Notch-1 Activates Nf-\(\text{К}b\) Activity in Cervical Cancer and Estrogen Receptor Negative Breast Cancer

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LOYOLA UNIVERSITY CHICAGO

NOTCH-1 ACTIVATES NF-kB ACTIVITY IN CERVICAL CANCER AND ESTROGEN RECEPTOR NEGATIVE BREAST CANCER

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR AND CELLULAR BIOCHEMISTRY

BY

YIN PENG

CHICAGO, IL

MAY 2010
ACKNOWLEDGMENTS

Everyone has an American dream, so do I. Coming to the United States and studying here was my wildest dream. I made it.

However, this trip was not easy. I could not have made it without the blessings from God. I had the best mentor in the world, Dr. Lucio Miele. He is smart, knowledgeable, passionate, positive and very encouraging. I could never express my appreciation enough for him. I also had the most supportive committee members: Dr. Manteuffel, Dr. Denning, Dr. Diaz and Dr. Schultz. I want to give my deepest gratitude for their critical suggestions and useful advices.

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Lab has been home for me for five years. I grew and fell, laughed and cried in the lab. I would like to extend my sincerest appreciation to my lab family members: Antonio,
Lynda, Paola, Clodia, Lu, Gwen, Jieun, Shilpa, Debby, Parul, Theresa and Sue. Antonio, Lynda and Paola were my direct supervisors who have taught me not only how to be a good scientist but also a great person. I also want to thank the Osipo lab, the Bocchetta lab and the Foreman lab for their invaluable advices and generous sharing.

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Five years in graduate school meant a five year long-distance relationship for me and my fiancé Yanjie. I could not survive graduate school without his continuous support and enormous patience.

Most importantly, I want to thank my family: my dad, mom and sister. My dad passed away because of lung cancer when I was little. I dedicated my cancer research to my dear father. I could never pay off my debt to my parents and sister who have always been there for me no matter what.

Love is what I received and gave in the course of my thesis work.
DEDICATED TO MY FAMILY
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<tr>
<td>ADAM</td>
<td>A Disintegrin and Metalloprotease</td>
</tr>
<tr>
<td>ANKR</td>
<td>Ankyrin repeats</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>Bid</td>
<td>BCL-2 interacting domain</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculoviral IAP repeat</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer susceptibility gene</td>
</tr>
<tr>
<td>CBF-1</td>
<td>C-repeat/DRE binding factor-1</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CDK9</td>
<td>Cyclin-dependent kinase 9</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>c-IAP</td>
<td>cellular inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>CIR</td>
<td>CSL-interacting repressor</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic-AMP responsive element binding</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF-1, Su (H) and LAG-1</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DLL</td>
<td>Delta-like</td>
</tr>
<tr>
<td>DR</td>
<td>Death receptor</td>
</tr>
<tr>
<td>DSL</td>
<td>Delta-Serrate-Lag2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GSI</td>
<td>Gamma secretase inhibitor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HATs</td>
<td>Histone acetyltransferases</td>
</tr>
<tr>
<td>HDACs</td>
<td>Histone deacetylases</td>
</tr>
<tr>
<td>HES</td>
<td>Hairy enhancer of split</td>
</tr>
<tr>
<td>HERP</td>
<td>Homocysteine-induced endoplasmic reticulum protein</td>
</tr>
<tr>
<td>Hey</td>
<td>Hair/E(spl)-related with YRPW motif</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>IKK</td>
<td>I kappa B kinase</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of kappa B</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAML1</td>
<td>Mastermind like 1</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>MIF</td>
<td>Migration inhibitory factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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NCOR  Nuclear receptor corepressor
NF-κB  Nuclear factor of kappa B
NIC  Notch intracellular
NIK  NF-κB inducing kinase
NLS  Nuclear localization signal
PCAF  p300 associated factor
PEST  Proline, glutamate, serine, threonine-rich
PI3K  Phosphatidylinositol 3-kinase
PIP3  Phosphatidylinositol triphosphate
PKC  Protein kinase C
PR  Progesterone receptor
PTEN  Phosphatase and tensin homolog
RHD  Rel-homology domain
RIP  Receptor interacting protein
ROS  Reactive oxygen species
RTKs  Receptor tyrosine kinases
SERM  Selective estrogen receptor modulator
SKIP  Ski interacting protein
SKP2  S-phase kinase-associated protein 2
Smac  Second mitochondria-derived activator of caspase
SMRT  Silencing mediator of retinoic acid and thyroid hormone receptor
SRC3  Steroid receptor coactivator 3
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TACE</td>
<td>TNFα-converting enzyme</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T-cell acute lymphoblastic leukemia/lymphoma</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>Tumor necrosis factor α-induced protein 3</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain</td>
</tr>
<tr>
<td>TRAF-2</td>
<td>TNFR associated factor 2</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked Inhibitor of Apoptosis Protein</td>
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ABSTRACT

Breast and cervical cancer are two common cancers that severely endanger women’s health. The fatality rates of breast and cervical cancer are ranked first and fifth, respectively, in cancer related deaths in women worldwide. Continuous dedication to cancer research is urgently required to elucidate the cancer cell survival mechanism that is responsible for disease aggressiveness and poor prognosis.

Emerging data indicate that Notch-1 is aberrantly regulated in human cervical and ER- breast cancer and inhibiting Notch-1 sensitizes cancer cells to apoptosis. However, the mechanism by which Notch-1 promotes cell survival is not known. NF-κB is also deregulated in these two cancers and might be the downstream signaling effector of Notch-1 to modulate cell survival and apoptosis. Here, we show that Notch-1 may provide cells with survival signal by activating NF-κB activity in cervical and ER- breast cancer.

First, we showed that Notch-1 activates and is required to maintain NF-κB activity in cervical cancer CaSki cells and ER- breast cancer MDA-MB-231 cells. Then, we found that Notch-1 activates NF-κB activity by interacting with IKKα/β and regulating IKKα/β kinase activity in CaSki cells stimulated with TNF-α.

Lastly, we demonstrated that Notch-1 activates NF-κB dependent gene expression in MDA-MB-231 cells through an Akt/IKK dependent pathway. We showed that Notch-1
and IKKα are required for the expression of a subset of NF-κB dependent genes. Notch activation mediated by Jagged-1 or EDTA activates Akt, which in turn activates the IKK complex. Notch activation mediated by Jagged-1 also induces NF-κB subunits, Pol II and IKKα recruitment to the NF-κB dependent gene promoters. Inhibiting Akt pharmacologically or genetically reduces the IKK activation and decreases NF-κB subunits, Pol II and IKKα recruitment to the NF-κB dependent gene promoters.

In summary, we showed that Notch-1 activates NF-κB activity in both cervical and ER- breast cancer cells, and we explored similarities and differences in the underlying mechanisms in the two cell types. Potentially, this study will provide a molecular rationale for targeting Notch-1, Akt and NF-κB in cervical and ER- breast cancer in the clinic.
CHAPTER I

INTRODUCTION

A. Background

1. Overview of cervical cancer and breast cancer

Cervical cancer is the second most frequently diagnosed neoplasm in women worldwide, constituting ~12% of all female cancers and causing ~250,000 deaths annually. Incidences of cervical cancer are higher in developing countries compared to developed countries (Sankaranarayanan et al., 2006). Persistent human papillomavirus (HPV) infections are now recognized as the major cause of cervical cancer. HPV types 16 and 18 together cause about 70% of cervical cancers (Munoz et al., 2003). The vaccine effective against these two HPVs has been licensed in the U.S. and the EU.

Cervical cancer was once the leading cause of death for women in the United States. During the past few decades, largely due to widespread use of the Papanicolaou test, incidence and mortality from cervical cancer have declined significantly. However, pressing demands still exist for developing more specific and more accurate screening tests. Understanding cervical cancer neoplastic formation will lay the foundation for
translating those molecular knowledge into developing more effective screening tests and treatments. There are several signal transduction pathways known to be deregulated in cervical cancer cells. Notch-1 and Nuclear Factor-kappa B (NF-κB) signaling pathways are two of those identified. In this thesis, we focused on the cross talk of Notch-1 and NF-κB signaling pathways in cervical cancer CaSki cells and the underlying mechanism whereby Notch-1 activates NF-κB.

Currently, breast cancer, particularly estrogen receptor alpha negative (ER-) breast cancer, has taken over cervical cancer and has become the leading cause of cancer related mortality in women worldwide (Wildiers et al., 2007). It is estimated that each year this disease is diagnosed in over one million people worldwide and is the cause of more than 400,000 deaths (O’Driscoll and Clynnes, 2006). The incidence of breast cancer has been increasing steadily from an incidence of 1:20 in 1960 to 1:8 women today. Early detection methods such as mammography and application of targeted therapy have improved the outlook of many breast cancer patients. However the morbidity and mortality rate remain high, partially because of low response rate in some subsets of breast cancers and the development of resistance to therapeutic drugs. Considerable effort has been dedicated into looking at the underlying mechanisms contributing to the low response rate and drug resistance. Not only in cervical cancer, but also in breast cancer, Notch-1 and NF-κB signaling pathways have been shown to be associated with poor outcome and drug resistance. In this thesis, we investigated the interplay of these two deregulated pro-proliferative signaling transduction pathways and the underlying mechanism responsible for their interaction in breast cancer.
Breast cancers can be classified into two groups: Estrogen Receptor positive (ER+) breast cancer, making up 70% of all breast cancers, or Estrogen Receptor negative (ER-) ones, constituting the remainder. Estrogen induces tumor cell growth through binding to ER and initiating hormone responsive gene expressions. The estrogen downstream proteins presumably promote mammary epithelial cell proliferation (Jordan, 1995). The current anti-hormone treatment tamoxifen, inhibits breast tumor proliferation by binding to ER but not conferring it into an active conformation, thus blocking downstream events. However, since tamoxifen targets the ER, it is not effective against ER- breast cancer.

Breast cancers usually progress from benign, ER+, estrogen dependent, noninvasive tumors to malignant, ER-, estrogen independent, invasive and metastatic ones (Clarke et al., 1990). ER- breast cancers are therefore clinically more aggressive and more difficult to treat than ER+ breast tumors (Jemal et al., 2002). Therefore in this thesis, we focused on the regulation of Notch-1 on pro-survival NF-κB signaling pathway in ER- breast cancer.

2. Notch-1 in cervical cancer and breast cancer

Emerging evidence has shown that Notch-1 and its ligands and targets have been associated with transformation of many types of tumors including cervical and breast cancer (Miele, 2006). Notch is a single-pass transmembrane receptor that participates in communication between adjacent cells (Nickoloff et al., 2002; Miele et al., 2006; Miele, 2006). The interaction between Notch and its ligands in a neighboring cell triggers Notch receptor activation. This conformational change resulting from ligand-receptor
interaction initiates two cleavages on Notch receptor, releasing an intracellular domain of Notch, (NIC) (Saxena et al., 2001). After NIC is released from the plasma membrane, it is translocated into the nucleus where it modulates gene expression primarily, but not exclusively, by binding to a ubiquitous transcription factor CBF-1 (Artavanis-Tsakonas et al., 1999). Notch-1 converts CBF-1 from a constitutive repressor into an activator by displacing a panel of co-repressors and recruiting a co-activator complex (Kao et al., 1998; Hsieh et al., 1999; Kurooka and Honjo, 2000; Oswald et al., 2001; Wu et al., 2000).

The target genes of Notch include hairy enhancer of split genes (HES) and HES-related repressor protein (HERP) (Iso et al., 2003), HEY family (Maier and Gessler, 2000), which are helix-loop-helix transcriptional regulators modulating differentiation, cell cycle mediator p21 (Rangarajan et al., 2001), and cyclin D (Ronchini and Capobianco, 2001) as well as NF-κB family (Cheng et al., 2001) and PPAR family (Garces et al., 1997; Nickoloff et al., 2002) transcription factors.

Accumulated evidence shows that Notch-1 is deregulated in cervical as well as breast cancer. Increased expression of Notch-1 and Notch ligands were reported in cervical carcinomas (Zagouras et al., 1995; Daniel et al., 1997; Gray et al., 1999). Accumulation of Notch-1 and its ligand Jagged1 and downregulation of Manic Fringe, a negative regulator of Notch signaling coincided with the progression of high-grade precursor lesions to invasive cervical cancers (Veeraraghavalu et al., 2004). More importantly, Notch-1 was shown to cooperate with HPV oncoproteins in transforming immortalized human keratinocytes (Rangarajan et al., 2001; Ramdass et al., 2006). HPV16 oncoproteins E6 and E7 increased Notch-1 levels and CBF-1 transcriptional
activity in primary mouse embryo cells (Weijzen et al., 2003). Taken together, Notch-1 is oncogenic in cervical cancer.

The accumulation of intracellular Notch and elevated Notch signaling were also observed in a variety of human breast carcinomas (Reedijk et al., 2005; Stylianou et al., 2006). Our lab has confirmed that Notch expression was deregulated in human breast cancer as opposed to normal breast cells. Ductal and lobular carcinomas consistently expressed high levels of Notch-1 and Notch-4 as compared to normal breast tissue. In addition, high level expression of Notch-1 or Notch-1 ligand Jagged-1 predicted poor overall survival in breast cancer patients, combination of both at high levels indicated even worse outlook (Reedijk et al., 2005; Dickson et al., 2007). Also, increased Notch signaling was sufficient to transform normal breast epithelial cells (Reedijk et al., 2005). Overall, the data support the idea that Notch plays an oncogenic role in human breast cancer. Recently, we and others have observed that high levels of Notch-1 and Jagged-1 were mostly found in ER- basal phenotype breast cancer (ER-, progesterone receptor (PR)-, Her2-) (Rizzo et al., 2008). We chose the breast cancer cell line MDA-MB-231 cells, which are ER-, PR- and Her2-, as our model system to study Notch-1 signaling.

There is increasing evidence that inhibition of Notch-1 sensitizes cervical and breast cancer cells to apoptosis, causes cell cycle arrest and decreases cellular transformation. Inhibiting Notch signaling by siRNA in CaSki cervical cancer cells abolished proliferation and anchorage-independent growth in vitro (Weijzen et al., 2002) and completely blocked the growth of CaSki xenografts in vivo (Weijzen et al., 2003). Inhibition of Notch by Gamma-secretase inhibitor (GSI) suppressed breast tumors growth (Pece et al., 2004) and reverted the transformed phenotype of human breast cancer cell
lines, including the ER- breast cancer cell line MDA-MB-231 (Stylianou et al., 2006). Our lab also showed that Notch-1 knockdown by siRNA or GSI caused G2 arrest and induced apoptosis in breast cancer cells. *In vivo*, GSI arrested the growth of MDA-MB-231 tumors and in combination with tamoxifen, caused regression of T47D:A18 (ER+ breast cancer cell) tumors (Rizzo et al., 2008). All these suggest that Notch signaling is a potential therapeutic target in cervical and breast cancer.

To date, the mechanism whereby Notch-1 promotes cell survival and/or proliferation and transformation is not clear. Reports suggest that Notch-1 may contribute to cell survival and proliferation via NF-κB dependent pathway in cervical and breast cancer. In this thesis, we will show that Notch-1 promotes cell survival in part by activating NF-κB activity in cervical and ER- breast cancer.

### 3. NF-κB in cervical cancer and breast cancer

NF-κB is a family of transcription factors with a critical role in regulating inflammation, cell survival, cell differentiation, and cell proliferation. The NF-κB subunits are inactive in the cytoplasm as a result of binding to inhibitory proteins of the IκB family (IκBs). In response to numerous stimuli: cytokines, chemokines, mitogens, bacterial and viral infection, an IκB kinase (IKK) complex is activated by IKK kinases (Karin, 1999). Activated IKK complex phosphorylates IκBs, leading to 26s proteasome mediated degradation of IκB. Degradation of IκB releases NF-κB, enabling it to translocate to the nucleus and rapidly induce a variety of genes encoding matrix
metalloproteinases, inflammatory and chemotactic cytokines, and antiapoptotic proteins (Li and Verma, 2002; Karin and Lin, 2002; Karin et al., 2002).

NF-κB is a major survival pathway that is highly relevant to cervical cancer. Previous studies have shown that NF-κB is constitutively activated in human cervical squamous cell carcinomas as compared to normal cervical epithelium. Increased nuclear staining of p50 and p65 and expression of NF-κB target genes have been detected in cervical cancer. Constitutive activation of NF-κB with nuclear accumulation of p50 and p65 is associated with cervical cancer progression (Nair et al., 2003; Prusty et al., 2005). Blocking NF-κB in cervical cancer cells may not only help eradicate tumor cells but also increase sensitivity to other conventional therapies. The cytotoxicity of arsenic trioxide (Woo et al., 2004; Wei et al., 2005) and celecoxib (Kim et al., 2004) on cervical cancer cells is mediated in part by NF-κB inhibition. Inhibition of NF-κB sensitizes cervical cancer cells to cisplatin induced apoptosis (Venkatraman et al., 2005) and enhances radiosensitivity of certain human cervical carcinoma cancer cells in vitro (Kamer et al., 2009).

In addition to cervical cancer, NF-κB was found to be highly active in ER- breast cancer cell lines as well as in ER- primary breast tumors. Constitutive DNA binding of NF-κB and elevated NF-κB concentration were also detected in extracts from poorly differentiated ER- primary tumors (Nakshatri et al., 1997; Bhat-Nakshatri et al., 1998; Biswas et al., 2000). Elevated NF-κB activation in ER- breast cancer has been suggested to be responsible for chemoresistance and metastatic growth. It was shown that active NF-κB protects the cells from apoptosis induced by TNF-α, ionizing radiation, or
daunorubicin. The protection resulted in radiation and drug resistance (reviewed in (Nakshatri et al., 1997).

NF-κB has been considered a potential therapeutic target for cervical and ER-breast cancer (Nakshatri et al., 1997; Biswas et al., 2000; Biswas et al., 2001; Biswas et al., 2004; Huang et al., 2009). Better understanding of the NF-κB signaling pathway will facilitate the development of more effective therapy for cervical and ER-breast cancer.

B. Specific Aims

Overall, emerging evidence indicated that Notch-1 was aberrantly regulated in human cervical and ER-breast cancer and inhibiting Notch-1 sensitized cancer cells to apoptosis. However, the mechanism whereby Notch-1 promotes cell survival and/or proliferation and transformation is not clear. Accumulating reports suggest that Notch-1 may contribute to cell survival and proliferation via the NF-κB dependent pathway in cervical and breast cancer, since Notch-1 and NF-κB are both deregulated in these two cancers and NF-κB provides pro-survival signal for cancer cells. It was shown that activated Notch signaling was accompanied by elevated NF-κB activity as determined by immunohistochemistry from cervical cancer patients’ samples. Constitutive activation of NF-κB with nuclear accumulation of p50 and p65 has been described to be associated with cervical cancer progression (Nair et al., 2003; Prusty et al., 2005). Aberrantly regulated Notch and NF-κB activity were also concurrently found in ER-breast cancer. Notch-1 and NF-κB activity were highly active in ER-breast cancer cell lines as well as ER-breast tumors as compared to ER+ counterparts. Elevated NF-κB activation in ER-
breast cancer has been suggested to be responsible for chemo-resistance and metastatic growth. Here, we hypothesize that Notch-1 promotes cell survival by activating NF-κB activity in cervical and ER- breast cancer.

To test this hypothesis we had 3 aims.

Aim 1: To determine whether Notch-1 activates NF-κB in cervical and ER- breast cancer.

Aim 2: To characterize the mechanism by which Notch-1 activates NF-κB in cervical cancer Caski cells.

Aim 3: To elucidate the mechanism by which Notch-1 activates NF-κB dependent gene expression in ER- breast cancer MDA-MB-231 cells.

In Aim 1, we showed that Notch-1 activates and is required to maintain constitutively active NF-κB activity in cervical cancer CaSki cells and ER- breast cancer MDA-MB-231 cells. NF-κB activity was determined by luciferase reporter assays under the conditions of either over-expressing Notch-1 or silencing Notch-1 by siRNA in both cervical cancer CaSki and ER- breast cancer MDA-MB-231 cells. It was found that down-regulation of Notch-1 causes a decrease in NF-κB dependent transcription activity and activation of Notch-1 leads to an increase in NF-κB activity in both cells.

In Aim 2, we showed that Notch-1 interacts with IKK complex and activating IKKa/β kinase activity in CaSki cells. The interaction between Notch-1 and IKK complex is shown by traditional Co-IP and rellina luciferase Co-IP. By Using in vitro kinase assay and western blot of phosphorylation of endogenous IkBα, we showed that IIKKα/β kinase activity induced by TNF-α is inhibited by either Notch-1 siRNA or GSI treatment.
In Aim 3, we were trying to elucidate the mechanism by which Notch-1 activates NF-κB dependent gene expression in ER- breast cancer MDA-MB-231 cells. We initially tested whether Notch-1 activates NF-κB by interacting with IKK complex and activating IKKα/β kinase activity the same as in cervical cancer cells, however, we could not detect a Notch-1/IKK physical interaction.

In order to explore the mechanism accounting for Notch-1 regulation of NF-κB in ER- breast cancer, we started gene expression studies in ER- breast cancer MDA-MB-231 cells. We found that both Notch-1 and IKKα are essential for basal and stimulated expression of some NF-κB dependent genes. As a well-known transcription factor, Notch-1 activates expression of target genes by binding to CBF-1, a DNA binding protein, and converting CBF-1 from a repressor to an activator. Binding of Notch-1 to CBF-1 facilitates the recruitment of transcription coactivators to the target gene promoters. Both Notch-1 and IKKα have nuclear functions. On the cIAP-2 promoter, IKKα has been shown to have nuclear kinase function to activate gene transcription by phosphorylating corepressor SMRT (silencing mediator of retinoid and thyroid hormone receptors) (Hoberg et al., 2004; Hoberg et al., 2006). We found that Notch-1 and IKKα were recruited to NF-κB dependent genes cIAP-2 and IκBα promoter in Caski cells. Furthermore, the IKKα recruitment was Notch-1 dependent on both promoters (Song et al., 2008). This promoted us to hypothesize that Notch-1 and IKKα together may exert regulation on NF-κB dependent gene promoters in MDA-MB-231 cells. Therefore, we explored the recruitment of Notch-1, IKKα and NF-κB on NF-κB dependent gene promoters in MDA-MB-231 cells by chromatin immunoprecipitation assays. We found that NF-κB subunits, Notch-1 and IKKα were recruited to NF-κB dependent gene
promoters however; Notch-1 was recruited later than NF-κB subunits and IKKα. NF-κB subunits and IKKα occupancy were Notch-1 dependent, suggesting that Notch-1 activates IKK complex and IKKα before being recruited to the promoter. What could be the possible way that Notch-1 activates IKK complex and IKKα before the nuclear function?

Akt might be the IKK upstream kinase responsible for NF-κB activation mediated by active Notch-1. Akt signaling pathway is involved in multiple cellular functions including promoting cell growth, increasing glucose uptake and oxidation, cell cycle progression and cell survival. Many studies showed that Notch-1 activates or is required for activation of Akt pathway in various systems (Nair et al., 2003; Liu et al., 2006; Graziani et al., 2008; Meng et al., 2009). Notch-1 has been shown to promote T-cell precursors survival, inhibit and rescue apoptosis, sustain transformation, drive tumor progression through a PI3K/Akt dependent pathway (Sade et al., 2004; Ciofani and Zuniga-Pflucker, 2005; Nair et al., 2003; Liu et al., 2006; Perumalsamy et al., 2009). In addition, NF-κB is an important downstream target of Akt activation. Akt promotes transactivation potential and phosphorylation of NF-κB through IKK (Ozes et al., 1999; Madrid et al., 2001). Akt is proposed to promote metastasis and angiogenesis through IKK in a manner that depends on NF-κB activation (Agarwal et al., 2005). Therefore, it is possible that Notch-1 activates NF-κB through Akt/iKK dependent pathway in MDA-MB-231 cells.

We showed that Notch activation mediated by Jagged-1 activated the IKK complex and induced phosphorylation and degradation of IκBα. We found that Akt was upstream of IKK in the NF-κB activation pathway mediated by Jagged-1. Inhibiting Akt pharmacologically or genetically reduced the IKK activation and decreased NF-κB
subunits, Pol II and IKKα recruitment to NF-κB dependent gene promoters. In agreement with the data from Notch-1 activation by Jagged-1, Notch-1 activation mediated by EDTA activated Akt/IKK/NF-κB pathway as well. We found that Akt activation was independent of canonical nuclear Notch-1 partner CBF-1, suggesting that cytoplasmic Notch-1 might have a role in NF-κB activation through an Akt/IKK/NF-κB axis.

In summary, we demonstrated that Notch-1 activates NF-κB activity in both cervical and ER- breast cancer and we explored similarities and differences in the underlying mechanisms in the two systems.

C. Significance of the study

The purpose of this work is to identify and characterize the relationship between two signaling pathways: Notch-1 and NF-κB in cervical and ER- breast cancer. These two pathways are crucial in regulating cell survival, apoptosis and proliferation. Studying their crosstalk will lay groundwork for clinical trials of Notch targeting therapeutic agents.
A. Overview of cervical cancer and breast cancer

1. Overview of cervical cancer

1.1 Introduction to cervical cancer

Cervical and breast cancer are most common cancers that severely endanger women’s health. Cervical cancer is the second most frequently diagnosed neoplasm in women worldwide, constituting ~12% of all female cancers and causing ~250,000 deaths annually. Incidences are higher in developing countries compared to developed countries (Sankaranarayanan et al., 2006).

Persistent human papillomavirus (HPV) infections are now recognized as the major cause of cervical cancer. HPV can be referred to as “low-risk” and “high-risk”. Low risk HPVs include: types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108. They rarely cause lesions that develop into cancer. High risk HPVs include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73. High risk HPVs are more likely to lead to the development of cancer. HPV types 16 and 18 together cause about 70% of cervical
cancers (Munoz et al., 2003). The vaccine effective against these two HPVs has been licensed in the U.S. and the EU.

Cervical cancer is considered as a sexually transmitted disease. Women who have many sexual partners (or who have sex with men or women who had many other partners) have a greater risk (Marrazzo et al., 2001). Use of condoms reduces, but does not always prevent transmission.

1.2 Cervical cancer stage and treatment

Cervical cancer evolves from morphologically distinctive non-malignant stages. Those stages can be classified in three-tier: cervical intraepithelial neoplasia (CIN) and two-tier systems: squamous intraepithelial lesion (SIL). Mild dysplasia as CIN1 corresponds to Low-grade Squamous Intraepithelial Lesion (LSIL). High-grade Squamous Intraepithelial Lesion (HSIL) includes moderate dysplasia CIN2 and severe dysplasia CIN3 (Chivukula et al., 2007).

Cervical cancer is treated by surgery in early stages and chemotherapy and radiotherapy in advanced stages of the disease. Cervical cancer was once the leading cause of death for women in the United States. However, during the past few decades, largely due to widespread use of the Papanicolaou test, incidence and mortality from cervical cancer have declined significantly. New approaches such as liquid-base cytology, automated slide analysis, physical parameters and visual inspection using acetic acid have been developed to improve the accuracy of a cervical screening test.
1.3 Biomarkers for cervical cancer

Understanding of the molecular signaling pathways in cervical neoplasia transformation provides the foundation for developing more specific and more accurate screening biomarkers. Those biomarkers include HPV E6, 7 proteins and cyclin E, protein required for cell cycle progression, minichromosome maintenance proteins (MCMs), proliferating cell nuclear antigen (PCNA), P16 and P21 (Martin et al., 2009). Notch-1 was shown to cooperate with HPV oncoproteins in transforming immortalized human keratinocytes (Ramdass et al., 2006; Rangarajan et al., 2001). Studying Notch-1 pro-survival signaling pathway could facilitate identification of a new biomarker for cervical cancer screening and prognosis. Thus, in this thesis, we will focus on elucidating the mechanism whereby Notch-1 promotes cell survival in cervical cancer.

2. Overview of breast cancer

2.1 Introduction to breast cancer and breast cancer treatment

According to the WHO, breast cancer has become the leading cause of cancer related mortality in women globally. About 1.3 million women will be diagnosed with breast cancer annually worldwide and about 465,000 will die from the disease. One out of eight women develops invasive breast cancer in the U.S.
Breast cancer is usually first treated by surgery. Based on the tumor size and cancer stage, lumpectomy or mastectomy including removal and examination of axillary lymph nodes are performed. In some cases, neo-adjuvant therapy (treatment before the surgery) may be administered to reduce tumor size before surgery. Breast cancers are also treated by radiotherapy, chemotherapy and hormone therapy after surgery to reduce recurrence and relieve pain.

2.2 ER positive versus ER negative breast cancer

Breast cancers can be classified into two groups: Estrogen Receptor positive (ER+) breast cancer, making up 70% of all breast cancers, or Estrogen Receptor negative (ER-) ones, constituting the remaining. Estrogen induces tumor cell growth through binding to ER and initiating hormone responsive gene expressions. The estrogen downstream proteins presumably promote mammary epithelial cell proliferation (Jordan, 1995). Consequently estrogen has become an obvious therapeutic target giving rise to the development of selective estrogen receptor modulators (SERMs) such as tamoxifen, raloxifene; aromatase inhibitors; and estradiol analogs like fulvestrant (Chakrabarti et al., 2004). However, since these hormone therapies target ER or estrogen, they are not effective against ER- breast cancer. Breast cancers usually progress from benign, ER+, estrogen dependent, noninvasive tumors to malignant, ER-, estrogen independent, invasive and metastatic ones. ER- breast cancers are therefore clinically more aggressive and more difficult for prognosis than ER+ breast tumors (Clarke et al., 1990). This is
why ER- breast cancer requires more dedicated research to improve the outlook and
treatment. We will study ER- breast cancer in this thesis.

2.3 Biomarkers for breast cancer

Besides ER, other genetic factors also have great impact on the choice of
treatment and prognosis for breast cancer. These genetic biomarkers include: Her2/neu,
BRCA, NF-κB, Notch-1 and Jagged-1. Her2 is the second member of the epidermal
growth factor family (EGFR). Her2 is one of the most important oncogenes in invasive
breast cancer. In about one of every three breast cancers, Her2 is amplified due to a gene
mutation. Her2-positive breast cancers tend to be more aggressive than other types of
breast cancer. They are also less responsive to hormone treatment and chemotherapy
(Lipton et al., 2002). However, Herceptin, a monoclonal antibody against Her2, was
developed and approved by FDA. It was shown to be very effective in killing these
cancer cells and decreases the risk of recurrence. When combined with chemotherapy,
Herceptin increased median survival time by about 25% compared to chemotherapy
alone (Baselga, 2001). Herceptin is one of the drugs designed based on molecular
therapeutic target. It has excellent clinical outcomes and moderate side effects. Herceptin
is a huge success of molecular oriented biomedical research.

In addition to HER2, BRCA1 and BRCA2 are two very important genes
associated with breast cancer, especially in hereditary breast cancer. The names BRCA1
and BRCA2 stand for breast cancer susceptibility gene 1 and breast cancer susceptibility
gene 2, respectively. They are tumor suppressors, whose function is to repair damaged DNA and maintain genomic integrity. More than 1,000 mutations in the BRCA1 and more than 800 mutations in the BRCA2 gene were found. Those mutations cause production of nonfunctional proteins. A woman’s lifetime risk of developing breast and/or ovarian cancer is greatly increased if she inherits a harmful mutation in BRCA1 or BRCA2 (Satagopan et al., 2001). Currently, women with BRCA1 mutations account for 5% of all breast cancer cases.

Genetic tests are available to check for BRCA1 and BRCA2 mutations. This facilitates the prevention, early detection and management of breast cancer for women with BRCA1 and BRCA2 mutations. Several options such as surveillance, prophylactic surgery, risk avoidance and chemoprevention, are available for managing cancer risk in individuals who have a harmful BRCA1 or BRCA2 mutation (Bouchard et al., 2004).

NF-κB is another factor that might contribute to tumorigenesis in breast cancer. NF-κB is a family of transcription factors with a critical role in regulating inflammation, cell survival, cell differentiation, and cell proliferation. We will discuss the detail of NF-κB activation in breast cancer in later section.

Notch-1 and its ligands overexpression have been shown to be poor prognosis markers for breast cancer. Again, we will talk in detail about Notch-1 signaling in breast cancer.
B. Overview of Notch signaling pathway

1. Notch structure

![Notch-1 structure](image)

Figure 2.1: Notch-1 structure. Notch receptors contain an extracellular subunit \( N^{EC} \) and a transmembrane subunit \( N^{TM} \). The extracellular subunit contains EGF-like repeats associated with ligand binding and three cysteine-rich Notch/LIN12 repeats (LIN). The transmembrane subunit contains two protein-protein interaction domains, the RAM domain (R, membrane-proximal RBP-J-associated molecule domain) and six ankyrin (ANK) repeats. RAM domain interacts with its transcriptional activator RBP-J/\( \kappa \)/CBF-1 and ANK repeats interacts with a different transcriptional factor MAML1. The transmembrane subunit also contains two nuclear localization signals (NLS) which flank ANK, a transactivation domain (TAD) and a PEST sequence (P, proline-, glutamate-, serine- and threonine-rich) which is important for mediating Notch degradation. Similar to the Notch receptor, Notch ligand has an extracellular domain composed of EGF-like repeats. They have a short intracellular portion with no recognizable domain structure.

Notch is a single-pass transmembrane receptor that participates in communication between adjacent cells (Nickoloff et al., 2002; Miele, 2006; Miele et al., 2006). The name for Notch originated because a partial loss of Notch function causes small notches in Drosophila wings. Notch signaling, conserved throughout evolution, plays a key role in
modulating cell fate decisions, cell proliferation, and cell death during development and postnatal life (Callahan and Raafat, 2001).

There are four Notch receptors (1 to 4) in the mammalian family (Blaumueller and Artavanis-Tsakonas, 1997), and five ligands: Jagged-1, Jagged-2, Delta-like-1,-3 and -4 (DLL1, DLL3 and DLL4) (Dunwoodie et al., 1997; Lindsell et al., 1995; Shawber et al., 1996). The structure and processing of Notch has been well characterized since the groups of Artavanis-Tsakonas and Young cloned the Notch gene in 1985 (Wharton et al., 1985). All Notch receptors contain an extracellular subunit $N^{EC}$ and a transmembrane subunit $N^{TM}$ (Rand et al., 2000). The extracellular subunit contains EGF-like repeats associated with ligand binding and three cysteine-rich Notch/LIN12 repeats (LIN) that prevent ligand-independent signaling. The transmembrane subunit contains two protein-protein interaction domains, the RAM domain (R, membrane-proximal RBP-J-associated molecule domain) and six ankyrin (ANK) repeats. RAM domain interacts with its transcriptional activator RBP-J-k/CBF-1 (Tamura et al., 1995) and ANK repeats interacts with a different transcriptional factor MAML1 (Mastermind-like1). The transmembrane domain also contains two nuclear localization signals (NLS) which flank ANK, a transactivation domain (TAD) and a PEST sequence (P, proline-, glutamate-, serine- and threonine-rich) which is important for mediating Notch degradation (Rechsteiner, 1988). Notch-1 structure is illustrated in Figure 2.1. Similar to the Notch receptor, Notch ligand has an extracellular domain composed of EGF-like repeats, which facilitate interactions with the Notch family. They also contain an amino-terminal structure called DSL (an acronym for Delta, Serrate and LAG-2) which is necessary for interaction with Notch.
receptors (Rebay et al., 1991). They have a short intracellular portion with no recognizable domain structure.

2. Notch processing

As shown in Figure 2.2, Notch proteins are derived from large polypeptide precursors. The precursor undergoes several enzymatic cleavage events while becoming a mature protein. The first proteolytic cleavage known as S1 is mediated by a furin-like protease (Logeat et al., 1998). This cleavage produces two fragments: one 180 kDa fragment that consists of the extracellular subunit of the protein (N^{EC}) and a 120 kDa, membrane-tethered, intracellular portion containing only a short extracellular piece known as N^{TM}. N^{EC} and N^{TM} are non-covalently associated on the cell surface until activation by a ligand (Rand et al., 2000). A specific ligand activates Notch signaling via binding to EGF repeats of Notch receptor from a neighboring cell, triggering the dissociating the N^{EC} from N^{TM}. The conformational change resulting from ligand-receptor interaction renders the extracellular stump of N^{TM} susceptible to the S2 cleavage by TACE (TNF-alpha converting enzyme), a member of a disintegrin and metallopeptase (ADAM) family (Brou et al., 2000). This cleavage event triggers presenilin, a catalytic subunit of a gamma-secretase complex, to conduct the S3 cleavage within the transmembrane sequence, releasing an intracellular domain of Notch, NIC (Saxena et al., 2001). NIC is considered the active form of Notch. Inhibiting gamma-secretase activity prevents the final cleavage of the Notch receptor, blocking Notch signal
transduction (Shi and Harris, 2006). After it is released from the plasma membrane, NIC is translocated into the nucleus where it modulates gene expression primarily, but not exclusively, by binding to a ubiquitous transcription factor CBF-1 (Artavanis-Tsakonas et al., 1999). Notch can be also activated without a ligand, since the noncovalent interaction between N$^{EC}$ from N$^{TM}$ is completely dependent on the Ca$^{2+}$-binding LIN repeats located in N$^{EC}$. Calcium chelators such as EDTA or mutation of the LIN repeats can deplete the interaction (Rand et al., 2000) and activate the Notch pathway without ligand stimulation.
Figure 2.2 Notch-1 processing

Figure 2.2 Notch-1 processing: Notch proteins are derived from large polypeptide precursors. The precursor undergoes several enzymatic cleavage events while becoming a mature protein. The first proteolytic cleavage known as S1 is mediated by a furin-like protease. This cleavage produces two fragments: one 180 kDa fragment that consists of the extracellular subunit of the protein (NEC) and a 120 kDa, membrane-tethered, intracellular portion containing only a short extracellular piece known as NTM. The conformational change resulting from ligand-receptor interaction renders the extracellular stump of NTM susceptible to the S2 cleavage by TACE (TNF-alpha converting enzyme), a member of a disintegrin and metalloprotease (ADAM) family. This cleavage event triggers presenilin, a catalytic subunit of a gamma-secretase complex, to enact the S3 cleavage within the transmembrane sequence, releasing an intracellular domain of Notch, NIC. NIC is considered the active form of Notch. Inhibiting gamma-secretase activity prevents the final cleavage of the Notch receptor, blocking Notch signal transduction.
3. Canonical Notch signaling

After it is released from the plasma membrane, NIC is translocated into the nucleus where it modulates gene expression primarily, but not exclusively, by binding to a ubiquitous transcription factor CBF-1 (Artavanis-Tsakonas et al., 1999). CBF-1 acts as a constitutive repressor at the promoter by recruiting co-repressors including SMRT (silencing mediator for retinoid and thyroid receptor), N-coR (nuclear receptor co-repressor), SKIP (Ski interacting protein), CIR (CSL-interacting repressor) and HDAC-1 (histone deacetylases-1) (Hsieh et al., 1999; Kao et al., 1998). The active NIC is thought to be initially recruited to the CBF-1: DNA complex by the RAM sequence of NIC, which has high affinity for the β-trefoil domain of CBF-1 (Lubman et al., 2007; Nam et al., 2006). The ANK domain of NIC further stabilizes DNA:CBF:NIC complex through the Rel-homology portion of CBF-1 to create a high affinity binding site for Mastermind-like 1 (MAML-1) (Nam et al., 2006, Wilson et al., 2006). Sequentially, MAML-1 recruits histone acetyltransferases, such as p300, which promote assembly of initiation and elongation complexes which, together with PCAF (p300 associated factor), cooperate to enhance transcriptional activation in the presence of p300 in vitro (Oswald et al., 2001; Wallberg et al., 2002). The net result of the Notch activation is converting CBF-1 from a constitutive repressor to a transcriptional activator by displacing the co-repressors and recruiting a co-activator complex.

The target genes of Notch include hairy enhancer of split genes (HES) and HES-related repressor protein (HERP) (Iso et al., 2003), HEY family (Maier and Gessler,
2000), which are helix-loop-helix transcriptional regulators modulating differentiation, cell cycle mediator p21 (Rangarajan et al., 2001), and cyclin D (Ronchini and Capobianco, 2001) as well as NF-κB family (Cheng et al., 2001) and PPAR family (Garces et al., 1997; Nickoloff et al., 2002) transcription factors. Since many Notch targets are themselves transcription factors, Notch activation can result in complex changes in gene expression profiles, potentially involving hundreds of genes. However, the consequences of Notch activation and the downstream genes that it affects are dependent on the cellular context in which they occur.
Figure 2.3 Canonical Notch signaling. After it is released from the plasma membrane, intracellular Notch NIC is translocated into the nucleus where it modulates gene expression by binding to a ubiquitous transcription factor CBF-1. CBF-1 acts as a constitutive repressor at the promoter by recruiting co-repressors including silencing mediator of retinoid and thyroid hormone receptors (SMRT), N-coR, and histone deacetylases. Nuclear Notch displaces the co-repressors and recruits a co-activator complex, that includes histone acetyltransferases PCAF, GCN5 or p300, and mastermindlike 1 (MAML1). The target genes of Notch include hairy enhancer of split genes (HES) and HES-related repressor protein (HERP), HEY family, cyclin D,c-Myc, as well as NF-κB family and PPAR family transcription factors.
4. Non-canonical Notch signaling

The CBF-1-dependent signaling pathway does not mediate all functions of Notch; mounting evidence points to the existence of CBF-1-independent Notch signaling pathways. One of these pathways involves the protein Deltex and possibly c-Jun N-terminal kinase (JNK, a member of the MAP kinase family) (Ramain et al., 2001; Zecchini et al., 1999). Deltex is an evolutionarily conserved cytoplasmic, zinc-finger protein which binds to the ankyrin repeats of Notch (Matsuno et al., 1998). This deltex and Notch complex does not enter the nucleus, but appears to inhibit JNK activity by decreasing its phosphorylation (Jang et al., 2004). The reduced JNK activity results in downregulation the phosphorylation of the E47 transcription factor (Ordentlich et al., 1998), which is important in transcription activation of B-cell specific immunoglobulin genes.

Recent evidence also suggests another cytoplasmic pathway of Notch signaling. Specifically, Notch has been shown to activate the PI3-kinase (PI3K)-Akt pathway in a variety of cells (Rangarajan et al., 2001; Nair et al., 2003). In T-cells, this is reported that Notch1IC forms a cytoplasmic complex with PI3K, and the non-receptor protein tyrosine kinase p56Lck, a Src-related enzyme (Sade et al., 2004). The complex appears to be membrane associated, but activates Akt, presumably through formation of a PIP3 intermediate. These observations suggest that Notch can act as a scaffold for kinase activation cascades independently of its nuclear effects.
In addition, there are several studies talking about Notch regulation on NF-κB activity without involvement of CBF-1. We will discuss these in detail in the section of Notch and NF-κB.

C. Notch-1 in cervical and breast cancer

1. Notch-1 in cervical cancer

Deregulated expression of Notch receptors, ligands and targets was observed in numerous solid tumors including cervical, head and neck, endometrial, renal, lung, pancreatic, ovarian, breast and prostate carcinomas, osteosarcoma, mesothelioma, gliomas, and medulloblastomas (Miele et al., 2006). In cervical cancer, increased expression of Notch-1 and Notch ligands was reported (Daniel et al., 1997; Gray et al., 1999; Zagouras et al., 1995). Low level of Notch-1 was expressed in normal cervical epithelium and higher level was detected in the membrane and cytoplasm of cells of precancerous cervical lesions. Accumulation of Notch-1 and its ligand Jagged-1 and downregulation of Manic Fringe, a negative regulator of Notch signaling coincided with the progression of high-grade precursor lesions to invasive cervical cancers (Veeraraghavalu et al., 2004). High level of Notch-1 was found in the nucleus in cervical cancer, indicating that Notch-1 was in cleaved and active form. More importantly, Notch-1 was shown to cooperate with HPV oncoproteins in transforming immortalized human keratinocytes (Ramdass et al., 2006; Rangarajan et al., 2001). These cells transfected
with E6, E7 and Notch-1 were able to generate xenograft tumors in nude mice (Chakrabarti et al., 2004). These observations were also extended to primary human keratinocytes (Lathion et al., 2003). Notch-1 levels and CBF-1 transcriptional activity were increased by HPV16 oncoproteins E6 and E7 in primary mouse embryo cells (Weijzen et al., 2003). Inhibiting Notch signaling by antisense silencing in CaSki cervical cancer cells abolished proliferation and anchorage-independent growth in vitro (Weijzen et al., 2002) and completely blocked the growth of CaSki xenografts in vivo (Weijzen et al., 2003). Collectively, these data have suggested a pivotal role of dysregulated Notch in transforming epithelial cells.

However, there was some controversy raised by Talora et al. They found that invasive and metastatic cervical cancers as well as cell lines derived from cervical cancer did not express Notch-1. Activated Notch signaling leads to growth arrest of cervical cancer derived cell lines (Talora et al., 2005; Wang et al., 2007).

It is difficult to compare data obtained with different antibodies by different groups, and immunohistochemistry is hardly quantitative. However, in a later study on a large quantity of clinical samples, Notch-1, its ligand Jagged-1 and its downstream targets were observed and were accompanied by activation of the NF-κB pathway (Ramdass et al., 2006). Moreover, Lathion et al showed that the effects of Notch-1 in cervical keratinocytes are strikingly dose-dependent: high levels of constitutively active Notch-1 suppress E6 and E7 as found by Talora et al, but moderate levels of Notch-1 promote keratinocyte transformation by E6 and E7.
The data from our lab also indicate that Notch-1 protein is present in most cervical carcinomas, in agreement with Ramdass et al. (Ramdass et al., 2006) and in cervical carcinoma lines, in agreement with Lathion et al. (Lathion et al., 2003). Notch-2 and 4 appear to be co-expressed with Notch-1. The pattern of Notch-1 expression appears to change from granular and membrane-associated in normal cells to diffuse and primarily cytoplasmic in cancer cells (Song et al., 2008). This may be responsible for apparent differences in published reports.

2. Notch-1 in breast cancer

2.1 Notch-1 expression in breast cancer

Notch-1 has been shown to be oncogenic not only in cervical cancer but also in breast cancer. Aberrant activation of Notch signaling was observed in a large number of human breast carcinomas. In Weijzen’s research, all seven human primary breast ductal carcinomas studied were positive for Notch-1, whereas normal breast ducts and lobules at the margins of the tumor sections exhibited little or no detectable Notch-1 staining (Weijzen et al., 2002). In another study, the accumulation of intracellular Notch and elevated Notch signaling were observed in a variety of human breast carcinomas (Reedijk et al., 2005; Stylianou et al., 2006). Our lab confirmed that Notch expression was deregulated in human breast cancer as opposed to normal breast cells. Ductal and lobular
carcinomas consistently expressed high levels of Notch-1 and Notch-4 as compared to normal breast tissue, which expressed Notch-1 at barely detectable levels.

Parr C et al showed that there were aberrant levels of Notch-1 and Notch-2 in breast cancer tissues compared with normal breast tissue by qualitative and quantitative RT-PCR analysis along with immunohistochemical staining. These authors suggested that a high level of Notch-1 may be associated with a poorer outlook for the breast cancer patient, while a high level of Notch-2 correlated to a higher chance of survival (Parr et al., 2004).

Numb, a negative regulator of Notch signaling, was lost in > 50% of human mammary carcinomas due to specific Numb ubiquitination and proteasomal degradation and its lower levels were found to be associated with grade and proliferation rate (Pece et al., 2004). In later study, Numb was shown to form a complex with p53 and the E3 ubiquitin ligase MDM2. This interaction resulted in prevention of ubiquitination and degradation of p53. Thus, Numb loss in breast cancer led to decreased p53 levels and increased chemoresistance (Colaluca et al., 2008).

Our lab showed that Notch-1 activity was aberrantly elevated in Herceptin resistant breast cancer cells compared to Herceptin sensitive cells. Her2 inhibition activated Notch-1 signaling which conferred cells resistance to Herceptin (Osipo et al., 2008).

The expression levels of Notch-1 and Jagged-1 have been shown to be prognostic markers for breast cancer patient survival. Reedijk et al showed that high-level expression of Notch-1 or Jagged-1 predicted poor overall survival in breast cancer
patients, combined higher levels of both indicated even worse outlook. The same group also showed high level Jagged-1 mRNA and protein predicted poor outcome in breast cancer and that Jagged-1 expression was positively associated with recurrence in lymph node-negative breast cancer (Reedijk et al., 2005; Reedijk et al., 2008; Dickson et al., 2007).

2.2 ER- breast cancer has highest Notch-1 activity

Reedijk group also observed that high levels of Notch-1 and Jagged-1 were mostly found in the ER- basal phenotype breast cancer (ER-, PR-, Her2-), which is the most aggressive tumor. This parallels with our lab finding that Notch activity negatively correlates with ER status. Specifically, Notch-1 activity was highest in ER-, Her2- cells (Rizzo et al., 2008). That was one of the reasons we chose MDA-MB-231 cells which are ER-, Her2- as our model to study the Notch-1 signaling.

Overall, the data support the idea that Notch plays an oncogenic role in human breast cancer.

2.3 Notch inhibition in breast cancer

Inhibition of Notch by pharmacological agents and molecular means has been shown to suppress breast cancer growth. Pharmacological inhibition of Notch suppressed Numb negative breast tumors growth (Pece et al., 2004). Attentuation of Notch signaling
by overexpressing Numb reverts the transformed phenotype of human breast cancer cell lines, including the ER- breast cancer cell line MDA-MB-231 (Stylianou et al., 2006). Blocking Notch by siRNA resulted in reduced proliferation by 80% and reduced anchorage-independent growth. Inhibition of ligand-mediated Notch signaling in xenografted Slug-positive/E-cadherin-negative breast cancers promoted apoptosis and inhibited tumor growth and metastasis (Leong et al., 2007).

Our lab also showed that in MDA-MB-231 cells, either Notch-1 knockdown or GSI decreased cyclin A and B1; caused G2 arrest, p53-independent induction of NOXA, and death. In T47D:A18 cells, the same targets were affected, and Notch inhibition potentiated the effects of tamoxifen. In vivo, GSI arrested the growth of MDA-MB-231 tumors. In combination with tamoxifen, GSI caused regression of T47D:A18 tumors (Rizzo et al., 2008). Moreover, we found that Notch-1 siRNA or GSI resensitized Herceptin-resistant BT747 cells to Herceptin (Osipo et al., 2008). All these findings suggest that Notch signaling is a potential therapeutic target in breast cancer.

A number of Notch inhibitors have been developed, such as antisense, siRNA, overexpressed Numb or deltex, monoclonal antibodies, soluble ligands, soluble decoys to EGFR 11 and 12, inhibitors of enzyme involved in glycosylation and pharmacological inhibitors of gamma-secretase. Gamma-secretase inhibitors (GSIs) have broad anti-neoplastic activity and immediate therapeutic potential. GSI has superior pharmacological properties compared to antibodies and siRNA and relatively cheaper. Several GSIs are developed in cooperation with research into drugs for Alzheimer’s disease. GSI is currently in phase III clinical trials. However, like virtually all small-
molecule agents, they also have non-specific effects since GSI target all Notch receptors and other substrates such as amyloid precursor protein. Given that Notch is a critical transcription regulator in many physiological events, inhibiting Notch might have widespread off target effects. Development of more specific inhibitor and identification of proper therapeutic doses and administration frequency are needed for more effective cancer therapy. In my experiments in the thesis, I used GSI as well as siRNA to complement each other.

2.4 Potential mechanism of Notch oncogenic function

The oncogenic role of Notch is implicated in cervical and breast cancer, however, the mechanism of its transforming function is not well understood. Most studies have focused on the ability of Notch to collaborate with other proteins to transform normal cells. Collaborating oncoproteins that have been identified include: adenovirus E1A (Capobianco et al., 1997), HPV E6 and E7 (Rangarajan et al., 2001), Ras (Fitzgerald et al., 2000), Myc (Girard et al., 1996), and SV40 large T antigen (Bocchetta et al., 2003). Notch, together with collaborative proteins, transform cells by promoting cell proliferation and survival, inhibiting differentiation, suppressing apoptosis.

Potentially oncogenic targets of Notch-1 include cyclin D1 and D3, cyclin A, SKP2 (S-phase kinase-associated protein 2), PI3K (phosphatidylinositol 3-kinase), Akt, Her2, NF-κB (nuclear factor-κB), β-catenin, signal transducers and activators of transcription-3, and hypoxia-inducible factor-1α (Miele et al., 2006). Here, in this thesis,
we investigated one of the downstream targets of Notch-1: NF-κB. NF-κB is a pro-survival signal in cervical and ER-breast cancer. We hypothesized that Notch-1 promotes cell survival by activating NF-κB activity.

D. Introduction to NF-κB

1. NF-κB members and activation

Nuclear Factor-kappa B (NF-κB), is a family of transcription factors with a critical role in regulating inflammation, cell survival, cell differentiation, and cell proliferation. In mammals, there are five members of the NF-κB family, including RELA (p65), RELB, c-REL, NF-κB1 (p105/p50), and NF-κB (p100/p52), which associate with each other to form different homodimers and heterodimers to regulate the expression of their downstream targets (Karin and Greten, 2005; Luo et al., 2005).

All family members are characterized by the presence of a Rel homology domain (RHD) which contains a nuclear localization sequence (NLS) and is involved in sequence-specific DNA binding, dimerization and interaction with the inhibitory IκB proteins. Members of the NF-κB family can be divided into two classes: one containing C-terminal transcriptional activation domains RelA/p65, RelB, and c-Rel, and the other which lacks the activation domains p50/p105 (NF-κB1) and p52/p100 (NF-κB2). A homodimer or heterodimer can potently transactivate NF-κB dependent promoters if it contains RelA/p65, RelB, or c-Rel, whereas p50 homodimer and p52 homodimer act as
repressors for gene transcription because of the absence of the activation domain (Hayden and Ghosh, 2004; Baeuerle and Baltimore, 1996).

There are two most frequently studied NF-κB pathways: the canonical (classic) and noncanonical (alternative). The canonical pathway of NF-κB activation is mediated by rapid phosphorylation of IκB α by the IKK complex. Take the predominant form of NF-κB, heterodimer of p65 and p50, for example. It is inactive in the cytoplasm as a result of binding to inhibitory proteins of the IκB family (IκBs). In mammalian cells, there are three principal IκBs, IκBα, IκBβ and IκBε, which function in part by using a core domain composed of six to seven ankyrin repeats to bind to the RHD and thereby masking the NLS (Perkins, 2007).

In response to numerous stimuli, such as cytokines, chemokines, mitogens, and bacterial and viral infections, an IκB kinase (IKK) complex is activated by IKK kinases (Karin, 1999). Activated IKK complex consisting of two catalytic subunits (IKKα and IKKβ) and an IKKγ regulatory subunit phosphorylates IκBs at the two conserved serine residues in the N-terminal domain, leading to 26s proteasome mediated degradation of IκB. Degradation of IκB releases NF-κB, enabling it to translocate to the nucleus and rapidly induce a variety of genes encoding matrix metalloproteinases, inflammatory and chemotactic cytokines, and antiapoptotic proteins (Karin et al., 2002; Karin and Lin, 2002; Li and Verma, 2002) as shown in Figure 2.4.

In contrast, the noncanonical pathway involves the upstream kinase NF-κB-inducing kinase (NIK), which activates IKKα homodimers instead of IKK complex in response to certain members of the TNF family. This pathway is mediated by
phosphorylation of p100 and subsequent processes to p52 through ubiquitin-dependent processing. The consequence of activation of the noncanonical pathway is p52/RelB heterodimer, which has a higher affinity for distinct κB elements and might therefore regulate a distinct subset of NF-κB targets (Karin and Greten, 2005; Luo et al., 2005).
Transcriptional Factors

- RelA/p65
- p50
- p52
- cRel
- RelB

Proteasome-mediated degradation

Figure 2.4 Canonical NF-κB Signaling Pathway

Stimulation

IKK-α
IKK-β
IKK-γ
IκB

Figure 2.4 Canonical NF-κB signaling pathway. In response to numerous stimuli, such as cytokines, chemokines, mitogens, and bacterial and viral infection, an IκB kinase (IKK) complex is activated by IKK kinases. Activated IKK complex consisting of two catalytic subunits (IKKα and IKKβ) and an IKKγ regulatory subunit phosphorylates IκBs at the two conserved serine residues in the N-terminal domain, leading to 26s proteasome mediated degradation of IκB. Degradation of IκB releases NF-κB, enabling it to translocate to the nucleus and rapidly induce a variety of genes encoding matrix metalloproteinases, inflammatory and chemotactic cytokines, and antiapoptotic proteins.
Generally speaking, NF-κB is considered as an oncogene in cancer. NF-κB contributes to oncogenesis by stimulating cell proliferation, inhibiting apoptosis, increasing metastasis and angiogenesis. The anti-apoptotic activity of NF-κB depends on gene induction. In fact, NF-κB induces the expression of a number of genes whose products can inhibit apoptosis; these include cellular inhibitors of apoptosis (c-IAPs), caspase-8-c-FLIP (FLICE inhibitory protein), A1, TNFR-associated factor 1 (TRAF1) and TRAF2. These anti-apoptotic proteins work in a coordinated manner to block apoptosis at multiple steps along the apoptotic signaling cascade. Although the majority of evidence provides overwhelmingly strong support to the anti-apoptotic function of NF-κB, there are few sporadic reports that NF-κB may contribute to induction of pro-apoptotic molecules. They include DR6 (death receptor 6) (Kasof et al., 2001), DR4, DR5 (Ravi et al., 2001) and Fas (Zheng et al., 2003). However, whenever the genes encoding death receptors are induced, so are the anti-apoptotic molecules that neutralize their pro-apoptotic activity. Thus, little physiological evidence exist for a pro-apoptotic function of NF-κB.

2. IKKα

Despite the fact that IKKα and IKKβ are very similar in structure, they have different roles in the NF-κB pathway and different NF-κB-independent functions. IKKβ appears to predominantly contribute to IκB α phosphorylation, while IKKα has more physiological functions than inducing IκB degradation and releasing of NF-κB.
IKKα is able to phosphorylate a number of substrates. For example, it phosphorylates p100 and leads to proteasome-dependent processing of p100 to p52 (Perkins, 2007). It also phosphorylates RelA at Ser 536 in macrophages and stimulates its degradation and thus promotes resolution of inflammation (Lawrence et al., 2005). In addition to phosphorylating components of the NF-κB and IKK pathways, IKKα regulates gene expression by modulating chromatin structure and facilitating recruitment of co-activators. The nuclear function of IKKα include the phosphorylation and activation of the estrogen receptor α, together with its co-activator protein SRC3 (steroid receptor coactivator 3) in breast cancer cells (Park et al., 2005) and phosphorylation and stabilization of the β-catenin oncoprotein (Albanese et al., 2003; Carayol and Wang, 2006) on cyclin D1 promoter. IKKα also phosphorylates H3 Ser10 through association with cyclic-AMP responsive element binding (CREB)-binding protein (CBP) (Anest et al., 2003; Yamamoto et al., 2003). Moreover, IKKα phosphorylates SMRT co-repressor and thus resulting in release of HDAC and facilitation of NF-κB subunits recruitment on NF-κB dependent gene promoters (Hoberg et al., 2004). This phosphorylation leads to de-repression of gene transcription. The same phenomenon is also detected on Notch-dependent promoters (Aguilera et al., 2004; Fernandez-Majada et al., 2007), indicating IKKα kinase function independent of classical NF-κB activation. In our lab, we found that IKKα is recruited to the promoters of NF-κB dependent gene c-IAP2 and IκB α in CaSki cells, presumably phosphorylating SMRT and derepressing gene transcription. The recruitment of IKKα on c-IAP2 and IκB α promoters is Notch-1 dependent, suggesting the crosstalk between NF-κB and Notch-1.
IKKα also has kinase-independent roles in the regulation of keratinocyte differentiation (Hu et al., 2001). Taken together, it can be concluded that IKKα activation may result in more widespread effects on cell signaling than previously realized.

3. NF-κB in cervical cancer

Recent immunohistochemistry data on a large series of 352 patients (Ramdass et al., 2006) indicate that activation of Notch signaling is observed in the majority of cervical cancers. The activation of Notch signaling is accompanied by activation of the NF-κB pathway. The Notch signaling is determined by expression levels of intracellular Notch-1, Hes1, CDK9 (cyclin-dependent kinase 9) and Jagged-1. The NF-κB activity is determined by nuclear staining for p50 and p65 and expression of NF-κB target genes. NF-κB is a major survival pathway that is highly relevant to cervical cancer. Previous studies have shown that NF-κB is constitutively activated in human cervical squamous cell carcinomas as compared to normal cervical epithelium. Constitutive activation of NF-κB with nuclear accumulation of p50 and p65 with cervical cancer progression has been described in clinical specimens (Prusty et al., 2005; Nair et al., 2003). The positive rates of NF-κB and BCL-2 based on immunohistochemistry were significantly higher in cervical cancer than in normal cervical tissue (Xia et al., 2005).

Blocking NF-κB in cervical cancer cells may not only help eradicate tumor cells but also increase sensitivity to other conventional therapies. The cytotoxicity of arsenic trioxide (Wei et al., 2005; Woo et al., 2004), celecoxib (Kim et al., 2004) and curcumin
and its analog (Divya and Pillai, 2006; Kasinski et al., 2008) on cervical cancer cells is mediated in part by NF-κB inhibition. Inhibition of NF-κB sensitizes cervical cancer cells to cisplatin induced apoptosis (Venkatraman et al., 2005). Inhibition of NF-κB by the proteasome inhibitor Velcade enhances radiosensitivity of certain human cervical carcinoma cancer cells in vitro (Kamer et al., 2009).

4. NF-κB in ER - breast cancer

Several studies suggest that NF-κB is important in breast cancer carcinogenesis initiation and progression. NF-κB was activated during neoplastic transformation of mammary epithelial cells such as HMEC and MCF-10A (Kim et al., 2000). Breast cancers usually progress from a hormone dependent, nonmetastatic, antiestrogen-sensitive phenotype to a hormone independent, antiestrogen and chemotherapy resistant phenotype. The latter phenotype is highly invasive and metastatic. This progression is accompanied by downregulation of ER expression, mutation of ER and altered regulation of ER responsive genes. NF-κB was found to contribute to breast cancer progression from ER+ breast cancer to ER- breast cancer. NF-κB was found to be highly active in ER- breast cancer cell lines as compared to ER + breast cancer cell lines, as well as in ER- primary breast tumors versus ER+ primary breast tumors. p100 subunit of NF-κB was overexpressed in a majority of breast cancer samples and breast cancer cell lines. Constitutive DNA binding of NF-κB and elevated concentration of NF-κB were also detected in extracts from poorly differentiated ER- primary tumors (Biswa...
Nakshatri et al., 1997; Bhat-Nakshatri et al., 1998). In addition, the transcription of NF-κB regulated genes was increased in breast tumors compared to adjacent normal tissues. These data imply that dysregulated NF-κB expression is associated with mammary carcinogenesis, especially in ER- breast cancer cells with more aggressive and metastatic phenotype.

Constitutive active NF-κB could be correlated with increased levels of members of epidermal growth factor receptor family EGFR, including Her2 in ER- cells (Biswas et al., 2000; Biswas et al., 2001; Biswas et al., 2004; Nakshatri et al., 1997) and linked to elevated expression and activity of IKK (Romieu-Mourez et al., 2001).

Elevated NF-κB activation in ER- breast cancer has also been suggested to be responsible for chemoresistance and metastatic growth. Chemotherapeutic drugs and γ irradiation usually caused apoptosis via activation of specific caspases. However, they also induce the activation of NF-κB. Active anti-apoptotic NF-κB protected the cells from apoptosis induced by chemotherapy reagent and radiation. This may explain the chemo-resistance and radiation resistance (reviewed in (Nakshatri et al., 1997)).

NF-κB has been considered a potential therapeutic target for ER- breast cancer (Biswas et al., 2000; Biswas et al., 2001; Biswas et al., 2004; Nakshatri et al., 1997). Inhibiting NF-κB activity by different methods, such as microinjection of IKBα; antibodies against RelA or NF-κB decoys (Sovak et al., 1997); inhibition of PKC; overexpression of dominant negative IKKβ (Biswas et al., 2001); an IκB α super-repressor; the use of a IKKγ-blocking peptide; application of IKK inhibitor cyclopentenone (Ciucci et al., 2006); or proteasome inhibitor bortezomib caused cell
apoptosis or tumor regression in ER- breast cancer \textit{in vitro} or \textit{in vivo}. Inhibition of NF-κB enhanced the sensitivity of tumor cells to apoptosis induced by chemo drugs and radiation. Therefore, simultaneous use of NF-κB inhibitors and chemotherapy or radiation might be advantageous.

5. Some important NF-κB dependent genes in cancer

   c-IAP2 (cellular inhibitor of apoptosis protein) is a member of inhibitor of apoptosis proteins (IAPs) family. Members of this family are characterized by the presence of a variable number of Baculoviral IAP repeat (BIR) motifs. c-IAP2 has three BIR domains, a RING domain and one CARD (CAspase Recruitment Domain) domain.

   c-IAP2 is one of the anti-apoptotic proteins regulated by NF-κB. Recent evidence indicated that c-IAP2 was frequently overexpressed in cancer and its expression level was implicated in contributing to tumorigenesis, chemoresistance, disease progression and poor patient survival (Hunter \textit{et al.}, 2007). It suppresses the apoptosis induced by both extrinsic and intrinsic pathways through direct inhibition of effecter caspases (caspases-3, -6, and 7) (Deveraux \textit{et al.}, 1997; Deveraux \textit{et al.}, 1998). It also promotes cancer cell survival by functioning as E3 ubiquitin ligases that maintain constitutive ubiquitination of the RIP1 (Receptor interacting protein 1) adaptor protein (Bertrand \textit{et al.}, 2008). Inhibition of multiple IAPs increased apoptosis in response to the Her2 antagonists Herceptin, Lapatinib or Gefitinib in Her2-overexpressing breast cancer
BT474 cells, or Gefitinib in EGFR overexpressing breast cancer MDA-MB-468 cells (Foster et al., 2009).

A20 is also called “tumor necrosis factor α-induced protein 3 (TNFAIP3)”. Its expression is mediated through activation of NF-κB. A20 inhibits TNF receptor signaling by mediating the ubiquitination and proteasomal degradation of RIP1 (Receptor interacting protein 1) which is essential for TNF-induced NF-κB activation (Vereecke et al., 2009). Depending on stimulus and cellular context, A20 could be anti-apoptotic or pro-apoptotic. A20 expression was found to be correlated with the differentiation stages of nasopharyngeal carcinoma (Codd et al., 1999) and associated with resistance to O6-alkylating agents in glioblastoma cells. It was proposed as a predictive marker associated with patient survival (Bredel et al., 2006). High A20 expression has also been shown to confer breast cancer cells resistance to apoptosis induced by TNF-α (Opipari, Jr. et al., 1992). Recently, Vendrell et al (Vendrell et al., 2007) showed that A20 was an E2 regulated gene. Its expression correlated with ER expression in both cell lines and tumor samples. Overexpression of A20 in ER+ breast cancer cells resulted in resistance to tamoxifen and hypersensitivity to E2. A20 was highly expressed in tamoxifen resistant cells and moreover it was overexpressed in more aggressive breast tumors (ER-, PR- and high histological grade). These data indicated that A20 was responsible for the development of tamoxifen resistance and associated in vivo with poor prognostic tumor marker. Thus, A20 was proposed a good marker for tumor aggressiveness in breast cancer.
6. TNF-α, apoptosis and cancer

Tumor necrosis factor (TNF) is a multifunctional cytokine with critical role in regulating cell survival, proliferation, differentiation, and death. TNF was first identified as an inflammatory cytokine with its ability to suppress tumor cell proliferation and induce tumor regression (Matthews and Watkins, 1978). While scientists were trying to utilize TNF as a powerful anticancer treatment, the paradoxical tumor-promoting effect of TNF became apparent. On one hand, TNF induces apoptosis. On the other hand, TNF stimulates proliferation, survival, migration, and angiogenesis in most cancer cells that are resistant to TNF-induced cytotoxicity, resulting in tumor promotion.

TNF receptor 1 (TNFR-1) mainly mediates TNF's cellular signaling in most cell types. TNFR-1 is a death domain (DD)-containing receptor capable of inducing cell apoptosis (Wajant et al., 2003). TNFR-1 forms a homotrimer to recruit TNFR-associated death domain (TRADD) after TNF, a natural homotrimer, binds to the receptors. TRADD recruits downstream adaptor proteins such as receptor interacting protein (RIP), TNFR associated factor 2 (TRAF-2), and Fas-associated death domain (FADD) to deliver signals for distinct signaling pathways. These adaptor proteins further recruit key molecules that contribute to intracellular signaling to activate NF-κB, mitogen activated protein kinases (MAPKs), and cell death (Aggarwal, 2003).

It is well established that TNF induces apoptosis in a variety of cell types. TNF induces apoptosis through extrinsic apoptosis and mitochondria-mediated (intrinsic) apoptosis pathway. The extrinsic apoptosis pathway is initiated from TNF binding to
TNFR1, provoking the recruitment of TRADD, RIP, FADD, and caspase-8. Active caspase-8 then activates the executor capsases-3 and -7, resulting in cell apoptosis. The mitochondria-mediated (intrinsic) apoptosis pathway is mediated by caspase-8 activating BCL-2 interacting domain (Bid), a BH3-only Bcl2 family member. Caspase-8 cleaves Bid to generate tBid, which promotes release of cytochrome c and second mitochondria-derived activator of caspase (Smac) from mitochondria to the cytosol. Cytochrome c forms a complex with apoptotic protease activating factor 1 (Apaf-1) and pro-caspase-9 to form apoptosome, leading to caspase-9-mediated activation of the executor caspases (Wang, 2001). Smac binds to and inhibits the inhibitor of apoptosis proteins to accelerate apoptosis. These proteins include c-IAP1, c-IAP2, X-linked Inhibitor of Apoptosis Protein (XIAP) and survivin.

However, the tumor-destructive activity of TNF was at its height, came the growing paradoxical observation that TNF was implicated in contributing to cellular transformation, survival, proliferation, invasion, angiogenesis, and metastasis. Emerging reports have shown that the serum TNF concentration is elevated in cancer patients (Ahmed et al., 2001; Ferrajoli et al., 2002). Moreover, the high TNF expression level in pre-cancerous and tumor cells is linked with the progression of malignant diseases such as chronic lymphocytic leukemia, Barrett's adenocarcinoma, prostate cancer, breast cancer, and cervical carcinoma (Ahmed et al., 2001; Ferrajoli et al., 2002; Tselepis et al., 2002; Michalaki et al., 2004; Garcia-Tunon et al., 2006). TNF has been shown to promote tumorigenesis and growth via activating NF-κB (Suzukawa et al., 2002; Hu et al., 2004) or a PKCα- and AP-1-dependent pathway (Arnott et al., 2002). TNF has also
been documented to promote tumor angiogenesis in U251 glioma cells (Nabors et al., 2003), ovarian cancer cells (Kulbe et al., 2007), a mouse lung metastasis model (Tomita et al., 2004). TNF enhances cancer invasion and metastasis by accelerating the epithelial-mesenchymal transition (EMT) (Bates and Mercurio, 2003; Bates et al., 2004; Chuang et al., 2008), inducing matrix metalloproteases (MMP-2, -3, -9, -12) (Esteve et al., 2002; Hagemann et al., 2004; Cheng et al., 2007) or α2β1 integrin (Montesano et al., 2005), activating focal adhesion kinase (FAK), up-regulating of macrophage migration inhibitory factor (MIF) and extracellular MMP inducer in tumor cells (Hagemann et al., 2005).

The paradoxical functions of TNF result from the existence of extensive crosstalk among TNF-induced pathways. For instance, TNF induces apoptosis through extrinsic and intrinsic pathways while it also enhances expression of anti-apoptotic proteins through NF-κB activation. Activative caspase-8 resulting from TNF stimulation mediates cell apoptosis and it also causes cleavage of the key NF-κB mediator RIP to terminate the cell survival signal (Lin et al., 1999). The mitochondria-released Smac blocks IAPs, leading to apoptosis. On the other hand, TNF induced NF-κB activation inhibits the apoptotic JNK activation and induces expression of anti-apoptotic and antioxidant genes, preventing cell death and promoting cancer cell proliferation (Wajant et al., 2003; Kamata et al., 2005). Other factors, such as cell type, concurrent stimulation of other cytokines, or the amount of reactive oxygen species (ROS) can shift the balance in favor of one pathway or another. Therefore, the balance between TNF-induced pro- and anti-survival signalings determines diverse functions of the end result.
E. Notch, Akt and NF-κB

1. Notch and NF-κB

So far, a number of reports have described the crosstalk between NF-κB and Notch. The effect of Notch-1 regulation on NF-κB activity has been conflicting. It has been suggested that Notch inhibits NF-κB activity in the nucleus via direct interaction mediated by the N-terminal portion of Notch-1 (Guan et al., 1996; Wang et al., 2001). Notch-1 was also shown to inhibit NF-κB activity by increasing IκBα expression via derepressing CBF-1 repressing effect on IκBα promoter in hepatic stellate cell (Oakley et al., 2003).

In contrast, more studies indicated that Notch receptors activate NF-κB. NF-κB activation involves a group of components along the signal transduction pathway. In fact, Notch has been shown to regulate NF-κB signaling components at multiple levels to activate NF-κB activity.

First of all, at the level of IKK upstream kinase, Notch-3 has been demonstrated to modulate PKC θ, which is a kinase upstream of IKK complex, to regulate NF-κB activation in thymocytes and T lymphoma cells of Notch-3 transgenic mice. Notch-3 can also increase association between IKKα and NIK, possibly leading to a further increase of the overall NF-κB activation (Bellavia et al., 2000; Bellavia et al., 2002). Notch-1 has been shown to activate Akt in many systems; we will discuss it in detail in later section.
Secondly, at the level of IKK complex, it is known that Notch-3 forms a complex with IKKα homodimers and activates cytoplasmic IKKα kinase activity (Bellavia et al., 2000; Felli et al., 2005; Vacca et al., 2006). Notch signaling activation mediated by Jagged-1 is reported to induce IKK kinase activity in human keratinocytes (Nickoloff et al., 2002). Similar effects were observed in murine erythroleukemia (MEL) cells undergoing erythroid differentiation (Jang et al., 2004). In this model, the authors showed that exposure of MEL cells to stromal cells expressing Notch ligand Jagged-2 caused rapid and short lived activation of NF-κB. Recently, it has been shown that overexpression of Notch-1IC activates NF-κB by interacting with the IKKα/β signalosome and enhancing IKKα/β kinase activity in a mouse model of T-cell acute lymphoblastic leukemia (T-ALL) and in human T-ALL cells (Vilimas et al., 2007).

Finally, at the level of NF-κB subunits, several different mechanisms have been elucidated: physical interaction between Notch-1 and p50/c-Rel; transcriptional regulation of NF-κB subunits and NF-κB dependent genes by Notch-1.

It is revealed that Notch-1 physically interacts with p50/c-Rel complexes. Through this interaction, Notch-1 competes with IκB α and prevents it from exporting NF-κB to the cytoplasm and promotes nuclear translocation of NF-κB (Shin et al., 2006).

In addition to physical interaction, Notch-1 is also shown to activate NF-κB by increasing transcription expression of NF-κB subunits and NF-κB dependent genes (Cheng et al., 2001; Oswald et al., 1998). Potential CBF-1 binding sites have been identified within the NF-κB sites in the promoter region of some genes, including the mouse major histocompatibility complex, β2-microglobulin, interleukin (IL) 6, NF-κB2,
and CYP2B1 (Brou et al., 1994; Israel et al., 1989; Kannabiran et al., 1997; Lee et al., 2000; Oswald et al., 1998). A subset of NF-κB binding sites (GGGAAA) may also contain an overlapping CBF-1 site (CTGGGA). Moreover, Oswald et al. (Oswald et al., 1998) demonstrated that CBF-1 binds to NF-κB binding sites in Bcl-3, IκBα, IFN-β. Cheng (Cheng et al., 2001) showed that Notch-1 upregulates the expression of p50, p65, RelB and c-Rel NF-κB subunits in murine bone marrow hematopoietic precursors.

Conversely, reports have shown that NF-κB also regulates Notch dependent signaling. c-Rel induced Jagged-1 expression, triggering the Notch signaling pathway in neighboring cells (Bash et al., 1999). Sequestration of p65 by IκBα translocated nuclear corepressor SMRT/N-CoR to the cytoplasm, abrogating SMRT/N-CoR mediated repression of Notch dependent promoters (Espinosa et al., 2002; Espinosa et al., 2003). IκBα was described to bind to the Hes-1 promoter and associate with transcriptional repression (Aguilera et al., 2004). IKKα could alleviate this transcription repression, presumably by phosphorylating IκB α. Nuclear IKKα also can phosphorylate SMRT on CBF-1 dependent promoter (Fernandez-Majada et al., 2007), leading to uncontrolled activation of Notch-mediated signaling. More recently, Notch-2 and NF-κB have been reported to cooperate in marginal zone B-cell development. In this model, NF-κB stimulates the expression of two known Notch targets, Hes-5 and Deltex-1 (Moran et al., 2007).
2. Notch and Akt

PI3K-Akt signaling pathway is involved in multiple cellular functions including promoting cell growth, increasing glucose uptake and oxidation, cell cycle progression and cell survival. The PI3K is activated by receptor tyrosine kinases and G protein coupled receptors to generate phosphatidylinositol triphosphate (PIP3) from phosphatidylinositol 4,5 biphosphate (PIP2). The accumulation of PIP3 at the membrane recruits the Akt kinase and induces its phosphorylation by PDK1 and the mTOR-Rictor kinases at Thr 308 and Ser 473, respectively (Sarbassov et al., 2005). In turn, Akt activates the phosphorylation of multiple downstream substrates including IKKα. Signaling by the PI3K-Akt pathway is terminated by the PTEN phosphatase, which dephosphorylates and thereby inactivates PIP3 (Bader et al., 2005).

Increasing number of reports demonstrate that Notch interplays with Akt in various systems. Notch is able to activate Akt and also inhibit Akt activity depending on the physiological context. In T-cells, Notch-1 has been shown to activate Akt via Src family protein tyrosine kinase p56lck (Sade et al., 2004). Notch signals promote T-cell precursors survival through maintenance of cellular metabolism via activation of the PI3K-Akt signaling pathway (Ciofani and Zuniga-Pflucker, 2005). Active Notch-1 inhibits p53-induced apoptosis and sustains transformation by HPV 16 E6 and E7 oncogenes through a PI3K/Akt dependent pathway (Nair et al., 2003). In melanoma, constitutive activation of Notch-1 drives primary melanoma toward a more aggressive phenotype in vitro and in vivo by activating PI3K/Akt pathway (Liu et al., 2006).
The mechanism by which Notch-1 activates has been studied. Notch-1 activates Akt by decreasing PTEN (phosphatase and tensin homolog) expression through Hes1 and Myc in TALL cells. Both Hes1 and Myc bind and regulate the PTEN promoter, with Hes1 working as a strong transcriptional repressor and Myc as a weaker transcriptional activator, so that the overall output downstream of Notch-1 activation is a controlled downregulation of PTEN transcripts (Palomero et al., 2007). More recently, Perumalsamy (Perumalsamy et al., 2009) showed another mechanism that cytoplasmic but not nuclear Notch-1 blocks apoptosis triggered by nutrition deprivation in Hela cells through mTOR, Rictor and Akt dependent signaling. The same effect was also observed in the cytokine-deprived condition in activated T cells.

Given the crucial role of Notch-1 promoting cell survival via Akt dependent signaling, inhibition of Notch-1 results in downregulation of PI3K/Akt activation and induction of cell apoptosis. In T-ALL cells, downregulation of Notch-1 by siRNA inhibits cell proliferation, induces cell cycle arrest and apoptosis by suppressing Akt activity. Using GSI to block oxaliplatin-induced activation of Notch-1 signaling enhances colon cancer cells chemosensitivity. This effect is achieved by prevention of induction of prosurvival Akt signaling (Meng et al., 2009). In gliomas cells, Notch-1 silencing inhibits cell growth, causes cell cycle arrest and apoptosis and reduces cell invasiveness by downregulating EGFR, PI3K/Akt (Xu et al., 2009).

Notch-1 has also been documented to inhibit Akt signaling. Constitutively active Notch-1 increases PTEN protein expression and leads to the loss of Akt phosphorylation in 293 HEK cells (Chappell et al., 2005). Notch-1 inhibits endothelial cell proliferation
by repressing MAPK and PI3K/Akt dependent pathway (Liu et al., 2006). Notch-1 inhibits Akt phosphorylation in T cell activation as reported by Eagar (Eagar et al., 2004).

On the other hand, several studies have shown the regulation of Akt in Notch-1 signaling. Notch-1 is required for Akt to transform melanocytes in vitro and activated by Akt through NF-κB activity (Bedogni et al., 2008). Inhibition of Akt results in the decreased protein expression of Notch-1 (Calzavara et al., 2008).

3. Akt and NF-κB

NF-κB is an important downstream outcome of Akt activation. It is reported that Akt is required for NF-κB activation induced by TNF-α (Ozes et al., 1999). Akt functions through IKK to promote the transactivation potential and phosphorylation of NF-κB (Madrid et al., 2001). Akt is proposed to promote metastasis and angiogenesis through IKK in a manner that depends on NF-κB activation (Agarwal et al., 2005).
CHAPTER III
MATERIAL AND METHODS

A. MATERIALS

1. Antibodies

The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): Notch-1 (C-20), IκB-α (H-4), Akt1 (C-20), Pol II antibody (H-224), Notch-4 (H-225) normal mouse IgG rabbit IgG, goat IgG, secondary anti-mouse antibody. The antibodies below were bought from Cell Signaling: active form of Notch-1 (Val1744), p-IKKα/β (2681), p-Akt Ser 473 (D9E) and p-IκBα(5A5). The antibodies below were obtained from IMGENEX (Sorrento Valley, CA): NF-κB p50 (IMG-5812A), p65 (IMG-150A) and IKKα (IMG-136A). The antibodies below were obtained from Abcam (Cambridge, MA): Human specific IKKα (ab4111) and IKKα (ab25108) for IP. The secondary anti-goat antibody was purchased from Vector Laboratories (Burlingame, CA). The secondary antibody anti-rabbit was from GE health care. The antibody against β-actin (AC-15) was from Sigma-Aldrich.
2. Cell culture

Caski and MDA-MB-231 cells from ATCC and LTK-Parental and LTK-Jagged-1 cells generously provided by Dr. G. Weinmaster (University of California at Los Angeles, Los Angeles, CA), were cultured in DMEM with 10% FBS.

3. Drugs

Gamma secretase inhibitor (GSI) cbz-Leu-Leu-Nle-CHO was purchased from Calbiochem (Cat# 565750). GSI MRK-003 was a kind gift from Dr. Peter Strack (Merck Inc.). Wortmannin was purchased from Sigma-Aldrich. Drugs were dissolved in DMSO and stored in aliquots at –20 °C. TNF-α (61606 BD Pharmingen) was aliquoted in BSA (1mg/ml).

4. Plasmids and siRNA

The pcDNA-Notch1IC constructs have been obtained from Dr. Anthony Capobianco, Cincinnati Childrens Hospital (Cincinnati, OH).

Dominant negative IKKα was kindly provided by G.E. Sonenshein, Boston University School of Medicine (Boston, MA).

The NF-κB firefly luciferase construct was kindly provided by B.J. Nickoloff, Loyola University Medical Center (Chicago, IL) and the Renilla Luiciferase construct PRL-TK (Cat#: E2241) was purchased from Promega (Madison,WI).
The CBF-1 luciferase construct was a generous gift from Dr. Tom Kadesch (University of Pennsylvania, Philadelphia PA). Four wild type CBF-1 consensus repeats were cloned in front of the minimal promoter and upstream the luciferase gene in the pGL2-basic vector.

Full length Notch-1 tagged with Renilla at C-terminus was kindly provided by Dr. Raphael Kopan (Washington University St. Louis, MO).

Control siRNA (sc-36869), Notch-1 (sc-36095) and IKKα siRNA (sc-29365) were from Santa Cruz Biotechnology (Santa Cruz, CA).

5. Kits

RNeasy Mini kit, RNase-Free DNase set kit, QIAquick PCR purification kit and QIA shredder were bought from Qiagen. First strand cDNA synthesis Kit was purchased from Fermentase Life Science Co., EU.

B. Methods

1. Dual luciferase assay

Caski or MDA-MB-231 cells were plated on 24 well plate and transfected with 0.4 µg of NF-κB luciferase reporter or CBF-1 luciferase reporter/well and 0.02 µg pRL-TK/well as the internal control. Luciferase assays (Dual-Luciferase Assay System,
Promega) were performed 48 hrs after transfection. Cells were washed twice with 1× PBS and lysed in 100 µl luciferase reporter buffer (Promega, Madison, WI). Ten µl of the cell lysate was transferred to a 96-well plate and luciferase assays were performed using the substrates Luciferin or Coelenterazine for Firefly luciferase or Renilla luciferase, respectively (Dual-Luciferase Assay System, Promega). The luciferase activity was quantified using a luminometer (Veritas, CA). The relative light units Luciferase values were normalized against renilla luciferase activity. At least three independent experiments were performed in duplicate.

2. Immunoprecipitation (IP), Co-Immunoprecipitation (Co-IP) and Quantitative Renilla Co-IP

For IP, MDA-MB-231 cells together with either LTK-PAR or LTK-JAG were lysed in RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP-40)) supplemented with a cocktail of protease inhibitors (Roche, IN) and (1×) phosphatase inhibitors (78420 pierce). The lysates were incubated on ice for 30 mins and centrifuged at 13000 rpm for 10 mins. The supernatants were precleared by incubating with 30 µl protein A/G beads (Calbiochem) for 1 hr with shaking. The cell lysate was immunoprecipitated with 2 µg of antibodies overnight followed by 2 hrs immunoprecipitation by 30 µl of beads. The beads were washed three times in RIPA buffer and were then boiled in Laemmli buffer and assayed by Western blot.
For Co-IP, the procedure is similar to IP. The differences are in the buffers and primary antibody used in the immunoblot. Lysis buffer contains 20 mM Hepes pH 7.9, 137 mM NaCl, 10% glycerol, 1% NP40, 2 mM EDTA, and protease inhibitors. Washing buffer has 20 mM Hepes pH 7.9, 137 mM NaCl, 10% glycerol, 0.5% NP40, 2 mM EDTA and protease inhibitors.

For Renilla Co-IP, quantitative Renilla immunoprecipitation was performed as described (Vooijs et al., 2004) previously. CaSki cells were transfected with 2.0 µg of Notch-1 luciferase construct. Forty-eight hours after transfection, cells were treated for 30 min with vehicle or TNF-α (10 ng/ml). The cells were washed with 2X PBS and lysed in Co-IP buffer (0.2M KCl, 25mM HEPES, pH 7.4, 1% Nonidet P-40, and 0.2mM EGTA) supplemented with a cocktail of protease inhibitors (Roche). The cell lysate was immunoprecipitated with 2 µg of antibodies or correspondent control IgG for 2 hrs followed by 2 hrs incubation with protein G plus/protein A Agarose beads (Calbiochem). After washing the beads with Co-IP buffer, luciferase activity in washed pellets was determined (Dual-Luciferase Assay System, Promega) and compared to total Renilla luciferase activity (pellet plus combined supernatants). Data are representative of three independent experiments, each conducted in triplicate.

3. Kinase assay

The kinase activity of the IKKα and the IKKβ subunits of the IKK complex was measured by kinase assay. Briefly the treated and untreated cells were lysed in lysis
buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 1% Triton X-100, 10% glycerol) supplemented with protease inhibitor cocktail (Roche), 1 mM Na-orthovanadate, 50 mM \(\beta\)-glycerophosphate and 1 mM DTT. The cell lysates were collected and pre-cleared with protein A/G sepharose beads rotating for 1 hr. The subunits were immunoprecipitated with antibodies specific to IKK\(\alpha\) or IKK\(\beta\) by using protein A/G sepharose beads. The beads were then washed three times with lysis buffer. Then 20 \(\mu\)l of kinase buffer supplemented with 10 \(\mu\)Curies of \(\gamma\)-P32 ATP and 2 \(\mu\)g of GST-IkB\(\alpha\) substrate (Millipore) was added to each tube. The tubes were incubated for 30 mins at 30 \(^\circ\)C with gentle shaking. The reaction was stopped by addition of 4X Nupage LDS Sample buffer (Invitrogen, CA) and the sample was boiled at 80 \(^\circ\)C for 5 mins. The samples were spun down, and the supernatant containing the substrates was then loaded and run on a 7% tris-acetate gel and gels were dried for 2 hrs. The amount of phosphorylated substrate was then detected and subjected to autoradiography.

4. Real time RT-PCR

Total RNA was isolated from either transfected or treated cells using RNeasy Mini kit (Qiagen) following the manufacture’s directions. Reverse transcription was performed with random hexamer primers or specific primers using the First strand cDNA synthesis Kit (Fermentase Life Science Co., EU) according to the manual. cDNAs were amplified by real time PCR using iTaq\textsuperscript{TM} SYBR Green Supermix with ROX (Bio-RAd, CA). Each reaction sample contained 12.5 ul Supermix, 0.25 \(\mu\)l of each primer at 50 \(\mu\)M,
10 µl H2O and 2 µl cDNA. Each sample was run in triplicate in a 96-well Optical Reaction plate (Applied Biosystems, CA). The PCR reaction conditions were: 50 °C for 2 mins, 95 °C for 5 mins, 35 cycles of (95 °C for 30 secs, 59 °C for 1 min, 72 °C for 1 min) followed by dissociation step. The data were analyzed by utilizing the 7300 system Software (Applied Biosystems, CA).

The primers used in real time PCR were: RPL13a forward and reverse respectively: ACAAGATAGGGCCCTCCA AT; CATAGGAAGCTGGG AGCAAG; cIAP-2 forward and reverse respectively: CCGGAAGAATAGAATGGC ACT; AACACAGCTTCAGCTTTTGC; A20 forward and reverse respectively: TATGGCTAAACC GGAACAGG; TCTCCTGCTCAGACACCTT; IkBa forward and reverse respectively: GAAGAAGGAGCGGCTACTGG; CTGCAGCTCCTTGACCATCT.

5. Quantitative Chromatin Immunoprecipitation (CHIP)

Quantitative ChIP analysis was carried out as described (Wu et al., 2005). Briefly, MDA-MB-231 cells were crosslinked with 1% formaldehyde for 10 mins at room temperature. Harvested cells were lysed in nucleus lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) for 10 mins. The lysate was sonicated on ice at 95% total power for 6 cycles of 12 pulses each. Between sample sonications, the samples were kept on ice to make sure low temperature. Lysates were centrifuged at 14,000 rpm for 10 mins at 4°C, and the supernatant was taken. Nine times volume of dilution buffer (10 mM Tris-
HCl, pH 8.1, 150 mM NaCl, 1 mM EDTA, 0.01% SDS, and 1% TritonX-100) based on the volume of the supernatant was then added to the supernatant, creating the IP solution.

The IP solution was pre-cleared with 60 μl of Salmon Sperm DNA/Protein G Agarose–50% Slurry (Upstate Biotechnology, VA) for 1 hr at 4°C with agitation. Following pre-clear, the IP solution was incubated with the indicated antibodies overnight.

The next day, immune complexes were recovered by incubation with 60 μl of Salmon Sperm DNA/Protein G Agarose – 50% Slurry for 2 hrs with gentle rocking. The agarose beads were pelleted by centrifugation for 2 mins at 2,000 rpm and washed for 5 mins for each wash at 4°C with rocking with the following buffers: one was in Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH=8.0)), one wash in High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl (pH=8.0)), one wash in LiCl Wash Buffer (0.25 M LiCl, 1% IGEPAL CA-630, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH=8.0)), and two washes in TE buffer (pH=8.0). After the final wash, the TE buffer was taken out by pipetting to insure no leftover. 250 μl of freshly prepared Elution Buffer was (1% SDS, 0.1 M NaHCO₃) added to the beads at room temperature. The immuno-complex was eluted by rocking for 15 mins and collected. Elution was repeated as described, yielding a total volume of 500 μl. Five μl of 5 M NaCl and 1 μl of 20 mg/ml Proteinase K (Invitrogen, CA) were added to each sample, which was incubated overnight at 65°C to break the formaldehyde crosslinks.
The next day, DNA was recovered by using the QIAquick PCR purification kit following the manufacturer’s instruction. The eluted DNA was analyzed by quantitative real-time PCR with the indicated primer pairs. The amounts of products were determined relative to a standard curve generated from a titration of input chromatin. Primers designed to amplify the NF-κB-site on c-IAP2 promoter were: (Forward) 5-TGTGTGTTATTACCGCTGG-3 and (Reverse) 5-GCGAGTCTCACGCTGTCTTTT-3; primers for NF-κB-site on A20 promoter were: (Forward) 5-CTGCAGAAAAACAACTGCGA and (Reverse) 5-GTGAGTCACCTGGGCATTTC-3. PCR was also performed with primers for β-globin gene (Forward) CCAGCCTTTATCCCAAACCATA, (Reverse): TATCATGCTCTTTTGCACCA as an internal control.

6. Transient DNA transfection and siRNA transfection

Transient DNA transfection: MDA-MB231 cells at 30-50% confluency were transiently transfected using FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, plasmid constructs (0.4-1 μg of DNA/well for 24-well plate or 6 μg of DNA for 8 cm dish) were diluted into serum-free media and mixed with FuGENE 6 Transfection Reagent (ratio of DNA and FuGENE 6: 1:3). Complexes were allowed to form for 15 mins at room temperature before adding to the cells, which were then incubated at 37 °C.
Forty-eight hrs after transfection, the expression level of target protein was determined by Western blot.

siRNA transfection: MDA-MB231 cells at 30-50% confluency were transiently transfected using Lipofectamine RNAiMAX (Invitrogen, CA). We followed the transfection protocol according to the manufacturer’s instruction. Briefly, Lipofectamine RNAiMAX was incubated in Opti-MEM medium for 5 mins at room temperature. siRNA were added to the Opti-MEM medium and incubated for 5 mins at room temperature. The siRNA and Lipofectamine RNAiMAX complex were mixed together and incubated for 20 mins at room temperature. The mixture was then added to cells. Forty-eight hrs after transfection, the expression level of target protein was determined by Western blot.

7. Western blots

Cells were washed twice with cold phosphate buffered saline (PBS) and collected in PBS using a cell scraper. Cells were pelleted in a centrifuge at 5,000 rpm for 5 mins at 4 °C. Cell pellets were lysed in radio-immunoprecipitation assay buffer (RIPA; 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40 (NP-40)) supplemented with fresh deoxycholic acid (0.5%) and a cocktail of protease inhibitors (Roche, IN) for 30 mins on ice. The lysates were kept on ice and sonicated at 50% total power on ice 4 times for 6 secs seconds each. Samples were then microcentrifuged at 4 °C for 10 mins at 14000 rpm. The supernatant was collected and protein concentration was determined using the BCA colorimetric assay (Pierce, IL).
For Western blot analysis, equal amounts of protein extracted from each sample (30-100 µg) were prepared by adding 4x sample buffer and 10x DTT (0.5 M) and subjected to 7% SDS-PAGE. The samples were loaded into pre-casted 7% NuPAGE Tris-acetate gel (Invitrogen). The resolved protein samples were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) by using Invitrogen’s buffering system at 30 volts for 1 hr at room temperature. The membranes were blocked with blocking buffer for 1 hr at room temperature. The membranes were then incubated at 4 °C overnight in the primary antibody diluted in blocking buffer. Membranes were washed 3 times in TBST for 10 mins each at room temperature followed by incubation in horseradish peroxidase labeled secondary antibody also diluted in blocking buffer for one hour at room temperature. The membrane blots were developed by chemiluminescent reagents (Supersignal, Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions and exposed to Fujifilm intelligent dark box (LAS-3000). After multiple probings of the membrane with different antibodies, the membranes were probed a final time with anti-actin antibody to confirm equal loading of the proteins.

8. Statistical analysis

For two sample comparisons, two-tailed unpaired Student’s t tests were used with α=0.05. When more than two samples were compared, one-way ANOVA was used (Student-Newman-Keuls method for multiple comparisons), with α=0.05. Excel software was used for statistical analysis.
CHAPTER IV
RESULTS

A. Aim 1: To determine whether Notch-1 activates NF-κB in cervical and ER-
breast cancer.

1. Introduction

Breast and cervical cancer are two common cancers that severely endanger
women’s health. Cervical cancer was once the leading cause of death for women in the
United States. However, during the past few decades, largely due to widespread use of
the Papanicolaou test, incidence and mortality from cervical cancer have declined
significantly. Currently, breast cancer particularly estrogen receptor alpha negative (ER-)
breast cancer has become the leading cause of cancer related mortality in women
worldwide (Wildiers et al., 2007). Cervical and breast cancer screening programs have
been established to detect cancer in earlier stages and help decrease mortality for both
cancers. However, based on the data from WHO (2006), the fatality rate of breast and
cervical cancer is still ranked first and fifth respectively in cancer-related death, in
women globally. Continuous dedication to cancer research is urgently required to
elucidate the cancer cell survival mechanism at the molecular level that is responsible for
disease aggressiveness and poor prognosis.

Emerging data indicate that misregulation of Notch-1 has been associated with
neoplastic transformation in cervical and breast cancer. In cervical cancer, increased
expressions of Notch-1 and Notch ligands were documented (Zagouras et al., 1995;
Daniel et al., 1997; Gray et al., 1999) and accumulation of Notch-1 and its ligand Jagged-
1 was found to be coincident with the progression of high-grade precursor lesions to
invasive cervical cancers (Veeraraghavalu et al., 2004). Not only in cervical cancer, but
also in a variety of human breast carcinomas, the elevated Notch signaling was also
observed (Reedijk et al., 2005; Stylianou et al., 2006). High level expression of Notch-1
and its ligand Jagged-1 predicted poor overall survival in breast cancer patients (Reedijk
et al., 2005; Dickson et al., 2007; Reedijk et al., 2008).

Increasing evidence reveals that inhibition of Notch-1 sensitizes cancer cells to
apoptosis, causes cell cycle arrest and decreases cellular transformation. Inhibiting Notch
signaling by antisense silencing in CaSki cervical cancer cells abolished proliferation and
anchorage-independent growth in vitro (Weijzen et al., 2002) and completely blocked the
growth of CaSki xenografts in vivo (Weijzen et al., 2003). Inhibition of Notch suppressed
breast tumors growth (Pece et al., 2004) and reverted the transformed phenotype of
human breast cancer cell lines, including the ER- breast cancer cell line MDA-MB-231
(Stylianou et al., 2006). Our lab also showed that Notch-1 knockdown by siRNA or GSI
causen G2 arrest and induced apoptosis in breast cancer cells.
However, the mechanism whereby Notch-1 promotes cell survival and/or proliferation and transformation is not clear. Accumulating reports show that NF-κB is also deregulated in these two cancers and might be the downstream signaling effector of Notch-1 to modulate cell survival and apoptosis. It was shown that activated Notch signaling was accompanied by elevated NF-κB activity as determined by immunohistochemistry from cervical cancer patients’ samples. Constitutive activation of NF-κB with nuclear accumulation of p50 and p65 has been described to be associated with cervical cancer progression (Nair et al., 2003; Prusty et al., 2005). Aberrantly regulated Notch and NF-κB activity are also concurrently found in ER- breast cancer. Notch-1 and NF-κB activity are highly active in ER- breast cancer cell lines as well as ER- breast tumors as compared to ER + counterparts. Elevated NF-κB activation in ER-breast cancer has been suggested to be responsible for chemoresistance and metastatic growth. Here, we hypothesize that Notch-1 promotes cell survival by activating NF-κB activity in cervical and ER- breast cancer.

Overall, previous literatures showed that Notch-1 was aberrantly regulated in human cervical and ER- breast cancer and inhibiting Notch-1 sensitized cancer cells to apoptosis. NF-κB was also deregulated in these two cancers and might be the downstream signaling effector of Notch-1 to modulate cell survival and apoptosis. We hypothesize that Notch-1 promotes cell survival by activating NF-κB activity in cervical and ER- breast cancer. Therefore, we have the first Aim: Aim1: to determine whether Notch-1 activates NF-κB in cervical and ER- breast cancer.

To investigate whether Notch-1 activates NF-κB in cervical and ER- breast cancer cells, we used the NF-κB driven luciferase reporter assay. We found that Notch-1 is required and sufficient to activate NF-κB activity in cervical and ER- breast cancer. Notch-1 silencing by siRNA resulted in approximately 50% decrease in NF-κB-driven luciferase activity in both cervical cancer cells CaSki and ER- breast cancer MDA-MB-231 cells at the basal level (Figure 4.1 A). In cervical cancer, Tumor Necrosis Factor (TNF)–α has been implicated in both direct and indirect control of HPV infection. HPV-harboring cervical keratinocytes constitutively produce active TNF-α (Malejczyk et al., 1992), and increased localized production of TNF-α in cervical cancer has been observed in vivo (Bequet-Romero and Lopez-Ocejo, 2000). Thus, we used TNF-α in CaSki cells to mimic the in vivo physiological condition. About 50% inhibition was also observed in TNF-α stimulated CaSki cells when transfected with Notch-1 siRNA (Figure 4.1 A). The siRNA and overexpression transfections were validated by western blots using the same lysates from used in luciferase experiments (Figure 4.1 A and C). In agreement with siRNA data, treatment of CaSki and MDA-MB-231 cells with a γ-secretase inhibitor (GSI) suppressed NF-κB reporter activity in a dose-dependent manner, with IC$_{50}$ between 1 and 2 µM (Figure 4.1 B). These results suggest that Notch-1 is required to sustain the constitutive active NF-κB activity in cervical and ER- breast cancer cells.

Conversely, we would like to determine how NF-κB activity changes when Notch-1 signaling is up-regulated in the cells. Intracellular Notch-1 after cleavage is
considered the active form of Notch-1. A plasmid encoding the intracellular Notch-1 was utilized to up-regulate Notch-1 activity. CaSki and MDA-MB-231 cells were transiently transfected with the active form of Notch-1 NIC. Overexpression of Notch-1 NIC led to a dose-dependent increase of NF-κB reporter activity up to a transfection dose of 0.2 µg plasmid DNA/10^5 cells (Figure 4.1C) in both cell lines. However, higher levels of Notch-1 expression resulted in lower stimulation, consistent with published observations in Jurkat cells (Guan et al., 1996; Wang et al., 2001). Representative western blots showed the transfection result of 0.2 µg plasmid DNA/10^5 cells. The data indicate that Notch-1 is not only required for full NF-κB activity but also sufficient to activate NF-κB activity in cervical cancer CaSki cells and ER- breast cancer MDA-MB-231 cells.

3. Conclusion of Aim 1

In Aim 1, we monitored NF-κB activity under the conditions of either over-expressing Notch-1 or silencing Notch-1 by siRNA in both cervical cancer CaSki and ER- breast cancer MDA-MB-231 cells. It is found that down-regulation of Notch-1 causes a decrease in NF-κB dependent transcription activity and activation of Notch-1 leads to an increase in NF-κB activity in both cells. In a word, data from Aim 1 showed that Notch-1 activates NF-κB activity in cervical cancer CaSki cells and ER- breast cancer MDA-MB-231 cells.
Figure 4.1: Notch-1 activates NF-κB activity in CaSki and MDA-MB-231 cells. (A) Cells were transfected with Control siRNA (CTL) or Notch-1 (N-1) siRNA. Twenty-four hrs later, they were transfected with NF-κB-luciferase reporter plasmid plus pTK-Renilla luciferase plasmid as internal controls. TNF-α was added to the medium 30 mins before harvest. (B) Cells were transfected with NF-κB-luciferase reporter plasmid plus pTK-Renilla luciferase plasmid. Twenty-four hrs after transfection, cells were treated with either DMSO or GSI with indicated doses for time shown (C) Cells were cotransfected with NF-κB-luciferase reporter plasmid plus pTK-Renilla luciferase plasmid as well as expression plasmid encoding intracellular Notch-1 NIC. The effects of Notch signaling modulation on NF-κB transcriptional activity were assessed by monitoring NF-κB promoter-driven luciferase activity. Relative luciferase activity was calculated considering 1.0 as the activity of cells transfected with empty vector. *P ≤ 0.05. Western blots from (A) show the effective downregulation of Notch-1 and Western blots from (C) show the overexpression of Notch-1 at 0.2 ug/10^5 cells. Data are representative of three independent experiments, each conducted in triplicate.
B. Aim 2: To characterize the mechanism by which Notch-1 activates NF-κB in cervical cancer CaSki cells: specifically determine whether Notch-1 interacts with IKKα and activates IKKα/β kinase activity.

1. Introduction

Since we knew from the results of Aim1 that Notch-1 is not only required for full NF-κB activity but also sufficient to activate NF-κB activity in cervical cancer CaSki cells and ER- breast cancer MDA-MB-231 cells, we asked what is the mechanism by which Notch-1 activates NF-κB activity in cervical cancer CaSki cells?

First of all, we looked at the classical NF-κB signaling transduction cascade. NF-κB subunits are sequestered in the cytoplasm by binding to inhibitory proteins (IκBs). This inhibition can be released by phosphorylation of IκBs by the IKK complex, consisting of IKKα, IKKβ and IKKγ. IKK complex could be activated by numerous stimuli such as cytokines, chemokines, mitogens, and bacterial and viral infection. Phosphorylation of IκBs results in degradation of IκBs and NF-κB subunits are allowed to translocate to the nucleus and bind to NF-κB consensus sequence to rapidly transactivate NF-κB target gene transcription. Therefore, there are multiple levels of NF-κB activity regulation modulated by IKK upstream kinases, IKK complex, IκBs and NF-κB subunits.

In fact, Notch has been shown to regulate NF-κB signaling components at multiple levels to activate NF-κB activity. At the level of IKK upstream kinase, Notch-3
has been demonstrated to modulate PKC θ (Bellavia et al., 2000; Felli et al., 2005; Vacca et al., 2006) to regulate NF-κB activation in thymocytes and T lymphoma cells of Notch-3 transgenic mice. Notch-1 has been shown to activate Akt which also sits upstream of IKK in a variety of cellular systems (Nair et al., 2003; Liu et al., 2006; Graziani et al., 2008; Meng et al., 2009).

Secondly, at the level of IKK complex, it is known that Notch-3 forms a complex with IKKα homodimers and activates cytoplasmic IKKα kinase activity (Bellavia et al., 2000; Felli et al., 2005; Vacca et al., 2006). Notch signaling activation mediated by Jagged-1 is reported to induce IKK kinase activity in human keratinocytes (Nickoloff et al., 2002). Recently, it has been shown that overexpression of intracellular Notch-1 NIC activates NF-κB by interacting with the IKKα/β signalosome and enhancing IKKα/β kinase activity in a mouse model of T-cell acute lymphoblastic leukemia (T-ALL) and in human T-ALL cells (Vilimas et al., 2007).

At last, at the level of NF-κB subunits, several different mechanisms have been elucidated: physical interaction between Notch-1 and p50/c-Rel; transcriptional regulation of NF-κB subunits and NF-κB dependent genes by Notch-1. It was found that Notch-1 physically interacts with IkBα and prevents it from exporting NF-κB to the cytoplasm and promotes nuclear translocation of NF-κB (Shin et al., 2006). In addition to physical interaction, Notch-1 is also shown to activate NF-κB by increasing transcription expression of NF-κB subunits and NF-κB dependent genes (Cheng et al., 2001; Oswald et al., 1998).
Given the regulation could occur on multiple levels, we chose to focus first on one regulation level: the activation of IKK complex. We hypothesize that Notch-1 interacts with IKKα/β and activates IKKα/β kinase activity to regulate NF-κB activity in CaSki cells.

2. Notch-1 interacts with IKKα/β kinase in cervical cancer CaSki cells.

To test the hypothesis that Notch-1 interacts with IKKα/β to regulate NF-κB activity in CaSki cells, the traditional Co-IPs (Co-immunoprecipitations) were conducted. Figure 4.2 A shows that IKKα could be readily immunoprecipitated from total cell extracts with Notch-1 antibody indicating the interaction between Notch-1 and IKKα. On the other hand, reverse immunoprecipitations with IKKα antibodies were also carried out to test the association. IKKα immunoprecipitations bring down a Notch-1 band migrating at approximately 98 kDa in both total and nuclear extracts. The identity of the Notch-1 form interacting with IKKα was determined by detecting Notch-1 with a specific antibody against active Notch-1 NIC (Cell Signaling, Danvers, MA, USA) that does not recognize uncleaved Notch-1. The same band was also immunoprecipitated by Notch-1 C-terminal antibodies (Santa Cruz) from nuclear lysate. This indicates that active intracellular Notch-1 interacts with IKKα. The fact that Notch-1 co-immunoprecipitates with IKKα in CaSki total cell extracts and nuclear extracts suggests a possible role of Notch-1 in modulating the cytoplasmic and nuclear functions of IKKα (Figure 4.2 B).
To confirm the interaction, we used a quantitative immunoprecipitation assay described by Vooijs et al. (Vooijs et al., 2004). This assay uses a full-length Notch-1 construct tagged at the C terminus with Renilla luciferase. By measuring luciferase activity specifically immunoprecipitated with antibodies to putative interacting proteins, this assay allows us to quantify putative interactions in a way not dependent on western blotting. Using this assay, Notch-1 was reproducibly immunoprecipitated with IKKα antibodies in CaSki cells treated with vehicle or TNF-α for 30 mins (Figures 4.2 C). This assay consistently detected a specific interaction with IKK β in TNF-α treated cells, although we didn’t observe the interaction between Notch-1 and IKK β in vehicle-treated cells (Figure 4.2 C and D). This may suggest that TNF-α affects the composition or stability of the IKK/Notch-1-containing complexes. Taken together, these data provide evidence that Notch-1 interacts with IKKα in cervical cancer CaSki cells.


In order to test the functional consequence of association between Notch-1 and IKK complex, we tested the IKK complex kinase activity from whole cell lysates by in vitro immunoprecipitation kinase assays using a commercially available glutathione S-transferase-full-length IκBα fusion protein as a substrate. Figure 4.3A shows that an IκBα kinase activity could be immunoprecipitated with antibodies to IKKα, IKKβ or Notch-1. Notch-1 knockdown did not inhibit basal IKKα- or IKKβ-immunoprecipitated kinase activity, but did inhibit basal Notch-1-associated activity. In TNF-α-stimulated cells,
Notch-1 knockdown markedly inhibited IκBα kinase activity immunoprecipitated with IKKα, IKKβ or Notch-1. TNF-α increased only the kinase activity immunoprecipitated with IKKβ antibodies, but not that immunoprecipitated with IKKα and Notch-1 antibodies. Consistent with the siRNA data, GSI treatment also inhibits IKKα, IKKβ, and Notch-1 immunoprecipitated kinase activity in a dose dependent manner in the presence of TNF-α. These results indicate that endogenous Notch-1 is indispensable for NF-κB activation and Notch-1 regulates IKK complex activity in TNF-α-stimulated CaSki cells.

Besides in vitro kinase assay, we also explored endogenous IκBα phosphorylation by western blotting, which was also an assessment to show the IKK complex activity. Figure 4.4A shows that siRNA-mediated knockdown of Notch-1 for 48 hrs reduced the basal levels of endogenous IκBα. Phospho-IκBα was very low under basal conditions and was also decreased compared to the control, most likely as a consequence of lower total protein levels. However, after 1 hr TNF-α stimulation, the phosphorylation of endogenous IκBα was virtually abolished by Notch-1 siRNA, and total IκBα levels were comparable to control. We interpreted this data to indicate that Notch-1 knockdown for 48 hrs inhibits basal NF-κB activity by undetermined mechanisms, reducing expression of NF-κB targets including IκBα (Oakley et al., 2003). After TNF-α stimulation, Notch-1 is required for IKK signalosome activation and IκBα phosphorylation, and Notch-1 knockdown prevents IκBα phosphorylation.

Additionally, we also suppressed the Notch signaling pharmaceutically by using GSI for 18 hrs, which blocks the final cleavage needed for Notch activation. Under these conditions, GSI did not significantly affect total IκBα expression, but like Notch-1
siRNA, GSI inhibited TNF-α-stimulated IκBα phosphorylation, which at 60 mins was abolished (Figure 4.4 B). These results indicate that endogenous Notch-1 modulates NF-κB activity in TNF-α stimulated CaSki cells at least in part by physically interacting with the IKK signalosome and regulating its IKK kinase activity.

4. Conclusion of Aim 2

In Aim 2, we showed that the Notch-1 associates with IKK complex by using traditional Co-IP and quantitative Co-IP. Notch-1 regulates IKK complex kinase activity in response to TNF-α stimulation in CaSki cells as demonstrated by the results from kinase assay and western blotting of phospho-IκBα. In summary, Notch-1 activates NF-κB activity possibly by interacting with IKKa/β and regulating IKKa/β kinase activity in CaSki cells stimulated with TNF-α.
Figure 4.2: Notch-1 associates with IKKα and the IKK signalosome in CaSki cells.

(A) Total cell lysates (TCLs) were immunoprecipitated (IP) with Notch-1 antibody from Santa Cruz (C-20) and analysed by western blotting (WB) for endogenous IKKα. Non-immune homologous IgG was used as negative control. (B) Reverse immunoprecipitation with IKKα antibody or control IgG in TCLs or nuclear extract (NL) of CaSki cells and detected with antibody specific to active Notch-1 NIC (Cell Signaling 2421). Immunoprecipitation with Notch-1 Santa Cruz C-20 confirmed band identity (rightmost lane). (C,D) Quantitative reverse immunoprecipitation in which cells were transfected with Notch-1/Renilla chimeric construct (Vooijs et al., 2004). Forty-eight hours after transfection, cells were treated for 30 mins with vehicle or TNF-α (10 ng/ml). Cells were lysed and immunoprecipitations were carried out with antibodies to IKKα, IKKβ or control IgG. Renilla luciferase activity (RLU) in washed pellets was determined and compared to total Renilla luciferase activity (pellet plus combined supernatants). Data are representative of three independent experiments. *P< 0.05 compared to IgG.
Figure 4.3: Notch-1 regulates IKKα/β kinase activity in CaSki cells.

(A) CaSki cells were transiently transfected with either Control (CTL) or Notch-1 (N-1) siRNA. Forty-eight hrs post-transfection, cells were treated with or without TNF-α (10 ng/ml) for 30 mins. Knockdown was verified by western blotting. For each condition, 500 mg whole-cell extract was immunoprecipitated with antibodies to IKKα, IKKβ or Notch-1 (C-20 Santa Cruz). Kinase activity was determined using glutathione S-transferase-IκBα as a substrate, followed by SDS–polyacrylamide gel electrophoresis and autoradiography.

(B) Cells were treated with either DMSO or different doses of GSI (µM) for 18 hrs and treated with TNF-α 10 ng/ml for 30 mins before harvest. The kinase assay was conducted the same as in (A). Data are representative of three independent experiments.
Figure 4.4: Inhibition of Notch-1 decreases IKKα/β kinase activity in CaSki cells.

(A) CaSki cells were transiently transfected with either Control siRNA (CTL) or Notch-1 (N-1) siRNA. Forty-eight hrs post-transfection, cells were treated with or without TNF-α (10 ng/ml) for 30 or 60 mins and analysed by western blotting for phospho-IκBα (p-IκBα) and total IκBα.

(B) CaSki cells were treated for 18 hrs with vehicle (DMSO) or 2 μM GSI and then stimulated with vehicle or TNF-α (10 ng/ml) for 30 or 60 mins and analysed by western blotting for phospho-IκBα and total IκBα.

(C) MDA-MB-231 cells have a more transient response to TNF-α, which is different from that in CaSki cells. Western blot of MDA-MB-231 cells transfected with either Control siRNA (CTL) or Notch-1 siRNA (N-1). Forty-eight hrs after transfection, cells were treated with TNF-α 10 ng/ml for 0 min, 30 mins before protein extraction. Data are representative of three independent experiments.
C. Aim 3: To elucidate the mechanism by which Notch-1 activates NF-κB activity in ER- breast cancer MDA-MB-231 cells.

1. Introduction

We knew that endogenous Notch-1 modulates NF-κB activity in CaSki cells at least in part by physically interacting with the IKK signalosome and regulating its IKK kinase activity. In order to elucidate the mechanism by which Notch-1 activates NF-κB activity in ER- breast cancer, we first tested if Notch-1 modulates NF-κB activity by physically interacting with the IKK signalosome as in CaSki cells.

However, we didn’t observe physical interactions between Notch-1 and IKKα/β and we didn’t see a reduction of IKKα/β kinase activity when cells were transfected with Notch-1 siRNA and treated with TNF-α for 30 mins, the same conditions that were used in CaSki cells. In addition, we found that the stimulation of NF-κB activity by TNF-α in MDA-MB-231 cells was more transient than that in CaSki cells. Specifically, in MDA-MB-231 cells, the phosphorylation of IκBα was barely detectable at 30 mins time point and total IκBα was degraded at 30 mins in both control and Notch-1 siRNA treated cells. This indicates that phosphorylation of IκBα occurred much earlier than 30 mins (Figure 4.4 B). While in CaSki cells, as shown in Figure 4.4 A, IκBα was phosphorylated at 30 mins time point and the phosphorylation continued to be accumulated after 1 hr TNF-α stimulation. At 1 hr time point, the total IκBα was still present in both control and Notch-
siRNA transfected cells. Taken together, it suggests that MDA-MB-231 cells have a much more transient NF-κB activation in response to TNF-α stimulation.

These different responses to TNF-α stimulation from two cell lines suggest that Notch-1 may utilize an alternative mechanism to regulate NF-κB activity in MDA-MB-231 cells. As we discussed previously in Aim2, there are multiple layers of regulation on NF-κB activity. Which level does Notch-1 regulate NF-κB activity in MDA-MB-231 cells?

2. Both Notch-1 and IKKα are required for a subset of NF-κB dependent gene expression.

MDA-MB-231 cells may use a different mechanism from CaSki cells to modulate NF-κB activity. In order to explore the mechanism accounting for Notch-1 regulation of NF-κB in ER- breast cancer, we started to explore the gene expression profiles from microarray data in MDA-MB-231 cells treated with either Notch-1 siRNA or GSI. A panel of NF-κB dependent genes is shown to be regulated by Notch-1. Table1 summarizes the list of genes that are down-regulated by Notch-1 siRNA compared to control siRNA. There are genes involved in cell cycle progression, such as cyclin D1, bcl-3; cell differentiation and cell to cell communication, such as interleukin 11; cell apoptosis such as c-IAP2; NF-κB regulator such as IκBα and A20.

We chose to study three important NF-κB dependent genes: cIAP-2, IκBα, and A20. cIAP-2 (cellular inhibitor of apoptosis protein) is one of the anti-apoptotic proteins
regulated by NF-κB. Recent evidence indicated that cIAP-2 was frequently overexpressed in cancer and its expression level was implicated in contributing to tumorigenesis, chemoresistance, disease progression and poor patient survival (Hunter et al., 2007). In addition, Notch-1 and IKKα have been shown to be recruited to the cIAP-2 and IkBα promoters in CaSki cells (Song et al., 2008). The gene expression of A20 is also transcriptionally activated through activation of NF-κB. High A20 expression has also been shown to confer breast cancer cells resistance to apoptosis induced by TNF-α. A20 is highly expressed in tamoxifen resistant cells and moreover it is over-expressed in more aggressive breast tumors (ER-, PR- and high histological grade). A20 is proposed to be a good marker for tumor aggressiveness in breast cancer (Vendrell et al., 2007).

Table1: Notch-1 regulates a subset of NF-κB dependent genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL3</td>
<td>0.54</td>
</tr>
<tr>
<td>BIRC3 (c-IAP2)</td>
<td>0.63</td>
</tr>
<tr>
<td>CASP1</td>
<td>0.61</td>
</tr>
<tr>
<td>CCND1</td>
<td>0.58</td>
</tr>
<tr>
<td>GJA1</td>
<td>0.68</td>
</tr>
<tr>
<td>HMOX1</td>
<td>0.64</td>
</tr>
<tr>
<td>ICAM1</td>
<td>0.73</td>
</tr>
<tr>
<td>IL11</td>
<td>0.46</td>
</tr>
<tr>
<td>PLAU</td>
<td>0.8</td>
</tr>
<tr>
<td>TLR4</td>
<td>0.65</td>
</tr>
<tr>
<td>TNFIAP3 (A20)</td>
<td>0.68</td>
</tr>
<tr>
<td>NFKBIA (IkBα)</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Figure 4.5: Notch-1 and IKKα are both required for a subset of NF-κB dependent gene expression in MDA-MB-231 cells.

To validate microarray data and investigate the functional role of Notch-1 in NF-κB activation in MDA-MB-231 cells, an independent quantitative RT-PCR was performed. Figure 4.5 shows that Notch-1 siRNA caused 50%, 20% and 80% reduction of cIAP-2, IκBα and A20 expression respectively. This is consistent with the microarray data. IKKα siRNA also results in significant reduction of three genes expression. This suggests that Notch-1 and IKKα are required for the expression of a subset of NF-κB dependent genes.

We also studied gene expression upon activation of Notch signaling by coculturing MDA-MB-231 cells with mouse fibroblasts expressing high level of Jagged-1.
(LTK-JAG). We chose this method because the coculture mimics the in vivo Notch activation by ligands interaction from adjacent stromal cells (Sade et al., 2004). Also, relevant to the biology of breast cancer, a high level of Jagged-1 was shown to be associated with poor patient survival (Reedijk et al., 2005). We tested cIAP-2, IκBα, and A20 transcript levels under coculture conditions using real time PCR with human specific primers. cIAP-2, IκBα, and A20 transcript levels were induced dramatically in LTK-JAG cocultured cells compared to that cocultured with parental cell (LTK-PAR) (Figure 4.6 A). It suggests that Notch-1 activation mediated by Jagged-1 activates a subset of NF-κB dependent gene expression.

To determine the functional role of Notch-1 and IKKα in LTK-JAG coculture mediated induction of NF-κB target gene expression, Notch-1 and IKKα were knocked down by specific siRNAs respectively. The gene expression of cIAP-2, IκBα, and A20 in the LTK-JAG coculture mediated induction was reduced markedly by either Notch-1 siRNA or IKKα siRNA, showing the requirement of both proteins in the gene activation mediated by LTK-JAG coculture (Figure 4.6 B-D). Interestingly, the induction is independent of the presence of another Notch family member Notch-4, confirming the specific involvement of Notch-1 and IKKα in the activation of target genes mediated by LTK-JAG coculture (Figure 4.6 B-D).
Figure 4.6: Coculture of MDA-MB-231 cells with LTK-JAG induces a subset of NF-κB dependent gene expression. (A) MDA-MB-231 cells were cocultured with mouse fibroblast parental LTK cells (PAR) or LTK cells with high expression of Jagged-1 (JAG) for 4 hrs. (B-E) MDA-MB-231 cells were transfected with either Control siRNA (CTL), Notch-1 (N-1), Notch-4 (N-4) or IKKα siRNA. Forty-eight hrs after transfection, cells were cocultured with LTK-PAR or LTK-JAG for 4 hrs. The relative transcript levels were determined. (E) Notch-1, Notch-4, and IKKα siRNA transfection were validated by Western blot. Data are representative of three independent experiments. * P < 0.05 compared to LTK-PAR; # P < 0.05 compared to CTL cocultured with LTK-JAG.
3. Notch-1 activation mediated by Jagged-1 induces NF-κB subunits and Notch-1 recruitment to NF-κB dependent gene promoters.

Understanding that Notch-1 activates NF-κB activity and both Notch-1 and IKKα are required for the expression of a subset of NF-κB dependent genes, we come back to the original question: what is the prominent mechanism responsible for the Notch-1 regulation of NF-κB dependent genes in MDA-MB-231 cells? We knew that the physical interaction between Notch-1 and IKK is not prominent in MDA-MB-231 cells and we turned to the canonical Notch-1 and novel IKKα signaling pathway for clues. As a well-known transcription factor, Notch-1 activates expression of target genes by binding to CBF-1, a DNA binding protein, and converting CBF-1 from a repressor to an activator. Binding of Notch-1 to CBF-1 facilitates the recruitment of transcription coactivators to target gene promoters. Not only does Notch-1 have nuclear function IKKα does too. On the cIAP-2 promoter, IKKα has been shown to have nuclear kinase function to activate gene transcription by phosphorylating corepressor SMRT (silencing mediator of retinoid and thyroid hormone receptors) (Hoberg et al., 2004; Hoberg et al., 2006). We found that Notch-1 and IKKα were recruited to NF-κB dependent genes cIAP-2 and IκBα promoter in Caski cells. Furthermore, the IKKα recruitment was Notch-1 dependent on both promoters (Song et al., 2008). This prompted us to hypothesize that Notch-1 and IKKα together may exert regulation on NF-κB dependent gene promoters in MDA-MB-231 cells. Therefore, we explored the recruitment of Notch-1, IKKα and NF-κB on NF-κB dependent gene promoters in MDA-MB-231 cells.
It is well known that NF-κB subunits are sequestered in the cytoplasm by IκBs. Activation of NF-κB requires degradation of IκBs, translocation of NF-κB subunits to the nucleus and transactivation of gene transcriptions. Since the cIAP-2 and A20 are induced remarkably by Jagged-1 mediated activation of Notch-1, NF-κB subunits are expected to be recruited to these promoters after coculture. We observed that NF-κB subunits p50 and p65 were recruited to cIAP-2 and A20 promoters prominently after about 3 hrs only when cocultured with LTK-JAG but not LTK-PAR as shown by quantitative ChIP experiments. The recruitment was abolished if we knocked down Notch-1, indicating the critical role of Notch-1 in recruiting or retaining NF-κB subunits to the gene promoters (Figure 4.7 A, B). Moreover, we found that Notch-1 was also recruited to cIAP-2 promoter at this time point and this association disappeared if Notch-1 siRNA was transfected, confirming the specificity of immunoprecipitation (Figure 4.8)
Figure 4.7: Coculture of MDA-MB-231 cells with LTK-JAG induces p50, p65 recruitment to the cIAP-2 and A20 promoter. (A-B) MDA-MB-231 cells were transfected with Control (CTL) or Notch-1 siRNA (N-1 siRNA) and 48 hrs later cocultured with mouse parental LTK cells (PAR) or LTK cells with high expression of Jagged-1 (JAG) for 2 hrs and 45 mins. ChIP assays against specific antibodies were performed. All ChIP data were expressed as relative fold induction of specific antibody over IgG control. All data were normalized to the internal control: unspecific binding to β-globin intron sequence. *P <0.05 compared to CTL cocultured with LTK-PAR. (C) Western blot of MDA-MB-231 transfected with Control (CTL) or Notch-1 siRNA (N-1 siRNA). Data are representative of three independent experiments.
Figure 4.8: Coculture of MDA-MB-231 cells with LTK-JAG induces Notch-1 recruitment to the cIAP-2 promoter.

4. Notch-1 activation mediated by Jagged-1 induces IKKα recruitment to NF-κB dependent gene cIAP-2 promoter.

IKKα has nuclear kinase activities independent of the IKK complex. IKKα has been shown to recruit to cIAP-2 promoter and phosphorylate corepressor SMRT and release SMRT from the promoter thereby de-repressing transcription (Hoberg et al.,
2004; Hoberg et al., 2006). We previously found that IKKα and Notch-1 are recruited to the cIAP-2 promoter and IKKα recruitment is Notch-1 dependent in CaSki cells (Song et al., 2008). Since we found that Notch-1 is recruited to the cIAP-2 promoter in MDA-MB-231, we were wondering if IKKα is also associated with the cIAP-2 promoter together with Notch-1 in these cells. Here, we failed to immunoprecipitate IKKα on cIAP-2 promoter at 3 hrs time point after coculture when Notch-1 is prominently associated with cIAP-2 promoter. However, IKKα was found to be recruited at an earlier time point, 1 hr after coculture, when Notch-1 was minimally recruited to the promoter (Figure 4.8 A and 4.9 A). It should be noted that the induced IKKα occupancy also required Notch-1 as illustrated in Figure 4.9 B, suggesting that Notch-1 activation mediated by Jagged-1 is necessary for IKKα recruitment.

IKKα nuclear kinase activity has been demonstrated to facilitate NF-κB subunits recruitment to the cIAP-2 promoter (Hoberg et al., 2006). We inhibited IKKα kinase activity by transfecting cells with dominant negative IKKα. Since IKKβ kinase is predominantly responsible for cytoplasmic IKK complex activity, applying dominant negative IKKα inhibits IKKα activity mostly while retaining normal IKKβ kinase activity. Figure 4.9 D illustrates that induced NF-κB subunits recruitment to the cIAP-2 promoter was inhibited by dominant negative IKKα, indicating that IKKα kinase activity was required for the induced NF-κB subunits recruitment to the cIAP-2 promoter.
Figure 4.9: Coculture of MDA-MB-231 cells with LTK-JAG induces IKKα recruitment to the cIAP-2 promoter.

(A) MDA-MB-231 cells were cocultured with mouse parental LTK cells (PAR) or LTK cells with high expression of Jagged-1 (JAG) for the indicated time. (B) MDA-MB-231 cells were transfected with Control (CTL) or Notch-1 siRNA (N-1 siRNA) and 48 hrs later cocultured with mouse parental LTK cells (LTK-PAR) or LTK cells with high expression of Jagged-1 (LTK-JAG) for 1 hr. (C) Western blot of MDA-MB-231 transfected with Control (CTL) or Notch-1 siRNA (N-1 siRNA). (D) MDA-MB-231 cells were transfected with EV or IKKα dominant negative construct (IKKα DN). Forty-eight hrs later cells were cocultured with PAR or JAG for 2 hrs and 45 mins. ChIP assays against specific antibodies were performed. All ChIP data were expressed as relative fold induction of specific antibody over IgG control. All data were normalized to the internal control: unspecific binding to β-globin intron sequence. *P <0.05 compared to LTK-PAR. Data are representative of three independent experiments.
5. Notch-1 is recruited to the cIAP-2 promoter later than NF-κB and IKKa.

As shown in Figure 4.10, we have a time course recruitment profile of NF-κB, Notch-1 and IKKa to the cIAP-2 promoter. At the 1 hr time point, both NF-κB subunits and IKKa but not Notch-1 are associated with the promoter. Notch-1 is recruited at about 3 hrs after coculture. However, both NF-κB subunits and IKKa recruitments require Notch-1 presence in the activation mediated by Jagged-1. This suggests that Notch-1 may activate NF-κB and/or IKKa before being recruited to the promoter. We then started looking for possible mechanisms through which Notch-1 may activate NF-κB and/or IKKa. Literature suggests us that Akt might be the IKK upstream kinase responsible for NF-κB activation mediated by active Notch-1.
Figure 4.10: NF-κB and IKKα are recruited to the cIAP-2 promoter before Notch-1.

(A-C) MDA-MB-231 cells were cocultured with mouse parental LTK cells (PAR) or LTK cells with high expression of Jagged-1 (JAG) for the indicated time. ChIP assays against specific antibodies were performed. All ChIP data were expressed as relative fold induction of specific antibody over IgG control. All data were normalized to internal control: unspecific binding to β-globin intron sequence. *P <0.05 compared to LTK-PAR cocultured. Data are representative of three independent experiments.
6. NF-κB activation by Jagged-1 correlates with activation of Akt, IKK complex and IKKα.

Many studies show that Notch-1 activates or is required for activation of Akt pathway in various systems (Nair et al., 2003; Liu et al., 2006; Graziani et al., 2008; Meng et al., 2009). Recently, Perumalsamy et al have demonstrated that cytoplasmic but not nuclear Notch-1 activates Akt to rescue cell apoptosis triggered by nutrition withdrawal (Perumalsamy et al., 2009).

In addition, NF-κB is an important downstream target of Akt activation. It is reported that Akt is required for NF-κB activation induced by TNF-α (Ozes et al., 1999). Akt functions through IKK to promote the transactivation potential and phosphorylation of NF-κB (Madrid et al., 2001). Akt is proposed to promote metastasis and angiogenesis through IKK in a manner that depends on NF-κB activation (Agarwal et al., 2005). Therefore, it is possible that Notch-1 activates IKK and/or IKKα via Akt in LTK-JAG cocultured cells.

In order to test whether Notch-1 activates IKK and/or IKKα via Akt, phosphorylation of Akt was determined by western blot. Interestingly, Akt was phosphorylated 15 mins after MDA-MB-231 cells were cocultured with LTK-JAG. IKK complex were also activated starting from 15 mins coculture and further activated in a time dependent manner up to 1hr, as evidenced by phosphorylated IKK α/β on serine 180/181. Consequently, the downstream target of IKK complex IκBα was phosphorylated and degraded beginning from 15 mins to 1hr (Figure 4.11 A). Total IKK and Akt expression levels were comparable in LTK-PAR and LTK-JAG cocultured cells.
Phosphorylation of IKKα at serine 180 has been shown to be linked to IKKα activation and IKKα chromatin associated kinase activity (Hoberg et al., 2004). We assessed IKKα activation by detecting the phosphorylation of IKKα at serine 180. The western blots were performed using whole lysates from the mixture of mouse and human cells. In order to confirm the phosphorylation of IKKα occurs in MDA-MB-231 cells, we immunoprecipitated IKKα using a human specific antibody. Human specific antibody failed to immunoprecipitate any IKKα in mouse cells, validating the human specificity and excluding the cross-reactivity (Figure 4.11 B). Our data show that IKKα was phosphorylated (activated) in LTK-JAG cocultured MDA-MB-231 cells in a time dependent manner.
Figure 4.11: Coculture of MDA-MB-231 cells with LTK-JAG induces activation of Akt and IKK complex and phosphorylation of IKKα on Serine 180

(A) MDA-MB-231 cells were cocultured with LTK-PAR (P) or LTK-JAG (J) for the indicated time. Western blots were carried out using the whole cell lysates containing MDA-MB-231 cells and mouse fibroblasts. (B) After coculture, 1000 mg of cell lysates were immunoprecipitated by human specific IKKα antibody. LTK-PAR cell lysates were immunoprecipitated as a negative control. p-IKKα on serine 180 and human specific IKKα were immunoblotted following IP. Data are representative of three independent experiments.
7. Wortmannin inhibits phosphorylation of IKK complex and IKKα induced by Notch-1 activation mediated by Jagged-1.

To test if Akt contributes to the phosphorylation of IKK complex and IKKα in the Jagged-1 induced NF-κB activation pathway, Akt was inhibited by incubating cells with the PI3K inhibitor wortmannin. Since the half life of wortmannin in the medium is only 10 mins, 50 nM of wortmannin was used 30 mins before, right before and 30 mins after overlaying LTK cells on a monolayer of MDA-MB-231 cells. As shown (Figure 4.12 A), Akt activation mediated by Jagged-1 was abolished by addition of wortmannin. Phosphorylation of IKK and IκBα induced by Jagged-1 was suppressed by wortmannin incubation, suggesting that Akt is upstream of IKK and IKKα in the NF-κB activation pathway mediated by Jagged-1. Total Akt, IKK and IκBα expression levels were comparable in cells treated with or without wortmannin.

Due to the complexity of a mixed coculture of cells from two different species, the pull down by human specific IKKα antibody was performed to confirm the specificity of Akt dependence of IKKα phosphorylation in MDA-MB-231 cells (Figure 4.12 B).
Besides pharmacological inhibition of Akt, we also utilized the dominant negative Akt to manipulate Akt activation. Figure 4.12 C illustrates that activation of Akt induced by Jagged-1 was abolished in cells transfected with dominant negative Akt. As a result, phosphorylation of IKK complex was also decreased compared to the control.
Overexpression of dominant negative Akt was validated by western blot of Akt. This infers that Akt is responsible for the activation of IKK complex and IKKα in the NF-κB activity induction mediated by Jagged-1.

8. **Wortmannin inhibits the p50, p65, IKKα and Pol II recruitment to the cIAP-2 promoter.**

Consistent with the previous observation that Akt is upstream of IKK complex and IKKα in the NF-κB activation pathway mediated by Jagged-1, wortmannin (Akt inhibitor) also inhibited the induced recruitment of p50, p65 and IKKα to cIAP-2 promoter (Figure 4.13 A). Pol II occupancy on cIAP-2 promoter was increased dramatically by LTK-JAG coculture, corresponding to the elevated p50, p65 and IKKα recruitment and the induction of cIAP-2 transcript. The increased Pol II occupancy was abrogated by wortmannin as well, confirming the pivotal function of Akt in mediating the NF-κB activation by Notch-1 (Figure 4.13).
Figure 4.13: Wortmannin inhibits the p50, p65, IKKα and Pol II recruitment to the cIAP-2 promoter. (A-B) 50 nM of wortmannin was added to MDA-MB-231 cells medium 30 mins before, right before and 30 mins after overlaying LTK cells to a monolayer of MDA-MB-231 cells. DMSO was used a vehicle control. MDA-MB-231 cells were cocultured with LTK-PAR and LTK-JAG in the presence or absence of wortmannin for 1 hr. ChIP assays against specific antibodies were performed. All ChIP data were expressed as relative fold induction of specific antibody over IgG control. All data were normalized to the internal control: unspecific binding to β-globin intron sequence. *P<0.05 compared to JAG cocultured. Data are representative of three independent experiments.

9. Notch-1 activation mediated by EDTA activates the Akt/IKK/NF-κB pathway.

Notch-1 can be activated not only by coculture but also transiently by incubation with EDTA (Rand et al., 2000). To confirm the activation of NF-κB by Notch-1 and exclude the possibility that NF-κB activation we observed in coculture experiments was from mouse fibroblast, we activated Notch-1 signaling by incubating cells with 5 mM EDTA. Notch-1 was activated and cleaved into active form of Notch-1 even 5 mins after
addition of EDTA (Figure 4.14 A). Active Notch-1 kept accumulating until 60 mins of incubation and possibly Notch-1 was modified as indicated by the slight band shift. Akt was also activated very quickly. Phosphorylation of Akt started 5 mins after addition of EDTA to the medium. Activation of Akt was transient and disappeared after 30 mins of incubation. IKK became active 5 mins after EDTA was added and continued to be active up to 15 mins, in agreement with the activation time profile of Akt. Consistently, IκBα was phosphorylated about 15 mins after incubation. The coincided activation time courses of Akt, IKK and IκBα indicate that Notch-1 might activate NF-κB through the Akt/IKK/IκBα signal transduction cascade.

So far, we have shown that Notch-1 activation induced by either coculture or EDTA incubation coincided with NF-κB activation through Akt/IKK/IκBα in MDA-MB-231 cells. We don’t know if Notch-1 is required for the NF-κB activation mediated by EDTA. In order to test this, Notch-1 was knocked down by siRNA. In control siRNA-transfected cells, EDTA activated Notch-1 as reflected by the cleaved Notch-1 accumulation determined by western blot. Notch-1 siRNA successfully abrogated the induction of active Notch-1. Notch-1 siRNA also caused complete abolishment of the induced phosphorylation of Akt and resulted in decreased phosphorylation of IKK complex and IκBα, compared to cells transfected with control siRNA (Figure 4.14 B). These results confirm the requirement of Notch-1 in the activation of NF-κB activity mediated by EDTA through an Akt/IKK dependent pathway.
Figure 4.14: Notch-1 activation mediated by EDTA activates the Akt/IKK/NF-κB pathway

A

Active Notch-1

p-Akt

Akt

p-IKKα/β

IKκα

p-IκB α

Total IκB α

Actin

0 5 15 30 60 120 EDTA 5 mM (mins)

B

Active Notch-1

p-Akt

Akt

p-IKKα/β

IKκα

p-IκB α

Total IκB α

Actin

si RNA:

CTL N-1 CTL N-1 CTL N-1 CTL N-1 EDTA 5 mM (mins)

0 5 15 30

Figure 4.14: Notch-1 activation mediated by EDTA activates the Akt/IKK/NF-κB pathway. MDA-MB-231 cells were incubated with 5 mM EDTA for the indicated time. After incubation, cells were lysed and western blots were conducted using whole cell lysates. (B) MDA-MB-231 cells were transfected with either Control siRNA (CTL) or Notch-1 (N-1) siRNA. Forty-eight hrs after transfection, cells were incubated with 5 mM EDTA for the indicated time. Whole cell lysates were used for blotting. Data are representative of three independent experiments.

10. Akt activation by Notch-1 is independent of CBF-1

It was demonstrated that Notch-1 activation mediated by either Jagged-1 or EDTA induces Akt activation very transiently. Does cytoplasmic or nuclear Notch-1 activate Akt/IKK/ NF-κB pathway? Does the activation require CBF-1? Given the fact that the activation of Akt by Notch-1 occurs minutes after stimulation, we hypothesized
the cytoplasmic Notch-1 activates Akt and this activation doesn’t require CBF-1 involvement. We used the CBF-1 siRNA to block canonical nuclear Notch-1 signaling. Down-regulation of CBF-1 did not change the phosphorylation status of Akt and IKK complex. This suggests that cytoplasmic not the nuclear Notch-1 activates Akt/IKK and Notch-1 doesn’t require CBF-1 to activate Akt/IKK. CBF-1 siRNA reduced the phosphorylation level of IκBα at 15 mins and 30 mins time points of EDTA incubation, possibly due to the down-regulated expression of total IκBα (Figure 4.15). The reduction in total IκBα expression is consistent with our finding in CaSki cells and previous literature observations (Oakley et al., 2003). These data indicate that Notch-1 regulates NF-κB activity in a CBF-1 dependent and independent manner.
Figure 4.15: Akt activation by EDTA is independent of CBF-1

Figure 4.15: Akt activation by EDTA is independent of CBF-1. MDA-MB-231 cells were transfected with either Control siRNA (CTL) or CBF-1 siRNA (CBF). Forty-eight hrs after transfection, cells were incubated with 5 mM EDTA for the indicated time. Whole cell lysates were used for immunoblotting. Data are representative of three independent experiments.
Interestingly, a function for non-nuclear Notch-1 has been reported in other systems. In T-cells, it is reported that NIC forms a cytoplasmic complex with PI3K and the non-receptor protein tyrosine kinase p56\textsuperscript{Leuk} to activate Akt (Sade et al., 2004). Recently, Perumalsamy et al have demonstrated that cytoplasmic but not nuclear Notch-1 activates Akt to rescue cell apoptosis triggered by nutrition withdrawal (Perumalsamy et al., 2009). Consistent with their data, here, our results suggest that cytoplasmic Notch-1 activates Akt independent of CBF-1.

11. Conclusion of Aim 3

In Aim 3, we have shown that NF-κB was activated by Notch-1 through Akt/IKK/NF-κB axis. First, we showed that Notch-1 and IKKα were both essential for basal and stimulated expression of some NF-κB dependent genes. Secondly, we demonstrated that NF-κB subunits, Notch-1 and IKKα were recruited to NF-κB dependent gene promoters however, Notch-1 was recruited later than NF-κB subunits and IKKα, suggesting a Notch-1 function before being recruited to the promoter. We showed that Notch activation mediated by Jagged-1 activated IKK complex and IKKα, induced phosphorylation and degradation of IκBα. We found that Akt was upstream of IKK complex and IKKα in the NF-κB activation pathway mediated by Jagged-1. Inhibiting Akt pharmacologically or genetically reduced the IKK activation and decreased NF-κB subunits, Pol II and IKKα recruitment to NF-κB dependent gene promoters. In agreement
with the data from Notch-1 activation by Jagged-1, Notch-1 activation mediated by EDTA activated Akt/IKK/NF-κB pathway as well. We proposed that the cytoplasmic, not the nuclear Notch-1 activates Akt since Akt activation was independent of canonical nuclear Notch-1 partner CBF-1.
CHAPTER V
DISCUSSION

A. Notch-1 regulation on NF-κB

The effect of Notch-1 regulation on NF-κB activity has been conflicting. It has been suggested that Notch-1 inhibits NF-κB activity in the nucleus via direct interaction mediated by the N-terminal portion of Notch-1 in T cells (Guan et al., 1996; Wang et al., 2001). Later Osborne group confirmed the interaction between p50/c-Rel and Notch-1 in T cells, however, they showed that through this interaction, Notch-1 activates the late not the early NF-κB activity following T cell receptor (TCR) activation by retaining the NF-κB subunits in the nucleus and preventing their translocation into the cytoplasm (Shin et al., 2005).

However, these observed discrepancies in regulation of NF-κB by intracellular Notch-1 (NIIC) might be due, in part, to the size of the NIIC construct used. The construct used by Wang and Guan is distinctly different from the physiological, in vivo-generated NIIC. Notch-1 protein is proteolytically cleaved by γ-secretase between amino acids G1743 and V1744 (Schroeter et al., 1998). Data from Dr.Osborne’s lab indicates that NIIC effects on NF-κB activity can be vastly different depending on the NIIC construct used (Cheng et al., 2001; Palaga et al., 2003). Therefore, we expect that size
discrepancies between active Notch constructs may lead to distinctly different effects in cells, and may account for the contradictory results previously reported.

In addition, the effect of Notch-1 overexpression on NF-κB highly depends on Notch-1 expression level and cellular context. Low amounts of Notch-1 stimulate NF-κB transcriptional activity while higher levels inhibit it as exhibited by Guan (Guan et al., 1996). We also observed the same effect in cervical cancer as well as in breast cancer cells, suggesting fine tuning of Notch-1 on NF-κB activity and a complicated regulation network. In addition to Notch-1 expression level, Notch-1 activation results in inhibition or activation of NF-κB activity depending on the cell type as well. As shown in our data, overexpression of Notch-1 induces NF-κB activation in CaSki and MDA-MB-231 cells. However, enforced expression of Notch-1 inhibits NF-κB activity in primary human keratinocyte (data not shown). In this thesis, we showed that modest overexpression of Notch-1 activates NF-κB activity in both cervical cancer CaSki cells and ER- breast cancer MDA-MB-231 cells.

B. Notch-1 regulation on NF-κB in cervical cancer

A role for Notch signaling in cervical cancer was suggested a decade ago (Zagouras et al., 1995), but has remained controversial. Both overexpression and loss of Notch-1 have been documented in cervical cancer. Increased expression of Notch-1 and Notch ligands were reported (Zagouras et al., 1995; Daniel et al., 1997; Gray et al., 1999). Low level of Notch-1 was expressed in normal cervical epithelium and higher level was detected in the membrane and cytoplasm of cells of precancerous cervical
lesions. Accumulation of Notch-1 and its ligand Jagged-1 and down-regulation of Manic Fringe, a negative regulator of Notch signaling coincided with the progression of high-grade precursor lesions to invasive cervical cancers (Schweisguth and Lecourtois, 1998; eeraraghavalu et al., 2004). More importantly, Notch-1 was shown to cooperate with HPV oncoproteins in transforming immortalized human keratinocytes (Rangarajan et al., 2001; Ramdass et al., 2006). These cells transfected with E6, E7 and Notch-1 were able to generate xenograft tumors in nude mice (Chakrabarti et al., 2004).

However, there was some controversy raised by Talora et al. They found that invasive and metastatic cervical cancers as well as cell lines derived from cervical cancer did not express Notch-1. Activated Notch signaling leads to growth arrest of cervical cancer derived cell lines (Talora et al., 2005; Wang et al., 2007).

It is difficult to compare data obtained with different antibodies by different groups, and immunohistochemistry is hardly quantitative. However, in a later study on a large quantity of clinical samples, Notch-1, its ligand Jagged-1 and its downstream targets were observed and were accompanied by activation of NF-κB pathway (Ramdass et al., 2006). Moreover, Lathion et al showed that the effects of Notch-1 in cervical keratinocytes are strikingly dose-dependent: high levels of constitutively active Notch-1 suppress E6 and E7 as found by Talora et al, but moderate levels of Notch-1 promote keratinocyte transformation by E6 and E7.

The data from our lab also indicate that Notch-1 protein is present in most cervical carcinomas, in agreement with Ramdass et al. (Ramdass et al., 2006) and in cervical carcinoma lines, in agreement with Lathion et al. (Lathion et al., 2003). Notch-2 and 4 appear to be co-expressed with Notch-1. The pattern of Notch-1 expression appears
to change from granular and membrane-associated in normal cells to diffuse and primarily cytoplasmic in cancer cells (Song et al., 2008). This may be responsible for apparent differences in published reports.

Regardless of the relative levels of Notch-1 compared to normal cells, our lab had previously shown that complete loss of Notch-1 is incompatible with survival in CaSki cervical cancer cells in vitro and in vivo (Weijzen et al., 2002; Weijzen et al., 2003). We now show that endogenously expressed Notch-1 is required to maintain constitutive NF-κB activity and modest over-expression of Notch-1 activates NF-κB activity in cervical cancer CaSki cells and suggest a novel mechanism for activation of this pathway by Notch-1.

Unlike T-cells, where Notch-1 activates NF-κB complexes primarily by enhancing their nuclear retention (Shin et al., 2005), in cervical cancer cells Notch-1 functions upstream of IKKα/β and serves as a necessary activation co-factor by forming a physical complex with it. In Aim 2, we found that Notch-1 appears to activate IKKα/IKKβ complex kinases activity, leading to phosphorylation of IκBα as illustrated by Figure 5.1. Notch-3 has been recently reported to bind and activate IKKα homodimers in murine thymocytes (Vacca et al., 2006). However, in that system Notch-3 binding results in NIK-independent activation of the cytoplasmic functions of IKKα, leading to the activation of p52/RelB heterodimers. Whether different Notch homologues modulate IKKα in different ways (i.e., by activating canonical kinase activity or promoting its kinase activity of phosphorylating p52/RelB), or alternatively Notch homologues regulate NF-κB by different mechanism remains to be established.
In CaSki cells, Notch-1/IKKα containing complexes are detectable in the nucleus as well. It is unclear whether Notch-1 and IKKα migrate to the nucleus as a complex or associate in the nucleus. However, later, we found that IKKα recruitment to NF-κB responsive promoter (Song et al., 2008) is Notch-1 dependent. This suggests that the two proteins may co-translocate to the nucleus.

The association of Notch-1 and IKKα in the nucleus suggests that Notch-1 may also activate the nuclear functions of IKKα. In recent years, evidence has been accumulating that IKKα has a number of nuclear functions within and beyond NF-κB pathway. These functions include the phosphorylation and activation of the estrogen receptor α, together with its co-activator protein SRC3 in breast cancer cells (Park et al., 2005) and phosphorylation and stabilization of the β-catenin oncoprotein (Albanese et al., 2003; Albanese et al., 2003; Carayol and Wang, 2006), phosphorylation of H3 Ser10 and phosphorylation of SMRT co-repressor. Through binding to IKKα, Notch-1 could be part of an IKKα-dependent enhanceosome that activates transcription at NF-κB-responsive, estrogen-responsive and other sites. Recent finding from our lab showed that Notch-1 activates ER dependent transcription via IKKα in ER+ breast cancer cells as shown in Figure 5.1. This further confirmed the observation of Notch-IKK complex and suggested potential critical functional role of Notch-IKK complex not only on NF-κB-responsive but also on estrogen dependent promoters.

On the other hand, is IKKα required for the canonical, CBF-1 mediated effects of Notch? The traditional mechanism of transcriptional activation by Notch-1 involves an exchange of SMRT-containing corepressor complexes for coactivator complexes (Kao et al., 1998; Lai, 2002; Miele, 2006), much like the transcriptional activation of NF-κB and...
the estrogen receptor. The mechanism whereby Notch-1 displaces SMRT-containing corepressor complexes from CBF-1 (Kao et al., 1998; Kadesch, 2000) is still unclear. Moreover, IKKα does associate with the Hes-1 promoter, a quintessential Notch/CBF-1 target, and de-represses it in part by phosphorylating promoter-associated IκBα (Aguilera et al., 2004). Nuclear IKKα can phosphorylate SMRT not only in association with NF-κB but also in association with CBF-1 (Fernandez-Majada et al., 2007) (Figure 5.1). This leads to deregulated activation of Notch-mediated signaling. Our lab showed that, at least in CaSki cells, IKKα is required for canonical CBF-1 activation (Song et al., 2008). This may be due to the mechanism that IKKα phosphorylates SMRT on CBF-1 dependent promoters as described by the Bigas group (Fernandez-Majada et al., 2007) and/or due to the upregulation of Notch ligands by NF-κB (Bash et al., 1999).
Figure 5.1 Notch-1 and IKKα interaction

Aim 2: Notch-1 forms a complex with IKK, activating IKK activity.

Figure 5.1 Notch-1 and IKKα interaction. In Aim 2, we showed that in cervical cancer cells Notch-1 functions upstream of IKKα /β and serves as a necessary activation co-factor by forming a physical complex with it. Notch-1 appears to activate IKKα homodimers and IKKα /IKKβ complex kinases activity, leading to phosphorylation of IκBα. Notch-1/IKKα containing complexes are detectable in the nucleus as well. Literature demonstrated that the interaction has been found not only on NF-κB but also on CBF-1 and ER dependent promoters. On NF-κB and CBF-1 dependent promoter, IKKα phosphorylates SMRT and derepresses gene transcription. On CBF-1 dependent promoter, IKKα also de-represses it in part by phosphorylating promoter-associated IκBα. On ER dependent promoter, IKKα phosphorylates and activates the ERα. Notch-1 may activate those nuclear IKKα activities through binding to it.
C. Notch-1 regulation on NF-κB in ER- breast cancer

Unlike in cervical cancer, Notch-1 oncogenic role in breast cancer has been studied and confirmed by several groups including us. Aberrant activation of Notch signaling was observed in a large number of human breast carcinomas. Our lab has confirmed that Notch expression was deregulated in human breast cancer as opposed to normal and hyperplastic breast cells. Ductal and lobular carcinomas consistently expressed high levels of Notch-1 and Notch-4 as compared to normal breast tissue, which expressed Notch-1 at barely detectable levels. Reedijk et al showed that high-level expression of Notch-1 or Jagged-1 predicted poor overall survival in breast cancer patients, combination of high level of both indicated even worse outlook. The same group also showed high level Jagged-1 mRNA and protein predicted poor outcome in breast cancer and Jagged-1 expression was associated with recurrence in lymph node-negative breast cancer (Reedijk et al., 2005; Dickson et al., 2007; Reedijk et al., 2008).

Inhibition of Notch by pharmacological agents and molecular means has been shown to suppress breast cancer growth and promote apoptosis (Leong et al., 2007). However the molecular mechanism responsible for the apoptosis induction was not known. In this thesis, we are the first to show that Notch-1 may contribute to cell survival by activating NF-κB activity in ER- breast cancer. We chose to use MDA-MB-231 cells as our studying model. Because MDA-MB-231 cells, which are ER- basal phenotype breast cancer (ER-, PR-, Her2-), harbor the highest level of Notch-1 and Jagged-1 (Rizzo et al., 2008). Moreover, this type of cancer has higher NF-κB activity compared to ER+ breast cancer cells.
We found that in CaSki cells Notch-1 modulates NF-κB activity by physically interacting with the IKK signalosome and regulates its IKK kinase activity. We could not detect a Notch-1 and IKK interaction in MDA-MB-231 cells. Nevertheless, we demonstrated that Notch-1 is required and sufficient to activate NF-κB activity in MDA-MB-231 cells.

In Aim 3, we found that NF-κB activation induced by Notch mediated through Jagged-1 or EDTA. Active Notch-1 activated Akt and IKK complex and caused phosphorylation and degradation of IκBα. Consequently, NF-κB subunits, Pol II, IKKα and Notch-1 were recruited to NF-κB dependent gene promoters. We also demonstrated that Akt was upstream of IKK complex and IKKα in the NF-κB activation pathway mediated by Jagged-1. Inhibiting Akt pharmacologically or genetically reduced the IKK activation and decreased NF-κB subunits, Pol II and IKKα recruitment to NF-κB dependent gene promoters. We showed that Akt activation was independent of canonical nuclear Notch-1 partner CBF-1. It is possible that a cytoplasmic Notch is responsible for Akt activation. The model from Aim 3 is illustrated in Figure 5.2.

An increasing number of reports demonstrate that Notch interacts with Akt in various systems. Notch-1 is able to activate Akt and also inhibit Akt activity depending on the physiological context. In T-cells, Notch-1 has been shown to activate Akt via Src family protein tyrosine kinase p56^lk (Sade et al., 2004). Notch signals promote T-cell precursors survival through maintenance of cellular metabolism via activation of the PI3K-Akt signaling pathway (Ciofani and Zuniga-Pflucker, 2005). Active Notch-1 inhibits p53-induced apoptosis and sustains transformation by HPV 16 E6 and E7 oncogenes through a PI3K/Akt dependent pathway (Nair et al., 2003). In melanoma,
constitutive activation of Notch-1 drives primary melanoma toward a more aggressive phenotype in vitro and in vivo by activating PI3K/Akt pathway (Liu et al., 2006).

The mechanism by which Notch-1 activates Akt has been studied. Notch-1 activates Akt by decreasing PTEN expression through Hes1 and Myc in TALL cells. Both Hes-1 and Myc bind and regulate the PTEN promoter, with Hes-1 working as a strong transcriptional repressor and Myc as a weaker transcriptional activator, so that the overall output downstream of Notch-1 activation is a controlled downregulation of PTEN transcripts (Palomero et al., 2007). More recently, Perumalsamy (Perumalsamy et al., 2009) showed another mechanism where cytoplasmic but not nuclear Notch-1 blocks apoptosis triggered by nutrition deprivation in HeLa cells through mTOR, Rictor and Akt dependent signaling. The same effect was also observed in the cytokine deprived condition in activated T cells. Here, we also showed that Notch-1 activated Akt in a CBF-1 independent manner, consistent with Perumalsamy’s finding. However, currently we do not know how exactly Notch-1 activates Akt. Notch-1 may interact with PI3K or mTOR in MDA-MB-231 cells. Elucidating the mechanism involved is one of our future directions.

Not only does Notch-1 regulate Akt, on the other hand, Akt also modulates Notch-1 signaling. Akt requires Notch-1 to transform melanocytes in vitro and Akt activates Notch-1 through NF-κB activity (Bedogni et al., 2008). Inhibition of Akt results in a decreased protein expression of Notch-1 (Calzavara et al., 2008). This is also confirmed in our lab. Inhibition of Akt by wortmannin or dominant negative Akt led to decrease in Notch-1 protein expression and CBF-1 dependent transcriptional activity in
T47D:C42 cells (ER- breast cancer cell line). Activation of Akt by overexpressing wild type or constitutive active Akt increased Notch-1 expression and activity.

There are intertwined Notch-1 and Akt signaling pathways. It would be very interesting to elucidate the crosstalk between Notch-1 and Akt as well as their association with NF-κB pathway.

D. Future directions and possible therapeutic implications

In the future, we would like to determine if there is additive or synergistic therapeutic effect using combination of GSI and current chemo drugs in vitro and in vivo. ER- breast cancer is usually more aggressive and more difficult to treat than ER+ breast cancer. In ER- breast cancer, Notch-1 and NF-κB are constitutively active. ER- breast cancer cells rely more on Notch-1 and NF-κB activity for survival compared to ER+ breast cancer cells. Here, we found that Notch-1 activates pro-survival NF-κB signaling pathway via Akt in ER- breast cancer MDA-MB-231 cells. ER- breast cancer is usually treated with chemotherapy and there is no specific target oriented therapy available. We are going to determine if inhibiting Notch-1 will sensitize ER- breast cancer cells to apoptosis induced by chemotherapy in vitro and in vivo. If there is synergistic therapeutic effect, the outlook of ER- breast cancer could be improved. The GSIs are currently undergoing clinical trials. It would be very promising to combine GSI and current chemo-drugs to treat ER- breast cancer patients.
E. Conclusions

In summary, we showed that Notch-1 activates NF-κB activity in both cervical and ER- breast cancer cells. We found that Notch-1 activates NF-κB activity by interacting with IKKα/β and regulating IKKα/β kinase activity in cervical cancer CaSki cells (Figure 5.1). We demonstrated that Notch-1 activates NF-κB dependent gene expression in ER- breast cancer MDA-MB-231 cells through an Akt/IKK dependent pathway (Figure 5.2). We explored similarities and differences in the underlying mechanisms in the two cell types. Potentially, this study will provide a molecular rationale for targeting Notch-1, Akt and NF-κB in cervical and ER- breast cancer in the clinic.
Figure 5.2 Notch-1 activates NF-κB through Akt in MDA-MB-231 cells. In Aim 3, we showed that Notch-1 is required and sufficient to activate NF-κB activity in MDA-MB-231 cells and Notch-1 activates NF-κB through the Akt/IKK dependent pathway. NF-κB activation induced by Notch mediated through Jagged-1 or EDTA activates Akt and IKK complex and causes phosphorylation and degradation of IκBα. There is evidence in the literature that not only does Notch-1 regulate Akt, on the other hand, Akt modulates Notch-1 signaling. Akt requires Notch-1 to transform melanocytes in vitro and Akt activates Notch-1 through NF-κB activity. Inhibition of Akt results in the decreased protein expression of Notch-1.
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VITA

The author, Yin Peng, was born September 8, 1982, in Changsha, China to Dongju Peng and Jie Wang. Yin attended a premier high school, Yali High School. Yin attended Sichuan University, where she was enrolled in a creative program, which gave her the opportunity to have two majors and to conduct research in the laboratory of Dr. Cao. Yin graduated from college with a B.S in Finance and a B.S in Biotechnology.

In August 2004, Yin entered the Molecular and Cellular Biochemistry Program at Loyola University Chicago. Driven by a great interest in cancer research, she joined the laboratory of Dr. Lucio Miele. Her work primarily focused on the regulatory role of Notch-1 in NF-κB pro-survival signaling pathway in cervical cancer and estrogen receptor negative breast cancer. Her work has been presented at national and international meetings. Yin was awarded the Arthur J. Schmitt Dissertation Fellowship for the 2008-2009 academic years. Yin served as a student representative for Molecular and Cellular Biochemistry Program in 2005-2006 and she was the president of Chinese Student and Scholar Association in 2008–2009. She organized several activities to improve the presence of the organization and help strengthen the Chinese community.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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