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Notch-1 and IGF-1 as Survivin Regulatory Pathways in Cancer: A Dissertation

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Mechanisms of Survivin Signaling Pathways in Cancer

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Abstract

The 21st century brought about a dramatic increase in knowledge about genetic and molecular profiles of cancer. This information has validated the complexity of tumor cells and increased awareness of "nodal proteins", but has yet to advance the development of rational targeted cancer therapeutics. Nodal proteins are critical cellular proteins that collect biological inputs and distribute the information across diverse biological processes. Survivin acts as a nodal protein by interfacing the multiple signals involved in mitosis and apoptosis and functionally integrate proliferation, cell death, and cellular homeostasis. By characterizing survivin as a target of both Type 1 Insulin-like Growth Factor (IGF-1) and Notch developmental signaling, we contribute to the paradigm of survivin as a nodal protein. The two signaling systems, Notch and IGF-1, regulate survivin by two independent mechanisms. Notch activation induces survivin transcription preferentially in basal breast cancer, a breast cancer subtype with poor prognosis and lack of molecular therapies. Activated Notch binds the transcription factor RBP-Jκ and drives transcription from the *survivin* promoter. Notch mediated survivin expression increases cell cycle kinetics promoting tumor proliferation. Inhibition of Notch in a breast xenograft model reduced tumor growth and systemic metastasis. On the other hand, IGF-1 signaling drives survivin protein translation in prostate cancer cells. Binding of IGF-1 to its receptor activates downstream kinases, mammalian target of rapamycin (mTOR) and p70 S6 protein kinase (p70S6K), which modulates survivin mRNA translation to increase the apoptotic threshold. The multiple roles of survivin in tumorigenesis implicate survivin as a rational target for the "next generation" of cancer therapeutics.

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4E-BP1	Eukaryotic initiation factor 4E binding protein 1
Ab	Antibody
ADAM	A distintegrin and metalloprotease
AIP	Aryl hydrocarbon receptor interacting protein
Akt	v-akt murine thymoma viral oncogene homolog, serine/threonine protein kinase, also known as protein kinase B
ALL	Acute lymphoblastic leukemia
A_{nm}	Absorbance at given wavelength
APC	Adenomatous polyposis coli, tumor suppressor
APC/C	Anaphase promoting complex/cyclosome
AURKB	Aurora B kinase
BIR	Baculovirus IAP repeat, conserved motif of $CX_2CX_{16}HX_{6-8}C$
BMP	Bone morphogenic protein
BRCA1	Breast cancer 1, early onset, tumor suppressor
Bub	Budding uninhibited by benzimidazole, tension sensor in spindle checkpoint
CBF-1	CREB binding protein, also known as RBP-Jκ
CDCA8	Borealin
CDE	Cell cycle dependent region, promoter element
CDK1	Cyclin dependent kinase 1, also known as cdc2
cDNA	Cloned DNA, coding region of organism's DNA
ChIP	Chromatin immunoprecipitation
CHR	Cell cycle homology region, promoter element
CPC	Chromosomal passenger complex
CpG	Cytosine and guanine separated by a phosphate, DNA regions high in cytosine and guanine typically near promoters of mammalian genes
Crm1	Nuclear exported, also known as XPO1 and exportin 1 in *Homo sapiens*
CSL	CBF-1, Suppressor of Hairless, LAG-1, also known as RBP-Jκ
DAB	3'-3'-diamino-benzidine, developing agent for immunohistochemistry
DEVD	z-Asp-Glu-Val-Asp, Caspase substrate
DMSO	Dimethyl sulfoxide, polar aprotic solvent
DNA	Deoxyribonucleic acid
DRB	5,6 dichloro-1-β-D-ribofuranosylbenzimidazole, transcriptional inhibitor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor, also known as ErbB1

eIF4E	Eukaryotic initiation factor 4E
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERK	Extracellular signal regulated protein kinase pathway
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSI	γ-secretase inhibitor
H_2O_2	Hydrogen peroxide
HBXIP	Hepatitis B-X-interacting protein
HER-2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, also known as ErbB2
Hsp	Heat shock protein
IAP	Inhibitor of apoptosis protein
IC_{50}	Inhibitory concentration at 50%
IGF-1	Type 1 insulin-like growth factor
IGF-1R	Type 1 insulin-like growth factor receptor
IgG	Immunoglobulin G
INCENP	Inner centromere protein
IRS	Insulin receptor substrate
Lef-1	Lymphoid enhancer binding factor-1, transcriptional enhancer
Mad	Mitotic arrest deficient, attachment sensor in spindle checkpoint
MAML	Mastermind-like, human homolog of Mastermind
MAPK	Mitogen activated protein kinase
MCAK	Microtubule-depolymerizing kinesin
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex 1(raptor) or 2(rictor)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, measures activity of mitochondrial reductase which correlated with metabolic activity, proliferation, and cellular viability
NF-κB	Nuclear factor of κ light polypeptide gene enhancer in B-cells, transcription factor
NIC	Notch intracellular domain
p70S6K	p70 S6 kinase
pAd	Adenoviral construct
PBS	Phosphate buffered saline
pcDNA	Mammalian expression vector
PCR	Polymerase chain reaction

PI3K	Phosphatidylinositol 3-kinase, protein kinase B
PKA	Cyclic AMP-dependent protein kinase A
pLuc	Luciferase reporter vector
PML	Promyelocytic leukemia, transcription factor and tumor suppressor
PP2A	Protein phosphatase 2A, serine/threonine phosphatase
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog, tumor suppressor gene
Rak	Fyn-related kinase, tyrosine kinase
RAN	Ras-related nuclear protein, small GTP binding protein
RBP-Jκ	Recombination signal binding protein for immunoglobulin Jκ, transcriptional factor also known as CBF-1, CSL
RING	Really interesting new gene, zinc finger motif
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SEM	Standard error of mean
Ser	Serine, amino acid
siRNA	Small interfering RNA
Smad	Mothers against decapentaplegic homologs, signaling proteins phosphorylated by TGF-β at SSXS motif
Sp1	Specificity protein, transcription factor
STAT	Signal transducer and activator of transcription, transcriptional activator
STS	Staurosporine
SW480	Colon adenocarcinoma cell line
TBE	TCF/Lef binding element, promoter element
TCF4	Transcription factor 4, basic helix-turn-helix transcription factor downstream of β-catenin
TGF-β	Transforming growth factor β
Thr	Threonine, amino acid
TSC2	Tuberous sclerosis complex
Tyr	Tyrosine, amino acid
Wnt	Wingless-type MMTV integration site family, secreted signaling proteins
WT	Wildtype
XIAP	X-linked inhibitor of apoptosis protein
zVAD	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone, pan-caspase inhibitor

Chapter 1. Introduction

The discovery of microarray technology, RNA silencing, and genomic sequencing provided a vast database of information about cancer, cancer genes, and cancer pathways (Vogelstein and Kinzler 2004; Lu, Getz et al. 2005; Bild, Yao et al. 2006; Wood, Parsons et al. 2007). Thus far, the information has not affected cancer patients who suffer from stagnant survival rates over the last thirty years (Ries, Melbert et al. 2008). Dozens of "targeted" therapies enter clinical trials, some like imatinib (Kohn, Bauer et al. 1999), tamoxifen (CRC 1992), trastuzumab (Baselga 2001) have indisputable benefits, but in practice few targeted therapies pass phase I or II clinical trials. Given the complexity of most tumors, "targeted" therapies may selectively disable one or two tumor pathways, but allow or even encourage tumor survival by activating alternate survival pathways (Kitano 2004). Even so, analysis of biological systems reveals that cancer networks rely on a handful of key molecules to regulate apoptosis, control proliferation, respond to growth inhibitory signals, and metastasize (Kitano 2004). Disabling these key molecules, or nodal proteins, destroys so many essential pathways that the cancer implodes from cellular stress. Survivin, an inhibitor of apoptosis protein (IAP), functions as one such protein. As expected from a nodal protein, survivin is a crucial molecule in cell death and cell division that affects numerous cellular processes like apoptosis, proliferation, differentiation, and metastasis. We identify Notch-1 and IGF-1 as two independent pathways that control survivin expression to drive tumorigenesis. The work reinforces the concept of survivin as a nodal protein. Selective targeting of these pathways may disable a key molecule required for tumor growth and survival.

Survivin in tumorigenesis

In an analysis of the human cancer transcriptome, survivin surfaced as the fifth highest transcript preferentially expressed in cancer cells (Velculescu, Madden et al. 1999). Survivin, an onco-fetal protein, is expressed during embryonic development but not in most normal adult tissue, and dramatically re-expressed in virtually all human tumors (Ambrosini, Adida et al. 1997). Furthermore, survivin is associated with stem cell gene signatures of mesenchymal (Taubert, Wurl et al. 2007), neuronal (Pennartz, Belvindrah et al. 2004), and skin (Marconi, Dallaglio et al. 2006) progenitor cells. Survivin homozygous knockout mice prematurely die at embryonic day 3.5 with defects of cell proliferation, spindle formation, and apoptosis (Uren, Wong et al. 2000; Conway, Pollefeyt et al. 2002) demonstrating the requirement of survivin during development. Knockdown of survivin results in dual phenotypes of mitotic defects, i.e. centrosomal abberations, multipolar spindles, and chromatin missegregation, and apoptosis, i.e. loss of mitochondrial membrane potential, caspase activation (Beltrami, Plescia et al. 2004). These characteristics may explain why overexpression of survivin in tumors is associated with poor prognosis, resistance to chemotherapy, and reduced patient survival (Fukuda and Pelus 2006). The role of survivin in elevating the apoptotic threshold while preserving genomic integrity is an essential feature of tumor development.

Survivin in Cell Division

Survivin is required for cell division. The functions of survivin in cell division fall into three main categories: participation in chromosomal passenger protein biology, participation in the spindle checkpoint, and regulation of microtubule dynamics.

Survivin is a chromosomal passenger protein (Adams, Earnshaw 2001) of which Aurora B (AURKB), inner centromere protein (INCENP), and borealin (CDCA8) are members. Chromosomal passenger proteins are conserved proteins that migrate to the inner centromeres at prometaphase and metaphase, subsequently to the spindle midzone at anaphase and finally to the midbody and cleavage furrow in telophase (Ruchaud, Carmena et al. 2007). The complex is required for correction of spindle attachment errors, organization of a bipolar spindle, and completion of cytokinesis (Ruchaud, Carmena et al. 2007). Survivin mislocalization disrupts the entire chromosomal passenger complex (CPC) since the localization and function of the CPC is highly interdependent on the recruitment of all subunits (Vagnarelli and Earnshaw 2004). Aurora B, a Ser/Thr kinase responsible for phosphorylation of key mitotic proteins, is the enzymatic component of this complex. Survivin serves as binding partner (Honda, Korner et al. 2003), a substrate (Wheatley, Henzing et al. 2004) and a regulator (Bolton, Lan et al. 2002; Chen, Jin et al. 2003) of this kinase.

The spindle checkpoint prevents anaphase onset by sensing the correct attachment of sister chromatids to the mitotic spindle. The sensor mechanism relies on inhibition of the anaphase promoting complex/cyclosome (APC/C) via recruitment of BubR1 to sense tension across the kinetochores, and Mad2 to sense microtubule attachments to the kinetochores. The spindle checkpoint can be activated in the absence of survivin, however, the localization of BubR1 and Mad2 to prometaphase kinetochores is impaired (Carvalho, Carmena et al. 2003; Lens, Wolthuis et al. 2003). In particular, survivin is crucial for a stable association of BubR1 to the kinetochore to maintain checkpoint activation in response to lack of tension (Carvalho, Carmena et al. 2003; Lens, Wolthuis

et al. 2003). As suggested by the interdependency of CPC localization, Aurora B depletion also impairs the spindle checkpoint (Hauf, Cole et al. 2003). The regulatory contribution of survivin to Aurora B serves as a secondary component of survivin in spindle checkpoint biology.

Finally, survivin has a role in microtubule dynamics. A population of survivin associates with polymerized microtubules (Li, Ambrosini et al. 1998; Beltrami, Plescia et al. 2004). Depletion of survivin destabilizes microtubules by increasing the rate of microtubule nucleation and catastrophe at multiple phases of the cell cycle, independently of Aurora B (Rosa, Canovas et al. 2006). Conversely, exogenous survivin hyper-stabilizes microtubules by reducing microtubule dynamics (Rosa, Canovas et al. 2006). At mitosis, survivin regulates both the number and stability of centrosomal microtubules (Rosa, Canovas et al. 2006) as well as the formation of kinetochore associated microtubules albeit most likely in distinct signaling pathways (Tulu, Fagerstrom et al. 2006). The regulation of microtubule dynamics by survivin also interfaces with the role of survivin in cell death. Phosphorylation of microtubule associated survivin on Thr34 by cyclin dependent kinase 1 (CDK1) protects cells from spindle poisons during mitosis (O'Connor, Grossman et al. 2000). A phosphorylation deficient survivin mutant (T34A) acts as a dominant negative isoform that prevents association of survivin with the mitotic spindle and induces mitotic cell death (O'Connor, Grossman et al. 2000).

Survivin in Cell Death

Survivin also plays a major role in regulating cell death. Survivin cooperatively interacts with adaptor or cofactor molecules as well as several members in the apoptotic

pathway to prevent cell death. Multiple pathways converge to regulate survivin expression contributing to the increased threshold to apoptotic stimulation.

Survivin is the smallest protein in the IAP family containing only one conserved baculoviral IAP repeat (BIR) domain, and lacking a commonly conserved RING finger domain at the carboxyl terminus (Ambrosini, Adida et al. 1997). The BIR domain serves as binding sites for important apoptotic regulators. Survivin, like most other IAPs, does not directly inhibit caspases nor exert its cytoprotection without intermolecular cooperation (Eckelman, Salvesen et al. 2006). Pools of survivin in the mitochondria associate with Smac, a pro-apoptotic inhibitor of IAPs (Sun, Nettesheim et al. 2005). In the cytosol, survivin interacts with Smac, possibly sequestering Smac and preventing the association with other IAPs (Song, Yao et al. 2003). Survivin associates with hepatitis B-X-interacting protein (HBXIP) forming a complex that inhibits caspase 9 and mitochondrial apoptosis, while survivin or HBXIP alone does not inhibit caspases (Marusawa, Matsuzawa et al. 2003). In a similar system, survivin interacts with X-linked inhibitor of apoptosis protein (XIAP), the sole IAP member that directly inhibits caspases (Eckelman, Salvesen et al. 2006), preventing ubiquitin mediated degradation of XIAP (Dohi, Beltrami et al. 2004).

The survivin-XIAP complex is also regulated in a mechanism dependent on compartmentalized phosphorylation of survivin (Dohi, Xia et al. 2007). Cyclic AMP-dependent protein kinase A (PKA) phosphorylates survivin on Ser20 in the cytosol but not the mitochondria (Dohi, Xia et al. 2007). Survivin phosphorylated on this residue loses its affinity for XIAP and the ability to protect cells from apoptotic stimuli (Dohi, Xia et al. 2007). Mitochondrial survivin is protected from the activity of PKA and

maintained in a dephosphorylated state by protein phosphatase 2A (PP2A) (Dohi, Xia et al. 2007). This pool of survivin preserves a high affinity for XIAP, prevents XIAP degradation and promotes tumor growth (Dohi, Beltrami et al. 2004; Dohi, Xia et al. 2007). In tumors, accumulation of survivin in the mitochondria serves as a mechanism to concentrate a key anti-apoptosis factor in an active, functional state (Dohi, Xia et al. 2007).

Survivin regulation is a point of convergence for pathways contributing to cellular homeostasis. Molecular chaperones in the heat shock protein (Hsp) family are responsible for protein folding, preventing protein aggregation, and managing the response to cellular stressors (Sreedhar and Csermely 2004). Hsp90, a molecular chaperone used by tumor cells to promote growth and survival, associates with survivin protecting the molecule from degradation (Beltrami, Plescia et al. 2004). Targeting the survivin-Hsp90 complex with antibodies or a Hsp90 inhibitor reduces survivin levels and mimics a survivin siRNA phenotype of cell cycle arrest and apoptosis (Beltrami, Plescia et al. 2004). The aryl hydrocarbon receptor interacting protein (AIP) is another component of the Hsp90 chaperone system that interacts with survivin to prevent proteasomal degradation of survivin (Kang and Altieri 2006). Knockdown of Hsp60, a chaperone responsible for transporting and refolding proteins into the mitochondria, depletes survivin in the mitochondria and induces mitochondrial membrane depolarization (Ghosh, Dohi et al. 2008). The mitochondrial chaperone network may protect mitochondrial survivin pools from degradation so it may exert its anti-apoptotic effect (Dohi, Xia et al. 2007; Kang, Plescia et al. 2007; Ghosh, Dohi et al. 2008).

In addition to regulation by molecular chaperones, survivin is regulated extensively by transcriptional elements in its promoter. The *survivin* promoter is a TATA-less promoter containing several Sp1 sites, a CpG island, three cell cycle dependent regions (CDE) and one cell cycle homology region (CHR) that control survivin expression throughout the cell cycle (Li and Altieri 1999). In cancer, the cell cycle periodicity of survivin expression is lost mainly by upregulation of *survivin* transcription. Several cancer survival pathways promote *survivin* gene transcription. The *survivin* promoter contains four putative NF-κB binding sites that can be activated by human T-cell leukemia virus to drive survivin transcription (Kawakami, Tomita et al. 2005). In colon cancer, the developmental Wnt/TCF-4/β-catenin cascade drives survivin expression protecting cells from UV induced apoptosis (Kim, Plescia et al. 2003). Other pro-survival cascades including the signal transduction and activator of transcription 3 (STAT3) cytokine driven transcription factor (Kanda, Seno et al. 2004), and the E2F transcription activators (Jiang, Saavedra et al. 2004) induce *survivin* expression. Conversely, tumor suppressor genes including APC (Zhang, Otevrel et al. 2001), p53 (Hoffman, Biade et al. 2002), and PML-4 (Xu, Zhao et al. 2004) negatively regulate the *survivin* promoter.

The diverse regulatory components of survivin and its central role in inhibiting cell death and ensuring genomic integrity suggests that multiple signals work collectively to modulate survivin. The goal of this work is to identify new networks where survivin may play a role. We looked at the developmental Notch pathway and the growth factor IGF-1 pathway.

Notch signaling

Notch signaling is a conserved developmental pathway used for cell to cell communication. Conventionally, the Notch signaling system controls the fate of stem cells as they progress along the differentiation process (Bray 2006). The Notch family consists of four Notch proteins (1-4) that differ in the number of EGF-like repeats, and presence of a trans-activation domain. Mammals have five classic Notch ligands, Delta-like-1,-3, -4 and Jagged-1, -2 that differ in the number of EGF-like repeats and the presence of a cysteine rich domain. Members of the Contactin family can also serve as a Notch ligand in oligodendrocytes (Hu, Ang et al. 2003; Cui, Hu et al. 2004) alluding to the possibility of aberrant Notch activation by non-classical ligands given a complex tumor microenvironment. Affinity and specificity between Notch ligands and Notch receptors is controlled via glycosylation of extracellular Notch (Fortini 2000; Moloney, Panin et al. 2000). Once the Notch receptor is engaged, two sequential cleavages ensue: an extracellular cleavage by an ADAM metalloprotease and an intracellular cleavage by γ-secretase. The γ-secretase cleavage releases Notch intracellular domain (NIC) into the cytosol. Upon nuclear translocation, NIC binds the transcriptional repressor RBP-Jκ, also known as CBP-1 and CSL, in a complex involving Mastermind (MAML) and p300 (Mumm and Kopan 2000). NIC binding converts RBP-Jκ from a transcriptional repressor to an activator, subsequently initiating transcription of a myriad of genes, most prominently the Hes family of transcription repressors (Table 1-1) (Imatani and Callahan 2000; Grabher, von Boehmer et al. 2006). Other NIC targets include ErbB2 (Haruki, Kawaguchi et al. 2005), NF-κB (Oswald, Liptay et al. 1998), c-myc (Sharma, Calvo et al. 2006; Weng, Millholland et al. 2006), and Slug (Timmerman, Grego-Bessa et al. 2004;

Leong, Niessen et al. 2007). Bcl-2 upregulation is attributed to a secondary pathway of Notch mediated RBP-Jκ independent signaling (MacKenzie, Duriez et al. 2004). The identity of Notch targets are still under active investigation since there are often tissue specific transcriptional targets.

The Notch signaling network often displays tissue specific effects. In skin, Notch signaling promotes cell cycle arrest (Rangarajan, Talora et al. 2001) while in other tissues Notch signaling increases cell cycle progression (Radtke and Raj 2003). A possible explanation is that in different tissues, Notch signals interact with different sets of transcription factors that control the transcripts sensitive to Notch regulation. NIC classically associates with RBP-Jκ, however, in certain tissues NIC may bind other co-activators such as Lef-1 to initiate downstream transcription (Ross and Kadesch 2001; Spaulding, Reschly et al. 2007). In another setting, collaborative signaling of Notch with other signaling cascades directly modulates cellular responses. Wnt co-activation with Notch is required for transformation of human mammary epithelial cells while Notch activation alone is insufficient to drive the process (Ayyanan, Civenni et al. 2006). This suggests that the signaling environment affects Notch function. For example, Notch signaling in various epithelial cells collaborates with TGF-β to promote cell cycle arrest (Niimi, Pardali et al. 2007). In contrast, Notch signaling in breast (Reedijk, Odorcic et al. 2005), T-cell acute lymphoblastic leukemia (ALL) (Weng, Ferrando et al. 2004), and pancreatic (Miyamoto, Maitra et al. 2003) cancer is correlated to proliferation, cell survival, and sometimes metastasis.

Mechanisms of Notch mediated tumorigenesis

The Notch signaling network is essential for stem cell maintenance in breast (Buono, Robinson et al. 2006), pancreas (Murtaugh, Stanger et al. 2003), intestine (Fre, Huyghe et al. 2005; Stanger, Datar et al. 2005), skin (Moriyama, Osawa et al. 2006; Schouwey, Delmas et al. 2006), and muscle (Conboy and Rando 2002). The same stem cell pathway may be commandeered for tumorigenesis. Activating mutations in Notch-1 is directly associated with pediatric and adult T-cell ALL (Weng, Ferrando et al. 2004; Mansour, Linch et al. 2006).

In breast cancer, Notch is of emerging importance. While many mouse models identify Notch deregulation as tumorigenic (Gallahan and Callahan 1987; Gallahan, Jhappan et al. 1996), the case in humans is only beginning to be unveiled. In an analysis of 11 human breast cancers and 11 human colon cancers, Notch-1 is found to be a mutational hotspot only in breast cancers (Wood, Parsons et al. 2007). In microarray studies, Notch-1 mRNA levels are associated with poorly differentiated tumors and lower chances of survival (van de Vijver, He et al. 2002). In addition, Notch-3 seems to have a role in proliferative control in HER-2 negative cell lines (Yamaguchi, Oyama et al. 2008) however, the specific downstream players are unknown.

Functionally, Notch most likely drives tumorigenesis via its many transcriptional targets which span numerous biological processes. Induction of NF-κB (Oswald, Liptay et al. 1998) and Bcl-2 (MacKenzie, Duriez et al. 2004) suggests a predisposition toward cellular survival upon Notch activation. Normal mammary epithelial cells stably transfected with NIC and RBP-Jκ resist drug-induced apoptosis by suppressing p53 apoptotic signals (Stylianou, Clarke et al. 2006). Notch activation in SW480 human

colon adenocarcinoma cells confers resistance to adriamycin, cisplatin, etoposide and taxol in a phosphatidylinositol 3-kinase (PI3K) dependent manner (Mungamuri, Yang et al. 2006). Notch activation may affect proliferation by modulating G1/S transition via direct transcriptional induction of cyclin D1 (Ronchini and Capobianco 2001) or controlling proteasomal degradation of $p27^{Kip1}$ (Sarmento, Huang et al. 2005). In metastasis, Notch activation induces Slug which in turn represses E-cadherin expression to mediate a epithelial to mesenchymal transition (Timmerman, Grego-Bessa et al. 2004; Leong, Niessen et al. 2007).

The functional parameters of Notch are very diverse. Therefore it is likely that this network may enlist nodal proteins such as survivin to exert its effects. Survivin, like Notch, correlates with stem cell signatures (Pennartz, Belvindrah et al. 2004; Marconi, Dallaglio et al. 2006; Taubert, Wurl et al. 2007), and regulates common pathways in cell cycle and cell death. Furthermore, survivin is largely transcriptionally regulated in cancer progression. Therefore, survivin may be controlled by transcriptional pathways such as Notch.

IGF-1 signaling

Binding of the Type 1 Insulin-like Growth Factor (IGF-1) to its cognate tyrosine kinase receptor Type 1 Insulin-like Growth Factor Receptor (IGF-1R) induces a conformational change in the receptor and activation of intrinsic tyrosine kinase activity that stimulates a mitogenic signaling cascade. The phosphorylated receptor transmits intracellular signals through protein substrates including the growth factor receptor bound protein (GRB) family, the SHC protein family, the insulin receptor substrate (IRS)

family, and the p85 regulatory subunit of PI3K. The receptor substrates triggers multiple independent signaling cascades including the PI3K/Akt pathway, the extracellular signal regulated protein kinase (ERK) pathway, and the mitogen activated protein kinase (MAPK) pathway (Craparo, O'Neill et al. 1995; Ryan and Goss 2008). PI3K/Akt signaling regulates many cellular processes including cell cycle regulation, apoptosis, cell growth, and protein synthesis.

Downstream of PI3K/Akt is the mammalian target of rapamycin (mTOR). The mTOR protein kinase is comprised of two distinct protein complexes each with different targets. The rapamycin sensitive mTOR complex 1 (mTORC1) contains raptor and responds to nutrient status, amino acid availability, oxidative stress, and growth factor stimulation by controlling protein synthesis, autophagy, and ribosome biogenesis (Hay and Sonenberg 2004). Activation of mTORC1 affects protein synthesis by phosphorylation of p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein (4E-BP1) (Hay and Sonenberg 2004). Phosphorylation of p70S6K by mTORC1 stimulates protein synthesis by activation of S6 ribosomal protein (Ruvinsky and Meyuhas 2006). The S6 ribosomal protein in association with the 18S ribosomal RNA forms the 40S ribosomal subunit required for translational initiation (Holland, Sonenberg et al. 2004). mTORC1 phosphorylation of 4E-BP1 prevents the inhibitory association of the protein with eukaryotic initiation factor 4E (eIF4E). Non-complexed eIF4E binds to 5’capped mRNA and recruits the mRNA to the ribosomal initiation complex (Pause, Belsham et al. 1994). The mTORC complex 2 (mTORC2) is less sensitive to rapamycin (Sarbassov, Ali et al. 2006) and contains rictor. This complex responds to insulin, growth factors, serum, and nutrient availability (Frias, Thoreen et al. 2006) to regulate the actin

cytoskeleton (Sarbassov, Ali et al. 2004) and the Akt feedback loop (Sarbassov, Guertin et al. 2005). As a regulator of protein synthesis, mTOR modulates cell size, cell proliferation and cell survival, all of which are key components of tumorigenesis.

IGF-1/Akt/mTOR in tumorigenesis

In large prospective studies of breast, colon, prostate and lung cancer, elevated serum IGF-1 levels have been correlated to a higher cancer risk (Ryan and Goss 2008). In prostate cancer, persistent IGF-1 signaling contributes to development of androgen independence by allowing cells to survive despite minimal androgen stimulation (Djavan, Waldert et al. 2001; Nickerson, Chang et al. 2001). Constitutive activation of IGF-1R confers anchorage independent growth (Kaleko, Rutter et al. 1990), and conversely, disruption of IGF-1R reduces spontaneous transformation of mouse embryonic fibroblasts (Sell, Rubini et al. 1993; Sell, Dumenil et al. 1994). Further downstream, Akt oncogenic signaling is a common driver of tumor formation and progression. Somatic alterations in the phosphatase and tensin homolog tumor suppressor gene (PTEN), occurring in 30-60% of human prostate cancer, potently activate the Akt/mTOR pathway (Gray, Phillips et al. 1995; Komiya, Suzuki et al. 1996). Elevation in survivin expression is correlated to PI3K/Akt/mTOR signaling, but the mechanisms remained unknown (Dan, Jiang et al. 2004). Based on this, the IGF-1/Akt/mTOR signaling cascade was explored for a mechanistic relationship between survivin and IGF-1 in prostate cancer.

Concluding Remarks

The validation of survivin as a nodal protein requires characterizing the roles of survivin in multiple networks. An Ingenuity Analysis of survivin displays the many signaling molecules that interact with survivin (Figure 1-1)(Altieri 2008). The survivin network rests in a cancer focused landscape with the majority of survivin interactors, regulators, and effectors being components of tumor development. The goal of this work is to identify and characterize the role of survivin in tumorigenic signaling cascades. As such, we have identified independent roles of Notch-1 and IGF-1 in regulation of survivin. The Notch-1 transcriptionally driven signaling pathway may use nodal proteins like survivin to execute its pleiotropic effects. We found Notch-1 transcriptionally targets the *survivin* promoter specifically in basal breast cancer, a clinical aggressive form of breast cancers. In contrast, the IGF-1 pathway regulates a novel translational mode of survivin expression in prostate cancer. The two pathways solidify survivin as a nodal protein. Targeting survivin or its key regulatory pathways may bypass issues of tumor heterogeneity and allow for selective killing of cancer cells.

RBP-Jκ Dependent Genes		
Hes family transcription factors	Murine Notch-1 activates transcription of Hes-1, a transcriptional repressor, through RBP-Jκ binding sites Cell type specific HERP1 and HERP2 activation by Notch-1	(Jarriault, Brou et al. 1995; Reya, Morrison et al. 2001)
ErbB2	RBP-Jκ mediate ErbB2 transcription augmented by Notch-1	(Haruki, Kawaguchi et al. 2005)
Pre T cell receptor α (pTα)	RBP-Jκ binds pTα enhancer element regulating specification of T cell lineage	(Reizis and Leder 2002)
Notch regulated ankryin repeat protein (NRARP)	NARP transcription via RBP-Jκ participates in inhibitory feedback look on Notch-1 signaling	(Krebs, Deftos et al. 2001; Yun and Bevan 2003; Pirot, van Grunsven et al. 2004)
Cyclin D1	NICD induction of cyclin D1 via RBP-Jκ correlate with transforming ability and S phase progression	(Ronchini and Capobianco 2001)
S-phase kinase associated protein 2 (SKP2)	Notch/ RBP-Jκ transcriptional activation of SKP2 downregulates $p21^{Cip1}$ and $p27^{Kip1}$ to modulate cell cycle progression	(Sarmento, Huang et al. 2005)
NF-κB	Notch-1 activation overcomes RBP-Jκ repression by NF-κB transcription via RBP-Jκ binding site	(Oswald, Liptay et al. 1998)
Myc	NIC targets c-myc in T-cell ALL models in mouse and man	(Sharma, Calvo et al. 2006; Weng, Millholland et al. 2006)
Slug	Jagged-1 mediates Notch targeting of Slug to drive EMT in breast epithelial cells, and cardiac endothelial cells	(Timmerman, Grego-Bessa et al. 2004; Leong, Niessen et al. 2007)
RBP-Jκ Independent Genes		
Bcl-2	Notch-1 and Notch-4 induce Bcl-2 independently of RBP-Jκ	(Deftos, He et al. 1998; MacKenzie, Duriez et al. 2004)

Table 1-1. Notch mediated RBP-Jκ dependent and independent genes

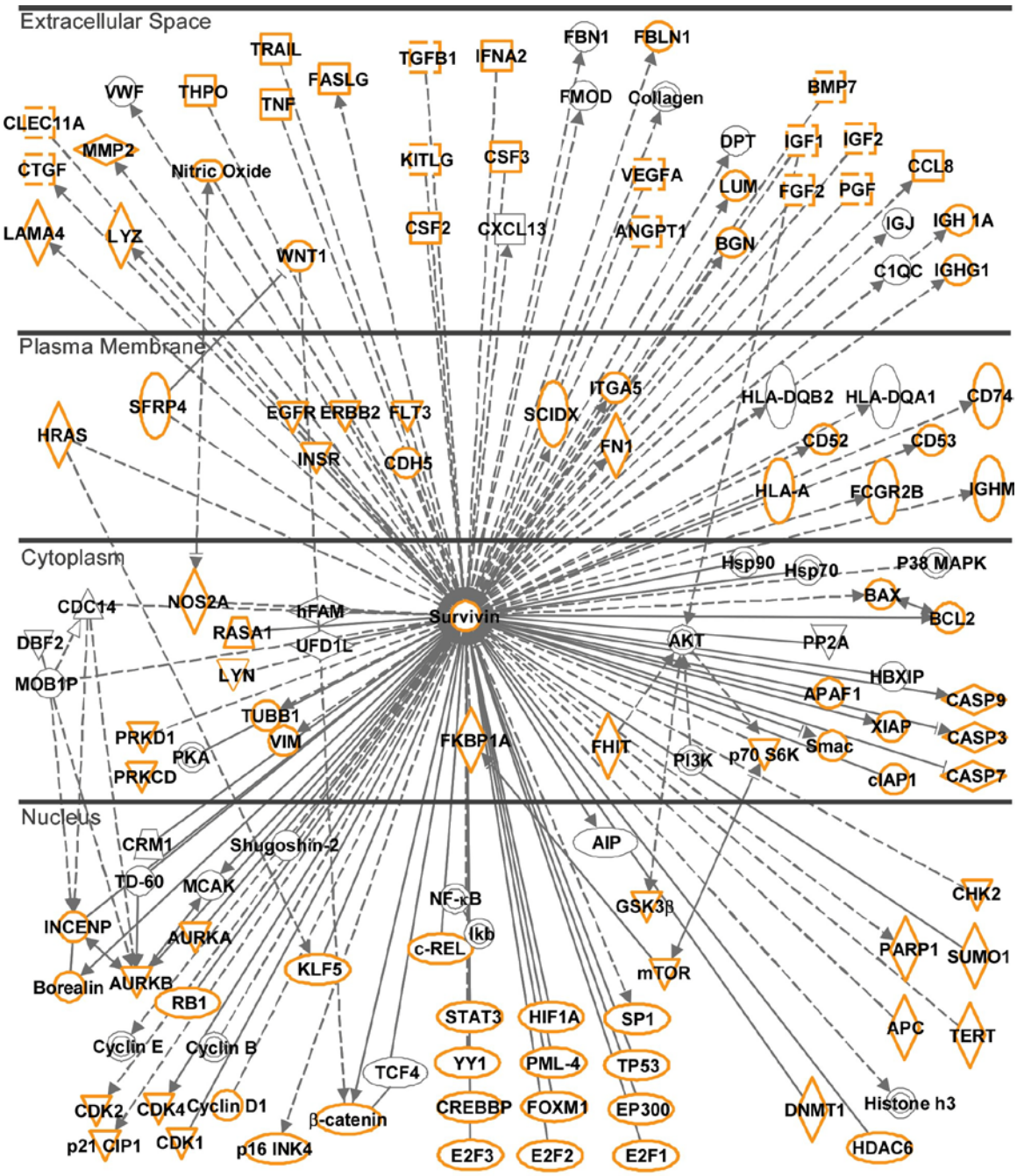

Figure 1-1. Survivin regulatory networks complied via Ingenuity Analysis. Highlighted molecules are genes known to contribute to tumorigenesis. (Altieri 2008)

Chapter 2. Notch survivin gene signature in basal-like breast cancer

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Chung-Cheng Hsieh, and Dario C. Altieri

This chapter represents work contributed by the listed authors. The text is a version of the manuscript submitted to Clinical Cancer Research on May 19, 2008 and is currently under review. Karl Simin, Dario Altieri, and I conceived and designed the study. Karl Simin and I collected and assembled the data. Karl Simin carried out the hierarchal clustering of Notch-1 on the 232 breast cancer samples from the University of North Carolina database. I carried out the immunohistochemical staining, assembly of the microarray datasets, descriptive statistical analysis for the cohorts, and the in vitro experiments. Minakshi Guha kindly constructed and shared the MDA-MB-231 stable cell line expressing GFP under the control of the 830 nt mouse *survivin* promoter. Qin Liu and Chung-Cheng Hsieh carried out the Pearson's correlation coefficient statistical analysis based on a random effects model for meta-analysis. All of us were involved in data analysis, interpretation, writing, and final approval of the manuscript.

Abstract

Basal-type, or "triple negative" breast cancer (lacking estrogen receptor, progesterone receptor, and HER-2 expression) is a high-risk disease for which no molecular therapies are currently available. We studied genetic signatures in basal breast cancer, potentially suitable for therapeutic intervention. We analyzed protein expression

by immunohistochemistry. A hierarchical clustering analysis was carried out on a microarray database of 232 breast cancer patients. Fifteen published datasets containing estrogen receptor (ER)-negative or -positive samples were subjected to meta-analysis for co-segregated gene expression. Experiments of plasmid transfection, gene silencing, and γ-secretase inhibition were carried out in ER-negative MDA-MB-231 model breast cancer cells. The developmental signaling regulator, Notch-1 was highly expressed in breast cancer, compared to normal tissue, and segregated with basal disease. Higher *Notch-1* levels correlated with progressively abbreviated overall survival, and with increased expression of *survivin*, a tumor-associated cell death and mitotic regulator implicated in stem cell viability. Analysis of Pearson's correlation coefficient indicated that *Notch-1* and *survivin* co-segregated in basal breast cancer. Notch-1 stimulation of MDA-MB-231 cells increased survivin expression, whereas targeting Notch by small interfering RNA (siRNA) or inhibition of γ-secretase activity suppressed *survivin* promoter activity, and reduced survivin protein levels. A *Notch-1*/*survivin* dual gene functional signature is a hallmark of basal breast cancer, and may contribute to disease pathogenesis. Antagonists of Notch and survivin currently in the clinic may be tested as novel molecular therapy of these recurrence-prone patients.

Introduction

The introduction of molecular "gene signatures" in breast cancer (Perou, Sorlie et al. 2000) provides important prognostic and predictive information (van 't Veer, Dai et al. 2002; Paik, Shak et al. 2004; Liu, Wang et al. 2007), and holds promise for "individualized" molecular therapy of these patients (Sotiriou and Piccart 2007).

However, certain subtypes of breast cancer continue to pose therapeutic challenges (Sotiriou and Piccart 2007). For example, basal breast cancer is breast cancer subtype characterized by histologic high-grade disease (Da Silva, Clarke et al. 2007), absence of HER-2 and receptors for estrogen (ER) and progesterone (Nielsen, Hsu et al. 2004), expression of basal cytokeratins (i.e. keratin-5), and proliferation-associated genes (Perou, Sorlie et al. 2000; Sotiriou, Neo et al. 2003), as well as defects in genomic gatekeepers, p53, or BRCA1 (Sorlie, Perou et al. 2001). While immunohistochemical diagnosis of basal breast cancer is straightforward (Nielsen, Hsu et al. 2004), these patients have limited therapeutic options: the response to mainstay chemotherapy is not uniform, and affected by the type of drugs used (Cleator, Heller et al. 2007), estrogen or HER-2 targeting is not indicated, and attempts to disable ancillary signaling pathways, for instance coordinated by the EGF receptor, have so far shown little promise (Baselga, Albanell et al. 2005). This adds to a high rate of relapses that is linked to shortened overall survival, and death from disease (Carey, Dees et al. 2007).

Although the cell of origin of basal breast cancer has not been firmly identified (Da Silva, Clarke et al. 2007), a link to the progenitor/stem cell compartment of the mammary epithelium has been proposed (Dontu, El-Ashry et al. 2004). In this context, developmental gene expression pathways that control the interplay between cell proliferation, survival, and differentiation (Clevers 2006) are candidates for stem cell-derived mammary tumorigenesis. One such pathway is centered on the Notch family of cell surface receptors (Bray 2006) that affects the mammary stem cell niche (Buono, Robinson et al. 2006), and has been associated with malignant transformation (Sansone, Storci et al. 2007), and aggressive tumor behavior (Reedijk, Odorcic et al. 2005).

Among the candidate effector molecules controlling stem cell viability is survivin, a dual regulator of cell division and apoptosis, broadly over-expressed in cancer (Altieri 2008). Consistent with its "onco-fetal" pattern of expression, survivin is essential for tissue homeostasis (Altieri 2008), and conditional knockout studies have suggested a potential critical role of this pathway in maintaining stem cell viability, at least in certain tissue compartments (Leung, Xu et al. 2007).

In this study, we used a combination of hierarchical clustering of a novel microarray dataset, meta-analysis of published gene profiling studies, and in vitro experiments of gene targeting to investigate a potential role of a Notch-1-survivin signaling axis in breast cancer.

Results

Differential expression of Notch in breast cancer

We began this study with preliminary experiments to look at the expression of Notch-1 in a representative collection of breast cancer specimens. An antibody to NIC intensely labeled the tumor cell population of invasive breast ductal carcinoma (Figure 2-1, *top panel*), infiltrating lobular carcinoma (Figure 2-1, *middle panel*), and invasive papillary carcinoma (Figure 2-1, *bottom panel*). In all tumor samples, Notch-1 reactivity was observed in both nuclei and cytoplasm of tumor cells (Figure 2-1). In contrast, matched normal breast tissues did not stain for activated Notch-1 (Figure 2-1, *right panels*), and a non-immune IgG gave no staining of normal or breast cancer samples (Figure 2-1, *insets*).

Expression of Notch in breast cancer microarray databases

We next analyzed the expression of Notch-1 in an established breast cancer patient cohort. Supervised hierarchical clustering of 232 cases of human breast cancer (Herschkowitz, Simin et al. 2007) using "Intrinsic Gene Analysis" revealed that higher expression of *Notch-1* segregated with basal breast cancer. Other known markers of the disease, including *keratin-5* and *-14*, and *kit*, were also highly correlated with *Notch-1* expression ($r \geq 0.58$) in this cohort (Figure 2-2A). One hundred twenty five patients with associated clinical outcome data were further analyzed. When stratified according to levels of *Notch-1* $\log_2$ transcript ratios, tumors with the highest quartile of *Notch-1* gene expression (1st Quartile) exhibited abbreviated overall survival with a median survival of 27 mo compared to the other groups, $p<0.001$ via Log-Rank test (Figure 2-2B). Seventy-two percent of tumors in this 1st Quartile (23/32) were classified as basal breast cancer, and the overall survival of these patients was approximately 50% lower than that of the remaining population ($p<0.02$). Conversely, reduced levels of *Notch-1* (2nd through 4th Quartile) were associated with better overall survival (Figure 2-2B). The percentage of basal breast cancers in these groups was 16% (2nd Quartile, 16/31), 19% (3rd Quartile, 19/31), and 3% (4th Quartile, 3/31), respectively.

Gene expression correlation in basal breast cancer

We next carried out a meta-analysis of published microarray datasets to identify genes associated with Notch-1, and potentially implicated in the molecular pathogenesis of basal breast cancer. We focused on survivin, a mitotic regulator and cell death inhibitor (Altieri 2008) over-expressed in breast cancer (van 't Veer, Dai et al. 2002), and

associated with unfavorable outcome (Paik, Shak et al. 2004), and keratin-5, a marker of basal epithelium, often linked to a progenitor/stem cell phenotype (Dontu, El-Ashry et al. 2004). Fifteen microarray datasets, mostly employing Affymetrix technology published between 2002 and 2007, fulfilled the search criteria (Table 2-1). The overall median age of patients was 55.2 years. The breast tumors examined were typically <5 cm, encompassing all grades, and included lymph node-positive and lymph node-negative disease. In one study (Zhao, Langerod et al. 2004) separate databases for lobular and ductal breast cancer were examined, bringing the datasets analyzed to a total of 16. Two studies (Zhao, Langerod et al. 2004; Saal, Johansson et al. 2007) did not contain downloadable *keratin-5* expression data, and one study (Yu, Ganesan et al. 2006) contained 68 out of 96 samples with *Notch-1* and *survivin* data, and 19 samples with *keratin-5* data. Table 2-2 shows the descriptive statistics of each cohort with respect to *Notch-1*, *survivin*, and *keratin-5* relative expression.

A novel dual gene signature in basal breast cancer

Analysis of 507 ER-negative and 1356 ER-positive breast cancer patients revealed that *keratin-5* associated with ER-negative breast cancers (Figure 2-3A) in 7 out of 13 data sets, and that *Notch-1* associated with ER-negative breast cancers in 9 out of 16 datasets (Table 2-2). Pooled estimates of Pearson's correlation coefficient between *Notch-1*/*keratin-5* were 0.3315 and 0.2043, for ER-negative and ER-positive breast cancers, respectively, p=0.04 (Figure 2-3A). Similarly, *survivin* and *keratin-5* co-segregated in ER-negative breast cancer, with a pooled estimate of Pearson's correlation coefficient of 0.1314 for ER-negative and -0.2408 for ER-positive breast cancers,

$p<0.0001$ (Figure 2-3B). Analysis of 604 ER-negative and 1463 ER-positive breast cancer patients revealed that *survivin* segregated with ER-negative tumors (two-tailed $p<0.05$) in 12 out of 16 cohorts (Table 2-2). The Pearson's correlation coefficients between *Notch-1* and *survivin* were 0.1804 and -0.0674 for ER-negative and ER-positive breast cancers, respectively, $p<0.0001$ (Figure 2-3C).

Notch-1 regulation of survivin expression

Recent studies have shown that survivin may function as a direct transcriptional target of Notch-1, thus controlling mitotic transition and resistance to apoptosis in breast cancer (Lee, Raskett et al. 2008). Transfection of model ER- breast cancer MDA-MB-231 cells with NIC resulted in increased survivin expression, by Western blotting (Figure 2-4A). Bcl-2, a known downstream target of Notch, and XIAP, a cofactor that is stabilized by survivin in protection from apoptosis, were also increased after NIC transfection (Figure 2-4A). In parallel experiments, targeting Notch by small interfering RNA (siRNA) resulted in a ~50% reduction in endogenous Notch-1 expression, by Western blotting (Figure 2-4B). This was associated with concomitant reduction of survivin levels in MDA-MB-231 cells, by Western blotting (Figure 2-4B), and reduction of cell viability (Supplemental Figure 2-5), whereas acute ablation of survivin by siRNA did not affect Notch-1 levels (Figure 2-4B). Activation of Notch signaling requires cleavage by the enzyme γ-secretase, and this step is inhibited by pharmacologic γ-secretase inhibitors (GSI). Treatment of MDA-MB-231 cells with a peptidyl GSI, z-Leu-Leu-Nle-CHO resulted in reduction of survivin, XIAP, and Bcl-2 expression levels, by Western blotting (Figure 2-4C), in agreement with recent observations (Lee, Raskett et al.

2008). To validate that survivin is a direct downstream transcriptional target of Notch (Lee, Raskett et al. 2008), we next stably transfected MDA-MB-231 cell line to express a GFP reporter gene under the control of the proximal, 830 nt mouse *survivin* promoter (MDA-MB-231 ms-830-GFP). Treatment of MDA-MB-231 ms-830-GFP stable cells with 1 μM of GSI was associated with a significant reduction of GFP expression, as assessed by flow cytometry (Figure 2-4D).

Discussion

In this study, we have shown that Notch-1 is preferentially expressed in breast cancer, as compared with normal tissues, segregates with basal disease, and correlates with abbreviated survival. In meta-analysis of multiple, independent microarray datasets, *Notch-1*, *survivin*, and *keratin-5* selectively co-associated with ER-negative *versus* ER-positive breast cancer patients. In model ER- breast cancer cells, survivin was validated as a direct transcriptional target of Notch, and targeting this pathway by genetic or pharmacologic approaches reduced the expression of survivin, as well as of other apoptotic regulators, including XIAP and Bcl-2.

These findings add to an in-depth molecular classification of breast cancer (Sotiriou and Piccart 2007), and in particular, basal breast cancer, a disease variant that still poses significant therapeutic challenges. In addition to high-risk genetics (Perou, Sorlie et al. 2000; Sotiriou, Neo et al. 2003) and aggressive histologic features (Da Silva, Clarke et al. 2007), it has been speculated that basal breast cancer may originate from a progenitor/stem cell compartment in the basal mammary epithelium. This is consistent with a proposed role of Notch in stem cell maintenance (Bray 2006), and potentially in

the early events of their transformation (Farnie, Clarke et al. 2007). Such a pathway may not be exclusively limited to breast cancer (Stylianou, Clarke et al. 2006), given that deregulated Notch signaling has been implicated as a "driver" of disparate malignancies (Bray 2006), promoting aberrant cell cycle progression (Ronchini and Capobianco 2001), and unfavorable outcome (Reedijk, Odorcic et al. 2005).

In this context, survivin appears ideally suited to function as a pleiotropic, *direct* Notch effector gene in clinically aggressive breast cancer (Paik, Shak et al. 2004). At the molecular level, this involves transcription from discrete RPB-Jκ binding element(s) in the *survivin* promoter upon Notch activation that results in transcriptional upregulation of survivin levels, inhibition of apoptosis, and acceleration of mitotic transitions selectively in ER- breast cancer cells (Lee, Raskett et al. 2008). Whether deregulation of a Notch-survivin signaling axis is preferentially operative in a progenitor/stem cell compartment is currently not known. However, it is intriguing that another developmental gene expression pathway, i.e. Wnt/β-catenin has been implicated in controlling survivin levels in intestinal crypt progenitor cells, potentially contributing to colon cancer (Kim, Plescia et al. 2003), and that survivin expression been consistently associated with 'stemness' gene signatures of mesenchymal (Taubert, Wurl et al. 2007), neuronal (Pennartz, Belvindrah et al. 2004), and skin (Marconi, Dallaglio et al. 2006) progenitor cells. Results of conditional knockout studies appear to support this model, as heterozygous deletion of survivin produced complete bone marrow ablation, loss of hematopoietic progenitor/stem cells, and rapid animal mortality (Leung, Xu et al. 2007). This pathway may have a clear link to human disease, as lineage-specific methylation and silencing of the *survivin* gene has been linked to bone marrow depletion in myelodsyplastic syndrome

(Hopfer, Komor et al. 2007). With respect to breast cancer, and consistent with recent observations (Lee, Raskett et al. 2008), Notch-dependent upregulation of survivin (and potentially Bcl-2 and XIAP) may broadly suppress apoptosis, deregulate cell cycle progression (Altieri 2008), and ultimately promote resistance to mainstay therapeutic agents in this disease, such as taxanes (O'Connor, Wall et al. 2002), and DNA damaging agents (Ghosh, Dohi et al. 2006).

Although the diagnosis of “triple negative”, basal breast cancer is straightforward (Nielsen, Hsu et al. 2004), these patients continue to pose therapeutic challenges because of the aggressive nature of the disease. Basal breast cancers often present in younger patients with a high grade disease. While the cancers respond initially to chemotherapy, the cancers are prone to relapse, reducing overall survival. Furthermore, these patients lack appropriate, molecularly-targeted agents for their disease (Cleator, Heller et al. 2007). Based on the findings presented here, it may be possible to envision antagonists of Notch (Shih Ie and Wang 2007), and survivin (Altieri 2008) as potential molecular therapy for basal breast cancer patients. Agents that interfere with Notch signaling inhibit the enzyme γ-secretase, which is responsible for the activating intracellular cleavage of Notch upon ligand binding at the cell surface (Bray 2006). Despite concerns of specificity (Fortini 2002), and potential intestinal toxicity (Wong, Manfra et al. 2004), GSI molecules are being tested as molecular therapy in leukemia patients harboring activating mutations in Notch (Shih Ie and Wang 2007). In our recent studies, systemic administration of a peptidyl GSI significantly inhibited breast cancer growth in vivo, and nearly completely abolished metastatic dissemination, with no detectable organ or systemic toxicity (Lee, Raskett et al. 2008). Antagonists of survivin are also available in

the clinic, producing encouraging patient responses and manageable toxicity in early phase clinical trials (Altieri 2008).

In summary, we have extended initial in vitro experiments (Lee, Raskett et al. 2008), and validated the existence of a functional Notch-1/survivin signaling axis selectively in patients with basal breast cancer. Targeting Notch-1 signaling in model breast cancer cells lowered survivin levels, resulting in pronounced anti-tumor effects (Lee, Raskett et al. 2008). Taken together with the stringent correlation reported here across disparate tumor series, this raises the possibility that basal breast cancer cells may selectively become dependent, or "addicted" to Notch/survivin signaling for their maintenance (Weinstein and Joe 2006). Although it is unclear to what extent "oncogene addiction" maintains the malignant phenotype in vivo (Jonkers and Berns 2004), antagonists of such pathways have produced impressive clinical responses, at least in certain patient subsets (Sharma, Bell et al. 2007). A similar rationale may be envisioned here for targeting Notch and survivin in basal breast cancer patients, especially if this pathway can be disabled in a progenitor/stem cell compartment, acting as a potential disease reservoir contributing to a high incidence of relapses.

Material and Methods

Immunohistochemistry

Nine cases of human breast cancer and matched normal breast tissue from the same patient were obtained from the University of Massachusetts Cancer Center Tissue Bank. Tissue sections (5-µm) from paraffin blocks were cut, deparaffinized in xylene, rehydrated, and baked overnight at 60°C. Slides were quenched for endogenous

peroxidase with 3% H_2O_2 in methanol for 20 min, and processed for antigen retrieval by pressure cooking in 9 mM sodium citrate, pH 6.0, for 20 min. Slides were washed in PBS, and incubated overnight at 4°C with a rabbit antibody to Notch-1 intracellular domain (NIC) or control IgG, rinsed, and incubated with a biotinylated anti-rabbit IgG for 10 min at 22°C. After addition of streptavidin-conjugated horseradish peroxidase, slides were incubated with 3'-3'-diamino-benzidine (DAB) for 3-10 min, and counterstained with hematoxylin, as described (Dohi, Beltrami et al. 2004).

Hierarchical clustering analysis of Notch-1 expression in breast cancer

The $\log_2$ Cy5/Cy3 ratios of 232 cases of human breast cancer and their associated clinical data were downloaded from https://genome.unc.edu (Herschkowitz, Simin et al. 2007). Only genes where the Lowess normalized intensity values in both channels were >30, and data in >70% samples were included for analysis. The gene set was further filtered to include only genes with Pearson's correlation coefficient >0.58 with *Notch-1* (n=101). Two-way hierarchical clustering was performed using Cluster v3 (Eisen, Spellman et al. 1998), and the results displayed using JavaTreeview (Saldanha 2004). Analysis of overall survival (Log-Rank test) was carried out using JMP 6.0 out on the subset of breast cancer patients in this cohort with available clinical data (n=125). Data were plotted for each quartile of normalized *Notch-1* $\log_2$ ratios, from highest (1^{st} Quartile) to lowest (4^{th} Quartile).

Meta-analysis of Oncomine microarray data

We reviewed Oncomine (http://www.oncomine.org) (Rhodes, Yu et al. 2004) for independent human breast cancer microarray datasets comparing ER-negative and ER-positive tumors. Databases from 15 studies were found to contain *Notch-1* and *survivin* relative expression data. The patient characteristics and analyses performed in each study are summarized in Table 2-1. Descriptive statistics including mean, standard error, and a two-tailed unpaired t-test were calculated for the comparisons between ER-positive and ER-negative samples within each study. Separately for ER-negative and ER-positive samples, a Pearson's correlation coefficient (r) was calculated for each study to measure levels of pair-wise co-expression between *Notch-1*, *survivin*, and *keratin-5*. The 95% confidence interval for r was calculated based on Fisher's Z transformation (Kleibaum, Kupper et al. 1998). In one study (Turashvili, Bouchal et al. 2007), a Fisher's Z transformation could not be performed for ER-negative samples (n=3), and an approximate variance for a Pearson's correlation coefficient was used to derive its 95% confidence interval. To summarize ER-specific results from the individual studies, Fisher's Z transformation and its variance were used in pooling correlation from different studies. Weighted-average of Fisher's Z transformation and its 95% confidence interval were first estimated based on a fixed-effect model, taking into account the variance associated with each study. ER-specific pooled estimate of Pearson's correlation coefficient and its 95% confidence interval were then derived from the estimates based on the Fisher's Z transformation. We applied a random-effect model for meta-analysis (DerSimonian and Laird 1986) to evaluate whether levels of co-expressions between

Notch-1, *survivin*, and *keratin-5* differ between ER-negative and ER-positive samples among the different studies.

Cells and reagents

The breast adenocarcinoma MDA-MB-231 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in culture as recommended by the supplier. An 830 nt fragment of the mouse survivin promoter fused upstream of a Green Fluorescence Protein (GFP) reporter gene (ms-830-GFP) was described previously (Xia and Altieri 2006). To generate a stable cell line expressing ms-830-GFP, MDA-MB-231 cells were transfected by Lipofectamine (Invitrogen), and selected in medium containing 1 mg/ml Zeocin (Invitrogen). A peptidyl γ-secretase inhibitor (GSI) z-Leu-Leu-Nle-CHO was purchased from Calbiochem (San Diego, CA), and characterized in recent studies (Lee, Raskett et al. 2008).

Transfections

A cDNA encoding activated NIC was characterized previously (Small, Kovalenko et al. 2001). MDA-MB-231 cells were transfected with pcDNA or NIC cDNA (2 μg) using 6 μl LipofectAmine (Invitrogen) in Opti-Mem medium (1 ml) (Gibco). The media was changed after 5 h, and cells were harvested after 24 h. For gene silencing experiments by small interfering RNA (siRNA), MDA-MB-231 cells were transfected with double stranded (ds) RNA oligonucleotide directed to Notch-1, survivin, or control non-targeted sequences using 10 μl HiPerfect (Gibco). Cells under the various conditions were harvested after 48 h, and analyzed by Western blotting.

Study	N	Median Age (years)	Microarray	Tumor Size	Lymph Nodes	Treatment	Stage/Grade
Chin et al, Cancer Cell, 2006	118	55.3 (SD=14.3)	Affymetrix	2.6 cm (SD=1.3)	67 LN positive 51 LN negative	60% Treated with tamoxifen 52% adjuvant chemotherapy 51% radiation treatment	26 Stage 1; 70 Stage 2; 14 Stage 3; 5 Stage 4 10 Grade 1; 42 Grade 2; 61 Grade 3; 5 unknown
Desmedt et al, Clinical Cancer Research, 2007	198	47 All < 61 yo	Affymetrix	<5 cm	Node negative		T1-T2
Ginestier et al, Clinical Cancer Research, 2006	55		Affymetrix			19 amplified for 20q13 36 unamplified for 20q13	Consecutive cases Unilateral localized invasive breast cancer
Hess et al, Journal of Clinical Oncology, 2006	133	Training set: 52 Range (29-79) Validation set: 50 Range (28-73)	Affymetrix			Preoperative weekly paclitaxel and fluorouracil-doxorubicin-cyclophosphamide chemotherapy	Stage I Stage II Stage III
Ivshina et al, Cancer Research, 2006	249 (Uppsala cohort)	62.3	Affymetrix	2.9 cm	35% node positive	30.3% Endocrine therapy 10.7% Chemotherapy 1.7% Combination therapy 58.8% No systemic therapy	68 Grade 1 126 Grade 2 55 Grade 3
Miller et al, PNAS 2005	251	62.1 (SD=13.9)	Affymetrix	22.4 mm (SD=12.5)	84/253 LN metastasis 160 node negative 9 unknown node status	143 no adjuvant therapy Otherws with systemic adjuvant therapy, and/or chemotherapy	
Minn et al, Nature 2005	82	55.8 (SD=13.5)	Affymetrix	3.68 cm (SD = 1.77cm)	Ave 3.5 (SD = 5.98) axillary LN	Adjuvant chemotherapy and/or hormonal therapy	
Richardson et al, Cancer Cell, 2006	39		Affymetrix				
Saal et al, PNAS, 2007	105	61 (range 26-77)	Non-Affymetrix	27 mm (range 2-50 mm)	65 (62%) LN positive	Treated uniformly with 2 years of adjuvant tamozifen	Stage II Primary breast cancer
Sotiriou et al, Journal of National Cancer Institute, 2006	119 (KJ125 dataset)	45% <50y 55% >50y	Affymetrix	61% < 2cm 39% >2cm	LN negative	No adjuvant systemic therapy	34 Grade 1 46 grade 2 28 Grade 3 17 N/A
Turashvili, BMC Cancer, 2007	10		Affymetrix				3 Grade I 5 Grade II 2 Grade III
Van De Vijver et al, NEJM, 2002	295	< 52	Non-Affymetrix	< 5 cm	151 LN negative 144 Ln positive	Modified radical mastectomy or breast conserving surgery	Stage I or II breast cancer
Wang et al, Lancet, 2005	286	54 (SD=12)	Affymetrix		Lymph node negative	No adjuvant treatment	
Yu et al, Clinical Cancer Reasearch, 2006	96 Only 68 with Notch-1 and survivin data	55 (SD = 10.9)	Affymetrix	37.7mm (SD = 17.9)	37.5% LN negative		2 Unknown Grade 5 Grade I 26 Grade II 63 Grade III
Zhao et al, Molecular Biology of the Cell, 2004	59 35 IDC 17 ILC 3 from each with unknown ER status	Ductal 53 (SD = 15.5) Lobular 63.5 (SD = 14.0)	Non-Affymetrix		Ductal 16 LN positive 16 LN negative 3 LN unknown Lobular 7 LN positive 7 LN negative 4 LN unknown		Ductal 5 Grade I 19 Grade II 11 Grade III Lobular 17 Grade II 1 Grade I

Table 2-1. Published datasets included in meta-analysis.
Sixteen datasets derived from an unbiased search of human breast cancer microarrays on Oncomine were identified that matched study criteria.

Study	N			Survivin			Notch-1			Keratin-5		
	Total	Subset		Mean	SEM	P	Mean	SEM	P	Mean	SEM	P
Chin et al, Cancer Cell, 2006	118	ER-	43	0.7723	0.0815	1.3528e-5	0.7793	0.0548	2.2315e-5	0.9844	0.1436	4.8935e-6
		ER+	75	0.3371	0.0437		0.5027	0.0245		0.1884	0.0678	
Desmedt et al, Clinical Cancer Research, 2007	198	ER-	64	0.7238	0.0478	1.4140e-8	0.3254	0.0389	3.9514e-4	0.6649	0.0996	1.1591e-2
		ER+	134	0.3278	0.0460		0.1658	0.0193		0.3773	0.0507	
Ginestier et al, Clinical Cancer Research, 2006	55	ER-	28	0.7621	0.1251	9.6885e-5	1.4343	0.0504	2.9332e-4	1.2587	0.2116	1.2193e-2
		ER+	27	0.4959	0.0954		1.1536	0.0520		0.5842	0.1492	
Hess et al, Journal of Clinical Oncology, 2006	133	ER-	51	0.0776	0.0451	2.0708e-3	0.2169	0.0454	1.2028e-1	0.5110	0.1706	1.1650e-4
		ER+	82	-0.0956	0.0309		0.1238	0.0384		-0.2216	0.0480	
Ivshina et al, Cancer Research, 2006	249 (Uppsala cohort)	ER-	34	0.4877	0.0847	1.0639e-7	0.3819	0.0589	1.6430e-2	0.7071	0.1683	1.4033e-1
		ER+	211	-0.0950	0.0408		0.2299	0.0137		0.4438	0.0479	
Miller et al, PNAS 2005	251	ER-	34	0.3701	0.0681	2.7800e-8	0.3011	0.0443	2.0727e-2	0.4714	0.1310	2.6379e-1
		ER+	213	-0.1337	0.0382		0.1905	0.0113		0.3165	0.0389	
Minn et al, Nature 2005	82	ER-	42	0.1969	0.0719	1.8086e-4	0.4515	0.0529	7.8967e-5	1.1060	0.1311	2.3908e-6
		ER+	57	-0.1821	0.0652		0.2036	0.0247		0.2624	0.1028	
Richardson et al, Cancer Cell, 2006	39	ER-	24	-0.1141	0.1363	6.3739e-3	1.1360	0.0621	1.2164e-1	1.1878	0.1436	4.6701e-3
		ER+	15	-0.6187	0.1089		1.0139	0.0457		0.6193	0.1225	
Saal et al, PNAS, 2007	105	ER-	60	-1.3798	0.1075	5.1426e-6	0.2043	0.0756	3.0330e-3			
		ER+	45	-2.1290	0.1121		-0.1145	0.0728				
Sotiriou et al, Journal of National Cancer Institute, 2006	119	ER-	34	0.4834	0.0320	2.5269e-3	0.2702	0.0565	1.3231e-4	0.9849	0.1107	9.3963e-3
		ER+	85	0.3691	0.0166		0.0181	0.0196		0.6560	0.0499	
Turashvili, BMC Cancer, 2007	10	ER-	3	0.1268	0.1688	2.533e-1	1.1991	0.1243	3.082e-1	0.6117	0.3735	6.7500e-1
		ER+	7	-0.2686	0.2733		1.0239	0.0709		0.3655	0.4358	
Van De Vijver et al, NEJM, 2002	295	ER-	69	0.3719	0.2218	1.8468e-12	0.6277	0.1148	1.418e-10	-0.7917	0.4073	1.7481e-5
		ER+	226	-1.7301	0.1580		-0.2900	0.0590		-2.8083	0.1822	
Wang et al, Lancet, 2005	286	ER-	77	0.0902	0.0459	2.2836e-8	0.4139	0.0282	2.2548e-11	0.8906	0.0819	3.2417e-7
		ER+	209	-0.2387	0.0318		0.1784	0.0151		0.3844	0.0459	
Yu, Clinical Cancer Research, 2006	68 (19)	ER-	26 (15)	-0.2546	0.0874	1.6884e-1	-0.2712	0.1090	1.9457e-4	1.8394 *	0.2312 *	2.2800e-2 *
		ER+	42 (4)	-0.4225	0.0831		0.7625	0.0431		0.9196 *	0.2431 *	
Zhao (Lobular), Molecular Biology of the Cell, 2004	16	ER-	4	-2.4418	0.2721	6.8507e-1	0.0416	0.4079	7.0107e-1			
		ER+	12	-2.3108	0.1269		-0.1367	0.1083				
Zhao (Ductal), Molecular Biology of the Cell, 2004	34	ER-	11	-1.4613	0.2938	1.4403e-1	0.3298	0.3722	1.7692e-1			
		ER+	23	-2.0013	0.1981		-0.2272	0.1104				

Table 2-2. Descriptive statistics of studies in meta-analysis.
Mean, SEM, and p-value between ER-negative and ER-positive breast cancers are shown for Notch-1, survivin, and keratin-5.

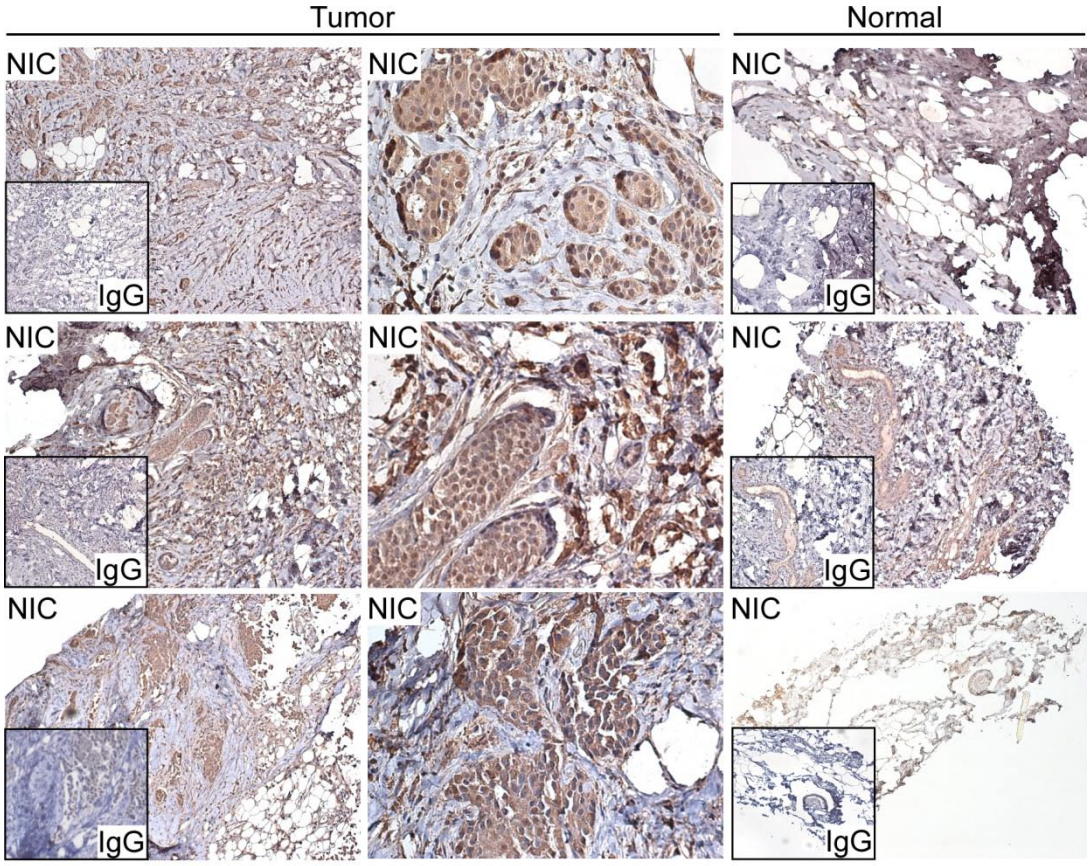


Figure 2-1. Differential expression of activated Notch-1 in breast cancer. Representative breast cancer cases of invasive ductal cell carcinoma (*top panel*), infiltrating lobular cell carcinoma (*middle panel*), and invasive papillary carcinoma (*bottom panel*), or normal breast (normal) were analyzed by immunohistochemistry. *Left panels*, 10x; *middle panels*, 40x, *right panels*, 10x.

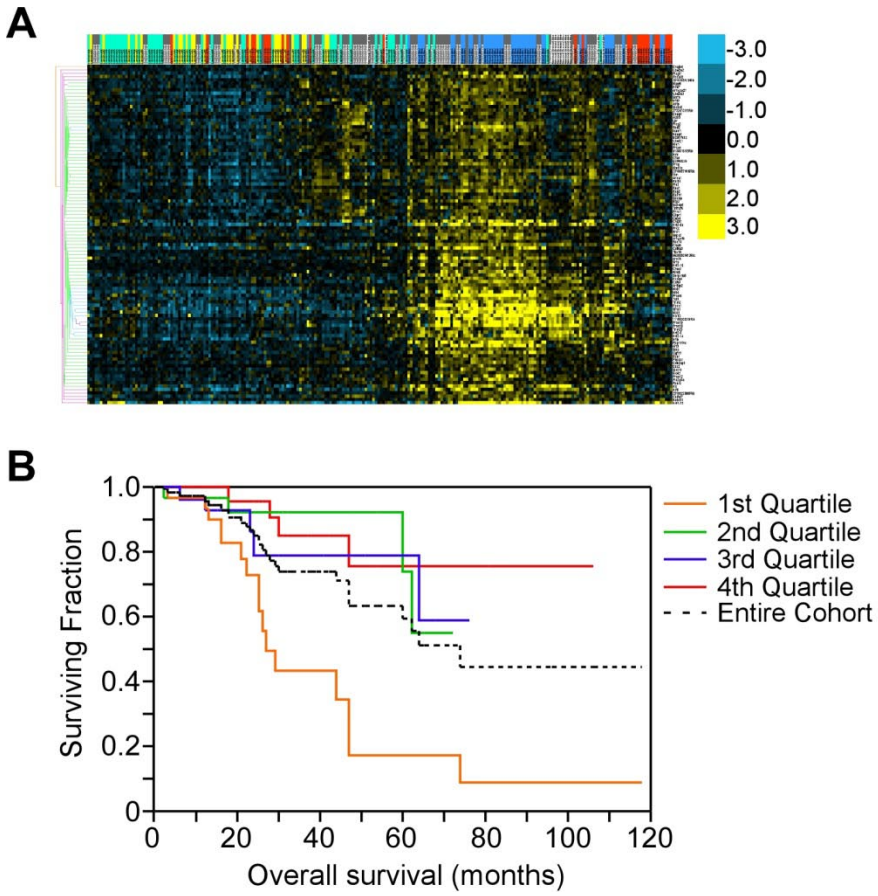


Figure 2-2. Notch-1 segregates with basal breast cancer.
A. Heat map of 232 cases of breast cancer. Color code: Luminal A, green; Luminal B, yellow; Normal Breast-like, white; HER-2+/ER-, red; Basal, blue; Unclassified, gray. B. Kaplan-Meier curves.

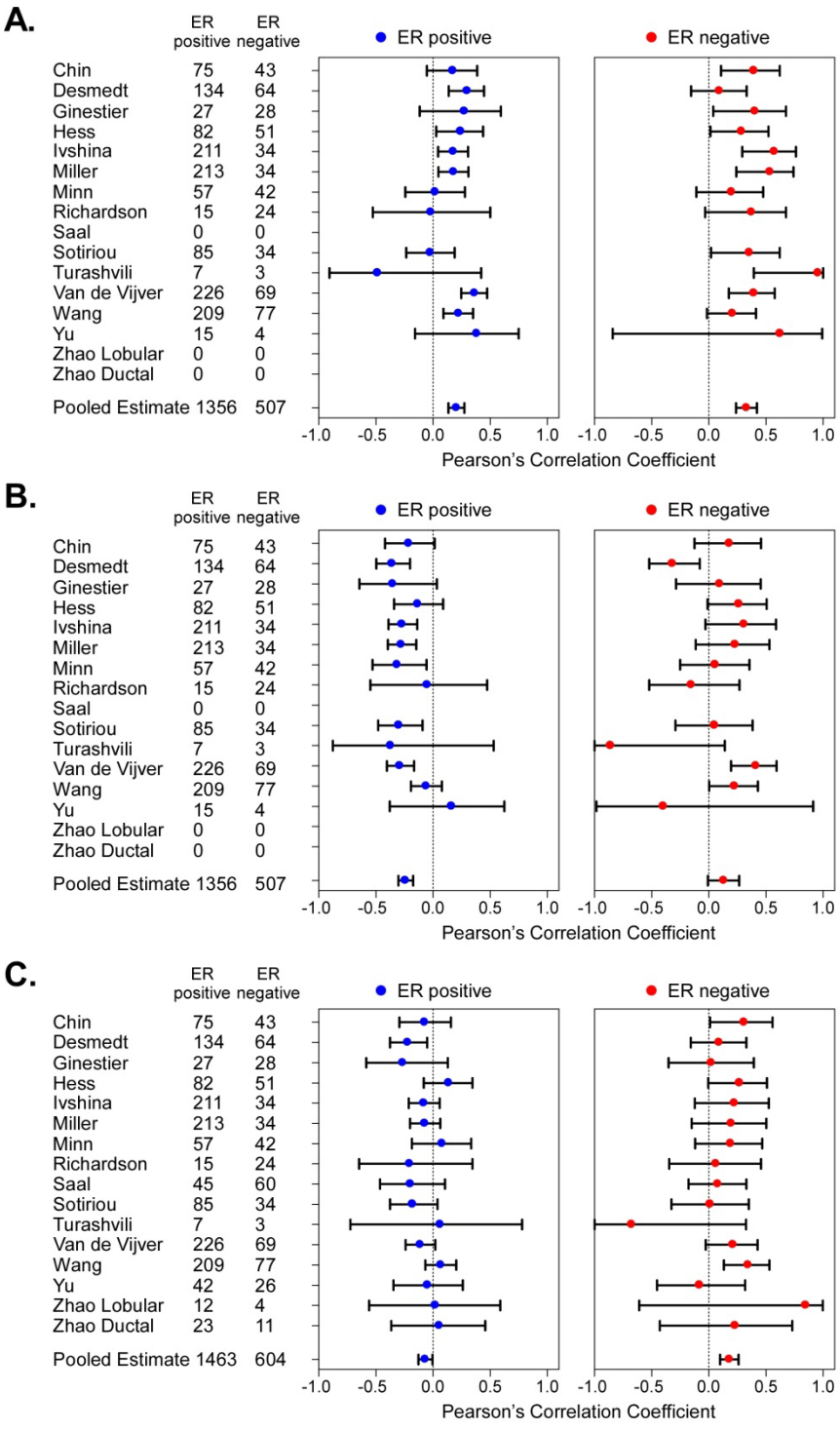


Figure 2-3. Co-segregation of Notch-1, survivin, and keratin-5 in breast cancer. Pearson's correlation coefficient and 95% confidence interval were calculated. A. *Notch-1*/*keratin-5*. B. *Survivin*/*keratin-5*. C. *Notch-1*/*survivin*.

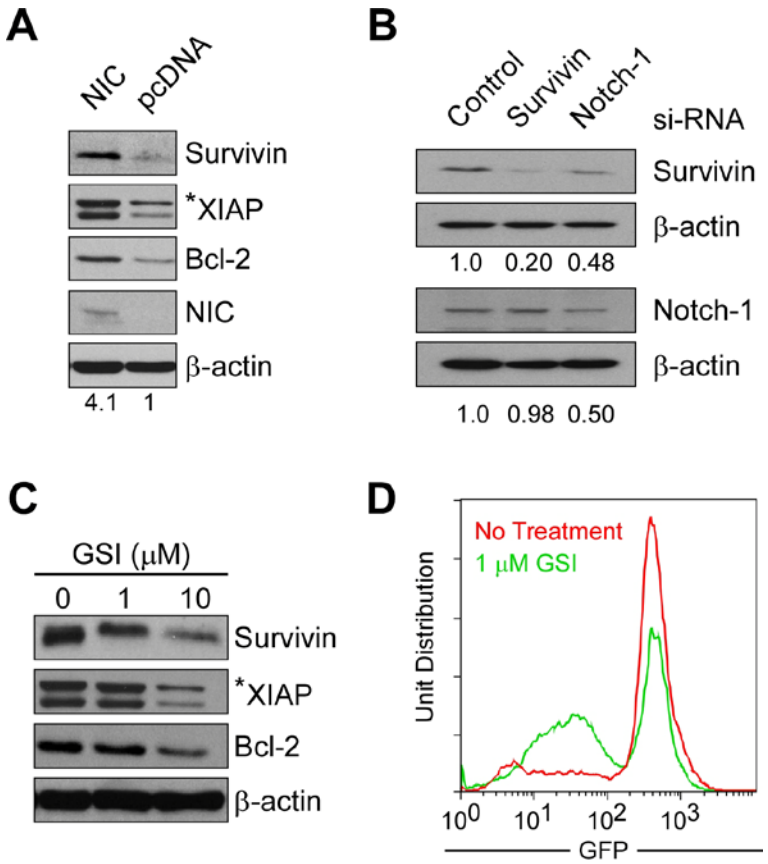
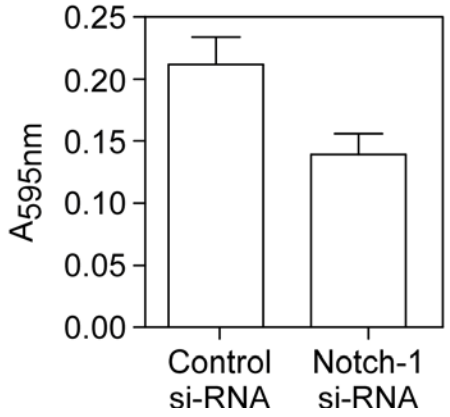


Figure 2-4. Notch-1 modulation of survivin expression.
A. Plasmid transfection. MDA-MB-231 cells were transfected with NIC or control pcDNA, and analyzed by Western blotting. B. siRNA transfection. MDA-MB-231 cells were transfected with control, survivin, or Notch-1 siRNA and analyzed by Western blotting. For panels A and B, numbers correspond to normalized densitometric quantification of survivin or Notch-1 protein bands. C. GSI-mediated Notch inhibition. MDA-MB-231 cells were treated with GSI for 24 h, and analyzed by Western blotting. D. GSI inhibits *survivin* promoter activity. MDA-MB-231 stably transfected with ms-830-GFP were treated with 1 μM GSI for 24 h, and analyzed for GFP expression by flow cytometry.



Supplemental Figure 2-5. Knockdown of Notch-1.
Metabolic MTT Assay of Notch-1 knockdown. Mean±SEM, n=4, p=0.04

Chapter 3. Molecular dependence of estrogen receptor negative breast cancer on a Notch-survivin signaling axis

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This chapter represents collaboration amongst the listed authors. The text is a version of the manuscript accepted by Cancer Research on May 20, 2008 (Lee, Raskett et al. 2008). I participated in all aspects of the project development. Christopher Raskett provided support in the in vivo murine experiments. Igor Prudovsky provided the initial Notch reagents. Dario Altieri helped conceive and design the study. I wrote the first drafts of the introduction, results, and material and methods. Dario Altieri revised the text while I provided feedback.

Abstract

Despite progress in the management of breast cancer, the molecular underpinnings of clinically aggressive subtypes of the disease are not well understood. Here, we show that activation of Notch developmental signaling in estrogen receptor (ER)-negative breast cancer cells results in direct transcriptional upregulation of the apoptosis inhibitor and cell cycle regulator, survivin. This response is associated with increased expression of survivin at mitosis, enhanced cell proliferation, and heightened viability at cell division. Conversely, targeting Notch signaling with a peptidyl γ-secretase inhibitor suppressed survivin levels, induced apoptosis, abolished colony formation in soft agar, and inhibited localized and metastatic tumor growth in mice, without organ or systemic toxicity. In contrast, ER+ breast cancer cells, or various

normal cell types, were insensitive to Notch stimulation. Therefore, ER- breast cancer cells become dependent on Notch-survivin signaling for their maintenance, in vivo. Therapeutic targeting of this pathway may be explored for individualized treatment of clinically aggressive, ER- breast cancer patients.

Introduction

Despite the success of "targeted" agents (Hudis 2007), including estrogen ablation (Jordan 2007), breast cancer remains a potentially deadly disease, marked by extraordinary molecular and clinical heterogeneity (Perou, Sorlie et al. 2000). Gene expression signatures have been useful to catalog the different molecular subtypes of breast cancer, and, in some cases, this information carries important prognostic (van 't Veer, Dai et al. 2002; Liu, Wang et al. 2007), and predictive (Paik, Shak et al. 2004) value to identify patients at risk of recurrent disease (Sotiriou and Piccart 2007). However, the molecular pathways underlying clinically aggressive variants of breast cancer, typically estrogen receptor (ER)-negative disease, have not been identified (Nahta, Yu et al. 2006), and "individualized" therapy for these patients based on molecular disease profile is not presently available.

Developmental signaling pathways, such as Notch, are critical for tissue specification and organ morphogenesis (Clevers 2006), and are frequently deregulated in cancer (Taipale and Beachy 2001). Although it remains to be seen to what extent these mechanisms are truly disease "drivers", mutations in Notch-1 (Weng, Ferrando et al. 2004) cause oncogene expression in a subset of T-cell acute lymphoblastic leukemia (Sharma, Calvo et al. 2006; Weng, Millholland et al. 2006), and deregulated Notch

activity may influence cellular transformation (Stylianou, Clarke et al. 2006), cell cycle dynamics (Ronchini and Capobianco 2001), progenitor/stem cell maintenance (Farnie, Clarke et al. 2007), and disease outcome (Reedijk, Odorcic et al. 2005) in breast cancer. Notch signaling initiates with the binding of extracellular ligands, Jagged-1, -2, Delta-like-1, -3, or -4, to Notch receptors at the cell surface. In turns, this results in proteolytic cleavage of Notch intracellular domain (NIC) by the enzyme γ-secretase, nuclear import of NIC, and recruitment of transcriptional modulators, including RBP-Jκ, to drive de novo expression of target genes (Bray 2006). Although it is clear that Notch and other developmental pathways control multiple downstream networks of cell proliferation, cell survival, and progenitor/stem cell maintenance (Clevers 2006), only a handful of target genes capable of orchestrating such pleiotropic responses has been identified, and their contribution to the cancer phenotype has remained largely elusive.

As a unique member of the Inhibitor of Apoptosis (IAP) gene family (Eckelman, Salvesen et al. 2006), *survivin* has emerged as a pivotal cancer gene with multiple roles in the regulation of mitosis, suppression of cell death, and enhanced adaptation to cellular stress (Altieri 2003). The sharp differential expression of survivin in cancer, as opposed to normal tissues, is largely controlled at the level of transcription, and several oncogenic pathways, including developmental signaling by Wnt/β-catenin (Kim, Plescia et al. 2003), have been shown to promote *survivin* gene expression. There is also evidence that *survivin* may be a critical gene in breast cancer, linked to aggressive disease (Paik, Shak et al. 2004), resistance to apoptosis (Gritsko, Williams et al. 2006), and modulation of ErbB2 signaling (Xia, Bisi et al. 2006).

In this study, we mapped novel molecular circuitries of breast cancer pathogenesis. We found that *survivin* is a novel target of Notch signaling, and this occurs preferentially in ER- breast cancer cells. Therapeutic targeting of a Notch-survivin axis produces strong anticancer activity, and is well tolerated in vivo, opening new opportunities for "individualized" therapy in clinically aggressive breast cancer subtypes.

Results

Notch-1 induction of survivin in estrogen receptor negative breast cancers

Preliminary meta-analysis of published microarray data (Sorlie, Perou et al. 2001; van de Vijver, He et al. 2002; Zhao, Langerod et al. 2004; Miller, Smeds et al. 2005; Minn, Gupta et al. 2005; Wang, Klijn et al. 2005; Chin, DeVries et al. 2006; Ginestier, Cervera et al. 2006; Hess, Anderson et al. 2006; Ivshina, George et al. 2006; Richardson, Wang et al. 2006; Sotiriou, Wirapati et al. 2006; Yu, Ganesan et al. 2006; Desmedt, Piette et al. 2007; Saal, Johansson et al. 2007; Turashvili, Bouchal et al. 2007) revealed that *Notch-1* and *survivin* co-segregated with ER- breast cancer cases, and correlated with abbreviated overall survival (unpublished observations). Consistent with this, transduction of ER- breast adenocarcinoma MDA-MB-231, HBL100, and Sum149 cells (Neve, Chin et al. 2006) with pAd-NIC, the active intracellular domain of Notch, resulted in a 1.5-2-fold increased survivin mRNA expression, as compared with pAd-GFP-treated cultures (Figure 3-1A). In contrast, Notch stimulation of ER+ breast cancer cells MCF-7 and T47D, or normal HMECs, did not modulate survivin mRNA levels (Figure 3-1A). In time-course experiments, pAd-NIC increased expression of survivin in ER- MDA-MB-231 cells, but not ER+ MCF-7 cells, by Western blotting (Figure 3-1B). Semi-

quantitative Western blotting verifies the selective increase of survivin after Notch activation in MDA-MB-231 cells compared to MCF-7 cells (Supplemental Figure 3-8). Conversely, Bcl-2 was comparably upregulated in both cell types (Wang, Zhang et al. 2006), whereas another survival factor, XIAP, was not significantly affected (Figure 3-1B). Even a prolonged, 72 h, stimulation of normal primary human mammary epithelial cells with pAd-NIC did not modulate survivin or XIAP expression (Figure 3-1C). Knockdown of Notch-1 by ~50% in MDA-MB-231 breast cancer cells corresponded with ~50% decrease in survivin protein levels (Figure 2-4D) and a 34% reduction in cellular viability (Supplemental Figure 2-5).

Notch-1 regulation of survivin gene expression

To determine whether Notch directly stimulated *survivin* gene expression, we next used an 830 bp fragment of the *survivin* promoter fused to Green Fluorescent Protein (GFP), i.e. ms-830-GFP (Xia and Altieri 2006), containing two putative RBP-Jκ sites (Bray 2006) at -543 and -355 (Figure 3-2A). Co-transfection of Notch-1 or Jagged-1 together with wild type ms-830-GFP resulted in strong expression of GFP, by Western blotting (Figure 3-2B), and quantitative fluorescence microscopy (Figure 3-2C, *upper panel*), as compared with pcDNA3-expressing cells (Figure 3-2C, *upper panel*). Mutation of the proximal RBP-Jκ site at -355 in ms-830-GFP (Figure 3-2A) abolished Notch-1 induction of GFP to background levels of control cultures (Figure 3-2C, *lower panel*). By EMSA, a radiolabeled probe of the -355 RBP-Jκ site formed DNA-protein complexes in MDA-MB-231 nuclear extracts (Figure 3-2D). This interaction was competed out by equimolar concentrations of unlabeled probe, or a canonical Hes RBP-

Jκ sequence, but not by an unrelated competitor (Figure 3-2D). Similarly, a radiolabeled Hes RBP-Jκ probe bound to MDA-MB-231 nuclear extracts, in a reaction also competed out by molar excess of unlabeled probe (Figure 3-2D). In contrast, a probe of the -543 survivin RBP-Jκ site did not form DNA-protein complexes in MDA-MB-231 extracts (data not shown), and was not further investigated. Consistent with these data, ChIP experiments using MDA-MB-231 cells transduced with pAd-NIC revealed that RNA polymerase II and RBP-Jκ were physically associated with the human survivin promoter in vivo at the putative RBP-Jκ binding site at -305 (Figure 3-3B).

Notch-1 regulation of cell proliferation

To determine the kinetics of Notch-1 induction of survivin, we next studied cell cycle transitions in synchronized MDA-MB-231 cells after transduction with pAd-NIC. In synchronized cultures expressing pAd-GFP, endogenous survivin was expressed in a cell cycle-dependent manner, peaking at mitosis, 8- to 12-h after thymidine release (Figure 3-4A), consistent with previous observations (O'Connor, Wall et al. 2002). In these cells, Notch-1 increased survivin with the same kinetics, in a reaction that was maximal at mitosis (Figure 3-4A). Quantification of cell cycle phases revealed that GFP-expressing cells approached S-phase 4 h after thymidine release, entered mitosis after 8 h, and completed cell division between 10 and 12 h, with re-accumulation in the next G1 phase by 14 h (Figure 3-4B). In contrast, Notch activation resulted in accelerated mitotic transitions, with 30% of NIC-expressing cells exiting mitosis 10 h after release, as compared with 17% of GFP-transduced cells, and 64% of Notch-1-stimulated cells re-entering G1 after 14 h, *versus* 40% of control cultures (Figure 3-4B). Accelerated mitotic

progression in Notch-stimulated MDA-MB-231 cells was associated with increased cell proliferation over a 6-d interval, as compared with pAd-GFP-expressing cultures (Figure 3-4C). In contrast, pAd-NIC or pAd-GFP did not induce changes in cell proliferation in ER+ MCF-7 cultures (Figure 3-4C).

We next asked whether Notch-1 induction of survivin was important for cell viability during this proliferative response. For these experiments, we transduced MDA-MB-231 cells with a dominant negative survivin mutant (pAd-T34A), which interferes with the mitotic function of survivin (O'Connor, Wall et al. 2002). Expression of pAd-T34A had no effect on synchronized, interphase cultures, but caused acute loss of cell viability as cells progressed through mitosis (Figure 3-4D), in agreement with previous observations (O'Connor, Wall et al. 2002). Notch stimulation reversed pAd-T34A-induced cell death, and preserved cell viability at mitosis (Figure 3-4D). In control experiments, pAd-NIC had no effect on MDA-MB-231 cell viability (Figure 3-4D). The higher number of cells with >4N DNA content after pAd-NIC transduction may reflect mitotic slippage and accumulation of chromosomal abnormalities due to accelerated mitotic transition.

Effect of Notch targeting on breast cancer viability

To test the impact of Notch signaling in breast cancer, we used a GSI z-Leu-Leu-Nle-CHO, which inhibits receptor cleavage and NIC generation (Bray 2006). GSI treatment reduced endogenous survivin levels in MDA-MB-231 cells, and this was associated with parallel suppression of Bcl-2, and XIAP (Figure 3-5A). In contrast, GSI minimally affected the expression of these anti-apoptotic molecules in MCF-7 cells

(Figure 3-5A). In addition, GSI had no effect on survivin levels in WS-1 fibroblasts or INT colonic epithelial cells, whereas XIAP was modestly reduced (Liu, Hsiao et al. 2007), and Bcl-2 was undetectable in these cells (Figure 3-5A). Treatment of MDA-MB-231 cells with a second, structurally independent GSI molecule, z-Ile-Leu-CHO, reduced Notch-1 expression, and attenuated survivin levels in MDA-MB-231 cells (Supplemental Figure 3-9).

Transduction of MDA-MB-231 cells with pAd control or pAd-NIC did not significantly affect cell viability, as determined by DEVDase, i.e. caspase, activity and multiparametric flow cytometry (Figure 3-5B, *top panels*). GSI treatment of these cells resulted in a 2-fold increase in the fraction of apoptotic cells, in a reaction nearly completely reversed by pAd-NIC (Figure 3-5B, *top panels*). In contrast, GSI did not significantly induce caspase activity in MCF-7 cultures, and transduction of these cells with pAd-NIC or pAd-Control had no further effect on cell viability (Figure 3-5B, *lower panels*). Consistent with these data, GSI induced concentration-dependent killing of multiple ER- breast cancer cell types, including MDA-MB-231, HBL100, Sum149 (Figure 3-5C, *left panel*) with IC_{50} <3.5 μM. Comparatively, ER+ cell types, MCF-7 and T47D, were less sensitive to GSI-induced killing, with IC_{50} concentrations of >10 μM and 5.907 μM, respectively (Figure 3-5C, *middle panel*). In addition, comparable concentrations of GSI did not affect the viability of three primary fibroblast cell lines, (WS-1, HFF, and HGF), normal INT epithelial cells, or primary human mammary epithelial cells (Figure 3-5C, *right panel*).

To further investigate the anti-tumor effects of GSI, we next tested the effect of Notch inhibition on a panel of tumor cell lines. Treatment of prostate adenocarcinoma

PC3, lung adenocarcinoma H1975, or squamous cell carcinoma A431 cells with GSI resulted in concentration-dependent cell death, quantitatively similar to that observed with MDA-MB-231 cells (Supplemental Figure 3-10). Conversely, prostate adenocarcinoma DU145, lung adenocarcinoma H460, colorectal adenocarcinoma HCT116, or glioblastoma U87MG cells were largely resistant to GSI-induced cell death (Supplemental Figure 3-10).

GSI induction of apoptosis in breast cancer cell lines

MDA-MB-231 cells exposed to GSI exhibited morphologic hallmarks of apoptosis, including chromatin condensation and fragmentation (Figure 3-6A, *left panel*), and concentration-dependent release of cytochrome c in the cytosol (Figure 3-6A, *right panel*). GSI-induced cell death under these conditions was fully reversed by a broad spectrum caspase inhibitor, zVAD, comparable to its inhibition of STS-induced apoptosis (Figure 3-6B). In contrast, GSI did not affect cell viability or cytochrome c release in MCF-7 cultures (Figure 3-6A). To test whether loss of survivin after Notch inhibition contributed to apoptosis, we transduced MDA-MB-231 cells with pAd-GFP or pAd-Survivin, and quantified GSI-induced cell death. In GFP-expressing cells, GSI induced a sustained G2/M arrest, and apoptosis (Figure 3-6C). Expression of survivin in these cells did not significantly affect the mitotic arrest imposed by Notch inhibition, but completely reversed GSI-induced cell death, whereas pAd-GFP was ineffective (Figure 3-6C).

Targeting Notch signaling for breast cancer therapy

GSI treatment of MDA-MB-231 cells abolished colony formation in soft agar (Figure 3-6D, *left panel*), whereas comparable concentrations of GSI were 3 orders of magnitude less effective in MCF-7 cells (Figure 3-6D, *right panel*). Survivin can rescue ablation of colony formation by low doses of GSI (<0.5 μM) (Supplemental Figure 3-11) but not at doses of GSI > 1 μM (data not shown). Next, we grew MDA-MB-231 cells as superficial tumors in immunocompromised mice, and treated the animals with vehicle or systemic GSI (3 mg/kg/daily i.p.). In control animals, MDA-MB-231 tumors grew at a steady exponential rate over a two week time interval (Figure 3-7A). In contrast, GSI treatment significantly inhibited tumor growth over a comparable time interval (Figure 3-7A). In addition, lungs from mice treated with vehicle exhibited a high density of epithelial micrometastases (Fraker, Halter et al. 1984), by hematoxylin-eosin staining of serial lung sections (Figure 3-7B, *left panel*). In contrast, GSI treatment inhibited formation of lung metastases, in vivo (Figure 3-7B, *right panel*).

Safety of Notch targeting for cancer therapy

Mice in the GSI group did not exhibit signs of systemic toxicity, and had no significant weight loss, as compared with vehicle-treated animals (Supplemental Figure 3-12). Histologic examination of lung, colon, pancreas, spleen, kidney, and liver was largely indistinguishable in control or GSI-treated mice (Figure 3-7C). Specifically, lung sections from GSI or control group showed a conserved architecture with a normal alveolar septum, absence of lymphatic dilatation, or interstitial inflammation (Figure 3-7C). With respect to the gastrointestinal tract, GSI treatment did not cause goblet cell

hyperplasia of colonic epithelium, and resulted in only mild fasciitis of pancreatic tissue, as compared with vehicle-treated control (Figure 3-7C). The liver architecture of hepatic nodules was fully preserved in GSI-treated animals, without steatosis or inflammatory infiltration, and extramedullary hematopoiesis was comparably observed in control- or GSI-treated animals (Figure 3-7C).

Discussion

In this study, we have shown that Notch signaling increases cell proliferation and promotes resistance to apoptosis preferentially in ER- breast cancer (Yehiely, Moyano et al. 2006), a disease subtype often marked by aggressive clinical behavior (Sorlie, Perou et al. 2001). A critical effector of this pathway was identified as survivin, a pleiotropic regulator of cell proliferation and inhibitor of apoptosis over-expressed in many cancers (Altieri 2003). Conversely, inhibition of Notch signaling with a peptidyl GSI downregulated survivin, inhibited cell proliferation, increased apoptosis, and suppressed the growth of localized and metastatic ER- breast cancer in mice, without overt toxicity.

Recently, there has been considerable interest in elucidating how developmental signaling pathways, including Notch (Bray 2006) may aberrantly contribute to tumorigenesis. Mounting evidence suggests that Notch deregulation may engender critical tumor hallmarks, including oncogene expression (Sharma, Calvo et al. 2006; Weng, Millholland et al. 2006), angiogenesis, (Keith and Simon 2007), stem cell maintenance (van Es, van Gijn et al. 2005), deregulated cell cycle progression (Ronchini and Capobianco 2001), and anti-apoptotic mechanisms (Beverly, Felsher et al. 2005; Liu, Hsiao et al. 2007). In this context, *survivin* fits well the pleiotropic requirements of a

Notch effector gene for its essential roles in mitosis (Lens, Vader et al. 2006), preservation of stem/progenitor cell homeostasis (Leung, Xu et al. 2007), inhibition of apoptosis (Dohi, Xia et al. 2007), and regulation of angiogenesis (Singh, Dhanalakshmi et al. 2005). Mechanistically, Notch stimulation resulted in direct activation of *survivin* gene transcription through at least one RPB-Jκ site (Bray 2006) in the *survivin* promoter. This is reminiscent of how another developmental signaling program, i.e. Wnt/β-catenin, directly induces *survivin* gene transcription in cancer (Kim, Plescia et al. 2003). Consistent with the known tissue specificity of Notch signaling that has been associated with malignant transformation (Weng, Ferrando et al. 2004), or tumor suppression (Nicolas, Wolfer et al. 2003) depending on the tissue, a Notch-survivin axis was preferentially operative in ER- *versus* ER+ breast cancer cells, and not at all detected in various normal cell types.

Three potential mechanisms may be envisioned for a role of survivin as a Notch target in clinically aggressive breast cancer (Sorlie, Perou et al. 2001). First, Notch-induced heightened survivin levels at mitosis may deregulate multiple mitotic checkpoints (Lens, Vader et al. 2006), and ultimately contribute to genetic instability and aneuploidy, in vivo. Second, this pathway may directly promote drug and radiation resistance. Higher survivin levels have been consistently linked to inhibition of apoptosis induced by DNA damaging agents (Ghosh, Dohi et al. 2006), as well taxanes (O'Connor, Wall et al. 2002), two mainstay therapeutic regimens in breast cancer. Third, survivin may operate as a Notch-regulated cytoprotective and/or mitotic factor to promote long-term persistence of breast cancer "stem cells" (Liu, Dontu et al. 2005; Buono, Robinson et al. 2006), potentially contributing to ductal carcinoma in situ (Farnie, Clarke et al.

2007), an idea consistent with the presence of survivin in “stemness” gene signatures (Taubert, Wurl et al. 2007), and its role in hematopoietic stem cell viability (Leung, Xu et al. 2007).

Although the introduction of “targeted” therapies (Hudis 2007), including anti-hormonal strategies (Jordan 2007), has significantly improved the survival of breast cancer patients, the prognosis of metastatic disease remains grim. This often involves ER- subsets of the disease, for instance triple-negative (ER, PR and HER-2), basal-like breast cancer, which is characterized by high recurrence rates (Sotiriou and Piccart 2007). Here, the increased sensitivity of ER- *versus* ER+ breast cancer cells to therapeutic Notch inhibition, in vitro and in preclinical models, suggests that these cells may become dependent, or “addicted” (Weinstein and Joe 2006) to Notch signaling. Although the importance of “oncogene addiction” in long-term maintenance of the neoplastic phenotype, in vivo, is debated, the notable clinical responses observed in subsets of cancer patients after targeting of specific cellular pathways support their pivotal role(s) as disease “drivers” (Sharma, Bell et al. 2007). A similar paradigm has been proposed for Notch (Roy, Pear et al. 2007), and GSIs have been pursued as therapy for potential “Notch-addicted” tumors, especially T-cell leukemia (Shih Ie and Wang 2007). Despite its rationale, this approach has not been without concerns. GSIs are not entirely specific for Notch, as they also affect other transmembrane proteins, including E-cadherin, EGFR and CD44 (Fortini 2002), and global inhibition of Notch receptors has been associated with serious toxicity, especially aberrant differentiation of intestinal epithelium, and T-cell development, in vivo (Wong, Manfra et al. 2004). The data presented here suggest a more encouraging scenario, as a prototype peptidyl GSI was safely administered

systemically to mice with effective anticancer activity, with negligible systemic or organ toxicity. Although this bodes well for potential further (pre)clinical development of similar compounds, it remains to be seen whether long-term GSI treatment will eventually result in pharmacologic resistance, as observed in leukemia (Weng, Ferrando et al. 2004).

In summary, we have uncovered a novel Notch-survivin signaling axis, preferentially exploited in ER- breast cancer cells. Although this pathway may contribute to worse clinical outcome, a potential "addiction" of ER- breast cancer cells to a Notch-survivin axis may open new prospects for individualized therapy of these recurrence-prone patients.

Material and Methods

Cell lines and viral transductions

Breast adenocarcinoma cell lines MCF-7, MDA-MB-231, and cervical carcinoma HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in culture as recommended by the supplier. Breast adenocarcinoma cell lines T47D, Sum149, and HBL100 were generously provided by Dr. Arthur M. Mercurio (University of Massachusetts Medical School). Primary human mammary epithelial cells (HMEC) were obtained from Lonza, Allendale, NJ. Primary human foreskin fibroblasts (HFF), human gingival fibroblasts (HGF), human epithelial fibroblasts (WS-1), and human intestinal epithelial cells (INT) (all from ATCC) were cultured per manufacturer's instructions, and used at early passages before the onset of senescence. A NIC construct has been characterized previously (Small, Kovalenko et al.

2001). For adenoviral transduction, various cell types ($1x10^5$) were incubated with replication-defective adenovirus pAd-NIC, pAd-GFP, or pAd-Control at multiplicity of infection of 50 for 4-8 h at 37°C, harvested at increasing time intervals, and processed for individual experiments.

Semi-quantitative RT-PCR

Total RNA was harvested using the RNeasy Kit (Qiagen, Valencia, CA). One μg of total RNA was reversed transcribed in the presence of SuperScript II polymerase plus random primer (Invitrogen, Carlsbad, CA). Survivin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified (28 cycles), and separated on 1.5% agarose gels. Band intensity normalized to GAPDH expression was quantified using Labworks 4.6 (UVP BioImaging Systems, Upland, CA).

Western blotting and antibodies

Cellular extracts were prepared in a lysis buffer containing 20 mM Tris pH 7.5, 0.5% DOC, 1% Triton, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, plus protease inhibitors (Roche Applied Science, Indianapolis, IN). Lysates were separated by SDS gel electrophoresis, and transferred to Immobilon membranes (Millipore, Billerica, MA). The following antibodies were used: survivin (1:1000) from NOVUS Biologicals, Littleton, CO), XIAP (1:500), GFP (1:500), cytochrome c (1:1000), and Cox-4 (1:5000) from BD Biosciences (San Jose, CA), Bcl-2 (1:500) from Santa Cruz Biotechnology (Santa Cruz, CA), V5 epitope for detection of NIC or Jagged-1 (1:5000)

from Invitrogen, NIC (1:1000) from Rockland (Gilbertsville, PA), and β-actin (1:5000) from Sigma (St. Louis, MO).

Analysis of survivin gene expression

A cDNA construct comprising the first 830 bp of the mouse *survivin* promoter upstream of the translational initiation codon fused to GFP (ms-830-GFP) was characterized previously (Xia and Altieri 2006). HeLa cells were co-transfected with ms-830-GFP plus Notch-1 or Jagged-1 cDNA by Lipofectamine. After 24 h, cells were analyzed for GFP expression by Western blotting, or fluorescence microscopy with image acquisition on an Olympus IX71 microscope outfitted with an Olympus Regina camera (Center Valley, PA). A putative RBP-Jκ binding site at position -355 in ms-830-GFP was mutated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with primer: 5'-GAACCTGCAGAGCACA**TGttA**CTTGCAGCGGACATGC-3'. The mutant construct (mutant ms-830-GFP) was confirmed by DNA sequencing, transfected in HeLa cells, and analyzed for GFP expression after Notch stimulation. For electrophoretic mobility shift assay (EMSA), MDA-MB-231 nuclear extracts were prepared using the CelLytic NuCLEAR extraction kit (Sigma) according to the manufacturer's instructions. cDNA sequences corresponding to the -355 region of ms-830-GFP (5'-GGAAGAACCTGCAG-AGCACA**TGGGA**CTTGCAGCGGACATGCT-3'), a random *survivin* promoter region (5'-TGCAACGCCAACCTGGGCTGTGTTCGGGGCATGCCCAGCCTG-3'), or an RBP-Jκ binding region in the human *Hes* promoter (5'-GTTACTG**TGGGA**AAGAAAG-TC-3') were synthesized. Probes (3.5 pmol) were made double-stranded by annealing at

equimolar concentrations, and 5' end-labeled with 1 μl of [γ^{32}P]ATP (3000 Ci/mmol) and 10 U of T4 polynucleotide kinase (NEB, Ipswich, MA) for 10 min at 37°C. MDA-MB-231 nuclear extracts were incubated with the reaction mixture (10 μl) containing radiolabeled DNA probes, 2 μg of poly(dI-dC) (Sigma), 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, 50 mM NaCl, 4% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 50 μg BSA, and various unlabeled competing oligonucleotides. After 30 min incubation at 22°C, samples were separated by electrophoresis on 4% non-denaturing polyacrylamide gels, and bands were visualized by autoradiography.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the ChIP-IT Express Kit (Active Motif, Carlsbad, CA) per the manufacturer's protocols. MDA-MB-231 cells were infected with pAd-NIC prior to fixation with 1% formaldehyde for 10 min. Cells were washed, lysed, and sonicated to reduce DNA lengths to a range of 300–600 bp. The chromatin/DNA complexes were incubated with antibodies to RNA Pol II (Active Motif), RBP-Jκ (Santa Cruz Biotechnology), or IgG (Active Motif) for 18 h at 4°C. The immune complexes were precipitated, eluted, reverse cross-linked and treated with proteinase K. The resulting DNA samples were amplified with primers to the RNA Polymerase II site in the *GAPDH* promoter (forward, 5' TACTAGCGGTTTTACGGGCG 3'; reverse, 5' TCGAACAGGAGGAGCAGAGAGCGA 3'), the -305 bp putative RBP-Jκ binding site in the human *survivin* promoter (forward, 5' ACCACGCCCAGCTAATTTTTG 3'; reverse, 5' CCTCGACTGCTTTCAAAGAACG 3'), and the RBP-Jκ binding site in the

Hes promoter (forward, 5' CGTGTCTCCTCCTCCCATTG 3'; reverse, 5' CCAGGACC-AAGGAGAGAGGT 3').

Cell cycle analysis

Cells ($1.5x10^5$) were synchronized at the G1/S transition by treatment with 2 mM thymidine for 18 h at 37°C. In some experiments, cells were released after 18 h, infected with pAd-NIC or pAd-GFP for 8 h, and resynchronized with 1 mM thymidine for another 18 h. Aliquots of synchronized cultures were collected at 0, 4, 8, 10, 12, 14 h after the second thymidine release, and analyzed for DNA content by propidium iodide staining and flow cytometry, or alternatively, by Western blotting. For single-color cell cycle analysis, cells were fixed in 70% ethanol for 18 h at -20°C, and analyzed by flow cytometry. Cell cycle populations were gated, and quantified using FlowJo software (TreeStar, Ashland, OR). A pAd-T34A survivin dominant negative mutant was characterized previously (Mesri, Wall et al. 2001). In some experiments, MDA-MB-231 cells were infected with the combinations pAd-NIC/pAd-Control, pAd-T34A/pAd-Control, or pAd-NIC/pAd-T34A, for 24 h, synchronized with 2 mM thymidine for 24 h, and released into S-phase. Cultures were collected after various time intervals, and analyzed for DNA content by propidium iodide staining, or, alternatively, by Western blotting.

Cell death assays

A γ-secretase inhibitor (GSI) z-Leu-Leu-Nle-CHO was purchased from Calbiochem (San Diego, CA), and dissolved in DMSO. In metabolic activity assays,

cells were treated with GSI or DMSO for 24 h, incubated with 50 μg/ml MTT for 2 h, and analyzed at A_{595}. In other experiments, cells treated with GSI or DMSO were spun onto slides, DNA was stained with DAPI, and cells with chromatin condensation/fragmentation were scored by fluorescence microscopy. For experiments of cytochrome c release, GSI-treated cells were harvested, fractionated into cytosolic extracts using the ApoAlert Cell Fractionation Kit (Clontech, Mountain View, CA), and analyzed for time-dependent release of cytochrome c, by Western blotting. Caspase activity was determined using the Capase-3/7 *In Situ* Assay Kit (Millipore) by multiparametric flow cytometry, in the presence or absence of a broad spectrum caspase inhibitor zVAD-fmk (American Peptides, Sunnyvale, CA, 20 μM).

Analysis of tumorigenesis

For soft agar colony formation, $1x10^4$ cells were treated with GSI (0.125–10 μM) for 24 h, suspended in 2 ml of DMEM/0.35% bactoagar, and plated onto 6-well tissue culture plates containing 2 ml DMEM/0.75% bactoagar as a bottom layer. After a 2-week incubation at 37°C, 5% CO_2, colonies were stained with 0.005% crystal violet and counted under high-power field. All experiments involving animals were approved by an Institutional Animal Care and Use Committee. For xenograft tumor studies, $5x10^6$ MDA-MB-231 cells were injected into each flank of 6- to 8-week-old female CB17 SCID/beige mice (Taconic, Hudson, NY). When tumors reached 150-175 mm^3 in volume, mice were randomized (3 animals/group, 6 tumors/group, 2 independent experiments) and treated daily with intraperitoneal (i.p.) injections of 3 mg/kg GSI or vehicle control. For experiments in vivo, z-Leu-Leu-Nle-CHO (American Peptides) was

synthesized to >99% purity, and dissolved in 25% Cremophor/PBS (Sigma). Mice were weighed daily, monitored for signs of pain and distress, and tumor sized measured in three dimensions with a caliper using the equation $LxW^2/2$ (mm^3). All animals were euthanized when tumor volume exceeded 1500 mm^3. At the end of the experiment, liver, spleen, lung, kidney, pancreas, and colon were harvested, fixed in formalin, and embedded in paraffin blocks for histology. For quantification of lung micrometastases, serial lung sections were cut 20 μm apart, stained with hematoxylin-eosin, and analyzed by light microscopy.

Statistical Analysis

Data were analyzed using the unpaired t-test on a GraphPad software program (Prism 4.0). All statistical tests were two sided. A *p* value of 0.05 was considered to be statistically significant.

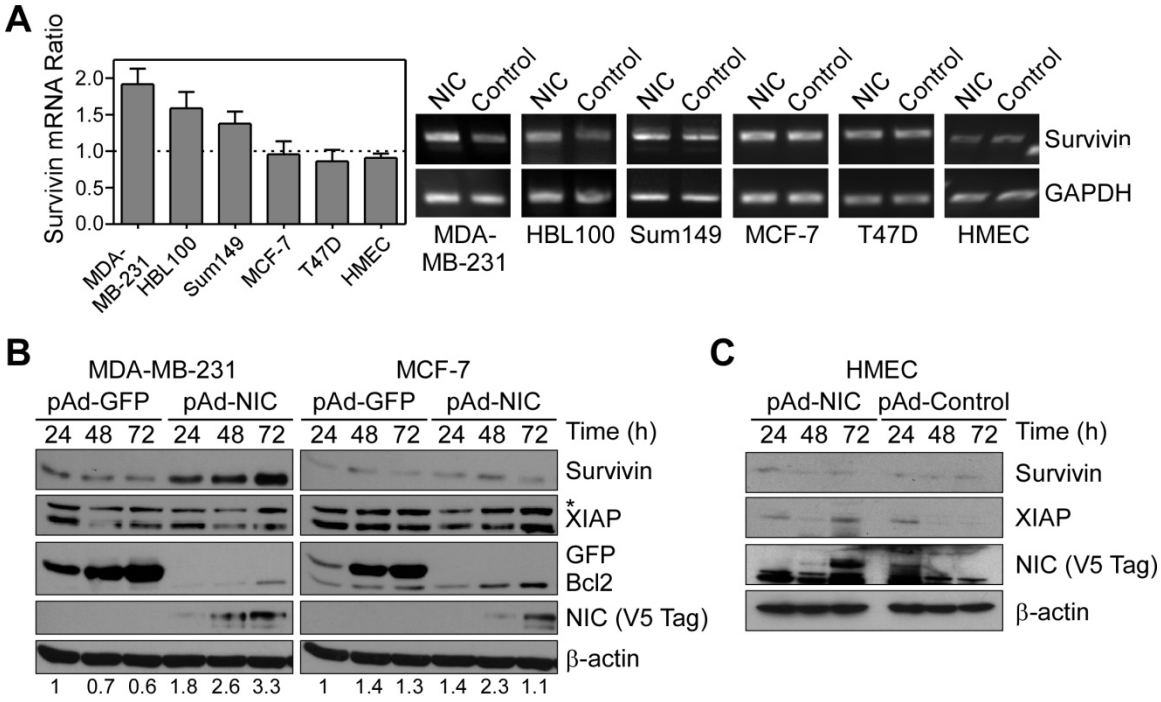


Figure 3-1. Regulation of survivin expression by Notch.
A. Semi-quantitative RT-PCR. A panel of ER- (MDA-MB-231, HBL100, Sum149), ER+ (MCF-7, T47D), or primary human mammary epithelial cells (HMEC) were transduced with pAd-NIC or pAd-Control, and assayed for survivin mRNA levels. B. Western blotting. Transduced MDA-MB-231 or MCF-7 cells were harvested at the indicated time intervals, and analyzed by Western blotting. C. Analysis of normal cells. Transduced primary HMEC were analyzed by Western blotting at the indicated time intervals. *, nonspecific. Numbers at the bottom of each panel correspond to normalized densitometric quantification of survivin protein bands.

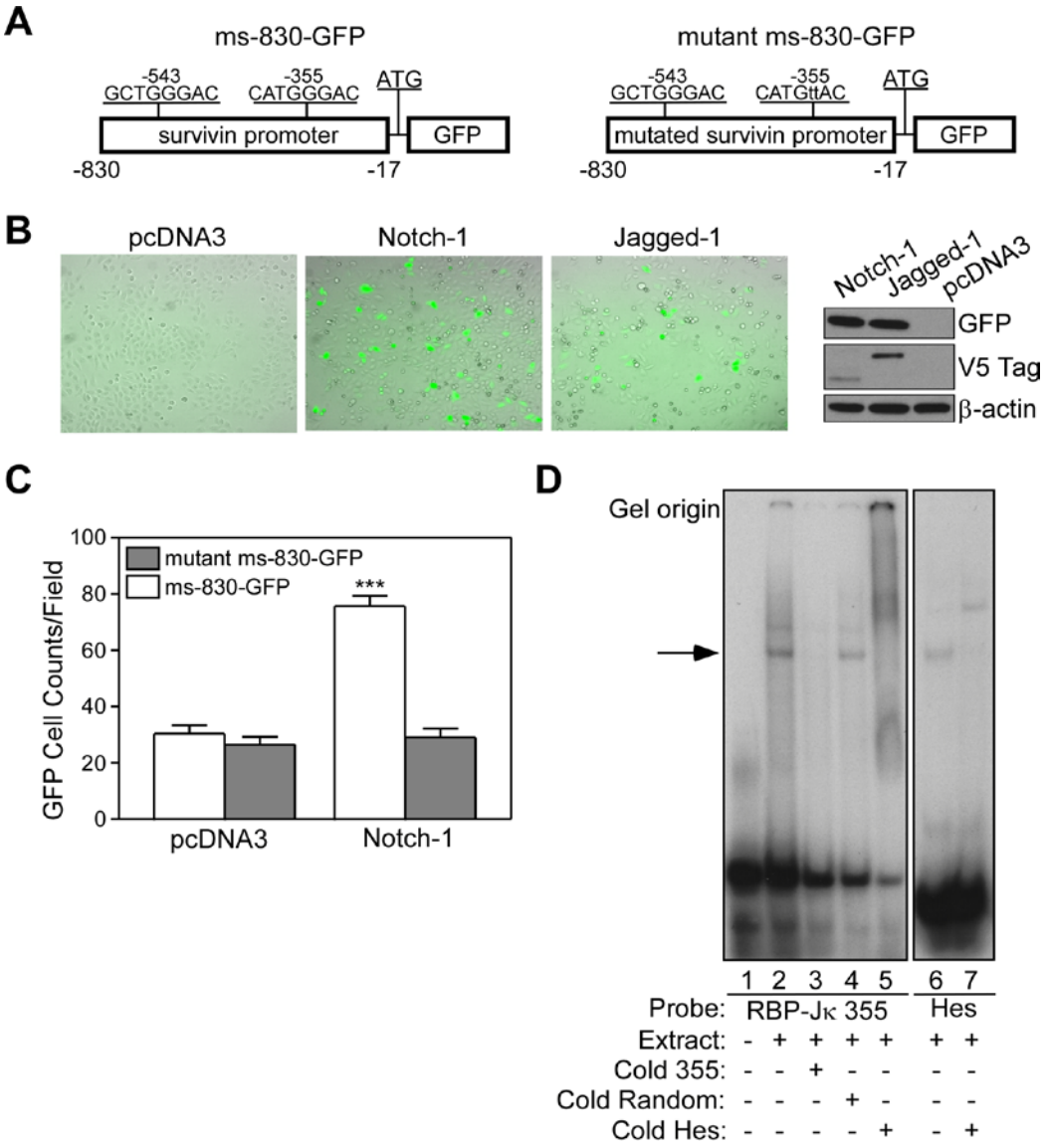


Figure 3-2. Notch regulation of survivin gene expression.
A. Maps of *survivin* promoter constructs (ms-830-GFP) and mutant survivin promoter construct (mutant ms-830-GFP). Two putative RBP-Jκ sites at -355 and -543 are indicated. B. Fluorescence microscopy (*left panels*). HeLa cells co-transfected with wild type ms-830-GFP plus pcDNA3, Notch-1, or Jagged-1 were analyzed by fluorescence microscopy (*left panels*). GFP expression was confirmed by Western blotting (*right panel*). C. Mutant ms-830-GFP co-transfections. Cells co-transfected with wild type or mutant ms-830-GFP plus pcDNA3 or Notch-1 were quantified for GFP-positive cells. Mean±SEM (n=10 high-power fields with approximately 200 cells/field) from two independent experiments. ***, $p<0.0001$. D. EMSA. Nuclear extracts from MDA-MB-231 cells were incubated with ^{32}P-γ ATP-labeled probe duplicating the *survivin* RBP-Jκ site at -355 (*left panel*), in the presence or absence of unlabeled competitors. An RBP-Jκ probe from the *Hes* promoter was used as a control (*right panel*). *Arrow*, position of a DNA-protein complex.

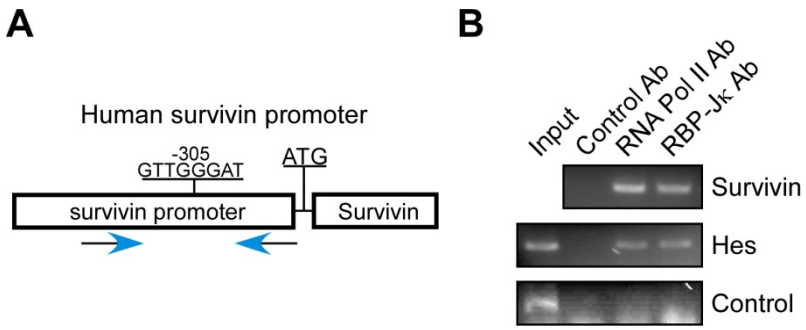


Figure 3-3. Chromatin immunoprecipitation of the human *survivin* promoter. A. Map of the human *survivin* promoter. Putative RBP-Jκ site at -305 in the human survivin gene is indicated. B. ChIP assay. Chromatin from MDA-MB-231 cells transduced with pAd-NIC was incubated with antibodies to RNA polymerase II (RNA Pol II), RBP-Jκ, or IgG, and the immunoprecipitated DNA was amplified with primers for the putative RBP-Jκ site in the human *survivin* promoter at -305, the RBP-Jκ site in the *Hes* promoter, or a non-specific promoter region.

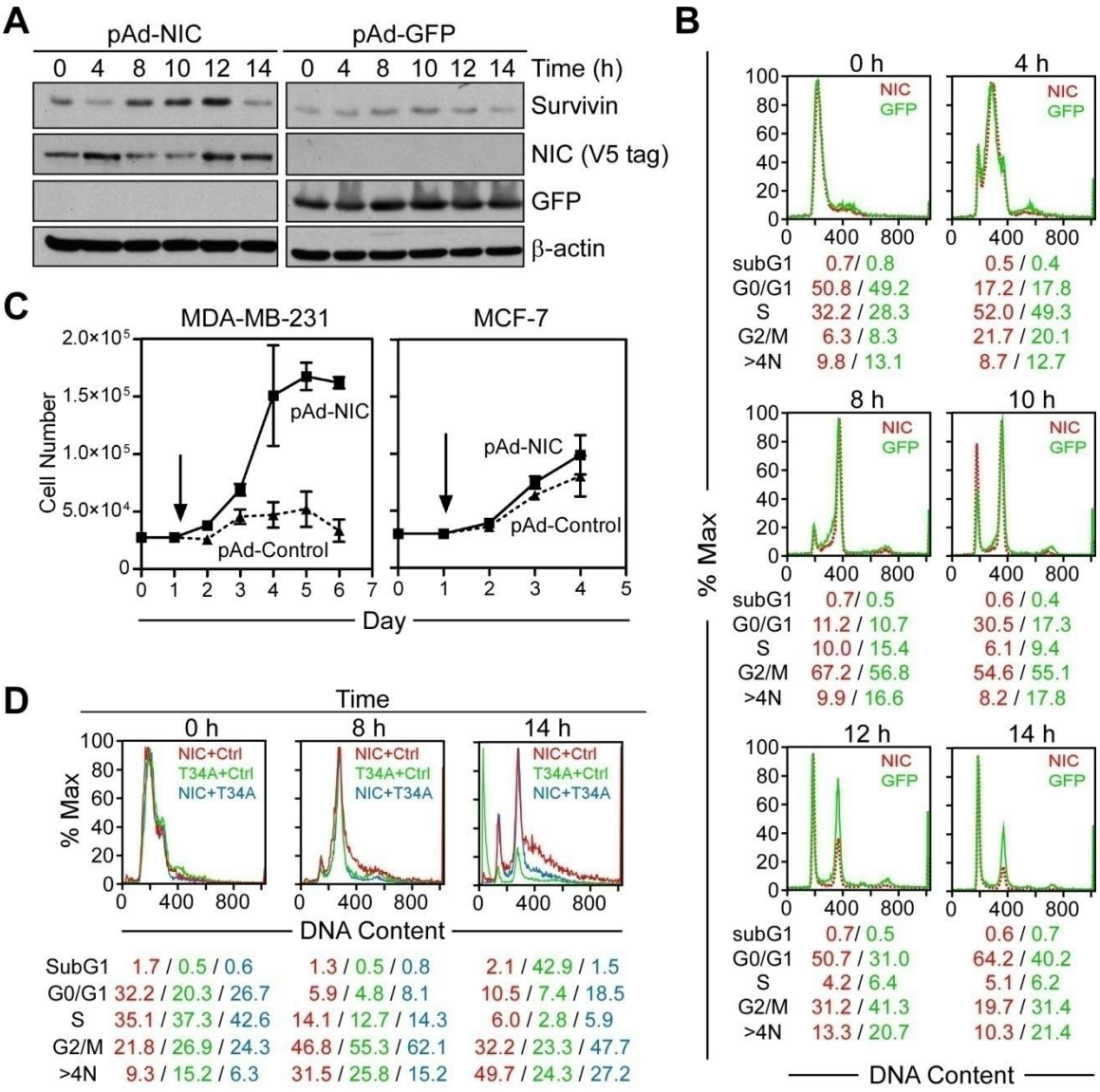


Figure 3-4. Effect of activated Notch-1 on cell cycle progression.
A. Thymidine synchronization. Transduced MDA-MB-231 cells were synchronized with 2 mM thymidine, and analyzed by Western blotting at the indicated time intervals after release. B. Cell cycle analysis. Synchronized and transduced MDA-MB-231 cells were analyzed for DNA content by propidium iodide staining and flow cytometry at the indicated time intervals. The percentage of cells in each cell cycle phase is indicated. C. Cell proliferation. Transduced MDA-MB-231 or MCF-7 cells were counted at the indicated time intervals, starting at d. 1 (*arrow*). Mean±SEM (n=3). D. Rescue from mitotic cell death. MDA-MB-231 cells were transduced with combinations of pAd-NIC, pAd-Control, and pAd-T34A, synchronized, and analyzed for DNA content by propidium iodide staining and flow cytometry. The percentage of cells in each cell cycle phase is indicated.

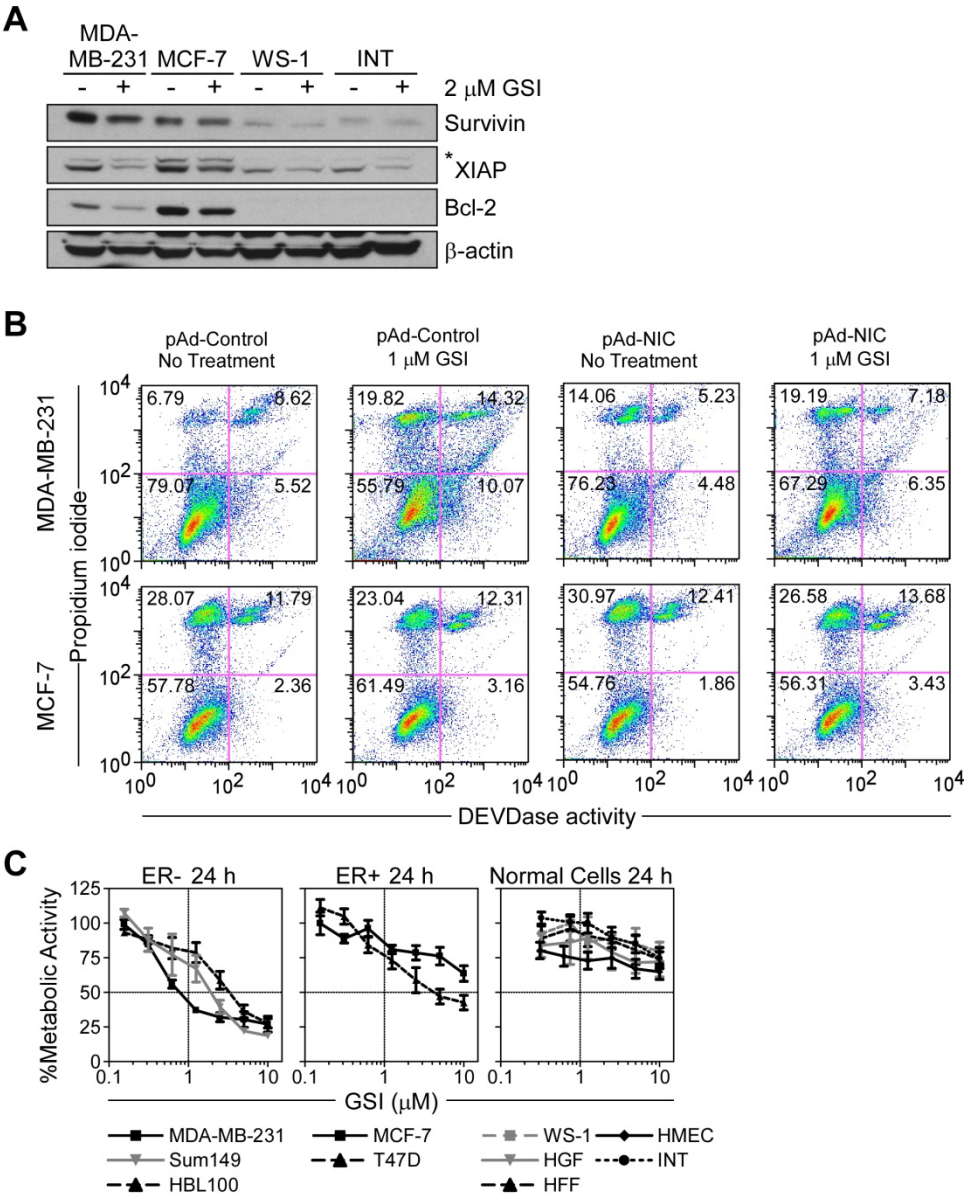


Figure 3-5. Effect of Notch inhibition on breast cancer cell viability.
A. Western blotting. The indicated breast cancer or normal cell types were treated with DMSO (-) or 2 μM GSI (+), harvested after 24 h, and analyzed by Western blotting. B. NIC rescue of GSI-induced cell death. Transduced MDA-MB-231 (top panels) or MCF-7 (*bottom panels*) cells were treated with 1 μM GSI or DMSO for 24 h, and analyzed for apoptosis by DEVDase activity. The percentage of cells in each quadrant is indicated. C. Cell viability. The indicated breast cancer (*left and middle panels*), or normal (*right panel*) cell types were treated with increasing concentrations of GSI (0.125–10 μM) for 24 h, and analyzed for cell viability by MTT. Mean±SEM (n=4). IC_{50} values for GSI-induced cell killing are as follows: MDA-MB-231, 1.196 μM; HBL100, 3.416 μM; Sum149, 2.030 μM; MCF-7, >10 μM; T47D, 5.709 μM; normal cell lines, >10 μM.

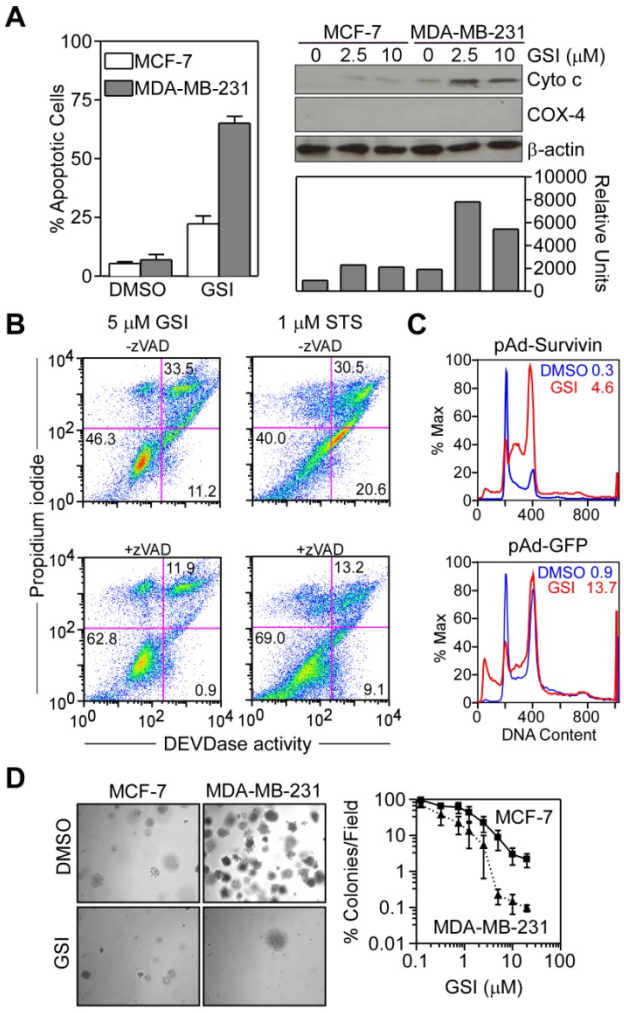


Figure 3-6. Induction of apoptosis by Notch inhibition.
A. Cell death. MCF-7 or MDA-MB-231 cells were treated with GSI (2.5 μM), harvested after 24 h, and analyzed for nuclear morphology of apoptosis by DAPI staining and fluorescence microscopy. *Left panel*, mean±SEM of 5-10 fields with an average of 60-30 cells/field). *Right panel*, release of mitochondrial cytochrome c in the cytosol by Western blotting. *Bottom right panel*, normalized densitometric quantification of cytochrome c protein bands. Cox-4 was used as a control mitochondrial marker. B. Caspase requirement. MDA-MB-231 cells were treated with 5 μM GSI or 1 μM STS, and analyzed for DEVDase activity after 24 h, in the presence or absence of zVAD. Percentage of cells in each quadrant is indicated. C. Survivin rescue. Transduced MDA-MB-231 cells were treated with 1 μM GSI for 24 h, and analyzed for DNA content. The percentage of cells with hypodiploid (sub-G1) DNA content is indicated. D. Colony formation assay. MCF-7 or MDA-MB-231 cells were treated with increasing concentrations of GSI (0.125–20 μM) for 24 h, plated onto semisolid medium. After 2 weeks, colonies (*left panels*) were stained with 0.005% crystal violet, and quantified by light microscopy (*right panel*). Mean±SEM (n=3). Six fields/GSI concentration were quantified per condition (*right panel*).

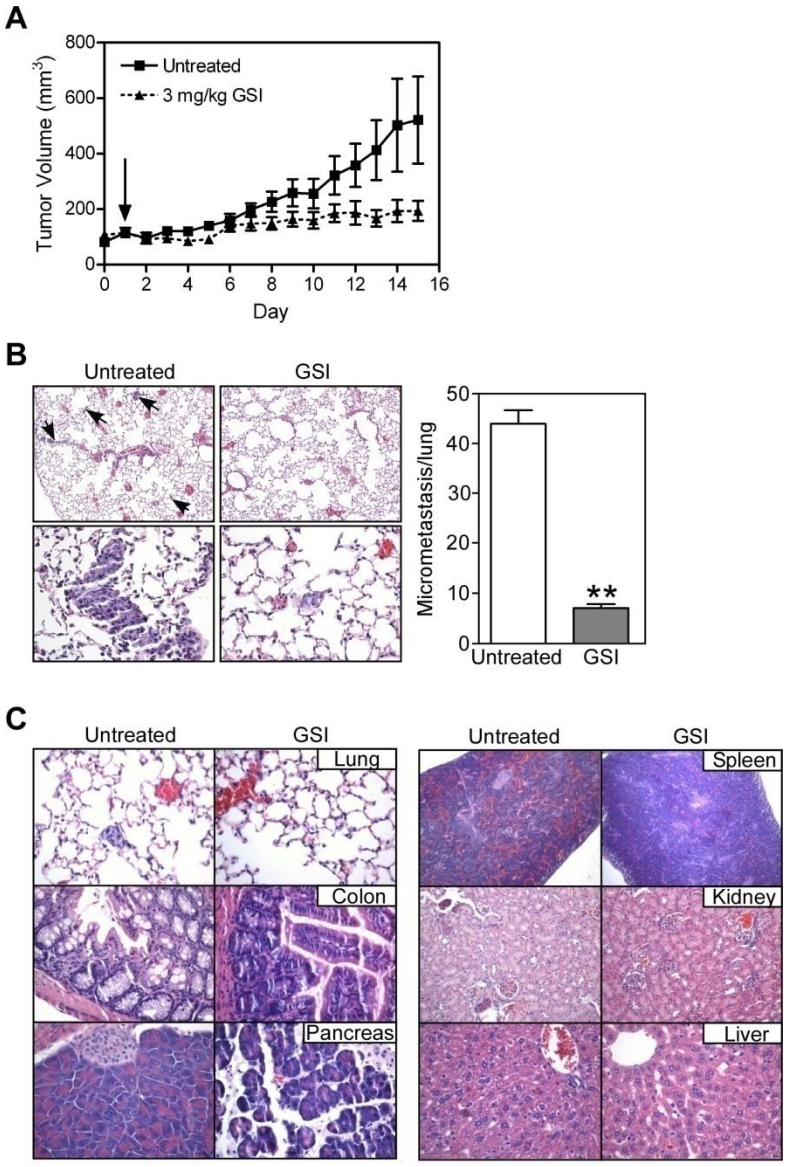
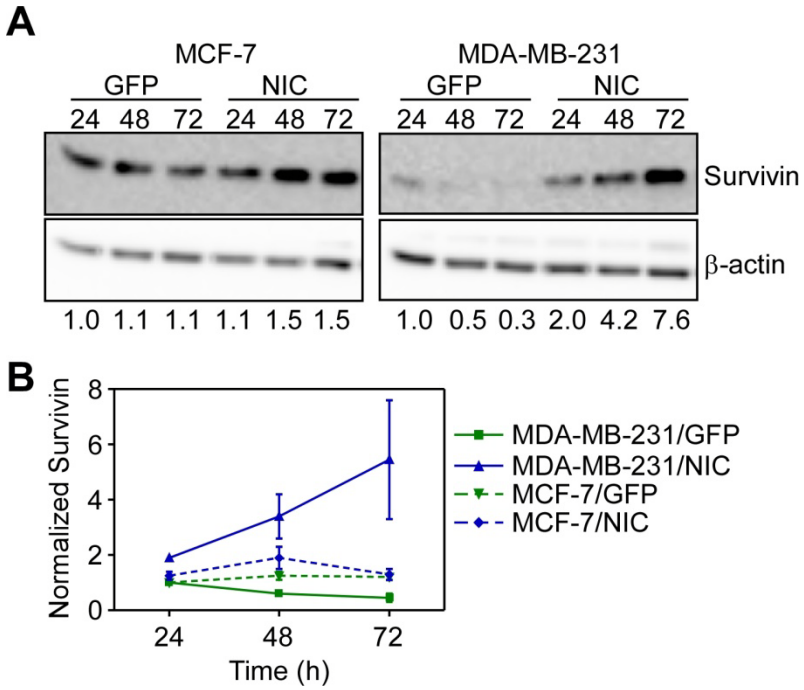
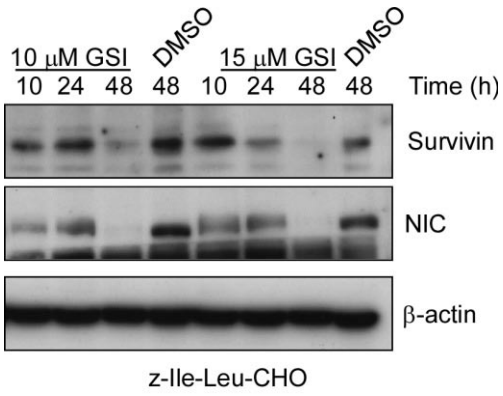


Figure 3-7. Anti-cancer activity of Notch inhibition.
A. Kinetics of tumor growth. CB17 SCID/beige mice were injected subcutaneously with 5x106 MDA-MB-231 cells into each flank. When tumors reached 150-175 mm3, mice (3 animals/group, 6 tumors/group) were treated with 3 mg/kg GSI daily or vehicle as i.p. injections. Arrow, start of GSI treatment. Mean±SEM of tumor volume at each indicated time point. B. Effect of Notch inhibition on lung metastases. Lung sections from vehicle- or GSI-treated animals were analyzed by hematoxylin-eosin staining (*left panel*), and lung micrometastases were quantified in serial tissue sections (20 μm apart) by light microscopy (*right panel*; mean±SEM from 5 independent fields. **, $p<0.0001$). Magnifications, *left panels*, 10x; *right panels*, 40x. C. Tissue histology. The indicated organs were collected from mice treated with vehicle (untreated) or GSI at the end of the experiment (14 d), paraffin-embedded, and analyzed by hematoxylin-eosin staining.

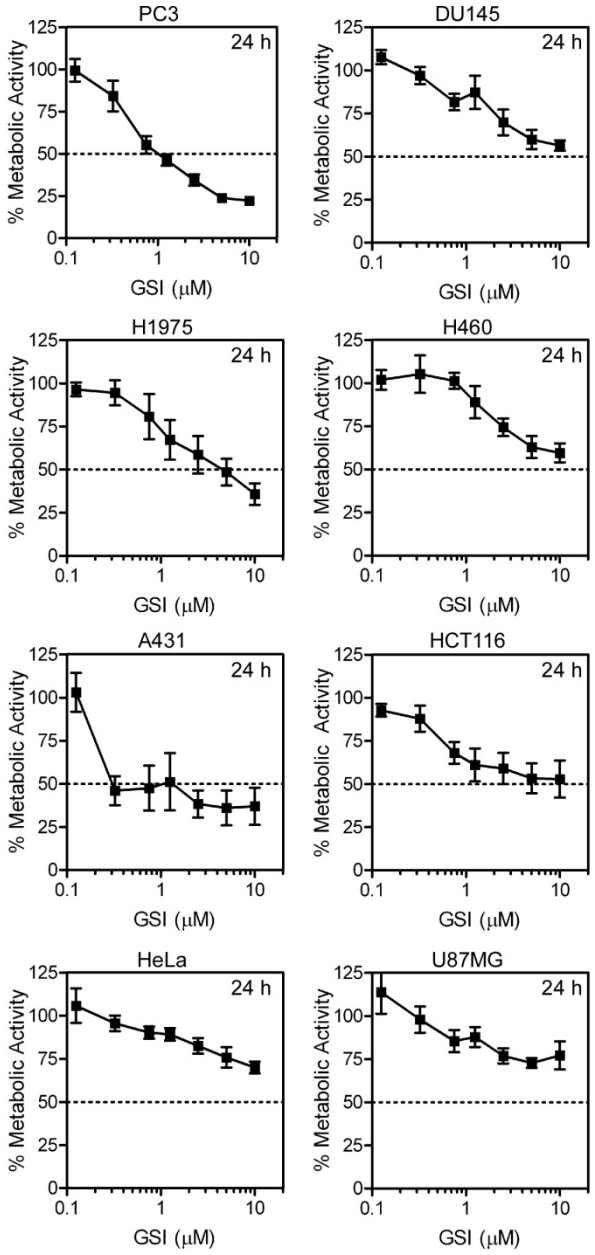
SUPPLEMENTAL FIGURES



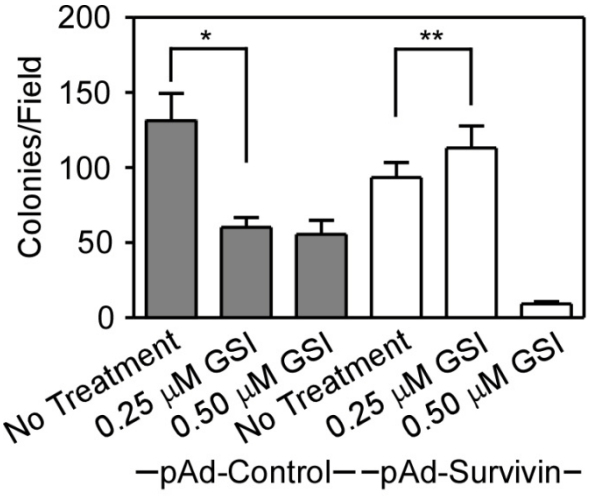
Supplemental Figure 3-8. Semi-quantitative Western blotting of Notch activation. A. MDA-MB-231 and MCF-7 cells were infected with pAd-NIC or pAd-Control and harvested after 24-72 h. B. Densitometric analysis.



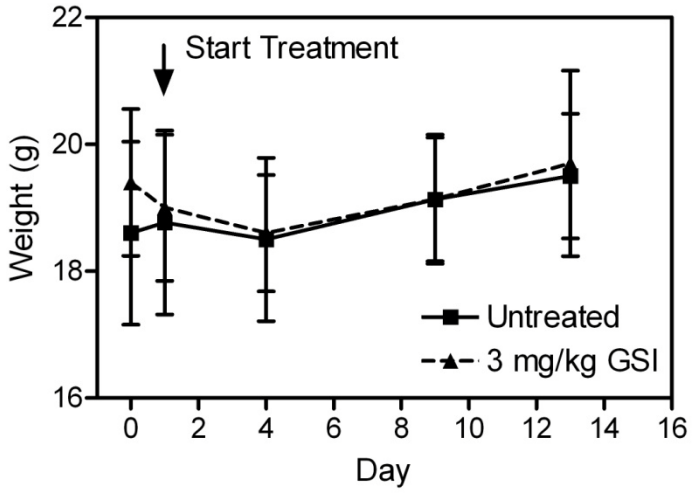
Supplemental Figure 3-9. Effect of second GSI.
MDA-MB-231 breast cancer cells were treated with 10 μM or 15 μM of z-Ile-Leu-CHO or control vehicle (DMSO) for the indicated time intervals, and analyzed by Western blotting.



Supplemental Figure 3-10. Effect of GSI on tumor cell viability.
The indicated tumor cell types were incubated with increasing concentrations of GSI, harvested after 24 h and analyzed for cell viability by an MTT assay. Mean±SEM (n=3).



Supplemental Figure 3-11. Rescue of colony formation by survivin after GSI. MDA-MB-231 cells were infected with pAd-survivin or pAd-Control and subsequently treated with low dosage GSI for 24 h. After two weeks, cells were stained with crystal violet and counted. Mean±SEM (n=6); *, p=0.004; **, p=0.29



Supplemental Figure 3-12. Animal body weight during GSI treatment.
Mice treated with vehicle (untreated) or 3 mg/kg GSI i.p. daily were weighted throughout the course of the experiment. *Arrow*, start of GSI treatment. Mean±SEM (n=6).

Chapter 4. Identification of novel IGF-1/survivin regulatory mechanism in prostate cancer

Valentina Vaira, Connie W. Lee, Hira L. Goel, Silvano Bosari,

Lucia R. Languino, and Dario C. Altieri

This chapter represents Valentina Vaira's thesis work on IGF-1 signaling in prostate cancer to which I contributed after she returned to Italy. The text is a version of the manuscript published online in Oncogene on October 30, 2006 (Vaira, Lee et al. 2007). I verified the induction of survivin by IGF-1 signaling, analyzed the cell cycle modulation of IGF-1 on prostate cancer cells, and determined the contribution of mTOR/survivin signaling to prostate cancer cell viability. Valentina Vaira and Dario Altieri wrote the text while I contributed to the material and methods section and provided feedback on the text.

Abstract

Survivin is a dual regulator of cell proliferation and cell viability over-expressed in most human tumors. Although strategies to lower survivin levels have been pursued for rational cancer therapy, the molecular circuitries controlling survivin expression in tumors have not been completely elucidated. Here, we show that stimulation with Type 1 Insulin-like Growth Factor (IGF-1) results in increased survivin expression in prostate cancer cells. This response is independent of de novo gene transcription, changes in mRNA expression, or modifications of survivin protein stability. Instead, IGF-1 induced

persistence and translation of a pool of survivin mRNA, in a reaction abolished by the mTOR (mammalian Target Of Rapamycin) inhibitor, rapamycin. Forced expression of the mTOR target p70S6K1 reproduced the increase in survivin expression in prostate cancer cells, whereas acute ablation of endogenous p70S6K1 by small interfering RNA downregulated survivin levels. Rapamycin, alone or in combination with suboptimal concentrations of taxol reduced survivin protein levels, and decreased viability of prostate cancer cells. Therefore, IGF-1/mTOR signaling elevates survivin in prostate cancer cells via rapid changes in mRNA translation. Antagonists of this pathway may be beneficial to lower an anti-apoptotic threshold maintained by survivin in prostate cancer.

Introduction

Deregulation of apoptosis leading to aberrantly increased cell survival is a hallmark of cancer (Hanahan and Weinberg 2000), and may result from increased expression of cell death antagonists or loss of cell death inducers (Hengartner 2000). Restoration of apoptotic pathways is being explored for rational cancer therapy (Reed 2003), and targeting survival proteins of the Bcl-2 (Cory and Adams 2002), or Inhibitor of Apoptosis (IAP) (Salvesen and Duckett 2002) gene family has been associated with tumor cell death, and in some cases, promising clinical responses (Reed 2003). Survivin (Ambrosini, Adida et al. 1997) has attracted attention as a unique IAP member (Salvesen and Duckett 2002) for its differential expression in tumors as opposed to normal tissues (Ambrosini, Adida et al. 1997), and a role in multiple pathways of tumor cell maintenance, including protection from apoptosis, cell division, the cellular stress response, and p53-dependent checkpoints (Altieri 2003).

Although the differential expression of survivin in tumors is influenced transcriptionally by oncogenic signaling (Altieri 2003), and loss of p53-mediated gene repression (Hoffman, Biade et al. 2002; Mirza, McGuirk et al. 2002), other, non-transcriptional regulatory mechanisms have been identified. These include rapid changes in survivin protein stability modulated by phosphorylation (O'Connor, Grossman et al. 2000), subcellular trafficking controlled by monoubiquitination (Vong, Cao et al. 2005), and dynamic exchange of survivin pools among individual subcellular compartments (Dohi, Beltrami et al. 2004). In prostate cancer, a plethora of molecular pathways has been associated with modulation of survivin levels, including extracellular matrix-integrin interactions (Fornaro, Plescia et al. 2003), antiandrogen therapy (Zhang, Latham et al. 2005), STAT3 activation (Nam, Buettner et al. 2005), and Smad/BMP-7 signaling (Yang, Lim et al. 2006), thus contributing to apoptosis resistance. In addition, activation of PI3 kinase/Akt signaling, which is a common hallmark of prostate cancer, has been consistently linked to increased survivin levels (Dan, Jiang et al. 2004), but the downstream target(s) of this response have not been identified.

Here, we studied a potential link between survivin expression and the mammalian Target of Rapamycin (mTOR) (Wullschleger, Loewith et al. 2006), a downstream effector of Akt with critical roles in cell growth, cell survival and adaptation to stress (Hay 2005).

Results

IGF-1 increases survivin protein expression in prostate cancer cells

Serum-deprived prostate adenocarcinoma DU145 cells exhibited low expression of endogenous survivin, which was induced by IGF-1 in a concentration-dependent manner, by immunoblotting (Figure 4-1A). IGF-1 induction of survivin was detectable as early as 12 h after stimulation (Figure 4-1A), and persisted throughout a 24 h (Figure 4-1B) or 48 h (Figure 4-1C) time interval. In contrast, mitogen stimulation of DU145 cells with FBS did not modulate survivin levels after 12 h (Figure 4-1A), but increased survivin expression at 24 and 48 h after treatment (Figure 4-1B and C). The effect of IGF-1 was specific for survivin, as FBS or IGF-1 did not modulate the expression of another IAP member, XIAP, or β-actin (Figure 4-1C). Finally, IGF-1 stimulation did not significantly affect the DNA content profile of DU145 cells at comparable time intervals (Figure 4-1D), suggesting that changes in survivin expression after exposure to IGF-1 were independent of cell cycle progression.

IGF-I receptor (IGF-1R) modulates survivin expression.

To determine the signaling requirements of IGF-1 modulation of survivin, we used MEF derived from IGF-1R null mice, which were stably transfected with WT or mutant IGF-1R (Romano, Prisco et al. 1999). Stimulation with IGF-1 resulted in increased survivin expression only in cells expressing WT IGF-1R (Figure 4-2A). In contrast, IGF-1 stimulation of cells expressing mutant receptor GR35 or GR48 did not modulate endogenous survivin levels (Figure 4-2A). In addition, IGF-1 stimulation of cells expressing WT IGF-1R resulted in strong receptor tyrosine phosphorylation (Figure

4-2B). In contrast, tyrosine phosphorylation was detected in GR35 cells, but completely absent in GR48 cells, and no receptor phosphorylation was observed without IGF-1 (Figure 4-2B). In addition, cells transfected with WT IGF-1R exhibited a transformed phenotype as judged by colony formation in soft agar, whereas expression of mutant receptor GR35 or GR48 did not result in colony formation (Figure 4-2C).

IGF-1 induction of survivin independent of transcription or protein stabilization

To investigate the mechanism(s) of IGF-1 induction of survivin, we first analyzed potential changes in survivin mRNA levels in untreated or stimulated cultures, by semi-quantitative PCR. In control experiments, addition of FBS to serum-deprived DU145 cells resulted in increased survivin mRNA expression (Figure 4-3A). In contrast, IGF-1 stimulation did not significantly increase survivin mRNA levels, as compared with untreated cultures (Figure 4-3A). We then transfected cells with *survivin* promoter-luciferase constructs pLuc-441 and pLuc-1430 encompassing most of the known transcriptional requirements for *survivin* gene expression. FBS stimulation of serum-deprived DU145 cells resulted in a 4- to 6-fold increased in β-galactosidase-normalized luciferase activity of both pLuc-441 and pLuc-1430 (Figure 4-3B), consistent with previous observations (Li and Altieri 1999). In contrast, concentrations of IGF-1 that maximally increased survivin protein levels did not significantly enhance the transcriptional activity of either *survivin* promoter, as compared with control cultures (Figure 4-3B). We next asked whether IGF-1 stimulation affected survivin protein stability. Exposure of serum-deprived DU145 cells to cycloheximide resulted in rapid disappearance of survivin levels, detected as early as 2 h after addition and throughout a

6-h time interval (Figure 4-3C). Although serum stimulation partially restored survivin levels in the presence of cycloheximide, treatment with IGF-1 had no effect (Figure 4-3C). In control experiments, IGF-1 did not affect XIAP expression in the presence or absence of cycloheximide (Figure 4-3C).

IGF-1 triggers survivin mRNA accumulation and translation

We next asked whether downstream inhibition of IGF-1 signaling by rapamycin affected survivin expression. Stimulation of serum-deprived DU145 cells with IGF-1 or FBS strongly increased survivin expression (Figure 4-4A), in agreement with the data presented above. Treatment of DU145 cells with rapamycin nearly completely abolished IGF-1 induction of survivin, whereas FBS stimulation of survivin was not significantly affected (Figure 4-4B). No modulation of XIAP was observed after IGF-1 or FBS stimulation, with or without rapamycin (Figure 4-4B), and no decrease in cell viability was observed at the concentrations of rapamycin used (Figure 4-6A, *left panel*).

Next, we treated IGF-1 or FBS-stimulated DU145 cells with the transcriptional inhibitor actinomycin D, and analyzed changes in survivin mRNA levels, with or without rapamycin. In these experiments, IGF-1 stimulation was associated with persistence of survivin mRNA levels over an 8-h time interval, in a reaction completely abolished by rapamycin (Figure 4-4C). In contrast, FBS treatment did not preserve survivin mRNA levels in the presence of actinomycin D (Figure 4-4C). Preservation of survivin mRNA levels in a rapamycin-sensitive response was also observed after treatment with an unrelated transcriptional inhibitor, DRB (Figure 4-4D), thus ruling out that the observed effect was due to non-specific activation of a cellular stress response.

Requirement of p70S6K in IGF-1 regulation of survivin

Next, we wanted to identify the molecular requirements of rapamycin-sensitive modulation of survivin. We transfected DU145 cells with cDNA encoding WT or rapamycin-insensitive p70S6K, a downstream substrate of mTOR. Expression of WT p70S6K substituted for IGF-1 stimulation in inducing survivin expression in serum-deprived DU145 cells (Figure 4-5A). Treatment of transfected DU145 cells with rapamycin partially reduced the extent of survivin induction by p70S6K (Figure 4-5A). Conversely, transfection of serum-deprived DU145 cells with rapamycin-insensitive p70S6K (T389EΔCT) resulted in strong upregulation of survivin in the absence of IGF-1, in a reaction unaffected by rapamycin (Figure 4-5B). In control experiments, FBS induction of survivin was unaffected by WT or rapamycin-insensitive p70S6K in the presence or absence of rapamycin (Figure 4-5A, B). Next, we ablated p70S6K by siRNA, and analyzed changes in survivin expression with or without IGF-1 stimulation. Serum-deprived DU145 cells transfected with control siRNA exhibited increased survivin expression after IGF-1 stimulation, as compared with untreated cultures (Figure 4-5C). In contrast, acute knockdown of p70S6K by siRNA completely abolished IGF-1-mediated increased survivin expression (Figure 4-5C). Conversely, siRNA knockdown of p70S6K did not affect FBS induction of survivin in DU145 cells (not shown).

Inhibition of mTOR sensitizes prostate cancer cells to combination therapy

To determine the impact of IGF-1 induction of survivin in prostate cancer cells, we treated DU145 cells with rapamycin, and investigated changes in cell viability. Exposure of serum-deprived DU145 cells to rapamycin did not induce loss of cell

viability for the first 24 h (Figure 4-6A). However, a 48 h treatment of DU145 cells with rapamycin resulted in ~50% decrease in cell viability, in a reaction fully reversed by IGF-1 stimulation, whereas FBS stimulation was ineffective (Figure 4-6B). Second, we combined rapamycin with established cytotoxic agents and investigated changes in survivin expression and cell viability. Treatment of DU145 cells with the combination of rapamycin plus a suboptimal concentration of taxol for 24 h resulted in nearly complete disappearance of survivin expression, as compared with either treatment alone (Figure 4-6B). In contrast, the combination of doxorubicin plus rapamycin was ineffective (not shown). IGF-1 stimulation of DU145 cells treated with the combination of taxol plus rapamycin failed to restore survivin expression, whereas FBS increased survivin levels to approximately 50% of those of untreated cultures (Figure 4-6B). Consistent with a critical reduction in the anti-apoptotic threshold maintained by survivin, the taxol plus rapamycin combination was more effective than either treatment alone in reducing DU145 cell viability in the presence of IGF-1 (Figure 4-6C).

Discussion

In this study, we have shown that IGF-1 stimulation of prostate cancer cells results in increased survivin expression, and that this pathway is mediated by stabilization and translation of a pool of survivin mRNA. Inhibition of mTOR (Wullschleger, Loewith et al. 2006) with rapamycin, alone or in combination with taxol, or molecular or genetic interference with its downstream target, p70S6K (Wullschleger, Loewith et al. 2006), abolished survivin increase by IGF-1, and decreased prostate cancer cell viability.

Because of its “crossroad” role in multiple essential pathways of tumor cell maintenance, and its differential expression in cancer as opposed to normal tissues, survivin is being actively pursued as a novel target for rational cancer therapy (Altieri 2003). A validating principle of this approach is that lowering intracellular survivin levels below a critical threshold using a variety of approaches, including antisense, dominant negative mutants, or siRNA sequences, has been consistently associated with arrest of cell proliferation, spontaneous apoptosis and sensitization to cell death stimuli, including cytotoxics and ionizing radiation (Altieri 2006). In this context, much attention has been devoted towards elucidating the molecular requirements of *survivin* gene transcription, but recent evidence points to additional, non-transcriptional mechanisms controlling survivin levels in tumor cells (Altieri 2003). One such pathway is centered on IGF-1 ligation to its cognate membrane receptor that has been shown to increase survivin expression in prostate (Zhang, Latham et al. 2005), myeloma (Stromberg, Ekman et al. 2006) and liver (Hopfner, Huether et al. 2006) tumor cell types, even though the underlying mechanism(s) of this response had remained elusive. As now reported here, this pathway depends on IGF-1R transforming potential (Sell, Dumenil et al. 1994), does not involve changes in cell cycle distribution, and is not associated with de novo *survivin* promoter activity or increased survivin protein stability. Conversely, IGF-1 modulates survivin levels by favoring stabilization and translation of a survivin mRNA pool through activation of the mTOR pathway.

Regulation of survivin expression has been linked in multiple experimental systems to increased Akt activity, a Ser/Thr kinase downstream of PI3 kinase signaling (Amaravadi and Thompson 2005). This is relevant for prostate cancer, where survivin is

highly expressed (Fornaro, Plescia et al. 2003; Krajewska, Krajewski et al. 2003), and Akt activity is commonly deregulated after loss of the inhibitory lipid phosphatase, PTEN (Majumder and Sellers 2005). In turn, increased Akt activity provides a broad anti-apoptotic environment through cytoplasmic trapping of cell death mediators (Datta, Dudek et al. 1997; Goswami, Burikhanov et al. 2005), modulation of NF-κB (Kane, Shapiro et al. 1999; Kane, Mollenauer et al. 2002), and repression of apoptotic inducers (Brunet, Bonni et al. 1999). In addition, Akt phosphorylates and inactivates the tuberous sclerosis complex, TSC2, a negative regulator of mTOR signaling (Wullschleger, Loewith et al. 2006). The data presented here identify a novel survival circuitry initiated by IGF-1/Akt signaling, and involving mTOR activation, p70S6K activation, and increased stability/translation of a survivin mRNA pool. This pathway is specific for IGF-1, as serum mitogens upregulated survivin in tumor cells independently of mTOR or p70S6K, and is centered on survivin, as XIAP (Salvesen and Duckett 2002), a IAP member whose levels are controlled by protein translation (Holcik, Yeh et al. 2000), was not affected.

The mTOR pathway constitutes a “sensor” network for environmental nutrients or stress conditions, thus affecting cell growth (cell volume), cellular proliferation, and activation of metabolic rescue pathways, i.e. autophagy (Wullschleger, Loewith et al. 2006). As a multifaceted mTOR effector, survivin appears ideally positioned to contribute to these responses, by favoring mitotic progression, resistance to apoptosis, and increased cellular adaptation to stress (Altieri 2003). Conversely, targeted inhibition of IGF-1/Akt/mTOR couples to cell cycle arrest and induction of apoptosis, which may be contributed by acute loss of survivin levels (Decker, Hipp et al. 2003; Hopfner,

Huether et al. 2006; Stromberg, Ekman et al. 2006), Forkhead-mediated transcription of the cyclin-dependent kinase inhibitor p27 (Wullschleger, Loewith et al. 2006), and activation of p53-dependent apoptosis (Levine, Feng et al. 2006).

In summary, these data reinforce the rationale of pharmacologic inhibition of IGF-1/Akt/mTOR signaling for prostate cancer therapy in humans (Hay 2005), and suggests that ablation of survivin mRNA translation may provide an additional strategy to remove an anti-apoptotic mechanism potentially contributing to aggressive tumor behavior, in vivo.

Materials and Methods

Cell culture conditions

Prostate adenocarcinoma DU145 and cervical carcinoma HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DU145 cells were cultured in high-glucose DMEM containing 1 mM sodium pyruvate, 10% heat inactivated fetal bovine serum (FBS) plus 100 U Pen-Strep antibiotic mixture, and maintained in a 5% CO_2 incubator at 37°C, as described (Fornaro, Plescia et al. 2003). All cell culture reagents were purchased from Invitrogen. IGF-1 was purchased from R&D System. Rapamycin, cycloheximide and the transcriptional inhibitors, actinomycin D and 5,6 dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were from Sigma. All working solutions were prepared in PBS, pH 7.4. For stimulation, $4x10^6$ DU145 cells were seeded in 6-well plates in 2 ml of serum-free DMEM for 24 h, and incubated with increasing concentrations of IGF-1 for 12-48 h in the presence or absence of rapamycin

(20 nM). When incubation reactions were prolonged to 48 h, IGF-1 and rapamycin were replaced daily in fresh medium.

Modulation of protein and mRNA expression

After IGF-1 stimulation, DU145 cells were harvested at increasing time intervals and solubilized in 150 μl of lysis buffer containing 20 mM Tris, pH 7.2, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA plus 5% protease inhibitor cocktail tab (Roche). Protein-normalized (50 μg) extracts were separated on 12% SDS polyacrylamide gels, transferred to PVDF membranes (Immobilon), and analyzed by immunoblotting, as described (Dohi, Beltrami et al. 2004). Antibodies to β-actin (1:5000, clone AC-15, Sigma), survivin (1:1000, NOVUS Biologicals), XIAP (1:500, BD Bioscience), or total p70S6K (1:500, Cell Signaling) were used with detection by chemiluminescence (Plus Reagents, Amersham Pharmacia).

For analysis of mRNA stability, serum-deprived DU145 cells were stimulated with medium, IGF-1 (100 ng/ml) or 10% FBS for 6 h at 37°C. Samples were incubated with 10 μM actinomycin D, or, alternatively, 50 μM DRB, harvested at 2-8 h time intervals, and processed for total RNA extraction (RNeasy® Mini, Qiagen, Valencia, CA). For analysis of protein stability, DU145 cells were incubated in serum-deprived conditions for 24 h, stimulated with medium, IGF-1 or 10% FBS for 18 h, followed by incubation with 10 μM cycloheximide. Samples were harvested at 2-6 h time intervals, and protein-normalized extracts were analyzed by immunoblotting.

Reverse transcription and semiquantitative PCR

Total RNA extracted from DU145 cells was reverse-transcribed (1 μg) in the presence of SuperScript II polymerase plus random primers (Invitrogen). Amplification of survivin (27 cycles) or GAPDH (32 cycles) cDNA was carried out by semi-quantitative PCR. Products were separated on 1.5% agarose gels and band intensity was quantified using Image J software followed by GAPDH normalization.

p70S6K transfection

DU145 cells were transfected with WT or rapamycin-resistant p70S6K mutant cDNA (4 μg) using LipofectAmine 2000 (Invitrogen, 6 μl) in Opti-Mem medium (1 ml) (Gibco). After 5 h, cells were incubated in serum-deprived conditions for 16 h at 37°C, stimulated with IGF-1 or 10% FBS for 24 h, and analyzed for changes in protein expression with or without rapamycin (20 nM), by immunoblotting. For gene silencing experiments by small interfering RNA (siRNA), DU145 cells were transfected with p70S6K-directed siRNA SMARTpool (M-003616-02-0010, Dharmacon Inc.), or control non-targeted dsRNA oligonucleotide (VIII) using LipofectAmine 2000. After 5 h, the medium was replaced with serum-free DMEM with or without IGF-1 (100 ng/ml) or 10% FBS. Samples were harvested after 24 or 48 h, and analyzed by immunoblotting.

Survivin promoter activity

DU145 or Hela cells were transfected with *survivin* promoter constructs upstream of a luciferase reporter gene (pLuc-441 and pLuc-1430) encompassing most of the transcriptional requirements for *survivin* gene expression (Li and Altieri 1999) plus 1 μg

LacZ cDNA. After transfection, cells were incubated in serum-deprived conditions for 16 h, stimulated with IGF-1 or 10% FBS for 12 h, and analyzed for β-galactosidase-normalized luciferase activity using a DXT 880 Multimode Detector luminometer (Beckman Coulter) at A_{405}.

Analysis of cell viability and cell cycle

Serum-deprived DU145 cells were seeded in 96-wells plates for 24 h, and stimulated with medium, IGF-1 (100 ng/ml) or 10% FBS in the presence or absence of rapamycin (20 nM) for 24 or 48 h. In some experiments, serum-deprived DU145 cells were treated with the combination of rapamycin plus taxol (25 μM) for 24 h, stimulated with IGF-1 or FBS, and harvested after 48 h. After addition of 20 μl of MTT solution (10% of total volume, Sigma) for 4 h at 37°C, samples under the various conditions tested were centrifuged at 3000 rpm for 10 min, suspended in 200 μl DMSO (Sigma) for 10 min at 22°C, and absorbance was determined at A_{570}. Alternatively, samples were analyzed for changes in survivin levels by β-actin-normalized quantitative immunoblotting. In other experiments, serum-deprived DU145 cells stimulated with FBS or IGF-1 were stained with propidium iodide and analyzed for DNA content at 12-48 h time intervals by flow cytometry.

Anchorage-independent cell growth

Cell growth in soft agar was assayed by scoring the number of colonies formed in DMEM containing 0.3% agarose with a 0.5% agarose medium underlay. R-, WT, GR35 or GR48 ($2x10^3$) cells were seeded on 60-mm diameter plates in triplicate. Cells were

fed with 1.5 ml of DMEM medium every 3 d. Colonies larger than 100 μm were counted after 14 d.

Statistical analysis

Data were analyzed with two-sided unpaired t tests in the GraphPad software package for Windows (Prism version 4.0). A p value of 0.05 was considered statistically significant.

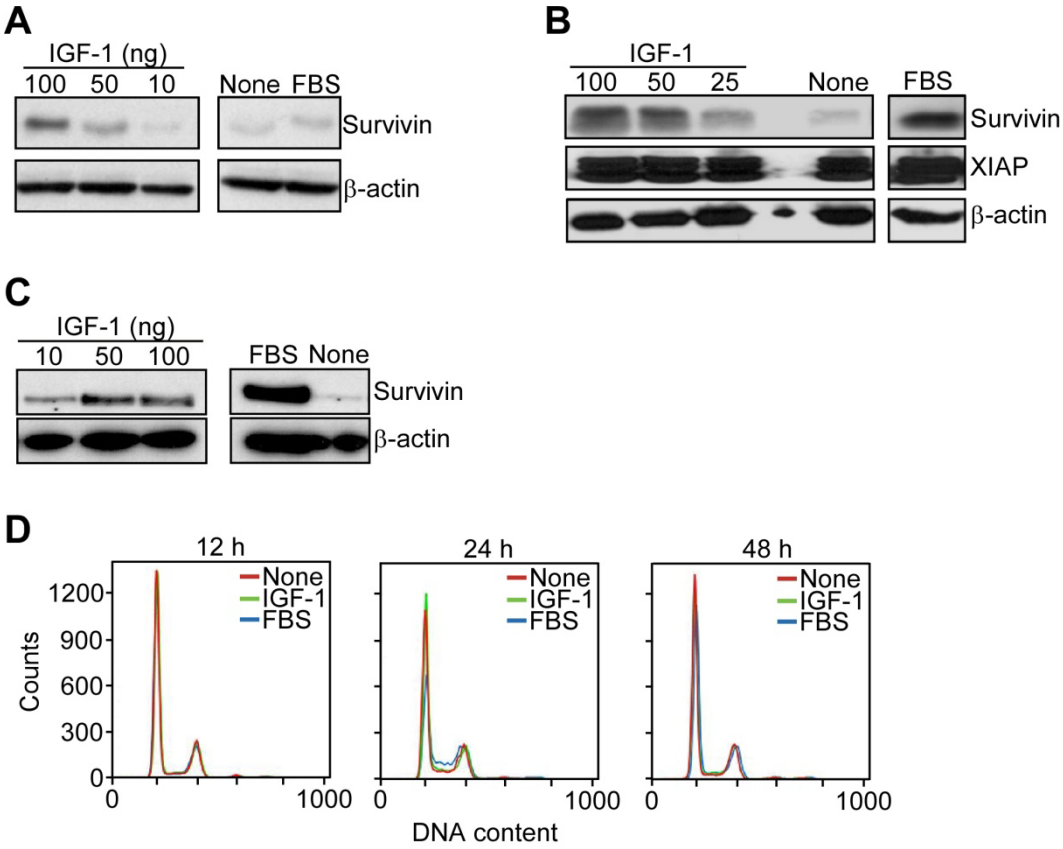


Figure 4-1. Regulation of survivin expression by IGF-1 stimulation.
A-C. Serum-deprived DU-145 cells were stimulated with the indicated concentrations of IGF-1 (ng/ml) or 10% FBS, and analyzed after 12 h (A), 24 h (B) or 48 h (C), by immunoblotting. D. Cell cycle analysis. Serum-deprived DU145 cells were stimulated with medium (None), 10% FBS or IGF-1 (100 ng/ml), and analyzed for DNA content by propidium iodide staining and flow cytometry at the indicated time intervals.

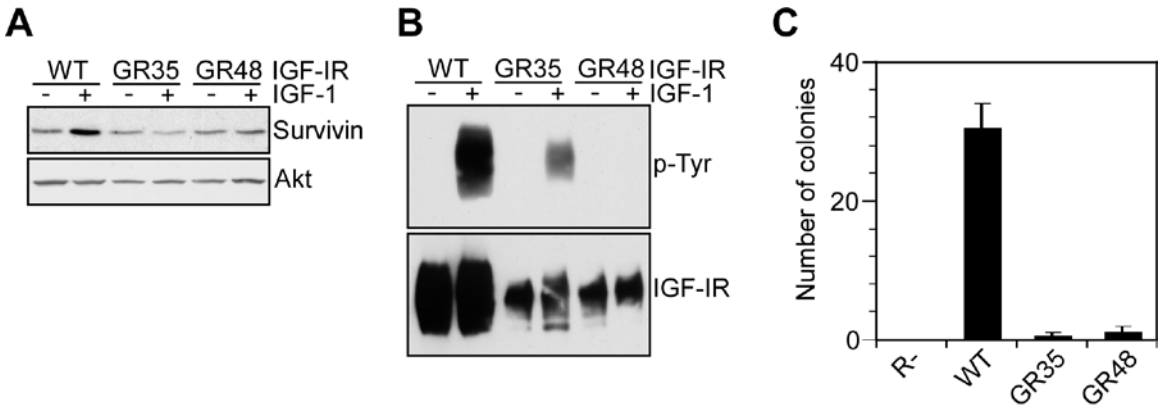


Figure 4-2. IGF-1R transforming potential required for IGF-1-induced survivin expression.

A. Modulation of survivin expression. MEF from IGF-1R null mice (R-) stably transfected to express WT, GR35 or GR48 mutant IGF-1R were stimulated with IGF-1, and analyzed by immunoblotting. B. IGF-1R tyrosine phosphorylation. Cells were stimulated with IGF-1 (100 ng/ml), and lysates were immunoprecipitated with an antibody to IGF-1R, followed by immunoblotting. p-Tyr, tyrosine-phosphorylated proteins. C. Colony formation assay. The indicated transfected cells were seeded in semisolid medium, and colonies larger than 100 μm were counted after 14 d. Data are the mean±SEM of triplicates.

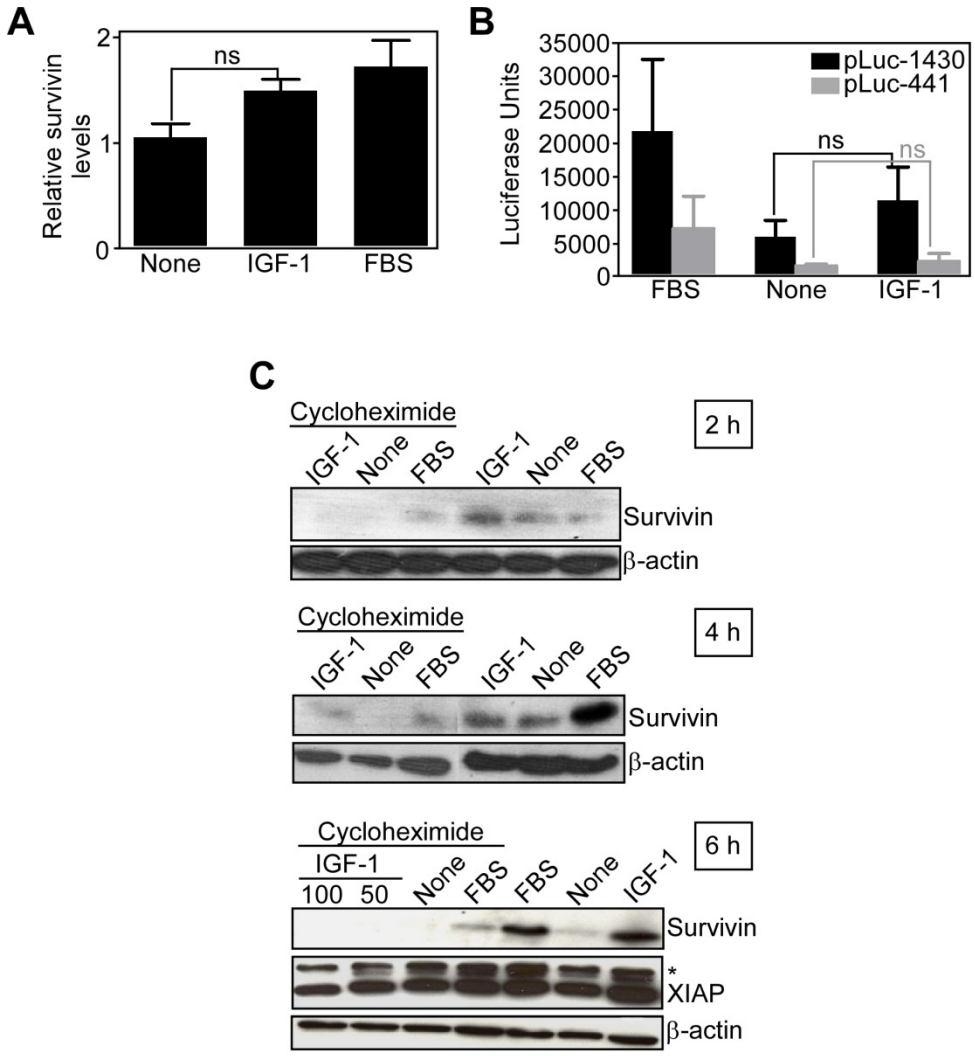


Figure 4-3. Requirements for IGF-1 induction of survivin.
A. Semi-quantitative PCR. Serum-deprived DU145 cells were stimulated with medium (None), IGF-1 (100 ng/ml) or 10% FBS, and analyzed for changes in survivin mRNA levels by GAPDH-normalized semi-quantitative PCR. Data are the mean±SD of two independent experiments. ns, not significant. B. Promoter analysis. DU145 cells were co-transfected with survivin-luciferase promoter constructs pLuc-441 and pLuc-1430, stimulated with IGF-1 (100 ng/ml) or 10% FBS for 12 h, and analyzed for β-galactosidase-normalized luciferase activity. Data are the mean±SD of two independent experiments. ns, not significant. C. Effect of cycloheximide on survivin levels. Serum-deprived DU145 cells were stimulated with IGF-1 (ng/ml) or 10% FBS, incubated with cycloheximide, and analyzed at the indicated time intervals by immunoblotting. *, non specific.

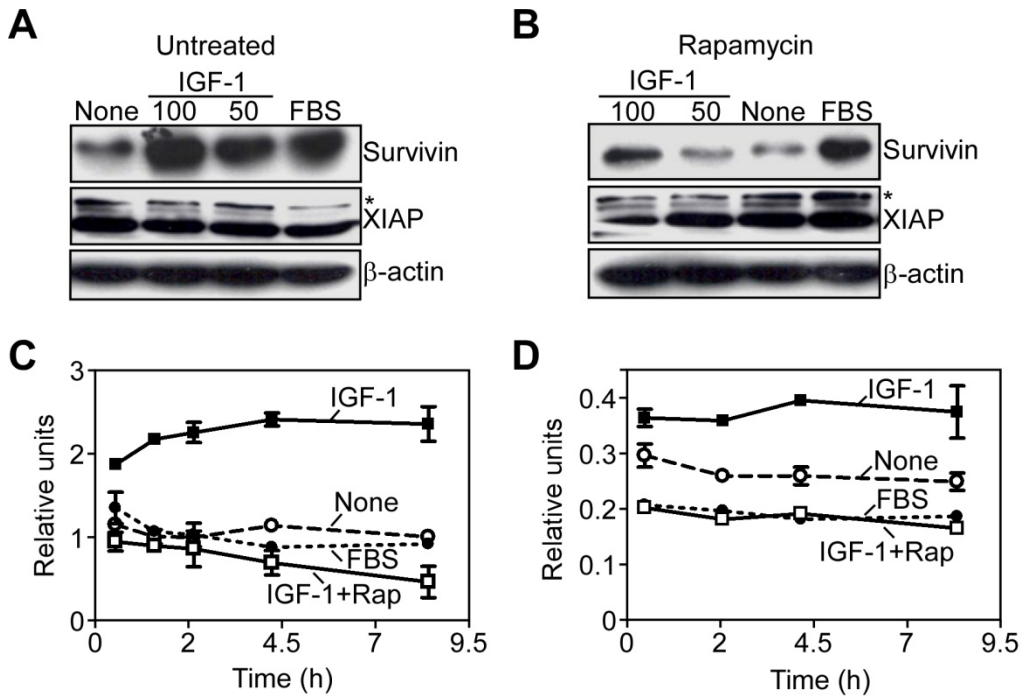


Figure 4-4. Modulation of IGF-1 induction of survivin by rapamycin.
A, B. Effect of rapamycin on survivin induction by IGF-1. Serum-deprived DU145 cells were stimulated with the indicated concentrations of IGF-1 (ng/ml) or 10% FBS, maintained in the absence (A) or presence (B) of rapamycin, and analyzed by immunoblotting after 24 h. *, non specific. C, D. Modulation of survivin levels after transcriptional inhibition. Serum-deprived DU145 cells were treated with the transcriptional inhibitors, actinomycin D (C), or DRB (D), stimulated with 10% FBS or IGF-1 (100 ng/ml) in the presence or absence of rapamycin, and analyzed at the indicated time intervals for GAPDH-normalized survivin mRNA expression by semi-quantitative RT-PCR. Data are the mean±SD of two independent experiments.

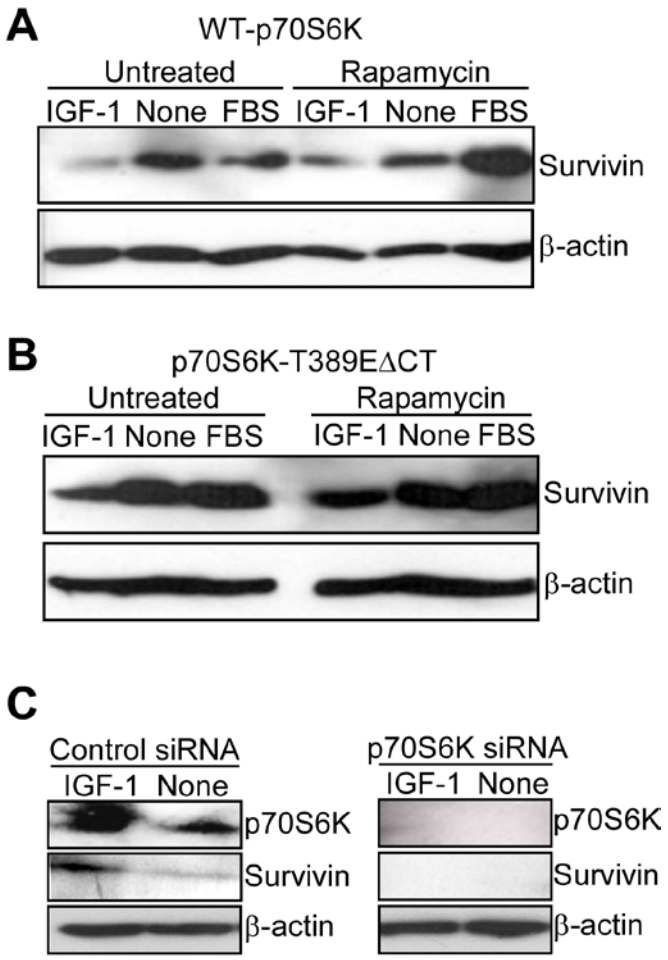


Figure 4-5. Requirement for p70S6K in IGF-1 modulation of survivin. Serum-deprived DU145 cells were transfected with WT (A) or T389EΔCT mutant (B) p70S6K cDNA, stimulated with IGF-1 (100 ng/ml) and analyzed in the presence or absence of rapamycin by immunoblotting after 24 h. C. siRNA silencing of p70S6K. Serum-deprived DU145 cells were transfected with control dsRNA oligonucleotide (*left panel*) or p70S6K-directed siRNA (*right panel*), treated with IGF-1 (100 ng/ml) or 10% FBS, and analyzed by immunoblotting after 48 h.

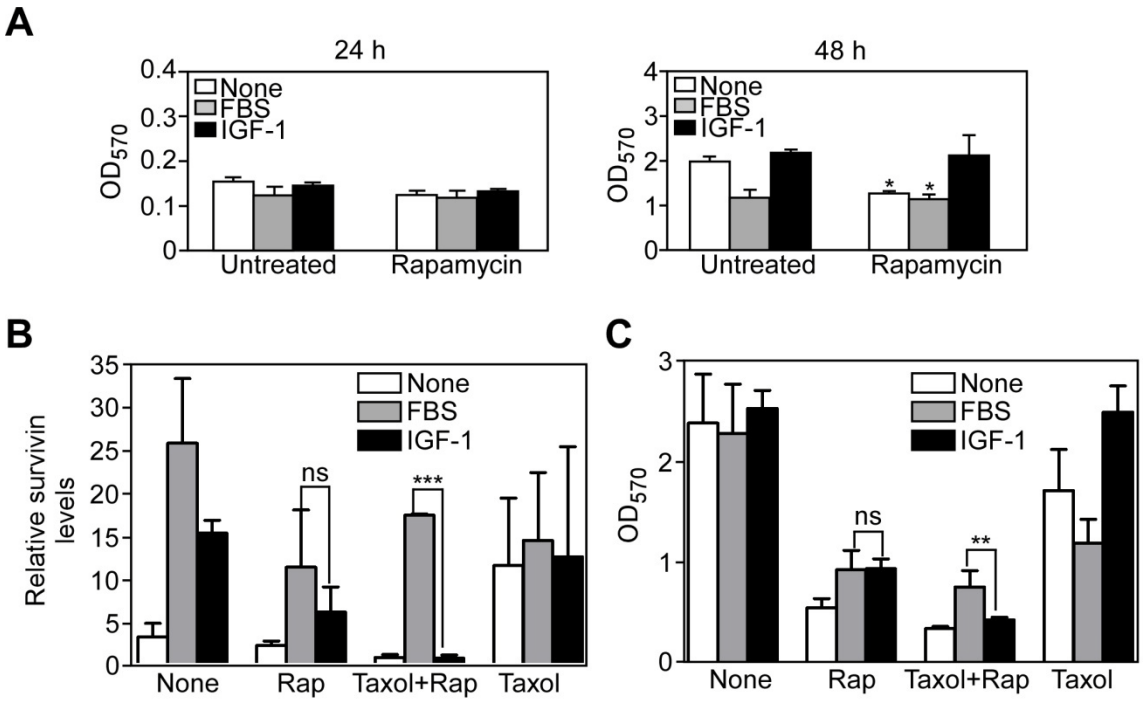


Figure 4-6. Effect of mTOR targeting on DU145 cell viability.
A. Rapamycin treatment. Serum-deprived DU145 cells were left untreated or treated with rapamycin, stimulated with 10% FBS or IGF-1 (100 ng/ml), and analyzed for cell viability by MTT after 24 h or 48 h. *, p = 0.021-0.032. Data are the mean±SEM of triplicates of a representative experiment out of at least two independent determinations. B. Modulation of survivin expression by taxol-rapamycin combination. Serum-deprived DU145 cells were treated with the combination of taxol plus rapamycin, harvested after 48 h and β-actin-normalized survivin levels were determined by quantitative immunoblotting and densitometry. ns, not significant; ***, p=0.0004. Data are the mean±SD of two independent experiments. C. Cell viability. The experimental conditions are as in B, except that DU145 cells were analyzed for cell viability by MTT. ns, not significant; p=0.0046. Data are expressed as mean±SEM of replicates of one experiment out of at least two independent determinations.

Chapter 5. Final Thoughts and Future Directions

Profiling of cancer cells overwhelmingly demonstrates the heterogeneity of human cancers (Velculescu, Madden et al. 1999; Griffin, Pole et al. 2003; Lu, Getz et al. 2005; Wood, Parsons et al. 2007), making targeting single molecules difficult in terms of cancer treatment. Despite this complexity, network analysis reveals that cells rely on a few critical nodes which serve as hubs to redistribute biological information (Ravasz, Somera et al. 2002; Albert 2005). Survivin, the cell death inhibitor and mitotic regulator, may act as one such hub. This body of work solidifies survivin as a nodal protein by identifying two novel signaling cascades, Notch and IGF-1, which contribute to survivin expression and tumorigenesis.

Notch signaling orchestrates a developmental pathway often exploited by tumors to regulate stem cell maintenance (van Es, van Gijn et al. 2005), modulate cell death (Beverly, Felsher et al. 2005; Liu, Hsiao et al. 2007), and control cell cycle progression (Ronchini and Capobianco 2001). We found that Notch-1 is preferentially expressed in breast cancer, as compared to normal tissue, and correlates with abbreviated disease survival. Both *Notch-1* and *survivin* selectively co-segregated in patients with basal breast cancer, a breast cancer subtype with poor prognosis and lack of molecular targeted therapies. Survivin was identified as a target of Notch-1 based on molecular studies of novel RBP-Jκ sites in the proximal *survivin* promoter (Lee, Raskett et al. 2008). Transcriptional activation of survivin by Notch signaling follows cell cycle periodicity of survivin, elevating survivin throughout the cell cycle, enhancing cell cycle kinetics and cell proliferation (Lee, Raskett et al. 2008). Targeting of Notch signaling via a γ-

secretase inhibitor decreases survivin expression, promotes cell death, inhibits local and systemic tumor growth in vivo (Lee, Raskett et al. 2008).

In our analysis of Notch-1 and survivin in breast cancer, we demonstrate that Notch-1 is overexpressed in breast tumors and correlated with poor prognosis (Figure 2-1 and Figure 2-2). However, we lack immunohistochemical data to demonstrate that survivin and Notch-1 are co-expressed in basal breast cancers. This can be resolved by scoring Notch-1 and survivin levels in immunohistochemical staining of known basal breast cancers. While the microarray analysis strengthens the co-segregation of Notch-1 and survivin in basal breast cancers, several issues remain. The association of *Notch-1* and *survivin* in basal breast cancers is based on the co-segregation of *Notch-1* or *survivin* with *keratin-5* in ER-negative cancers (Figure 2-3). Formally however, lack of ER is not completely interchangeable with basal breast cancers (Cleator, Heller et al. 2007). Furthermore, *keratin-5* expression is one of multiple markers for basal breast cancer therefore using solely *kertain-5* and ER status as basal markers limits the strength of the study. To reinforce the *Notch-1*/*survivin* association, other Notch-1 targets can be assayed for co-expression with *survivin*. Based on preliminary analysis of Oncomine data, the Notch target *c-myc* is selectively increased in ER-negative versus ER-positive breast cancers. However, *Hes-1* and *Hey-1*, two other Notch targets, are not overexpressed in ER-negative versus ER-positive breast cancers (data not shown). Ideally, microarray data from each study should be individually downloaded and re-clustered to determine the association of *Notch-1* and *survivin* with basal breast cancers. Despite these flaws, the strength of the existing study lies in the number of patients and

the collective trend demonstrating the association of *Notch-1*, *survivin*, *keratin-5*, and ER-negativity.

The significant but modest Pearson's correlation coefficients between *Notch-1*/*survivin*, *survivin*/*keratin-5*, and *Notch-1*/*keratin-5* may reflect the inaccuracies of microarray analysis in heterogeneous tumor populations (Figure 2-3). Microarray analysis solely ascertains the predominant gene transcript levels within this cellular assortment. Meanwhile, subpopulations of tumor cells with basal characteristics may be masked by a predominantly luminal population. Laser microdissection or sorting of homogenized tumors for basal, luminal, Her2/neu, and stromal populations followed by microarray analysis may provide a better perspective of gene correlations.

Microarray analysis also fails to account for tumor variability over time. In vivo, a tumor population fluctuates in composition depending on external factors. Stem-like cells with Notch expression and a basal breast cancer phenotype increase in population after stimulation with radiation or interleukin-6 (Phillips, McBride et al. 2006; Sansone, Storci et al. 2007). Furthermore, treatment with trastuzumab may reorganize cellular signals to render the cells responsive to GSI inhibition (Osipo, Patel et al. 2008), possibly by encouraging a shift to a basal phenotype. The factors that regulate plasticity of tumor cells have yet to be determined. Possibly, cellular reorganization requires the participation of stem cell transcription factors like Oct-4, Nanog and activation of stem cell pathways like Notch. Notch activation creates a pro-survival subcellular environment through survivin induction as well as direct changes in transcription patterns. Microarray of tumors before and after any external stimuli may reveal genetic

patterns associated with drug response, and aggressive disease (Ramaswamy and Golub 2002).

Basal breast cancers possess embryonic stem cell like qualities (Ben-Porath, Thomson et al. 2008). Likewise, survivin is associated with stem cell gene signatures (Pennartz, Belvindrah et al. 2004; Marconi, Dallaglio et al. 2006; Taubert, Wurl et al. 2007) and the Wnt/β-catenin developmental pathway controls survivin expression in colonic progenitor cells (Kim, Plescia et al. 2003). Survivin is also expressed in $CD44^{+}/CD24^{-/low}$ putative breast cancer progenitor cells (Ponti, Costa et al. 2005). Notch-1 mediated transcription of *survivin* suggests a larger role of survivin as a candidate target in stem cell biology. As such, the embryonic transcription factor Oct-4 indirectly enhances *survivin* transcription via collaboration from other transcription factors like STAT3 (Guo, Mantel et al. 2008) and possibly Notch/RBP-Jκ. In these stem cell populations, transcriptional activation of *survivin* may elevate the apoptotic threshold, protect the cells from environmental stress, and ensure genomic fidelity during mitosis. As the isolation of stem cells and cancer stem cells improve, we will be able to determine the functions of survivin and Notch in stem cell populations.

A key aspect of our Notch-1/survivin axis is our reliance on ER status as a differentiating marker. In retrospect, other characteristics, like expression of E-cadherin, also delineate the two groups (Table 5-2). Expanding the analysis in additional cell lines or primary breast cancers may help identify the fundamental biomarkers or breast cancer subtypes that associates with the Notch/survivin transcription axis. The contributions of ER, E-cadherin, or other biomarkers to survivin regulation also compound the issue of Notch driven survivin transcription. E-cadherin expression represses *survivin*

transcription by promoting caveolin-1 sequestration of β-catenin, a transcriptional activator of *survivin* (Torres, Tapia et al. 2007). Similarly, estrogen signaling may influence survivin transcription although estrogen responsive elements have yet to be identified in the *survivin* promoter. Therefore, the effects of Notch-1 on *survivin* transcription may be augmented by existing survivin regulatory pathways in these cells. Most likely, cancer cells enlist multiple pathways to fine tune survivin expression.

Notch activation drives transcription by converting RBP-Jκ from a transcriptional repressor to an activator. RBP-Jκ recognizes the DNA consensus sequences GTGGGAA and TTCCCAC (Ling, Hsieh et al. 1994; Tun, Hamaguchi et al. 1994; Lam and Bresnick 1998). The *survivin* promoter in mouse and man lack the complete 7 bp consensus sequence but contain several TGGGA elements which slightly deviate from the canonical motif. Despite this difference, RBP-Jκ physically associates with the *survivin* promoter during active transcription suggesting flexibility in the RBP-Jκ binding motif, at least in cancer cells (Figure 3-2 and Figure 3-3). The TGGGA element also serves as a partial NF-κB binding element (Oswald, Liptay et al. 1998). Coincidentally, *survivin* transcription is also regulated by NF-κB (Kawakami, Tomita et al. 2005). Therefore, the cellular context in terms of transcriptional co-factors or external stimuli potentially functions as a switch between Notch and NF-κB driven transcription of *survivin*. Given the overlap between RBP-Jκ and NF-κB binding elements, it is possible that Notch and NF-κB signals can non-discriminately activate both NF-κB and RBP-Jκ transcription sites. This can be tested by assessment of *survivin* transcription in the context of Notch inactivation and NF-κB stimulation, or vice versa. Substitution of transcription factors

from independent signaling pathways may be a novel component of tumor or stem cell plasticity.

Our analysis of Notch targets focused selectively on survivin and other anti-apoptotic molecules. However, Notch signaling primarily had a functional significance on cell cycle modulation. Of note, the Notch targets cyclin D1 (Ronchini and Capobianco 2001) and SKP2 (Sarmento, Huang et al. 2005) can accelerate G1 and S-phase progression (Table 1-1). Therefore, survivin may not be the sole modulator of Notch driven cell cycle progression. Future experiments will be needed to determine whether Notch can induce cyclin D1 and SKP2 transcription in breast cancer cells. Likely, Notch affects multiple genes regulating multiple phases of the cell cycle including mitotic entry and exit. In our experiments, G1 and S-phase progression was less dramatically enhanced by Notch activation, as noted by overlapping cell cycle profiles prior to mitosis (Figure 3-4B). The excess of survivin at mitosis induced by Notch signaling possibly ensures proper alignment of chromosomes at the metaphase plate, formation of a functional bipolar spindle, and execution of cytokinesis (Beltrami, Plescia et al. 2004).

Furthermore, the ability of Notch to rescue cells from mitotic cell death caused by T34A survivin attests to the specificity of Notch/survivin axis to protect mitotic progression (Figure 3-4D). However, Notch activation elevates survivin protein levels at nearly all phases of the cell cycle suggesting that a second, non-mitotic role of Notch mediated survivin expression. Notch, like survivin, is reported to protect cancer cells from a myriad of chemotherapeutic agents (Altieri 2003; Dohi, Beltrami et al. 2004; Stylianou, Clarke et al. 2006). In addition to protecting mitotic progression, Notch

mediated survivin expression may promote chemotherapeutic resistance and encourage survival of rare cancer progenitor populations. Exogenous survivin alone prevents cell death induced by GSI although GSI reduced expression of two other survival proteins, Bcl-2 and XIAP, suggesting the importance of survivin in the Notch survival signal (Figure 3-6C and Figure 3-5A). While Notch is thought to inhibit apoptosis by interfering with ubiquitin mediated degradation of XIAP (Liu, Hsiao et al. 2007), we did not observe consistent elevation of XIAP protein levels after Notch transduction (Figure 2-4A and Figure 3-1A). This suggests that XIAP downregulation by GSI, at least in our breast cancer cells, is a result of protein instability caused by survivin depletion (Dohi, Okada et al. 2004). To complete our understanding of the Notch/survivin survival pathway, exogenous survivin can be used to rescue Notch-1 ablation, bypassing criticisms of GSI off-target effects. Possibly, the mitotic and apoptotic effects of Notch depletion overlap. To address this possibility, we can treat synchronized cells with GSI and characterize the cell death and cell cycle profiles in an attempt to segregate the two functions of survivin in the Notch pathway.

Activating Notch mutations, which bypass juxtacrine ligand stimulation, are prevalent in T-cell ALL (Weng, Ferrando et al. 2004). However, such mutations have yet to be described in human breast cancer, although genomic analysis suggests that the Notch locus is a mutational hotspot (Wood, Parsons et al. 2007). Therefore, we must assume that ligand association initiates the Notch pathway. In our studies, we observed elevated survivin protein expression in cells stimulated by Jagged-1 ligand (data not shown). Like, Notch-1, Jagged-1 has been associated with decreased overall survival and increased metastasis in human breast cancers (Reedijk, Odorcic et al. 2005; Reedijk,

Pinnaduwage et al. 2007). Normal breast tissues express Jagged-1, -2, and Delta-like-4, any of which may engage the Notch receptor (Stylianou, Clarke et al. 2006). In normal tissue, Notch signaling directs cell fate decisions by lateral signaling which compel equipotent cells to express either the Notch receptor or the ligand (Radtke and Raj 2003). However, tumor cells lose this regulatory component and express both receptor and ligand. Translated in vivo, this implies that tumor, or stromal, cells can act as both activator and receiver of Notch signaling. Furthermore, F3/contactin (Hu, Ang et al. 2003) and excess calcium (Raya, Kawakami et al. 2004) can promiscuously activate the Notch receptor leading to aberrant signaling in a tumor environment.

While our studies were limited to the Notch-1 receptor, other Notch receptors also activate RBP-Jκ transcription and promote breast cancer development. In particular, Notch-3 may regulate cell proliferation in ErbB2 negative cells (Yamaguchi, Oyama et al. 2008). Intriguingly, overexpression of Notch-2 is correlated with low grade cancers and improved survival (van de Vijver, He et al. 2002). Recruitment of different transcriptional co-factors may explain the contradictory effects of Notch-2. Finally, Notch-4 inhibition with a Notch-4 specific antibody reduces formation of mammospheres derived from primary ductal carcinoma in situ (Farnie, Clarke et al. 2007). Characterizing the tumorigenic potential of each Notch receptor will aid in the development of receptor specific therapeutics, potentially with reduced side effects.

In the context of breast cancers, Notch targeting is hotly pursued as a cancer therapeutic. GSI treatment in our xenograft basal breast cancer model reduces local and systemic disease (Figure 3-7). Notch signaling in certain cell types can induce epithelial mesenchymal transition and metastasis (Timmerman, Grego-Bessa et al. 2004; Leong,

Niessen et al. 2007) while survivin expression is correlated to metastatic disease (van 't Veer, Dai et al. 2002; Paik, Shak et al. 2004). Further work will be needed to characterize the molecular contributions of survivin to invasion and metastasis. While GSI treatment inhibited tumor growth, a limitation in our studies is the absence of in vivo tracking of Notch inhibition. Attempts to stain tumor sections with survivin were unsuccessful (data not shown) but alternative methods may be used to monitor Notch activity. Whole spleens can be assayed for Notch activity in T-cells (Palaga, Miele et al. 2003). Alternatively, tumors can be resected at earlier timepoints to facilitate immunohistochemical staining for Notch, survivin, or caspase activity.

Notch inhibition with GSI carries a concern of severe toxicities. GSI treated mice lose transient amplifying cells in the intestine leading to goblet cell metaplasia within the colonic crypt (Milano, McKay et al. 2004; Wong, Manfra et al. 2004). Furthermore, Notch signaling is a critical component of T-cell development (Robey, Chang et al. 1996) and peripheral T-cell function (Palaga, Miele et al. 2003). While GSI toxicities may be a result of the mouse background, the type of GSI, and the purity of the GSI, the potential toxicities are concerning and warrant scrutinization in human studies. However, GSI treatment in our mice show limited toxicities with effective tumor inhibition suggesting that GSI can be safety administered (Figure 3-7). GSI trials in human breast cancer (NCT00106145 and NCT00645333) encourages optimism as side effects, while severe, are likely to be manageable with intermittent dosing (Krop, Kosh et al. 2006).

In contrast to the Notch-survivin transcriptional signaling axis, analysis of IGF-1 signaling in prostate cancer uncovered a post-transcriptional regulatory component of survivin expression. Early state prostate cancer is dependent on androgens for growth

and survival, therefore, the main treatment options center on androgen removal by surgery, radiation or hormone ablation. As the disease progresses, the prostate cancer cells develop insensitivity to androgen signaling and the cancer recurs. IGF-1 signaling increasing survivin may contribute to the development of androgen independence by elevating the apoptotic threshold. In prostate cancer cells, IGF-1 stimulation increases expression of survivin by stabilization and translation of survivin mRNA (Vaira, Lee et al. 2007). Interfering with signals downstream of IGF-1 with IGF-1R mutants, rapamycin or gene silencing of p70S6K abrogated IGF-1-survivin signaling and sensitized prostate cancer cells to traditional cytotoxics (Vaira, Lee et al. 2007). This translational mechanism of survivin expression is independent of transcriptional regulation or protein stabilization of survivin and unaffected by cell cycle progression. The rapid tuning of survivin levels allows cells to respond instantaneously to fluctuations in the tumor microenvironment. Persistent activation of IGF-1/survivin signaling provides the tumor with an increased threshold to apoptotic induction, promoting the development of anti-cancer therapy resistance and heralding the onset of clinically aggressive disease (Oh, Jin et al. 2008).

Although our study focused on IGF-1R homodimerization, the IGF-1R receptor can dimerize with several other receptors. Heterodimerization of IGF-1R with EGFR (Morgillo, Woo et al. 2006), ErbB2 (Nahta, Yuan et al. 2005), and the insulin receptor (Pandini, Frasca et al. 2002) is reported to contribute to drug resistance. IGF-1R and ErbB2 heterodimerization in breast cancers cells results in resistance to trastuzumab (Nahta, Yuan et al. 2005). In non-small cell lung cancer, the heterodimerization of IGF-1R to EGFR promotes drug resistance to erlotinib by stimulating mTOR dependent

protein synthesis of survivin (Morgillo, Woo et al. 2006). Heterodimerization of IGF-1R in prostate cancer may trigger accumulation of survivin protein in response to anti-hormonal therapies. This may be a mechanism for prostate cancer cells to evolve toward androgen independence. Furthermore, heterodimerization may confer tissue or cell type specificity to the IGF-1R/survivin pathway.

The IGF-1 signal transduction pathway can activate many pathways including the MAP kinase pathway, the ERK pathway, and the PI3K pathway (Craparo, O'Neill et al. 1995; Ryan and Goss 2008). In acute myeloid leukemia, both the ERK and PI3K pathway induces survivin mRNA and protein (Carter, Milella et al. 2001). In prostate cancer, IGF-1 predominantly regulates survivin translation through downstream signaling via PI3K. However, given the importance of the MAPK and ERK pathways in tumorigenesis, they may be explored for a contribution to survivin regulation in the context of IGF-1 signaling.

The current study utilizes transcriptional and protein synthesis inhibitors to track mRNA translation (Figure 4-4). While these methods are sufficient, more rigorous procedures can complement the existing work. Northern blotting of survivin mRNA associated with polyribosomes can reveal translation patterns of survivin splice variants under conditions of IGF-1 stimulation (Li, Bor et al. 2006). Survivin splice variants have a conserved N-terminal suggesting that the same promoter elements regulate their protein expression (Sampath and Pelus 2007). Therefore, post-transcriptional mechanisms like IGF-1 regulation of mRNA translation may regulate expression of survivin splice variants. Preferential translation of splice variant survivin-ΔEx3 may provide enhanced apoptotic threshold since the splice variant can interact with Bcl-2 to inhibit caspase 3

(Wang, Sharp et al. 2002). Likewise, regulation of survivin translation may control intracellular survivin availability by influencing subcellular localization (Dohi, Beltrami et al. 2004), phosphorylation (Dohi, Xia et al. 2007) or ubiquitination (Vong, Cao et al. 2005) which can be tracked with ^{35}S-methionine. Pulse-chase labeling with ^{35}S-methionine will also provide a precise analysis of protein stability.

The IGF-1 translational control of survivin was inhibited by rapamycin which is primarily a mTORC1 inhibitor. However, evidence suggests that prolonged treatment with rapamycin could also inhibit mTORC2 activity (Sarbassov, Ali et al. 2006). Immunoblotting IGF-1 stimulated or rapaymcin inhibited cells for phosphorylated p70S6K1 can identify mTORC1 activity. To monitor mTORC2 activity, cell lysates can be analyzed for Akt activity although this may be a cell type dependent effect (Sarbassov, Ali et al. 2006). Alternatively, we may immunoprecipitate the mTOR complex to determine the raptor/rictor ratio to assess mTORC1 or mTORC2 activity.

The two pathways highlight the tissue specificity of survivin regulators. IGF-1 regulates survivin translation in prostate cancer cells (Vaira, Lee et al. 2007) and non-small cell lung cancer (Morgillo, Woo et al. 2006) but not in MCF-7 or MDA-MB-231 breast cancer cells (data not shown). This may reflect the differences in the microenvironments of each cancer tissue, the dominance of signaling pathways in particular cancer types, or the variability of genetic landscapes in different tumors. Our analysis of GSI sensitivity across different cell types suggests that other tumor types may also rely on Notch/survivin signaling (Supplemental Figure 3-10). Comparatively, PC3 prostate cancer cells, H1975 non-small cell lung cancer cells, and A431 squamous skin carcinoma cells are more sensitive to GSI than DU145 prostate carcinoma cells, H460

large cell lung cancer cells, HCT-116 colon carcinoma cells, HeLa cervical carcinoma cells, or U87MG glioblastoma cells (Supplemental Figure 3-10). However, a brief analysis of p53 status, EGFR expression, ER/PR/Her2 expression, and IGF-1R status does not reveal any pattern as to why some cell respond to IGF-1 while others respond to Notch (Table 5-2). A more comprehensive genomic comparison may reveal patterns associated with Notch or IGF-1 mediated survivin regulation.

Even so, further work will be required to determine why survivin regulation shows tissue specificity. One explanation is that changes in the transcriptional environment may affect survivin regulatory pathways. The tumor suppressor p53, which is frequently lost or mutated in tumor progression, represses the *survivin* promoter by recruiting chromatin deacetylation complexes (Mirza, McGuirk et al. 2002), and via direct inhibition of the *survivin* promoter (Hoffman, Biade et al. 2002). Similarly, differences in promoter associated transcription factors, chromatin structure, and DNA methylation patterns may help explain tissue specificity. In the future, a detailed identification of transcription factors, histones, and chromatin modifications associated with the survivin promoter can be determined using ChIP on chip technology.

Furthermore, Notch mediated expression of survivin was selectively activated in tumor cells compared to normal cell lines (Figure 3-1B and C). The IGF-1-survivin upregulation was not tested on normal cell types, but may follow a similar paradigm. This suggests that survivin regulatory pathways are different in normal versus tumor tissues. Multiple aberrant pathways may drive survivin expression in tumor cells because tumors rely on the dual nature of survivin to bypass mitotic regulation and overcome apoptotic stress (Altieri 2003). Fluctuations of survivin levels inflicted by survivin

antagonists may significant impair tumor viability. Conversely, normal tissues are less affected by changes in survivin levels because a minimal level of survivin is needed during mitotic progression. Since the majority of work analyzing the functions of survivin in mitotic regulation and apoptotic inhibition was conducted in tumor cells, characterization of survivin in normal tissue may reveal differences that can be manipulated to selectively target survivin in tumor tissue.

Our studies thus far suggest that cancers are "addicted" (Weinstein and Joe 2006) to survivin for survival. Basal breast cancers are "addicted" to the Notch/survivin signaling axis. The basal breast cancer cell types were more sensitive to GSI treatment compared to luminal breast cancer cell types and normal cell types (Figure 3-5C). In these cells, depletion of Notch resulting in reduced survivin becomes a fatal event. Androgen independent prostate cancers are possibly "addicted" to the survival environment created by IGF-1 modulation of survivin. This suggests that survivin regulation may be coupled to other "addictive" pathways depending on the tumor type. These partner pathways may include EGFR in non-small cell lung cancer and Wnt/β-catenin in colorectal cancer (Kim, Plescia et al. 2003). Identification of survivin partner pathways will reveal key "oncogenic addiction" pathways which can be targeted to propel us toward tailored cancer therapy. While these concepts require further characterization, survivin directed anti-sense molecules, dominant negative viruses, and transcriptional repressors are all in various phases of clinical development (Table 5-1) (Altieri 2008). Preliminary results are promising with phase I clinical trial data demonstrating tumor regression with minimal side effects (Tolcher, Antonia et al. 2006).

Existing data overwhelmingly place survivin in the center of a tumor network. Many regulators, protein interactors, or upstream signals of survivin are tumor suppressors or oncogenes exploited by tumor cells (Figure 1-1)(Altieri 2008). This work identifies two additional pathways that regulate survivin in tumorigenesis. IGF-1 signaling promotes translation of survivin mRNA while the Notch developmental pathway transcriptionally controls *survivin* expression. The integration of survivin into these networks strengthens the argument that survivin functions as a nodal protein. This nodal property of survivin is the rationale for developing survivin antagonists that may bypass tumor heterogeneity by debilitating multiple tumor specific pathways.

Therapy	Function	Stage
Molecular Antagonists		
Survivin RNAi	Survivin shRNA cocktail	Planned preclinical
Survivin Ribozyme	Hammerhead ribozyme targeting exon 1 of survivin	Planned preclinical
LY2181308	Survivin anti-sense drug	Phase II
Gene Therapy		
Dominant negative mutants C84A, T34A	Interfering mutants of survivin	Preclinical
Survivin gene promoter targeting	Survivin gene promoter driving cytotoxic gene expression	Planned preclinical
Immune Therapy		
Survivin Peptide Vaccine	immunotherapy	Phase I/II
Autologous CTL pulsed with survivin primed dendritic cells	Vaccine primed autologous T cells Survivin Sur1M2 peptide vaccine	Phase I/II
Small Molecule Inhibitors		
YM155	Inhibit survivin mRNA transcription	Phase II
EM1421	Inhibit Sp1 regulated survivin expression, Tetra-O-methylnordihydro-guaiaretic acid	Phase I
Flavopirodol	Inhibit cdc2 phosphorylation of survivin	Phase I/II
Shepherdin	Survivin derived antagonists	Preclinical

Table 5-1. Survivin antagonists in clinical development.
(Altieri 2008)

Cell Lines	Cell Type	GSI Sensitivity	ERα	PR	ErbB2	IGF-1R	PTEN	E-cadherin	P53	EGFR
MCF-7	Breast adenocarcinoma	No	+	+	-/low	+	-	+	+	+
MDA-MB-231	Breast adenocarcinoma	Yes	-	-	+/low	+	-	-	Mutant	+
HBL100	Breast carcinoma	Yes	-	-	-/low	+	+	-	+	+
Sum149	Breast carcinoma	Yes	-		-	+		-	+	+
T47D	Breast infiltrating ductal carcinoma	No	+	+	+low	+	+	+	Mutant	+
HeLa	Cervical carcinoma	No			+		+		+	+
HCT116	Colon carcinoma	No			-/low	+	+	inducible	+	+
U87MG	Grade III Brain Glioblastoma-astrocytoma	No			-	+	-		+	+
H1975	Lung Adenocarcinoma, NSCLC	Yes					+		+	Heterozygous missense mutation
H460	Lung carcinoma, Large cell lung cancer	No			+	+	+		+	+
DU145	Prostate adenocarcinoma Androgen dependent	No	-		-/low	+	+	+	Mutant	+
PC3	Prostate grade IV adenocarcinoma Androgen dependent	Yes	-		-/low	+	-	-	-	+
A431	Squamous cell carcinoma	Yes			+	+	+		Mutant	+

Table 5-2. Characteristics of select cancer cell lines.

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