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14. ABSTRACT:

The goal of this proposal was to evaluate the role of splicing regulators upregulated in triple negative breast cancer in the tumorigenesis process and identify potential therapeutic targets. We previously identified three splicing factors, SRPK1, KHDRBS3 and SRSF12, that were expressed at higher levels in triple negative breast cancer compared to other breast cancer subtypes. We proposed to evaluate these splicing factors for their ability to support the tumorigenic properties of basal type breast cancer cells, and the formation of tumors in mouse model of triple negative breast cancer.

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Introduction The goal of this proposal was to evaluate the role of splicing regulators upregulated in triple negative breast cancer in the tumorigenesis process and identify potential therapeutic targets. We previously identified three splicing factors, SRPK1, KHDRBS3 and SRSF12, that were expressed at higher levels in triple negative breast cancer compared to other breast cancer subtypes. We proposed to evaluate these splicing factors for their ability to support the tumorigenic properties of basal type breast cancer cells, and the formation of tumors in mouse model of triple negative breast cancer.

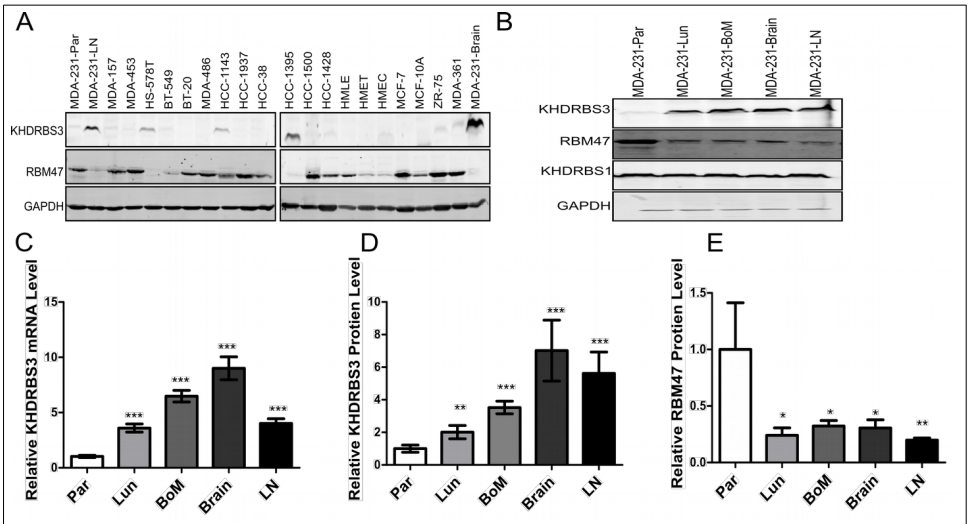

Figure 1. Expression of KHDRBS3 in human breast cancer cell lines. (A) KHDRBS3 is expressed in basal type cell lines and its expression is mutually exclusive with RBM47, a luminal cell type specific RNA binding protein. **(B)** Elevated KHDRBS3 levels in metastatic derivatives (MDA-231-Lun, MDA-231-BoM, MDA-231-Brain, MDA-231-LN) of the MDA-MB-231 (MDA-231-Par) cell line. Quantification of the relative mRNA levels of KHDRBS3 **(C)** and protein levels of KHDRBS3 **(D)** and RBM47 **(E)**

We showed that despite the fact that KHDRBS3 levels correlated with the metastatic potential of MDA-MB-231, and CN34 cells, the protein is dispensable. Depletion of KHDRBS3 in the highly metastatic MDA-MB-231LN cells did not affect cell growth and migration in vitro, and tumor formation and metastasis in mouse xenografts. Strikingly knockout of the KHDRBS3 gene in mice reduced more than two fold the rate of breast cancer formation in the C3.1-TAg model of triple negative breast cancer. These data suggest that KHDRBS3 is required in the early stages of tumor initiation.

We did not pursue in vitro experiments with SRPK1 as data showing requirement for SRPK1 in cell line mobility *in vitro*and metastasis *in vivo* was published shortly after the proposal was submitted [1]. We used the resources allocated for this work to carry out the experiments with KHDRBS3 knockout mice that were outlined above and were not part of the original proposal. In mice, homozygous deletion of SRPK1 is lethal. For this reason we used heterozygous deletion of SRPK1 to evaluate if reduced gene dosage will affect tumor formation. The results showed that reduction of the SRPK1 gene dosage did not affect tumor formation in the C3.1-TAg mouse model.

We were unable to carry out the proposed work on SRSF12 due to the lack of suitable antibodies. The commercially available anti-SRSF12 antibodies failed to recognize the protein. We made two attempts to raise antibodies against two different epitopes of SRFS12. In both cases we failed to produce antiserum that would react with the recombinat SRSF12 protein. At that point we exhausted the available epitopes that can produce antibodies to SRSF12 that do not react with the closely relates SRSF10 protein.

Keywords

Pre-mRNA splicing, breast cancer, KHDRBS3, SRPK1, SRSF12, metastasis

Accomplishments

Major goals

Major Task 1 *Determine the effect of SRPK1, KHDRBS3 and SRSF12 on the malignant properties of the basal type breast cancer cells in vitro.*

Major Task 2 *Determine the effect of SRPK1, KHDRBS3 and SRSF12 on tumor initiation and growth in xenografts.*

Major Task 3 *Demonstrate that reduced SRPK1 and **KHDRBS3** levels suppress tumor initiation, growth and lung metastasis*

Major Task 4 *Analyze SRPK1, KHDRBS3 and SRSF12 expression in Splicing factor expression in normal and malignant tissue samples*

Major Task 5 *Analysis of the regulation of splicing factor expression by the PI3K pathway and cMyc*

Major Task 6 *Analysis of the regulation of alternative splicing in mammary basal epithelial cells by SRPK1, KHDRBS3 and SRSF12*

Specific objectives and activities

Major Task 1 *Determine the effect of SRPK1, KHDRBS3 and SRSF12 on the malignant properties of the basal type breast cancer cells in vitro.*

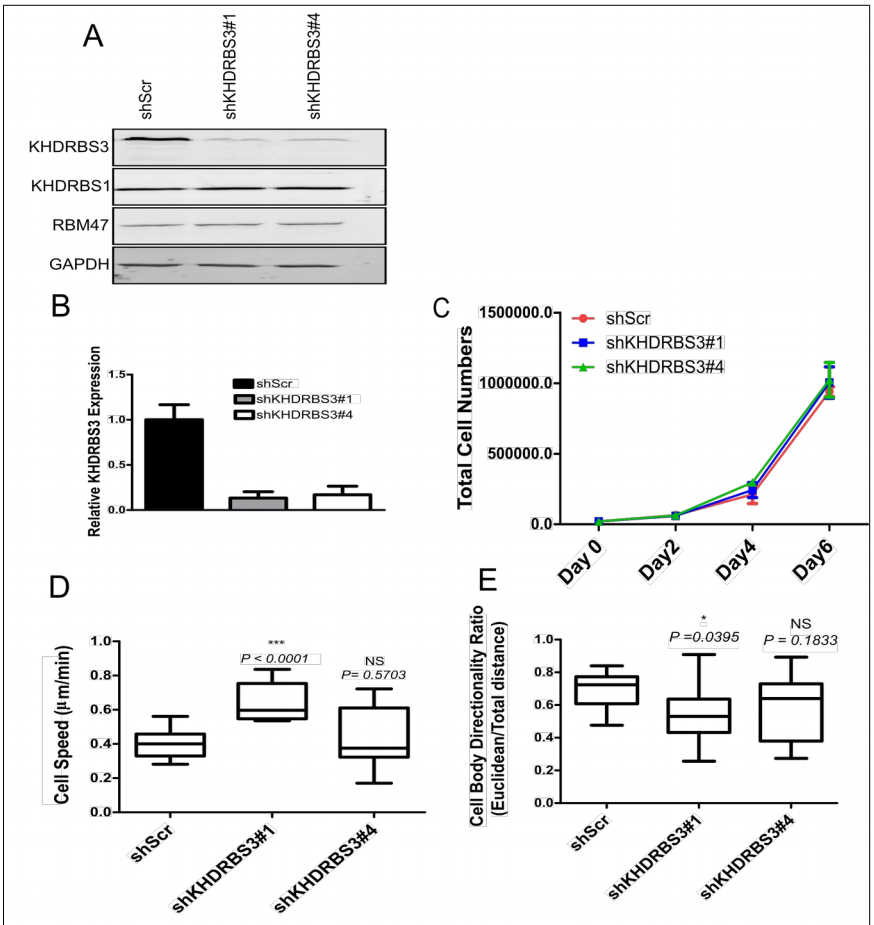

Figure 2. KHDRBS3 is dispensable in MDA-MB-231 cells. (A) and **(B)** Depletion of KHDRBS3 in MDA-MB-231LN cells by two independent shRNA constructs: shKHDRBS#1 and shKHDRBS#4. **(C)** Knockdown of KHDRBS3 does not affect cell proliferation. **(D)** and **(E)** effect of KHDRBS3 depletion on the speed and directionality of MDA-MB-231LN chemotacsis. Cell speed is affected by shKHDRBS3#1, however this is likely an off-target effect as shKHDRBS3#4 which depleted the protein to similar levels has no effect.

We examined the expression of KHDRBS3 in a panel of breast cancer cell lines (Figure 1A). As expected we observed expression of the protein in basal type cell lines. We did not detect KHDRBS3 in MDA-MB-468 cells, one of the cell lines we proposed to use in this project, despite their classification as a basal type. Instead MDA-MB-468 cells expressed the luminal RNA binding protein RBM47. We observed correlation of KHDRBS3 expression with the metastatic potential of MDA-MB-231 cells. KHDRBS3 transcript and protein levels were upregulated in MDA-MB-231 cells selected for metastasis to the lymph node (MDA-MB-231-LN), bone marrow (MDA-MB-231-BoM), lung (MDA-MB-231-Lu) and brain (MDA-MB-231-BR) (Figure 1B). Analysis of publicly available RNASeq data showed that elevated KHDRBS3 RNA levels are also associated with metastatic properties of at least one more breast cancer cell line, CN34.

After knockdown of KHDRBS3 in MDA-MB-231-LN cells (Figure 2A and B)we did not observe significant change in the growth rate (Figure 2C). Chemotaxis through collagen gel using FBS as chemoattractant (Figure 2D and E) and migration in Boyden chamber assays (not shown) were also not affected by the knockdown. While we see some changes in cell migration in cells expressing shKHDRBS3#1 we do not consider these to be due to the depletion of KHDRBS3 as shKHDRBS3#4 which is as efficient in depleting the protein has no effect on the migration properties. The effect of shKHDRBS3#1 is likely due to off-target effects, consistent with its significantly larger impact on overall gene expression compared to shKHDRBS3#4 (See Major Task 6).

We decided not to carry out in vitro experiments for SRPK1 as nearly identical work was recently completed by Dr. van de Water's group, convincingly showing that SRPK1 is critical for cell migration in vitro, and tumor growth and metastasis in xenograft models [1]. We redirected resources planned for this research towards expanding Major Task 3 by adding a KHDRBS3 knockout model which become available in 2016 [2]. This mouse model allowed us to evaluate the effect of KHDRBS3 on tumor initiation, which is not possible with the xenograft approach. In addition, these experiments allowed us to evaluate the effect of KHDRBS3 in conditions more closely resembling human tumors and rule out potential artifacts due to the nature of the cultured cell line models.

Commercial antibody to SRSF12 obtained from SIGMA did not recognize the recombinant portein on western blot. Antibodies from other vendors recognize epitopes common between SRSF12 and SRSF10 and are unable to distinguish between the two proteins. With the assistance of Pacific Immunology and Thermo Scientific we tried to produce antibodies to peptide antigens derived from SRSF12. Both attempts failed to produce an antibody that would react with recombinant SRSF12 protein. We exhausted the regions of the SRSF12 protein that are suitable for producing an antibody that would be selective to SRSF12 and not cross-react with the related SRSF10 protein. Consequently, we lacked the means to monitor SRSF12 protein levels and experiments under **Tasks 1, 2, 4, 5, and 6,** that involve SRSF12 could not be carried out. We therefor consider this task to be completed to the extend feasible.

Major Task 2 *Determine the effect of SRPK1, KHDRBS3 and SRSF12 on tumor initiation and growth in xenografts.*

We used the two KHDRBS3 knockdown lines to evaluate the effect of KHDRBS3 depletion on tumor growth and metastasis in mouse xenograft models. Orthotopic injections of the cell lines in the mammary fat pad of NSG mice were used to determine to effect of the knockdown on tumor growth. We monitored the tumor growth by bioluminescence and caliper measurements during a five-week time course and determine the weight of the tumor at the end of the experiment. We did not observe any effect of the KHDRBS3 knockdown on tumor growth (Figure 3).

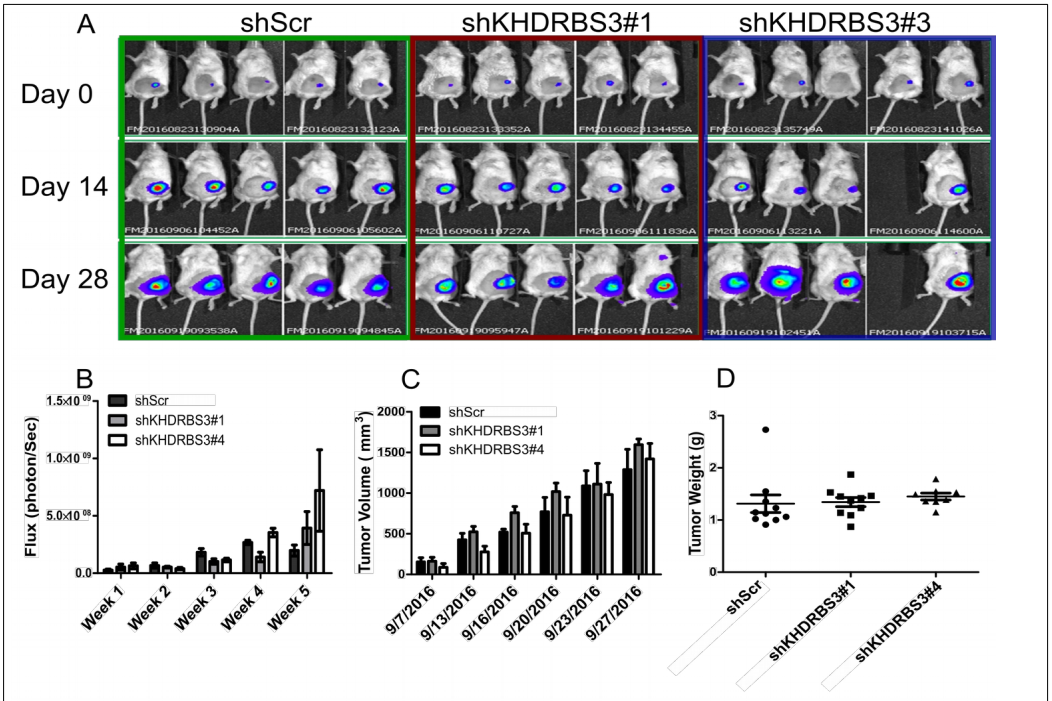

Figure 3. KHDRBS3 knockdown does not affect tumor formation in orthotopic mouse xengraft model of MDA-MB-231LN cells. (A) Images recordin luminescence from MDA-MB-231LN cells at the day of injection, and 2 and 4 weeks after the injection. **(B)** Quantification of the tumor luminescence readings over the course of five weeks. **(C)** Caliper measurement of the tumor volume. **(D)** Tumor weight five weeks after injection of MDA-MB-231 cells in the mammary fat pad.

To determine the effect on the ability of the knockdown cell lines to colonize distant tissues and metastasize from the primary site we analyzed the formation of lung metastasis after tail vein injection or spontaneous migration from the orthotropic injection site (Figure 4). Bioluminescence measurements and histology examination of the lungs failed to detect significant difference between the KHDRBS3 knockdowns and the control (Figure 4). We also examined the spontaneous metastasis to the lung from our orthotopic fat pad injections shown on Figure 3. The orthotopic tumors readily metastasized to the lung (Figure 4E) and both the primeray tumor and the metastasis preserved the knockdown of KHDRBS3 (Figure 4D). Regardless we did not detect significant change in the rate of metastasis between the control and the KHDRBS3 knockdowns.

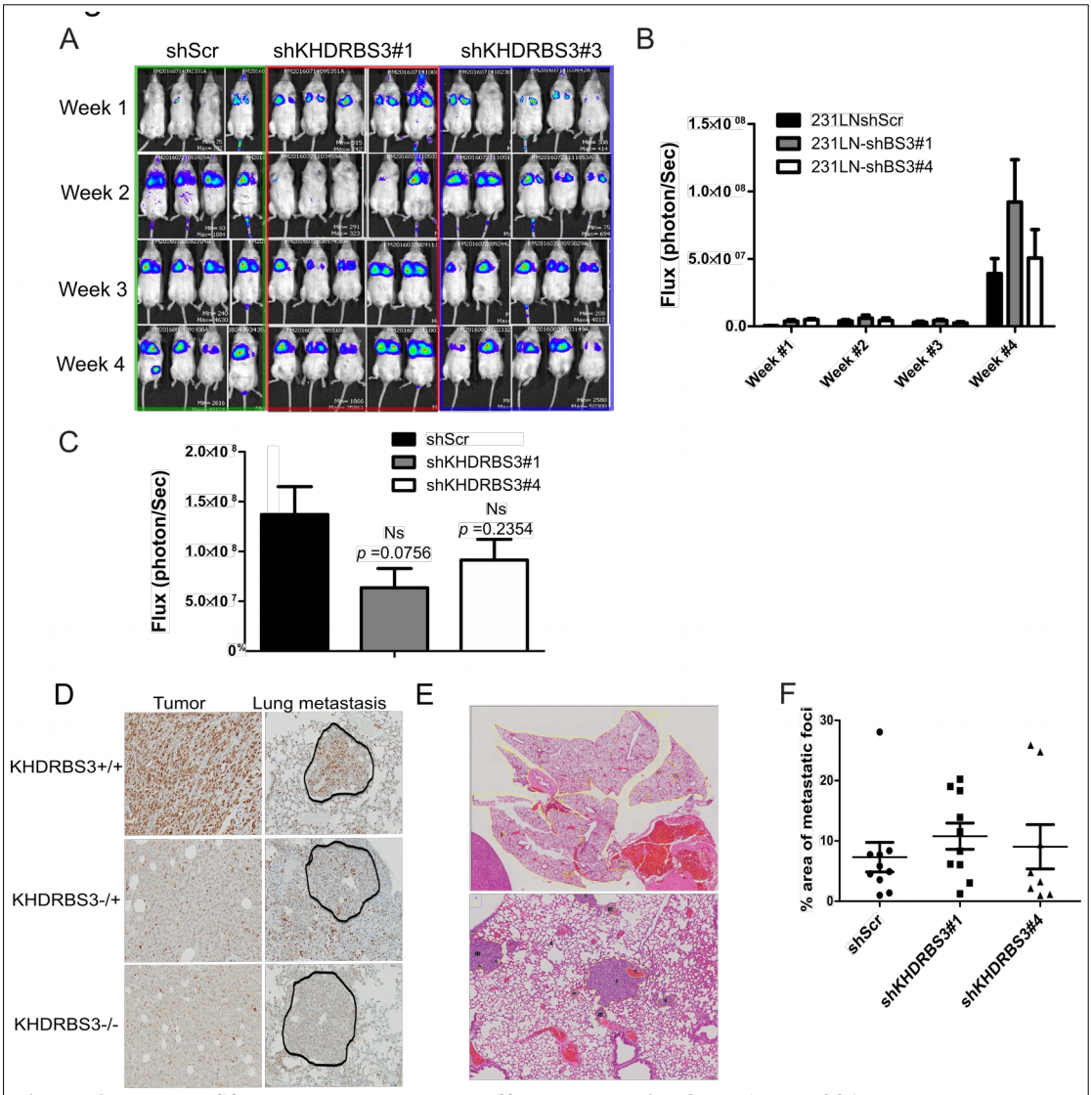

Figure 4. KHDRBS3 knockdown does not affect metastasis of MDA-MB-231LN cells to the lung. (A) Luminescence images of MDA-MB-231LN cells in the lungs after tail vein injection. **(B)** Quantification of the in vivo luminescence in the lungs after tail vein injection. **(C)** Quantification of the ex-vivo luminescence in the lungs four weeks after the tail vein injection. **(D)** Immunohistochemistry showing KHDRBS3 expression in the primary orthotopic tumors and corresponding metastasis to the lung. **(E)** H&E staining of lungs with spontaneous metastasis from orthotopic tumors. The areas being quantified are oulined and marked. **(F)** Area of metastatic foci in the lung derived from orthotopic tumors compared to the tissue area of the section.

The work related to KHDRBS3 under **Task 2** was completed as proposed. We did not perform work on SRPK1 as the results of substantially the same experiments showing requirement for SRPK1 in metastasis were published shortly after we submitted our application [1]. The freed resources were redirected toward the use of KHDRS3 knockout mouse model. Lack of SRSF12 antibody prevents us from carrying out the experiments related to this protein. We therefore consider this task to be completed to the extend feasible.

Major Task 3 *Demonstrate that reduced SRPK1 and KHDRBS3 levels suppress tumor initiation, growth and lung metastasis*

Under this task we examined the effect of reduced SRPK1 gene dosage or elimination of KHDRBS3 on tumor formation in the C3.1-Tag mouse model of triple negative breast cancer. We back-crossed each of the three alleles, KHDRBS3 knockout, SRPK1 knockout and C3.1-Tag for five generation into SV129 background.

We then generated four experimental groups with the following genotypes: C3.1-Tag in wild type background, C3.1-Tag; KHDRBS3(+/-); C3.1-Tag; KHDRBS3(-/-), and C3.1-Tag; SRPK1(+/-). Each group consisted of 33 to 46 virgin female mice. Homozygous KHDRBS3 knockout reduced the rate of mammary gland tumor formation by more than two fold. This reduction in tumor formation is statistically significant: X^2=11.98, p-value=0.00054. Heterozygous loss of one allele of KHDRBS3 and SRPK1 did not affect the rate of tumor formation. Our data shows that KHDRBS3 is supporting mammary gland tumor formation. As the protein can be knocked out in mice without producing a significant adverse phenotype, it is likely that targeting it for breast cancer treatment will produce only minor side effects. We consider this task to be completed.

Major Task 4 *Analyze SRPK1, KHDRBS3 and SRSF12 splicing factor expression in normal and malignant tissue samples*

We analyzed 123 breast cancer tissue samples obtained from the WVU Tissue bank and commercial sources. Of these 29 samples were triple negative and 94 expressed at least one hormonal receptor (ER, PR or Her2). KHDRBS3 expression was detected by immunohistochemistry. Of 29 triple negative breast cancers 20 had detectable expression of KHDRBS3 protein (67 %), while only 20% (19 out of 94) expressed KHDRBS3. This a significant enrichment (X^2=24.3268, p-value < 0.00001), that demonstrates that the elevated KHDRBS3 RNA levels on triple negative breast cancer produce corresponding increase in protein expression.

Evaluation of SRPK1 on over 300 patient tumors was published shortly after this grant application was submited. This analysis did not show association with tumor type but demonstrated that elevated SRPK1 levels correlate with poor prognosis and increased metastasis. Consequently, we opted not to duplicate this research. Instead we redirected the is grant application resources towards expanding **Major Task 3** with the addition of KHDRBS3 knockout mouse.

Evaluation of SRSF12 expression was thwarted due to the lack of suitable antibodies. We consider this task completed to the extent feasible.

	Animals with mammary gland tumors	Animals in the group	% Animals with tumors	X^2 statistics
C3.1-Tag; Wild Type	31	46	67%	Control group
C3.1-Tag; SRPK1(+/-)	32	42	76%	X^2=0.84, p-value=0.36 (ns)
C3.1-Tag; KHDRBS3(+/-)	27	33	82%	X^2=2.04, p-value=0.15 (ns)
C3.1.Tag; KHDRBS3(-/-)	10	35	29%	X^2=11.98, p-value=0.00054

Table 1. Rate of breast cancer formation within 12 months of age. X^2 statistics compare each group to the control C3.1-Tag; Wild Type group.

Major Task 5 *Analysis of the regulation of splicing factor expression by the PI3K pathway and cMyc*

We examined the role of PI3K and MAPK pathways in controlling KHDRBS3 expression. Figure 5 shows outline of the experiments. We used inhibitors to block PI3K and MAPK signaling in the MDA-MB-231LN cell that express high levels of KHDRBS3. We also performed

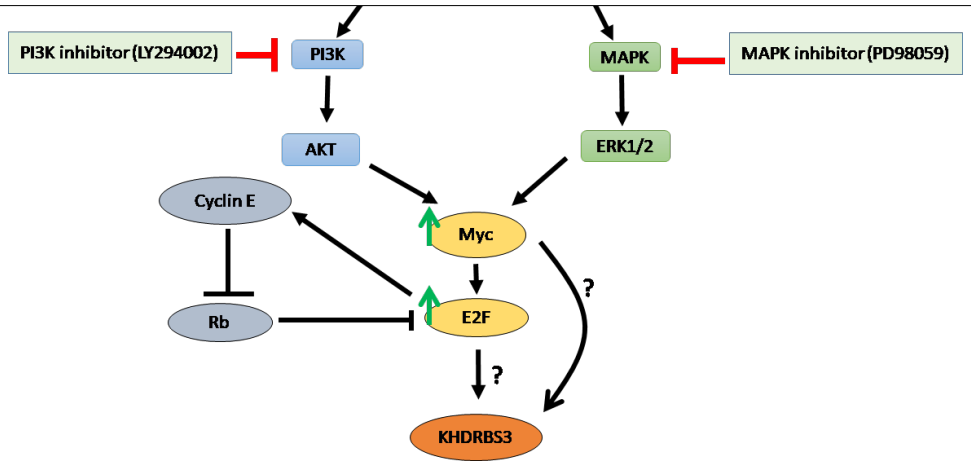

Figure 5. Signaling pathway and experimental design. Growth signal supporting tumor growth are transmitted through the PI3K and MAPK pathways to activate Myc and E2F. We used PI3K and MAPK inhibitors to block these pathways in cell expressing high levels of KHDRBS3. In cells not expressing KHDRBS3 we simulated the signaling pathway activation by expressing downstream targets (Myc, E2F).

the reciprocal experiments where the downstream targets of the two pathways Myc and E2F in MDA-MB-231 cells that express low levels of KHDRBS3. Neither the inhibition of the signaling pathways (Figure 6) nor the expression of the downstream targets (Figure 7) affected the KHDRBS3 levels. We conclude that KHDRBS3 is not controlled by the PI3K and MAPK pathways.

We examined the role of PI3K and MAPK pathways in controlling Srpk1 expression. Figure 1 shows outline of the experiments. We used inhibitors to block PI3 kinase (PI3K) and MAPK/ERK signaling in the MDA-MB-231LN cell line. Neither the inhibition nor the activation of the signaling pathways affected the Srpk1 protein levels. We conclude that Srpk1 is not controlled by the PI3K and MAPK pathways.
Lack of suitable SRSF12 antibodies precludes us from examining its regulation. This task was completed to the extent feasible.

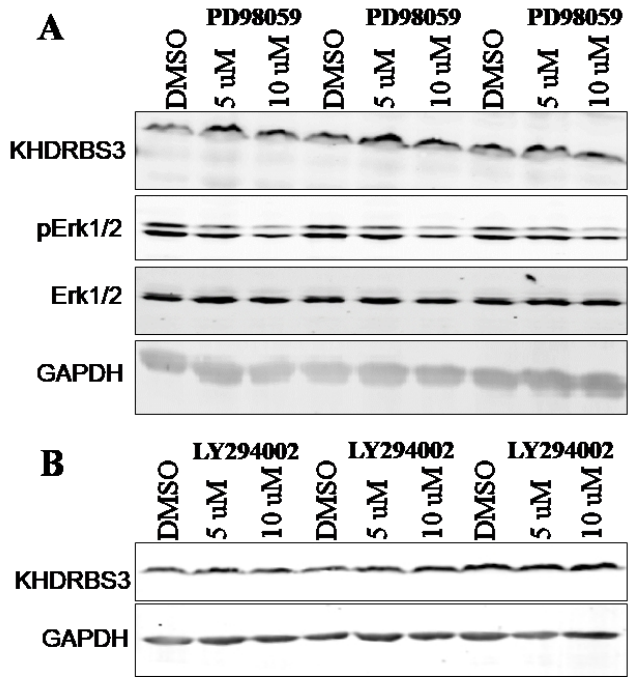

Figure 6. Inhibition of MAPK (**A**) and PI3K (**B**) in MDA-MB-231LN cells does not affect KHDRBS3 protein levels.

Major Task 6 *Analysis of the regulation of alternative splicing in mammary basal epithelial cells by SRPK1, KHDRBS3 and SRSF12*

We analyzed effect of KHDRBS3 and SRPK1 knockdown on splicing in metastatic derivatives of the MDA-MB-231 line by RNA-Seq. We did not see effect of the knockdown on alternative splicing. Gene expression analysis showed dramatic effect of one of the KHDRBS3 shRNAs (shKHDRBS3#1) on gene expression (450 genes showed altered expression levels). We attribute these changes to off-target effects as the second shRNA (shKHDRBS3#4), which is as effective in knocking down the protein affected the expression of only 39 genes. Analysis of alternative splicing showed a minimal effect of KHDRBS3 knockdown on splicing with only two exons located in the ALDH3B1 and SRPK1 showing significant change in inclusion levels in both shRNA knockdowns. The lack of significant effect on gene expression in splicing is consistent with the absence of significant phenotypic differences between the control cells and the KHDRBS3 knockdowns. SRPK1 was previously reported not to have effect on splicing when knocked down in breast cancer cells. We reanalyzed the published raw data and confirmed the lack of effect on splicing.

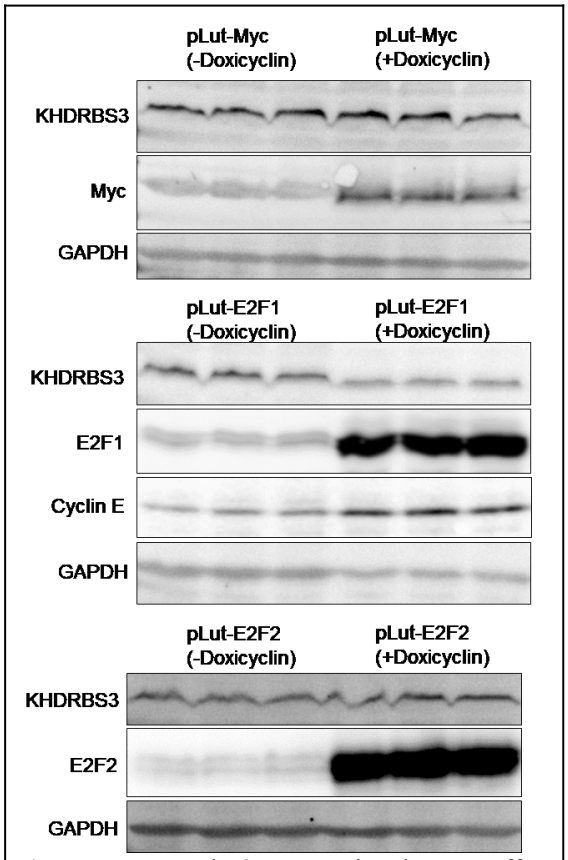

Figure 7. Myc and E2F expression does not affect KHDRBS3 protein levels.

Impact

The research under this project demonstrated that the KHDRBS3 RNA binding protein is supporting mammary tumor formation in a mouse model of triple negative breast cancer. KHDRBS3 does not appear to be regulated by oncogenic pathways common to triple negative breast cancer, and does not change the properties of already transformed cells. Thus KHDRBS3 expression is likely a prerequisite that enables oncogenic transformation. It remains to be determined if knockout of KHDRBS3 in already formed tumors impacts tumor growth in vivo. If this proves to be the case KHDRBS3 will be the first representative of a novel calss of targets for treatment of breast cancer: RNA binding proteins.

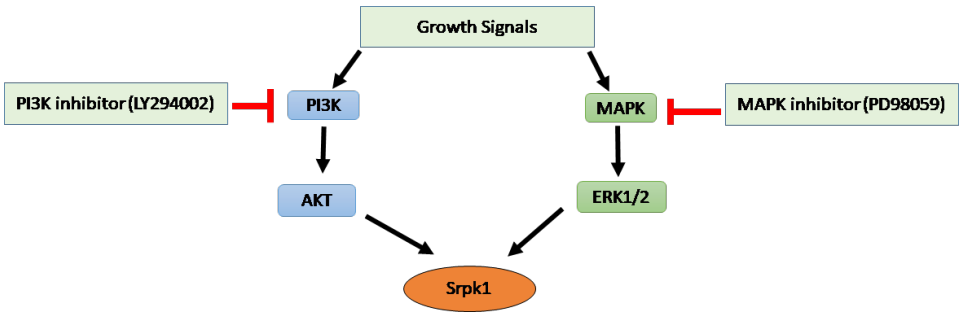

Figure 8. Signaling pathway and experimental design. Growth signal supporting tumor growth are transmitted through the PI3K and MAPK pathways potentially controlling Srpk1 expression. We used PI3K and MAPK inhibitors to block these pathways, or activated them by supplying fetal bovine serum and epidermal growth factor. We then analyzed the effect of these treatments on Srpk1 expression by western blot.

Changes/Problems

Changes:

After submission of the proposal, research substantially similar to the experiments we proposed was published by others. We decided the duplicated the published work is unnecessary. In addition, due to the lack of suitable SRSF12 antibody we were unable to cary out the proposed work related to this protein. The resources that were freed allowed us to test a mouse knockout model of KHDRBS3 for its impact on tumor formation.

Encountered problems:

1. Delayed onset of tumor formation in C3.1-Tag animals. In our hands the C3.1-Tag mice developed tumors considerably later and with more variable time frame than the published literature suggested. This could be due to the SV129 genetic background that we used, or to varibaility in the stock of this line in Jackson labs. We solved this problem by extending the project with one year and increasing the size of the animal groups to address the increase variability.
2. Lack of working SRSF12 antibody. This was a major obstacle as we were unable to find suitable commercial source or generate the antibody ourselves. The lack of this critical reagent prevented us from carrying out the research related to SRFS12. We redirected the resources towards using KHDRBS3 knockout mice to test the role of KHDRBS in tumor formation in vivo.

Products

Nothing to report

Participants & Other Collaborating Organizations

Participants:
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Nearest person months worked: 3

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Project Role: Graduate Student
Nearest person months worked: 7

Changes in active other support:
Nothing to report

Other Organizations:
Nothing to report

Special reporting requirements

Nothing to report

Appendices

Nothing to report

References

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