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Characterization of IKBKE as a Breast Cancer Oncogene

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14. ABSTRACT Previous work in the Hahn Lab has identified <i>IKBKE</i> as a novel breast cancer oncogene that is capable of human mammary cell transformation. Amplification and overexpression of IKKepsilon was observed in a significant percentage of human breast cancer cell lines and primary tumor samples. Additionally, breast cancer cell lines carrying the <i>IKBKE</i> amplicon showed decreased viability in response to IKKepsilon suppression by shRNA. Our work has also demonstrated that IKKepsilon is a non-canonical IKK (IkappaB kinase) family member that activates the NF-kappaB pathway and that this activity is essential for cell transformation. However, it is known that IKKepsilon does not participate in the canonical IKK complex to activate NF-kappaB signaling. Though recent work has sought to identify the downstream targets of IKKepsilon, the mechanism of its upstream regulation is not well-understood. Thus, I propose to further our understanding of IKKepsilon function by investigating the upstream regulation of IKKepsilon – specifically, the role of ubiquitination in IKKepsilon-mediated cell transformation. In addition, I am to investigate the role of IKKepsilon in breast cancer initiation and maintenance <i>in vivo</i> through the generation of a constitutive and an inducible transgenic mouse model.				
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Introduction

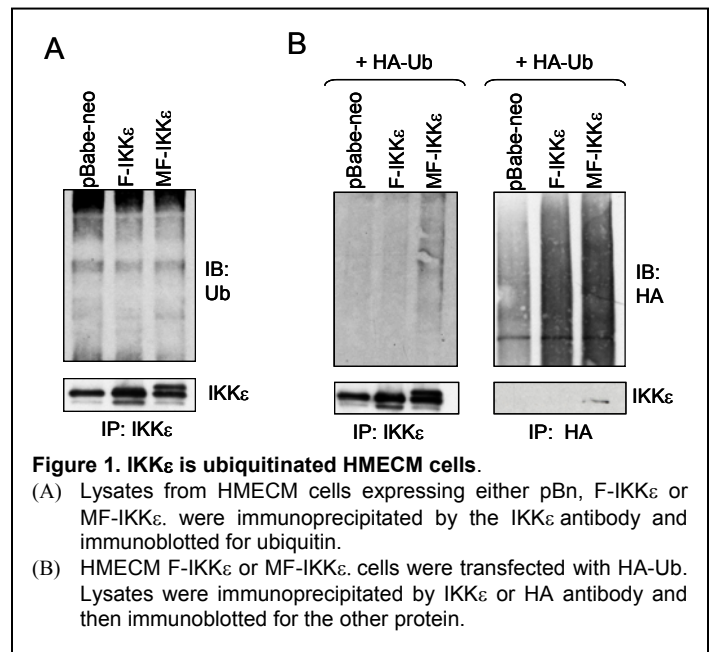
I have made significant progress in both Specific Aims that were originally laid out in my Statement of Work. In this Progress Report, I will report the studies and results that I have conducted over the past two year towards accomplishing those aims.

Specific Aims

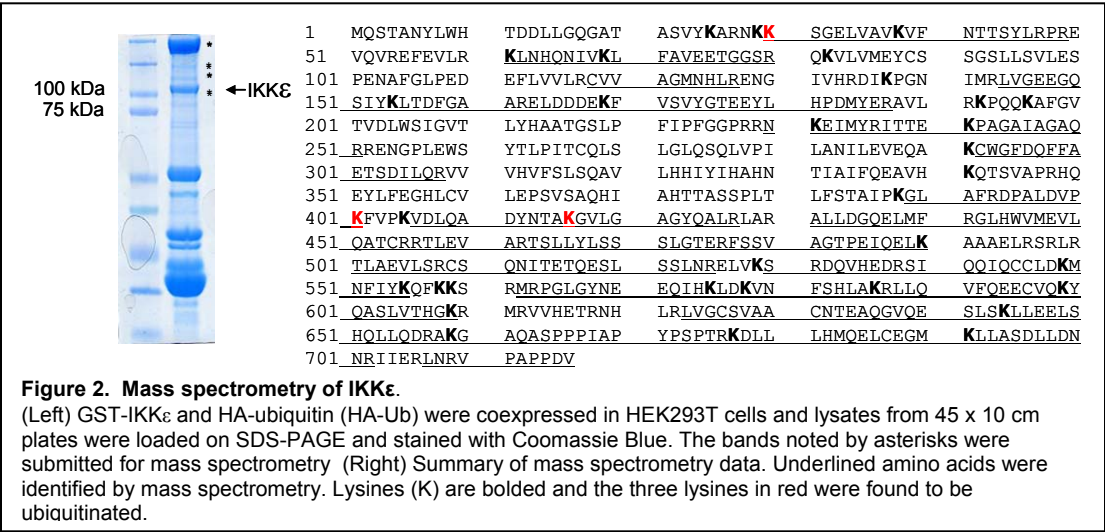
1. Investigate the role of ubiquitination in IKK ϵ -mediated cell transformation
 - a. Confirm and characterize IKK ϵ ubiquitination in the context of mammary cell transformation
 - b. Identification of the IKK ϵ ubiquitin-accepting residues
 - c. Determine the functional relevance of IKK ϵ ubiquitination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

Specific Aim 1a: I have confirmed that IKK ϵ ubiquitination occurs in a mammary epithelial cell context. I immunoprecipitated IKK ϵ from human mammary epithelial cells that stably overexpress activated MEK (HMECM) along with either Flag-tagged (F-IKK ϵ) or myristolated Flag-tagged (MF-IKK ϵ) IKK ϵ and was able to detect an endogenously ubiquitinated species of MF-IKK ϵ by immunoblotting for ubiquitin (Figure 1A). In parallel, I transiently transfected the same cells with HA-tagged ubiquitin, performed an immunoprecipitation for IKK ϵ and was able to successfully detect an ubiquitinated ladder of MF-IKK ϵ by immunoblotting for HA. I performed the reverse IP/Western experiment, and was also able to immunoblot for IKK ϵ after immunoprecipitation by HA antibody (Figure 1B). Interestingly, I was only able to confirm IKK ϵ ubiquitination in the MF-IKK ϵ transformed HMECM cells and not in the F-IKK ϵ transformed cells. Previous characterization of these cell lines has shown that MF-IKK ϵ exhibits a much more robust transformation phenotype in HMECM cells than the F-IKK ϵ counterpart. Perhaps this variation in transformation phenotype is related to the ubiquitination status of IKK ϵ in these cells.

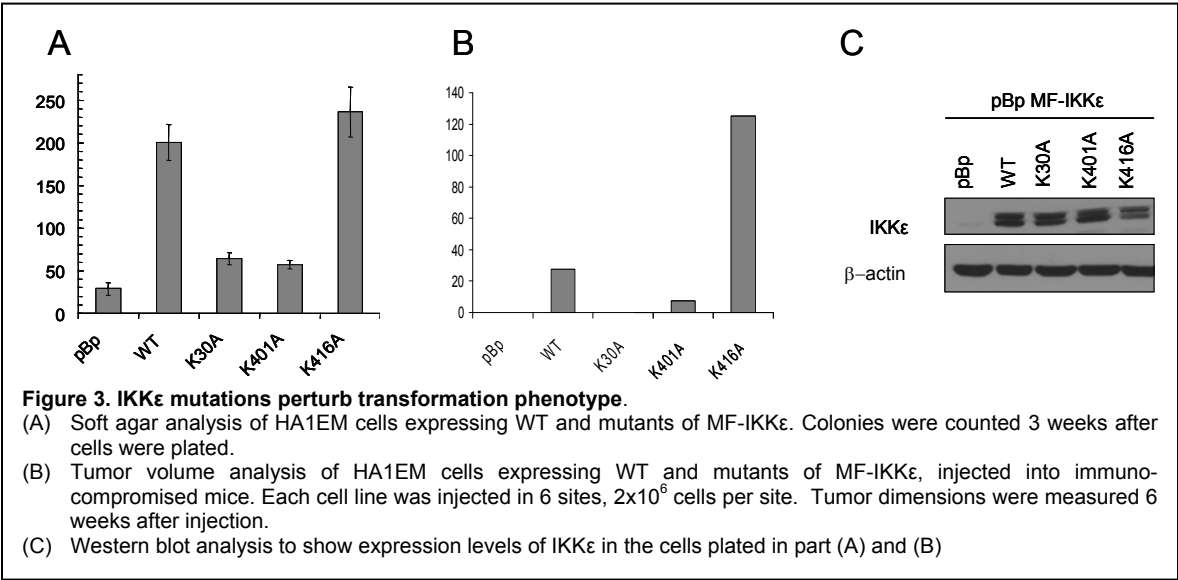


Specific Aim 1b: I have successfully identified three lysine residues in IKKε that are subject to ubiquitination by mass spectrometry. I transiently cotransfected GST-tagged IKKε and HA-tagged ubiquitin in HEK293T cells and performed a GST immunoprecipitation. The immunoprecipitates were then



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: I have generated site-specific lysine-to-alanine 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

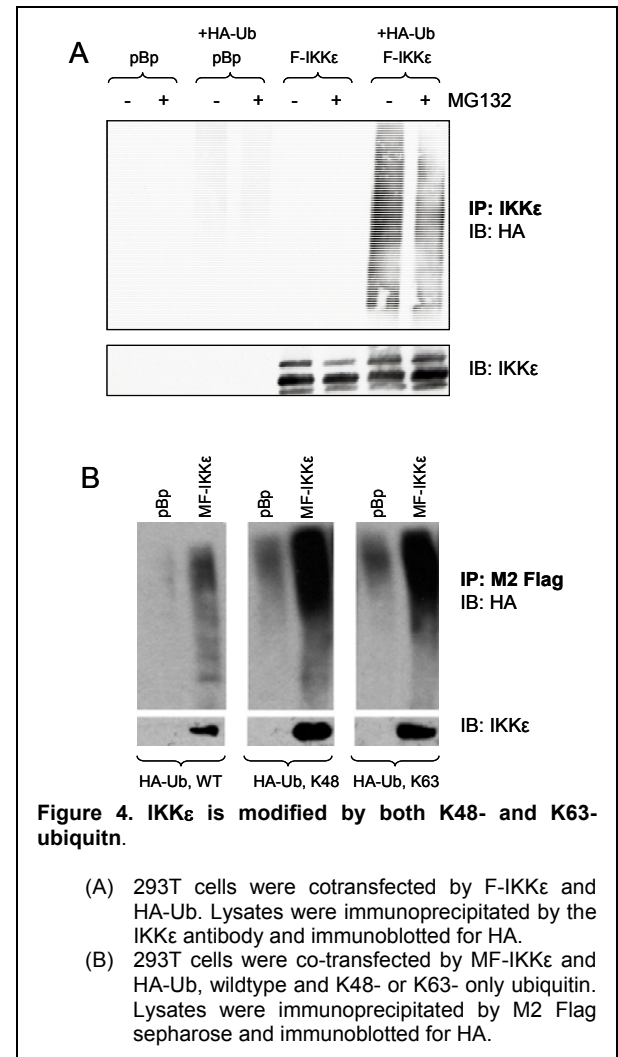


express these mutant constructs. These mutants were assessed for transformation capacity by both soft agar analysis (Figure 3a) as well as injection into immunocompromised mice (Figure 3b). From this analysis, I have determined that the K30 and K401 residues are essential for IKKε-mediated transformation. However, it appears that mutation of the K416 residue does not perturb the transformation phenotype of IKKε.

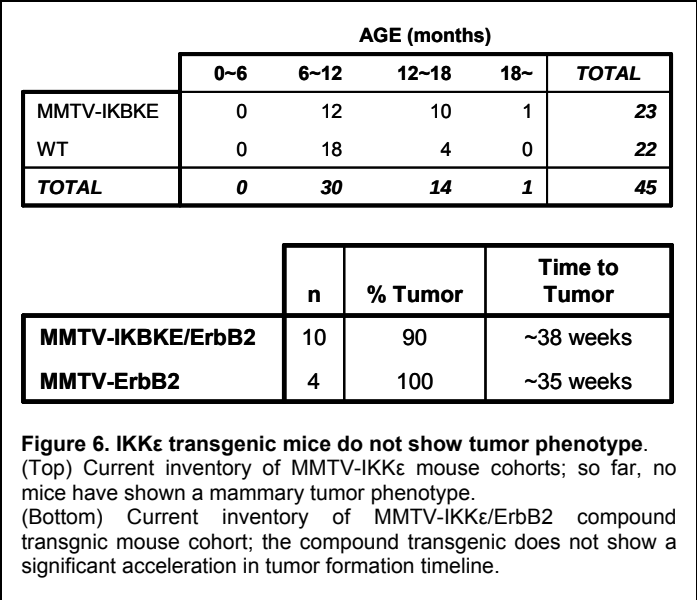
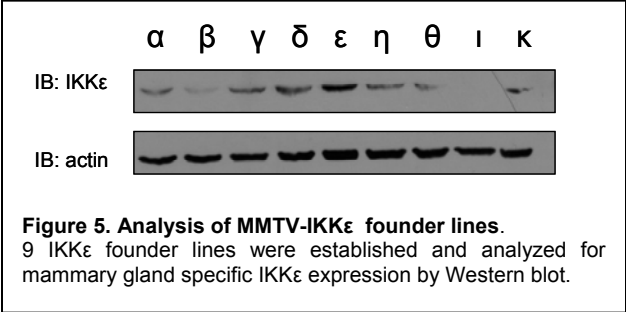
Ongoing Studies: Having established that IKK ϵ is modified by ubiquitination, my next aim will be to characterize what type of ubiquitination is occurring. It has been well-established that there are two types of ubiquitin modifications that both play important roles in the NF- κ B pathway – K48- and K63- linked ubiquitination. My preliminary studies of IKK ϵ show that proteasome inhibitor treatment does not affect the levels of IKK ϵ protein expression (Figure 4a), this indicates that IKK ϵ is undergoing a form of non-degradative ubiquitin modification. I, next, cotransfected mutant HA-ubiquitin constructs, in which all other lysines except K48 or K63 are replaced with Arg, with MF-IKK ϵ into 293T cells. I immunoprecipitated for IKK ϵ using M2 Flag affinity sepharose and immunoblotted for HA (Figure 4b). These results indicate that IKK ϵ is subject to modification by both K48- and K63- linked ubiquitination.

My next step will be to determine what type of modification is occurring at each of the residues within IKK ϵ that were identified in Aim 1b. I intend to do this by using a combination of the IKK ϵ mutants generated in Aim 1c and the HA-ubiquitin mutants that were described above. In addition, I would like to determine the functional consequences of IKK ϵ ubiquitination. To assess this, I will perform IKK ϵ kinase assays to determine if IKK ϵ kinase activity is affected, as well as look at the induction of well-known NF- κ B downstream effectors to assess the effects on NF- κ B activation.

Finally, since it is well-known that the IKK proteins act in large multi-protein complexes, I would like to determine the binding partners of IKK ϵ in the context of mammary cell transformation. In collaboration with the CeMM Laboratory in Vienna, Austria, we will use mass spectrometry techniques to determine the protein binding partners of wild-type and mutant IKK ϵ in the context of cell transformation.



Specific Aim 2a: I have successfully generated a MMTV-IKK ϵ transgenic mouse model. By genotyping analysis, 9 founder mice were identified. These founders were bred out and assessed for mammary gland-specific expression of IKK ϵ by immunoblot (Figure 5). From this analysis, 2 founder lines with varying levels of IKK ϵ expression were chosen and are currently being expanded and followed. In addition, all of the original founder mice were retained



and monitored. We currently have a cohort of 23 MMTV-IKK ϵ mice of varying ages, the oldest of which are ~18 months in age (Figure 6, top). In addition, 3 of the original founder female mice have died from natural causes at an average age of ~20 months, no mammary tumor phenotypes have yet been observed in any of the MMTV-IKK ϵ mice. Those founder lines that have been expanded were also bred to MMTV-ErbB2 mice, which are known to have a delayed mammary gland tumor formation phenotype. These MMTV-IKK ϵ /MMTV-ErbB2 bitransgenic mice were observed to determine if there is a synergistic tumor formation phenotype. Our data, so far, show that IKK ϵ does not cooperate with ErbB2 to accelerate tumor formation (Figure 6,

bottom). This result is somewhat expected because IKK ϵ and ErbB2 are effectors of the same pathway and, therefore, we would not expect them to synergize to accelerate tumor formation.

We have also obtained WAP-Cre and p53 floxed mice from the Mouse Models of Human Cancer Consortium (MMHCC) and are now in the process of crossing the MMTV-IKK ϵ mice into a mammary-specific p53 null background. Our preliminary results in soft agar have shown that IKK ϵ alone can induce colony formation in the mouse fibroblast cell line NIH3T3. These cells are p53 deficient, which provides good support that the introduction of IKK ϵ in the context of p53 deficiency will result in cooperativity and an accelerated tumor formation phenotype. Currently, we have a total of 55 WAP-Cre/p53floxed double positive mice (Figure 7) and are in the process of breeding them with the MMTV-IKK ϵ mice.

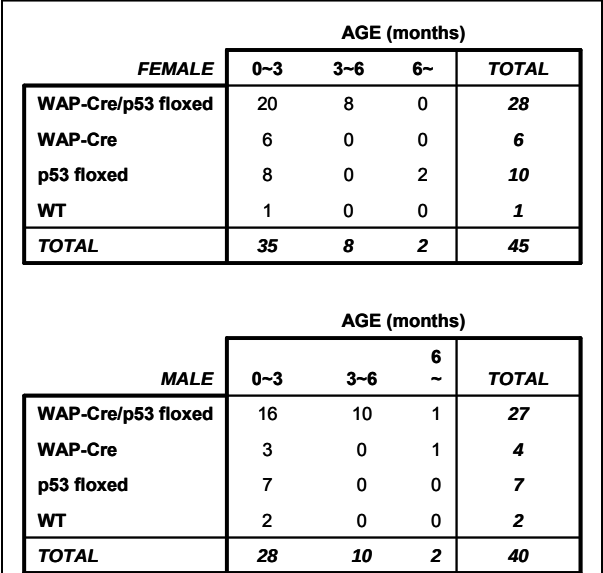
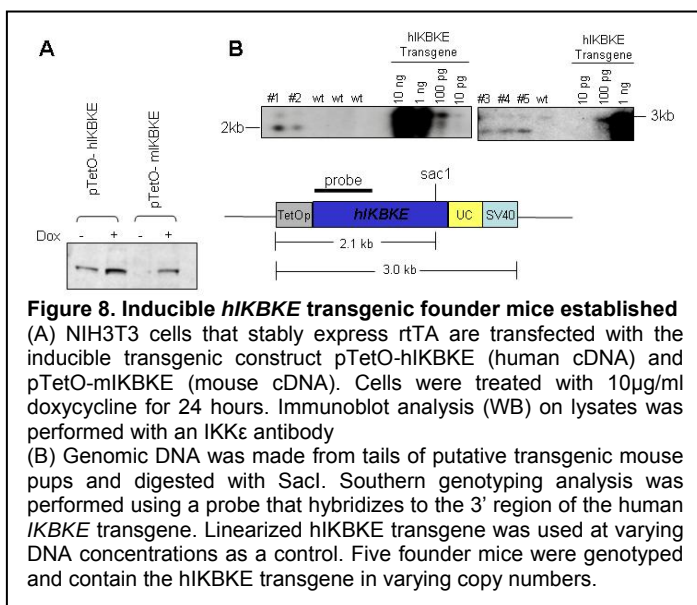


Figure 7. Current cohort of WAP-Cre/p53 floxed mice.



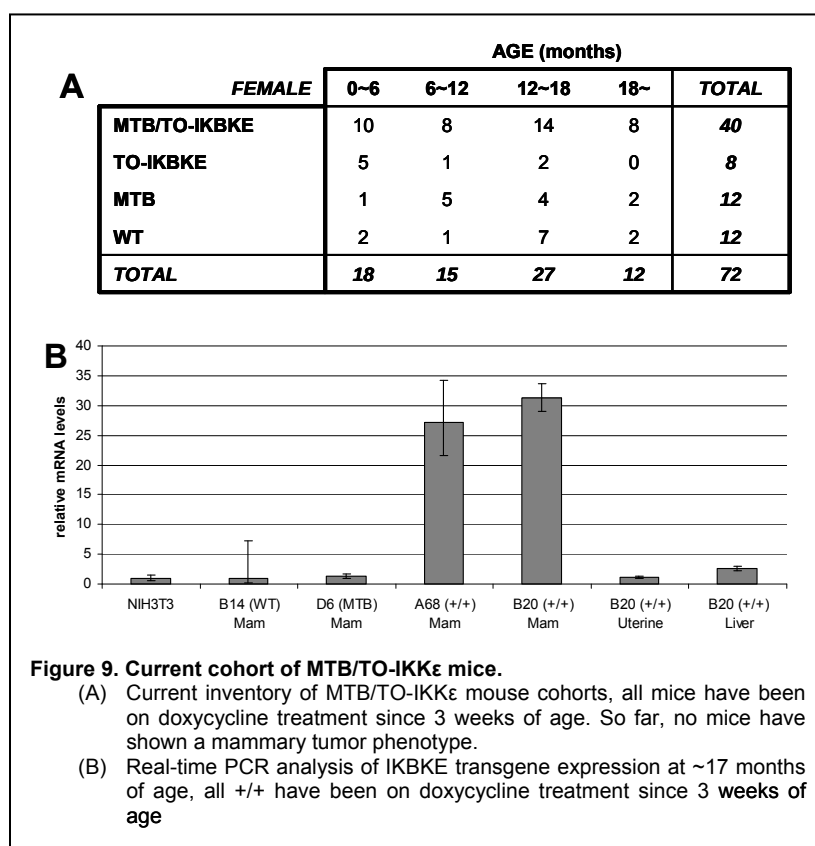
diet. We have assessed for IKKε expression at three timepoints thus far: immediately after genotyping analysis (3 weeks age), 21 days after doxycycline diet (6 weeks age) and finally, at the sacrifice date of the oldest mice (17 months of age). We have observed robust and specific transgene expression in these mice, all the way through to the final timepoint (Figure 9b). Thus far, these mice have not yet shown a tumor formation phenotype. Therefore, we are also currently breeding them into the WAP-Cre/p53 floxed line to determine if IKKε expression will accelerate tumor formation in a mammary-specific p53 deficient background.

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
- Generated cell lines that stably express the IKKε mutant constructs
- Demonstrated that IKKε lysine mutants show a distinct change in transformation phenotype *in vitro* and *in vivo*

Specific Aim 2b: We have successfully generated a doxycycline-inducible MMTV-rtTA/TetO-IKKε (MTB/TO-IKKε) bitransgenic mouse model. By genotyping analysis, 5 founder mice were identified (Figure 8). After analysis for doxycycline-inducible mammary gland IKKε expression, 2 founder lines with varying levels of IKKε expression were chosen and are currently being expanded and followed.

We currently have 40 female MTB/TO-IKKε mice (Figure 9a). All MTB/TO-IKKε bitransgenic mice are put on a doxycycline diet starting at the age of 3 weeks and have since remained on this



- Generated founder mice for the constitutive MMTV-IKK ϵ transgenic mouse model
- Generated founder mice for the inducible MMTV-rtTA/TetO-IKK ϵ transgenic mouse model
- Generated compound transgenic MMTV-IKK ϵ /MMTV-ErbB2 mouse model
- Generated compound transgenic MMTV-IKK ϵ /WAP-Cre/p53^{fl/fl} mouse model

Reportable Outcomes

- Developed HA1EM mutant IKK ϵ cell lines: both F-IKK ϵ and MF-IKK ϵ with the following mutations K30A, K401A, K416A
- Developed four new animal models: MMTV-IKK ϵ and MMTV-rtTA/TetO-IKK ϵ , MMTV-IKK ϵ /MMTV-ErbB2, MMTV-IKK ϵ /WAP-Cre/p53^{fl/fl}

Conclusion

Over the course of the past two years, I have made significant progress in all aspects of my Specific Aims as laid out in my original Statement of Work.

I have been able to identify three residues of IKK ϵ that undergo modification by ubiquitination, and I have been able to show that the mutation of these residues is able to perturb the IKK ϵ -mediated transformation phenotype. However, my mutational studies have also indicated that there is a more complex and dynamic role for IKK ϵ ubiquitination than I had originally hypothesized. I believe that IKK ϵ is undergoing a combination of both Lys48- and Lys63- linked ubiquitination, and that this combination of modifications is serving to tightly regulate both the levels of IKK ϵ protein in the cell as well as its enzymatic activation. I propose to do a series of IP/Western experiments that utilize the Lys48- and Lys63- only ubiquitin mutants in combination with the IKK ϵ mutants, in order to determine the exact nature of these modifications. In addition, to understand how the perturbation of the various residues of IKK ϵ affects its function, I will assay these mutants for IKK ϵ kinase activity as well as NF- κ B activation. Finally, to understand how IKK ϵ functions in complex to mediate cell transformation, in collaboration with the CeMM Laboratories, I will determine the protein binding partners of both wildtype and mutant IKK ϵ in the context of cell transformation using a mass spectrometry approach.

I have also successfully generated both a constitutive and inducible transgenic mouse model of mammary-specific IKK ϵ expression. Thus far, these mice have not shown a robust tumor formation phenotype. In parallel, I have bred the constitutive MMTV-IKK ϵ with MMTV-ErbB2 mice to determine if there is a synergistic tumor formation phenotype – these mice, thus far, have also not shown any tumor acceleration. Therefore, as originally laid out in my Statement of Work proposal, I am currently working to generate a cohort of WAP-Cre/p53 floxed and will cross the MMTV-IKK ϵ mice into this mammary-specific p53 null background to determine if IKK ϵ promotes tumor formation/acceleration in a p53 deficient background.