK ϵ followed by immunoblot for ubiquitin showed that IKK ϵ 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 IKK ϵ normally undergoes K63-linked ubiquitination at K30 and K401 under physiological conditions.

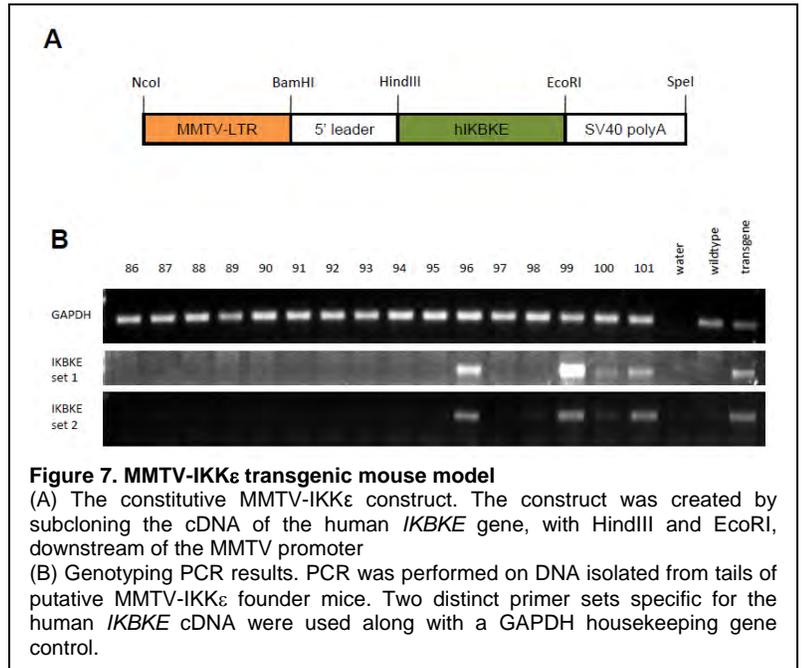
My most recent work on this project has been to determine the E3 ubiquitin ligase that is responsible for IKK ϵ modification. Through a thorough literature search, I was able to determine that the TRAF family of proteins was a relevant family of E3 ubiquitin ligase proteins which are known to associate with various NF- κ B 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 IKK ϵ ubiquitination. In HA1EM cells expressing F-IKK ϵ and MF-IKK ϵ , I was able to demonstrate that IKK ϵ 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 IKK ϵ and TRAF2. My results indicate that all three of the proteins do indeed form a complex. Next, I



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

Specific Aim 2: In my previous progress reports, I have reported that we had successfully generated a MMTV-IKK ϵ 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-IKK ϵ 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.



We will shortly move ahead with our original intended experiments to determine if IKK ϵ 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-IKK ϵ 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 IKK ϵ causes tumor growth acceleration in these mice.

Key Research/Training Accomplishments

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

- Demonstrated that IKK ϵ is being modified by K63-linked ubiquitination
- Identified TRAF2 as a putative E3 ligase that catalyzes the ubiquitination of IKK ϵ
- Generated founder mice for the constitutive MMTV-IKK ϵ 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 IKK ϵ point mutants: K30R, K401R, K41R and K30R K401R
- Established HA1EM cell lines that constitutively express F- and MF-IKK ϵ point mutants
- Established two new animal models: MMTV-IKK ϵ and WAP-Cre/p53^{fl/fl}

Conclusion

Taken altogether, the data that I have summarized in this report offer significant evidence that IKK ϵ 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 IKK ϵ kinase function and subsequent ability to activate NF- κ B. Most importantly, I have shown that these two residues are essential for the ability of IKK ϵ 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 IKK ϵ . These results offers new insights into the regulation of IKK ϵ , a known breast cancer oncogene, allowing for the future development of more targeted therapeutics against IKK ϵ .

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

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