# A Kras Driven Murine Model Elucidates Oncogenic Role of FADD in lung cancer

Paul Joseph, Brittany Bowman, Stefanie Galbán, Alnawaz Rehemtulla

*Department of Radiation Oncology, University of Michigan, Ann Arbor, Michigan*

## Abstract

Lung adenocarcinoma is the most common type of non-small cell lung cancer, causing more than 500,000 deaths per year worldwide [5]. This research aims to investigate the role of Fas-associated protein with death domain (FADD) in lung adenocarcinomas in hopes of identifying a clinical biomarker and/or therapeutic target for the disease. Recent studies in lung cancer have indicated a correlation between phosphorylated FADD (p-FADD), induction of Nuclear Factor-KappaB (NF-κB) and poor clinical outcome [6]. The precise role of FADD in these cancers is not well understood. Here we aimed to elucidate its role in cancer by using a genetically engineered mouse model, which allowed for tissue specific FADD deletion simultaneous to activation of oncogenic Kras$^{G12D}$. This model enabled evaluation of differential lung cancer development in the presence or absence of FADD expression. Infecting the lungs with adenovirus expressing Cre recombinase allowed for inducible excision of FADD simultaneous to Kras$^{G12D}$ activation. After treatment with Cre recombinase, mice consistently developed lung tumors in the presence of FADD expression, while in mice wherein FADD was simultaneously deleted, tumor progression was inhibited. In addition, fibroblasts isolated from FADD deficient lungs grew more slowly in culture than fibroblasts from lung tissue expressing FADD. These findings support our hypothesis that FADD is required for lung tumor cell growth and provide impetus for targeting FADD as a therapeutic for lung adenocarcinoma.

**Background**

Lung cancer causes more deaths of both men and women worldwide than any other type of cancer [13, 14]. The American Cancer Society has predicted that over 220,000 new patients will be diagnosed with lung cancer in 2013, and 165,000 Americans will die from it this year [14]. Adenocarcinoma is the most common type of non-small cell tumor, accounting for over 40% of all lung malignancies [13]. Their metastatic tendencies, slow growth and asymptomatic presentation in peripheral lung tissues make adenocarcinomas especially deadly [5].

Recent explorations into the cellular basis for lung cancer have focused on the activation of oncogenes and the loss of tumor suppressors [9]. These opposing gene types comprise a tightly regulated system of checks and balances. When mutations are introduced, these genes may become weaker or stronger, disturbing this delicate balance. When Kras becomes mutated, it acts through the cell proliferation pathway and actuates unwarranted proliferation [15]. In many cancers, these effects are exacerbated by loss of essential tumor suppressor genes. For example, protein kinase C (PKC) is a tumor suppressor that normally acts to restrict signaling from mutant Kras. When PKC undergoes a loss of function mutation, Kras is further enabled and homeostasis is further disturbed [10].

Apoptosis is an indispensible homeostatic mechanism that allows for the proper growth of eukaryotic organisms. In *C. elegans*, apoptosis plays a crucial role during an invariable developmental process in which 131 of the total 1090 healthy cells undergo a highly regimented pattern of programmed cell death [2]. Apoptosis plays a crucial role by eliminating self-reactive immune cells, by sculpting organs during embryogenesis, and by destroying damaged and cancerous cells. In the extrinsic apoptosis pathway, ligands such as APO-3, TRAIL, TNF-α/β, and Fas bind to corresponding membrane bound death receptors which transmit their signals through an intracellular death domain that binds to a diverse array of proteins [2]. Fas-associated protein with death domain (FADD) is a cytosolic adapter protein that utilizes its C-terminal death domain to bind the cytosolic death domain of trimerized Fas, TRAIL and other receptors [11]. Binding to the death domain unmasks the N-terminal effector domain of FADD allowing it to cleave procaspase-8, initiating the serine protease cascade leading to apoptosis [11].

Counteracting the cell's apoptotic pathways are proliferation pathways initiating cell growth and division. When one of the proteins in this pathway is mutated, it can lead to loss of cell cycle control, which is a hallmark of cancer [9]. For this reason, the genes and mutations involved in cell growth are of great interest to those studying cancer. While FADD is primarily known as part of the apoptotic pathway, its role in promoting cell division has recently been characterized [4, 11]. Phosphorylated FADD (p-FADD) is necessary for progression through metaphase of mitosis [4]. FADD's role in both apoptosis and cell proliferation makes it a complex yet promising target for cancer researchers.

Recent research has correlated increased FADD concentrations with lung cancer and poor clinical prognosis [4]. However, it is unknown if this change in FADD levels is a cause of, or a response to the cancer. Also, there is disagreement on whether FADD is acting as a tumor suppressor or an oncogene [1, 4]. We hypothesized that FADD's oncogenic potential in lung cancer stems from its role in

regulating cell cycle. FADD acts in an oncogenic capacity by allowing lung cells to progress unchecked through the cell cycle, which is a highly common oncogenic mechanism.

In order to create a model system that is faithful to human adenocarcinomas, we used a mouse model in which tumor development and FADD expression were under our control. These criteria were met by combining two previously described mouse models. To create a tumor inducible system, a mutation in the Kras cell proliferation pathway was employed. Kras is an intracellular signal transducer responsible for transmitting growth and proliferation signals [12, 15]. Activating mutations in Kras have been implicated in nearly 25% of lung tumors [15]. Characterization of these mutations indicates that a missense mutation at the 12th codon causes constitutive activation of downstream cell proliferation pathways, eventually leading to cancer [12]. To achieve tissue specific and inducible oncogenic Kras$^{G12D}$ expression a mouse model carrying a knock-in allele with a stop codon preceding the mutant Kras (Kras$^{G12D}$) was employed [8]. Treatment with Cre recombinase recombined the DNA at the loxP sites flanking the stop codon, thereby removing the stop and enabling transcription of oncogenic Kras$^{G12D}$.

Mice without FADD die in utero, thus requiring an alternative strategy to study FADD's requirement for tumor formation [19]. Exogenous expression of a conditional FADD transgene overcomes this hurdle and has been developed by Zhang et al [19]. These mice were further crossed with the inducible mutant Kras model described by above, thereby achieving a mouse model wherein FADD deletion and Kras$^{G12D}$ activation represent simultaneous events in lung pathogenesis [18]. Using this mouse model allowed us to create mice with lung tumors and variable FADD genotypes. Comparing these experimental cohorts allowed us to isolate the variable of FADD expression in lung cancers.

Lung cancer causes more deaths than any other form of cancer [5, 14]. Efforts to diagnose and treat this devastating disease have been largely unsuccessful. In search of a new biomarker and/or therapeutic target for lung cancer, this research aimed to investigate the role of FADD in cancer progression. To accomplish this, a mutant Kras driven mouse model with conditional FADD expression was employed. We were able to experimentally demonstrate the success of Kras$^{G12D}$ mediated lung cancer induction and FADD suppression upon infection with Cre recombinase using histology and Western blot analysis. FADD transgene excision led to decreased growth potential of murine lung fibroblasts in tissue culture. These results confirm our initial hypothesis that FADD promotes cancer and may serve as a therapeutic target in the treatment of lung cancer. The proposed mechanism for FADD's role in tumorigenesis involves deregulation of cell cycle leading to self-stimulated growth, a hallmark of cancer [9]. Future goals of this research involve mechanistically investigating FADD's oncogenic role in the cancer cell and evaluating FADD as a therapeutic target.

**Results**

*Characterization of novel Kras$^{G12D}$ driven mouse model with conditional FADD expression*

Having a mouse model in which lung cancer is readily and consistently inducible is crucial to designing experimental directives. To do this, we used a well-characterized mutation in the Kras cell signaling protein [12]. This conditionally activatable allele of oncogenic Kras harbors a missense mutation at codon 12, thus interfering with GTPase activity and leading to constitutive signaling down the proliferative MAP-Kinase pathway leading to cancer [8, 18]. To attain tissue specific Kras$^{G12D}$ expression a mouse model was used, where Kras$^{G12D}$ was knocked in along with a loxP mediated excisable stop codon (Figure 1 A). Once mice reached adulthood, treatment with adenovirus Cre lead to successful removal of the preceding stop codon, and enabled transcription of the mutant Kras (Figure 1 A). Due to the difficulty of specifically identifying mutant Kras in a Western Blot, we probed for p-ERK as a surrogate protein target. p-ERK actuates downstream cell proliferation mechanisms initiated by Kras, and is found to be in higher concentration when Kras$^{G12D}$ is activated.

In order to study the impact of FADD in developing lung cancers, it was necessary to regulate FADD protein levels in the cell. To do this, a mouse model was attained from the Zhang lab that had FADD knocked out of one or both alleles [19]. Since FADD is essential for normal development, a conditional transgene was inserted containing a FADD:GFP fusion gene. Previous research demonstrated FADD's ability to functionally tolerate this insertion [19, 21]. In order to combine the two models discussed above, mice from each strain were crossed (Figure 1 B). Four experimental cohorts were successfully established, as confirmed by genotyping PCRs (data not shown). As depicted in Figure 1C, western blot analysis using a GFP antibody confirmed successful transgene depletion in lung tissue. Endogenous FADD protein expression status was confirmed in tissue acquired from FADD ko/ko and FADD ko/+ mice by using anti-FADD antibody.

By cross breeding previously distinct murine lines, we were able to successfully establish a Kras driven lung cancer model with conditional FADD expression. Using Western blots to analyze gene expression allowed us to confirm the success of this breeding and to separate the resultant mice into four distinct experimental cohorts (Figure 1 B).

*Induction of lung cancer following Kras$^{G12D}$ activation*

To demonstrate that the effects of mutant Kras are observed in the mouse and not only in tissue culture, Kras$^{G12D}$ was activated in the lungs of mice whose lungs were then evaluated by histology. The experimental cohort was selected to have wild type and mutant Kras expressing mice, as well as having normal FADD expression levels in all mice. This allowed us to isolate the variable of Kras$^{G12D}$ activation. Analysis of lung histology after Kras$^{G12D}$ activation illustrates the tumorigenicity of mutant Kras. Kras$^{G12D}$ mice developed severe tumors and hyperplasias, while wild type Kras mice appeared healthy (Figure 2 A).

Next, it was necessary to confirm the expression profiles of the mice used in these experiments. In order to confirm activation of oncogenic Kras$^{G12D}$ in mouse lung tissue by Western blot and
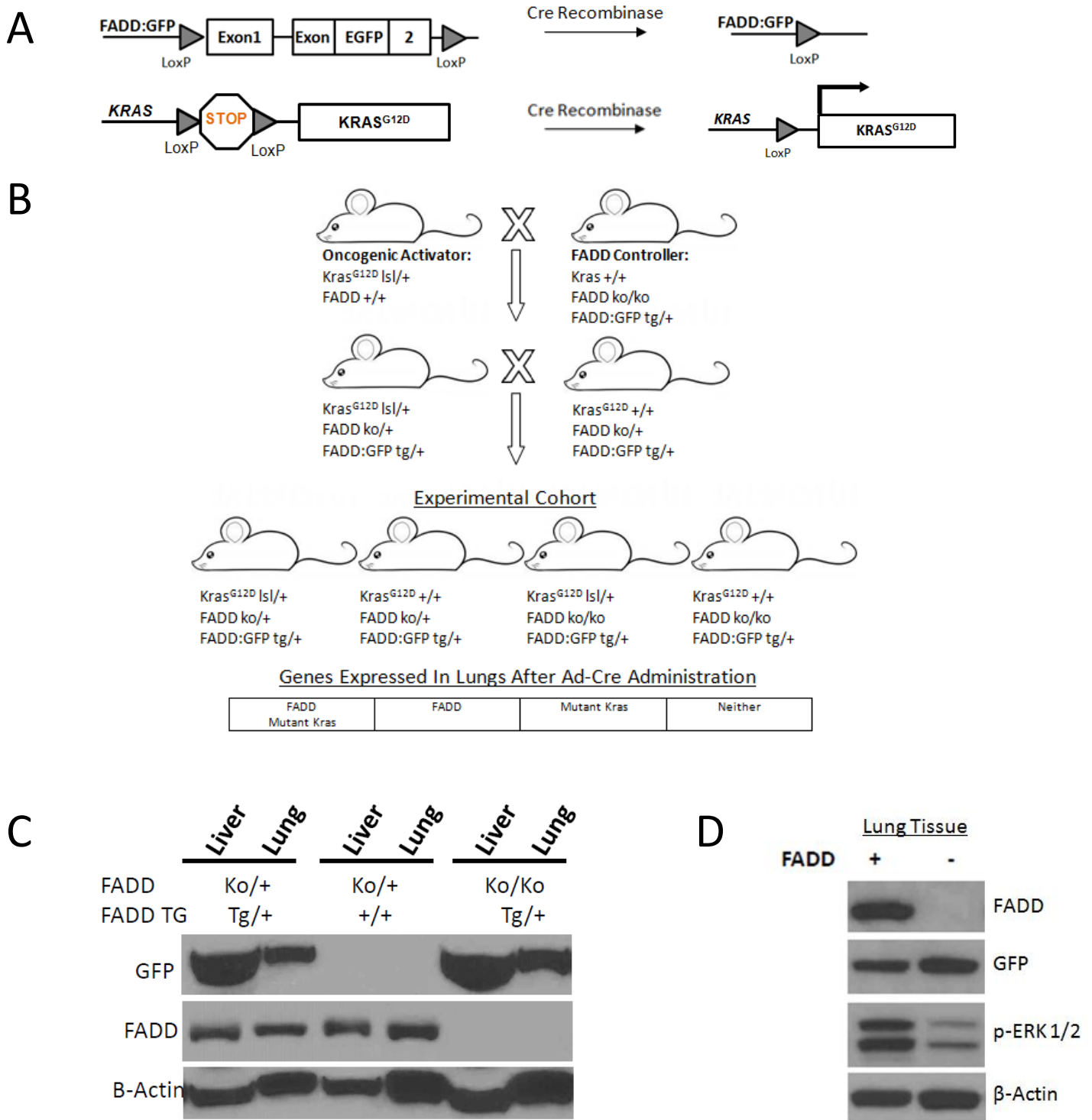
**Figure 1:** Developing the mouse model. **A.** Gene schematic for the FADD:GFP transgenic construct and Kras$^{G21D}$ knock in construct. loxP mediated excision of transgene by Cre recombinase is indicated. Kras$^{G21D}$ knock in construct containing an excisable STOP codon with flanking loxP sites. Transcription is enabled by Cre recombinase as indicated. **B.** Establishment of experimental cohort. Kras$^{G12D}$ lsl/+ denotes knock in of mutant Kras with loxP flanked STOP codon. **C.** Western Blot of liver and lung tissues demonstrating endogenous and transgenic FADD expression after intranasal instillation with Ad-Cre. **D.** Western Blot of lung tissue demonstrating p-ERK activity as a surrogate for Kras$^{G12D}$ expression.
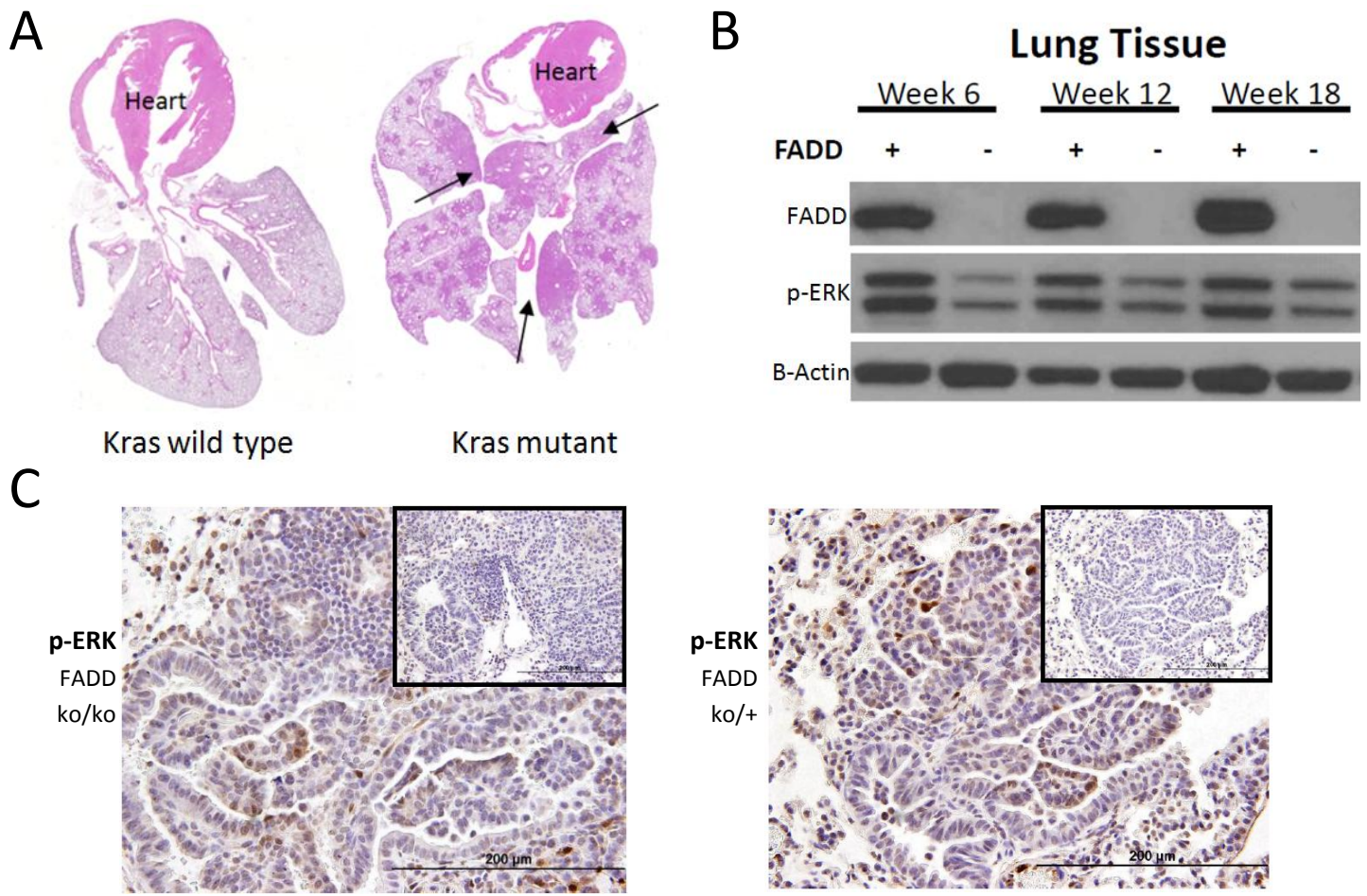
**Figure 2:** Oncogenic Kras activation. **A.** Microscope scans of haematoxylin and Eosin Y stained murine lung cross-sections after treatment with Ad-Cre. Arrows indicate lesions in the Kras$^{G12D}$ lungs. **B.** Western blot demonstrating lower p-ERK concentration in murine lung tissues lacking FADD. Two distinct bands represent the two subunits of p-ERK. **C.** p-ERK staining of murine lung cross-sections taken at 40X magnification. Brown precipitate demarks p-ERK. Inlay is a control stained with secondary antibody only.

immunohistochemistry, it was necessary that we had an antibody specific to mutant Kras specifically, and not total Kras. The difference between these proteins is only a single amino acid. Since this antibody has not been commercially produced yet, we selected a surrogate protein downstream of Kras that increases in concentration upon Kras activation: p-ERK (Figure 2 G). Since mutant Kras is constitutively signaling for proliferation, p-ERK should be in higher concentration in tissues where Kras$^{G12D}$ is being expressed.

In order to further demonstrate that Kras activation leads to p-ERK up-regulation, immunohistochemistry was carried out on cross-sections of lung tissue acquired from the mice of our experimental cohort. This approach allowed for spatial determination of p-ERK expression, and demonstrated a strong association between Kras induced lesions and p-ERK expression in tissues from both genotypes (Figure 2 C).

Precise quantification of p-ERK was not possible by the immunohistochemistry technique, so we turned to the Western blot as a more quantifiable approach. Through these studies of p-ERK in our FADD knockout model, we serendipitously discovered a connection between FADD expression and p-ERK expression (Figure 1 D). In lung tissues where FADD had been knocked out, we noticed that p-ERK concentrations were significantly lower (Figure 2 B). These results were observed in multiple mice at multiple time points, illustrating the persistence of this result.

*Isolation of mouse embryonic fibroblasts*

In order to develop a tissue culture model in which the role of FADD can be mechanistically determined, mouse embryonic fibroblasts (MEFs) were isolated from developing embryos after 9 days of gestation. This in vitro approach allowed for preliminary determination of the role of FADD in cell growth (Figure 3 A). MEFs with the desired phenotypes were successfully isolated and grown up in cell culture. Western blots were used to assess expression patterns which allowed us to separate cell lines into distinct experimental cohorts (Figure 3, B).

A frequent problem associated with Cre recombination is that it is nearly impossible to transfect and attain successful recombination in 100% of cells [7]. Having non-recombined cells in culture alongside the recombined cells will skew results in the Western blots and cell growth assays to follow. In an attempt to gain higher percentage of recombination, we exposed cells to multiple treatments with Ad-Cre at a high multiplicity of infection. While this approach improved overall recombination percentages, it was unsuccessful at excising all of the transgene (Figure 3C). In order to definitively segregate our transformed cells, we focused on using the GFP tag that was being expressed in any non-recombined cells. Frequency associated cell sorting (FACS) allowed for selection of cells that fluoresce at a certain wavelength. In this case, we want to isolate cells that do not fluoresce since these cells must have had their FADD:GFP transgene excised by Cre recombinase (Figure 3 C). Figure 3 D clearly demonstrates the principles of FACS and the purpose it served in our experiment. The GFP+ control is a non-Cre treated sample of lung fibroblasts. Patterning of the quantification data clearly demonstrates a shift of cells from GFP+ to GFP- after treatment with Ad-Cre.
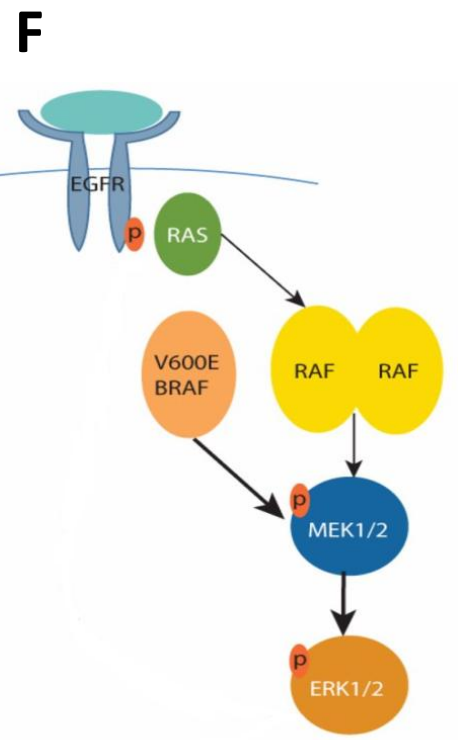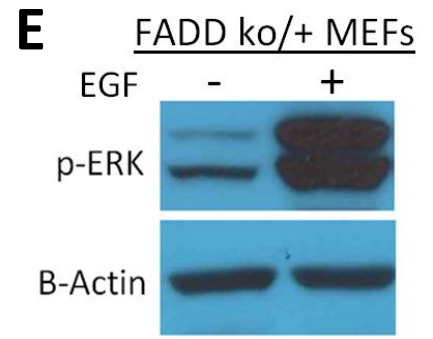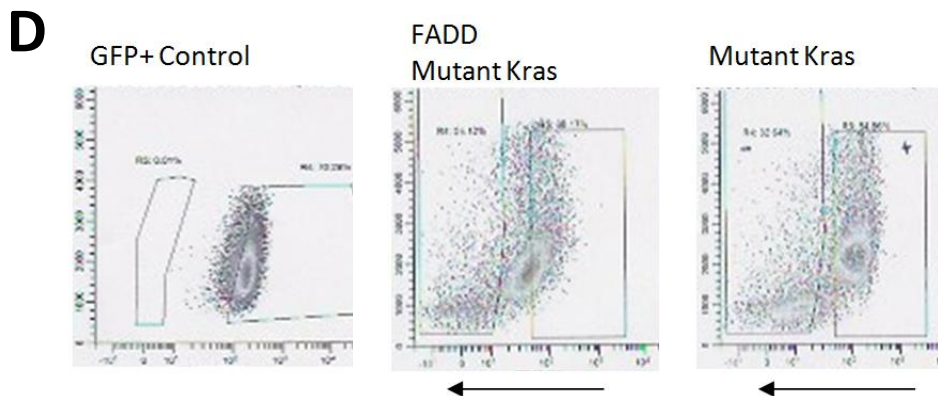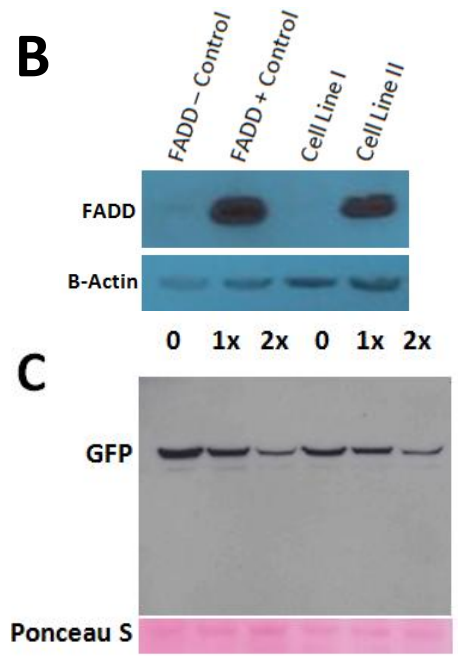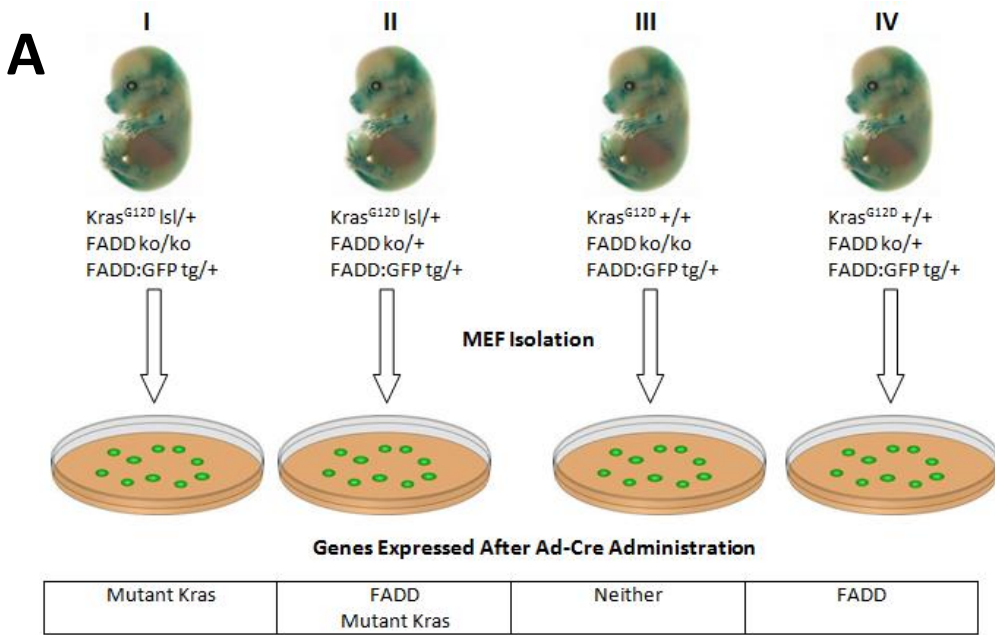
**Figure 3:** Isolation of mouse embryonic fibroblasts **A.** Schematic depicting isolation of MEFs from experimental cohort and subsequent treatment with adenovirus encoding Cre recombinase. **B.** Western blot demonstrating genotypes of MEFs from cohorts I and II. FADD + and - control MEF lines attained through collaboration with Astar Winoto, UC Berkeley. **C.** Western blot demonstrating effectiveness of Cre at removing transgene. Cells were exposed to 0, 1, or 2 rounds of infection as labeled. **D.** Following Ad-Cre infection, Cre recombined cells were isolated via FACS. Positive control represents cells sorted before Ad-Cre infection that are actively expressing GFP. Arrows demonstrate shift of GFP negative cells that have been recombined by Cre recombinase. The box drawn closer to the y-axis represents cells that do not fluoresce. **E.** Western blot showing change in p-ERK expression after treatment of MEFs with epidermal growth factor (inducing EGFR based activation of Kras). **F.** Pathway demonstrating correlation between Kras activation and ERK phosphorylation.

In order to demonstrate this correlation experimentally, MEFs were treated with serum supplemented with epidermal growth factor (EGF).  EGF is known to bind to the membrane receptor EGF-R, which then activates Kras [2].  Western blots demonstrate that upon treatment with EGF, p-ERK concentrations increase dramatically, confirming that p-ERK concentration is dependent upon Kras activation.  This indicates that p-ERK is a viable surrogate protein to represent the activation state of Kras (Figure 2 F).

*FADD increases lung fibroblast proliferation*

In order to develop an in vitro system that is faithful to lung cancer development, we isolated lung fibroblasts from the four murine lines described in Figure 4.  Lung fibroblasts were successfully grown up in culture and treated with Ad-Cre in order to activate Kras$^{G12D}$ and excise the FADD:GFP transgene.  As expected, cells expressing wild type Kras in both alleles (cell lines III and IV) died after approximately 5 passages in culture, while cells expressing Kras$^{G12D}$ on one allele have been immortalized.  The difference in growth rate between these strains was apparent when visualizing the number of cells in culture (Figure 4 E).  Since not all cells on the plate were infected by the adenovirus, it was again necessary to screen for successful transformants using FACS.

First, we tried infecting the cells with successive rounds of treatment with Ad-Cre.  While these additional treatments reduced the amount of transgene expressing cells in our culture, they were unable to infect all cells on the plate.  This result was evidenced by the persistence of transgene detected by Western blot (similar to Figure 3 C).  In order to establish an entirely transgene negative cell population, we employed frequency associated cell sorting (FACS) after infection with Ad-Cre.  Since cells that had been infected successfully would no longer be expressing the FADD:GFP fusion protein, we selected for cells that did not fluoresce with GFP.  Protein from this GFP negative cell population was analyzed via Western blot, and loss of transgene was confirmed (Figure 4 C).

In order to characterize the cancerous potential of our newly attained FADD deficient cell line, we assayed for growth rate using the crystal violet growth assay.  In this assay, the ability of plated cells to retain crystal violet stain is used as a metric for cell population.  This method was chosen because standard cell counting protocols were more tedious and produced less precise results.  The FADD deficient cell line grew more slowly and reached a lower maximum population than the FADD heterozygous cell line (Figure 4 D).  The phases of cell growth, from exponential to stationary to death, are clearly observed in both strains.  However, the cell line expressing FADD undergoes faster exponential growth, reaching the stationary phase with a higher total cell population (Figure 4 D).
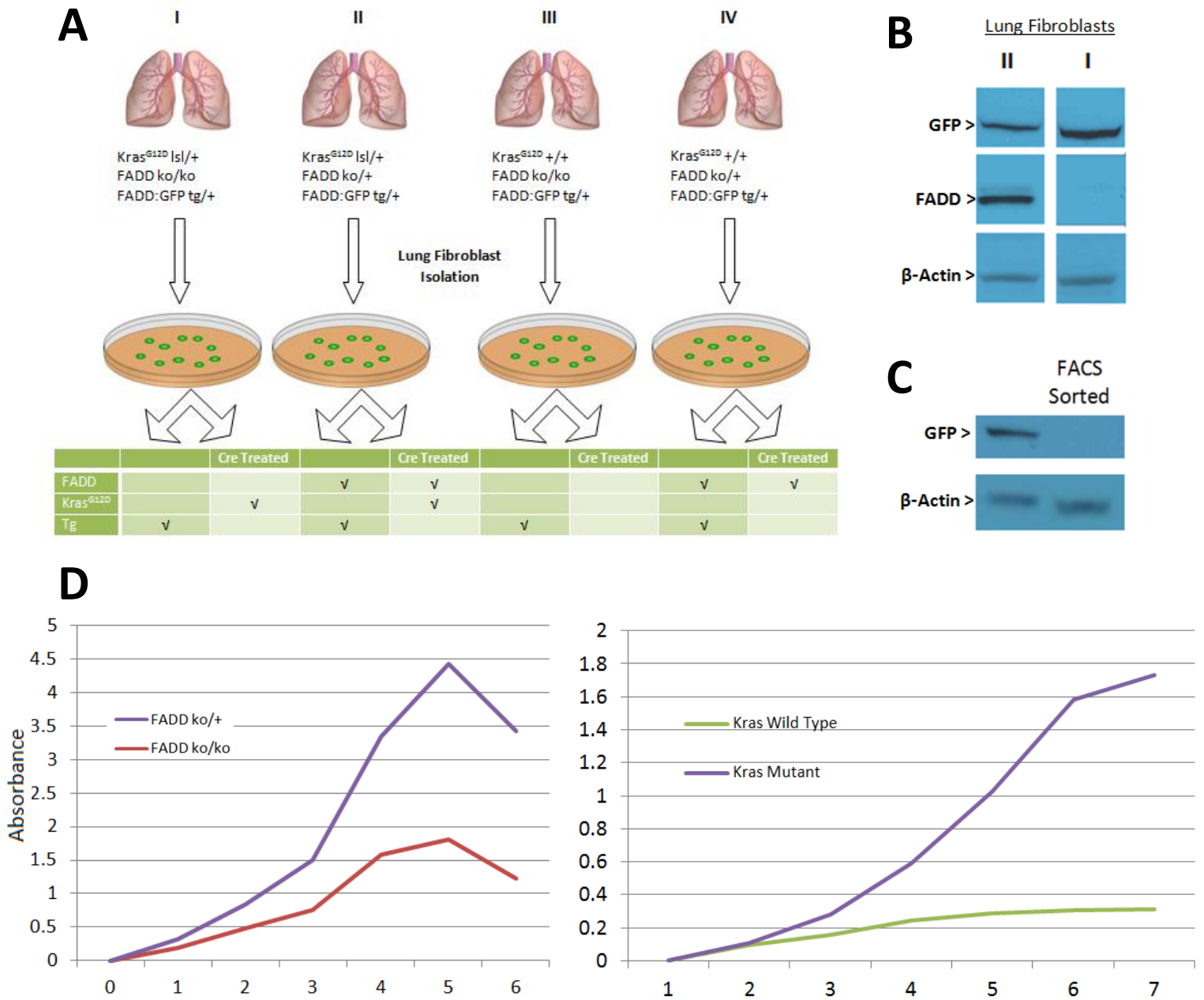
**Figure 4:** Establishment of murine lung fibroblast cell lines. **A.** Isolation of lung fibroblasts and treatment with adenovirus encoding Cre recombinase. Check marks indicate actively expressed genes. **B.** Western blot demonstrating expression patterns in cultured lung fibroblasts. **C.** Western blot demonstrating effectiveness of FACS in separating successful transformants from non-successful transformants. **D.** Crystal violet retention as a surrogate for cell growth rate. Shown are cell growth differences secondary to either loss of FADD or activation of mutant Kras. **E.** Images of lung fibroblasts taken from tissue culture after treatment with Ad-Cre and FACS.

10

**Materials and Methods**

1.) **Mouse Model**

   FADD knockout mice with the conditional FADD:GFP transgene were attained in collaboration with researchers at the Kimmel Cancer Center [19]. Inducible Kras[G12D] mice were attained in collaboration with Dr. Tyler Jacks [18]. Establishment of the Kras driven FADD knockout model with the FADD:GFP transgene was completed with in house breeding and genotyping. Mutant Kras activation and FADD:GFP excision was achieved in a single step by addition of adenovirus Cre into the lungs. Administration of Ad-Cre was achieved by intranasal injection, during which the mice inhale the virus particles. The inhaled solution was composed of 1 mL of Modified Eagle Medium (MEM: Life Sciences), $1.2 \times 10^{11}$ plaque forming units of Adenovirus encoding Cre recombinase, and 5 uL of 2M $CaCl_2$ which precipitated the virus. Mice were anesthetized with isoflurane and held in the supine position with the head above the feet. The Ad-Cre solution was added drop wise to the nostril. All experiments involving animals were carried out in accordance with protocols approved by the University Committee on the Use and Care of Animals at the University of Michigan (UCUCA).

   Assessing the expression patterns of lung tissue by the Western blot allowed confirmation of dual recombination. Transgene expression was assessed using α-GFP (Biovision 3999-100) and Kras activation was assessed with α-p-ERK (Biovision), a protein downstream of Kras[G12D] that increases in concentration when mutant Kras is activated. Protein was released from tissues by Dounce homogenization in RIPA lysis buffer supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche) and phosphatase inhibitors (PhosSTOP, Roche). For tissue culture samples, cells were scraped from the plate in PBS and incubated for 30 minutes in RIPA lysis buffer. Insoluble portions were pelleted, while all soluble protein was quantified via the Lowry assay (Biorad). Equal quantities of protein were resolved on 12% Bis-Tris gels (Invitrogen) and transferred onto 0.20 μm nitrocellulose membranes (Invitrogen). Membranes were blocked for 1 hour in tris-buffered saline with Tween 20 (TBST) containing 5% milk then incubated with primary overnight at 4°C before incubation for 1 hour with the appropriate horseradish peroxidase conjugated secondary antibody. All washes were carried out for 30 minutes in fresh TBST. ECL substrate enabled detection of peroxidase activity by chemiluminescence. Primary antibodies used were α-FADD (Epitomics), α-GFP (Biovision), α-p-ERK (Biovision), or HRP conjugated-β-Actin (Biovision).

2.) **Histology**

   Mice were sacrificed by $CO_2$ asphyxiation followed by cervical dislocation. Thoracic cavity was exposed by incisions along upper midline and transverse lines followed by removal of ventral rib cage. Lungs were perfused with 10 mL phosphate buffered saline (PBS) solution by injection into the right ventricle. Following perfusion, heart and lungs were removed and samples of lung were snap-frozen for subsequent protein and DNA analysis. Remaining lung tissue was either fixed in formalin for histology or processed for lung fibroblast isolation.

   After 48 hours in formalin, histology samples were placed in 70% ethanol and sent to the Tissue Core at the University of Michigan Comprehensive Cancer Center to be cut and affixed to slides using standard techniques. Washes with xylenes then ethanol removed paraffin wax and re-hydrated the tissue to allow for staining. Antigenic sites were retrieved from formalin induced

cross-links by boiling in citrate buffer (Biogenex). 2% $H_2O_2$ was used to block any endogenously existing peroxidase enzymes. After blocking in 5% BSA, primary antibody was added by the hydrophobic barrier method for an overnight incubation (α-FADD, α-p-ERK). Biotinylated secondary antibody was added for 45 minutes at room temperature, followed by treatment with peroxidase conjugated avidin which targeted the biotin on the secondary antibody with high affinity (ABC, Vector). Incubation with peroxidase substrate (DAB, Vector) yielded a brown precipitate demarking the protein of interest. Gross morphology of tissue sections was observed by staining with Gill's haematoxylin (Sigma-Aldrich) and 25% Eosin Y solution. Slides were immersed in haematoxylin for 3 minutes before washing with warm tap water, distilled water, 95% ethanol, then immersion in 25% Eosin Y for 30 seconds. Tissues were then dehydrated by washes in ethanol then xylenes. Coverglass was affixed with Cytoseal 60 (Thermo Scientific).

3.) **Isolation of Cell Lines**

Murine lung fibroblast cells were isolated by mechanical dispersion of perfused lung tissue with a sterile scalpel followed by a 30 minute shaking incubation with filtered collagenase media (14mL DMEM, 1mg DNase (Roche), 200mcg Collagenase A (Roche)). Further mechanical digestion was achieved by repeated aspiration through an 18 gauge needle. Cells were then pelleted and transferred to MEF media in 10 cm plates to be incubated at 37°C for up to 10 passages. Adenovirus harboring the gene for Cre recombinase (Ad-Cre) was attained from the Vector Core at the University of Michigan and was added directly to tissue cultures at a high multiplicity of infection. FACS sorting was performed by the Flow Cytometry Core at the University of Michigan Biomedical Sciences Research Building.

To isolate MEFs, pregnant females were sacrificed after 9 days of gestation by $CO_2$ asphyxiation and the abdominal cavity was exposed using sterile surgical scissors. Both uterine horns containing the fetuses were removed and placed in PBS on ice. Each embryo was removed from its amniotic sack and the head and red tissue containing liver precursor cells was excised, leaving only fibroblast cells which were mechanically dispersed using curved iris scissors. 4 mL of trypsin was added for a 20 minute incubation at 37°C followed by transfer to a new dish containing specialized MEF media (93% Dulbecco's modified Eagle medium, 5% Fetal Bovine Serum, 1% Non-Essential Amino Acids, and 1% Penicillin/Streptomycin/Glutamine solution (Life Sciences)).

4.) **Cell Growth Assay**

Cells were grown in triplicate on 12-well tissue culture treated plates. 17,000 cells were suspended in 2 mL of specialized MEF media. Every 24 hours following, a plate was assayed for cell population as follows. Cells were fixed to the plate with 4% paraformaldehyde for 15 minutes, then stained for 20 minutes with crystal violet solution (0.1% crystal violet, 10% ethanol). Unabsorbed stain was removed by 3 rinses with distilled water. Absorbed stain was eluted with 2mL of 10% acetic acid solution and absorbance of the resultant crystal violet/acetic acid solution was measured at 590 nm with a single beam spectrophotometer.

## Discussion

It is known that FADD plays distinct roles in both apoptosis and cell cycle regulation [2, 11]. In apoptosis, FADD propagates death inducing signals from membrane receptors to downstream effectors such as caspase 3, NF-κB, PARP, and CAD [2]. In the cell cycle, phosphorylation of FADD at serine 194 is an essential step for the spindle fiber formation necessary for completion of mitosis [1]. Recent studies have associated elevated levels of p-FADD with poor clinical outcome in lung cancer patients [4]. After associating FADD with lung cancer, it became necessary to evaluate its exact role in these cancers. Since FADD acts in both an apoptotic and proliferative capacity in the cell, it is equally possible that FADD is causing the tumors as it is trying to ameliorate them. We approached this dilemma by establishing a lung cancer model in which FADD expression is a variable. With this model, we demonstrated that FADD acts as an oncogene by promoting unregulated cell growth through the cell cycle.

Establishing a lung cancer model faithful to the human disease has been a priority of this research thus far. The murine model is a result of careful breeding protocols using mice from collaborators Dr. Tyler Jacks and Dr. Yuhang Zhang [18, 19]. The combination of these models provides an elegant system which offers us the opportunity to mechanistically investigate the role of FADD in lung cancer. The combination of a Kras$^{G12D}$ driven lung cancer with conditional FADD expression through a transgene is a novel approach. This innovative mouse strain was further used to establish cell culture model systems of embryonic fibroblasts (MEFs) and lung fibroblasts with different genotypes. These cell lines provide the basis for most experimentation offered thus far.

Using lung fibroblasts, we were able to demonstrate that FADD deficient cell lines grew more slowly than equivalent cells with FADD. Since FADD helped cells to grow more aggressively in culture, it can be concluded that FADD is acting in an oncogenic capacity. Mechanistically, this conclusion suggests that FADD acts in cancer cells in one of two likely ways. First, it is possible that FADD abandons its role as an apoptosis signaling protein. This would result in the loss of essential apoptosis signals that are sent to tumorigenic cells by the immune system. If FADD enabled the cell to ignore these signals, it is conceivable that this cell would then be capable to developing into a tumor. Second, FADD's role as an oncogene may be a result of its malfunction in the cell cycle. If FADD promotes cell cycle progression beyond the confines of deliberate cell growth and proliferation, then it may lead to tumor development. Regardless of the mechanism, these results confirm our hypothesis that FADD acts as an oncogene by enabling proliferation of lung fibroblasts in lung adenocarcinomas.

Thus far, analysis of lung cross-sections by histology have been successful at implicating Kras$^{G12D}$ activation in tumor development, but the impact of FADD is less clear. Preliminary data confirms expectations that FADD increases the number and severity of tumors and lesions, but this data is not yet statistically significant. Immunohistochemistry performed on these cross-sections has been useful in linking p-ERK expression to mutant Kras expression. The unexpected finding that p-ERK is found to be in lower concentrations in FADD null tissues is likely to be a result of cell cycle regulation. Since FADD plays an important role in progressing through the cell cycle, cells without FADD are not able to proceed through the cell cycle as efficiently. P-ERK is known to play a role in promoting cell cycle progression, so

it is therefore up-regulated in dividing cells. Since FADD null cells do not divide as often, p-ERK is not expressed in as high of concentrations.

In order to more extensively characterize the role of FADD in lung cancer, this mouse model is currently being used to evaluate tumor growth in vivo as it relates to FADD expression. A significant amount of work has already been invested in histological analysis of lungs from each experimental group at several time points. We hope to use histology to scale the severity of the lung cancer. This ranking can then be cross-referenced with the genotype of the sample in hopes of demonstrating the effects of FADD on lung cancers in vivo. Essentially, these results are expected to mirror those observed in the lung fibroblast culture, in which FADD presence acted as an oncogene by exacerbating tumors. We have experimented with several mechanisms for scaling tumor burden. Computational algorithms have been used to calculate functional lung volume from a cross section of the lung. Having a lower functional lung volume is indicative of more advanced tumors that have penetrated farther into the healthy viscera of the lungs. Another approach that has proven very successful in scaling tumor burden is computer tomography (CT) scanning of 3-dimensional lung space. Preliminary results from other investigators in the Rehemtulla laboratory have drawn a strong correlation between FADD and more severe lung cancers using this method (Data not shown). Another approach that is in progress is staining of histology samples with Masson's Trichome. This stain is capable of identifying collagen bundles in the lung that are characteristic of tumorous lesions. Lungs with more collagen can therefore be assumed to have a greater tumor burden. Finally, immunohistochemical analysis targeting Ki-67 will provide a diagnostic for cell cycle activity. Ki-67 is a protein that is present only during active phases of the cell cycle. Since accelerated cell growth and division is a hallmark of cancer, Ki-67 will serve as a tumor indicator. Determining the distribution of Ki-67 in a lung cross-section will allow us to determine where the tumors are growing and how severe they may be.

The second reason for focusing on histology is the potential for associating increased FADD concentration with the localization of the tumors themselves. Thus far, all protein has been taken from whole lung homogenates. This means that the origin of the increased FADD may be from tumor tissue or healthy tissue. It would strengthen the existing results if we could confirm that FADD up-regulation is a tumor specific phenomenon. Thus far, none of the FADD antibodies have been effective at targeting FADD in our immunohistochemistry protocol.

Future directions of this research will involve a more in-depth evaluation of the post-transcriptional modification of FADD. Orthotopic tumor models are designed to illuminate the characteristics of cells grown in vitro by introducing them to an in vivo environment. This experiment involves injecting cells from tissue culture back into athymic mice. Previous research has correlated p-FADD, not total FADD, with poor clinical prognosis in lung cancer [4, 20]. In order to study how the phosphorylation state of FADD impacts the cellular environment, experiments are being designed using mice that express a non-phosphorylatable form of FADD. This mutant FADD, with a mutation at the phosphorylation site at serine 191 will enable the evaluation of mice that are unable to phosphorylate FADD. Additionally, mutating the phosphorylation site at serine 191 to become constitutively phosphorylated will allow for amplification of p-FADD's effects on the cell. Both mutant forms of FADD will be transfected into cultured lung fibroblasts without endogenous FADD expression. These cells will

then be injected into mice via the tail vein, where they will target the lungs and develop into tumors there. Subsequent analysis of these tumors will provide invaluable insight in the effects of FADD phosphorylation.

## References

1.  Alappat, Elizabeth C., Christine Feig, Benjamin Boyerinas, Jörg Volkland, Martin Samuels, Andrea E. Murmann, Andrew Thorburn, Vincent J. Kidd, Clive A. Slaughter, and Stephanie L. Osborn. "Phosphorylation of FADD at Serine 194 by CKIα Regulates Its Nonapoptotic Activities." *Molecular Cell* 19.3 (2005): 321-32. Print.

2.  Ashkenazi, Avi. "Death Receptors: Signaling and Modulation." *National Center for Biotechnology Information*. U.S. National Library of Medicine, 28 Aug. 1998. Web. 31 Mar. 2013.

3.  Beer, David G., and Sharon Kardia. "Gene-expression Profiles Predict Survival of Patients with Lung Adenocarcinoma." *Nature Medicine* 8.8 (2002): 816+. Print.

4.  Bhojani, Mahaveer S., Alnawaz Rehemtulla, David Beer, and Brian Ross. "Nuclear Localized Phosphorylated FADD Induces Cell Proliferation and Is Associated with Aggressive Lung Cancer." *Cell Cycle* 4.11 (2005): 1478-481. Print.

5.  "Cancer - United States Cancer Statistics (USCS) Data - 2009 Top Ten Cancers." *National Program for Cancer Registries*. Center for Disease Control and Prevention, n.d. Web. 15 Feb. 2013.

6.  Chen, G., and Alnawaz Rehemtulla. "Phosphorylated FADD Induces NF- kB, Perturbs Cell Cycle, and Is Associated with Poor Outcome in Lung Adenocarcinomas." *Proceedings of the National Academy of Sciences* 102.35 (2005): 12507-2512. Print.

7.  DuPage, Michel, Alison L. Dooley, and Tyler Jacks. "Conditional Mouse Lung Cancer Models Using Adenoviral or Lentiviral Delivery of Cre Recombinase." *Nature Protocols* 4.8 (2009): 1064-072. Print.

8.  Guerra, Carmen, Alberto J. Schuhmacher, Marta Cañamero, Paul J. Grippo, Lena Verdaguer, Lucía Pérez-Gallego, Pierre Dubus, Eric P. Sandgren, and Mariano Barbacid. "Chronic Pancreatitis Is Essential for Induction of Pancreatic Ductal Adenocarcinoma by K-Ras Oncogenes in Adult Mice." *Cancer Cell* 11.3 (2007): 291-302. Print.

9.  Hanahan, D., and R. Weinberg. "The Hallmarks of Cancer." *Cell* 100.1 (2000): 57-70. Print.

10. Harper, N. "Protein Kinase C Modulates Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis by Targeting the Apical Events of Death Receptor Signaling." *Journal of Biological Chemistry* 278.45 (2003): 44338-4347. Print.

11. Imtiyaz, H. Z., X. Zhou, H. Zhang, D. Chen, T. Hu, and J. Zhang. "The Death Domain of FADD Is Essential for Embryogenesis, Lymphocyte Development, and Proliferation." *Journal of Biological Chemistry* 284.15 (2009): 9917-926. Print.

12. Kim, Y. "Correlation of K-ras Mutation and K-ras Protein Expression in Non-small Cell Lung Cancer." *Lung Cancer* 29.1 (2000): 206. Print.

13. "Lung Cancer Statistics." *Centers for Disease Control and Prevention*. Centers for Disease Control and Prevention, 20 Dec. 2012. Web. 20 Feb. 2013.

14. "Lung Cancer Statistics." *WebMD*. WebMD, n.d. Web. 15 Feb. 2013.

15. Riely, G. J., J. Marks, and W. Pao. "KRAS Mutations in Non-Small Cell Lung Cancer." *Proceedings of the American Thoracic Society* 6.2 (2009): 201-05. Print.

16. Wang, Hanxiao. "Molecular Imaging Reveals a Role for AKT in Resistance to Cisplatin for Ovarian Endometrioid Adenocarcinoma." *Clinical Cancer Research* 19 (2013): 158-69. *American Association for Cancer Research*. Web. 12 Jan. 2013.

17. Winslow, Monte, and Tyler Jacks. "Genetic Mouse Models of Cancer." *Carcinogenesis and Cancer Genetics*. N.p.: n.p., n.d. 129-38. Print.

18. Xue, W., E. Meylan, T. G. Oliver, D. M. Feldser, M. M. Winslow, R. Bronson, and T. Jacks. "Response and Resistance to NF- B Inhibitors in Mouse Models of Lung Adenocarcinoma." *Cancer Discovery* 1.3 (2011): 236-47. Print.

19. Zhang, Jianke, and Astar Winoto. "Fas-Mediated Apoptosis and Activation Induced T-cell Proliferation Are Defective in Mice Lacking FADD/MORT1." *Letters to Nature* 392 (1998): 296-300. Web.

20. Zhang, Jing, Dapeng Zhang, and Zichun Hua. "FADD and Its Phosphorylation." *IUBMB Life (International Union of Biochemistry and Molecular Biology: Life)* 56.7 (2004): 395-401. Print.

21. Zhang, Yuhang, and Stephen Rosenberg. "Fas-associated Death Domain (FADD) Is a Negative Regulator of T-cell Receptor–mediated Necroptosis." Journal of Immunology, 23 Feb. 2005. Web. 12 Feb. 2012.