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TITLE: Novel Epigenetic Reprogramming to Inhibit or Reverse EMT in Lung Cancer

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Fort Detrick, Maryland 21702-5012

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14. ABSTRACT
Carcinoma of the lung is the leading cause of cancer deaths in the US. The malignant properties of lung cancer, including invasion, metastasis and drug resistance, are driven in part by the epithelial- mesenchymal transition (EMT), which makes this disease very difficult to treat. We hypothesized that TGFβ links NRP2-dependent signaling to upregulation of EZH2, an epigenetic modifier, and that EMT results from gene expression changes via a pathway of NRP2, ERK, ZEB1 and EZH2. We proposed to test the validity of this linkage, particularly the connection between NRP2, ZEB1 and EZH2, and to evaluate lung cancer susceptibilities to EZH2 inhibitors in combination with other epigenetic modifiers.

15. SUBJECT TERMS
None listed

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Carcinoma of the lung is the leading cause of cancer deaths in the US. The malignant properties of lung cancer, including invasion, metastasis and drug resistance, are driven in part by the epithelial-mesenchymal transition (EMT), which makes this disease very difficult to treat. We hypothesized that TGFβ links NRP2-dependent signaling to upregulation of EZH2, an epigenetic modifier, and that EMT results from gene expression changes via a pathway of NRP2, ERK, ZEB1 and EZH2. We proposed to test the validity of this linkage, particularly the connection between NRP2, ZEB1 and EZH2, and to evaluate lung cancer susceptibilities to EZH2 inhibitors in combination with other epigenetic modifiers.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

TGFβ, NRP2, EZH2, PRC2, Histone-H3K27, ZEB1, ERK, epithelial-mesenchymal transition, lung cancer, SCLC, NSCLC

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Goal 1: Test hypothesis that a TGFβ-NRP2-ERK-ZEB1-EZH2-EMT signaling axis exists.
Task 1: Generate TGFβ-exposed cells for chromatin crosslinking; immunoprecipitate ac- or me-H3K27 cross-linked chromatin; perform qPCR for selected promoters; express ZEB1 alone or +/- EGF +/- U0126 & analyze for H3K27-me3 changes by ChIP;
Task 2: Perform RNA sequencing to identify TGFβ modulated genes affected by NRP2 inhibition; create CRISPR knockouts of NRP2 in NSCLC lines; expose lines to TGFβ, isolate RNA, perform RNAseq; conduct Bioinformatics analysis of data

Major Goal 2: Test single & combined epigenetic inhibitors for ability to block EMT.
Task 3: Measure EMT-assoc. gene expression (migration & invasion) in TGFβ-exposed cell line after treatment with epigenetic inhibitor. Confirm specific effects with knockout of targeted epigenetic regulators; Test reversibility of EMT-associated drug resistance using epigenetic inhibitors in HCC4006-ER cells; test selected epigenetic knockdowns in xenografts for anti-tumor and anti-metastatic efficacy. Obtain local IRB/IACUC and HRPO/ACURO approval

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met.

Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

This study generated extensive results that are beyond the space limitation of this form. Please see the complete report, consisting of 9 pages of data and descriptive text, submitted as Appendix 1.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Our results have not yet been published, therefore Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Nothing to Report. Since the project is over, there will not be another reporting period. However, the exciting results obtained will be used in two ways. 1) as the preliminary data for a grant proposal to pursue the findings in more detail and mechanistically; 2) as the core of a manuscript describing the inter-relationship between TGFβ, NRP2 and EZH2.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our study is significant in two ways. 1) The data unequivocally demonstrate the importance of the NRP2 receptor system for responses of lung cancer cells to TGFβ. Fully 45% of TGFβ responsive genes were influenced by knockdown of NRP2a, NRP2b or both. 2) Over 10% of TGFβ responsive genes were dependent upon EZH2, and nearly all of these were modulated by genetic manipulation of NRP2 isoforms. Given that both TGFβ and NRP2b are crucial to metastasis, the understanding that a major effect of NRP2 is on the TGFβ transcriptome, and that this requires EZH2, are major findings. The potential impact of this study on our understanding of metastasis in lung tumors is therefore quite high.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Published evidence has linked NRP2 expression to multiple cancers, including melanoma, breast cancer and colorectal cancer. We speculate that the regulatory relationships uncovered in this Concept Award may be equally applicable to these other diseases. In addition, TGFβ is implicated in other disease types, such as fibrosis, and the NRP2 isoforms and EZH2 may have important roles in these contexts as well.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

We have discussed the issues faced by this project within the report in Appendix 1. Briefly, we found that EZH2 inhibition had (at best) minor effects on cell biological parameters and gene expression, when examined *via* individual genes by qRT-PCR. We therefore focused effort on Goal #1, Approach 2, RNA sequence analysis of TGFβ treated cells bearing NRP2 isoform-specific knockdowns. This was always part of our project so it is not a change in that sense, but rather a shift in emphasis. By performing the RNA sequencing on cells pretreated or not with an EZH2 inhibitor, we were able to combine the most important aspects of Goals 1 and 2 into one global analysis with a highly significant outcome.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Problems are discussed in the Appendix 1 report. Briefly, these were identified and dealt with as noted above. As the Project has been completed, all future plans will depend on additional funding.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

An animal experiment was included at the end of Goal 2, time permitting. We applied for and received approval for this work from both the local IACUC as well as the ACURO office. However, the experimentation did not progress to this point and the xenograft experiment was not performed.

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  1. Dimou A, Dincman, T., Evanno, E., Gemmill, R.M., Roche, J., Drabkin, H.A. : Epigenetics during EMT in lung cancer: EZH2 as a potential therapeutic target. Cancer Treatment and Research Communication, Accepted, (2017). YES
  2. Roche J, Gemmill RM, Drabkin HA: Epigenetic Regulation of the Epithelial to Mesenchymal Transition in Lung Cancer. Cancers 9, (2017). 5532608. YES
  3. Noman MZ, Van moer K, Marani V, et al: CD47 is a direct target of SNAI1 and ZEB1 and its blockade activates the phagocytosis of breast cancer cells undergoing EMT. OncoImmunology:00-00, (2017). YES

  dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each

one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

The RNA seq dataset will be deposited in an appropriate publically available database , such as the Gene Expression Omnibus (GEO) maintained by the NCBI. Deposition will occur concurrently with publication.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name: *Mary Smith*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: Harry A. Drabkin
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 0.6 calendar months
Contribution to Project: PI of this project, all aspects
Funding Support: 1 I01 BX003333-01A2

Name: Robert M. Gemmill
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): 0000-0003-0747-5984
Nearest person month worked: 1.2 calendar months
Contribution to Project: Co-PI of this project, oversight of all experimental work
Funding Support: 1 I01 BX003333-01A2

Name: Patrick Nasarre
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 2.4 calendar months
Contribution to Project: Performed growth assays and RNA sequencing analysis
Funding Support: 1 I01 BX003333-01A2

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

The following changes have taken place:

1. Dr. Drabkin has retired from MUSC.
2. Due to Dr. Drabkin's retirement, the VA Merit award to him was terminated. Thus all funding for our group has ended.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Appendix 1 contains data and a detailed text description of the project's results.

APPENDIX 1

Detailed Description of Results and Interpretations

Introduction:

In previous studies, we found that NRP2 was upregulated during TGFβ-driven EMT in lung cancer cells and that NRP2 knockdown substantially inhibited invasive tumor growth and EMT features. Moreover, we found that an uninvestigated isoform, NRP2b, was predominantly involved in this process. The EMT process includes suppression of epithelial genes; moreover, epigenetic changes mediated by Polycomb Repressor Complex 2 (PRC2) have been implicated in this process. Thus, we **hypothesized** that NRP2 acts upstream of EZH2, the catalytic subunit of the PRC2 complex, and that NRP2b knockdown would impair TGFβ-driven EMT in a manner that was dependent on EZH2.

Like other malignant diseases, lung cancer encompasses a spectrum of pathologic mechanisms and drivers, such that only a subset would be expected to respond to TGFβ in the manner described above. Furthermore, TGFβ is involved in numerous processes including growth regulation, inflammation, wound repair, fibrosis, immune regulation, migration and invasion. Similarly, only a subset of these responses would probably be regulated by NRP2.

The Specific Aims in this Concept Award were crafted, first to test the hypothesis that a signaling axis existed from TGFβ to EZH2 and EMT that involved NRP2, and second to test whether EZH2 inhibitors, alone and in combination, could alter cell biological parameters including the EMT. Two approaches were proposed for achieving the first Goal; 1) perform chromatin immunoprecipitation (ChIP) and measure changes in histone H3K27 methylation status in specific genes in response to manipulations of TGFβ, NRP2 and ZEB1; 2) perform RNA sequencing analysis on lung cancer cell lines to define genes sensitive to TGFβ in a NRP2 and EZH2-dependent manner. Initial results from Goal 2 (described first below), showed relatively minor effects of EZH2 inhibition on a series of 11 lung cancer cell lines. We subsequently examined changes in gene expression in these lines using real-time RT-PCR with modest changes attributable to EZH2. These initial observations convinced us that the second approach for Goal 1, global analysis of genes influenced by TGFβ, EZH2 and NRP2, was required before any chromatin immunoprecipitation experiments or combination therapies should be undertaken. We realized that the fundamental hypothesis of this project needed to be tested directly and globally, and that the second approach for Goal 1 was the most productive way to do this. We subsequently focused our effort on Task 2, RNAseq analysis of three lung cancer cell lines genetically manipulated to differentially express NRP2 isoforms. These cells were analyzed following treatment with TGFβ, +/- pre-treatment with an EZH2 inhibitor. This approach thus combined aspects of both Goals 1 and 2 and was a spectacular success. The results have provided a foundation for new proposals as well as a logical pathway to complete the goals originally set forth in this Concept Award.

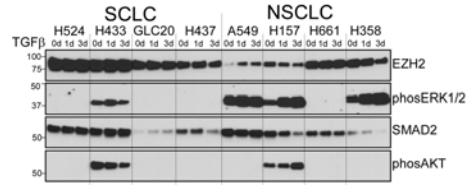

Fig. 1. Western blot survey for EZH2 protein expression in 8 lung cancer cell lines.

Major Activities:

Responsiveness of Lung Cancer cell lines to inhibition of EZH2. To begin an analysis of TGFβ, NRP2 and EZH2 interactions, we first verified that EZH2 was expressed at the protein level in our lung cancer cell lines (**Fig. 1**). Although variable, EZH2 was highly expressed in most lines. Five NSCLC and six SCLC lung cancer cell lines were then treated with TGFβ, using control and

specific NRP2 isoform shRNA knockdowns in some, as part of Goal 2. Treated cells were interrogated for changes in EMT markers, EZH2 and SOX4, a reported upstream regulator of EZH2, using quantitative RT-PCR. Because some tumors are dependent on EZH2, we also treated cell lines with GSK126, a small molecule inhibitor of EZH2, and monitored growth and viability. Of interest, the SCLC cell lines (H433, H524, GLC20, H740, H437, H345) were strikingly non-responsive to TGFβ stimulation. This lack of response included the classical TGFβ-responsive gene, PAI1, as well as EMT markers (E-cadherin, N-cadherin), NRP1, the NRP2 a/b isoforms, SOX4 and EZH2. As reported by others and verified in **Fig. 1**, SCLCs express high levels of EZH2. However, GSK126 had only a modest effect on growth with substantial inhibition observed only in two lines (GLC20, H524) and at high (20 μM) concentration (**Fig 2**). These studies were performed using six different initial cell densities (1000-6000 cells/well) and four different drug concentrations (2-20 μM). Of note, most EZH2 inhibitors, including GSK126, have considerably less effect on the related enzyme, EZH1, which very recently has been shown to be a critical target in VHL mutated kidney cancer. To our knowledge, EZH1 has not been examined in SCLC. The fact that high concentrations of GSK126 are required to inhibit the growth of SCLC cell lines suggests that EZH1 might be a relevant target in this disease. The SCLC cell lines were not further addressed in this Concept Award, but will be pursued in other studies.

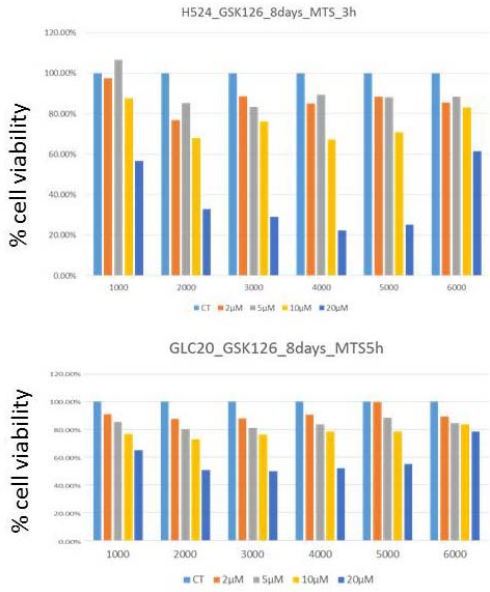

Fig. 2. Inhibition of SCLC cell lines H524 & GLC20 by EZH2 inhibitor GSK126.

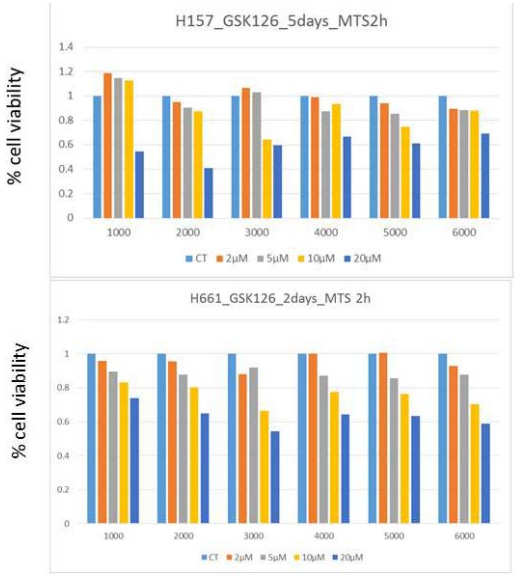

Fig. 3. Inhibition of NSCLC cell lines H157 & H661 by EZH2 inhibitor GSK126.

When 6 NSCLC cell lines were tested by viability assays for sensitivity to GSK126, we found that NSCLC did not differ substantially from the SCLCs. There was some evidence of increasing growth inhibition at higher doses, and the depth of effect was sometimes higher. **Fig. 3** shows two examples, H157 and H661. We had proposed to test different combinations of inhibitors, but we spent more time than anticipated with single agent GSK126 treatments at various doses, durations and cell densities to obtain both reproducible data and in an attempt not to miss a particular set of conditions that would be more effective.

Changes in gene expression in response to TGFβ and EZH2 inhibition are sensitive to NRP2 status. At the transcriptional level, the NSCLC cell lines were uniformly responsive to TGFβ, at least involving the upregulation of PAI1 (SERPINE1), a classic TGFβ response gene. This analysis was carried out with quantitative real-time PCR (qRT-PCR). **Fig. 4** shows the response of PAI1 to TGFβ+/-EPZ011989 in H1975 cells. There are two notable effects illustrated in **Fig. 4**. First, the relative induction of PAI1 was influenced by knockdown of the NRP2 isoforms, with shNRP2b increasing the induction while shNRP2a blunted induction. Second, in all cases, the EZH2 inhibitor, EPZ011989, reduced the induction mediated by TGFβ. However, the effects of EPZ appeared to be independent of NRP2 isoform-specific knockdowns. These results support the notion that NRP2 isoforms and EZH2 activity are important for normal TGFβ responses but did not show a clear relationship between NRP2 and EZH2.

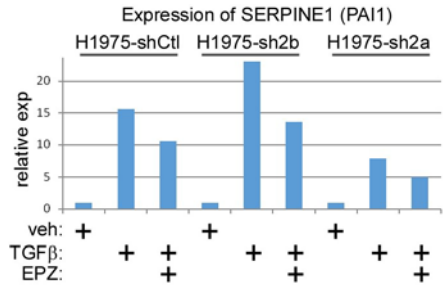

Fig. 4. PAI1 (SERPINE1) induction in H1975 by TGFβ

We then asked whether altered gene expression could be observed for other genes, again using qRT-PCR to measure expression changes. Although H460 was an exception, most NSCLC lines (H358, H661, H1975, H157, A549) responded to TGFβ with upregulation of SOX4, a transcription factor important for EMT and EZH2. However, at the mRNA level, EZH2 was unaffected. Among the Neuropilins, NRP2 (especially NRP2b) was often upregulated (in 3/5 lines), whereas NRP1 showed little change. **Fig. 5** shows the relative expression of 5 genes in H1975 cells in response to TGFβ. Interestingly, the NRP2 genotype appears to influence the TGFβ-mediated induction of SOX4, particularly its response to the EZH2 inhibitor, EPZ011989. Thus, in control cells, EPZ blunted the induction of SOX4, while this effect was lost when NRP2b was knocked down and completely reversed when NRP2a was knocked down. The repression of E-cadherin expression was also affected by these combinations, with EPZ enhancing suppression in shControl cells but not when NRP2a was knocked down. In contrast, NRP2b knockdown led to stronger inhibition of E-cadherin by TGFβ alone. These findings supported our overall hypothesis that TGFβ responses leading to EMT (E-cadherin loss, SOX4 gain) were linked to both NRP2 isoform expression and to EZH2 activity. At the same time, it became clear that a strategy based upon candidate genes was very limited in its ability to illuminate this relationship further.

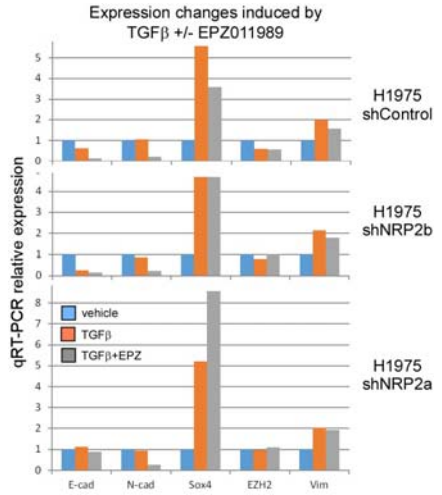

Fig. 5. Relative expression of 5 genes in H1975 shControl, shNRP2b or shNRP2a cells in response to TGFβ+/-EPZ011989.

RNA sequencing analysis supports the TGFβ-NRP2-EZH2-EMT axis. During the course of these experiments, we reasoned that it would be possible to explore our overall hypothesis on a global level and in a robust manner that directly addressed the possible relationship between TGFβ, the NRP2 isoforms, EZH2 and its target genes. By pretreating control or NRP2 isoform

knockdown cells with the EZH2 inhibitor, EPZ011989, followed by subsequent exposure to TGFβ, we could interrogate RNAseq data for genes that were responsive to EZH2 inhibition in a NRP2-dependent manner. This strategy combined aspects of Goals 1 and 2, and provided a means to test the central hypothesis of our project. The results are presented below; not only do they support our central hypothesis, they also support NRP2, and especially NRP2b, as a therapeutic target in lung cancer and thus warrant further exploration.

To directly determine if there was a regulatory relationship between NRP2 and EZH2/PRC2, we turned to the global RNA sequencing approach encompassed in Goal 1, Task 2. Importantly, the use of the specific EZH2 inhibitor, EPZ011989, allowed us to identify genes which were selectively affected by EZH2/PRC2 activity in a manner that was dependent upon NRP2 isoforms and in the context of TGFβ exposure. The results also revealed a substantially larger role for NRP2b in the TGFβ-induced transcriptome than we had anticipated.

RNA sequencing. The experimental design utilized A549 cells bearing shControl, shNRP2a and shNRP2b knockdown constructs, each cultured under three conditions; 1) vehicle, 2) TGFβ (5ng/ml, 24h), 3) or pre-treated for 7 days with EPZ011989 (1 μM; EPZ henceforth) followed by 24h TGFβ (5ng/ml). The long EPZ pre-treatment was included to allow time for methylation status of histone H3-K27 residues to change and become established as a result of blocking EZH2 activity. The efficacy of the EPZ treatment was revealed by its ability to alter the EMT morphology of A549 cells exposed to TGFβ for 24h. As shown in **Fig. 6**, EPZ pre-treatment partially blocked the spindle shape induced by TGFβ, an effect that was most readily apparent in the shNRP2b cells (middle panels, compare left to right). Following treatments, A549 cell RNA was isolated from two independent biological replicates representing all 9 culture conditions. RNA was purified using the RNeasy Mini Plus kit according to manufacturer's directions.

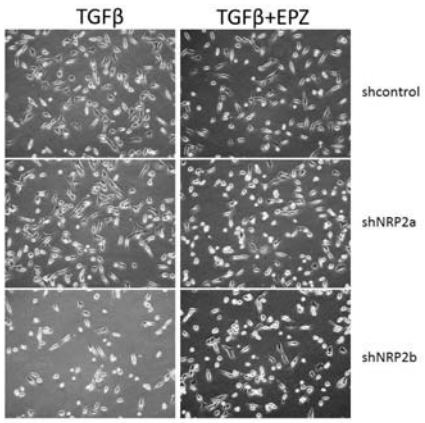

Fig. 6. EPZ pre-treatment of A549 cells partially blocks the spindle shape characteristic of the EMT, especially in shNRP2a cells.

RNAseq pipeline: RNAseq utilized the Illumina HiSeq 2500 at the MUSC Genomics Core. Polyadenylated (poly A^+) RNA was enriched from total RNA, reverse-transcribed and converted to a strand-specific sequencing library. Each sample was then sequenced to a depth of 50 million reads. Adapters were trimmed by CutAdapt (http://journal.embnet.org/index.php/embnetjournal/article/view/200) or Trimmomatic. Fold-change estimation and hypothesis testing for differential expression was then performed using DESeq2. Gene ontology and pathway analyses were performed using Advaita as well as ToppGene web-based tools.

Using a 2-fold cutoff, we found 958 genes were induced while 665 were repressed by TGFβ in control cells. EZH2 inhibition resulted in an additional 152 genes being induced and 211 genes being suppressed. However, of the genes originally induced or suppressed by TGFβ, 194 required EZH2 activity for this response (110 upregulated genes were lost; 84 down regulated genes were lost). An additional 77 genes required EZH2 to moderate the induction or enhance the suppression mediated by TGFβ (by 2-fold or more) – that is, induction was at least 2-fold higher when EZH2 was inhibited or suppression was 2-fold greater when EZH2 was inhibited.

These observations are consistent with EZH2/PRC2 being an important factor in the TGFβ response, as expected.

In cells knocked down for NRP2 isoforms, intriguingly altered expression patterns were noted by simple inspection with many examples of an apparent complex regulatory interplay between NRP2 isoforms and EZH2 with specific effects on specific genes. For example, the gene **SH3TC2** was not affected by TGFβ in control cells, but was *suppressed* 9-fold by EPZ, suggesting that EZH2 activity is necessary to retain baseline expression. However, knocking down either NRP2 isoform rendered this gene sensitive to TGFβ repression, with no evidence of additive effects with EPZ. Initial examination of the dataset suggested that many genes responded to the experimental manipulations in a similar manner. However, it was also clear that several additional distinct patterns of response were present in the data. We wanted to systematically identify genes with altered responses to the EZH2 inhibitor, and to classify these into similar groups, reasoning that these would provide mechanistic insights into the roles of NRP2 isoforms and EZH2-dependent changes in gene expression.

To this end, we calculated the expression difference (Δ^{exp}) observed for each gene between vehicle and EPZ011989-treated cultures of the three genotypes, A549-shControl, -shNRP2a and -shNRP2b, all exposed to TGFβ for 24h. For genes which responded to EPZ pre-treatment similarly, regardless of the underlying NRP2 knockdown status, the Δ^{exp} values were very similar. However, for genes like **SH3TC2**, whose responses were obviously distinct in NRP2 knockdown cells, these values were substantially different. By calculating the standard deviation of the three Δ^{exp} values for each gene, and sorting for S.D. values, we prepared a list of loci most differentially influenced by EPZ in a NRP2-dependent manner. Of the first 200 genes on this list, over 20% showed a pattern of response similar to SH3TC2. The second most common pattern, also seen in 20%, consisted of genes *induced* by EPZ, but not in cells knocked down for either NRP2 isoform, suggesting that NRP2a and "b" were required for EZH2/PRC2 to repress. Collectively, there were 23 distinct patterns of altered gene expression identified among the 812 genes most sensitive to the EPZ-NRP2 combination. Below we provide a brief synopsis of the six most relevant patterns along with an exemplar and a hypothetical model for each.

1) The most prevalent pattern 1 is exemplified by **SH3TC2**, whose responses to EPZ are represented graphically in **Fig. 7A**. Genes with this pattern appear to require EZH2 for normal

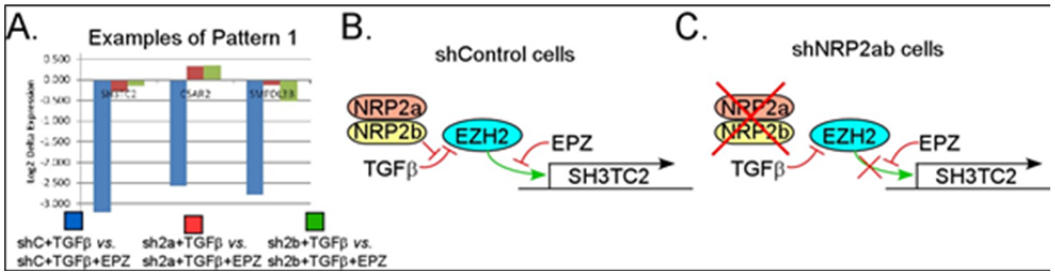

Fig. 7. Most abundant pattern of gene expression changes with shNRP2 & EZH2i

expression and are repressed by EZH2 inhibition (**Fig. 7B**). However, response to EZH2i is lost if either NRP2a or NRP2b is knocked down, and the gene becomes sensitized to TGFβ-induced repression (**Fig. 7C**). This is consistent with a model in which TGFβ signaling would inhibit the action of EZH2, but is prevented in the presence of NRP2. Thus when NRP2a or NRP2b are knocked down, the latent repression mediated by TGFβ is uncovered, reducing SH3TC2

expression. Approximately 22% of genes showed a similar pattern, and two of these (C5AR2, SMPDL3B) are included in **Fig. 7A** for comparison.

2) The second commonest pattern is exemplified by **FCRL4**, whose responses to EPZ are represented graphically in **Fig. 8A**. Genes with this pattern are inhibited by EZH2, and thus induced by EZH2 inhibition (**Fig. 8B**). However, this response to EZH2i is missing if either NRP2a or NRP2b is knocked down (**Fig. 8C**). Most of these genes are not responsive to TGFβ. These findings are consistent with a model in which NRP2a and NRP2b somehow maintain the capability of EZH2 to inhibit gene expression, while TGFβ signaling appears uninvolved. When either NRP2a or NRP2b is knocked down, EZH2 loses its repressive capability, rendering EPZ impotent with no effect on expression. 20% of genes showed a similar pattern, and two of these (CLEC5A, LOC101448202) are included in **Fig. 8A** for comparison.

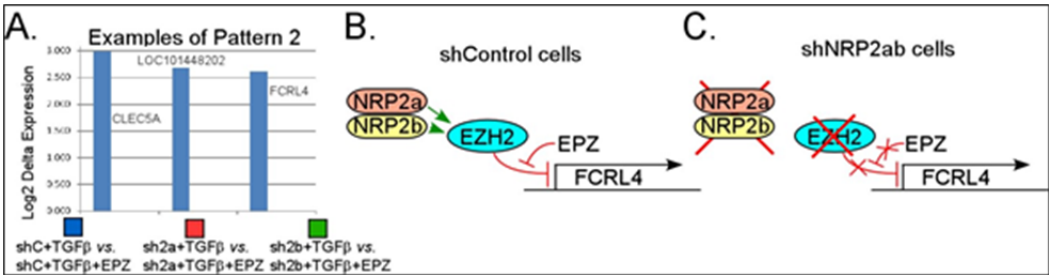

Fig. 8. Second pattern of gene expression changes with shNRP2 & EZH2i

3) The third pattern is exemplified by **FABP4**, whose responses to EPZ are represented graphically in **Fig. 9A**. Genes with this pattern are unaffected by EZH2 inhibition or by TGFβ in control cells (**Fig. 9A, B**). However, when cells are knocked down for either NRP2a or NRP2b, EZH2 comes into play. These genes become sensitized to EPZ and are induced by the EZH2 inhibitor (**Fig. 9C**). These findings are consistent with a model in which NRP2a and NRP2b prevent EZH2 from affecting certain genes (**Fig. 9B**). However, in the absence of either NRP2a or NRP2b, regulation by EZH2 is now imposed to inhibit gene expression. Thus in the absence of NRP2, these genes are up-regulated in response to the EZH2 inhibitor. TGFβ signaling appears to not be involved. 16% of genes showed a similar pattern, and two of these (DCN, COL3A1) are included in **Fig. 9A** for comparison.

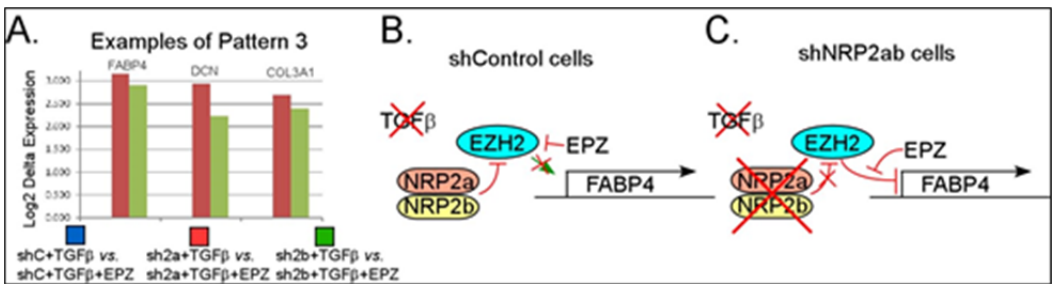

Fig. 9. Third pattern of gene expression changes with shNRP2 & EZH2i

4) The fourth most abundant pattern is exemplified by **LCE3D**, whose responses to EPZ are represented graphically in **Fig. 10A**. Genes displaying this response pattern may be induced or not by TGFβ but are only weakly affected (at most) by EZH2 inhibition in control cells (**Fig. 10A**). However, in cells knocked down for NRP2a specifically, EZH2i strongly induces expression. This does not happen when NRP2b is knocked down, indicating that this group

shows highly differential responses depending on which isoform is present. These findings are consistent with a model in which EZH2 has at most a minor role in regulating expression in control cells (**Fig. 10B**). However, EZH2 comes into play when NRP2a is knocked down (**Fig. 10C**) but not in cells knocked down for NRP2b (**Fig. 10D**).

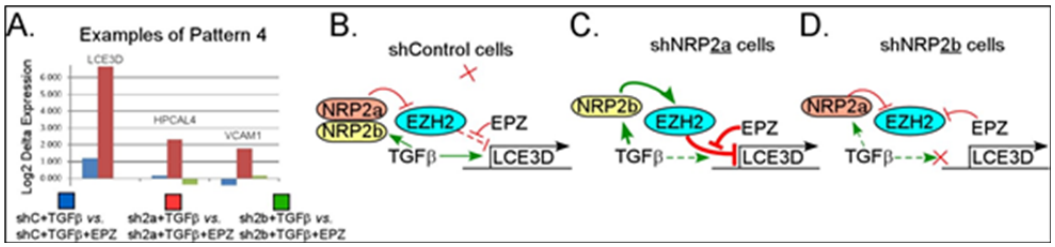

Fig. 10. Fourth pattern of gene expression changes with shNRP2 & EZH2i

These results suggest that NRP2b alone is important to bring EZH2 to bear on repression of these genes. In the absence of NRP2b, these genes become unresponsive to both TGFb and EZH2 inhibition. 7% of genes showed a similar pattern, and two of these (HPCAL4, VCAM1) are included in **Fig. 10A** for comparison.

5) The fifth pattern (2) is exemplified by **TREM1**, whose responses to EPZ are represented graphically in **Fig. 11A**. Genes displaying this response pattern may be induced or not by TGFβ but are only

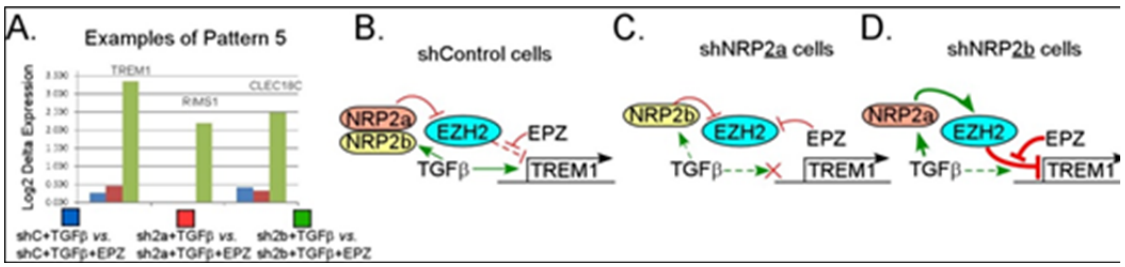

Fig. 11. Fifth pattern of gene expression changes with shNRP2 & EZH2i

weakly affected (at most) by EZH2 inhibition in control cells (**Fig. 11A**). However, in cells knocked down for NRP2b, EZH2i strongly induces expression, but not when NRP2a is knocked down. This group is similar to the previous Group 4, showing a highly differential response depending on which isoform is present, but the specifics are reversed, with genes becoming responsive to EPZ only when NRP2b is missing. These findings are consistent with a model in which EZH2 has a minor role in regulating expression in control cells (**Fig. 11B**), similar to the minor role posited for Group 4 (**Fig. 10B**). Again, EZH2 only comes into play when NRP2b is knocked down (**Fig. 11D**) but not in cells knocked down for NRP2a (**Fig. 11C**). These results suggest that NRP2a alone is important to bring EZH2 to bear on repression of these genes. In the absence of NRP2a, these genes become unresponsive to both TGFβ and EZH2 inhibition. 7.5% of genes show a similar pattern, and two of these (RIMS1, CLEC18C) are included in Fig. 11A for comparison.

6) The sixth and final pattern to be described here is exemplified by **ODAM**, whose responses to EPZ are represented graphically in **Fig. 12A**. Genes displaying this response pattern may or may not respond to TGFβ. However, they are induced by EZH2 inhibition in control cells and in NRP2a knockdown cells, but not following NRP2b knockdown (**Fig. 12A**). This is another group

with a highly differential response depending on which isoform is present, but in this case, the response is extended to control cells expressing endogenous NRP2 isoforms. The salient feature in this group is that response to EPZ requires the presence of NRP2b. These findings are consistent with a model in which EZH2 has a major role in regulating expression in control

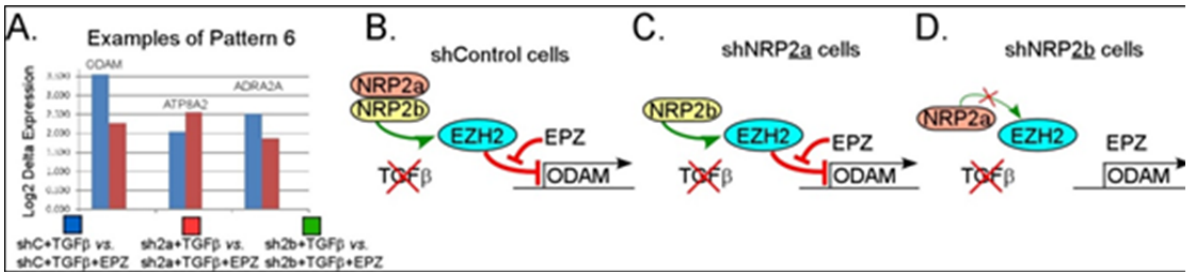

Fig. 12. Sixth pattern of gene expression changes with shNRP2 & EZH2i

cells (**Fig. 12B**) and in NRP2a knockdown cells (**Fig. 12C**) but not in NRP2b knockdown cells (**Fig. 12D**). 6% of genes show a similar pattern, and two of these (ATP8A2, ADRA2a) are included in **Fig. 12A** for comparison.

In addition to the patterns of expression changes described above, there were a number of less frequent patterns that were also informative that included opposing effects. For example, there were genes for which EPZ induced expression in NRP2a knockdown cells but had the opposite effect in NRP2b knockdowns. The reverse pattern was also observed. Overall, we interpret these results to indicate there is a deep, but complex, relationship between EZH2 and the NRP2 receptor system (NRP2b, NRP2a), which essentially confirms the hypothesis proposed in this Concept Award.

Table I provides a synopsis of the number of genes in each of the 23 categories (patterns) identified in our analysis. Distinctions between categories were established by differential effects of TGFβ, EZH2 inhibition and knockdown of NRP2a or NRP2b.The entries of “activated” or “repressed” indicate the effects of EZH2 inhibition. Thus 73 genes were uniquely repressed in NRP2b knockdown cells by EPZ while 96 genes were activated by EPZ in the same cells. This activated gene set is described as Pattern #5 in the text above. Note that only 6 patterns are described in detail in the text above.

TABLE I Gene Numbers and Expression Patterns

# of Genes	shCTL	shNRP2a	shNRP2b	Pattern in text
73			repressed	
96			activated	5
43		repressed		
18		repressed	repressed	
1		repressed	activated	
87		activated		4
1		activated	repressed	
54		activated	activated	3
181	repressed			1
15	repressed		repressed	
3	repressed		activated	
13	repressed	repressed		
13	repressed	repressed	repressed	
4	repressed	activated		
1		activated	activated	
128	activated			2
1	activated		repressed	
20	activated		activated	
1	activated	repressed		
1	activated	repressed	repressed	
27	activated	activated		6
1	activated	activated	repressed	
30	activated	activated	activated	
812	total genes			
7071	genes with no EZH2 effect			

In summary, these results indicate that nearly all genes influenced by EZH2 inhibition were also sensitive to the exact status of NRP2 isoforms. Some genes required both NRP2b and NRP2a in order for EPZ to alter their expression. Others were specific for either NRP2a or NRP2b while only a very few showed opposite effects. Collectively, these results demonstrate that NRP2 isoforms are deeply imbedded in the regulatory responses mediated by EZH2 and PRC2. Mechanistically, we do not know how this is accomplished, although we can speculate that signaling downstream of TGFβ is affected by the NRP2 isoforms in a manner that has differential impact on EZH2. Further dissection of this mechanism is well beyond the scope of this Concept Award. However, the data generated here will provide the necessary foundation

for research applications designed to illucidate this mechanism as well as exploit this new knowledge for therapeutic gain, the ultimate goal of all this effort.

One final point is that our RNAseq data also firmly demonstrate the existence of a bidirectional relationship between TGFβ signaling and NRP2. While TGFβ preferentially induces NRP2b, these RNAseq data confirm that both NRP2b and NRP2a contribute to the TGFβ transcriptome. As shown in **Fig. 13**, NRP2b was required for the induction or suppression of over 45% of the genes responding to TGFβ in A549 cells [(250+516)/1623]. Using a 2-fold cutoff, 1623 genes were significantly altered in A549 cells by exposure to TGFβ (24h). NRP2b was required for TGFβ to modulate 766 of these genes, either alone (250) or in combination with NRP2a (516). GO-term enrichment analysis (biological process) revealed that the 250 genes specifically requiring NRP2b alone were associated with pathways important for "migration", in agreement with previous transwell migration assays. GO-terms linked to the 516 gene set, requiring both NRP2b and NRP2a, were associated with "cell cycle" or "proliferation". Predominant GO-terms associated with the other subsets are listed in **Fig. 13**. The small number of genes requiring NRP2a uniquely (47), suggested that the TGFβ transcriptome was more prominently influenced by NRP2b.

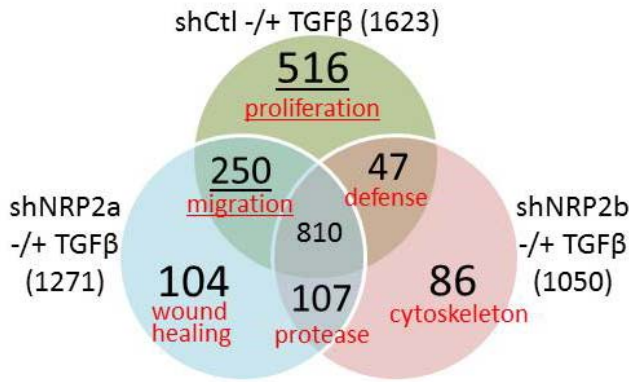

Fig. 13. Venn diagram of gene numbers regulated by TGFβ (24h) in A549 cells knocked-down for NRP2 isoforms [shCtl (green), shNRP2a (blue) & shNRP2b (pink), 2-fold cutoff, pAdj <0.1]. Primary GO biological processes linked to each subset are in red.

APPENDIX 1

Detailed Description of Results and Interpretations

Introduction:

In previous studies, we found that NRP2 was upregulated during TGFβ-driven EMT in lung cancer cells and that NRP2 knockdown substantially inhibited invasive tumor growth and EMT features. Moreover, we found that an uninvestigated isoform, NRP2b, was predominantly involved in this process. The EMT process includes suppression of epithelial genes; moreover, epigenetic changes mediated by Polycomb Repressor Complex 2 (PRC2) have been implicated in this process. Thus, we **hypothesized** that NRP2 acts upstream of EZH2, the catalytic subunit of the PRC2 complex, and that NRP2b knockdown would impair TGFβ-driven EMT in a manner that was dependent on EZH2.

Like other malignant diseases, lung cancer encompasses a spectrum of pathologic mechanisms and drivers, such that only a subset would be expected to respond to TGFβ in the manner described above. Furthermore, TGFβ is involved in numerous processes including growth regulation, inflammation, wound repair, fibrosis, immune regulation, migration and invasion. Similarly, only a subset of these responses would probably be regulated by NRP2.

The Specific Aims in this Concept Award were crafted, first to test the hypothesis that a signaling axis existed from TGFβ to EZH2 and EMT that involved NRP2, and second to test whether EZH2 inhibitors, alone and in combination, could alter cell biological parameters including the EMT. Two approaches were proposed for achieving the first Goal; 1) perform chromatin immunoprecipitation (ChIP) and measure changes in histone H3K27 methylation status in specific genes in response to manipulations of TGFβ, NRP2 and ZEB1; 2) perform RNA sequencing analysis on lung cancer cell lines to define genes sensitive to TGFβ in a NRP2 and EZH2-dependent manner. Initial results from Goal 2 (described first below), showed relatively minor effects of EZH2 inhibition on a series of 11 lung cancer cell lines. We subsequently examined changes in gene expression in these lines using real-time RT-PCR with modest changes attributable to EZH2. These initial observations convinced us that the second approach for Goal 1, global analysis of genes influenced by TGFβ, EZH2 and NRP2, was required before any chromatin immunoprecipitation experiments or combination therapies should be undertaken. We realized that the fundamental hypothesis of this project needed to be tested directly and globally, and that the second approach for Goal 1 was the most productive way to do this. We subsequently focused our effort on Task 2, RNAseq analysis of three lung cancer cell lines genetically manipulated to differentially express NRP2 isoforms. These cells were analyzed following treatment with TGFβ, +/- pre-treatment with an EZH2 inhibitor. This approach thus combined aspects of both Goals 1 and 2 and was a spectacular success. The results have provided a foundation for new proposals as well as a logical pathway to complete the goals originally set forth in this Concept Award.

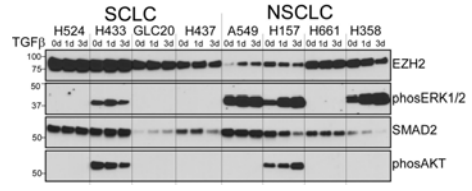

Fig. 1. Western blot survey for EZH2 protein expression in 8 lung cancer cell lines.

Major Activities:

Responsiveness of Lung Cancer cell lines to inhibition of EZH2. To begin an analysis of TGFβ, NRP2 and EZH2 interactions, we first verified that EZH2 was expressed at the protein level in our lung cancer cell lines (**Fig. 1**). Although variable, EZH2 was highly expressed in most lines. Five NSCLC and six SCLC lung cancer cell lines were then treated with TGFβ, using control and

specific NRP2 isoform shRNA knockdowns in some, as part of Goal 2. Treated cells were interrogated for changes in EMT markers, EZH2 and SOX4, a reported upstream regulator of EZH2, using quantitative RT-PCR. Because some tumors are dependent on EZH2, we also treated cell lines with GSK126, a small molecule inhibitor of EZH2, and monitored growth and viability. Of interest, the SCLC cell lines (H433, H524, GLC20, H740, H437, H345) were strikingly non-responsive to TGFβ stimulation. This lack of response included the classical TGFβ-responsive gene, PAI1, as well as EMT markers (E-cadherin, N-cadherin), NRP1, the NRP2 a/b isoforms, SOX4 and EZH2. As reported by others and verified in **Fig. 1**, SCLCs express high levels of EZH2. However, GSK126 had only a modest effect on growth with substantial inhibition observed only in two lines (GLC20, H524) and at high (20 μM) concentration (**Fig 2**). These studies were performed using six different initial cell densities (1000-6000 cells/well) and four different drug concentrations (2-20 μM). Of note, most EZH2 inhibitors, including GSK126, have considerably less effect on the related enzyme, EZH1, which very recently has been shown to be a critical target in VHL mutated kidney cancer. To our knowledge, EZH1 has not been examined in SCLC. The fact that high concentrations of GSK126 are required to inhibit the growth of SCLC cell lines suggests that EZH1 might be a relevant target in this disease. The SCLC cell lines were not further addressed in this Concept Award, but will be pursued in other studies.

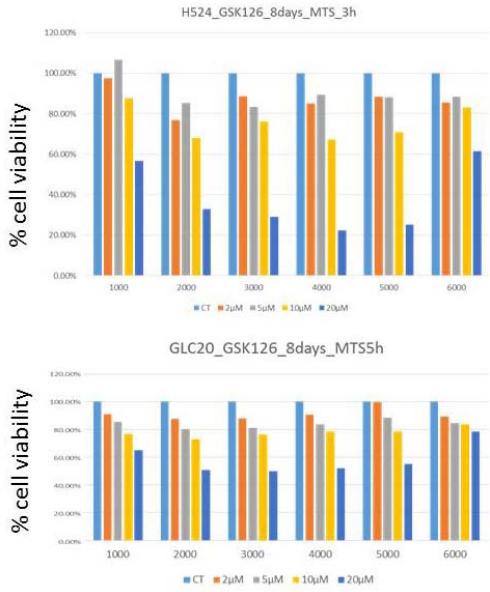

Fig. 2. Inhibition of SCLC cell lines H524 & GLC20 by EZH2 inhibitor GSK126.

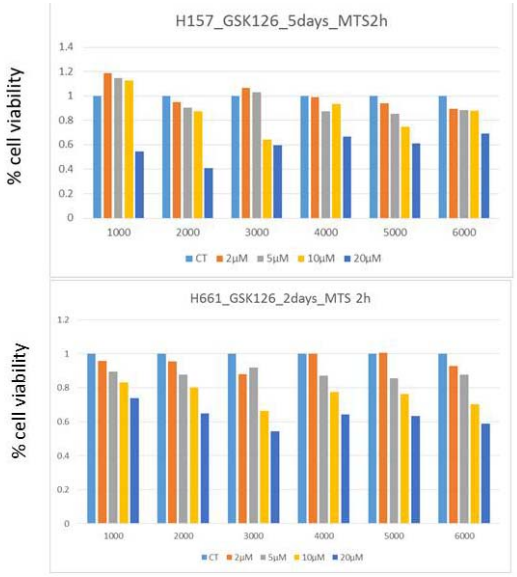

Fig. 3. Inhibition of NSCLC cell lines H157 & H661 by EZH2 inhibitor GSK126.

When 6 NSCLC cell lines were tested by viability assays for sensitivity to GSK126, we found that NSCLC did not differ substantially from the SCLCs. There was some evidence of increasing growth inhibition at higher doses, and the depth of effect was sometimes higher. **Fig. 3** shows two examples, H157 and H661. We had proposed to test different combinations of inhibitors, but we spent more time than anticipated with single agent GSK126 treatments at various doses, durations and cell densities to obtain both reproducible data and in an attempt not to miss a particular set of conditions that would be more effective.

Changes in gene expression in response to TGFβ and EZH2 inhibition are sensitive to NRP2 status. At the transcriptional level, the NSCLC cell lines were uniformly responsive to TGFβ, at least involving the upregulation of PAI1 (SERPINE1), a classic TGFβ response gene. This analysis was carried out with quantitative real-time PCR (qRT-PCR). **Fig. 4** shows the response of PAI1 to TGFβ+/-EPZ011989 in H1975 cells. There are two notable effects illustrated in **Fig. 4**. First, the relative induction of PAI1 was influenced by knockdown of the NRP2 isoforms, with shNRP2b increasing the induction while shNRP2a blunted induction. Second, in all cases, the EZH2 inhibitor, EPZ011989, reduced the induction mediated by TGFβ. However, the effects of EPZ appeared to be independent of NRP2 isoform-specific knockdowns. These results support the notion that NRP2 isoforms and EZH2 activity are important for normal TGFβ responses but did not show a clear relationship between NRP2 and EZH2.

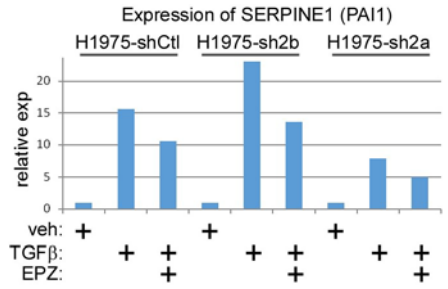

Fig. 4. PAI1 (SERPINE1) induction in H1975 by TGFβ

We then asked whether altered gene expression could be observed for other genes, again using qRT-PCR to measure expression changes. Although H460 was an exception, most NSCLC lines (H358, H661, H1975, H157, A549) responded to TGFβ with upregulation of SOX4, a transcription factor important for EMT and EZH2. However, at the mRNA level, EZH2 was unaffected. Among the Neuropilins, NRP2 (especially NRP2b) was often upregulated (in 3/5 lines), whereas NRP1 showed little change. **Fig. 5** shows the relative expression of 5 genes in H1975 cells in response to TGFβ. Interestingly, the NRP2 genotype appears to influence the TGFβ-mediated induction of SOX4, particularly its response to the EZH2 inhibitor, EPZ011989. Thus, in control cells, EPZ blunted the induction of SOX4, while this effect was lost when NRP2b was knocked down and completely reversed when NRP2a was knocked down. The repression of E-cadherin expression was also affected by these combinations, with EPZ enhancing suppression in shControl cells but not when NRP2a was knocked down. In contrast, NRP2b knockdown led to stronger inhibition of E-cadherin by TGFβ alone. These findings supported our overall hypothesis that TGFβ responses leading to EMT (E-cadherin loss, SOX4 gain) were linked to both NRP2 isoform expression and to EZH2 activity. At the same time, it became clear that a strategy based upon candidate genes was very limited in its ability to illuminate this relationship further.

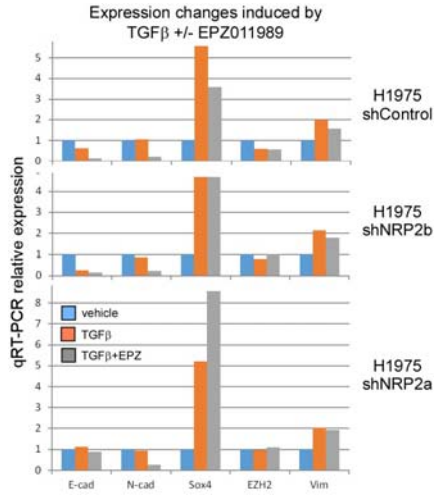

Fig. 5. Relative expression of 5 genes in H1975 shControl, shNRP2b or shNRP2a cells in response to TGFβ+/-EPZ011989.

RNA sequencing analysis supports the TGFβ-NRP2-EZH2-EMT axis. During the course of these experiments, we reasoned that it would be possible to explore our overall hypothesis on a global level and in a robust manner that directly addressed the possible relationship between TGFβ, the NRP2 isoforms, EZH2 and its target genes. By pretreating control or NRP2 isoform

knockdown cells with the EZH2 inhibitor, EPZ011989, followed by subsequent exposure to TGFβ, we could interrogate RNAseq data for genes that were responsive to EZH2 inhibition in a NRP2-dependent manner. This strategy combined aspects of Goals 1 and 2, and provided a means to test the central hypothesis of our project. The results are presented below; not only do they support our central hypothesis, they also support NRP2, and especially NRP2b, as a therapeutic target in lung cancer and thus warrant further exploration.

To directly determine if there was a regulatory relationship between NRP2 and EZH2/PRC2, we turned to the global RNA sequencing approach encompassed in Goal 1, Task 2. Importantly, the use of the specific EZH2 inhibitor, EPZ011989, allowed us to identify genes which were selectively affected by EZH2/PRC2 activity in a manner that was dependent upon NRP2 isoforms and in the context of TGFβ exposure. The results also revealed a substantially larger role for NRP2b in the TGFβ-induced transcriptome than we had anticipated.

RNA sequencing. The experimental design utilized A549 cells bearing shControl, shNRP2a and shNRP2b knockdown constructs, each cultured under three conditions; 1) vehicle, 2) TGFβ (5ng/ml, 24h), 3) or pre-treated for 7 days with EPZ011989 (1 μM; EPZ henceforth) followed by 24h TGFβ (5ng/ml). The long EPZ pre-treatment was included to allow time for methylation status of histone H3-K27 residues to change and become established as a result of blocking EZH2 activity. The efficacy of the EPZ treatment was revealed by its ability to alter the EMT morphology of A549 cells exposed to TGFβ for 24h. As shown in **Fig. 6**, EPZ pre-treatment partially blocked the spindle shape induced by TGFβ, an effect that was most readily apparent in the shNRP2b cells (middle panels, compare left to right). Following treatments, A549 cell RNA was isolated from two independent biological replicates representing all 9 culture conditions. RNA was purified using the RNeasy Mini Plus kit according to manufacturer's directions.

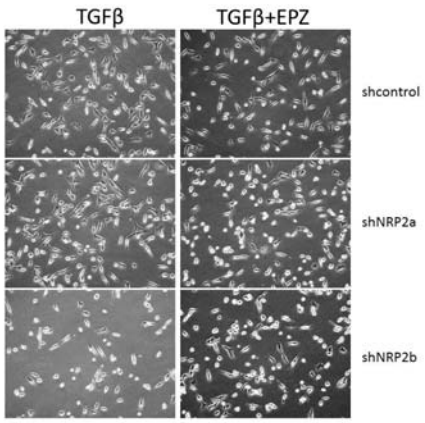

Fig. 6. EPZ pre-treatment of A549 cells partially blocks the spindle shape characteristic of the EMT, especially in shNRP2a cells.

RNAseq pipeline: RNAseq utilized the Illumina HiSeq 2500 at the MUSC Genomics Core. Polyadenylated (poly A^+) RNA was enriched from total RNA, reverse-transcribed and converted to a strand-specific sequencing library. Each sample was then sequenced to a depth of 50 million reads. Adapters were trimmed by CutAdapt (http://journal.embnet.org/index.php/embnetjournal/article/view/200) or Trimmomatic. Fold-change estimation and hypothesis testing for differential expression was then performed using DESeq2. Gene ontology and pathway analyses were performed using Advaita as well as ToppGene web-based tools.

Using a 2-fold cutoff, we found 958 genes were induced while 665 were repressed by TGFβ in control cells. EZH2 inhibition resulted in an additional 152 genes being induced and 211 genes being suppressed. However, of the genes originally induced or suppressed by TGFβ, 194 required EZH2 activity for this response (110 upregulated genes were lost; 84 down regulated genes were lost). An additional 77 genes required EZH2 to moderate the induction or enhance the suppression mediated by TGFβ (by 2-fold or more) – that is, induction was at least 2-fold higher when EZH2 was inhibited or suppression was 2-fold greater when EZH2 was inhibited.

These observations are consistent with EZH2/PRC2 being an important factor in the TGFβ response, as expected.

In cells knocked down for NRP2 isoforms, intriguingly altered expression patterns were noted by simple inspection with many examples of an apparent complex regulatory interplay between NRP2 isoforms and EZH2 with specific effects on specific genes. For example, the gene **SH3TC2** was not affected by TGFβ in control cells, but was *suppressed* 9-fold by EPZ, suggesting that EZH2 activity is necessary to retain baseline expression. However, knocking down either NRP2 isoform rendered this gene sensitive to TGFβ repression, with no evidence of additive effects with EPZ. Initial examination of the dataset suggested that many genes responded to the experimental manipulations in a similar manner. However, it was also clear that several additional distinct patterns of response were present in the data. We wanted to systematically identify genes with altered responses to the EZH2 inhibitor, and to classify these into similar groups, reasoning that these would provide mechanistic insights into the roles of NRP2 isoforms and EZH2-dependent changes in gene expression.

To this end, we calculated the expression difference (Δ^{exp}) observed for each gene between vehicle and EPZ011989-treated cultures of the three genotypes, A549-shControl, -shNRP2a and -shNRP2b, all exposed to TGFβ for 24h. For genes which responded to EPZ pre-treatment similarly, regardless of the underlying NRP2 knockdown status, the Δ^{exp} values were very similar. However, for genes like **SH3TC2**, whose responses were obviously distinct in NRP2 knockdown cells, these values were substantially different. By calculating the standard deviation of the three Δ^{exp} values for each gene, and sorting for S.D. values, we prepared a list of loci most differentially influenced by EPZ in a NRP2-dependent manner. Of the first 200 genes on this list, over 20% showed a pattern of response similar to SH3TC2. The second most common pattern, also seen in 20%, consisted of genes *induced* by EPZ, but not in cells knocked down for either NRP2 isoform, suggesting that NRP2a and "b" were required for EZH2/PRC2 to repress. Collectively, there were 23 distinct patterns of altered gene expression identified among the 812 genes most sensitive to the EPZ-NRP2 combination. Below we provide a brief synopsis of the six most relevant patterns along with an exemplar and a hypothetical model for each.

1) The most prevalent pattern 1 is exemplified by **SH3TC2**, whose responses to EPZ are represented graphically in **Fig. 7A**. Genes with this pattern appear to require EZH2 for normal

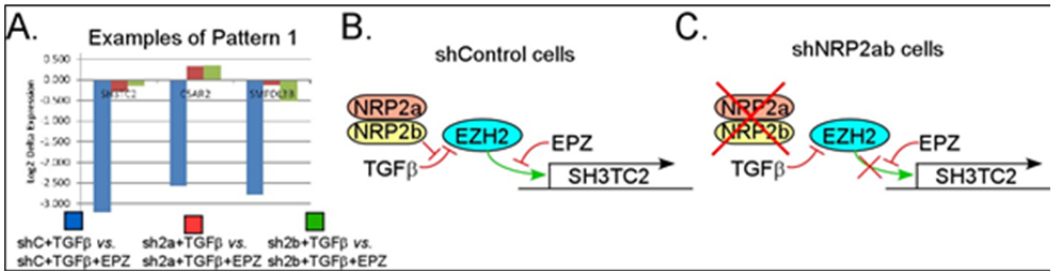

Fig. 7. Most abundant pattern of gene expression changes with shNRP2 & EZH2i

expression and are repressed by EZH2 inhibition (**Fig. 7B**). However, response to EZH2i is lost if either NRP2a or NRP2b is knocked down, and the gene becomes sensitized to TGFβ-induced repression (**Fig. 7C**). This is consistent with a model in which TGFβ signaling would inhibit the action of EZH2, but is prevented in the presence of NRP2. Thus when NRP2a or NRP2b are knocked down, the latent repression mediated by TGFβ is uncovered, reducing SH3TC2

expression. Approximately 22% of genes showed a similar pattern, and two of these (C5AR2, SMPDL3B) are included in **Fig. 7A** for comparison.

2) The second commonest pattern is exemplified by **FCRL4**, whose responses to EPZ are represented graphically in **Fig. 8A**. Genes with this pattern are inhibited by EZH2, and thus induced by EZH2 inhibition (**Fig. 8B**). However, this response to EZH2i is missing if either NRP2a or NRP2b is knocked down (**Fig. 8C**). Most of these genes are not responsive to TGFβ. These findings are consistent with a model in which NRP2a and NRP2b somehow maintain the capability of EZH2 to inhibit gene expression, while TGFβ signaling appears uninvolved. When either NRP2a or NRP2b is knocked down, EZH2 loses its repressive capability, rendering EPZ impotent with no effect on expression. 20% of genes showed a similar pattern, and two of these (CLEC5A, LOC101448202) are included in **Fig. 8A** for comparison.

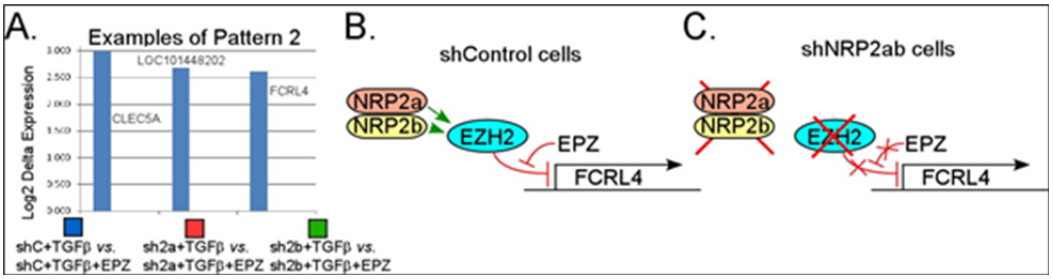

Fig. 8. Second pattern of gene expression changes with shNRP2 & EZH2i

3) The third pattern is exemplified by **FABP4**, whose responses to EPZ are represented graphically in **Fig. 9A**. Genes with this pattern are unaffected by EZH2 inhibition or by TGFβ in control cells (**Fig. 9A, B**). However, when cells are knocked down for either NRP2a or NRP2b, EZH2 comes into play. These genes become sensitized to EPZ and are induced by the EZH2 inhibitor (**Fig. 9C**). These findings are consistent with a model in which NRP2a and NRP2b prevent EZH2 from affecting certain genes (**Fig. 9B**). However, in the absence of either NRP2a or NRP2b, regulation by EZH2 is now imposed to inhibit gene expression. Thus in the absence of NRP2, these genes are up-regulated in response to the EZH2 inhibitor. TGFβ signaling appears to not be involved. 16% of genes showed a similar pattern, and two of these (DCN, COL3A1) are included in **Fig. 9A** for comparison.

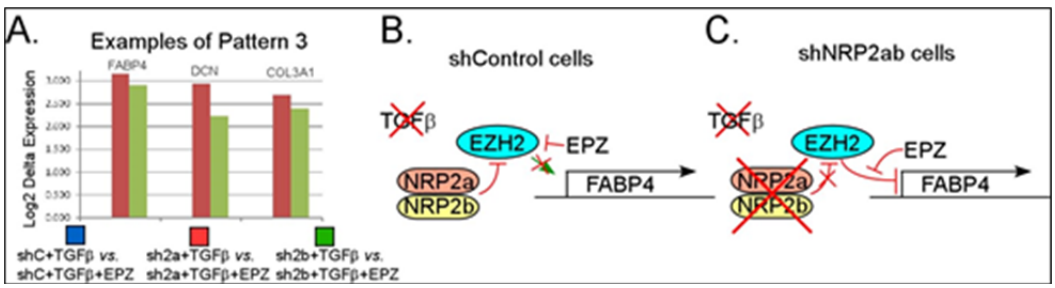

Fig. 9. Third pattern of gene expression changes with shNRP2 & EZH2i

4) The fourth most abundant pattern is exemplified by **LCE3D**, whose responses to EPZ are represented graphically in **Fig. 10A**. Genes displaying this response pattern may be induced or not by TGFβ but are only weakly affected (at most) by EZH2 inhibition in control cells (**Fig. 10A**). However, in cells knocked down for NRP2a specifically, EZH2i strongly induces expression. This does not happen when NRP2b is knocked down, indicating that this group

shows highly differential responses depending on which isoform is present. These findings are consistent with a model in which EZH2 has at most a minor role in regulating expression in control cells (**Fig. 10B**). However, EZH2 comes into play when NRP2a is knocked down (**Fig. 10C**) but not in cells knocked down for NRP2b (**Fig. 10D**).

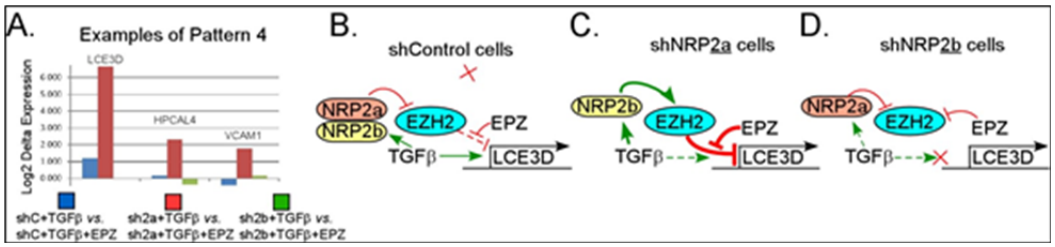

Fig. 10. Fourth pattern of gene expression changes with shNRP2 & EZH2i

These results suggest that NRP2b alone is important to bring EZH2 to bear on repression of these genes. In the absence of NRP2b, these genes become unresponsive to both TGFb and EZH2 inhibition. 7% of genes showed a similar pattern, and two of these (HPCAL4, VCAM1) are included in **Fig. 10A** for comparison.

5) The fifth pattern (2) is exemplified by **TREM1**, whose responses to EPZ are represented graphically in **Fig. 11A**. Genes displaying this response pattern may be induced or not by TGFβ but are only

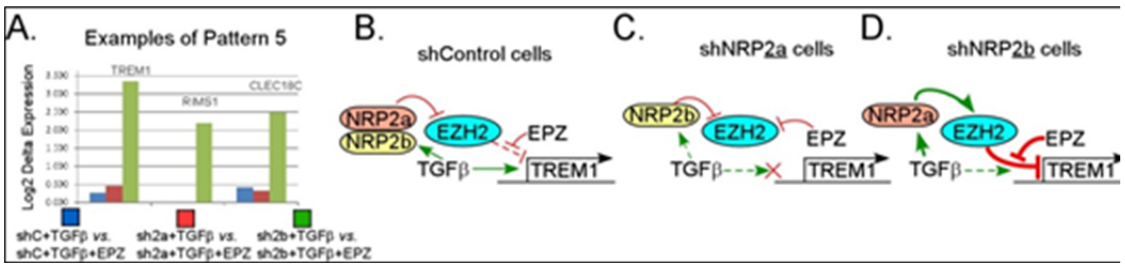

Fig. 11. Fifth pattern of gene expression changes with shNRP2 & EZH2i

weakly affected (at most) by EZH2 inhibition in control cells (**Fig. 11A**). However, in cells knocked down for NRP2b, EZH2i strongly induces expression, but not when NRP2a is knocked down. This group is similar to the previous Group 4, showing a highly differential response depending on which isoform is present, but the specifics are reversed, with genes becoming responsive to EPZ only when NRP2b is missing. These findings are consistent with a model in which EZH2 has a minor role in regulating expression in control cells (**Fig. 11B**), similar to the minor role posited for Group 4 (**Fig. 10B**). Again, EZH2 only comes into play when NRP2b is knocked down (**Fig. 11D**) but not in cells knocked down for NRP2a (**Fig. 11C**). These results suggest that NRP2a alone is important to bring EZH2 to bear on repression of these genes. In the absence of NRP2a, these genes become unresponsive to both TGFβ and EZH2 inhibition. 7.5% of genes show a similar pattern, and two of these (RIMS1, CLEC18C) are included in Fig. 11A for comparison.

6) The sixth and final pattern to be described here is exemplified by **ODAM**, whose responses to EPZ are represented graphically in **Fig. 12A**. Genes displaying this response pattern may or may not respond to TGFβ. However, they are induced by EZH2 inhibition in control cells and in NRP2a knockdown cells, but not following NRP2b knockdown (**Fig. 12A**). This is another group

with a highly differential response depending on which isoform is present, but in this case, the response is extended to control cells expressing endogenous NRP2 isoforms. The salient feature in this group is that response to EPZ requires the presence of NRP2b. These findings are consistent with a model in which EZH2 has a major role in regulating expression in control

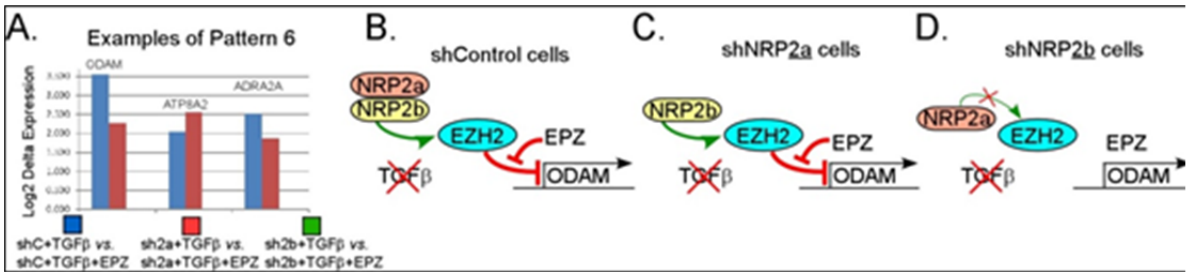

Fig. 12. Sixth pattern of gene expression changes with shNRP2 & EZH2i

cells (**Fig. 12B**) and in NRP2a knockdown cells (**Fig. 12C**) but not in NRP2b knockdown cells (**Fig. 12D**). 6% of genes show a similar pattern, and two of these (ATP8A2, ADRA2a) are included in **Fig. 12A** for comparison.

In addition to the patterns of expression changes described above, there were a number of less frequent patterns that were also informative that included opposing effects. For example, there were genes for which EPZ induced expression in NRP2a knockdown cells but had the opposite effect in NRP2b knockdowns. The reverse pattern was also observed. Overall, we interpret these results to indicate there is a deep, but complex, relationship between EZH2 and the NRP2 receptor system (NRP2b, NRP2a), which essentially confirms the hypothesis proposed in this Concept Award.

Table I provides a synopsis of the number of genes in each of the 23 categories (patterns) identified in our analysis. Distinctions between categories were established by differential effects of TGFβ, EZH2 inhibition and knockdown of NRP2a or NRP2b.The entries of “activated” or “repressed” indicate the effects of EZH2 inhibition. Thus 73 genes were uniquely repressed in NRP2b knockdown cells by EPZ while 96 genes were activated by EPZ in the same cells. This activated gene set is described as Pattern #5 in the text above. Note that only 6 patterns are described in detail in the text above.

TABLE I Gene Numbers and Expression Patterns

# of Genes	shCTL	shNRP2a	shNRP2b	Pattern in text
73			repressed	
96			activated	5
43		repressed		
18		repressed	repressed	
1		repressed	activated	
87		activated		4
1		activated	repressed	
54		activated	activated	3
181	repressed			1
15	repressed		repressed	
3	repressed		activated	
13	repressed	repressed		
13	repressed	repressed	repressed	
4	repressed	activated		
1		activated	activated	
128	activated			2
1	activated		repressed	
20	activated		activated	
1	activated	repressed		
1	activated	repressed	repressed	
27	activated	activated		6
1	activated	activated	repressed	
30	activated	activated	activated	
812	total genes			
7071	genes with no EZH2 effect			

In summary, these results indicate that nearly all genes influenced by EZH2 inhibition were also sensitive to the exact status of NRP2 isoforms. Some genes required both NRP2b and NRP2a in order for EPZ to alter their expression. Others were specific for either NRP2a or NRP2b while only a very few showed opposite effects. Collectively, these results demonstrate that NRP2 isoforms are deeply imbedded in the regulatory responses mediated by EZH2 and PRC2. Mechanistically, we do not know how this is accomplished, although we can speculate that signaling downstream of TGFβ is affected by the NRP2 isoforms in a manner that has differential impact on EZH2. Further dissection of this mechanism is well beyond the scope of this Concept Award. However, the data generated here will provide the necessary foundation

for research applications designed to illucidate this mechanism as well as exploit this new knowledge for therapeutic gain, the ultimate goal of all this effort.

One final point is that our RNAseq data also firmly demonstrate the existence of a bidirectional relationship between TGFβ signaling and NRP2. While TGFβ preferentially induces NRP2b, these RNAseq data confirm that both NRP2b and NRP2a contribute to the TGFβ transcriptome. As shown in **Fig. 13**, NRP2b was required for the induction or suppression of over 45% of the genes responding to TGFβ in A549 cells [(250+516)/1623]. Using a 2-fold cutoff, 1623 genes were significantly altered in A549 cells by exposure to TGFβ (24h). NRP2b was required for TGFβ to modulate 766 of these genes, either alone (250) or in combination with NRP2a (516). GO-term enrichment analysis (biological process) revealed that the 250 genes specifically requiring NRP2b alone were associated with pathways important for "migration", in agreement with previous transwell migration assays. GO-terms linked to the 516 gene set, requiring both NRP2b and NRP2a, were associated with "cell cycle" or "proliferation". Predominant GO-terms associated with the other subsets are listed in **Fig. 13**. The small number of genes requiring NRP2a uniquely (47), suggested that the TGFβ transcriptome was more prominently influenced by NRP2b.

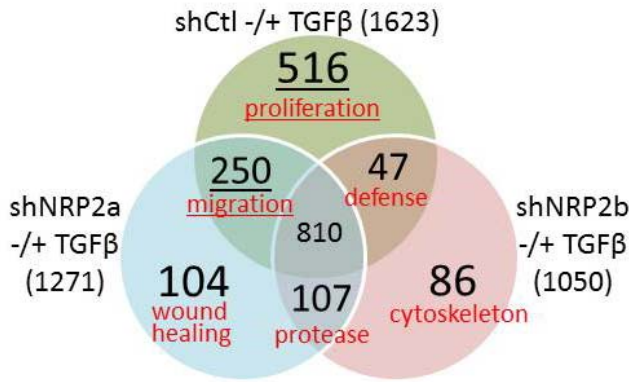

Fig. 13. Venn diagram of gene numbers regulated by TGFβ (24h) in A549 cells knocked-down for NRP2 isoforms [shCtl (green), shNRP2a (blue) & shNRP2b (pink), 2-fold cutoff, pAdj <0.1]. Primary GO biological processes linked to each subset are in red.