Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-231-2825.

1. **Grantee Institution:** Thomas Jefferson University

2. **Reporting Period (start and end date of grant award period):** 1/1/2011-12/31/2014

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Theodore F. Taraschi, PhD

4. **Grant Contact Person's Telephone Number:** 215-955-3900

5. **Grant SAP Number:** 4100054872

6. **Project Number and Title of Research Project:** 4 -Targeting Endoplasmic Reticulum Stress (ERS) Signaling for Overcoming Lung Cancer Resistance

7. **Start and End Date of Research Project:** 1/01/2011-12/31/2014

8. **Name of Principal Investigator for the Research Project:** Adam Dicker, MD, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 699,714.32

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Lu, Bo	Professor	18.461% Yr 1; 20% Yrs. 2-3; 10% Yr 4	$178,780.17
Sun, Yunguang	Research Associate	26.154% Yr 1; 40% Yrs 2-3; 14.615% Yr 4	$85,806.97
Fan, Yongjun	Research Associate	37.923% Yr 1; 24.539% Yr 2	$44,843.31
Wang, Yong	Postdoctoral Fellow	50.769% Yr 2; 88% Yr 3; 50.769% Yr 4	$91,223.55
Dicker, Adam Paul	Professor & Chair	1% Yrs 1-3; 0.777% Yr 4	$11,847.97

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____x_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the

research?

Yes___x_____ No___

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds awarded:
Targeting IGFBP3R for therapeutic resistance of lung cancer	☒NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	10/2012	$275,000	Not funded

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____x_____ No_____

If yes, please describe your plans:

R01 in 2015

12. Future of Research Project. What are the future plans for this research project?

To use a novel mouse model of lung cancer with or without IGFBP3 that was created through the CURE funding, we will develop novel biomarkers and therapeutics to predict and overcome therapeutic resistance of lung cancer.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____ No___x_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other				
Unknown				
Total				

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

    Yes_____ No___x_____

    If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

    Yes_____ No__x_____

    If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. **Collaboration, business and community involvement.**

    16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes____x_____ No_____

If yes, please describe the collaborations:

We have received reagents from Dr. Youngman Oh from Medical College of Virginia to investigate the role of IGFBP3 receptor.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No___x_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No_____x_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project

work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project Title and Purpose

Targeting Endoplasmic Reticulum Stress (ERS) Signaling for Overcoming Lung Cancer Resistance - Lung cancer patients die as a consequence of therapeutic resistance and treatment failure. One of the important features of cancer resistance is the inability to undergo apoptosis. Ongoing research from this laboratory supports the idea of inducing ER stress-mediated autophagy as a way of combating resistant lung cancer cells. Therefore we will investigate the underlying mechanisms and preclinical concepts of using ER stress activators for enhancing radiotherapy in treating lung cancer.

Project Overview

Tumor cells, as a result of transformation or tumor microenvironment, have dysregulated signaling pathways that control cell survival or death. This enables the cells to evade programmed cell death (apoptosis) following treatment with cancer therapies. In every tumor, there is a population of cells that harbors multiple genetic defects in a major form of apoptosis. These cells are enriched following cytotoxic chemotherapy such as cisplatin and become a source of failure due to therapeutic resistance. This laboratory has identified an alternative death program, autophagy (self-eating), which can be triggered in cells that are defective in apoptotic signaling and that overcome tumor resistance to ionizing radiation (IR). The translational potential of this concept uses model systems of several chemical agents that increase IR-induced cell death, even in the presence of defective apoptotic machinery. In these systems, the radio-sensitizing mechanism is dependent upon autophagy mediated by PERK-dependent endoplasmic reticulum stress (ERS) signaling. This project will extend this work using *in vitro* and *in vivo* cancer models, and lung cancer cell clones that are resistant to cisplatin and IR.

The hypothesis is that in tumor cells lacking functionally intact apoptosis, ERS regulates the IR response through autophagic pathways. To validate this theory, both cancer cells with known apoptotic gene defects and cancer cells fully resistant to cisplatin will be used to determine the key molecules that regulate ERS and autophagy signaling. The following four aims are proposed:

(1) Determine if the regulation of ERS is a significant molecular target of the cellular damage response that occurs during IR-induced autophagy;
(2) Determine the mechanism by which caspases 3/7 regulate ERS signaling in the IR response;

(3) Determine whether ERS inducers increase IR sensitivity in *in vitro* cancer models;
(4) Determine whether ERS inducers increase IR sensitivity in *in vivo* cancer models.

Summary of Research Progress

The success of radiation therapy in lung cancer is affected by a variety of factors, including location, size, grade, extent of invasion, and individual tumor characteristics. Resistance to treatment, which can be an intrinsic characteristic of the tumor, but can also be induced by initial rounds of treatment, is a very real concern. Understanding the changes induced by radiation therapy that induce subsequent resistance is crucial to developing mechanisms to subvert this resistance.

Radiation resistant H460 cells (RR-H460 cells) were selected per the following protocol. Briefly, after H460 cells were treated with 20Gy using a PanTak 310-keV X-ray machine and cells were cultured for seven days. The surviving cells were trypsinized and cultured in 0.8% methylcellulose that was supplemented with 20ng/mL EGF (BD Biosciences), bFGF, and 4µg/mL insulin (Sigma). EGF, bFGF (20ng/mL), and insulin (4µg/mL) were added every second day for 14 days to allow the cells to form spheres. Spheres were diluted with PBS to make a single-cell suspension and then plated in 100mm dishes with RPMI 1640 supplemented with 10% FBS. A single plaque was chosen for expansion and subsequent characterization. Radiation resistance was confirmed by a clonogenic assay. Cells were irradiated with 0–6Gy (dose rate of 1.8Gy/min) using ^{137}Cs irradiator (J.L. Shepherd and Associates). After irradiation, cells were incubated at 37°C for 8–10 days. Cells were fixed for 15m with 3:1 methanol/acetic acid and stained for 15m with 0.5% crystal violet (Sigma) in methanol. After staining, colonies were counted by the naked eye (cut-off of 50 viable cells). Figure 1 demonstrates that this cell model (RR H460) has the property of radiation-resistant.

To discover novel mechanisms of radiation resistance, limited sequencing was performed on the resistant cell line and compared with the parent H460 cell line. A sample from the expanded clone was run on an Ion 314 chip in an Ion Torrent PGM System. Sequencing analysis revealed a novel deletion mutation at the end of the DNA binding domain of p53 in the RR H460 cell model (Figure 2). The parental H460 lung cancer cell line has intact p53. This 4 base deletion generates a stop codon, resulting in a truncated protein missing the C-terminal ~100 amino acids. Sanger sequencing of the same DNA samples confirmed the above finding.

p53 operates as a cell cycle monitor; it has been implicated in the regulation of both the G1/S and G2/M checkpoints via p21. It can also induce the caspase cascade ending with the cleavage of caspase 3 and apoptosis (G). To determine the impact of this deletion mutant on p53 signaling and its function, we examined the levels of active p53, p21 and apoptosis by caspase 3 cleavage at baseline, as well as after 6Gy. The presence of the deletion mutant was confirmed by the truncated p53 in the RR H460 cells (Figure 3A). In addition, the parent and derivative cell lines were found to have comparable levels of p21, total caspase 3 and cleaved caspase 3. Levels of p53 and phosphorylated p53 were also comparable, but the radiation resistant samples showed two bands corresponding to normal and mutant p53 and their phosphorylated counterparts. Notably, the expression of p21 and cleaved caspase 3 is delayed in the RR cell line. The impact on p21 transcription was initially determined by mRNA analysis of p21 expression, which

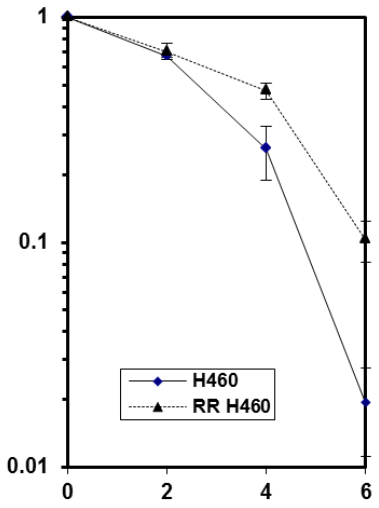
indicates that in the hours immediately after irradiation, the transcription is initially higher in the RR cell line, but by 24h post-irradiation the levels are similar in both the RR and parent cell lines (Figure 3C). The data suggest the absence of overall impact by this deletion mutant when wild-type p53 is present.

To determine the potential effects of the deletion mutant when wild-type p53 is absent, we overexpressed the deletion mutant in a p53-null H1299 lung cancer cell line. When WT or mutant p53 was overexpressed in the parental H460 cell line, comparable levels of p53 and phosphorylated p53, p21, total caspase 3 and cleaved caspase 3 were expressed (Figure 3A); however, transfection of H1299 cells with mutant-overexpressing plasmid did not induce p21 expression, whereas those given WT p53 produced a significant amount, as expected (Figure 3B). This finding was also confirmed at the mRNA level (Data not shown).

Furthermore, we determined the ability of the deletion mutant to transcriptionally activate a luciferase reporter gene construct containing a p53-binding element. The ability of p53 to bind its consensus elements is critical to its function. Examination of H460 cells transfected with WT p53 showed nearly twice the luciferase expression as the control, whereas cells transfected with the mutant p53 had slightly lower expression than the control (Figure 4A). When the same vectors were applied to naturally p53-deficient H1299 cells, the sample treated with the mutant p53 showed a small amount of luciferase activity, approximately 3x that of the vector, whereas the WT p53 induced 25x as much (Figure 4B). These luciferase assays demonstrated the inability of mutant p53 to bind its consensus elements, as evidenced by the failure to initiate downstream gene transcription.

Last, we examined the impact of this deletion mutant on cell survival by an MTS assay. 48h or 72h after transfection with WT p53, the survival rate for H1299 cells was 50%, whereas transfection with mutant p53 resulted in over 90% survival (Figure 5). In H460 cells, survival was over 90% regardless of p53 mutation status, showing definitively that the novel mutation did not improve cell survival.

In summary, we have generated a radiation resistant cell line with a unique mutation resulting in the deletion of the tetramerization domain. While this mutation may cause a loss of function in the resulting protein, it also prevents the mutant protein from binding with residual normal p53, but does not create a dominant negative. Functional characterization of a radiation-induced p53 mutation in the p53-competent H460 lung cancer cell line does not implicate it in the development of radiation resistance, whereas transfection of the mutated protein into p53-deficient H1299 cells indicates its ability to abrogate appropriate effects.



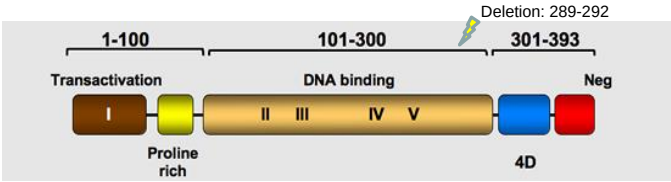


Figure 2. Cartoon representation of the functional domains of the p53 protein. RR460H cells contain a deletion of amino acids 289-292, which are located in the DNA binding domain of the protein.

Figure 1. Radiation resistance in H460 cells. Clonogenic assays with increasing concentrations of Gy show that cells with the H460 mutation are highly resistant to radiation treatment.

A) B) C)

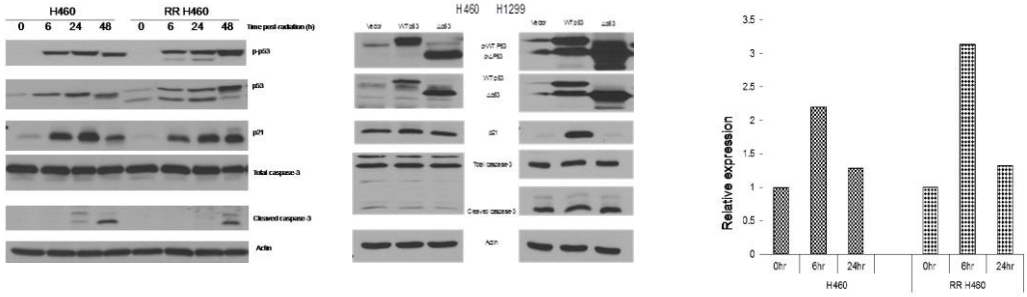


Figure 3. Effects of the H460 deletion mutant when p53 is absent from cells. A) When WT or mutant p53 was overexpressed in the parental H460 cell line, comparable levels of p53 and phosphorylated p53, p21, total caspase 3 and cleaved caspase 3 were expressed. B) Transfection of H1299 cells with mutant-overexpressing plasmid did not induce p21 expression, whereas those given WT p53 produced a significant amount, as expected. C) In the hours immediately after irradiation, transcription is initially higher in the RR cell line (6hr), but by 24h post-irradiation, the levels are similar in both the RR and parent cell lines.

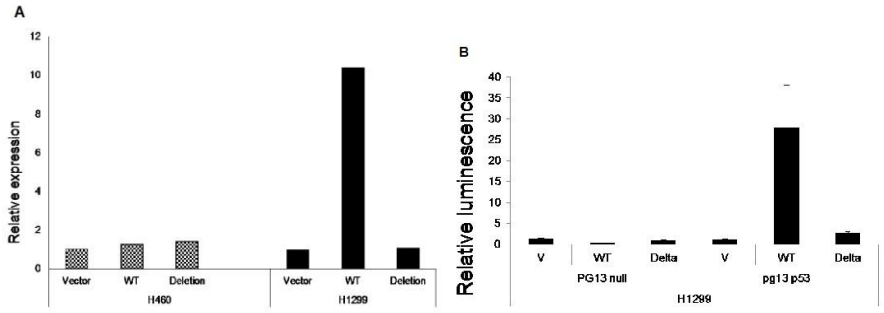


Figure 4. The ability of p53 to bind to its consensus elements. A) Examination of H460 cells transfected with WT p53 showed nearly twice the luciferase expression as the control, whereas cells transfected with the mutant p53 had slightly lower expression than the control. B) When the same vectors were applied to naturally p53-deficient H1299 cells, the sample treated with the mutant p53 showed a small amount of luciferase activity, approximately 3x that of the vector, whereas the WT p53 induced 25x as much.

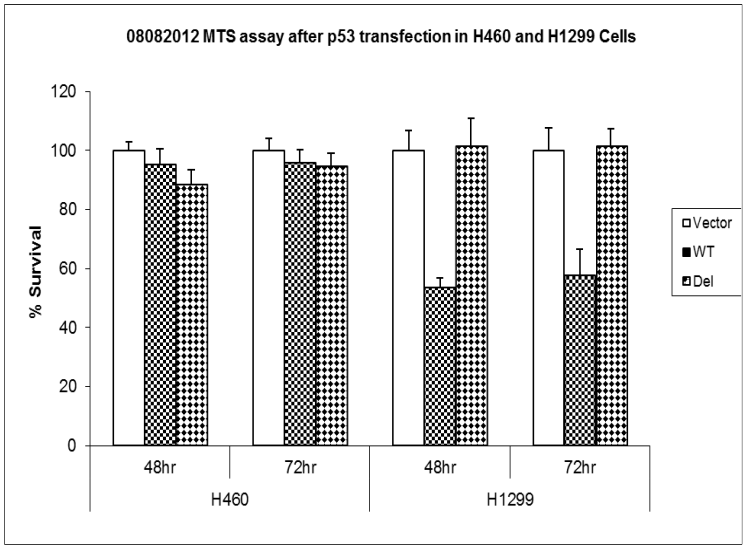


Figure 5. The impact of the H460 deletion on cell survival. 48h or 72h after transfection with WT p53, the survival rate for H1299 cells was 50%, whereas transfection with mutant p53 resulted in over 90% survival. In H460 cells, survival was over 90% regardless of p53 mutation status, showing definitively that the novel mutation did not improve cell survival

Overexpression of IGFBP-3 inhibits survival in HCC2429 cells

We previously reported that IGFBP-3 was significantly down regulated in cisplatin-resistant lung cancer cells, making it a molecule of interest in exploring treatment-induced lung cancer resistance. To study the effect of IGFBP-3 on the survival of two lung cancer cell lines (HCC2429 cells and H460 cells), we used adenoviral vector to overexpress IGFBP3. Ad-IGFBP-3 and its control virus, Ad-EV, were used to infect these lung cancer cells. Western analyses demonstrated that IGFBP-3 was highly expressed 72h after infection in both HCC2429 cells and H460 cells (Figure 6).

IGFBP-3 inhibits cell survival partially through blocking IGF-1 signaling

IGFBP-3 binds to IGF-1 and prevents IGF-1 from activating IGF-1R and its downstream signaling. To determine whether IGFBP-3 inhibition of cell survival is dependent upon its interaction with IGF-1, we used an IGFBP-3 ^{GGG} mutant that has no binding affinity to IGF-1 (Figure 7A). 72 h after HCC2429 cells were exposed to Ad-IGFBP-3 infection, we observed a 40% survival reduction in HCC2429, whereas only a 20% survival reduction was seen following the exposure of Ad- IGFBP-3 ^{GGG} (Figure 7B). In contrast, neither virus affected survival of H460 lung cancer cells (Figure 7B). We then probed the expression of various signaling proteins 72 hours after Ad-IGFBP-3 infection. Baseline signaling is different between the two cell lines. HCC2429 cell showed active phosphorylated IGF-1R and Akt, but no active STAT3, in contrast to H460 cells. Both cell models have high levels of phosphorylated ERK. When IGFBP-3 is overexpressed, active IGF-1R and ERK were suppressed in HCC2429 cells. Neither alteration was seen in the H460 cell line. Overexpression of the non IGF-1-binding mutant partially decreased ERK phosphorylation in HCC2429 cells (Figure 7C).

Overexpression of IGFBP-3 increases susceptibility to cisplatin treatment in lung cancer cells

Since IGFBP-3 inhibits lung cancer cell survival and is reduced in cisplatin-resistant lung cancer cells, we further explored its potential to enhance the efficacy of cisplatin chemotherapy. HCC2429 cell survival is decreased to 60% by either cisplatin treatment or IGFBP-3 overexpression, whereas either treatment decreased H460 cell survival by ~40% and ~20%, respectively (Figure 8A). For H460 cells, the survival reduction was associated with a decrease in STAT3 phosphorylation (CDDP and IGFBP-3 OE) and a decrease in Akt phosphorylation (CDDP) (Figure 8B). For HCC2429 cells, we observed decreased IGF1R phosphorylation and STAT3 expression following IGFBP-3 overexpression, and Akt phosphorylation following CDDP or IGFBP-3 OE. Changes in ERK phosphorylation were as follows: 1) an increase after CDDP treatment or 2) a decrease after IGFBP-3 OE (Figure 8B). Combining IGFBP3 and CDDP decreased survival of HCC2429 by 80% and by 45% in the setting of H460. In H460 cells, the combined treatment abrogated STAT3 phosphorylation and decreased Akt phosphorylation. In HCC2429 cells, STAT3 expression, Akt phosphorylation and ERK phosphorylation were greatly attenuated.

48h after treatment, caspase-3 cleavage was noted in H460 cells treated with either CDDP or IGFBP-3 overexpression and became more pronounced after the combined treatment. CDDP-treated HCC2429 cells showed significant increases in caspase-3 cleavage, whereas IGFBP-3 overexpression had a mild effect. Combination treatment resulted in a significant increase of caspase-3 cleavage (Figure 8C).

IGFBP-3-overexpression induces ER stress, and IGFBP-3-induced apoptosis is dependent on GRP78

We hypothesized that the observed therapeutic efficacy of IGFBP3 is mediated by ER stress signaling. Therefore, the ER stress markers GRP78 and phospho-eIf2a were probed and found to be induced 48h following Ad-IGFBP-3 infection in both HCC2429 and H460 cells (Figure 9A).

We then used GRP78 siRNA to determine whether GRP78 is responsible for IGFBP-3 induced apoptosis. GRP78 knockdown attenuated IGFBP-3 induced apoptosis in HCC2429 cells (Figure 9B). These results suggest that IGFBP-3-overexpression induces ER stress, and IGFBP-3-induced apoptosis is dependent on GRP78.

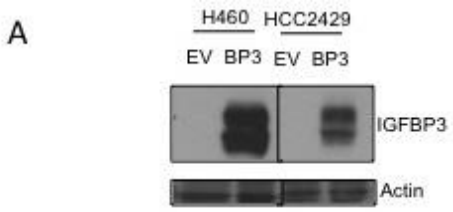


Figure 6: Overexpression of IGFBP-3 inhibits survival of HCC2429 cells. A) HCC2429 and H460 cells were infected with Ad-IGFBP-3 or a control virus, Ad-EV. 72 h later, cells were collected and protein was extracted, immunobloting was performed using IGFBP-3 antibody. Western analyses demonstrated that IGFBP-3 was highly expressed 72h after infection in both HCC2429 cells and H460 cells

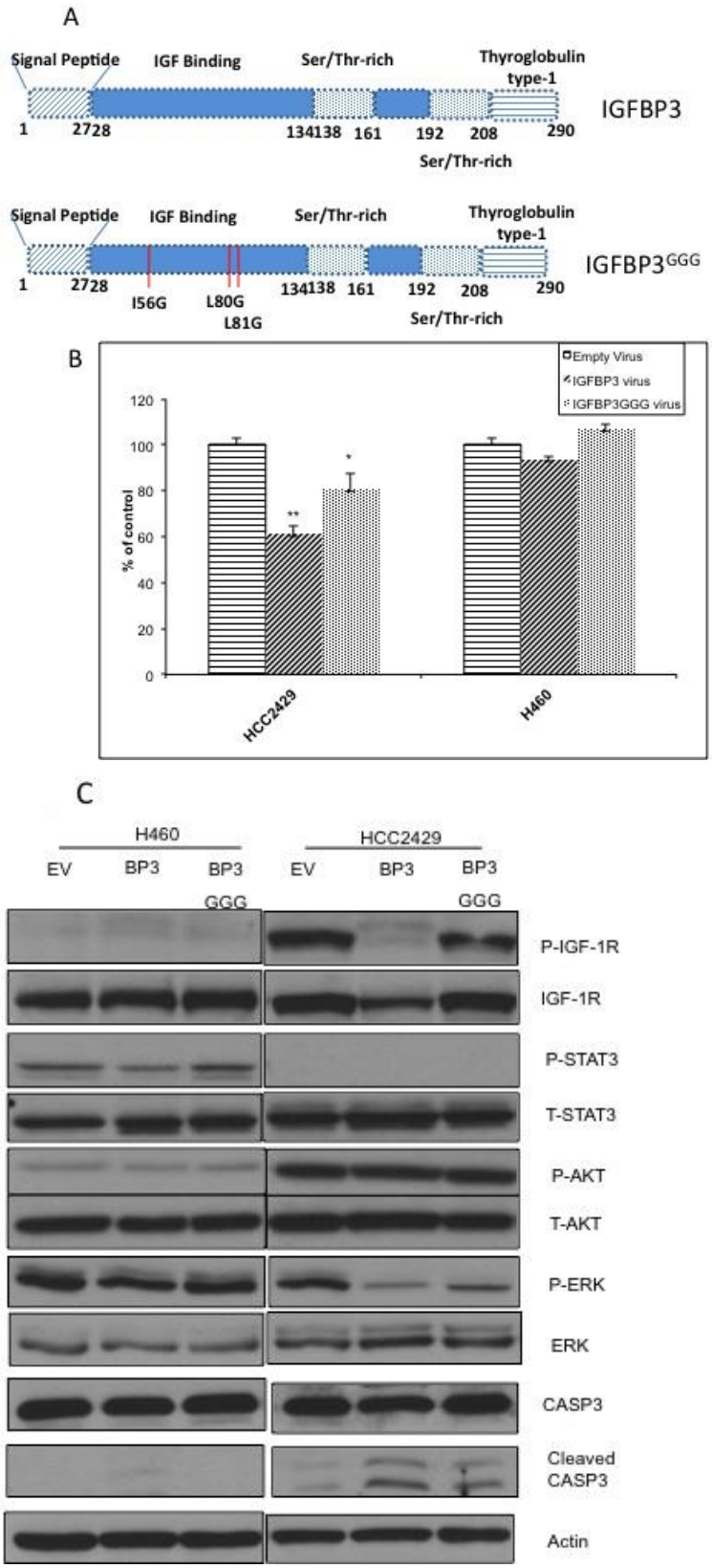


Figure 7: IGFBP-3 inhibits cell survival partially by blocking IGF1 signaling. A) schematic structure of IGFBP-3 and its mutant, IGFBP-3^{GGG}. B) HCC2429 and H460 cells were infected with Ad-IGFBP-3, Ad-IGFBP-3 mutant and Ad-EV. MTS assay was performed 72 hours after infection. C) HCC2429 and H460 cells were infected with Ad-IGFBP-3, Ad-IGFBP-3 mutant and Ad-EV, 72hr post infection, cells were collected and protein samples were extracted. Various signaling proteins were probed. As shown in Figure 7C, baseline signaling is different in the two cell lines. HCC2429 cell showed active phosphorylated IGF-1R and Akt, but no active STAT3, in contrast to H460 cells. Both cell models have high level of phosphorylated ERK. When IGFBP-3 is overexpressed, active IGF-1R and ERK were suppressed in HCC2429 cells. Neither alteration was seen in the H460 cell line. Overexpression of the non IGF-1-binding mutant partially decreased ERK phosphorylation in HCC2429 cells.

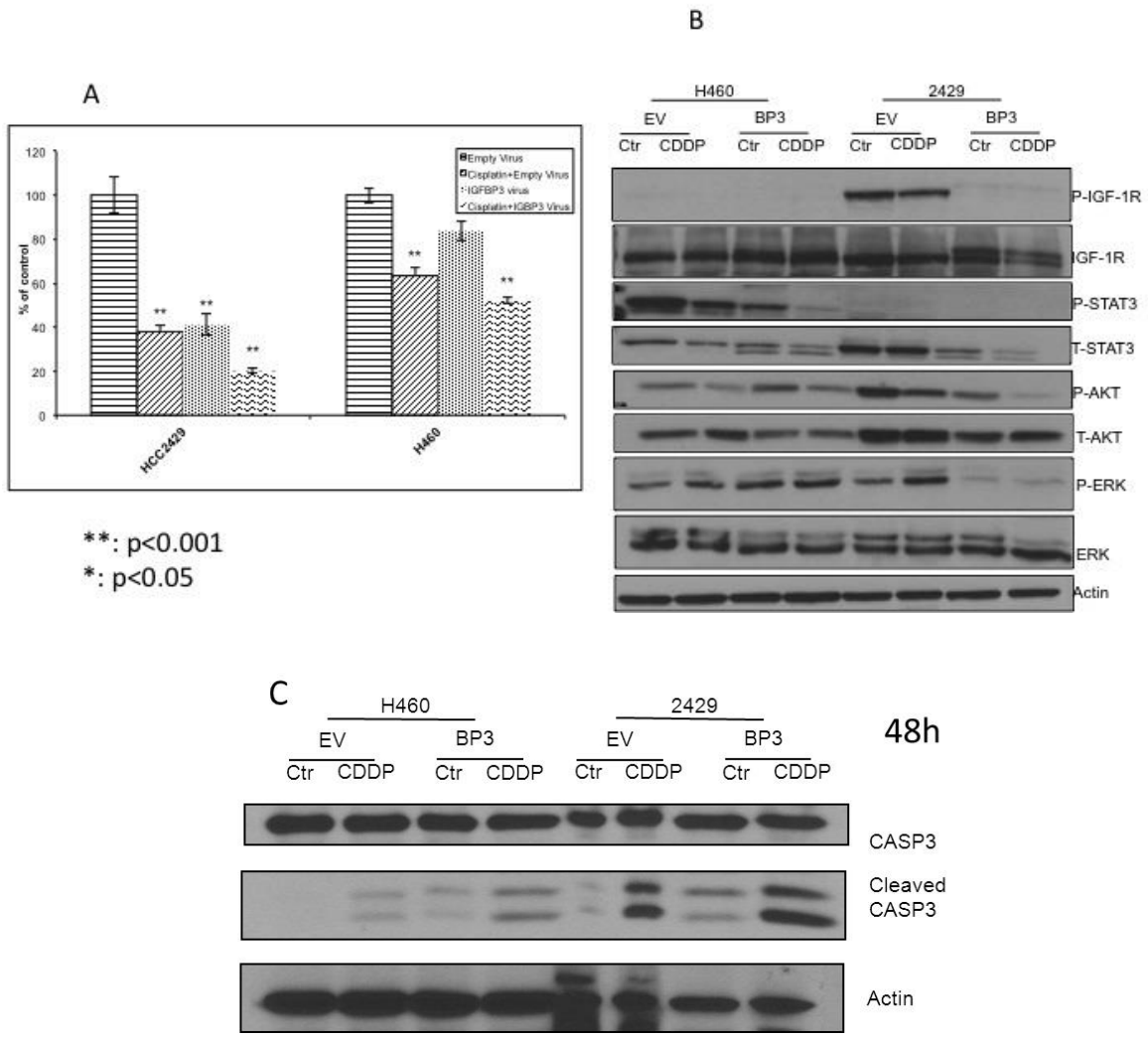


Figure 8: Overexpression of IGFBP-3 increases susceptibility to cisplatin treatment in lung cancer cells. A) HCC2429 and H460 cells were infected with Ad-IGFBP-3 and Ad-EV; 72 hr post infection, cells were treated with 20 µM cisplatin. MTS assay was performed 24 hr after incubation. B) HCC2429 and H460 cells were infected with Ad-IGFBP-3 and Ad-EV; 72 hr post infection, cells were treated with 20 µM cisplatin. Cells were collected for protein extraction and immunoblotting. C) 48h after treatment, caspase-3 cleavage was noted in H460 cells treated with either CDDP or IGFBP-3 overexpression and became more pronounced after the combined treatment. CDDP-treated HCC2429 cells showed significant increases in caspase-3 cleavage, whereas IGFBP-3 overexpression had a mild effect. Combination treatment resulted in a significant increase of caspase-3 cleavage.

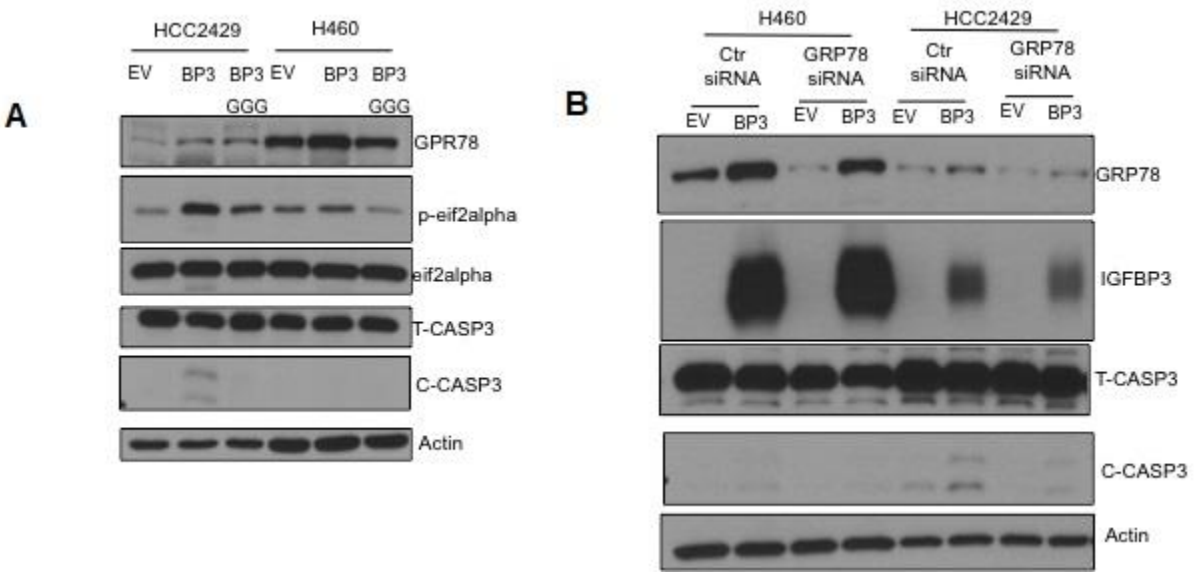


Figure 9: IGFBP-3-overexpression induces ER stress, and IGFBP-3-induced apoptosis is dependent on GRP78. A) HCC2429 and H460 cells were infected with Ad-IGFBP-3 (BP3), Ad-IGFBP-3 mutant (BP3 GGG) and Ad-EV (EV). The ER stress markers GRP78 and phospho-eIf2a are induced following Ad-IGFBP-3 infection. B) To determine whether GRP78 is responsible for IGFBP-3 induced apoptosis, HCC2429 and H460 cells were transfected with GRP78 siRNA or a control (Ctr) siRNA. 24 hrs after transfection, cells were infected with either Ad-IGFBP-3 or Ad-EV. Cells were collected 72 hrs later. GRP78 knockdown attenuated IGFBP-3 induced apoptosis in HCC2429 cells. These results suggest that IGFBP-3-overexpression induces ER stress, and IGFBP-3-induced apoptosis is dependent on GRP78.

Determine the role of IGFBP-3 in carcinogenesis and in the therapeutic response of lung cancer

Our data from lung cancer cell lines suggested that lung cancer cells express significantly lower levels of IGFBP3 when they develop resistance to cisplatin and IR. Recombinant IGFBP3 or re-expression of IGFBP3 reverses the resistant phenotype and reprograms resistant cancer cells for apoptotic cell death. We hypothesized that loss of IGFBP3 accelerates progression of lung cancer and confers therapeutic resistance. To test this hypothesis *in vivo*, we crossed the k-ras-driven (Kras-G12D) transgenic lung cancer model (Stock No.008185, Jackson Laboratory) with IGFBP3 knockout mice (provided to us by Lexicon Pharmaceuticals) to determine whether loss of IGFBP3 changes the tumor behavior and its therapeutic response to both cisplatin and ionizing radiation.

We generated Kras-G12D driven lung tumors in IGFBP-3 knockout mice by mating the k-ras G12D mutant transgenic mice with IGFBP-3 knockout mice (viable and without phenotypes) (Figure 10A). Genotyping identified Kras-G12D&IGFBP-3$^{-/-}$ offspring (Figure 10B). We assessed lung tumor progression by imaging, including micro-CT scans or examinations of tumor tissues.

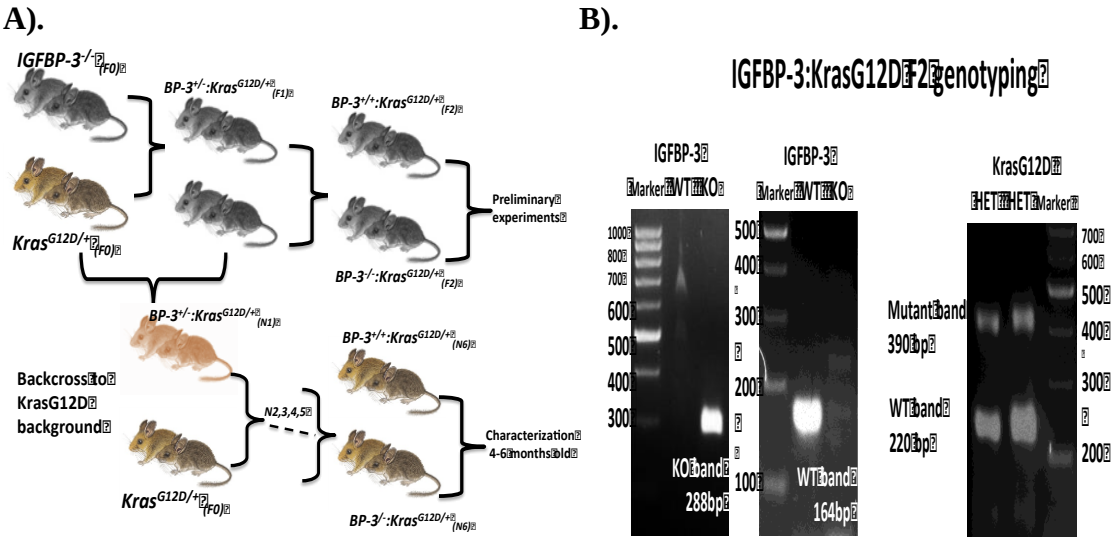


Figure 10. Generation and identification of the IGFBP3$^{-/-}$ KrasG12D$^{+/-}$ mouse lung cancer model: A) Mouse breeding strategy. B) Mouse genotyping of IGFBP3 and K-Ras G12D in F2 mice of various genotypes.

IGFBP-3 Knockout accelerates KrasG12D-induced lung cancer growth

To determine lung tumor burden of IGFBP3$^{+/+}$Kras$^{G12D+/-}$ vs. IGFBP3$^{-/-}$Kras$^{G12D+/-}$ mice, mice of both genotype were sacrificed when they reached 4-6 months of age. HE staining confirmed that all lesions are adenocarcinoma and that the incidences are similar (Figure 11A). We measured number and size of lung tumors through a variety of approaches. Lung tumor nodules were dissected from the tumor-bearing lungs. The tumor nodules less than 3mm vs. greater than 3mm were counted and graphed. IGFBP3$^{-/-}$Kras$^{G12D+/-}$ mice have more and larger tumor nodules (Figure 11B). We measured the weight of tumor-bearing lungs, as well as the weight of dissected tumors, from mice of the two genotypes. IGFBP3$^{-/-}$Kras$^{G12D+/-}$ mice bear heavier tumor load (Figure 11C and 11D).

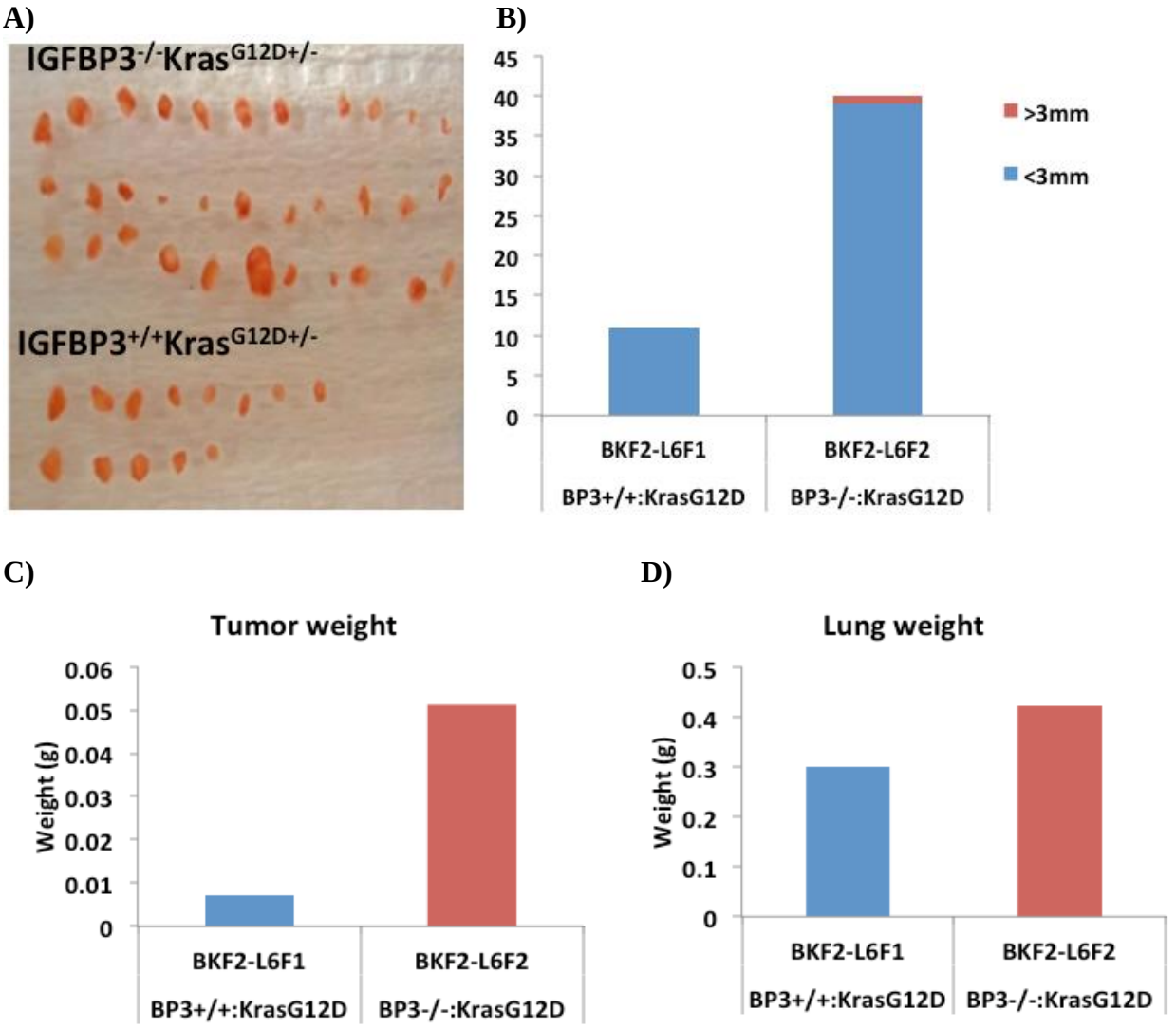


Figure 11. Assessment of tumor volumes in a IGFBP3 deficient KrasG12D mouse lung cancer model: A) Tumor nodules dissected from tumor-bearing lungs. B) Number of all dissected tumors (<3mm vs. >3mm). C) Weight of dissected tumors and D) weight of tumor-bearing lungs from IGFBP3$^{+/+}$Kras$^{G12D/+/-}$ or IGFBP3$^{-/-}$ Kras$^{G12D/+}$ mice. IGFBP3$^{-/-}$Kras$^{G12D+/-}$ mice bear heavier tumor load.

Increased radiographic tumor volumes in IGFBP3$^{-/-}$ KrasG12D$^{+/-}$ mice

To determine the tumor burden in live mice, we performed micro-CT scans on these mice, contoured the tumor nodules and calculated tumor volumes. There was a 5-fold reduction of total tumor volume in IGFBP3$^{+/+}$ Kras$^{G12D/+}$mice compared with IGFBP3$^{-/-}$ KrasG12D$^{+/-}$ mice (Figure 12).

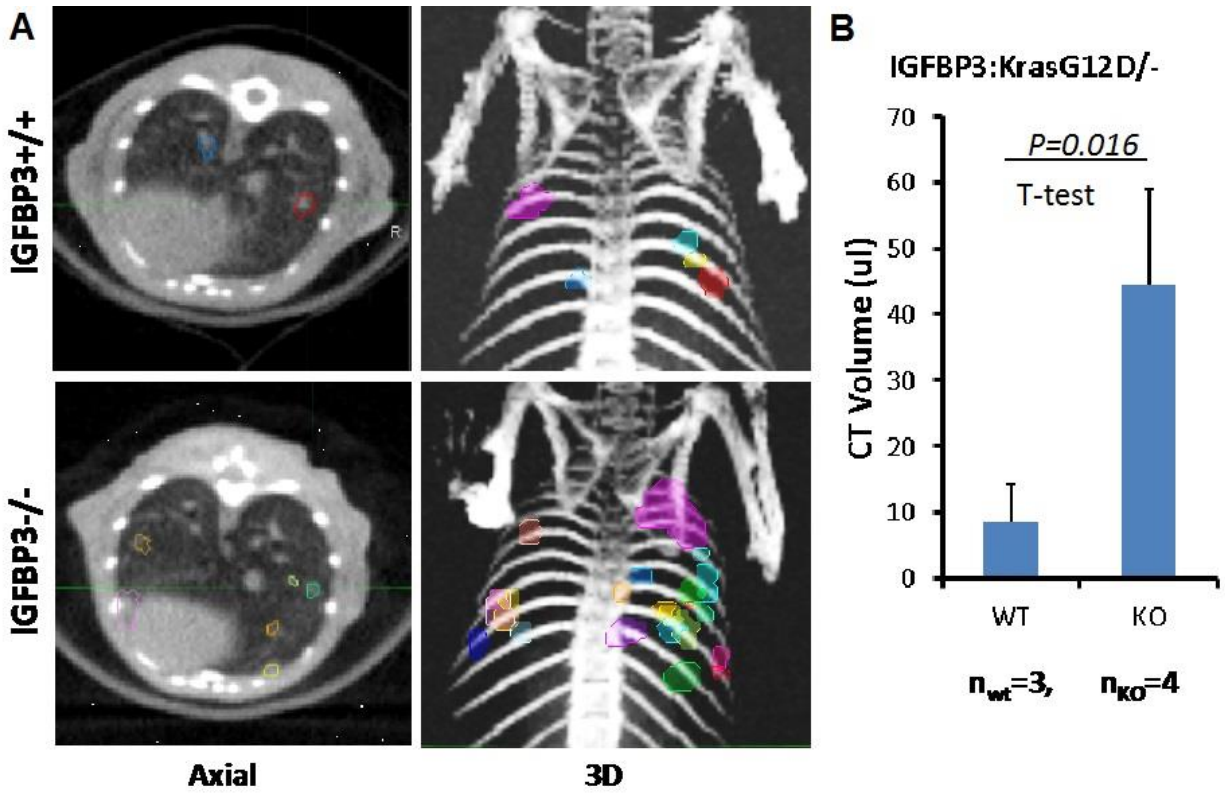


Figure 12. Increased radiographic tumor volumes in the IGFBP3 deficient/ KrasG12D mice. A) Representative micro-CT image. Left: Axial CT images; Right: color-rendered 3D representations of tumor volumes from sagittal and coronal planes. B) Tumor volumes are significantly decreased in IGFBP3$^{+/+}$ Kras$^{G12D/+}$ (WT) mice (n=3) compared with IGFBP3$^{-/-}$ Kras$^{G12D/+/-}$ (KO) mice (n=4).

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___x__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___x__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___x__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes_____x____ No_____

If yes, please describe your plans:

We have a manuscript under preparation for the journal of Cancer Research.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

We have demonstrated both in human lung cancer cell lines and in genetically engineered models of lung cancer that IGFBP3 is an essential modulator of lung cancer growth and its therapeutic resistance.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No____x_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No__x_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors. Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Lu, Bo M.D., Ph.D.	POSITION TITLE Professor
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Shanghai Medical University	M.D.	1981-1987	Medicine
Baylor College of Medicine	Ph.D.	1988-1993	Cell and Mol Biology
Univ. of Pittsburgh School of Medicine	Internship	1996-1997	
Univ. of Southern California School of Med.	Residency	1997-2002	Radiation Oncology

A. Positions and Honors

Positions and Employment

Faculty Appointments:

7/1/02-2/19/08 Assistant Professor, Department of Radiation Oncology, Vanderbilt University School of Medicine, Nashville, TN

4/1/07-2/19/08 Assistant Professor, Department of Cancer Biology, Vanderbilt University, Nashville, TN

2/20/08-1/31/11 Ingram Associate Professor of Cancer Research, Departments of Radiation Oncology and Cancer Biology, Vanderbilt University School of Medicine, Nashville, TN

2/1/11-present Professor (Tenured), Department of Radiation Oncology, Thomas Jefferson University, Philadelphia, PA

B. Selected Publications:

1. Carolyn Cao, Jeffrey Albert, Ling Geng, Alan Sandler, Percy S. Ivy, David H. Johnson, and **Bo Lu**. Vascular Endothelial Growth Factor Tyrosine Kinase Inhibitor AZD2171 and Fractionated Radiotherapy in Mouse Models of Lung Cancer. *Cancer Res 2006 66: 11409-11415.*

2. Kwang Woon Kim, Robert W. Mutter, Carolyn Cao, Jeffrey M. Albert, Michael Freeman, Dennis E. Hallahan, and **Bo Lu.** Autophagy for cancer therapy through inhibition of proapoptotic proteins and mTOR signaling. *J Biol Chem. 2006 Dec 1; 281(48):36883-90.*

3. Jeffrey Albert, Carolyn Cao, Alan Sandler, David Johnson, Jennifer Low, Mace Rothenberg and **Bo Lu**. Inhibition of poly(ADP-ribose) polymerase enhances cell death and improves tumor growth delay in irradiated lung cancer models. *Clin Cancer Res 2007 13: 3033-3042.*

4. Kwang Woon Kim, Misun Hwang, Luigi Moretti, Jerry J. Jaboin, Yong I. Cha and **Bo Lu** Autophagy upregulation by inhibitors of caspase-3 and mTOR enhances radiotherapy in a mouse model of lung cancer. *Autophagy* 2008 Jul 1;4(5):659-68.
5. Kwang Woon Kim*, Luigi Moretti*, **Bo Lu.** Essential role of caspase3/7 in regulating ER stress-mediated autophagy through the PERK pathway following radiation. *Plos One* 2008 May 28;3(5):e2275.
6. Kim KW, Moretti L, Mitchell LR, Jung DK, **Lu B**. Combined Bcl-2/mammalian target of rapamycin inhibition leads to enhanced radiosensitization via induction of apoptosis and autophagy in non-small cell lung tumor xenograft model. *Clin Cancer Res*. 2009 Oct 1;15(19):6096-105.
7. Luigi Moretti, David Yu, and **Bo Lu.** Prognostic Factors for Resected Non-Small Cell Lung Cancer with pN2 Status: Implications for Postoperative Radiotherapy. *Oncologist*, 2009 Nov, 14(11):1106-15.
8. Schleicher S, Moretti L and **Lu B**. Progress in the unraveling of the endoplasmic reticulum stress/autophagy pathway and cancer: implications for future therapeutic approaches. *Drug Resist Updat*, 2010 Jun;13(3):79-86.
9. Kwang Woon Kim, Luigi Moretti, Lauren Mitchell and **Bo Lu.** Endoplasmic Reticulum Stress Mediates Radiation-Induced Autophagy via PERK-eIF2α in Caspase-3/7 Deficient Cells. *Oncogene*, 2010 Jun 3;29(22):3241-51.
10. Yunguang Sun, Nicolas Giacalone, and **Bo Lu**. Inhibition of JAK2 Signaling by TG101209 Enhances Radiotherapy in Lung Cancer Models. *J Thorac Oncol. 2011 Apr;6(4):699-706*.
11. Bo Li, Luigi Moretti and **Bo Lu**. A Novel Bioluminescent Orthotopic Mouse Model for Lung Cancer. *Radiat Res. 2011 Jun 10. [Epub ahead of print]*.
12. Yunguang Sun, Nicolas Giacalone, and **Bo Lu**. Terameprocol (tetra-O-methyl nordihydroguaiaretic acid), an inhibitor of Sp1-mediated survivin transcription, induces radiosensitization in non-small cell lung carcinoma. *J Thorac Oncol*, 2011 Jan;6(1):8-14.
13. Li W, Li B, Giacalone NJ, Torossian A, Sun Y, Niu K, Lin-Tsai O, **Lu B**. BV6, an IAP Antagonist, Activates Apoptosis and Enhances Radiosensitization of Non-small Cell Lung Carcinoma In Vitro. J Thorac Oncol. 2011 Nov;6(11):1801-9.
14. Yunguang Sun, Siyuan Zheng, Christina Speirs, Zhongming Zhao and **Bo Lu.** Gene profiling of lung cancer stem-like cells reveals IGF pathway as a promising target for therapeutic resistance. *Int J Radiat Oncol Biol Phys*, 2012 Mar 1;82(3):e563-72.
15. Sun Y, Nowak KA, Zaorsky NG, Winchester CL, Dalal K, Giacalone NJ, Liu N, Werner-Wasik M, Wasik MA, Dicker AP, **Lu B** <u>ALK inhibitor PF02341066 (crizotinib) increases sensitivity to radiation in non-small cell lung cancer expressing EML4-ALK.</u> Mol Cancer Ther. 2013 May;12(5):696-704.