

ABSTRACT

J. Makenzie Nutter. INTERRELATED ROLE OF NOTCH SIGNALING AND mTORC PATHWAYS IN PROSTATE CANCER CELL SURVIVAL AND GROWTH. (Under the direction of Dr. Fred E. Bertrand). Department of Oncology, July 2014.

Prostate cancer is currently the second highest leading cause of cancer death in men. Notch is a transmembrane receptor protein that is part of a signaling pathway necessary in the normal development of the prostate. Notch1 signaling has been shown to be lost in prostate adenocarcinoma. One of prostate cancer's biggest risk factors is age and mTOR has been shown to be linked to longevity and age related diseases. mTOR exists as two complexes, mTORC 1/2, whose key functions are to control cell survival, metabolism, and growth. mTORC1/2 are often overexpressed in cancer. It was also reported that the mTORC1 pathway became inactivated when Notch1 signaling was inhibited in prostate cancer cell line PC-3. Herein, we suggest a link between Notch1 signaling and mTOR pathway activity which led to experiments with DU145 cells manipulated to have decreased Notch1 expression. The data shows that loss of Notch1 signaling causes decreased expression of the mTORC1 component Raptor as well as decreased phosphorylation of mTORC1 downstream target 4E-BP1 in conditions of cell stress. The mTORC2 pathway exhibited decreased phospho-mTOR (Ser2481) in normal conditions and decreased Rictor signaling in both normal and serum starved conditions when Notch1 expression was lost. The data also suggests there is less G β L in conditions of stress in Notch1 knockdown cells. We hypothesize that downregulation of Notch1 signaling leads to the dysfunction of both mTOR pathways.

INTERRELATED ROLE OF NOTCH SIGNALING AND mTORC PATHWAYS IN
PROSTATE CANCER CELL SURVIVAL AND GROWTH

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PROSTATE CANCER CELL SURVIVAL AND GROWTH

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To all those who never stopped believing in and encouraging me.

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CHAPTER 1

INTRODUCTION

Prostate cancer is the second leading cause of cancer related deaths in men in the United States (Siegel et al, 2013). It is estimated 1 in 6 men will get prostate cancer in their lifetime, with 6 in 10 men at risk for the cancer once over the age of 65. Various therapies exist now such as chemotherapy that are successful in treating primary prostate cancer still in the organ, but the main issue that accounts for such a high mortality rate is that tumors tend to progress to metastatic cancer which currently has no curative therapies (Wang et al., 2010).

In the past few decades, research has arisen showing that Notch signaling has a role in how cancers function. Notch was initially discovered as a gene that caused a “notch” in the wings of *Drosophila melanogaster* (Shellenbarger and Mohler, 1975). It is well understood now that Notch is a transmembrane heterodimeric receptor protein family of four, called Notch1/2/3/4. A human Notch homolog was first documented in 1991 in a case of T-cell acute lymphoblastic leukemia (T-ALL) in which a chromosomal translocation caused a truncated form of Notch that was constitutively active. It was originally called translocation-associated Notch-1 homolog, or TAN-1 (Ellisen et al., 1991).

Notch is conserved in many species. In mammals, Notch has five ligands: Jagged1/2 and Delta-like 1/3/4 (Dll1/3/4) (Fleming, 1998). When a Notch receptor on one cell binds to its ligand on an adjacent cell, a conformation change occurs. This stimulates a series of cleavage events. The conformational change in the Notch receptor reveals an S2 cleavage site that tumor necrosis factor- α converting enzyme

(TACE) will cleave in the Notch extracellular domain (Mumm and Kopan, 2000). Directly after, S3 cleavage occurs within the Notch transmembrane domain by the γ -secretase complex which consists of presenelin-1/2, nicastrin, Pen-2, and Aph-1 (Edbauer et al., 2003). The Notch intracellular domain (NICD) is released into the cytoplasm and can then translocate to the nucleus (Struhl, 1998).

Once inside the nucleus, the NICD will bind to the transcriptional repressor C protein binding factor 1/Suppressor of Hairless/Lag1 (CSL) which converts it to a transcriptional activator (reviewed in Leong and Gao, 2008). This can be seen in Figure 1.1. Notch signaling is therefore responsible for directing expression of its downstream target Hairy/Enhancer of Split 1 (HES1). HES1 is from a family of basic helix loop helix transcription factors that acts as a transcriptional repressor when activated by Notch (Iso et al., 2003).

Normal Notch signaling is crucial for cell differentiation, proliferation and growth. Notch signaling is also critical for postnatal tissue development and adult tissue maturation, and serves as a key regulator of normal prostate development (Artavanis et al., 1999; Wang et al., 2006). The function of Notch in cancer has been shown to have both an oncogenic and a tumor suppressive role. Overexpressed Notch1 receptor signaling has been observed in cancers such as T-ALL (Ellisen et al., 1991), B-cell derived Hodgkin lymphoma (Jundt et al., 2002), and in solid tumors of the brain, cervix, pancreas, and breast (Leong, 2006). A tumor suppressive role for Notch1 signaling has been seen mainly in skin as well as neuroendocrine tumors such as carcinoids (Nicolas et al., 2003; Kunnimalaiyaan et al., 2005). It is clear from the various reports that Notch signaling varies between two crucial functions among cancers. More research is critical

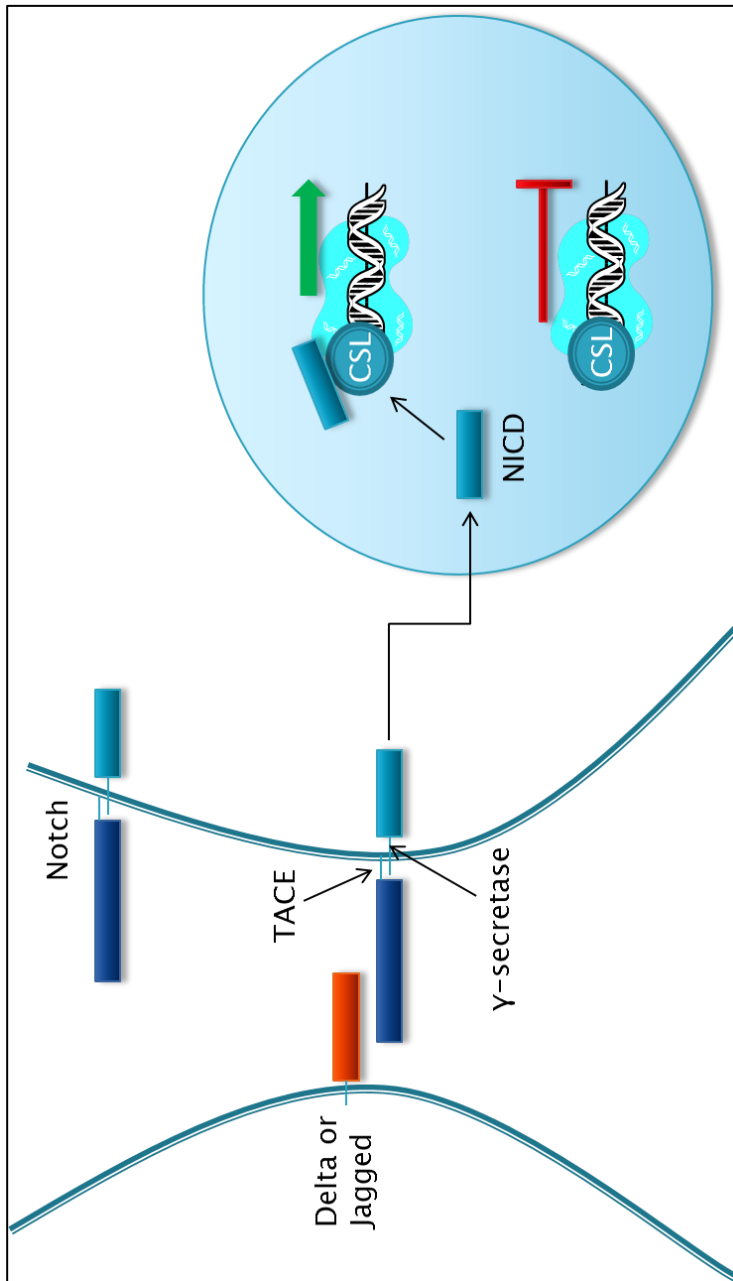
to understanding how Notch functions for cancers such as prostate cancer with high diagnosis and mortality rates. However, it is not clearly understood if Notch promotes or inhibits tumorigenesis in the prostate.

Loss of phosphatase and tensin homolog (PTEN) expression and/or function is observed in up to 60% of clinical prostate cancer cases (Li et al., 1997). PTEN is a tumor suppressor gene that has been shown to be regulated by Notch1 signaling, in that PTEN gene expression goes up when Notch1 signaling is overexpressed (Chappell et al., 2005; Whelan et al., 2009). PTEN has three known functions, as a lipid phosphatase (Maehama and Dixon, 1998), a protein phosphatase (Li et al., 1997; Myers et al., 1997), and a phosphorylation substrate (Torres et al., 2003). Most commonly, PTEN is known as a negative regulator of the proto-oncogenic phosphoinositide (PI) 3-Kinase/Akt pathway.

Briefly, PTEN functions as a lipid phosphatase to target active phosphatidyl inositol 3,4,5-triphosphate (PIP₃) and remove the phosphate at the 3' position of the inositol ring, converting it to the inactive precursor PIP₂ so that downstream signaling to other pro-survival effector proteins like Akt is prevented (reviewed in Paez and Sellers, 2003). The PI3-K/Akt pathway normally exerts survival and proliferative signals in cells when not bound to PTEN (Salmena et al., 2008). When PTEN is lost in cancers, this allows for constitutive activation of the PI3-K/Akt pathway which helps the cancer cells survive and grow (Vivanco & Sawyers, 2002).

Along with the dysregulation of the PI3-K/Akt pathway, the mammalian target of rapamycin (mTOR) is often overexpressed in cancer (Alvarez et al., 2003; Yu et al., 2009). mTOR is a protein Ser-Thr kinase involved in the control of cell growth and

Figure 1.1: Notch signaling pathway. Upon binding to a ligand from an adjacent cell, the Notch receptor undergoes two cleavage events. The first cleavage event, known as S2, is performed by an ADAM metalloprotease called TACE which cleaves the extracellular portion of the Notch receptor. S3 cleavage then occurs using the γ -secretase complex which cleaves in the intermembrane to release the Notch intracellular domain (NICD) which can then translocate to the nucleus, bind to the transcriptional repressor CSL, and allow transcription to occur.



proliferation (Harris et al., 2003). mTOR exists as two distinct complexes with very distinct functions. mTOR complex 1 (mTORC1) forms when mTOR, Raptor (regulatory associated protein of TOR) and GβL (also known as LST8) join together (Jacinto & Hall, 2003). mTORC1 has the ability to phosphorylate downstream targets that are necessary for translation to occur and activation of this pathway ultimately leads to protein synthesis and cell growth, seen in Figure 1.2.

mTOR complex 2 (mTORC2) was discovered in 2004 and forms a complex with mTOR, Rictor (rapamycin insensitive component of TOR), and GβL (Sarbasov et al., 2004). Its functions have not been clearly defined, but it is now understood that mTORC2 plays a role in cytoskeleton formation (Jacinto et al., 2004) and in phosphorylation of Akt at Serine 473 (Sarbasov et al., 2005). This particular phosphorylation of Akt at Ser473 is not necessary for normal functioning. Akt plays an important role in the mTOR pathways in that it both affects and is affected by mTOR depending on which complex is formed and functional.

mTOR is a nutrient and metabolic sensor that is activated when nutrients and growth factors, such as insulin, are readily available (Fingar & Blenis, 2004). When nutrients are present, the PI3-K/Akt pathway activates. Akt will phosphorylate and inactivate the tuberous sclerosis complex 1 (TSC1) which is a tumor suppressor responsible for suppressing mTOR activity (Gao et al., 2002, Inoki et al., 2002). From here, mTORC1 can go on to phosphorylate its downstream targets 4E-BP1 (Graves et al., 1995) and p70 S6 kinase (S6K) (Chung et al., 1992). In the case of 4E-BP1, phosphorylation from mTOR causes it to bind less tightly to its target eIF4E (Lin et al., 1994) which allows eIF4E to form complexes important for cap-dependent translation

(Beretta et al., 1996). mTORC1 mediated phosphorylation of p70 S6K has several translational control functions which include positively regulating helicase activity (Shahbazian et al., 2006) and enhancing translation of newly spliced mRNAs (Ma et al., 2008).

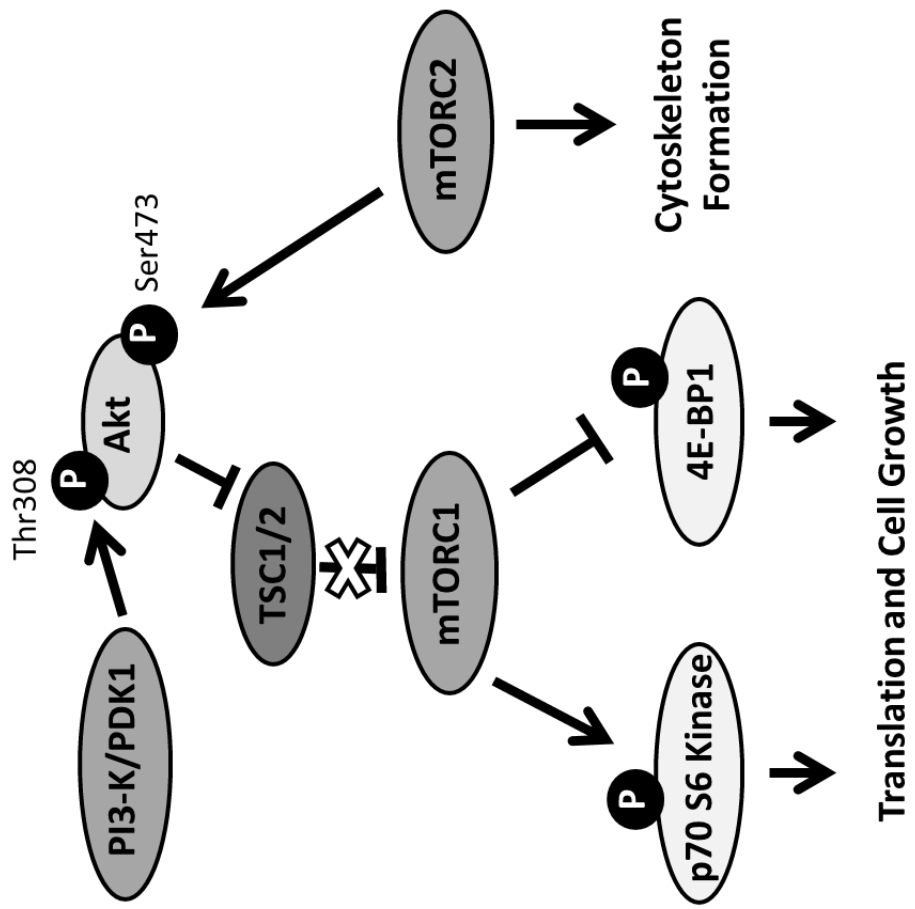
Much less is known about mTORC2. Recent data has suggested very specific roles for it in both prostate cancer upon loss of PTEN in mice (Guertin et al., 2009) and in regulating glucose metabolism with a suggested role in the development of type II diabetes (Kumar et al., 2008). Its most well-known roles are in cytoskeleton formation and specific, but not necessary for function, phosphorylation of Akt at Ser473. However, it is still not clear what upstream regulators there are for this pathway. Several studies recently however have suggested Notch signaling plays a specific role in mTOR functions.

Knockdown of Notch1 and its ligand Jagged1 in the prostate cancer cell line PC-3 has been shown to inhibit prostate cancer cell growth and lead to apoptosis due to inactivation of the Akt and mTOR pathways (Wang et al., 2010). Another study has shown that mTOR regulates cell differentiation by serving as a positive regulator of Notch signaling (Ma et al., 2010). A study on mutant T-ALL cells showed that by inhibiting PI3-K/mTOR pathways, Notch signaling was increased and that there was Notch induced resistance to PI3-K/mTOR inhibition (Shepherd et al., 2013). These have important implications of the treatment of cancers and diseases with overexpressed Notch signaling with use of combinational Notch/mTOR inhibitor drug therapies.

In this present study, Notch1 signaling was knocked down in DU145 metastatic prostate tumor cells. These cells, called DU/shN1, were used to examine the effects of

Notch1 loss on the mTOR pathways. Loss of Notch1 resulted in decreased expression of downstream target S6 ribosomal protein and in phosphorylation of 4E-BP1 in both normal and serum starved conditions. Loss of Notch1 expression also played a role in decreasing mTOR signaling through decreased expression of the key complex components Raptor and Rictor. The data also suggests there is less GβL in conditions of stress in Notch1 knockdown cells.

Figure 1.2: mTOR signaling pathway. When PI3-K is activated, it will phosphorylate Akt at Thr308. Phosphorylated Akt binds and deactivates TSC1/2. TSC1/2 can no longer repress mTOR signaling and the complexes can form. mTOR complex 1 is phosphorylated at Ser2448 and will phosphorylate 4E-BP1 to inactivate it and p70 S6 Kinase to activate it, both of which events lead to cell proliferation and growth. mTOR complex 2 is phosphorylated at Ser2481 and can affect actin cytoskeleton formation and also loop back to phosphorylate Akt at Ser473 which helps to maintain Akt activity.



CHAPTER 2

METHODS

Cell lines

DU145 cells were purchased from the ATCC. DU145 cells were originally derived from brain metastasis of a prostate cancer patient, and are thus considered representative of metastatic prostate cancer (Stone et al., 1978). Cells were maintained in RPMI supplemented with 10% heat inactivated fetal bovine serum (FBS) (Cellgro; Herndon, VA) at 37° C with 5% CO₂, penicillin (10 units/ml), and streptomycin (10 µg/µl) (Invitrogen; Carlsbad, CA). The stable transformed cell lines, DU PLK 1.0 and DU/shN1, were maintained as described above and also treated with puromycin (2 µg/ml) (Sigma; St. Louis, MO). For serum starved cells, 1.5 x 10⁶ parental DU145, DU PLK 1.0, or DU/shN1 cells were plated on tissue culture plates in complete RPMI medium and cultured for 6 hours. Next, media was removed and cells were rinsed twice with phosphate buffer saline (PBS) and cultured in serum free RPMI. Cells were incubated overnight and harvested 16 hours later for protein analysis. For insulin treatment, cells were serum starved as described above and before harvesting were treated with bovine insulin (2 µl/10ml) (Sigma; St. Louis, MO) and incubated for 10 minutes before being harvested for protein collection.

Lentiviral packaging

shRNA lentiviral constructs against Notch1 were purchased from Open Biosystems (Lafayette, CO). Lentiviruses were packaged per manufacturer's instructions. Briefly, viral constructs were packaged with an amphotropic coat in 293 cells. Forty eight hours post-transfection, viral supernatant was collected and used to transduce DU145 cells. Transduced DU145 cells were selected in 2µg/ml of puromycin.

Cell Dividing Time assay

Two hundred thousand cells from the following cell lines were plated into individual cell culture plates containing complete RPMI: DU145, DU PLK 1.0, and DU/shN1. After 96 hours, the cells were fully trypsinized and counted with a hemacytometer to determine the total number of cells in the plate. The duplication time was calculated using the following formula: $d = t * \ln(2) / \ln(N_t / N_0)$ where N_t is the number of cells at the time of counting, N_0 is the initial amount of cells, t is the hours of growth, and d is the duplication time in hours.

Western blot analysis

Total cell lysate protein was quantitated based on cell number and prepared in 1 ml RIPA lysis buffer per 3 million cells. Lysates were stored at -80° C. Twenty μ l of total cellular protein from each sample was loaded per well and separated on 8% or 12% SDS-PAGE gels, transferred to PVDF membranes, and blocked in 1% bovine serum albumin (BSA) at room temperature for 1 hour. Blots were probed with primary phospho-specific antibodies (1:1000 dilution in 1% BSA) or total protein recognizing antibodies (1:1000) overnight at 4° C, and either β -tubulin (hybridoma) or β -actin (1:5000) as loading controls. Horseradish peroxidase (HRP) conjugated secondary antibodies (anti-rabbit or -mouse) diluted (1:5000) in TBST (25mM Tris-HCl [pH 8.0], 125mM NaCl, 0.02% Tween-20) were used to detect protein-bound primary antibody. Horseradish peroxidase chemiluminescence substrate (Pierce; Rockfield, IL) and radiography film were used to visualize protein bands. All primary antibodies can be found in Table 2.1.

Table 2.1: Primary Antibodies

Antibody Name	Company	Catalog #
mTOR	Cell Signaling	2972
p-mTOR (Ser2448)	Cell Signaling	5536
p-mTOR (Ser2481)	Cell Signaling	2974
Notch1 (D6F11)	Cell Signaling	4380
Akt (pan) (11E7)	Cell Signaling	4685
p-Akt (Ser473) (D9E)	Cell Signaling	4060
4E-BP1 (53H11)	Cell Signaling	9644
p-4E-BP1 (Thr37/46)	Cell Signaling	2855
S6 Ribosomal Protein	Cell Signaling	2217
Raptor (24C12)	Cell Signaling	2280
Rictor (53A2)	Cell Signaling	2114
p-Rictor (Thr1135)	Cell Signaling	3806

Transwell migration assay

Transwell inserts (Costar; Corning, NY) were placed in a 24 well plate, with three transwells for each cell line for each experiment. Cells were washed with PBS then brought up in serum free RPMI media to a dilution of 1×10^6 cells/ml. Then, 100 μ l was added to the top of the transwells and incubated for ten minutes at 37° C. A stock of RPMI with 20% serum was made and at the end of the incubation period, 600 μ l of the stock was added to the bottom of each transwell chamber to serve as a chemoattractant for the cells. The 24 well plate was then incubated overnight at 37° C. The next day,

cells were removed from the top layer of the transwells and fixed in 70% ethanol for ten minutes then removed and allowed to dry for five minutes. Cells were then placed in 0.2% crystal violet for ten minutes to stain cells that had migrated through the transwell membrane. Transwells were then washed in dH₂O and allowed to dry before counting.

CHAPTER 3

RESULTS

Generation of DU145 cells with decreased expression of Notch1. In order to study the effects of loss of Notch1 on the mTOR complexes, we generated DU145 cells that stably express a shRNA to abrogate Notch1 expression (DU/shN1) as well as DU145 cells that express the empty vector alone (DU PLK 1.0). Stably transfected cells were isolated using puromycin treatment with every cell passage due to the vector containing a gene that confers puromycin resistance to the cells. Loss of Notch1 was verified by Western blot analysis using an anti-Notch1 antibody (Cell Signaling), seen in Figure 3.1.

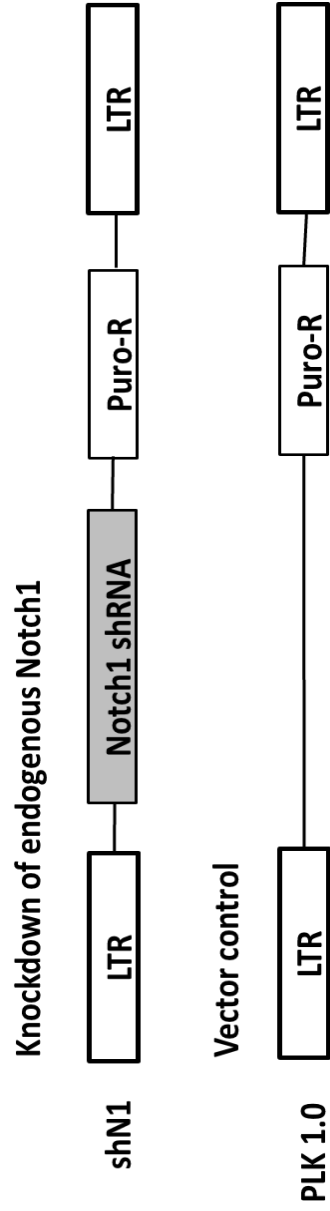
DU/shN1 cells have slower dividing time and decreased migratory ability. A cell dividing time assay was conducted as previously described to determine if there were any differences due to down regulation of Notch1. Three experiments were run on each cell line. On average, DU/shN1 cells were at least three hours slower to divide than DU PLK 1.0 cells and at least four hours slower to divide than DU145 cells, seen in the chart in Figure 3.2A. A student t-test confirmed these differences were not statistically significant.

Transwell migration assays were also performed as previously described to test migratory ability of cells with knockdown of Notch1. Results indicate that DU/shN1 cells have a significantly decreased ability to migrate when compared to DU145 and DU PLK 1.0 cells, which can be seen in the chart of Figure 3.2B. Statistical significance was determined by student t-test.

Downregulation of Notch1 causes no change in mTOR expression. Due to the reduction in cell dividing time and migratory ability when Notch1 expression was reduced, we next examined the mTOR pathway. Figure 3.3 shows the results of

Figure 3.1: Establishment of stable Notch1 knockdown DU145 cells. A) Lentiviral constructs. The constructs used are lentiviral constructs in which shN1 contains the silencer for downregulating Notch1 signaling and PLK 1.0 is the empty vector control. Constructed DU/shN1 and DU PLK 1.0 cells were purified using puromycin as previously described which was included in the vector constructs. **B) Confirmation of Notch1 knockdown.** Stable knockdown of Notch1 signaling was confirmed by Western blot.

A) Lentiviral Constructs



B) Notch1 Knockdown

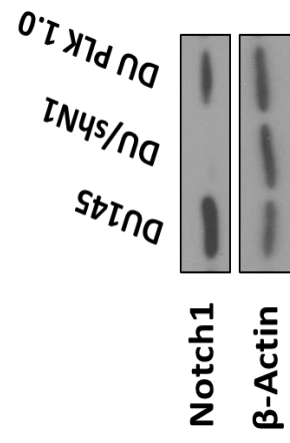
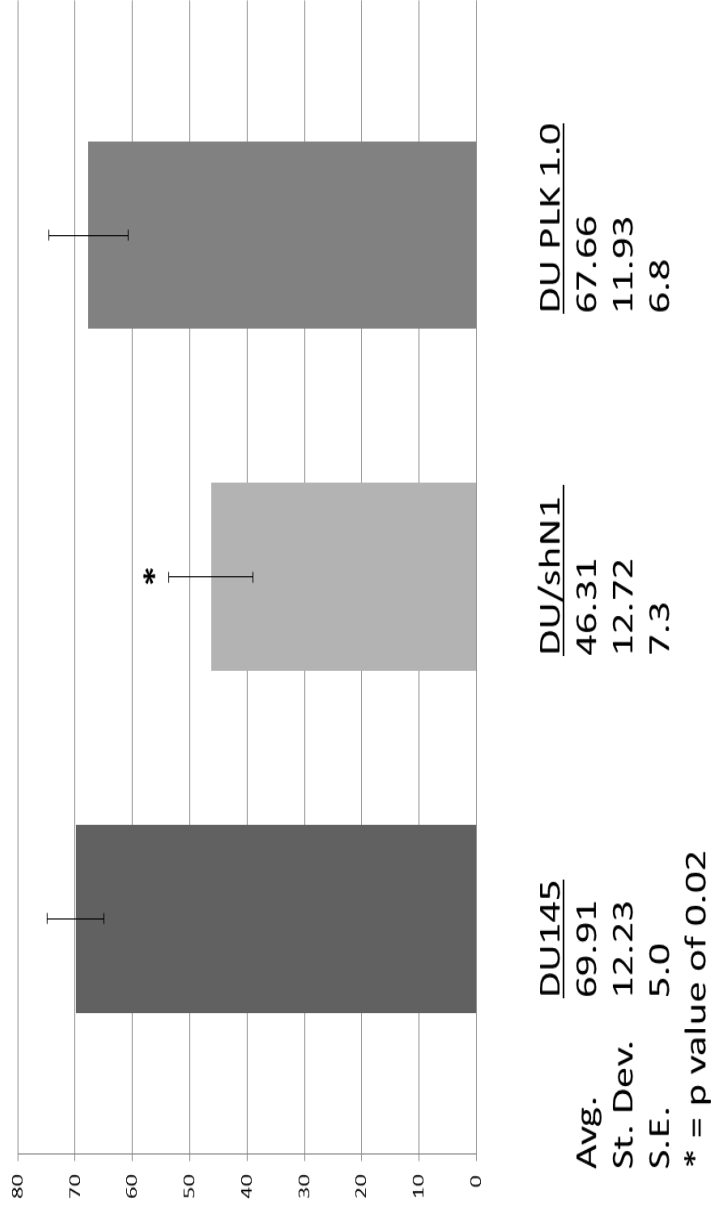


Figure 3.2: DU/shN1 cells have slower dividing time & decreased migratory ability. A) Cell Dividing Time. The chart lists the results of three consecutive cell dividing time assays for all three cell lines. DU/shN1 cells on average grew four hours slower than DU145 control cells and three hours slower than the DU PLK 1.0 vector control cells. Student t-tests reveal these are not statistically significant differences. **B) Migration Assay.** Three migration assay sets of three wells per cell line were performed. The (*) denotes a statistically significant decrease in migratory ability of DU/shN1 cells from DU145 cells as determined by a T-test where a p value less than 0.05 is considered statistically significant. Avg. = Average, St. Dev. = Standard Deviation, S.E. = Standard Error

A) Cell Dividing Time

Cell Dividing Time	DU145	DU PLK 1.0	DU/shN1
Trial 1	24 h	25 h	28 h
Trial 2	20.5 h	21.5 h	24.5 h
Trial 3	21.5 h	22.5 h	25.5 h
Average	22 h	23 h	26 h

B) Migration Assay

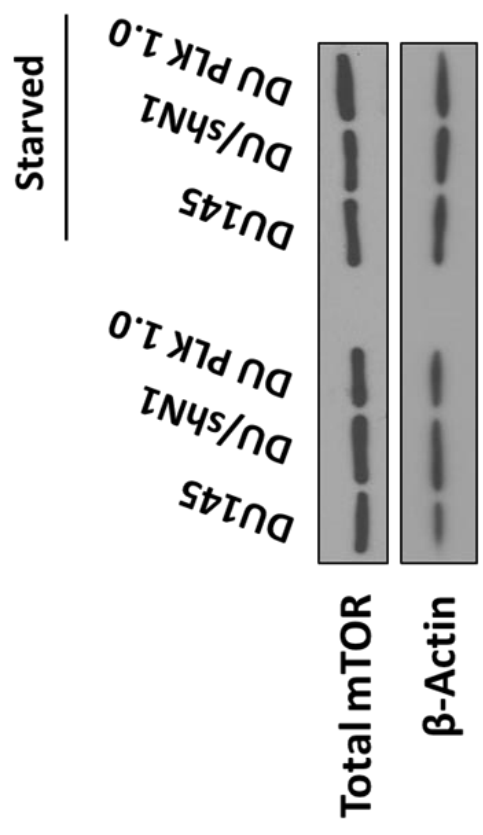


Notch1 knockdown on total mTOR expression. No significant differences in total mTOR expression were observed between cell lines in either control conditions or serum starved conditions.

Downstream targets of mTORC1 show altered signaling in DU/shN1 cells in serum starved conditions. Downstream targets of mTORC1 were next observed to see if the changes in cell growth and migratory ability were due to components responsible for translation in Notch1 knockdown cells. Results in Figure 3.4A show p-4E-BP1 expression was decreased in DU/shN1 cells in the serum starved condition, suggesting that cells without Notch1 expression are more sensitive to nutrient deprivation through the mTOR pathway. In Figure 3.4B, results show that total S6 ribosomal protein decreases in DU/shN1 cells when compared to both controls, although there appears to be a modest decrease in DU PLK 1.0 cells as well. Due to the observed evidence that mTOR signaling may be decreased as seen in a reduction of p-4E-BP1, we examined the phosphorylation status of mTOR which is required for complex assembly and expression of Raptor and Rictor, key components of mTORC1 and mTORC2, respectively.

Expression of mTORC1&2 complex components, Raptor and Rictor, is reduced in DU/shN1 cells. Figure 3.5A shows the results of Notch1 knockdown on the mTORC1 pathway components. Although phosphorylation of mTOR at Ser2448, which is generally a mTORC1 specific site, remains the same, there is a reduction of Raptor expression upon serum starvation of DU/shN1 cells. This loss of a necessary component to the complex could lead to the dysregulation of mTORC1's downstream targets. However, there also appears to be a decrease of Raptor expression in DU145

Figure 3.3: Loss of Notch1 causes no changes in mTOR expression. Cells were lysed in normal conditions and serum starved overnight conditions to test if the mTOR expression was decreased at any point when Notch1 signaling was knocked down. There were no differences in expression levels between cells in either condition.

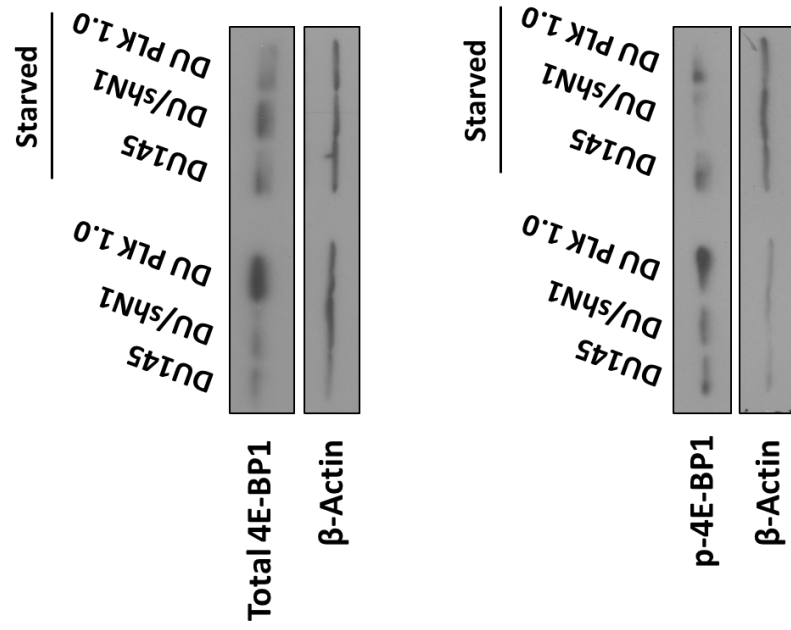


cells when compared to DU PLK 1.0 cells, so the degree to which Notch1 is involved is less clear. For mTORC2, the results seen in Figure 3.5B show that both mTOR phosphorylated at Ser2481, an mTORC2 specific site, and Rictor have decreased signaling in normal conditions. In serum starved conditions, it appears that mTOR overexpresses phosphorylation at Ser2481, but Rictor expression remains reduced. These results can also explain reduced mTOR signaling, through reduction of Rictor and failure to form the mTORC2 complex.

Preliminary data shows a decrease in G β L, a key component to both the mTORC1 and mTORC2 complex, in DU/shN1 cells. G β L is a key component in the formation of both mTOR complexes, and is lost in DU/shN1 cells in starved + insulin conditions. This result also needs to be further studied, as there is less in DU145 cells than in DU PLK 1.0 cells. This result can be seen in Figure 3.6.

Figure 3.4: mTORC1 mediated phosphorylation of downstream targets decreased in Notch1 knockdown cells in serum starved conditions. A) Total 4E-BP1 expression is marginally increased in DU/shN1 cells in serum starved conditions when compared to controls. This result is confirmed in that there is a loss of phosphorylated 4E-BP1 in Notch1 knockdown cells in starved conditions. **B)** Total S6 ribosomal protein signaling is lost in DU/shN1 cells in serum starved conditions when compared to both controls.

A)



B)

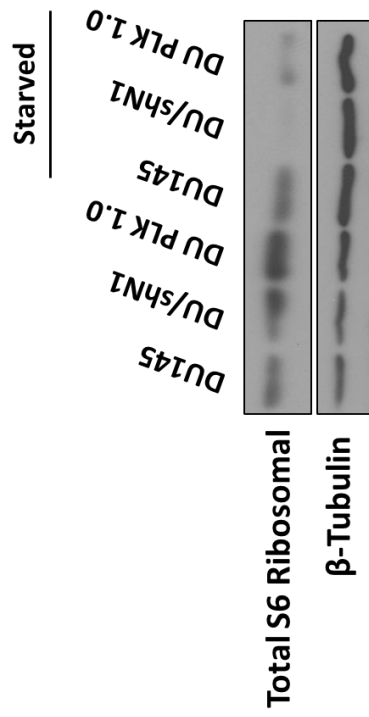
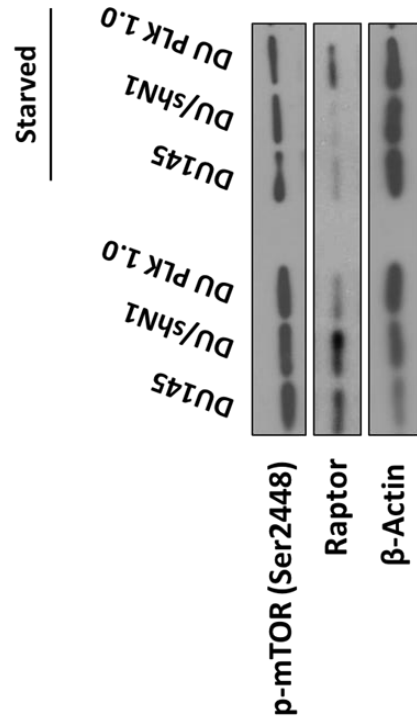


Figure 3.5: Downregulation of Notch1 causes dysregulation of both mTOR complexes' components. A) Loss of Notch1 causes decreased Raptor expression in conditions of cell stress. Raptor expression is lost in Notch1 knockdown cells in serum starved conditions. **B) Decreased Notch1 correlates with decreased Rictor expression in normal and serum starved conditions.** In normal conditions, DU/shN1 cells have decreased phosphorylation of mTOR at Ser2481 when compared to both controls although differences in expression when compared to DU145 cells appear minimal. In normal conditions, Rictor expression is also reduced in DU/shN1 cells. Upon serum starvation, phosphorylation of mTOR at Ser2481 increases but Rictor expression still remains less active when compared to controls.

A) mTORC1



B) mTORC2

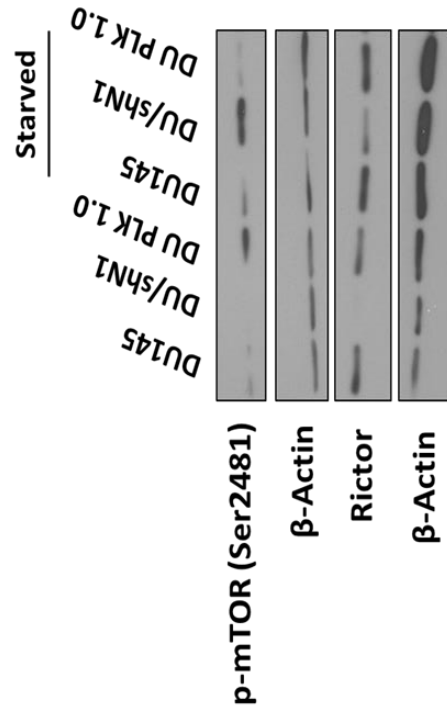
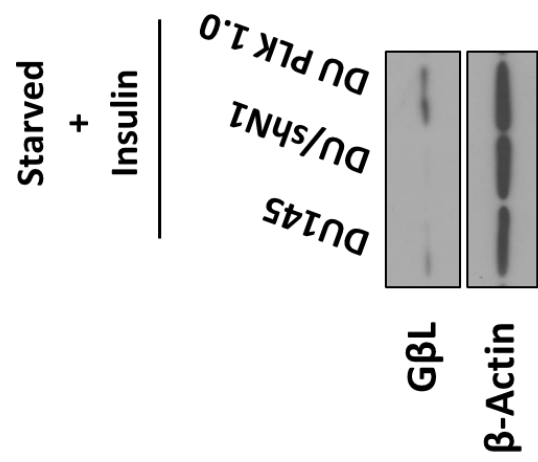


Figure 3.6: Preliminary data reveals a decrease in G β L expression in serum starved + insulin condition of DU/shN1 cells. G β L has decreased expression in Notch knockdown cells in starved + insulin conditions when compared to both controls, although there is a slight decrease in expression in DU145 cells when compared to DU PLK 1.0 cells.



CHAPTER 4

DISCUSSION

To further examine the interaction between Notch1 signaling and the mTOR pathways, the human metastatic prostate cancer cell line DU145 was transduced with a shRNA lentivirus to reduce Notch1 expression (DU/shN1). DU145 cells were also transduced with a control vector (DU PLK 1.0). Notch1 knockdown was verified by Western blot analysis. Initial observations of the newly transfected cell lines demonstrated that Notch1 knockdown resulted in a slower cell dividing time and a decreased ability to migrate. Changes to cell morphology were not observed. These results led to the hypothesis that loss of Notch1 signaling was inhibiting a pathway responsible for cell growth and proliferation.

mTOR is commonly overexpressed in cancer. mTOR is responsible for controlling cell growth and proliferation. Current research suggests that there is a link between Notch signaling and mTOR in cancers (Wang et al., 2010; Shepherd et al., 2013), although the precise mechanism is not clearly understood. By knocking Notch1 expression down, our findings indicate mTOR loses some degree of functionality.

The effects of downregulated Notch1 on mTOR are intriguing. Although there is no change in total mTOR expression, expression of the mTORC2 component Rictor is reduced. Most of the disrupted signaling is seen in conditions of cell stress due to serum starvation. Serum starving cancer cells in vitro is a way to mimic metabolically stressed cells in vivo to see how they adapt to a new environment (Levin et al., 2010). Adaptation is an important characteristic of cancer cells, for without it the cells would not be able to survive or metastasize. Serum starvation may also serve as a way to “prime” cells for

death (Braun et al., 2011). Stress in a cell induces activation of many survival pathways in order to restore the cell's health, yet these pathways must be controlled to prevent aberrant effects.

When Notch1 signaling is lost, there is a decrease in expression of Raptor, phospho-4E-BP1, and S6 ribosomal protein under serum starvation. However, the fact that mTOR expression remains the same suggests that Notch1 does not play a role in mTOR directly but with its components. The fact that the downstream targets of mTORC1 also have decreased expression indicates that the complex is disrupted.

There is a decrease of phosphorylation of 4E-BP1 seen in DU/shN1 cells in starved conditions when compared to either control. This loss of phosphorylation by the mTOR complex allows for 4E-BP1 to remain active and prevent cap dependent translation (Beretta et al., 1996). In the case of p70 S6 kinase, the other downstream target of mTORC1, this research was unable to see it on a Western and instead looked at p70 S6 kinase's direct downstream target S6 ribosomal protein. Results show there is a loss of total S6 protein signaling in starved conditions of DU/shN1 cells. Without S6 protein, several translational control functions may be lost, as mentioned previously.

A decrease in cell growth is seen in the cell growth assays in which DU/shN1 cells have slower growth rates than both DU145 and DU PLK 1.0 cells, although t-test shows that these are not statistically significant differences. Cells also have a decreased migratory ability as seen by the transwell migration assays which do have statistical significance. The results of both assays support a disruption in mTOR complex formation.

A preliminary look into GβL shows a very interesting result. It appears that in conditions of serum starvation plus addition of insulin, there is no detectable expression of GβL in DU/shN1 cells. Preliminary probing of GβL expression in normal and serum starved conditions are inconclusive at this time. Further investigation is needed to confirm this result, but it would explain why both mTOR complexes are not functioning correctly, in that it is a key component to both mTOR complexes.

A study in 2009 showed that Notch1 signaling played a non-canonical role in activating mTORC2 in HeLa cells. This was believed to activate an anti-apoptotic pathway when cells underwent stress due to serum starvation (Perumalsamy et al., 2009). This paper would be consistent with the findings that Notch signaling is overexpressed in many types of cancer. The more Notch signaling, the less likely a cancer cell is to die during its metastatic phase or when treated with chemotherapeutic drugs. Those studies are consistent with our findings in that as we lose Notch signaling we observe tumor suppressive effects.

All of these results lead to a very interesting potential for new combinational drug therapy for prostate cancer. This research suggests that when Notch1 signaling is knocked down, DU/shN1 cells lose migratory ability and have a slower growth rate. The changes in the mTORC2 pathway when Notch1 is decreased are especially intriguing because there is currently very little literature in what are upstream effectors of this specific pathway. There are drugs currently available used to inhibit both Notch and mTORC1 signaling individually to treat cancer. It may be possible to treat with both a mTOR inhibitor and Notch inhibitor, in which both mTORC1 and Notch are inhibited. If Notch1 truly is an upstream effector of mTORC2, mTORC2 signaling will be knocked

out as well and cancer cells will lose an entire major pathway responsible for growth and survival. Further research is needed to test the hypothesis that Notch-1 may be an upstream effector of mTORC2.

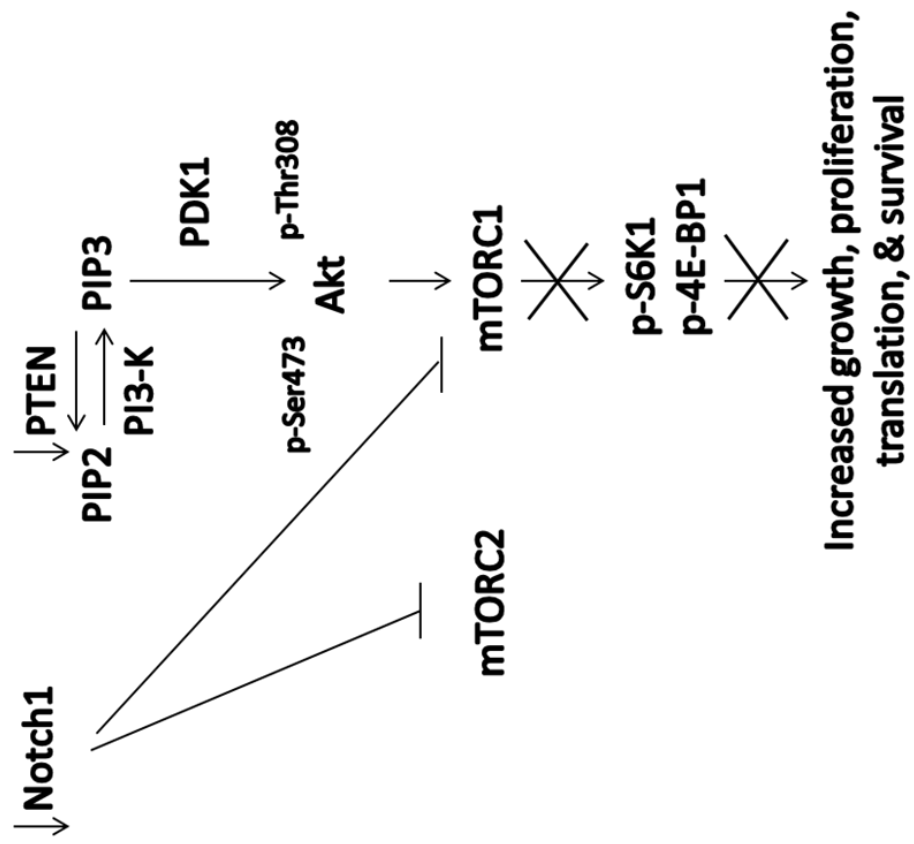
Figure 4.1 proposes a simplified model of what we believe the research is showing. Notch1 signaling has already been suggested to play a role in regulating PTEN which serves as a key component in the PI3-K/Akt/mTOR survival pathway. In most prostate cancers, PTEN function is lost and therefore the PI3-K/Akt/mTOR pathway is activated and can lead to uncontrollable cell growth and proliferation. Herein, we suggest that there is a very specific balance to Notch1 signaling in prostate cancer. Previous research has suggested that overexpression of Notch1 in DU145 cells causes an increase in PTEN expression which would have a tumor suppressive effect, but this research shows that significant loss of Notch1 also has a tumor suppressive effect in the DU145 cell line.

In our model, Notch signaling and PTEN are signaling low enough to the point that the PI3-K/Akt/mTOR pathways still function. When Notch1 signaling is further decreased, mTOR complex formation is negatively impacted and mTORC1 loses most of its kinase functions to the extent cell growth is slowed and migratory ability is lessened. Further studies should be performed to look into the loss of G β L and its link to Notch signaling.

A proposed experiment for future research in this area would be to look at DU145 cells treated with Notch and mTOR signaling inhibitors in combination as well as individually. Looking at individual and combined treatment would allow for us to see if there are differences in cell death rate and how the mTOR pathways change based on

the drug treatment. If Notch1 is a true effector of the mTORC2 pathway directly, it could be the key to knocking out the entirety of one of the most overexpressed pathways in prostate cancer.

Figure 4.1: Proposed Notch signaling model. This simplified model shows the suggested role of Notch signaling in prostate cancer. On the right hand side, Notch plays a role in maintaining inactive PTEN so that the PI3-k/Akt/mTOR pathway is active and this leads to cell growth, proliferation, translation, and survival through the mTORC1 pathway. When Notch1 expression is lost, Notch plays a role in mTOR complex formation to the extent mTORC1 loses some kinase activity, especially in serum starved conditions.



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