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Identification of Novel STAT3 Target Genes Associated with Oncogenesis

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by

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DEDICATION

This dissertation is dedicated to my husband, Peter Haviland. Thank you for making me promise you that I would never give up!

I would also like to give special thanks and appreciation to Dr. Richard Jove for being a never ending source of encouragement and support despite all the odds. You are a true gift to the scientific community and a graduate student is blessed to have you as their mentor.

Thanks also go to my parents, Keith and Lyn Radbourne, for their unceasing support and faith in me throughout my whole life.

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NOTE TO READER

The original of this document contains color that is necessary for understanding the data. The original dissertation is on file with the USF library in Tampa, Florida.

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APP	acute phase protein
Bcl-2	B-cell lymphoma/leukemia-2
bp	base pairs
Cdk	Cyclin-dependent kinase
cDNA	complimentary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CHX	Cyclohexamide
DNA	deoxyribonucleic acid
E2F	Early 2 factor
EMSA	Electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
IL-6	interleukin-6
IRF	IFN regulatory factor
JAB	Jak-binding proteins
kDa	kilodalton
Mcl-1	Myeloid cell leukemia-1
NES	Nuclear export/exclusion sequence
NLS	Nuclear localization sequence

PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PIAS	protein inhibitor of activated STATs
PKC	Protein kinase C
P/S	Penicillin/streptomycin
Rb	Retinblastoma
RT	room temperature
SIE	Serum-inducible element
siRNA	Small inhibitory RNA
SOCS	suppressor of cytokine signaling
SSI	STAT-induced STAT inhibitor
STAT	signal transducer and activator of transcription
StIP1	STAT3-interacting protein
ul	micro liter

ABSTRACT

Cytokine and growth factor signaling pathways involving STAT3 are frequently constitutively activated in many human primary tumors, and are known for the transcriptional role they play in controlling cell growth and cell cycle progression. However, the extent of STAT3's reach on transcriptional control of the genome as a whole remains an important question. We predicted that this persistent STAT3 signaling affects a wide variety of cellular functions, many of which still remain to be characterized.

We took a broad approach to identify novel STAT3 regulated genes by examining changes in the genome-wide gene expression profile by microarray, using cells expressing constitutively-activated STAT3. Using computational analysis, we were able to define the gene expression profiles of cells containing activated STAT3 and identify candidate target genes with a wide range of biological functions. Among these genes we identified Necdin, a negative growth regulator, as a novel STAT3 target gene, whose expression is down-regulated at the mRNA and protein levels when STAT3 is constitutively active. This repression is STAT3 dependent, since inhibition of STAT3 using siRNA restores Necdin expression. A STAT3 DNA-binding site was identified in the Necdin promoter and both EMSA and chromatin immunoprecipitation confirm binding of STAT3 to this region. Necdin expression has previously been shown to be down-regulated in a melanoma and a drug-resistant ovarian cancer cell line. Further analysis of Necdin expression demonstrated repression in a STAT3-dependent manner in human melanoma, prostate and breast cancer cell lines.

These results suggest that STAT3 coordinates expression of genes involved in multiple metabolic and biosynthetic pathways, integrating signals that lead to global transcriptional changes and oncogenesis. STAT3 may exert its oncogenic effect by up-regulating transcription of genes involved in promoting growth and proliferation, but also by down-regulating expression of negative regulators of the same cellular processes, such as Necdin.

CHAPTER 1: INTRODUCTION

Signal Transduction and Oncogenesis

Normal cells have a network of molecular controls that tightly regulate growth and proliferation, preventing cell division in the absence of key environmental stimuli, such as mitogenic growth factors and signals from the extracellular matrix (ECM). Cancer cells have typically lost some of the molecular controls that regulate normal cell division, allowing them to divide in an unregulated manner even in the absence of extracellular cues (Hanahan and Weinberg, 2000).

Oncogenesis, or carcinogenesis, is the process by which normal cells are transformed into cancer cells. The initiation and promotion of cancer is a complex, multi-step process characterized by progressive cellular and genetic changes that reprogram a cell and lead to uncontrolled cell division and the formation of a malignant mass (Weinstein, 1987). Despite the fact that neoplastic development is a highly complex process, cancer cells do exhibit certain hallmarks or biological capabilities which are acquired during oncogenesis. These hallmarks include: sustaining a proliferative signal; evading growth suppressors; resisting cell death; enabling replicative immortality; inducing angiogenesis and activating invasion and metastasis (Hanahan and Weinberg, 2000), as well as the contribution of the tumor microenvironment (Hanahan and Weinberg).

The underlying trait of oncogenesis is genomic instability, which usually begins with changes in the expression of particular genes (proto-oncogenes and tumor suppressor genes), caused by mutations in DNA. This destabilization of the genome

during carcinogenesis results in changes in gene activity and stability (Vogelstein *et al.*, 2000) and affect many genes involved in cell cycle control, DNA damage responses and checkpoints, as well as DNA repair. The expression and activity of growth factors and their receptors and signaling molecules are often affected, thus disrupting the tightly controlled and orderly signal transduction processes that regulate cell growth and division.

Oncogenes are mutated versions of normal cellular genes (proto-oncogenes), which are capable of transforming a cell. They may contribute to the growth of a tumor by causing a cell to divide in an unregulated manner, particularly in the absence of normal growth signals. In contrast, tumor suppressor genes (TSGs) act as protective genes that usually limit or block one step in the development of tumors. A mutation in a TSG, or deletion of the gene, can predispose an individual to cancer by causing the loss of function of the tumor suppressor protein encoded by the gene (Knudson, 2002).

Oncogenic mutations are usually 'dominant', requiring a mutation in only one allele in order for the cellular phenotype to be affected. Unlike oncogenes, changes in tumor suppressor genes are usually recessive. Tumor suppressor genes follow a 'two-hit hypothesis' (Knudson, 1971), whereby both of the alleles that code for a particular gene just be affected before the biological function is sufficiently affected and the phenotype of the cell is altered.

Signal transduction pathways are the processes whereby the cell mediates the sensing and processing of stimuli and are essential for development, cell differentiation and homeostasis (Hanahan and Weinberg, 2000). These cascades act as molecular circuits capable of detecting, amplifying and integrating a diverse array of extracellular signals to generate appropriate intracellular responses. For example, an extracellular signaling molecule activates a receptor in the cell membrane, initiating a cascade of signaling events within the cell in response. In a two-step process, the extracellular

signaling molecule binds to a specific receptor on the cell membrane followed by the stimulation of a second messenger within the cell which propagates the signal into the cell to elicit a full physiological response (Taga and Kishimoto, 1997).

Multiple signal transduction pathways exist within a normal cell and their dysregulation is frequently associated with the malignant phenotype. The JAK (Janus tyrosine kinase)-STAT (Signal Transducer and Activator of Transcription) pathway is a classic example of an evolutionarily conserved signaling cascade that becomes disrupted in oncogenic cells (Darnell *et al.*, 1994). The JAK family tyrosine kinases and latent cytoplasmic transcription factor STATs coordinate to transform a wide array of intracellular and environmental stimuli into targeted gene expression, resulting in distinct phenotypic alterations (Darnell, 1996).

Signal Transducers and Activators of Transcription

Signal transducers and activators of transcription (STATs) are a family of latent transcription factors that normally become activated in response to various extracellular polypeptide ligands, including many cytokines and growth factors, through cytokine receptors, receptor tyrosine kinases, as well as various non-receptor tyrosine kinases, such as c-Src. STATs were originally identified as signal transduction molecules activated during the study of interferon signaling (Shuai *et al.*, 1993). STAT3 was originally discovered as being activated during signaling by IL-6 (Zhong et al., 1994). Since then IL-6 signaling through the JAK-STAT pathway has been well characterized (Aaronson and Horvath, 2002).

Seven mammalian STAT family members have now been identified and characterized, STAT1, 2, 3, 4, 5a, 5b and 6 (Ihle, 1996). They share similar structural features and mechanisms of activation. Localized in three chromosomal clusters, the family of transcription factors may have evolved by gene duplication (Copeland *et al.*,

1995). The STAT proteins consist of 750-850 amino acids and have several conserved domains that are required for STAT function (Figure 1):

The N-terminal 130 amino acid region of STAT proteins is necessary for the formation of tetramers via STAT dimer-dimer interaction, thus stabilizing DNA-binding at weak promoter-binding sites. This may occur in promoters with closely spaced tandem STAT binding sites. There is evidence that STAT1, STAT4, and STAT5 form higher order complexes (dimer:dimer or higher) on promoters where there are two or more neighboring STAT binding sites (John *et al.*, 1999; Vinkemeier *et al.*, 1996; Xu *et al.*, 1996). Cooperation between the dimers exists to allow the interaction, and is lost if the N-terminal domain of the STATs is deleted (Vinkemeier *et al.*, 1996; Xu *et al.*, 1996; Zhang *et al.*, 1999b).

The adjacent coiled-coil domain, between the N-terminal and DNA-binding domains, contains four long helices and allows interaction with other transcription factors and regulatory proteins, such as the interaction between STAT1 N-terminal and the histone acetyltransferase CBP/P300 (Zhang *et al.*, 1996). STAT3-mediated gene transcription is also enhanced by the binding of the transcription factor c-Jun to the coiled-coil region of STAT3. This region may also be involved in STAT3 recruitment to the receptor leading to tyrosine phosphorylation and downstream signaling, since mutation of Asp170 or, to a lesser extent, Lys177 in the alpha-helix 1 results in diminished binding of STAT3 to the receptor and also reduced STAT3 tyrosine phosphorylation (Zhang *et al.*, 2000a).

The DNA-binding domain is in the center of the STAT molecules and determines the specificity of binding of the different family members. All STATs bind to similar DNA sequences (TTN_5AA), most likely due to the highly conserved amino acid sequences of the DNA binding domains. Analysis of STAT binding to synthetic oligonucleotides revealed differences in the binding affinity between STAT proteins (Horvath *et al.*, 1995),

demonstrating that the space between the palindromic TT-AA core affects the selective binding of the STATs to their respective DNA elements. For example, a 4 bp core separating TT-AA results in selective binding of STAT3 dimers, whereas a 6 bp core leads to preferential binding of STAT6. Those sequences with a 5 bp core can bind several STAT members, although may demonstrate a preference towards one particular STAT protein. In addition, the specificity of DNA binding may also be affected by the composition of the STAT dimers, for examples STAT1-STAT3 heterodimers can bind different DNA elements to STAT 1 or STAT3 homodimers, leading to a further level of control and complexity (Horvath *et al.*, 1995).

The Src-Homology 2 (SH2) domain in the C-terminus functions to recruit STATs to tyrosine phosphorylated receptors and is also required for homo- and hetero-dimerization. Upon ligand stimulation, JAK-mediated phosphorylation of receptor tyrosine docking sites enables recruitment of STATs to the receptor and resultant STAT phosphorylation. This critical phosphotyrosine residue is located around amino acid 700 (Tyr 701 for STAT1 and Tyr705 for STAT3) adjacent to the SH2 domain and is required for STAT activation via reciprocal SH2-phosphotyrosine interactions between STAT monomers. The negatively charged phosphate of the tyrosine residue at the C-terminal end of the SH2 domain is stabilized by the positively charged arginine residue at the N-terminal of the partner STAT SH2 domain. These residues are critical for dimer formation, since mutation of either the tyrosine or arginine residues abolishes STAT dimerization (Yuan *et al.*, 2005).

At the C-terminus of the molecule is the transcriptional activation domain (TAD), required for transcriptional activation of target genes. STAT1, STAT3 and STAT4 share a conserved amino acid sequence in the C-terminus (LPMSP), in which the leucine and serine residues are required to achieve maximum transcriptional activity (Kovarik *et al.*, 2001; Sun *et al.*, 2006). Following cytokine or growth factor stimulation, the serine

residue becomes phosphorylated, which is a critical event for high levels of transcription (Zhang *et al.*, 1995). Interestingly, STAT1β and STAT3β, which both lack C-terminal regions, demonstrate reduced transcriptional activity (Dewilde *et al.*, 2008). Interaction of CBP/P300 with both STAT1 and STAT3 C-terminal regions has previously been described (Zhang *et al.*, 1996).

The linker region, between the DNA-binding and SH2 domains may be important for regulating transcriptional activity, since mutations in the linker region of STAT1 form a protein which can be tyrosine phosphorylated, dimerize, translocate to the nucleus and bind to DNA but fails to completely activate gene transcription (Yang *et al.*, 2002).

Figure 1. General structure of the STAT protein family. The STAT proteins contain functional protein domains.

Alternatively spliced isoforms have been described, apart from STAT2. STATs 1,3, (Darnell, 1997; Ihle and Kerr, 1995; Maritano *et al.*, 2004), 4 (Hoey *et al.*, 2003) and 5 (Wang *et al.*, 1996) are expressed as two isoforms, designated as α and β, which have different transcriptional activities.

Two forms of STAT3 exist: full length, wild-type STAT3 alpha (p92) and a truncated version STAT3 beta (p83) (de Koning *et al.*, 2000), both derived from the same gene by alternative mRNA splicing. Sequences in the 3' untranslated region of the STAT3 gene were previously identified as important modulators of RNA splicing and determine the balance between α and β isoforms. STAT3β lacks the 55 residue C-

terminal transactivation domain, which is replaced by seven alternative C-terminal residues (Caldenhoven *et al.*, 1996) and is expressed is a variety of cell types (Chakraborty *et al.*, 1996). STAT3β has the Tyr705 residue critical for dimerization, but lacks the ser727 residue. It can act as a dominant negative, although there is evidence to suggest that it may regulate distinct genes itself.

Activation of STATs in Normal Signal Transduction

Signal transducers and activators of transcription (STATs) are a family of latent transcription factors that are usually present in an inactive form in the cytoplasm and become activated by tyrosine phosphorylation in response to various extracellular polypeptide ligands, including many cytokines and growth factors, through cytokine receptors, receptor tyrosine kinases, as well as various non-receptor tyrosine kinases, such as c-Src and members of the Janus kinase (JAK) families (Figure 2). Once phosphorylated (on a single C-terminal tyrosine residue), STATs form homo- or hetero-dimers by the interaction of the SH2 domain of one monomer with the phosphorylated tyrosine residue of the other monomer (Figure 1). The dimers then translocate to the cell nucleus, bind to specific promoter sequences of target genes and activate their transcription. The STAT proteins are subsequently de-phosphorylated and return to the cytoplasm, thus terminating the signaling pathway (Haspel *et al.*, 1996).

Various modes of activation have been described for STATs:

Classical JAK-STAT pathway. STATs become activated during cytokine signaling. Cytokine binding to receptors leads to dimerization of the receptors followed by activation of the receptor-associated Janus Kinases (JAKs). The JAKs then phosphorylate tyrosine residues in the intracellular domain of the receptor to provide docking sites for latent cytoplasmic STATs to bind (e.g. pYXXQ in gp130 receptor for

STAT3 binding). STATs then bind the receptor via their SH2 domain allowing JAKs to phosphorylate the STATs on a specific tyrosine residue in their cytoplasmic tail. Reciprocal binding of this pTyr in one monomer to the SH2 domain of a partner monomer allows homo- or hetero-dimerization of the proteins. Once released from the receptor, the dimers translocate to the cell nucleus and bind to specific DNA sequences to activate the transcription of cytokine-responsive genes (Akira, 1997).

Growth factor receptors. STATs are also activated directly by receptors with intrinsic tyrosine kinase activity or indirectly via JAKs. Such receptors include the EGF, PDGF and FGF receptors (Garcia *et al.*, 1997; Ruff-Jamison *et al.*, 1994; Sahni *et al.*, 1999)

Non-receptor tyrosine kinases. Non-receptor tyrosine kinases, such as v-Src, v-abl, v-Sis, v-Fps (Silva, 2004; Turkson *et al.*, 1998) and polyoma virus middle T antigen can induce constitutive STAT activation (Garcia *et al.*, 1997).

G-protein coupled receptors (GPCR). GPCRs, including chemokine receptors, can activate STAT1 and STAT3 upon chemokine binding e.g. MCP-1 and RANTES receptors (Buettner *et al.*, 2007; Ram and Iyengar, 2001).

Adaptor proteins. Activation of STATs can be mediated by other adaptor proteins which serve to bring JAKs in close proximity to STATs.

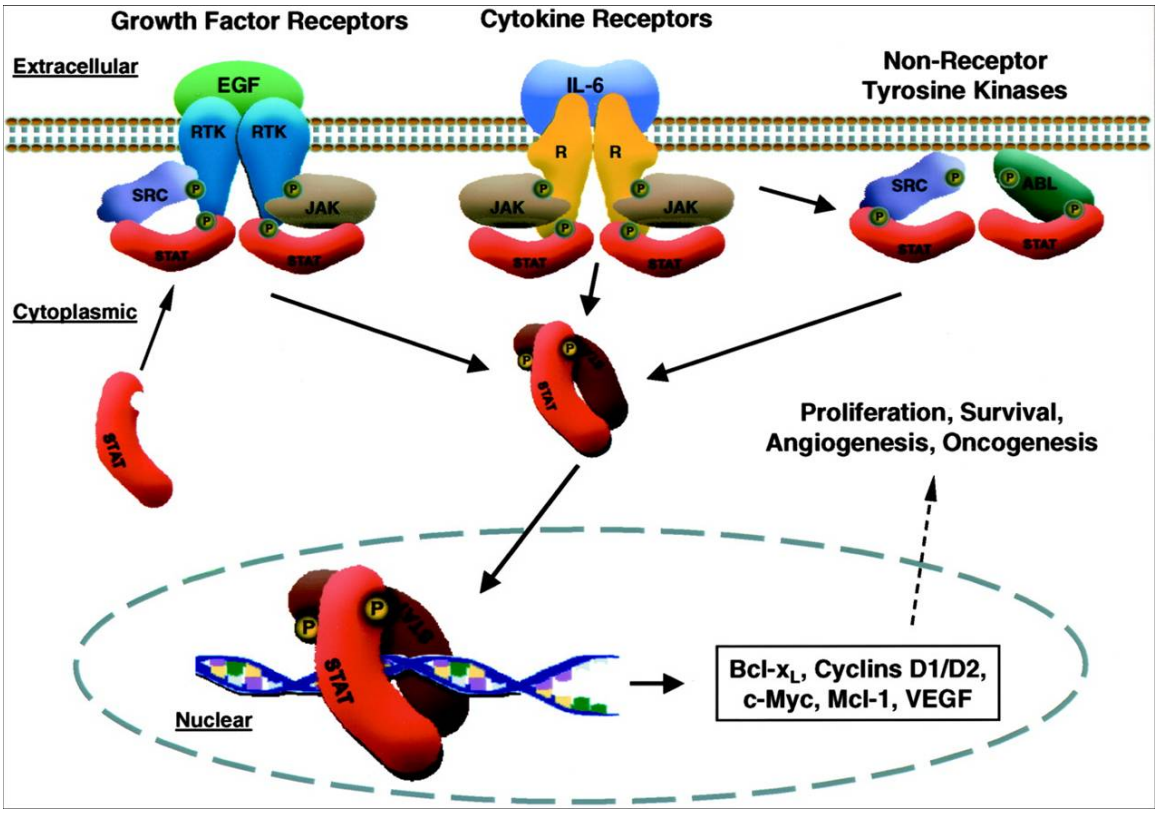

Figure 2. Normal and oncogenic STAT signaling pathways. Stimulation of cells with growth factors or cytokines results in dimerization of their cognate receptors and activation of intrinsic receptor tyrosine kinase activity (as shown for the EGF receptor tyrosine kinase, *RTK*) or activation of receptor-associated tyrosine kinases such as JAKs (as shown with the IL-6 cytokine receptor, *R*). Both receptor intrinsic and associated tyrosine kinases can subsequently phosphorylate the receptor cytoplasmic tail to provide docking sites for the recruitment of monomeric, non-phosphorylated STATs via their SH2 domain. Once STATs are recruited to activated tyrosine kinases, they become themselves substrates for tyrosine phosphorylation. Although receptor-associated tyrosine kinases such as JAKs and Src can cooperate in STAT activation by both growth factor and cytokine receptors, oncogenic forms such as Src and Abl can also phosphorylate STATs independently of receptor engagement. Phosphorylation of STAT monomers induces their dimerization via reciprocal phosphotyrosine-SH2 domain interactions and translocation of STATs to the nucleus, where the dimers bind to specific STAT DNA-response elements and directly regulate gene expression. In normal cells, STAT-mediated gene regulation is both transient and tightly regulated, whereas constitutive activation of STATs, in particular Stat3 and Stat5, is associated with permanent changes in the expression of genes that control fundamental cellular processes subverted in oncogenesis. STATs are proposed to participate in oncogenesis through up-regulation of genes encoding apoptosis inhibitors (*Bcl-xL, Mcl-1*), cell cycle regulators (cyclins D1/D2, c-Myc), and inducers of angiogenesis (VEGF). Adapted and reprinted by permission from the American Association for Cancer Research: Buettner *et al.,* Activated STAT Signaling in Human Tumors Provides Novel Molecular Targets for Therapeutic Intervention. Clinical Cancer Research, 2002, Vol. 8, #4: 945-954. (Buettner *et al.*, 2002).

Serine phosphorylation of STATs

As previously mentioned, STATs are phosphorylated prior to dimerization and activation, and this is required for DNA-binding activity. Additional modifications to the STAT proteins, such as phosphorylation of serine residues are required to reach maximum transcriptional activity. Phosphorylation of a serine residue in the C-terminal transcriptional activation domain, corresponding to Ser-727 in both STAT1 and STAT3, enhances the transcriptional activity of these STATs (Wen and Darnell, 1997; Wen *et al.*, 1995). The mechanism of transcriptional enhancement may not be completely understood, but may include interactions between STATs and co-activator proteins (Decker and Kovarik, 1999) which enhance gene transactivation.

There is evidence that serine phosphorylation can occur via members of the mitogen-activated protein kinases (MAPK) family (Schaeffer and Weber, 1999) including extracellular signal-regulated kinases (ERKs) (Chung *et al.*, 1997b; David *et al.*, 1995; Kuroki and O'Flaherty, 1999; Ng and Cantrell, 1997), c-Jun N-terminal kinase (JNK) (Lim and Cao, 1999; Turkson *et al.*, 1999) and $p38^{mapk}$ (p38) (Gollob *et al.*, 1999; Turkson *et al.*, 1999). Protein kinase C (PKC) may also play a role (Jain *et al.*, 1999).

The serine phosphorylation site in both STAT1 and STAT3, (-Pro-Met-Ser-Pro-), conforms to the MAPK consensus sequence, -Pro-X-Ser/(Thr)-Pro- (Schaeffer and Weber, 1999). Cell-type specific expression of the individual serine kinases along with their interactions with individual STAT members most likely affects serine phosphorylation status and is complex

In contrast, repression of STAT signaling by serine phosphorylation has also been noted, suggesting that the kinases play a dual role, both enhancing and repressing STAT activity under certain conditions. This may be due to serine phosphorylation inhibiting STAT tyrosine residue phosphorylation (Chung *et al.*, 1997b); negative feedback effect of the serine kinase on upstream tyrosine kinases (Sengupta *et al.*,

1998) or even an indirect effect from STAT proteins preferentially interacting with serine kinases versus tyrosine kinases (Jain *et al.*, 1998; Jain *et al.*, 1999; Lim and Cao, 1999). However, such repression can occur when the serine kinases are in a 'superactive' state (Jain *et al.*, 1998), as found with ERK: moderate levels of ERKs enhance, yet overexpression of ERKs inhibit, STAT3 transcriptional activity (Turkson *et al.*, 1999).

Nuclear Import and Export of STAT Proteins

STATs do not exhibit a classical nuclear localization sequence (NLS), despite the fact that the interferon-induced nuclear importation of STATs is mediated via the importin/Ran system (McBride *et al.*, 2002). A structural region in the DNA-binding domain of STAT1 and STAT2, rich in arginine and lysine residues, is required for nuclear import (Melen *et al.*, 2001). These conserved regions are required to be present in both STAT monomers for nuclear import to occur, since dimers with one wild-type STAT and one STAT mutated in the arginine/lysine region fail to translocate to the nucleus upon stimulation, thus such a dimer acts as a dominant negative. Previous work also suggests that the adapter protein importin-α3 binds to STAT3 and is required for nuclear import (Liu *et al.*, 2005), however, the importin-α/importin-β1/Ran mechanism has also been shown to traffic STAT3 to the nucleus (Cimica *et al.,* 2011)

STAT1 also has a nuclear export signal (NES), located adjacent to the NLS amino acids 400-409) (Mowen and David, 2000). STAT1 nuclear export is regulated by the CRM1 export protein and is Leptomycin B (LMB)-sensitive. STAT3 is also exported from the nucleus in an LMB-sensitive process, allowing STAT3 to accumulate in the nucleus (Bhattacharya and Schindler, 2003). This accumulation is independent of tyrosine phosphorylation, suggesting that a "basal" STAT3 signaling pathway exists. STAT3 contains three NES elements, two of which, STAT3 (306-318) and STAT3 (404-

414), correspond to those previously identified in STAT1, as well as a third NES, STAT3(524-535). STAT3 (306-318) appears to be important in the rapid nuclear export of STAT3 seen following stimulation, whereas the STAT3 (404-414) and STAT3 (524-535) have an important role in regulating basal nuclear export. Unphosphorylated, latent STAT3 shuttles constitutively between cytoplasm and nucleus. Mutation of a putative NLS or NES sequence did not impair nucleocytoplasmic shuttling of latent STAT3 (Bhattacharya and Schindler, 2003).

The N-terminal domain (amino acids 1-125) was found to be essential for formation of unphosphorylated STAT3 dimers, but not for assembly of tyrosine-phosphorylated STAT3 dimers. In resting cells, the monomeric N-terminal deletion mutant (STAT3-ΔNT) shuttles faster between the cytoplasm and nucleus than the wild-type STAT3, indicating that dimer formation is not required for nucleocytoplasmic shuttling of latent STAT3 (Vogt *et al.,* 2011).

Negative Regulation of STAT Signal Transduction

Since the JAK/STAT pathway plays such a critical role in cell signaling, there are multiple fine-tuning mechanisms that control STAT activation both spatially and temporally. Under normal circumstances STAT activation is transient and is controlled by several pos-translational mechanisms, not at the level of gene transcription, including (i) negative feedback proteins (ii) expression of nuclear inhibitors of STAT signaling (iii) activation of tyrosine and/or serine phosphatases (iv) receptor internalization and (v) protein degradation (Greenhalgh and Hilton, 2001; Kile *et al.*, 2001; Wormald and Hilton, 2004).

Suppressors of Cytokine Signaling (SOCS). Suppressors of cytokine signaling comprise a family of inhibitors which also act on the JAK-STAT pathway as negative feedback regulators (Masuhara *et al.*, 1997). Also known as JAK-binding proteins (JAB) or STAT-induced STAT inhibitors (SSIs), SOCS are induced by cytokine stimulation and inhibit phosphorylation of receptors by interaction with the kinase domain of JAKs. For example, IL-6 is capable of inducing transcription of SOCS3, which inhibits phosphorylation of gp130 by interaction with the kinase domain of JAK 2. (Endo *et al.*, 1997). The SOCS family consists of seven members, SOCS 1-7, of which SOCS-1 and SOCS-3 are the most studied. Structurally, the SOCS proteins share several similarities including a central SH2 domain, a highly homologous C-terminal region (SOCS-box), and an N-terminal region of varied length and a highly variable amino acid sequence.

SOCS-1 inhibits signaling by a wide range of cytokines including IL-6, IL-4, LIF, GH, TPO, prolactin, interferons (especially IFN-g) and stem cell factor (kit ligand). SOCS-1 binds directly to the kinase domain (JH1) of JAKs to inhibit kinase activity, as well as JAK-mediated phosphorylation of downstream substrates, such as the receptor and STATs (Endo *et al.*, 1997). Both the SH2 domain and the N-terminal region of SOCS-1 are required for inhibitory activity, while the SOCS box appeared to be dispensable [104]. However, the SOCS box is conserved in all SOCS proteins, suggesting an important physiological role, possibly involving SOCS proteolytic degradation (Kamura *et al.*, 1998; Zhang *et al.*, 1999a).

SOCS-3 inhibits many of the same cytokine/receptor systems as SOCS-1, but also downregulates IL-2, IL-3 and leptin signal transduction (Bjorbaek *et al.*, 1999; Cohney *et al.*, 1999). At high concentrations SOCS-3 interacts with JAKs, however, it has a much lower affinity for binding JAKs compared to SOCS-1 (Nicola and Greenhalgh, 2000). Instead, SOCS-3 appears to inhibit cytokine signaling by associating with the gp130 receptor directly (Nicholson *et al.*, 2000).

The N-terminal domains of SOCS-1 and SOCS-3 are interchangeable without loss of function, suggesting that both proteins may inhibit JAK kinase activity via this domain, and that the SH2 domain either binds JAKs (SOCS-1) or the gp130- receptor (SOCS-3) (Nicola and Greenhalgh, 2000).

Protein Inhibitors of Activated STAT (PIAS). Protein inhibitors of activated STAT3 (PIAS) have been shown to associate with STATs and prevent binding of the activated nuclear STATs to DNA (Chung *et al.*, 1997a). PIAS1 was cloned using a yeast two-hybrid assay and shown to specifically inhibit STAT1 binding to DNA. (Liu *et al.*, 1998). PIAS3 was then identified in a mouse thymus library screened with a human EST clone related to PIAS1. Both proteins bind *in vivo* to the N-terminal region of their target activated STAT dimers and block binding of the transcription factors to their target gene promoters, thus preventing STAT-mediated gene transcription. However, the PIAS proteins do not cross-inhibit other STAT proteins e.g. PIAS3 is a specific inhibitor of STAT3 only and will not inhibit STAT1 activity.

PIAS proteins are constitutively expressed in a variety of tissues (Greenhalgh and Hilton, 2001; Kile *et al.*, 2001; Wormald and Hilton, 2004). They have conserved putative zinc-binding motifs [C2-(X)21-C2 (Chung *et al.*, 1997a). PIAS3 was shown to bind to STAT3 via the conserved proline, isoleucine, asparagine, isoleucine, threonine (PINIT) domain of PIAS3 (Mautsa *et al.*), however they may also bind to other proteins, especially transcription factors. The PIAS proteins function as SUMO (small ubiquitin-like modifier)-E3 ligases which catalyzes the covalent attachment of a SUMO protein to their specific target substrates (Rytinki *et al.*, 2009).

Dephosphorylation of STAT3. Tyrosine phosphorylation of proteins is a rapid and reversible mechanism that is often used in cell signaling to indicate activation of the

target protein. STATs are one class of proteins activated by phosphorylation as previously discussed. Therefore, one of the most obvious mechanisms of STAT regulation is via phosphatases. For example, SHP-1 has been shown to suppress cytokine signaling systems and is composed of two SH2 domains following by the phosphatase domain. SHP-1 is thought to function by direct binding to cytokine receptors and dephosphorylating signaling components (Ram and Waxman, 1997).

Receptor Internalization. Cytokine signaling begins with activation of plasma membrane receptors, however, these receptors do not remain permanently at the membrane. The receptors are removed from the cell membrane via endocytosis, which has been studied in depth for the gp130 receptor. IL-6 has been shown to downregulate its own receptor. Following binding of IL-6, the gp130 receptor is quickly internalized within 60 minutes, leading to a complete depletion of IL-6 surface binding receptors (Zohlnhofer *et al.*, 1992) Since *de novo* synthesis of the gp130 receptor is required to re-populate the membrane, this suggests that following internalization, gp130 is degraded.

Proteolysis. Protein degradation plays a very limited role in controlling STAT signaling. Proteosome inhibitors had little effect on the turnover rate of STAT1 and STAT3 (Heinrich *et al.*, 1998) and half-life studies have shown that STAT3 has a long half-life >8 h, which is not reduced by stimulation with IL-6. However, limited proteolytic processing of STATs by serine proteases (Azam *et al.*, 1997) or the cysteine protease calpain (Hendry and John, 2004; Oda *et al.*, 2002) generates C-terminally truncated STAT proteins that are able to negatively regulate STAT3-, STAT5- and STAT6-mediated signaling. Designated as STATγ, these molecules function as dominant negative regulators of transcription.

STAT3 is also cleaved by caspases at multiple sites (Darnowski *et al.*, 2006) forming cleavage fragments. Such cleavage reduces STAT3-DNA binding, STAT3-driven luciferase reporter activity and represses expression of STAT3-dependent genes (Darnowski *et al.*, 2006). Caspase cleavage of STAT3 was also demonstrated to increase sensitivity to apoptotic stimuli. Thus, proteolytic cleavage of STAT3 reduces its expression and leads to the production of cleavage fragments which can modulate STAT3 transcriptional activity.

Biological Functions of STAT Proteins

The STAT family protein structure is highly conserved, yet there are distinct differences both in primary sequence and in function. STATs are ubiquitously expressed, apart from STAT4, whose expression is restricted to including spleen, heart, brain, peripheral blood cells, and testis (Yamamoto *et al.*, 1997).

The control of normal physiological processes by STAT family members has been based on studies using homozygous deletion or tissue-specific, conditional mouse knockouts of each STAT family member (Akira, 1999). The different family members have been shown to be involved in mediating a variety of biological functions in diverse cell types.

STAT1-deficient mice have an impaired ability to respond to interferons and become susceptible to infections from bacterial and viral pathogens (Durbin *et al*., 1996; Meraz *et al*., 1996), indicating that STAT1 is required for interferon signaling as well as innate immunity.

Homozygous deletion of the gene encoding either STAT2 (Kimura *et al*., 1996) or STAT3 (Takeda *et al*., 1997), proteins is embryonic lethal, establishing a critical role for both STATs in normal development. STAT3-null animals exhibit mortality at day 6.5 to 7 during early development, therefore tissue-specific, Cre-Lox systems are more

commonly used to generate STAT3-null conditions. Mice lacking STAT3 expression in keratinocytes suggest a role for STAT3 signaling in control of cell motogenesis, as it pertains to wound healing (Sano *et al*., 1999). Furthermore, in contrast to wild-type littermates, IL-6 fails to prevent apoptosis in T lymphocytes from mice deficient for STAT3 signaling, demonstrating that STAT3 is required in hematopoietic cells for IL-6 signaling as well as anti-apoptosis (Catlett-Falcone *et al.*, 1999)Takeda *et al*., 1998).

Targeted disruption of the STAT4 (Kaplan *et al*., 1996; Thierfelder *et al*., 1996) or STAT6 (Shimoda *et al*., 1996; Takeda *et al*., 1996; Thierfelder *et al*., 1996) genes in mice demonstrates that these STATs are required for IL-12- or IL-4-induced proliferation of activated T lymphocytes, respectively.

STAT5 has also been shown to be important in lactation and hematopoiesis (Akira, 1999; Frank, 1999). The STAT5A and STAT5B genes are highly related both at the genetic and protein levels, however, targeted disruption of either gene exhibits a tissue-specific phenotype that is also gene-specific. For example, STAT5A knockout mice exhibit defects in mammary gland development and lactation during pregnancy (Liu *et al*., 1997), while STAT5B knockout mice display sexually dimorphic patterns of liver gene expression (Udy *et al*., 1997). Additionally, female mice lacking both STAT5A and STAT5B proteins are infertile and double-knockout mice indicate a role for STAT5 proteins in proper immune function (Teglund *et al*., 1998).

These studies indicate that STAT proteins are required for regulation of a diverse array of cellular functions, which are also affected by the spatial and temporal expression of the respective STAT proteins.

Activation of STATs in Oncogenesis

In oncogenesis, the signal transduction pathways involving STATs are often disrupted and since STATs play a critical role in a remarkable variety of biological

processes, it is of note that the dysregulation of STAT signaling pathways is becoming more frequently associated with oncogenesis. Mounting evidence suggests that constitutively active STATs play a role in cellular transformation induced by multiple oncogenes, as well as progression of human tumors.

Constitutive activation of STATs 1, 3 and 5 have been demonstrated in a variety of human tumors and cell lines (Turkson and Jove, 2000), (Table 1) including solid tumors and blood tumors. This persistent signaling of specific STATs, in particular STAT3 and STAT5, has been shown to stimulate cell proliferation and prevent apoptosis in various tumor cell lines, through upregulating a number of target genes, such as c-Myc, cyclins and bcl-x. In contrast, inhibition of constitutively activated STAT3 or STAT5 leads to growth suppression or apoptosis (Buettner *et al.*, 2002).

Table 1. Activation of STATs in human cancers

Tumor Type	STAT(s) Activated
Solid Tumors	
Melanoma	STAT3
Prancreatic cancer	STAT3
Prostate cancer	STAT3
Lung cancer	STAT3
Renal cell carcinoma	STAT3
Ovarian cancer	STAT3
Head and neck cancer	STAT1, STAT3, STAT5
Breast cancer	STAT1, STAT3, STAT5
Blood Tumors	
Multiple myeloma	STAT1, STAT3
Leukemias:	
Acute myelongenous leukemia (AML)	STAT1, STAT3, STAT5
Chronic myelogenous leukemia (CML)	STAT5
Large granular lymphocyte luekemia (LGL)	STAT3
HTLV-I-depdendent	STAT3, STAT5
Erythroleukemia	STAT1, STAT5
Lymphomas:	
Mycosis fungoides	STAT3
EBV-related/Burkitt's	STAT3
Cutaneous T-cell lymphoma	STAT3
Non-Hodgkins lymphoma (NHL)	STAT3
Anaplastic large-cell lymphoma (ALCL)	STAT3

Based on references cited in (Turkson and Jove, 2000)

Interaction of STATs with other proteins

Following research investigating STAT regulated transcription, it is now clear that STATs function as part of multi-protein enhanceosomes to stimulate activation of their target genes. The association of STATs with a number of nuclear proteins has previously been described (Paulson *et al.*, 1999; Zhang *et al.*, 1996), for example, the association of STATs with co-activator proteins, such as CBP/p300, which bridge

between activated STATs and the basal transcription machinery (Paulson *et al.*, 1999;. CBP/p300 is an important regulator of chromatin remodeling, since it contains a histone acetyltransferase (HAT) domain and interacts with a wide range of transcription factors (Kadonaga, 1998). CBP/p300 has been shown to interact with STAT1, via both N- and C-terminals of STAT1 (Zhang *et al.*, 1996), as well as STAT5 (Pfitzner *et al.*, 1998)

STATs have also been shown to interact with hormone receptors, mini-chromosome maintenance proteins and members of the AP-1 and IFN regulatory factor (IRF) families (Bromberg and Darnell, 2000; Horvath *et al.*, 1995; Zhang *et al.*, 1996)

STAT3-interacting protein (StIP1) has been identified and may serve as a scaffold protein to facilitate the interaction between JAKs and STAT3 (Collum *et al.*, 2000).

The interaction of STAT3 in the nucleus with the zinc-finger protein Gfi-1 has also been reported, leading to enhanced IL-6 induced transcription (Rodel *et al.*, 2000).

Many gene promoters contain binding sites for multiple transcription factors and STAT3 has been shown to cooperate with multiple transcription factors to regulate transcription of target genes, including SMAD1, AP-1, Sp-1. In contrast, some promoters have overlapping binding sites for transcription factors, which cannot be occupied by both transcription factors at the same time. For example, overlapping binding sites for STAT3 and NF-κB are found on promoters of several acute phase proteins (APPs). IL-1 and IL-6, two early-response cytokines expressed during an acute inflammatory response regulate the expression of APPs in the liver. IL-1 stimulates transcription via NF-κB, whereas IL-6 induces its target genes via STAT1 and STAT3. Since both STAT3 and NF-κB are active during the inflammatory response, they could both potentially bind the overlapping element in the alpha2-macroglobulin promoter and may regulate each other via competition for the binding site (Zhang and Fuller, 1997).

The Role of STAT3 in Cancer

STAT3 activation has been seen at a high frequency in a wide variety of solid tumors, including those of the breast, head and neck, leukemias, lymphomas, melanomas, pancreas, prostate, ovary, lung and brain (Bowman *et al.*, 2000; Turkson and Jove, 2000)

In normal cells, the activation of STAT3 is a highly regulated, transient process and has a duration of minutes to several hours, at which point the STAT3 homodimers are inactivated by de-phosphorylation. However, constitutive activation of STAT3 has been associated with malignant transformation, including transformation by v-Src (Yu *et al.,* 1995; (Cao *et al.*, 1996), Eyk (Zong *et al.*, 1996) and other oncogenic tyrosine kinases. Evidence has shown that constitutively active STAT3 is required for v-Src transformation (Bromberg *et al.*, 1998) and is alone sufficient to induce transformation and tumor formation in nude mice (Bromberg *et al.,* 1999). Moreover, persistent STAT3 activation is frequently detected in human tumors, both in patient samples and tumor cell lines. The critical role of constitutively active STAT3 in various human cancers has been well established for a number of tumor types, including breast (Garcia *et al.*, 2001), prostate (Mora *et al.*, 2002), and head and neck squamous cell carcinoma (Song and Grandis, 2000). STAT3 is the family member that most frequently shows unregulated, persistent activation in human tumors and human cancer cell lines.

STAT3 itself has not been found to be mutated in human tumors, but it is activated by various upstream oncogenic proteins and is required for their transforming ability. For example, STAT3 is activated in cells transformed by v-Src (Bromberg *et al.*, 1998) or v-Abl oncogenes and has been shown to be required for the transforming ability of v-Src (Danial *et al.*, 1995; Turkson *et al.*, 1998; Yu *et al.*, 1995).

STAT3 activation can also be induced by a variety of growth factors and cytokines which are commonly over-expressed in cancer cells. These include Epidermal Growth Factor (EGF) (Garcia *et al.*, 1997; Ruff-Jamison *et al.*, 1994), Platelet Derived Growth Factor (PDGF) (Bowman *et al.*, 2001), as well as the cytokine, IL-6 (Akira, 1997). As such, STAT3 plays a central role in facilitating many oncogenic signaling pathways.

STAT3 activation has been associated with both chemoresistance and radioresistance, and mediates these responses via interaction with other transcription factors, including nuclear factor kappa B, Hypoxia-inducible factor-1 and peroxisome proliferator activated receptor-gamma (Aggarwal *et al.*, 2009).

STAT3-regulated genes

Following tyrosine phosphorylation and subsequent translocation to the nucleus, STAT3 binds to promoters containing the consensus sequences TT(N_4)AA or TT(N_5)AA (Horvath *et al.*, 1995). To date, STAT3 has been show to regulate a handful of target genes, including genes which are involved in cell survival, proliferation, inhibition of apoptosis, angiogenesis, metastasis and immune evasion.

So far genes that have been identified as being directly regulated by STAT3, and may contribute to oncogenesis, are involved in cell cycle control and proliferation, inhibition of apoptosis and angiogenesis.

STAT3 Regulation of Cell Growth and Proliferation. Dysregulated cell growth and proliferation is a hallmark of cancer (Hanahan and Weinberg, 2000) and STAT3 plays a major role in such uncontrolled growth by activating target gene such as Cyclin D1, p21WAF1/CIP1, as well as the proto-oncogenes c-Myc and c-fos, all of which play a role in proliferation, particularly in cancer (Kiuchi *et al.*, 1999; Sinibaldi *et al.*, 2000). For example, Cyclin D1, encoded by the CCND1 gene, is a G1/S specific protein belonging

to the cyclin family and capable of forming a complex with CD4 or CD6, whose activity is required for the cell cycle G1/S transition (Reddy, 1994). Overexpression of this gene is observed frequently in a variety of human tumors and contributes to oncogenesis (Motokura and Arnold, 1993).

p21 / WAF1 also known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1 is a protein that in humans is encoded by the CDKN1A gene located on chromosome 6 (6p21.2). A potent cyclin-dependent kinase inhibitor, p21 inhibits the activity of cyclin-CD2 or –CDK4 complexes to regulate cell cycle progression (Reddy, 1994). Transcriptional regulation of p21 was demonstrated in v-Src transformed cells (Sinibaldi *et al*., 2000) where coordinate increases in p21, cyclin D1 and cyclin E resulted in an increase in cyclin/CDK/p21 complexes. Overexpression of p21 and cyclins D1 and E most likely gives v-Src transformed cells sufficient active cyclin/CDK complexes to proceed through the cell cycle.

STAT3 Regulation of Cell Survival and Apoptosis. Regulation of cell survival and apoptosis are also disrupted in cancer cells and STAT3 has previously been shown to increase expression of the pro-survival proteins Bcl-XL, Bcl-2 (B-cell lymphoma 2) (Fujio *et al.*, 1997; Grad *et al.*, 2000)and Mcl-1 (Puthier *et al.*, 1999), as well as Survivin (IAP) (Diaz *et al.*, 2006; Gritsko *et al.*, 2006). Frequently overexpressed in human tumors, these genes play a critical anti-apoptotic role in a number of cancers, including in a number of cancers, including melanoma, breast, prostate, and lung carcinomas.

STAT3 Regulation of Angiogenesis and Metastasis. Metastasis is a major cause of morbidity and mortality in cancer patients. Many of the molecular mechanisms regulating metastasis have been elucidated and commonly involve various signal transduction pathways. STAT3 has been identified as a central regulator of tumor

metastasis (Devarajan and Huang, 2009) and STAT3 target genes are involved in multiple steps of metastasis. We have already discussed the role of STAT3 in regulating genes involved in cell survival and self-renewal, however, STAT3 also controls transcription of genes involved in invasion, angiogenesis and tumor cell immune evasion.

Hypoxia is a state of oxygen deprivation and a characteristic of tumors that are actively growing and occurs as a result of rapid oxygen consumption by the cancer cells, combined with an insufficient supply of oxygen. Rapidly growing solid tumors outgrow their blood supply, leaving hypoxic regions where the oxygen concentration is lower than surrounding, healthy tissue (Vaupel, *et al.,* 2001). As a result, hypoxia triggers specific pathways associated with homeostasis in order to maintain cellular metabolism and allow the tumor to continue to grow. The transcription factor HIF1-α is a critical mediator required for oxygen homeostasis (Semenza *et al.,* 1999) and HIF1-α expression is know to be elevated under hypoxic conditions (Hockel, 2001). Under normoxia conditions, HIF1-α activity is regulated by proteasomal degradation (Cockman, 2000; Ohh *et al.,* 2000), however, under hypoxic conditions, degradation is reduced and stabilized HIF1-α is targeted to the nucleus and forms a heterodimer with HIF1-β. The complex then binds specific hypoxia response elements (HRE) in the promoters and enhancers of HIF1 target genes. Among these target genes is vascular endothelial growth factor (VEGF). STAT3 can also be activated via SRC kinase through a Notch signaling pathway (Lee MCR 2009) leading to downstream expression of HIF1-alpha.

Degradation and remodeling of the extracellular matrix (ECM) are essential steps in tumor invasion and metastasis. The role of matrix metalloproteinases (MMPs) in metastasis is well established (Coussens, 1996; Stetler-Stevenson 2006; Chamber and Matrisian, 1997; Pollet, 1998; Curran and Murray, 1999). MMPs are key proteolytic

enzymes involved in invasion and metastasis, as well as contributing to angiogenesis by facilitating the migration of endothelial cells through the ECM in response to angiogenic stimuli (Liotta, 1991; Moses, 1997; Stetler 2001). STAT3 regulates the expression of MMP-2 and was shown to correlate with their expression, tumor invasiveness and metastasis (Xiw, 2004).

STAT3 in Inflammation and Immune Evasion. Inflammatory conditions can increase the risk of cancer (Mantovani *et al.,* 2010) and inflammatory components are present in the microenvironment of tumors that are not even related epidemiologically to inflammation. STAT3 has been shown to orchestrate the molecular pathways that link inflammation and cancer (Kato, 2011).

STAT3 is constitutively expressed in diverse tumor-infiltrating immune cells (Yu *et al.,* 2007) leading to the inhibition of a pro-inflammatory cytokine response, reduced cytokine production and also the release of factors that actively down-regulate the immune response.

However, STAT3 has been shown to repress expression of nitric oxide synthase (NOS) by direct interaction with NF-KB, to terminate NO production by activated iNOS following exposure to pro-inflammatory stimuli. NOS enzymes catalyze the reaction between oxygen and L-arginine substrates to produce L-citrulline and NO, which can cause unintended injury to host cells during glomerulonephritis and other inflammatory diseases (Yu *et al.*, 2002).

Identifying Novel STAT3 Target Genes

Cytokine and growth factor signaling pathways involving STAT3 are frequently constitutively activated in many different human primary tumors, and are best known for the transcriptional role they play in the controlling cell growth and cell cycle progression.

However, the extent of STAT3's reach on transcriptional control of the genome as a whole remains an important question. We predicted that this persistent STAT3 signaling affects a wide variety of cellular functions, many of which still remain to be characterized.

STAT3 is a latent cytoplasmic transcription factor, induced by a variety of upstream signals, including growth factors, cytokines and non-receptor tyrosine kinases. Upon activation by tyrosine phosphorylation, STAT3 forms dimers, which translocate to the nucleus and regulate transcription of target genes. Under normal physiological conditions, STAT3 activity is tightly controlled; however, intracellular signaling pathways involving STAT3 are frequently constitutively activated in many different human primary tumors. We and others have shown that constitutive activation of STAT3 provides cancer cells with growth and survival advantages and enhances tumor angiogenesis and metastasis. Recent studies have also indicated that STAT3 activation contributes to tumor immune evasion. These findings indicate that aberrant STAT3 signaling affects a wide variety of fundamental cellular functions through multiple mechanisms.

To date, up-regulated expression of numerous STAT3 target genes has been identified, including VEGF (Niu *et al.*, 2002), Bcl-2, Bcl-xL (Zushi *et al.*, 1998), p21, Cyclin D1 (Sinibaldi *et al.*, 2000) and survivin (Gritsko *et al.*, 2006). These STAT3 target genes have generally been identified on an individual basis, while few studies have attempted to identify large numbers of STAT3 regulated genes (Alvarez *et al.*, 2005; Dauer *et al.*, 2005; Paz *et al.*, 2004; Sekkai *et al.*, 2005; Snyder *et al.*, 2008). Our goal was to take a broad approach to identify novel STAT3 regulated genes involved in oncogenesis by examining changes in the genome-wide gene expression profile by microarray, using cells expressing constitutively-active STAT3.

Experimental Activation of STAT3

STAT3 signaling pathways can be induced by many ligands and oncoproteins, however, these ligands may also induce other STAT family proteins simultaneously e.g. Epidermal Growth Factor induces both STAT1 and STAT3. In order to identify genes which are regulated by STAT3 specifically, we chose techniques which preferentially induced STAT3 only.

Activation of STAT3 by IL-6. IL-6 is a pleiotropic cytokine and mediates multiple biological functions within the cell including cell survival, growth, growth arrest and differentiation. Cells stimulated by IL-6 can respond in a variety of different ways as a result of the activation of various signaling pathways and the induction of specific target gene expression.

IL-6 signals through the JAK/STAT pathway and also the Ras/MAPK pathway (Kishimoto, 1994) Kishimoto 1995). STAT3 was originally identified as a signal transduction molecule that is activated during signaling by IL-6 (Zhong *et al.,* 1994). Since then IL-6 signaling through the JAK-STAT pathway has been well characterized (Aaronson and Horvath, 2002).

The JAK/STAT pathway is used by all neuropoietic cytokines, interferons and other cytokines (Darnell *et al.*, 1994; Lutticken *et al.*, 1994; Stahl and Yancopoulos, 1994). Gp130 is the common subunit for the receptor complexes for the IL-6 cytokine family (including IL-6, LIF, CNTF, OnM, IL-11 and CT-1) (Hirano *et al.*, 1994). These cytokines are referred to as 'IL-6-type cytokines' since they induce similar and overlapping physiological responses.

IL-6 signals via a receptor consist of a ligand binding α-chain and a signal transducing chain, and binding of IL-6 leads to the receptor leads to homodimerization of gp130 and activation of the receptor-associated Janus Kinases (JAKs). The JAKs then

phosphorylate tyrosine residues in the intracellular domain of the receptor to provide docking sites for latent cytoplasmic STATs to bind (e.g. pYXXQ in gp130 receptor for STAT3 binding). STATs then bind the receptor via their SH2 domain allowing JAKs to phosphorylate the STATs on a specific tyrosine residue in their cytoplasmic tail. Reciprocal binding of this pTyr in one monomer to the SH2 domain of a partner monomer allows homo- or hetero-dimerization of the proteins. Once released from the receptor, the dimers translocate to the cell nucleus and bind to specific DNA sequences to activate the transcription of cytokine-responsive genes.

JAKs are a family of non-receptor tyrosine kinases from the Janus kinase family (JAK1-3 and Tyk2) with molecular masses of 120-140 kDa. JAK3 is mainly expressed in hematopoietic cells, whereas JAK1, JAK2 and Tyk2 are widely expressed and associated with the cytoplasmic domain of gp130. Ligand activation of the receptor causes receptor homodimerization and reciprocal tyrosine phosphorylation, resulting in activation of the JAKs. Activated JAKs then phosphorylate the cytoplasmic tail of gp130 on specific tyrosine residues, which then act as docking sites for SH2 domain-containing proteins, such as the STATs.

IL-6 specifically induces recruitment of STAT1 and STAT3 to these phosphorylated sites, and the STATs are then phosphorylated by the activated JAKs. Upon activation, the STATs translocate to the nucleus and directly control target gene expression. STAT3 is known to upregulate many IL-6 responsive genes, including immediate early genes (Lord 1991, Nakajima and Wall 1991, Yuan 1994) and also acute phase response genes (Wegenka 1993).

Activation of STATs following IL-6 stimulation is rapid, usually with a maximum accumulation of STATs within the nucleus within 30 minutes, followed by rapid inactivation via dephosphorylation (Haspel *et al.*, 1996). However, in tumor cells IL-6 induction of persistent STAT3 activity is achieved as a result of a positive feedback loop

wherein STAT3 causes transcription of the gene S1PR1 (sphingosine-1-phosphate receptor-1). S1PR1 is upregulated in STAT3-positive tumors and acts as a G-coupled receptor for the lysophospholipid sphingosine-1-phosphate (S1P). In turn, the S1pr1 protein enhances STAT3 activity and IL-6 expression, thus completing the loop (Lee *et al.*)

Activation of STAT3 by v-Src. STAT3 activity is also known to be induced by cellular transformation by the activated product of the *v-src* oncogene, v-Src (Yu *et al.*, 1995). The cellular and viral versions of the gene were shown to differ in the carboxy terminal region with v-Src having amino acid substitutions and deletions. The absence of the regulatory Tyr530 residue causes v-Src to be a constitutively active kinase. Turkson *et al* (Turkson *et al.*, 1998) demonstrated that v-Src activates STAT3 in transformed mouse fibroblasts and that this activation is required for cell transformation, resulting in STAT3-specific gene regulation. NIH3T3 and Balb/c-3T3 cells stably transfected with a plasmid containing Moloney murine leukemia virus long terminal repeats and the v-Src gene (pMvSrc) were kindly provided by Dr. D. Shalloway (Cornell University, New York, USA). pMvSrc was constructed by ligating the Schmidt Ruppin A v-Src fragment from plasmid pN4 into the *Bgl*II site of the pEVX plasmid. The fragment contains 276 base pairs of pBR322 DNA followed by 2.8 kb of Rous Sarcoma virus (SRV) (Johnson *et al.*, 1985).

Activation of STAT3 by expression of STAT3-C. STAT3 activity is tightly regulated under normal conditions and requires upstream stimulation for STAT3 phosphorylation and activation. In order to express STAT3 in a constitutively active manner within cells, we used the mutant STAT3-C construct. STAT3-C is an artificially-engineered, constitutively dimerized STAT3 molecule (Bromberg *et al.*, 1999). The

STAT3-C expression vector encodes a constitutively active mutant form of the STAT3 protein that was created using site-directed mutagenesis to substitute two cysteine residues in the C-terminal loop of the SH2 domain of STAT3. The encoded monomers dimerize spontaneously, independent of tyrosine phosphorylation, forming a permanent homodimer that is capable of binding to DNA and activating transcription. STAT3-C is able to cause cellular transformation of immortalized fibroblasts and the formation of tumors in nude mice (Bromberg *et al.*, 1999). Balb/c-3T3 cells stably transfected with pRc/CMV-STAT3C-Flag and selected with G418 for stable expression of STAT3-C were provided by Dr. H. Yu (Cancer Immunotherapeutics Program, City of Hope Comprehensive Cancer Center, Duarte, California, USA). The STAT3-C transfectants stably express the STAT3-C protein, which is capable of binding DNA and stimulating target gene expression.

Identifying Changes in Gene Expression

High-density oligonucleotide expression array technology is a widely used method to analyze global gene expression levels within cells. The Affymetrix GeneChip® system is one of the most reliable and commonly used oligonucleotide microarray systems. The system uses oligonucleotides of 35 base pairs that are used to probe genes. Typically each gene us represented by 16-20 pairs of oligonucleotides, referred to as a *probe set.* Each pair consists of a perfect match (PM) probe and a mismatch probe (MM). The mismatch probe is created by changing the center (13^{th}) base, to create a probe which has non-specific binding. The PM and MM are referred to as a probe pair.

To identify potential novel STAT3-regulated genes, we examined global gene expression patterns in cell lines harboring persistently active STAT3. Gene expression profiles in such cells are likely to be representative of the genetic profile of a cancer cell

with aberrant STAT3 expression, as compared to inducing STAT3 activity transiently using exogenous stimulation, such as IL-6 or transient transfection (Paz *et al.*, 2004).

Previous studies have used microarrays to identify STAT3 target genes under certain conditions, such as those which regulate inflammation and wound healing (Dauer *et al.*, 2005), differentiation (Snyder *et al.*, 2008), transformation (Paz *et al.*, 2004), embryonic stem cells (Sekkai *et al.*, 2005) and STAT3 target genes in human tumors (Alvarez *et al.*, 2005). In this study we aimed to expand on these prior examinations of STAT3-regulated gene expression to examine global STAT3 target genes, regardless of cell type or other influencing conditions.

Pathway Analysis of Genes

Microarrays assess simultaneous changes in transcript levels on an individual basis, resulting in a long list of genes which have significantly changed transcript levels when compared to control cells. However, these changes in gene expression do not occur as independent events within the cell, but are controlled in a coordinated manner and are often interconnected. Pathway Analysis is an unbiased method to determine whether differentially expressed genes, and the proteins they encode, are enriched in particular pathways, giving insight into the biological meaning of the changes observed.

Functional Analysis of Genes

The transcriptional profile of a cell expressing constitutively-active STAT3 is predicted to be very different compared to a cell where STAT3 is under tight regulation. Our initial hypothesis was that constitutive activation of STAT3 within cells leads to permanent changes in global gene expression patterns. We predict that STAT3 promotes widespread changes in gene expression, including both direct and indirect targets, involving multiple signaling pathways and involving a broad range of genes.

Analysis of the biological meaning behind the large lists of genes generated by microarrays can be very difficult. To determine the functional classification of the differentially expressed genes identified, the "Functional Annotation" tool in the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Database (http://david.abcc.ncifcrf.gov/) (Dennis *et al.*, 2003; Huang da *et al.*, 2009) is very useful. The DAVID Knowledgebase integrates information from multiple databases for a particular gene and allows the identification of enriched biological themes, especially Gene Ontology (GO) terms, as well as the discovery of enriched functionally-related gene groups, leading to meaningful biological interpretations of changes in the gene expression profile of a cell.

Necdin – A Negative Growth Regulator

Through computational analysis of our microarray data, we identified Necdin, a negative growth regulator, as a novel potential STAT3 target gene. Necdin is a potent growth suppressor that is predominantly expressed in post-mitotic neurons (Aizawa *et al.*, 1992; Hayashi *et al.*, 1995; Maruyama *et al.*, 1991; Uetsuki *et al.*, 1996). Necdin expression has been shown to be down-regulated both carcinoma cell lines and primary tumors (Chapman and Knowles, 2009), suggesting that repression of Necdin expression may have a role in oncogenesis.

We verified that Necdin mRNA expression inversely correlates with STAT3 activity in cells expressing constitutively-active STAT3 and that STAT3 directly regulates the expression of Necdin at the promoter level. In addition, Necdin expression in human tumor cell lines is inversely correlated with activation of endogenous STAT3. Our findings provide further evidence for a role of Necdin as a physiological target of STAT3, demonstrating that computational analysis of microarray data can be used to identify potential STAT3 target genes for further investigation.

Necdin Protein family

Necdin was originally identified in 1991 by the Yoshikawa lab in Japan (Maruyama *et al.*, 1991) as a novel DNA sequence isolated from a subtraction cDNA library of murine P19 embryonal carcinoma cells treated with retinoic acid to induce neural differentiation of the stem cells. As a result, the name, Necdin, stands for "neurally differentiated embryonal carcinoma cell-derived factor)".

Necdin is a member of the melanoma-associated antigen (MAGE) superfamily of proteins. The MAGE proteins are expressed in melanoma cells and act as antigens which are recognized by cytolytic T lymphocytes. They have been shown to have roles in a number of cellular processes, including cell cycle regulation and cell death (Barker and Salehi, 2002). Three groups of related MAGE genes have been identified in clusters on the X chromosome (MAGE-A genes are located at Xq28; MAGE-B genes at Xq21 and MAGE-C genes at Xp26-27) (reviewed by (Chomez *et al.*, 2001). In humans, 10 genes have been identified which lie outside of the MAGE-A, B or C clusters, which includes the Necdin gene NDN.

The MAGE family is divided into two groups of phylogenetically distinct branches (Barker and Salehi, 2002). Type I proteins include MAGE-A, B and C group proteins and Type II proteins which include the MAGE genes identified outside these clusters and include the gene Necdin (NDN).

Necdin Protein structure

The Necdin genes encodes a novel protein sequence of 325 amino acids in mice and 321 amino acids in humans, with an overall homology of 82% between species. The proteins are highly conserved (91%) in the central-to-carboxyl terminal region (amino acids 101-325) but less conserved (62%) in the amino-terminal region (residues

1-100) (Nakada *et al.*, 1998). It is of interest to note that Necdin contains regions of extremely acidic and basic residues. Residues 7-65 are proline-rich and highly acidic, whereas amino acids 117-160 are highly basic.

The MAGE family of proteins is characterized by a large central region termed the MAGE homology domain (MHD). Necdin has a MAGE Homology Domain (MHD) located at amino acids 160-170. This region may be involved in protein-protein interactions. MAGEL2, a MAGE-related sequence, encodes a protein that is homologous to Necdin and has been found to map closely to the position of the NDN gene.

Amino acids 191-222 may mediate nuclear localization, but Necdin does not have a traditional nuclear localization sequence.

In its native state, Necdin seems to assemble with other proteins through multiple protein-protein crosslinking (Maruyama, 1996). Necdin can also form homo-oligomers, confirmed by co-immunoprecipitation of differentially tagged Necdin proteins from transfected COS cells (Tcherpakov *et al.*, 2002).

NDN Gene Structure and Regulation of Expression

Located on human chromosome 15q11.2-q12 and mouse chromosome 7C, the gene coding for Necdin is unusual in that it does not contain any introns and its upstream region does not have a classical TATA or CAAT box (Uetsuki *et al.*, 1996). Necdin is an imprinted gene. The promoter region of Necdin contains a CpG island which is maternally imprinted, leading to expression only from the paternal allele.

Little is known about the regulation of Necdin expression. A study by Lui *et al* (Liu *et al.*, 2009) identified NDN as a target gene of p53, demonstrating that the NDN promoter contains a p53 binding site and that NDN is a direct transcriptional target of p53. Furthermore, Necdin is an activator of p53 in the hematopoietic stem cells (HSC)

used in the study, thus regulating HSC quiescence. Necdin expression has also been found to be regulated by NSCL-1 and NSCL-2, however further research is required to understand the mechanisms behind the regulation of Necdin expression (Kruger *et al.*, 2004). However, it has been established that Necdin is expressed in a cell-type specific manner and its expression is finely controlled, both temporally and spatially, as well as control of allelic expression by imprinting. The maternal allele is genomically imprinted and so expression from this allele is silenced. Expression of Necdin occurs solely from the paternal allele (Jay *et al.*, 1997). Disruption of the paternal allele in NDN knock out mice leads to post-natal lethality, approximately 30 hours after birth (Gerard *et al.*, 1999).

Necdin was shown to be expressed during brain development (Maruyama *et al.*, 1991) and post-mitotic neurons, but not in other non-neuronal tissues. Necdin is abundantly expressed in the hypothalamus, midbrain, pons and medulla oblongata but only in low levels in neocortical neurons. Necdin is also expressed at a high level in peripheral neurons, such as the sympathetic nervous system and retinal neurons and abundantly in skeletal myoblasts during myotube formation. During development, Necdin is constitutively expressed in mouse brain neurons from early embryogenesis (expressed in forebrain as early as E10) and continues to be expressed until adulthood (Maruyama *et al.*, 1991).

Necdin Localization

Whilst Necdin has no nuclear localization sequence, amino acids 191-222 may mediate nuclear localization of the protein (Taniura *et al.*, 2005) Immunohistochemical studies demonstrated that Necdin was predominantly expressed in the nucleus of differentiated neurons in the mouse brain (Aizawa *et al.*, 1992; Maruyama *et al.*, 1991) but not in proliferative neuron-like stem cells that originate from tumors (neuroblastoma and pheochromocytoma) (Aizawa *et al.*, 1992). Necdin is expressed and localized in the

nucleus in differentiated, post-mitotic neurons in the central and peripheral nervous system of mice. The fact that Necdin is able to interact with p53 and E2F1 also suggests that Necdin functions within the nucleus (Taniura *et al.*, 1999; Taniura *et al.*, 1998). However, another study showed that a large amount of Necdin is also in the cytoplasm of differentiated neurons, with a clear translocation into the nucleus under specific physiological changes (Niinobe, 2000)

Since Necdin does not have a specific organelle targeting sequence, its distribution within the cell may be dependent on the localization of its target proteins. Necdin localization is known to change when Necdin interacts with different proteins. The subcellular distribution of Necdin alters when it interacts with E2F1 and P75NTR (Kuwako *et al.*, 2004; Tcherpakov *et al.*, 2002). For example, in cells transfected with HA-tagged Necdin alone, the majority of the signal was in the cytoplasm, specifically in the soluble fraction. However, when HA-tagged Necdin was co-expressed with Myc-tagged EBNA3C, Necdin was predominantly nuclear, with some localization to the periphery of the cytoplasm/cell membrane component (Kaul et al).

Biological Functions, Mechanisms, and Regulation

Necdin has been shown to have various biological functions within the cell:

Negative growth regulator. The biological functions of Necdin are still being elucidated. To date Necdin expression has been shown to cause cell cycle arrest in NIH3T3 and is capable of suppressing the growth of Rb-deficient SAOS-2 osteosarcoma cells, suggesting that Necdin can act as a substitute for pRb in these cells (Taniura *et al.*, 2005; Taniura *et al.*, 1998). Necdin, therefore, is functionally similar to pRb but the two proteins are structurally different.

Necdin is a potent growth suppressor that is predominantly expressed in post-mitotic neurons (Aizawa *et al.*, 1992; Hayashi *et al.*, 1995; Maruyama *et al.*, 1991; Uetsuki *et al.*, 1996). Expression of FLAG-tagged Necdin in melanoma cells suppressed growth as demonstrated by a 30% reduction in [^{3}H] thymidine incorporation 4 days after transfection (Hoek *et al.*, 2004).

Necdin as a transcription factor. Necdin is likely to be a DNA binding protein, as shown by micrococcal nuclease digestion and may possibly regulate transcriptionally active genes involved in cellular differentiation and proliferation. This DNA-binding ability may be coordinated directly by both the amino and acidic regions of the protein and may be involved in a complex with both histones and DNA or may be modulated through interaction with other transcription factors, such as p53 and E2F1 (Taniura *et al.*, 1998).

Necdin may also act as a transcriptional repressor by binding to guanosine-rich sequences known as GN boxes. GN boxes are recognized by the Sp family of transcription factors and Necdin was shown, in an *in vitro* assay, to repress Sp-1 dependent transcriptional activity of a mouse c-*myc* P1 promoter via the GN box (Matsumoto, 2001). This suggests that Necdin can bind to these multiple guanosine clusters present in promoters of target genes, including cell cycle related genes, allowing Necdin to regulate their expression and, thus, cell proliferation.

The role of Necdin in development. In contrast to highly restricted expression in mouse, Necdin expression in human cells is much broader. Necdin has been shown to be expressed in a diverse range of fetal and adult tissue, including brain, lung, liver and kidney (Jay *et al.*, 1997). This suggests that the multi-functional roles of Necdin may be relevant in many tissue types.

Necdin has been demonstrated to regulate polarization of the cytoskeleton during development and lack of NDN expression impairs cell migration of murine and human fibroblasts by impairing polarity initiation via a Cdc42-myosin-dependent pathway (Bush and Wevrick, 2008).

Necdin as a tumor suppressor gene. Necdin has been postulated to be a potential tumor suppressor gene (Chapman & Knowles, 2009), functionally similar to pRb and capable of repressing the cell cycle proteins (see Protein-Protein Interactions section). In line with this claim, Necdin expression has also been shown to be downregulated in several tumor types and cell lines. However, further research is required to validate the function of Necdin as a tumor suppressor gene.

Cell Differentiation. Necdin is expressed during neuronal differentiation *in vivo* and *in vitro* (Kuwako *et al.*, 2004) in which many cells undergo apoptosis and protects them from apoptosis. Necdin may serve as an intrinsic anti-apoptotic protein to prevent neuronal precursors and postmitotic neurons from E1F1 induced death. Necdin is also expressed during terminal differentiation of sensory neurons that are dependent on NGF (Takazaki *et al.*, 2002).

Necdin Protein-Protein Interactions

Necdin has been shown to interact with various molecules with diverse functions:

p53. Yeast two-hybrid and *in vitro* binding assays demonstrated that Necdin binds the transactivation domain of p53 in the amino-terminal region of p53, between the MDM2-binding site and the proline-rich domain. Amino acids 35-62 of p53 are required for this binding (Taniura *et al.*, 1998).

Necdin binding to p53 appears to modulate the activity of p53. The Necdin/p53 complex is competent for DNA binding and is able to repress transcription of a p21/WAF1 luciferase reporter but Necdin does not have an inhibitory effect on p53-regulated growth suppression. Necdin can block p-53 induced apoptosis in U2OS cells, suggesting that Necdin has a protective effect. Necdin therefore appears to exert a negative effect on the transactivation domain and apoptosis, via the proline-rich domain.

Acetylation of p53 leads to transcription of pro-apoptotic genes. Necdin is capable of mediating an interaction between p53 and SRT1 histone deacetylase, leading to p53 deacetylation and consequent inhibition of p53-regulated apoptosis in cortical neurons (Hasegawa 2008).

SV40 large T antigen. The Simian Virus 40 large T antigen can bind both p53 and pRb during cellular transformation (Ludlow, 1993) and studies showed that the SV40 large T antigen could also bind to Necdin (Ohman Forslund and Nordqvist, 2001; Taniura *et al.*, 1998) . Indeed, Necdin co-immunoprecipitated with the large T antigen in nuclear extract from SV40-transformed COS-1 monkey kidney cells that were transfected with Necdin. Necdin binds to the amino-terminal region of the large T antigen (amino acids 84-708, T), with which both p53 and pRb interact (Taniura *et al.*, 1998).

Adenoviral E1A. Whilst both pRb and p53 bind to the SV40 large T antigen, only pRb is known to bind to the adenovirus E1A viral oncoprotein. p53, however, interacts with the adenoviral E1B protein. Necdin also interacts with the adenovirus E1A viral oncoprotein at the same location as pRb (amino acids 1-185), whereas p53 was not able to bind (Taniura *et al.*, 1998).

E2F1. Since Necdin and pRb show similar binding characteristics, it is not surprising that Necdin interacts with the carboxy-terminal of E2F1 (amino acids 55-430). This domain contains the cyclin A binding site, DNA binding region as well as the transactivation domain of E2F1. Necdin represses E2F1–dependent transactivation *in vivo* (Taniura 1998) by interacting with the transactivation domain of E2F1.

E2F1 has previously been suggested to function as a pro-apoptotic factor in postmitotic neurons (Azuma-Hara 1999, Hou 2000). Necdin, however, suppressed E2F1-induced apoptosis in differentiated neuroblastoma cells (Taniura 1998, Kobayashi 2002). Necdin is also expressed during neuronal differentiation in vivo and in vitro (Kuwako 2004) in which many cells undergo apoptosis. Necdin may serve as an intrinsic anti-apoptotic protein to prevent neuronal precursors and postmitotic neurons from E1F1 induced death.

It would appear from deletion mutants, that the central region of Necdin (amino acids) 83-292) is required for interaction with SV40 large T antigen, E1A oncoprotein and E2F1 (Taniura 1998).

E2F4. Insulin receptor substrate proteins regulate the interaction of Necdin with E2F4, which results in repression of the peroxisome-proliferator-activated receptor gamma (PPARgamma) transcription via a cyclic AMP response element binding protein (CREB)-dependent pathway (Tseng *et al.*, 2005). This interaction plays a role in regulating brown preadipocyte differentiation.

HIF-1α. Necdin has been shown to associate with the main transcriptional regulator involved in hypoxia, HIF-1α and may directly regulate its activity (Moon *et al.*, 2005). Necdin binds to the oxygen dependent degradation (ODD) domain of HIF-1α

reducing the transcriptional activity of HIF-1α under hypoxic conditions. Necdin may also play a role in HIF-1α degradation. As such, Necdin demonstrates an anti-angiogenic function.

Role of Necdin in Disease

Prada-Willi Syndrome (PWS). The human Necdin gene, NDN, is located on chromosome 15q11.2-q12 (Nakada *et al.*, 1998), a region that is involved in the pathogenesis of the neurodevelopmental disease Prader-Willi syndrome (PWS). PWS is a complex and progressive disease. PWS symptoms include hypotonia (poor muscle tone), mental retardation and developmental delay, obesity and respiratory problems. PWS is caused by either a large *de novo* deletion on the paternal chromosome 15, maternal disomy 15 or an imprinting defect, all of which result in lack of expression of imprinted genes that are active on the paternal chromosome only (Kanber *et al.*, 2009).

Necdin is an imprinted gene, the maternal allele being hypermethylated at the promoter region CpG islands, resulting in silencing of transcription. As a result, expression of Necdin occurs solely from transcription of the paternal allele, which remains hypomethylated, as shown in mice (Hanel and Wevrick, 2001). However, Necdin is is not expressed at all in PWS (Jay *et al.*, 1997; MacDonald and Wevrick, 1997; Sutcliffe *et al.*, 1997). Necdin lies in a region of the genome that is commonly deleted in PWS and is likely the candidate gene to play a role in the syndrome.

Human Malignancy. Necdin expression has also previously been show to be repressed in multiple tumor types. Oncomine analysis (www.oncomine.org) demonstrated that Necdin has reduced expression in bladder, melanoma, cervical,

prostate, lung, breast and ovarian cancer, when compared to normal tissue (Chapman & Knowles 2009). In agreement with these data, Necdin expression was shown to be suppressed in a drug-resistant ovarian carcinoma cell line (Varma *et al.*, 2005) and also in melanoma (Hoek *et al.*, 2004). In the melanoma study, Necdin was shown to be have reduced expression in six of eight melanoma cell strains tested. This could confer a growth advantage for tumors. Furthermore, Necdin in melanoma cells is localized to a different compartment in the cytoplasm compared to normal melanocytes. This indicates that Necdin may be inactivated by sequestration in these cells.

Together, these results suggest that the multi-functional role of Necdin may be significant in the development of an array of different tissue types and that repression of Necdin expression may play a role in tumorigenesis, possibly even acting as a tumor suppressor gene (Chapman and Knowles, 2009).

Necdin may also play an indirect role in patients with cancer. Late stage cancer patients often experience cachexia, which presents as progressive wasting of skeletal muscles, loss of weight, fatigue, weakness and loss of appetite. Necdin has been show to be expressed in the muscles of cachectic mice and functions to protect the muscle fibers from tumor-induced wasting by inhibiting the TNFα cachetogenic signaling pathways at various levels (Sciorati *et al.*, 2009)

Summary and Rationale

Hypothesis

Aberrant STAT3 activity is expected to result in permanent genetic alterations that participate in the development of a malignant phenotype. The genes affected include regulators of cell cycle progression and cell proliferation, angiogenesis, survival and apoptosis. We hypothesize that a universal gene expression pattern exists which is characteristic of aberrant STAT3 activity in malignant cells that may be clinically relevant.

Rationale

- Aberrant STAT3 activity is expected to result in permanent genetic alterations that play a role in the development of a malignant phenotype.
- Global gene expression patterns exist that are associated with constitutive STAT3 activity, as well as tissue specific gene expression profiles e.g. breast vs. prostate cells.
- The genes regulated by STAT3, when constitutively transcribed, are likely to play a major role in the development of malignancy through dysregulation of cell cycle progression, cellular proliferation and survival, angiogenesis and apoptosis.

Objective

The overall objective of this study was to identify global gene expression patterns associated with activation of STAT3 and to identify candidate STAT3 primary target genes for further analysis in their role in oncogenesis.

Aim 1. To evaluate the efficiency of currently available methods for activating STAT3 activity with the potential for gene expression analysis.

Aim 2. To identify new STAT3 regulated genes in mouse fibroblasts using microarrays.

Aim 3. To directly confirm and further investigate putative STAT3 primary target genes.

CHAPTER 2: MATERIALS AND METHODS

Cell Culture and Reagents

NIH3T3 and Balb/c-3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum and 1% penicillin and streptomycin. NIH3T3 and Balb/c-3T3 v-Src transformed counterparts (Yu *et al.*, 1995) as well as Balb/c-3T3 cells stably transfected with STAT3-C were grown in DMEM supplemented with 5% bovine calf serum and 1% penicillin and streptomycin. MDA-MB-231, MDA-MB-468 and MCF7 cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM/10% fetal bovine serum supplemented with 1% penicillin and streptomycin. PC3 and A375 cells were obtained from ATCC and maintained in RPMI/10% fetal bovine serum supplemented with 1% penicillin and streptomycin.

Human recombinant IL-6 was obtained from BD Pharmingen (San Jose, CA, USA). Cells treated with IL-6 were plated at 1 x 10^6 cells per 10 cm plate and allowed to adhere for 24 hours prior to serum-starvation (0.1% bovine calf serum) for 3 hours. The cells were then treated with either IL-6 (10 ng/ml) or cycloheximide (CHX) alone (10 ug/ml) or pre-treated with CHX for 30 min, followed by the addition of IL-6 (10 ng/ml) for 1 hour.

STAT1 (E23X) and STAT3 (C20X) antibodies were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. CPA-7 was a generous gift from Dr. Nick Lawrence (Turkson *et al.*, 2004).

Expression Vectors

pRc/CMV-STAT3C-Flag (constitutively activated STAT3) was a generous gift from J. Bromberg and J. Darnell (Bromberg *et al.*, 1999). The v-Src plasmid expression vector, pMvSrc, has been described previously (Johnson *et al.*, 1985). Transient transfections were performed using Lipofectamine PLUS (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

siRNA Transfections

siRNA directed specifically against STAT3 and a non-targeting control siRNA were obtained from Dharmacon RNA Technologies (Chicago, IL, USA). Cells were transfected using RiboJuice transfection reagent (Novagen, Gibbstown, NJ, USA) as per the supplier's instructions. At 48 h after initial transfection, non-adherent cells were washed off and the remaining cells were harvested for Western blot.

Isolation of RNA

At each of 3 passages, cells from the five 10 cm dishes were pooled and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA in the aqueous phase of the TRIzol reagent was further purified using the RNeasy RNA clean-up procedure (Qiagen Inc., Valencia, CA, USA). The quality of the total RNA was assessed by agarose gel electrophoresis and RNA concentration analyzed on an Agilent 2100 Bioanalyzer.

Quantitative Real-time PCR

For real-time PCR, total RNA was isolated as detailed above. An aliquot of the same RNA used for microarray analysis, was used for Real-Time PCR using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) according to the

manufacturer's instructions. TaqMan® Gene Expression Assay Mm02524479_s1* was used to analyze Necdin expression and Eukaryotic 18S rRNA (4319413E, Applied Biosystems) was used as an endogenous control. Data were analyzed using SDS software version 2.2.2 and exported into an Excel spreadsheet.

Preparation of labeled RNA for hybridization

Total RNA (1 µg) was then used for microarray analysis using Affymetrix Mouse Genome 2.0 GeneChips, according to the manufacturer's protocol. Briefly, the poly(A) RNA was specifically converted to cDNA, amplified then labeled with biotin following the procedure initially described by Van Gelder *et al*. (Van Gelder RN, 1990).

First-strand cDNA synthesis was carried out using the Superscript Choice System (Invitrogen, Carlsbad, CA) with the T7 promoter/oligo (dT) primer (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24-3') (Genset Corp., La Jolla, CA). Following annealing, the rest of the cDNA synthesis reaction was prepared such that the final reaction contains 5 ug RNA, 100 pmol T7-(T)24 primer, 500 µM each dNTP, 10 mM DTT, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, and 200 U of Superscript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA). The reaction was incubated for 1 hr at 42°C. A second-strand cDNA synthesis was performed at 16°C for 2 hr in a total volume of 150 µL, using 10U of *E.coli* DNA ligase, 40 U of *E. coli* DNA polymerase I, and 2 U of *E. coli* RNase H in the presence of 200 µM of each dNTP, 10 mM (NH4)SO4, 1.3 mM DTT, 26.7 mM Tris-HCl, pH 7.0, 100 mM KCl, 5 mM MgCl2, and 150 µM β-NAD+ (Invitrogen). Following the second-strand DNA synthesis, 10 U of T4 DNA Polymerase (Invitrogen) was added and the samples incubated an additional 5 min at 16°C. The reaction was stopped by the addition of 0.5 M EDTA and subsequently extracted with an equal volume of phenol/chloroform/isoamyl alcohol. The double-stranded DNA (dsDNA) will then be precipitated with the addition of

0.5 volumes of 7.5 M NH4 Acetate and 2.5 volumes of ice-cold 100% ethanol. The dsDNA then serves as a template for a transcription reaction performed with the GeneChip IVT Labeling kit according to manufacturer's instructions (Affymetrix Corp., Santa Clara, CA) which incorporates biotinylated UTP into the transcripts. The Biotin-labeled RNA was purified using RNeasy columns (Qiagen) and fragmented to a size of 35 to 200 bases by incubating at 940 C for 35 minutes in fragmentation buffer (40 mM Tris-acetate, pH 8.1/100 mM potassium acetate/30 mM magnesium acetate). The integrity of the starting material and the products of each reaction were monitored on agarose gels to assess the size distribution of the products and compare them to the starting material.

Array Hybridization and Scanning

The hybridization solution consisted of 20 µg of fragmented RNA and 0.1 mg/ml sonicated herring sperm DNA, in 1x MES buffer (containing 100 mM MES, 1 M Na+, 20 mM EDTA, and 0.01% Tween 20). In addition the hybridization solutions were spiked with known concentrations of RNA from the bacterial genes, BioB, BioC, and BioD, and one phage gene, Cre, as hybridization standards. The hybridization mixtures were heated to 99°C for 5 min followed by incubation at 45°C for 5 min before injection of the sample into a probe array cartridge. All hybridizations were carried out at 45°C for 16–17 h with mixing on a rotisserie at 60 rpm. Following hybridization, the solutions were removed and the arrays were rinsed with 1x MES. Subsequent washing and staining of the arrays was carried out using the GeneChip Fluidics station protocol EukGE_WS2, which consists of 10 cycles of 2 mixes per cycle with non-stringent wash buffer (6x SSPE, 0.01% Tween 20) at 25°C followed by 4 cycles of 15 mixes per cycle with stringent wash buffer (100 mM MES, 0.1 M Na+, and 0.01% Tween 20) at 50°C. The probe arrays were then stained for 10 min in streptavidin-phycoerythrin solution (SAPE)

[1x MES solution, 10 µg/ml SAPE (Molecular Probes, Eugene, OR), and 2 µg/µl acetylated BSA (Invitrogen) at 25°C. The post-stain wash was 10 cycles of 4 mixes per cycle at 25°C. The probe arrays were treated for 10 min with an antibody solution [1x MES solution, 2 µg/µl acetylated BSA, 0.1 µg/µl normal goat IgG (Sigma Chemical, St. Louis, MO), 3 µg/µl biotinylated goat-anti-streptavidin antibody, (Vector Laboratories, Burlingame, CA) at 25°C. The final wash consisted of 15 cycles of 4 mixes per cycle at 30°C. Following washing and staining, probe arrays were scanned once at 1.5-µm resolution using the Affymetrix GeneChip Scanner 3000. Scanned output files were visually inspected for hybridization artifacts.

Normalization of Microarray Data

When using high density oligonucleotide microarrays, the aim is usually to determine how RNA populations may differ in expression in response to experimental conditions. Variations in global gene expression patterns usually result in a change in cell phenotype and are referred to as “biological variation”. However, the observed expression of genes can also include variation introduced through non-biological or “experimental variation”, which includes sample preparation, array production and processing, including labelling, hybridization and scanning. The raw data from the scanned chips therefore needs to be appropriately normalized to reduce unwanted variation between chips and allow data from different arrays to be compared in a meaningful way.

Microarray analysis using the Affymetrix GeneChip® system yields a CEL file for each GeneChip run. Each CEL file contains the expression level data for all the probesets on the chip. The first step in preparing the microarray data for analysis is referred to as normalization. This process adjusts the individual hybridization intensities to balance them appropriately, allowing meaningful biological comparisons to be made

between chips. Normalization is similar to adjusting expression levels measured by Northern blot or quantitative Real Time PCR where expression of a particular gene is adjusted relative to the expression of one or more reference genes whose expression level us assumed to be constant between experimental samples. Consequently, normalization takes into account different quantities of starting RNA, differences in labelling or detection of the fluorescent dyes and any systematic biases in the measured expression levels for any probe.

Normalization methods usually involve the selection and calibration of data derived from genes that are known not to be affected by experimental conditions. In this study, we used the Robust Multichip Average (RMA) method of normalization established by Irizarry *et al.* (Irizarry *et al*., 2003). RMA consists of three steps: a background adjustment, quantile normalization and finally summarization. Background correction adjusts for background noise and processing effects; cross-hybridization and adjusts estimated expression values to fall on a proper scale. Quantile normalization is a simple and fast algorithm which normalizes the data so that the quantiles of each chip of equal.

The CEL files were normalized using Robust Multichip Average (RMA) (Irizarry *et al.*, 2003) using RMAExpress software using background correction, quantile normalization and Median Polish summarization.

Significance Analysis of Microarrays

In order to identify changes in gene expression caused by v-Src or STAT-3C expression, CEL files for three Balb/c-3T3 control chips were either normalized with the CEL files for the three v-Src chips or normalized separately with the CEL files for the three STAT3-C chips. The expression sets were then exported to a Microsoft Excel spreadsheet, formatted for analysis by the Significance Analysis of Microarrays (SAM)

add-in tool for Excel (Tusher *et al.*, 2001). SAM was performed twice: first to identify differentially expressed genes between control Balb/c-3T3 cells and Balb/c-3T3 cells expressing pMvSrc, then again to identify differentially expressed genes between control Balb/c-3T3 cells and Balb/c-3T3 cells expressing pRcCMV-STAT3-C.

The options selected for SAM analysis were as follows: Response Type: two-class, unpaired data (Class 1 – Balb/c-3T3, Class 2 – v-Src or STAT3-C); Data logged: logged (base 2); Weblink Option: Accession number; Number of Permutations: 100; Imputation engine: N/A – no missing data in experiment; Random number seed: generate random number seed. This produced a list of Affymetrix probeset IDs differentially expressed in cells expressing v-Src and also for STAT3-C as compared to control cells. We accepted all probesets identified by SAM as differentially regulated by at least 1.5-fold.

Overlap of the Two Microarray Data Sets.

Microarray analysis and subsequent SAM generated two lists of differentially expressed genes: one list identified genes differentially expressed between control Balb/c-3T3 cells and cells transfected with v-Src, and the second list contained genes differentially expressed between control Balb/c-3T3 cells and cells transfected with STAT3-C. Genes common to both lists are most likely to be directly regulated by STAT3. Probeset IDs in common between the two lists were identified using the Excel VLOOKUP function. The probesets identified were then processed by Affymetrix NetAffx to yield a list of genes. SAM analysis generates a Score (T-statistic) for each probeset on each list. Probesets common to both lists were ranked using the average of the two Score values generated from the v-Src and STAT3-C SAM analysis.

The microarray data have been deposited in the Gene Expression Omnibus (GEO) Database at http://www.ncbi.nlm.nih.gov/geo (GEO accession no. GSE22251).

Computational Analysis of Microarray Data

We analyzed and categorized the differentially expressed genes identified by SAM using the Functional Annotation tool in the DAVID Bioinformatics Database (Dennis *et al.*, 2003; Huang da *et al.*, 2009). Pathway Analysis was carried out using the MetaCore Analysis Suite v 5.2 build 17389 GeneGO Maps program (GeneGO, Inc, New Buffalo, MI) to identify signaling pathways that were enriched in the list of differentially expressed genes.

Nuclear Extract Preparation and EMSA

For the detection of DNA-binding activity of STAT3 by EMSA, nuclear protein extracts were prepared using high-salt extraction as described previously (Garcia *et al.*, 1997). For standard EMSA, nuclear protein (5 µg) was incubated with ^{32}P-radiolabeled double-stranded DNA oligonucleotides containing a high-affinity variant of the sis-inducible element (hSIE; sense strand, 5'-AgCTTCATTTCCCTgAAATCCCTA-3') derived from the c-*fos* gene promoter, which binds activated STAT3 and STAT1 proteins as a positive control (Kreis *et al.*, 2007; Wagner *et al.*, 1990).

Supershift assays were performed using anti-STAT3 polyclonal antibodies (C20X, Santa Cruz Biotechnology) to identify STAT3. 2 µL of the concentrated STAT3 antibody was pre-incubated with 5 µg nuclear protein for 20 min at room temperature before adding the radiolabeled probe (30 min, 30°C). Samples were then separated by non-denaturing PAGE and detected by autoradiography.

For competition EMSA, nuclear extract was incubated with a series of unlabeled NDN oligonucleotides containing putative STAT3 binding sites, added in a molar excess, prior to adding ^{32}-P-labeled hSIE oligonucleotide. In addition, a wild-type oligonucleotide probe derived from the Necdin gene promoter (STAT3 consensus DNA-binding

sequence italicized) was used as follows: wild-type Necdin/–558 (sense strand, 16-mer), 5'-CTAC*TTCTAgAA*-3'.

Western Blot Analysis

Whole cell lysates were prepared in boiling sodium dodecyl sulphate (SDS) sample buffer and equal amounts (100 µg) of total protein were run on a 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membrane, washed with PBS/0.2% Tween 20, and incubated in 1x PBS/0.2% Tween 20/5% milk overnight with anti-phospho-STAT3 antibody (STAT3 Tyr705, Cat. #9131, Cell Signaling, Boston, MA, USA), or anti-STAT3 antibody to an epitope in the C-terminus of full-length STAT3-alpha (sc-482, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Necdin antibody (ab18554, Abcam, Cambridge, MA, USA). The membrane was then washed with PBS/0.2% Tween 20, incubated for 1 h at room temperature with alkaline phosphatase–linked anti-rabbit secondary antibodies, and visualized using ECL Western Blotting Detection Reagents (Amersham, Pittsburgh, PA, USA). For detection of ß-actin, the blot was stripped with stripping buffer [2% SDS, 62.5 mmol/L Tris (pH 6.8), 0.7% ß-mercaptoethanol] and re-blotted with anti-ß-Actin (A5441, Sigma) for 1 h at room temperature and visualized as described. Bands were detected by autoradiography. For densitometry, images were digitally scanned and optical density of the bands was quantified using Scion Image (Scion Corporation, Frederick, MD) and normalized to control.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed using a kit from Upstate as described by the supplier. Briefly, 2 million v-Src 3T3 cells were treated with formaldehyde for 10 minutes at room temperature. Cells were collected by scraping,

lysed and the DNA sheared by ultrasonication (Bioruptor XL, Diagenode). Immunoprecipitations were performed with the following antibodies (4.0 µg): anti-total STAT3 (sc-482, Santa Cruz Biotechnology), and anti-rabbit Ig G (sc-2027, Santa Cruz Biotechnology) as a control. Subsequently, cross-links were reversed, and bound DNA was purified. PCR was performed using NDN/-558 specific primers: Fwd: 5`-CATgAgAgACTgTTAggTATC-3` and Rev: 5`-CTATAgATTTgggCTCTCCAT-3`. Control primers were also used as follows: Fwd: 5'- TAg AAC CTA ggA ATg CCA ACA-3' and Rev: 5'- gAT ACC TAA CAg TCT CTC ATg-3'.

CHAPTER 3: RESULTS

PART I: Induction of STAT3 Activity in Mouse Fibroblasts

As previously described in the introduction, STAT3 can be activated by various upstream signalling molecules, including cytokines, non-receptor tyrosine kinases and constitutively active mutants, however, these ligands may also induce other STAT family proteins simultaneously e.g. Epidermal Growth Factor induces both STAT1 and STAT3. In order to identify genes which are regulated by STAT3 specifically, we chose techniques which to preferentially induced STAT3 only in mouse fibroblast cells. We chose to examine STAT3 activation in cells using IL-6 stimulation to induce transient phosphorylation of STAT3. We also chose to examine constitutive STAT3 activation in cells stably transfected with v-Src or STAT3-C.

IL-6 Induces STAT3 DNA Binding in Mouse Fibroblasts

Human recombinant IL-6 was used to induce STAT3 activation in Balb/c-3T3 mouse fibroblasts. Cells were seeded at 1 $x10^6$ per 10cm plate for 24 hours before serum starvation (0.1% bovine calf serum) for 3 hours prior to stimulation. Cells were then treated with increasing doses of human recombinant IL-6 for 30 minutes. Nuclear extracts were then prepared from cells as previously described Src (Yu *et al.*, 1995) and analyzed by Electrophoretic Mobility Shift Assay (EMSA) using a radioactive oligonucleotide (hSIE) that specifically detects activated STAT1 and STAT3.

As shown in Figure 3A, IL-6 activates endogenous STAT proteins in Balb/c-3T3 cells in a dose-responsive manner. Supershift assays, using antibodies to STAT1 and

STAT3-alpha confirm that IL-6 induces STAT3 DNA binding activity (Fig. 3B Lane 4), but not STAT1 (Lane 3). This is critical for specifically identifying STAT3-regulated genes, since the presence of active STAT1 would confound the results by activating additional signaling pathways.

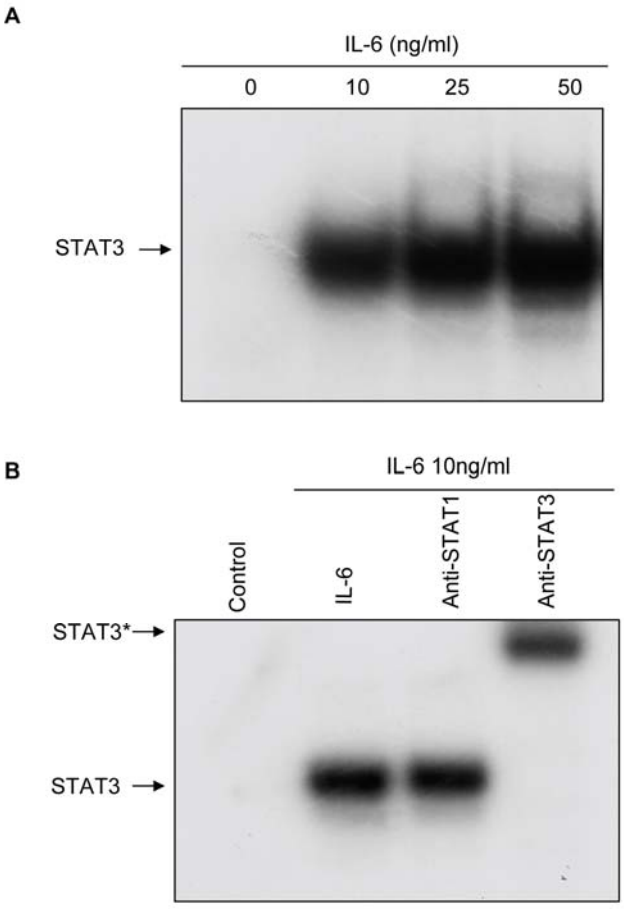

Figure 3. IL-6 induces STAT3 DNA binding in Balb/c-3T3 cells. **A**. Nuclear extracts prepared from cells treated with IL-6 at the doses indicated for 30 min were incubated with the ^{32}P-labeled hSIE oligo-nucleotide probe and analyzed by EMSA. **B**. Identification of specific STAT proteins activated by IL-6 in Balb/c-3T3 cells by antibody supershift analysis: nuclear extracts pre-incubated with STAT1 or STAT3-alpha specific antibodies were incubated with ^{32}P-labeled hSIE. * Antibody-shifted STAT3.

We then investigated the activation of STAT3 by IL-6 stimulation in NIH3T3 mouse fibroblast cells. As before, NIH3T3 cells were serum starved for 3 hours prior to treatment with increasing concentrations of IL-6.

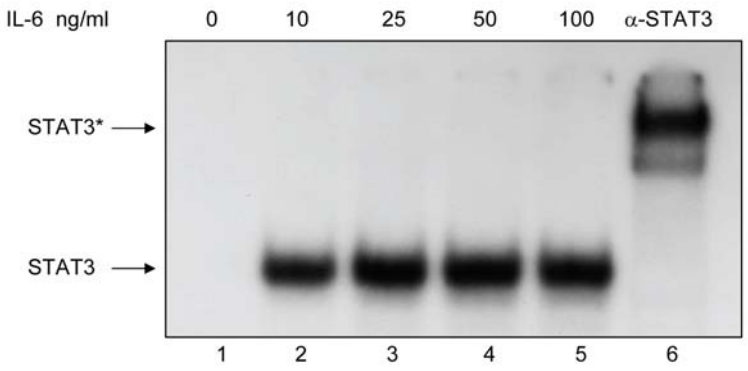

Figure 4. IL-6 induces STAT3 DNA binding in NIH3T3 cells. Nuclear extracts prepared from cells treated with IL-6 at the doses indicated for 30 min were incubated with the ^{32}P-labeled hSIE oligo-nucleotide probe and analyzed by EMSA. *, "supershifting" was achieved using anti-STAT3 antibodies added to the reaction to confirm the presence of STAT3 in the complex.

As shown in Figure 4, STAT3 activation shows maximum saturation at a treatment dose of 25 ng/ml and higher doses do not increase STAT3 binding at 30 min. We therefore treated NIH3T3 cells with doses of IL-6 lower than 25 ng/ml to see a dose response (Figure 5).

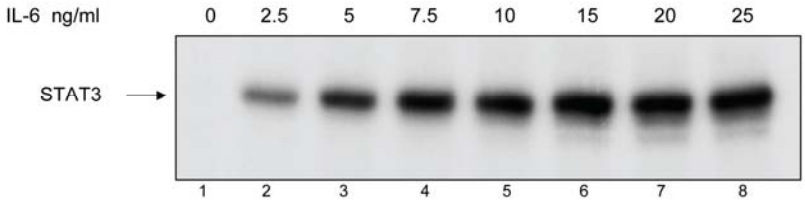

Figure 5. IL-6 induces STAT3 DNA binding in NIH3T3 cells in a dose responsive manner. Nuclear extracts prepared from cells treated with IL-6 at the doses indicated for 30 min were incubated with the ^{32}P-labeled hSIE oligo-nucleotide probe and analyzed by EMSA.

Figure 5 demonstrates that NIH3T3 cells show a dose response to IL-6 at lower doses than Balb/c-3T3 cells, achieving maximal STAT3 activation at 25 ng/ml. This indicates that this cell line is more sensitive to IL-6 stimulation than Balb/c-3T3 cells.

Kinetics of IL-6 Response in Mouse Fibroblasts

Activation of STATs is rapid, usually with a maximum accumulation of STATs within the nucleus within 30 minutes, followed by rapid inactivation via dephosphorylation (Haspel *et al.*, 1996). We therefore examined the kinetics of STAT3 phosphorylation in mouse fibroblasts.

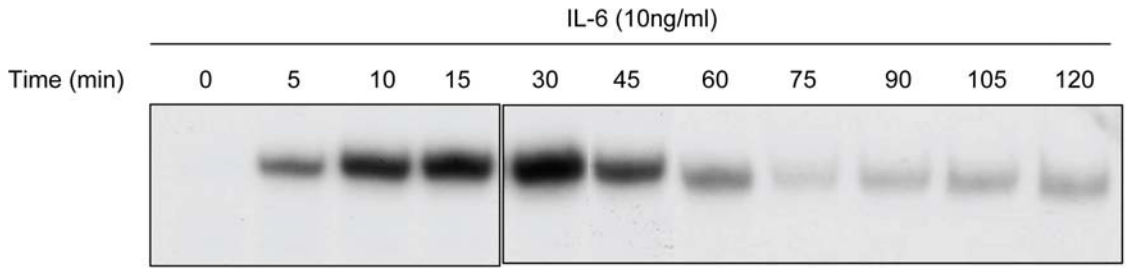

Figure 6. IL-6 induces STAT3 phosphorylation in Balb/c-3T3 cells in a time-dependent manner. Balb/c-3T3 cells were treated with IL-6 (10 ng/ml) for 0-120 minutes. Nuclear protein was collected, incubated with the ^{32}P-labeled hSIE oligo-nucleotide probe and analyzed by EMSA.

Figure 6 demonstrates that maximal induction of active STAT3 occurs at 30 minutes post IL-6 treatment, then decreases over time as STAT3 is deactivated. However, from 90 minutes onwards, there seems to be a slight increase in STAT3 phosphorylation, possibly suggesting a second wave of STAT3 activation. To verify this, we examined STAT3 activation past the 120 minute time point for cells treated with a single dose of IL-6. Figure 7 shows an early induction of STAT3 phosphorylation as expected, followed by a decrease in activated STAT3. Interestingly, a second surge in

STAT3 phosphorylation occurs from 3 hours onwards, despite no further IL-6 being added to the cells.

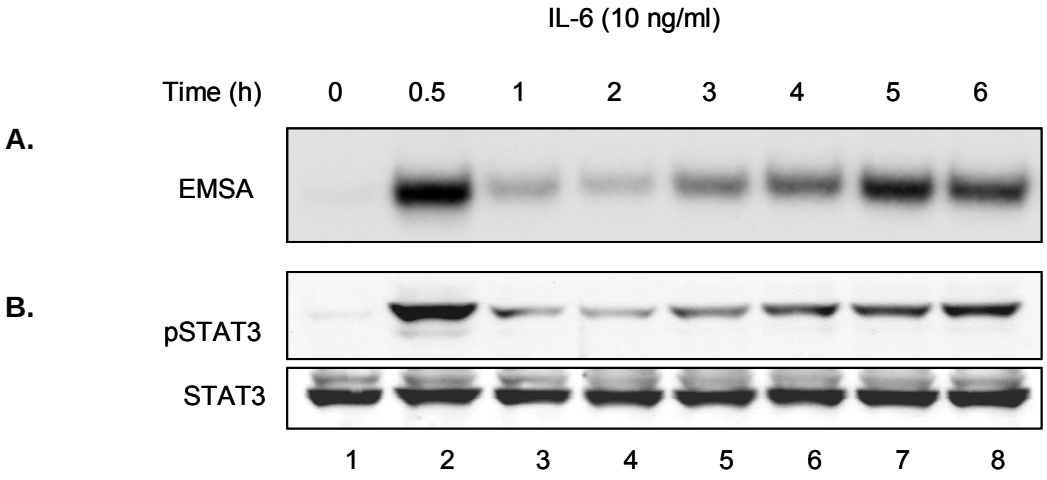

Figure 7. Single dose IL-6 treatment induces STAT3 phosphorylation in Balb/c-3T3 cells at multiple time points. A. Nuclear extracts were prepared from cells treated with IL-6 (10 ng/ml) for the time indicated and were incubated with the ^{32}P-labeled hSIE oligo-nucleotide probe then analyzed by EMSA. **B.** Total protein was collected from cells treated with IL-6 (10 ng/ml) for the times indicated and equal amounts of total protein (100ug) were loaded on a 10% SDS-polyacrylamide gel, electrophoresed and immunoblotted for pSTAT3 and total STAT3.

We then compared the kinetics of STAT3 activation in Balb/C-3T3 cells to that of NIH3T3 cells. Figure 8 demonstrates that a similar induction of STAT3 occurs in NIH3T3 cells treated with IL-6, reaching maximum induction at 30 minutes, which rapidly degrades by 60 minutes.

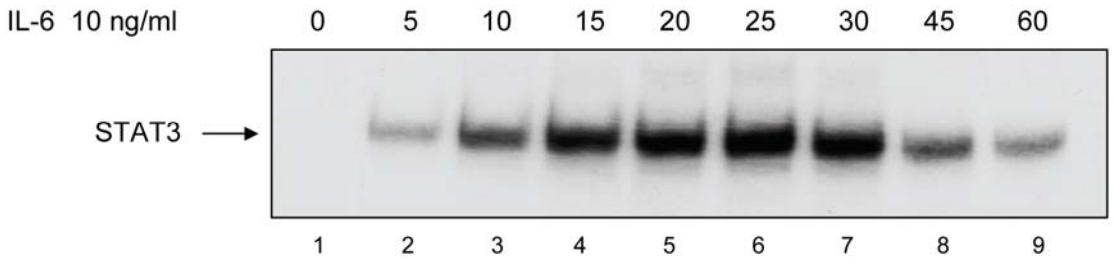

Figure 8. IL-6 induces STAT3 phosphorylation in NIH3T3 cells in a time-dependent manner. NIH3T3 cells were treated with IL-6 (10 ng/ml) for 0-60 minutes. Nuclear protein was collected, incubated with the ^{32}P-labeled hSIE oligo-nucleotide probe and analyzed by EMSA.

We then examined whether a similar second wave of STAT3 activation occurred in these cells. Figure 9 indicates that after a maximum activation at 30 minutes, followed by rapid degradation of the signal, STAT3 phosphorylation does indeed increase again at 3 hours.

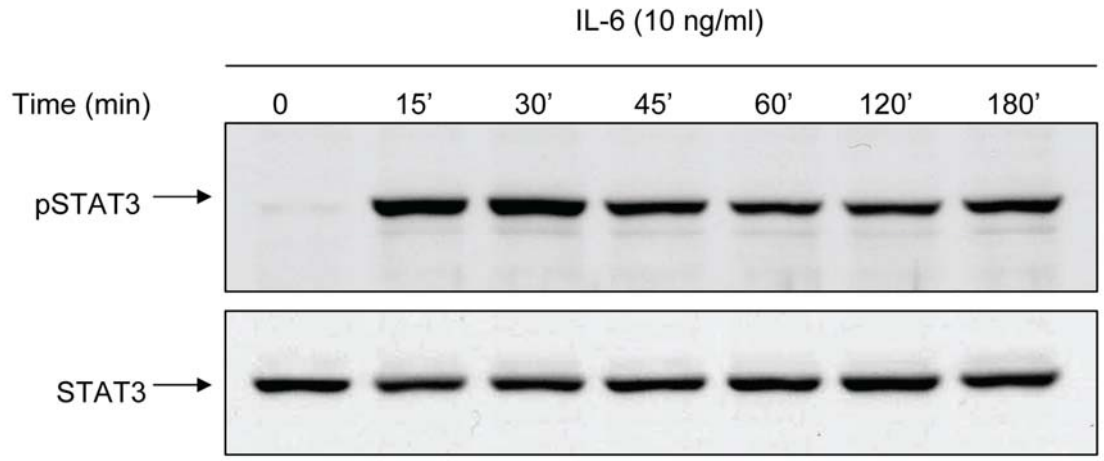

Figure 9. Single dose IL-6 treatment induces STAT3 phosphorylation in Balb/c-3T3 cells at multiple time points. Total protein was collected from cells treated with IL-6 (10 ng/ml) for the times indicated and equal amounts of total protein (100ug) were loaded on a 10% SDS-polyacrylamide gel, electrophoresed and immunoblotted for pSTAT3 and total STAT3.

STAT3 Activation in the absence of *de novo* protein synthesis

Cycloheximide (CHX), produced by the bacterium *Streptomyces griseus*, is widely used as an inhibitor of protein biosynthesis in eukaryotic organisms. It exerts its effect by interfering with the translocation step in protein synthesis, blocking translational elongation. In order to examine the requirement of protein synthesis for STAT3 activation, we pre-treated Balb/c-3T3 cells with CHX for 30 minutes prior to stimulating the cells with IL-6. In Figure 10, STAT3 activation does indeed occur in the absence of protein synthesis, both at 30 and 60 minutes following treatment. Induction of phospho-STAT3 is even stronger in cells pre-treated with CHX at both time points.

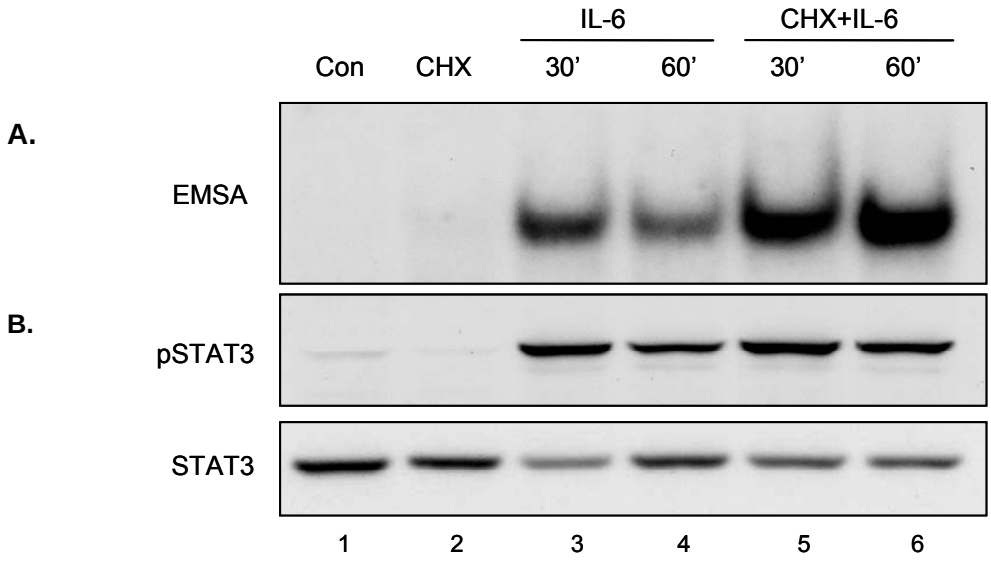

Figure 10. IL-6 stimulates STAT3 activation in the absence of *de novo* protein synthesis. **A.** Cells were pre-treated with CHX (10 ug/ml) followed by IL-6 (10 ng/ml) for the time indicated. Nuclear extracts were prepared and incubated with the ^{32}P-labeled hSIE oligo-nucleotide probe then analyzed by EMSA. **B.** Total protein was collected from cells treated with IL-6 (10 ng/ml) for the times indicated and equal amounts of total protein (100ug) were loaded on a 10% SDS-polyacrylamide gel, electrophoresed and immunoblotted for pSTAT3 and total STAT3.

v-Src Transformation Induces Constitutive STAT3 Activation in Mouse Fibroblasts

STAT3 is also known to be induced by cellular transformation by v-Src (Yu *et al.*, 1995). NIH3T3 and Balb/c-3T3 cell lines transformed by v-Src were provided by Dr. D. Shalloway (Cornell University, New York). Compared to their parental, non-transformed lines, v-Src transformed cells exhibit high levels of constitutive STAT3 activation (Figure 11), which was confirmed by supershift EMSA assay. Both cell lines exhibit comparable high levels of STAT3 activation.

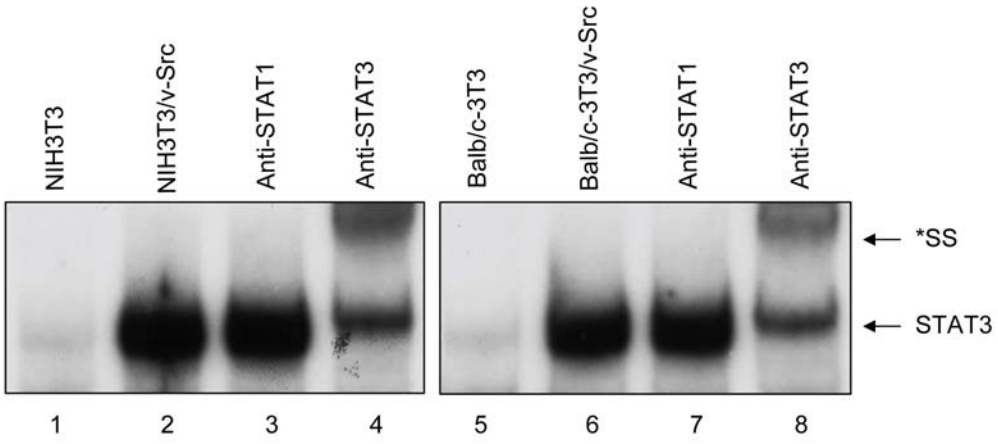

Figure 11. v-Src transformation induces constitutive STAT3 activity in mouse fibroblasts. Nuclear extracts were harvested from transformed and untransformed NIH3T3 and Balb/c-3T3 cells, incubated with the ^{32}P-labeled hSIE oligo-nucleotide probe and analyzed by EMSA. *, "supershifting" was achieved using anti-STAT3 antibodies added to the reaction to confirm the presence of STAT3 in the complex.

Activation of STAT3 Signaling by STAT3-C

A constitutively-activated STAT3 molecule (called STAT3-C) was genetically engineered and is capable of dimerization in the absence of tyrosine phosphorylation, migrate to the nucleus and bind to STAT3 response elements in promoters to induce gene expression (Bromberg *et al.*, 1999).

Balb/c-3T3 parental cells and Balb/c-3T3 cells stably expressing the constitutively active STAT3-C mutant expression vector or v-Src were collected and nuclear extracts prepared. Figure 12 shows an EMSA demonstrating STAT3 binding activity in cells expressing either v-Src or STAT3-C (lanes 2 and 4), which is absent in parental cells. When compared to STAT3 binding capability in Balb/c-3T3 cells stably transfected with v-Src (lane 2), the STAT3-C cells show lower, yet significant STAT3 activity.

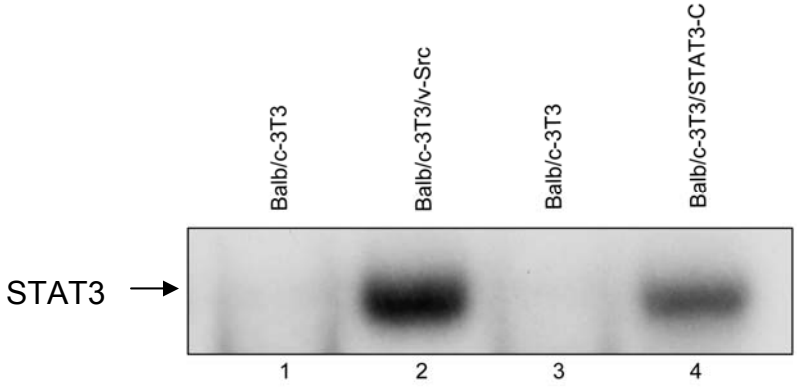

Figure 12. Mouse fibroblasts stably expressing v-Src or STAT3-C show constitutive STAT3 activity. Nuclear extracts were harvested from transformed and untransformed Balb/c-3T3 cells, incubated with the ^{32}P-labeled hSIE oligo-nucleotide probe and analyzed by EMSA.

Summary

STAT3 activation can be induced by multiple mechanisms, including cytokines, growth factor receptors, non-receptor tyrosine kinases and constitutively active mutants. We chose to examine activation of STAT3 in mouse fibroblasts using transient stimulation with IL-6 and constitutive activation of STAT3 via v-Src and STAT3-C stable transfection.

IL-6 induces STAT3 activation in a dose- and time-dependent manner in mouse fibroblasts. IL-6 does not induce STAT1 activity in these cells, making them useful to study gene expression profiles regulated by STAT3. The experiments revealed differences in the sensitivity of the two cell lines to IL-6 treatment, with NIH3T3 cells being more sensitive to IL-6. Stimulation of NIH3T3 cells with IL-6 resulted in maximal STAT3 activation at lower doses than seen in Balb/c-3T3 cells.

Both cell lines, however, exhibited similar kinetics in response to IL-6 treatment. IL-6 induced a maximal STAT3 activation at 30 minutes, followed by a rapid degradation of the signal with a surge in STAT3 activation at a later time point, despite no further IL-6 treatment. The rapid degradation of the STAT3 signal post-stimulation is most likely due to the activation of negative regulators of STAT3, such as SOCS3. Once SOCS3 has down-regulated the STAT3 activation, it is likely to be degraded itself, thus resulting in a later surge of STAT3 activity as IL-6 continues to signal via the gp130 receptor.

Pre-treating the cells with CHX prior to IL-6 stimulation blocked *de novo* protein synthesis. This ensured that IL-6 was stimulating direct activation of latent STAT3 monomers present in the cytoplasm, but also prevented any other proteins not already present from being translated. In cells pre-treated with CHX, we saw an induction of STAT3 activity as expected, which was higher than IL-6-only treated cells at both 30 and 60 minutes. This higher level of STAT3 induction is most likely due to the inhibition of protein synthesis, particularly in relation to negative feedback proteins such as SOCS3.

v-Src and STAT3-C have both been previously characterized in constitutively activating STAT3 (Bromberg *et al.*, 1999; Yu *et al.*, 1995). We confirmed this in the mouse fibroblasts we used for these experiments, however, STAT3-C did not appear to induce constitutive STAT3 activity to the same level as v-Src.

In summary, we examined three well characterized methods for activating STAT3 using mouse fibroblast cells. These cell lines were used for subsequent experiments to study gene expression patterns in the presence of active STAT3.

Part II: Analysis of STAT3-Regulated Gene Expression

Cytokine and growth factor signaling pathways involving STAT3 are frequently constitutively activated in many different human primary tumors, and are best known for the transcriptional role they play in the controlling cell growth and cell cycle progression. However, the extent of STAT3's reach on transcriptional control of the genome as a whole remains an important question. We predicted that this persistent STAT3 signaling affects a wide variety of cellular functions, many of which still remain to be characterized.

To date, up-regulated expression of numerous STAT3 target genes has been identified, including VEGF (Niu *et al.*, 2002), Bcl-2, Bcl-xL (Zushi *et al.*, 1998), p21, Cyclin D1 (Sinibaldi *et al.*, 2000) and survivin (Gritsko *et al.*, 2006). These STAT3 target genes have generally been identified on an individual basis, while few studies have attempted to identify large numbers of STAT3 regulated genes (Alvarez *et al.*, 2005; Dauer *et al.*, 2005; Paz *et al.*, 2004; Sekkai *et al.*, 2005; Snyder *et al.*, 2008). Our goal was to take a broad approach to identify novel STAT3 regulated genes involved in oncogenesis by examining changes in the genome-wide gene expression profile by microarray, using Balb/c-3T3 cells expressing active STAT3.

Identification of Potential STAT3 Target Genes Expressed Upon IL-6 Stimulation

Balb/c-3T3 cells were treated with IL-6 for 1 h, with or without CHX pre-treatment. RNA was collected from control cells (no treatment or CHX only) and treated cells (IL-6 only or CHX+IL-6) from 3 consecutive passages. At each passage, cells from five 10cm plates were pooled and RNA collected and purified. The RNA was hybridized to Affymetrix Mouse Genome 430 2.0 GeneChip microarrays and the data analyzed to identify differentially expressed genes under the different conditions.

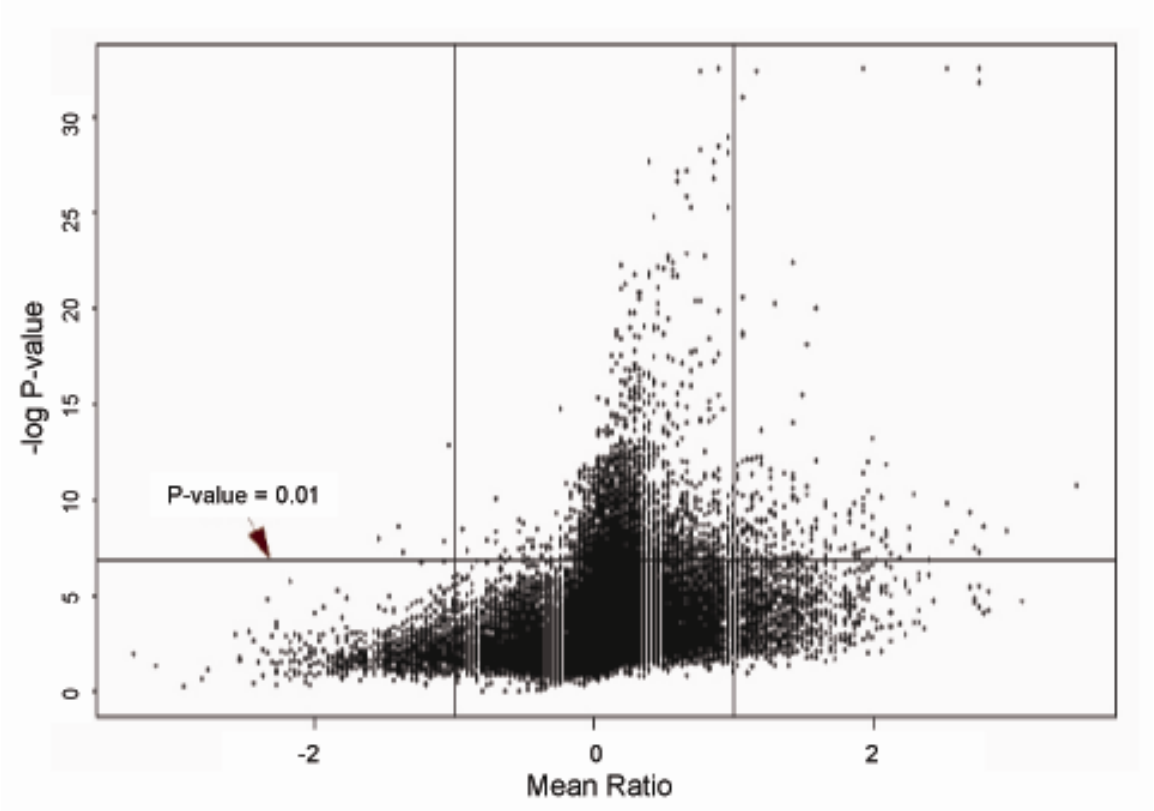

Figure 13. Volcano plot of genes induced by IL-6 at 1 h in mouse fibroblasts. RNA was extracted from Balb/c-3T3 cells serum starved for 3 hours prior to treatment with IL-6 for 1 h. The experiment was performed in triplicate and the RNA collected separately. Purified RNA from each experiment was prepared and hybridized to individual Affymetrix Mouse 2.0 GeneChips. Following analysis using Affymetrix MAS 5.0 software and a 2-tailed test for significance, the differentially expressed genes were plotted on a volcano plot.

A volcano plot is a type of scatter plot used to identify changes in large data sets using replicate data. It plots significance (-log P-value) on the y-axis versus $\log_2$ fold-change (Mean Ratio) on the x-axis allowing many thousands of replicate data points (Affymetrix Probesets) between two conditions to be viewed, in this case untreated cells versus cells treated with IL-6. By combining the p-value statistical test with the magnitude of the fold-change, visual identification of statistically significant data points is made simple. The two regions of interest, demonstrating the differentially expressed

genes that are most highly significant, are found towards the top of the plot and to either the far left (underexpressed genes) or to the far right (overexpressed genes). These data points represent values that display large magnitude fold-changes as well as high statistical significance. The upper middle region shows data points with less than 2-fold difference, but are statistically significant. The lower middle region shows data points that have less than 2-fold difference and are not statistically significant.

As can be seen in Figure 13, few data points represent probe sets that were underexpressed, or downregulated, compared to control (top left quadrant), whereas many more data points were overexpressed, or upregulated, compared to control (top right quadrant) in cells treated with IL-6 for 1 h. From previous studies, this is to be expected, since most genes identified to date as regulated by STAT3 are activated in the presence of active STAT3.

In cells pre-treated with cycloheximide (CHX) prior to IL-6 stimulation, the volcano plot (Figure 14) shows fewer significant data points located in the top quadrants. Only one gene is significantly underexpressed (downregulated) as shown in the top left quadrant and many fewer genes are overexpressed (upregulated) as seen in the top right quadrant. These data plots indicated the extent to which gene expression profiles were altered in mouse fibroblasts with IL-6 stimulation, demonstrating that the expression of most genes remained unaltered at the 1 h time point.

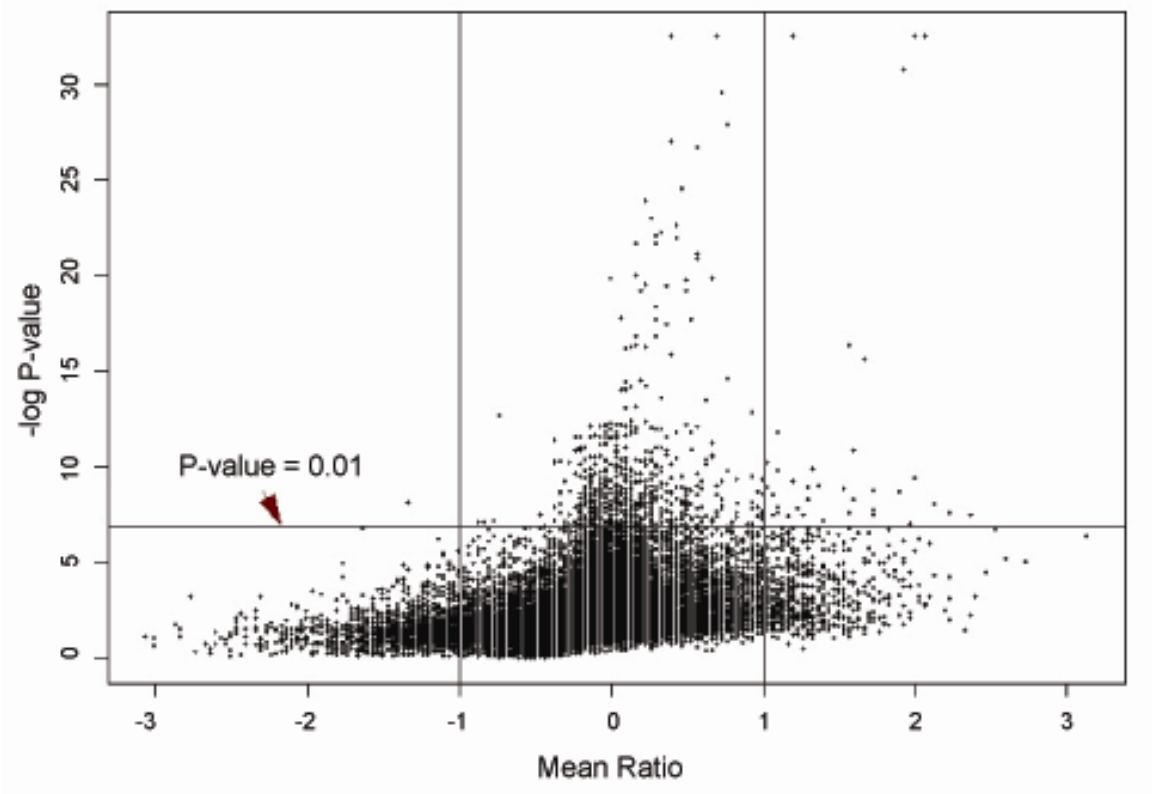

Figure 14. Volcano plot of genes induced in mouse fibroblasts by IL-6 at 1 h in the presence of cycloheximide. RNA was extracted from Balb/c-3T3 cells serum starved for 3 hours prior to treatment with CHX for 30 min then IL-6 for 1 h. The experiment was performed in triplicate and the RNA collected separately. Purified RNA from each experiment was prepared and hybridized to individual Affymetrix Mouse 2.0 GeneChips. Following analysis using Affymetrix MAS 5.0 software and a 2-tailed test for significance, the differentially expressed genes were plotted on a volcano plot.

Identification of Genes Induced by IL-6 Activation of STAT3

The most significant data points from the above analysis were plotted on 3-dimensional dot plots. Data points which were identified as being highly significantly altered in all 3 experimental replicates converge on the lower corner of the plot.

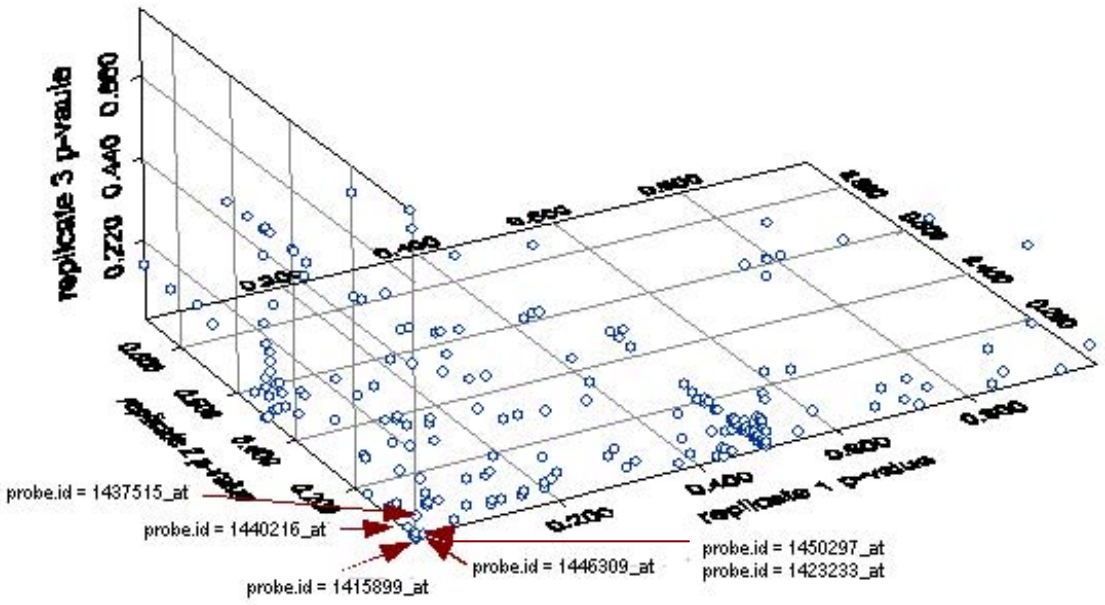

Figure 15. Most significant genes induced by IL-6 compared to control. Genes identified from the previous experiments having composite p-value <0.001 and mean log signal >1 were plotted on a dot plot according to p-value.

The most significantly differentially expressed genes are as follows (indicated by arrows): SOCS3 (1415899_at), Cebpd (1423233_at), unknown EST (1446309_at), IL-6 (1450297_at), Ifitm5 (interferon induced transmembrane protein 5) (1440216_at) and a gene sequence similar to the prolactin family genes (1437515_at).

Table 2. Average fold-change of the most significant genes upregulated by IL-6.

Probe ID	Gene	Av. Fold change (log2)	p-value
1416576_at	Cish3 (SOCS3)	2.27	0.00002
1455899_x_at	Cish3 (SOCS3)	2.07	0.00002
1456212_x_at	Cish3 (SOCS3)	1.93	0.00002
1423233_at	Cebpd	1.93	0.00002

Table 2 shows the top significantly differentially expressed probesets with fold-changes greater than 1.5 induced upon IL-6 stimulation (note: the most significant genes were the same for both IL-6 only and CHX+IL-6 treatment). Both SOCS3 and CEBPD are known IL-6 regulated genes. Although the other genes had significant p-values, their fold-change was <1.5, therefore the change in expression is unlikely to have a large impact on the cell and they are not included in the table.

The most significant genes differentially expressed in cells treated with CHX and IL-6 were also plotted (Figure 16). Two genes were identified as being significantly differentially expressed between CHX treated cells and cells treated with CHX then stimulated with IL-6 for 1 h: Probeset 1416576_at: Cish3 (SOCS3) with an average fold-change of 2.0 (p-value 0.00002) and probeset 1426730_a_at: prolactin family gene Prl2b1 (prolactin family 2, subfamily b, member 1) with an average fold-change of 1.67 (p-value 0.005).

Summary

Treatment of mouse fibroblasts with IL-6 for 1 h induced a number of significant genes. Pre-treatment with CHX inhibited expression of some of those genes. This is to be expected since *de novo* protein synthesis is inhibited. The data points shown in Figure 15 represent those genes which are direct targets of STAT3 and do not require other proteins or transcription factors to be produced in order for the genes to be

transcribed. SOCS3, Cebpd and IL-6 are previously known targets of IL-6 activation of the JAK-STAT pathway. As previously mentioned, SOCS3 is known to be rapidly induced by IL-6 as an immediate early gene (Starr *et al.*, 1997). SOCS3 inhibits JAKs by binding to the kinase domain and inhibiting their tyrosine kinase activity. The early induction of SOCS3 fits with the previous results in this study demonstrating a down-regulation of STAT3 phosphorylation after 30 minutes of IL-6 stimulation. This rapid loss of signal is most likely due to the upregulated expression of SOCS3, as shown by microarray.

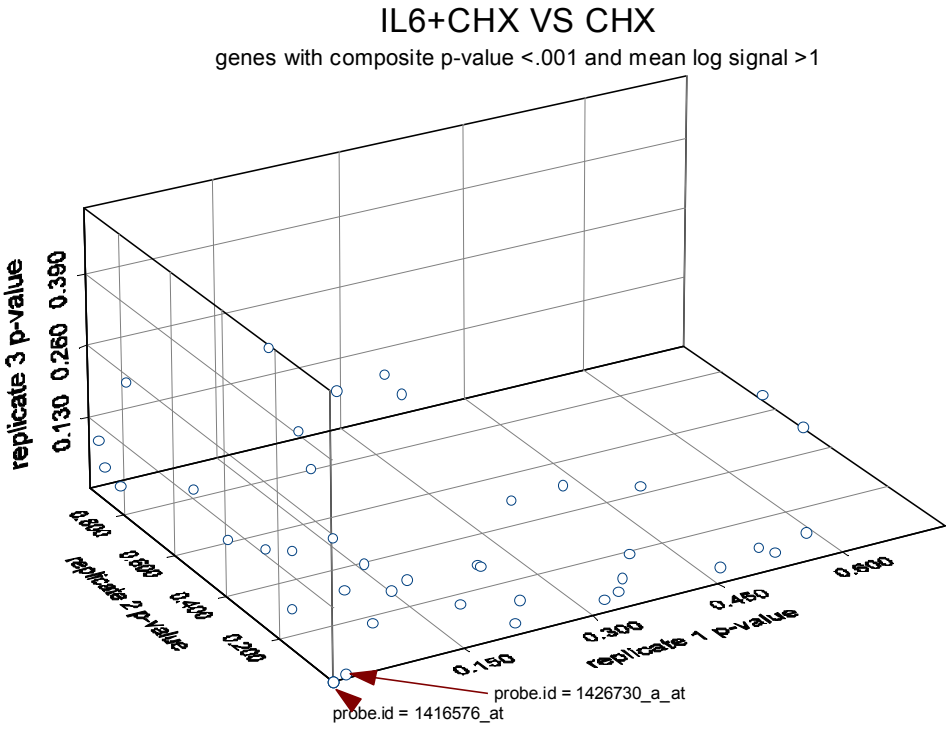

Figure 16. Most significant genes induced by IL-6 in the presence of CHX compared to CHX control. Genes identified from the previous experiments having composite p-value <0.001 and mean log signal >1 were plotted on a dot plot according to p-value.

Cebpd (CCAAT/enhancer binding protein (C/EBP), delta) is a bZIP transcription factor which can bind as a homodimer to certain DNA regulatory regions. It can also form heterodimers with the related protein CEBP-alpha. A known target of IL-6 signaling via the JAK-STAT pathway (Yamada *et al.*, 1997), CEBPD is important in regulating genes involved in immune and inflammatory responses.

In conclusion, microarray analysis of IL-6 induced genes in the presence or absence of CHX demonstrated the proof of concept that STAT3-regulated genes can be identified using microarrays, however the genes identified were already known to be regulated by IL-6 via the JAK-STAT pathway. Using an early time points such as 1 h, we were unable to identify any novel STAT3-regulated genes that were differentially expressed.

Identification of STAT3 Target Genes in Cells Expressing v-Src and STAT3-C

To identify potential novel STAT3-regulated genes, we examined global gene expression patterns in cell lines harboring persistently active STAT3. Gene expression profiles in such cells are likely to be representative of the genetic profile of a cancer cell with aberrant STAT3 expression, as compared to inducing STAT3 activity transiently using exogenous stimulation, such as IL-6 or transient transfection (Paz *et al.*, 2004).

Balb/c-3T3 cells were chosen for this study, since parental cells and cells stably expressing both v-Src and STAT3-C were available. RNA was harvested from normal Balb/c-3T3 cells with low levels of endogenous STAT3 activity, to serve as a control. RNA was also extracted from Balb/c-3T3 cells stably transfected with either v-Src, known to induce persistent activation of STAT3 (Garcia *et al.*, 1997; Zhang *et al.*, 2000b), or the constitutively active mutant, STAT3-C (Bromberg *et al.*, 1999). Triplicate samples were collected, one each from three consecutive passages. At each passage, cells from five 10cm plates were pooled and RNA collected and purified. Each RNA sample was hybridized to a single Affymetrix Mouse Genome 430 2.0 GeneChip.

Significance Analysis of Microarrays (SAM) (Tusher *et al.*, 2001) was used to identify differentially expressed genes between parental Balb/c-3T3 cells and cells stably transfected with either v-Src or STAT3-C. We accepted all genes identified by SAM as differentially regulated by at least 1.5-fold (Yan *et al.*, 2002).

Overlap of the Two Microarray Data Sets

Microarray analysis and subsequent SAM generated two lists of differentially expressed genes: one list identified genes differentially expressed between control Balb/c-3T3 cells and cells transfected with v-Src, and the second list contained genes differentially expressed between control Balb/c-3T3 cells and cells transfected with

STAT3-C. Genes common to both lists are most likely to be directly regulated by STAT3. These genes were identified by cross-referencing the data in the two lists using the Microsoft Excel VLOOKUP function.

While v-Src transformed cells have constitutively active STAT3, Src also stimulates other STAT3-independent pathways (Brunton and Frame, 2008; Frame, 2002; Frame, 2004; Odajima *et al.*, 2000). In contrast, target genes activated by STAT3-C are limited to direct binding of the activated protein to STAT3 consensus sites in DNA. Therefore, using cells stably transfected with either v-Src or STAT3-C allowed us to control for clonal variations, as well as divergence in signaling pathways depending on the mechanism of STAT3 activation. The use of multiple microarray replicates in our approach further increases confidence in the results. This allowed us to identify a set of common genes as targets of STAT3. The data were further validated by the identification of several previously characterized STAT3-regulated genes, including CCND1, p21 (Sinibaldi *et al.*, 2000), VEGFA (Niu *et al.*, 2002), and Mcl-1 (Puthier *et al.*, 1999). The most significantly over-expressed (induced) and under-expressed (repressed) genes are listed in Table 3 and Table 4, respectively (Top 50 genes are listed in Tables A-1 and A-2).

To date, the majority of studies have examined putative STAT3 target genes which are up-regulated or over-expressed when STAT3 is active. STAT3 has been shown to activate transcription of many genes involved in oncogenesis, cell survival, tumor progression and metastasis. STAT3 has also previously been shown to repress the transcription of a handful of genes, including p53 (Niu *et al.*, 2005) and nitric oxide synthase (Saura *et al.*, 2006) However, our results demonstrate that STAT3 is capable of repressing expression of a much larger number of genes. This novel discovery has the potential to profoundly impact the biology of cells harboring constitutively active STAT3.

Table 3. Most Significant Probesets Over-Expressed Common to v-Src and STAT3-C

				v-Src Data		STAT3-C Data		
Accession	**Affy Probeset**	**Gene Name**	**Gene Description**	**Score(d)**	**Fold Change**	**Score(d)**	**Fold Change**	**Av. Score**
NM_024223	1417311_at	Crip2	cysteine rich protein 2	51.343	17.4918	72.639	26.6976	61.991
NM_021451	1418203_at	Pmaip1	phorbol-12-myristate-13-acetate-induced protein 1	39.681	31.3643	34.058	47.1463	36.869
NM_009701	1418818_at	Aqp5	aquaporin 5	21.732	22.2635	45.193	94.0883	33.463
AF352788	1451527_at	Pcolce2	procollagen C-endopeptidase enhancer 2	36.947	19.9492	27.024	31.4550	31.986
AV066880	1452592_at	Mgst2	microsomal glutathione S-transferase 2	29.291	15.1149	34.651	37.3030	31.971
BF235516	1420842_at	Ptprf	protein tyrosine phosphatase, receptor type, F	16.848	20.1766	44.849	20.1217	30.848
NM_013867	1415936_at	Bcar3	breast cancer anti-estrogen resistance 3	26.309	11.7696	33.804	22.3994	30.056
BI251808	1416613_at	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	21.870	47.4091	36.953	84.8654	29.412
NM_053132	1449527_at	Pcdhb7	protocadherin beta 7	25.742	18.8861	29.613	16.6966	27.677
AF022072	1425458_a_at	Grb10	growth factor receptor bound protein 10	29.734	22.6049	23.021	30.5514	26.378
BB041811	1455900_x_at	Tgm2	transglutaminase 2, C polypeptide	33.990	5.1505	16.141	19.8117	25.065
NM_011577	1420653_at	Tgfb1	transforming growth factor, beta 1	38.605	4.2769	11.154	2.2493	24.879

The top genes ranked by Average Score identified by SAM as being upregulated in common by v-Src and STAT3-C

Table 4. Most Significant Probesets Under-Expressed Common to v-Src and STAT3-C

				v-Src Data		STAT3-C Data		
Accession	**Affy Probeset**	**Gene Name**	**Gene Description**	**Score(d)**	**Fold Change**	**Score(d)**	**Fold Change**	**Av. Score**
AF081260	1418070_at	Cdyl	chromodomain protein, Y chromosome-like	-72.232	0.0548	-47.865	0.0832	-60.049
NM_010882	1415923_at	Ndn	necdin	-58.571	0.0081	-54.087	0.0102	-56.329
AW743020	1435382_at	Ndn	necdin	-30.525	0.0135	-73.974	0.0122	-52.249
NM_009866	1450757_at	Cdh11	cadherin 11	-32.836	0.0055	-52.719	0.0048	-42.778
BB074430	1437853_x_at	Ndn	necdin	-16.328	0.1440	-67.114	0.1065	-41.721
AV228782	1434261_at	Sipa1l2	signal-induced proliferation-associated 1 like 2	-43.072	0.0715	-38.883	0.1002	-40.978
BB259670	1437284_at	Fzd1	frizzled homolog 1 (Drosophila)	-16.598	0.0997	-61.622	0.0879	-39.110
AW743020	1435383_x_at	Ndn	necdin	-40.515	0.0120	-29.383	0.0141	-34.949
BB125261	1448293_at	Ebf1	early B-cell factor 1	-46.495	0.0688	-19.975	0.0796	-33.235
NM_007993	1460208_at	Fbn1	fibrillin 1	-38.299	0.0180	-26.476	0.0200	-32.388
AV124445	1455792_x_at	Ndn	necdin	-30.296	0.0151	-32.708	0.0189	-31.502
NM_011581	1422571_at	Thbs2	thrombospondin 2	-33.293	0.0148	-26.721	0.0124	-30.007

The top genes ranked by Average Score identified by SAM as being down-regulated in common by v-Src and STAT3C.

One such gene which appears to be repressed by STAT3 is the negative growth regulator Necdin. Necdin was one of the most significantly repressed genes identified (Table 4) and five Affymetrix probesets corresponding to NDN were ranked in the list of the top 12 most significantly repressed probesets, suggesting that Necdin is significantly repressed when STAT is constitutively active. This demonstrates that computational analysis of microarray data can be used to identify potential STAT3 target genes for further investigation.

Pathway Analysis Reveals Known and Novel Functions of STAT3

Pathway Analysis is an unbiased method to determine whether differentially expressed genes, and the proteins they encode, are enriched in particular pathways, giving insight into the biological meaning of the changes observed.

We subjected the list of differentially expressed genes in common between v-Src and STAT3-C expressing cells to the MetaCore™ Analysis Suite (GeneGO) and compared them to known biological pathways in the MetaBase™ database. Using this analysis we were able to identify known STAT3 pathways, including the JAK/STAT pathway and Angiotensin/STAT pathway. This provides support for the use of such analyses to identify novel pathways that may also be regulated by STAT3.

Cell adhesion and cytoskeletal remodeling were among the most significantly enriched pathways identified from the differentially expressed genes (Table 5). The role of STAT3 in cytoskeletal remodeling has previously been reported (Gao and Bromberg, 2006). Functional analysis of the genes we identified in cytoskeletal remodeling processes, indicates that STAT3 regulates genes involved in protein phosphorylation, signaling (MAPKK and Ras pathways), as well as response to hypoxia and cell migration.

We also examined the genes regulated by STAT3 in cell adhesion and demonstrated that proteins involved in cell-matrix adhesion and cell-cell adhesion, particularly focal adhesion formation, were particularly enriched when STAT3 is constitutively active, as well as several genes in the integrin cell adhesion pathway. As such, we show that computational analysis of microarray data can identify both known and novel pathways regulated by STAT3.

Table 5. Enriched Pathways in Genes Differentially Expressed by STAT3-C and v-Src

Cell process	Count	%	p-Value
Cell adhesion - Integrin-mediated cell adhesion and migration	19	42.22	5.56E-05
Cytoskeleton remodeling	32	33.33	6.09E-05
Development - Angiotensin signaling via STATs	13	52.00	6.25E-05
Cell adhesion, cytokine and chemokine mediated signaling pathway	31	33.33	7.93E-05
Transcription - Ligand-dependent Transcription of Retinoid Target Genes	15	46.88	8.03E-05
Proteolysis	12	50.00	1.97E-04
Cytoskeletal remodeling and cell adhesion - Integrin outside in signaling	18	39.13	2.79E-04
Development - WNT signaling, degradation of beta-catenin	10	50.00	6.83E-04
G-protein signaling - RhoA regulation pathway	14	41.18	7.26E-04
Immune response - IFN alpha/beta signaling pathway	11	45.83	9.32E-04

Over-represented pathways identified using the MetaCore Analysis Suite (GeneGO Inc.)

Functions of Induced Genes.

To determine the functional classification of the differentially expressed genes identified by SAM, we performed Functional Annotation using the tool in the DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov/) (Dennis *et al.*, 2003; Huang da *et al.*, 2009). A wide range of target genes were altered by STAT3 activation, including genes involved in multiple pathways regulating biological and cellular processes, metabolism, protein localization and transport, as well as organ and system development (Table 6, Figure 17). Genes within these categories include many involved in cell growth and maintenance, such as lipid, nucleotide and protein synthesis, metabolism and/or localization (including VLDLR, APOL6, AK5, MTAP, UPP1, POP5, NUPL1, SEC61B, VDP) as well as signal transduction, all of which are required to promote cell growth and proliferation.

STAT3 has a well characterized role in regulating gene transcription, however, we also show through Functional Analysis, that STAT3 controls the expression of genes involved in cellular processes required to transport the proteins and regulate their subcellular localization. This supports our hypothesis that STAT3 coordinates multiple pathways within the cell and reveals that STAT3 has wide-ranging effects, controlling multiple cellular pathways involved in fundamental biological processes. Our results suggest that STAT3 orchestrates transcription, translation, transport and localization leading to wide reaching effects on cell growth, proliferation and survival. In contrast to previous studies of STAT3 target genes, we demonstrated that STAT3 regulates a diverse array of genes in both a positive and negative manner. Most genes regulated by STAT3 that have been identified to date demonstrate increased expression in cells where STAT3 is activated. However, our results also show that STAT3 signaling causes repression of many genes, including Necdin, which could profoundly impact the biology of cells harboring constitutively active STAT3.

Table 6. Functional Enrichment (based on GO Biological Process) in Genes Differentially Expressed in Common by STAT3-C and v-Src using DAVID.

GO Category Level	GO Term	GO Category*	Count	p Value
GOTERM_BP_3	GO:0050794	Regulation of cellular process	535	1.64E-14
GOTERM_BP_3	GO:0043283	Biopolymer metabolic process	627	5.89E-12
GOTERM_BP_3	GO:0048519	Negative regulation of biological process	170	3.05E-09
GOTERM_BP_3	GO:0008104	Protein localization	140	5.76E-09
GOTERM_BP_3	GO:0045184	Establishment of protein localization	131	9.26E-09
GOTERM_BP_3	GO:0048523	Negative regulation of cellular process	158	1.96E-08
GOTERM_BP_3	GO:0009653	Anatomical structure morphogenesis	187	2.40E-08
GOTERM_BP_3	GO:0015031	Protein transport	123	3.79E-08
GOTERM_BP_3	GO:0048513	Organ development	213	7.26E-08
GOTERM_BP_3	GO:0048731	System development	248	7.76E-07

The top 10 functionally enriched categories ranked by p-Value for GOTERM_BP_3 identified by DAVID

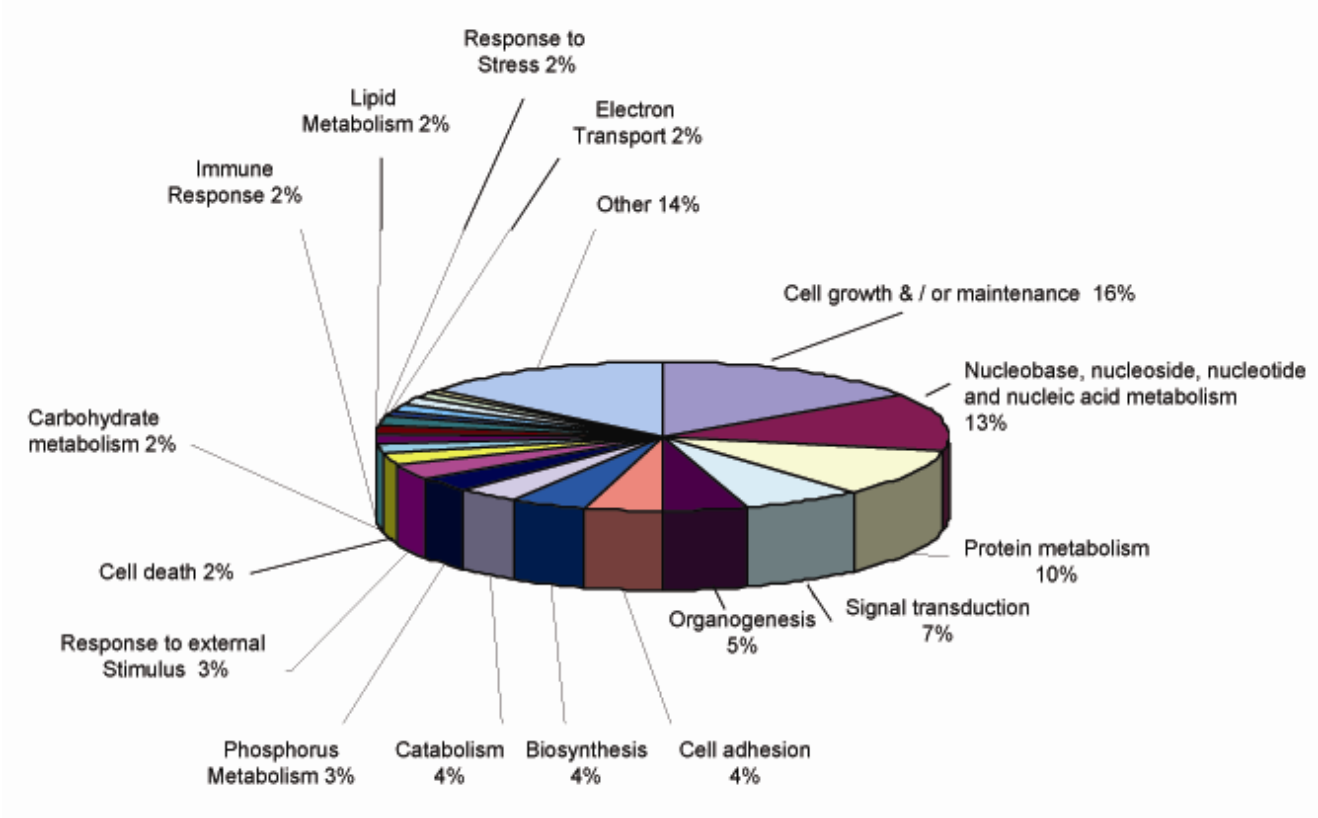

Figure 17. Biological Processes Regulated by STAT3-C. Top significant genes differentially expressed in cells expressing constitutively active STAT3-C grouped according to biological process.

Part III: Necdin is a Novel STAT3 Target Gene

Through computational analysis of our microarray data, we identified Necdin, a negative growth regulator, as a novel potential STAT3 target gene. Necdin is a potent growth suppressor that is predominantly expressed in post-mitotic neurons (Aizawa *et al.*, 1992; Hayashi *et al.*, 1995; Maruyama *et al.*, 1991; Uetsuki *et al.*, 1996). Necdin expression has been shown to be down-regulated both carcinoma cell lines and primary tumors (Chapman and Knowles, 2009), suggesting that repression of Necdin expression may have a role in oncogenesis.

Constitutively Activated STAT3 Blocks Necdin mRNA Expression

Microarray analysis of global gene expression patterns produces a large list of potential target genes. Identifying true potential target genes from that list for further investigation is a critical decision. NDN, the gene encoding Necdin, a negative growth regulator (Hayashi *et al.*, 1995) and member of the MAGE family of melanoma-associated tumor antigens, was identified as one candidate STAT3-regulated gene. In Table 2, the genes identified as down-regulated in the presence of STAT3 activity were ranked according to significance (Score (d)). Necdin was one of the most statistically significantly down-regulated genes. Of even greater importance, is the fact that 5 Affymetrix probesets corresponding to NDN are ranked in the top 12 most significantly repressed probesets (Table 4). This indicates that, based on the statistical analysis alone, Necdin is highly likely to be down-regulated in expression when STAT3 is active in the cell. These data, together with the fact that Necdin has not previously been suggested as a potential STAT3 target gene, prompted us to select Necdin for further analysis.

We first examined Necdin gene expression in the microarray samples we collected, including cells treated with IL-6 (+/-CHX), v-Src and STAT3-C. Figure 18

demonstrates that Necdin expression levels are indeed lower in cells expressing v-Src and STAT3-C, however, Necdin expression is high in control cells, as well as cells treated for only 1 hour with IL-6 (+/-CHX).

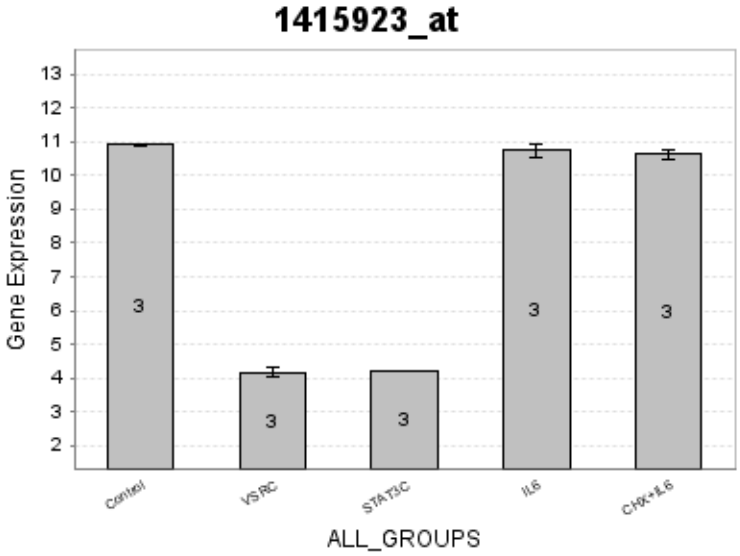

Figure 18. Necdin expression in cells with activated STAT3. Microarray Analysis of Necdin mRNA expression levels. RNA from Balb/c-3T3 cells stably expressing either pMvSrc or pRc-STAT3-C or treated with IL-6 (+/- CHX) for 1 h was isolated, processed and hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips. All microarray experiments were done in triplicate independent experiments, and the results are presented for each probe set as average fold change in RNA expression. Data for two different probesets are presented. The signal intensity of the parental Balb/c-3T3 cells was set to 100%.

We set out to verify the computational analysis and confirm whether Necdin is in fact a physiological STAT target gene. When compared with normal control cells, analysis of the microarray data demonstrated that NDN expression was consistently repressed in the cell lines expressing v-Src or STAT3-C, indicating that NDN is a candidate STAT3-regulated gene in both of these cell lines (Fig. 19A). Figure 19B. confirms that NDN mRNA expression is dramatically down-regulated in v-Src and STAT3-C expressing cells as measured by quantitative Real-Time PCR.

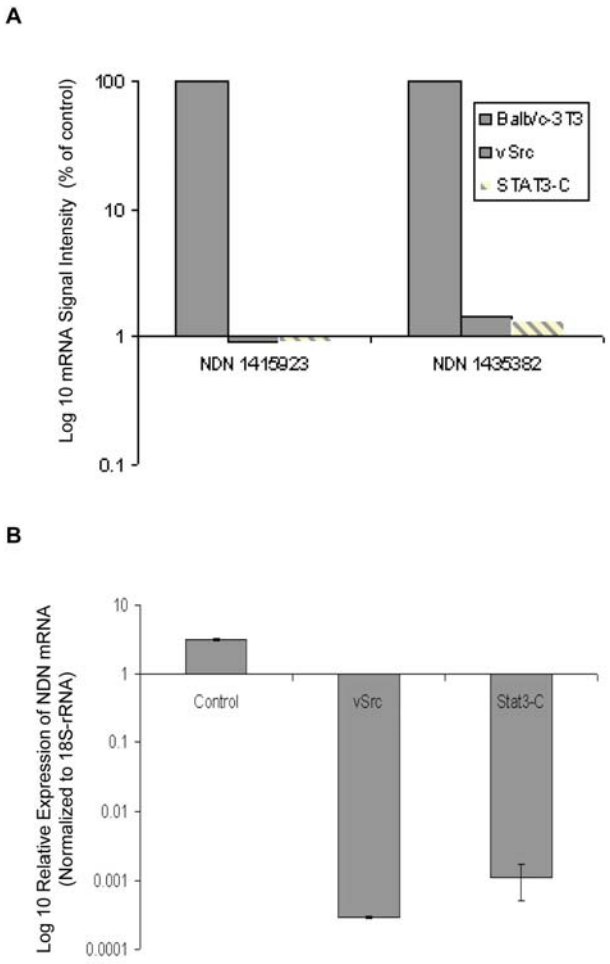

Figure 19. **Analysis of Necdin expression in cell lines stably expressing v-Src or STAT3-C**. **A.** Microarray Analysis of Necdin mRNA expression levels. RNA from Balb/c-3T3 cells stably expressing either pMvSrc or pRc-STAT3-C was isolated, processed and hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips. All microarray experiments were done in triplicate independent experiments, and the results are presented for each probe set as average fold change in RNA expression. Data for two different probesets are presented. The signal intensity of the parental Balb/c-3T3 cells was set to 100%. **B.** Real-time PCR analysis. RNA samples used for microarray analysis were measured for Necdin mRNA expression using Real-time PCR with gene-specific primers and fluorescent-labeled probe (Taqman® Gene Expression Assays, Applied Biosystems). RNA expression was normalized to 18S rRNA. n = 3 independent experiments.

Repression of Necdin mRNA Expression is STAT3 Dependent

NIH-3T3 cells stably expressing v-Src express high levels of active STAT3. These v-Src 3T3 cells were treated with either control siRNA or two different doses of STAT3-specific siRNA. Cells treated with control siRNA maintain high levels of STAT3 and have low levels of Necdin expression (Fig. 20, lane 1). As expected, STAT3 siRNA effectively inhibited expression of total STAT3 (Fig. 20, lanes 2 and 3). In these cells the expression of the STAT3 protein was inhibited in a dose-dependent manner, and Necdin expression was restored in a manner consistent with STAT3 knockdown in this cell line. These results suggest that repression of Necdin is dependent on activated STAT3.

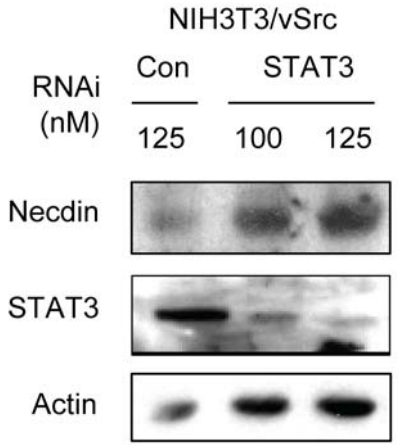

Figure 20. Inhibition of STAT3 activity correlates with Necdin expression. Western. NIH3T3 cells stably expressing v-Src were seeded (2.5 x 10^5) in 6 cm tissue culture plates in complete medium 24 h before transfection. Cells were then transfected with either 125 nM control siRNA or 100 nM or 125 nM STAT3 siRNA. At 48 h after transfection, total protein was harvested and equal amounts of total protein (100□g) were loaded on a 10% SDS-polyacrylamide gel, electrophoresed and immunoblotted for Necdin (polyclonal, Abcam ab18554), phosphorylated STAT3 (p-STAT3, Cell Signaling 9131), total STAT3 (Santa Cruz, sc-482) and anti-actin (monoclonal, Sigma A-4551) proteins.

Activated STAT3 binds to the NDN promoter *in vitro*

To determine whether STAT3 directly regulates Necdin transcription, we analyzed the sequence of the mouse NDN promoter (Uetsuki *et al.*, 1996) for potential STAT3 binding sites. STAT3 consensus sites have been defined as palindromic sequences with the common sequence 5`-TT($N_{4-6}$)AA-3` (Ehret *et al.*, 2001). Our analysis identified several candidate STAT3 binding sites throughout the 1500 base pairs upstream of the transcriptional start site. Double-stranded oligonucleotide probes were generated for all the potential binding sites and tested in a competition EMSA (Figure 21) for their ability to compete for the binding of STAT3 against a high affinity variant of the STAT3 binding site in the c-*fos* promoter (hSIE) (Wagner *et al,* 1990; Yu *et al.*, 1995).

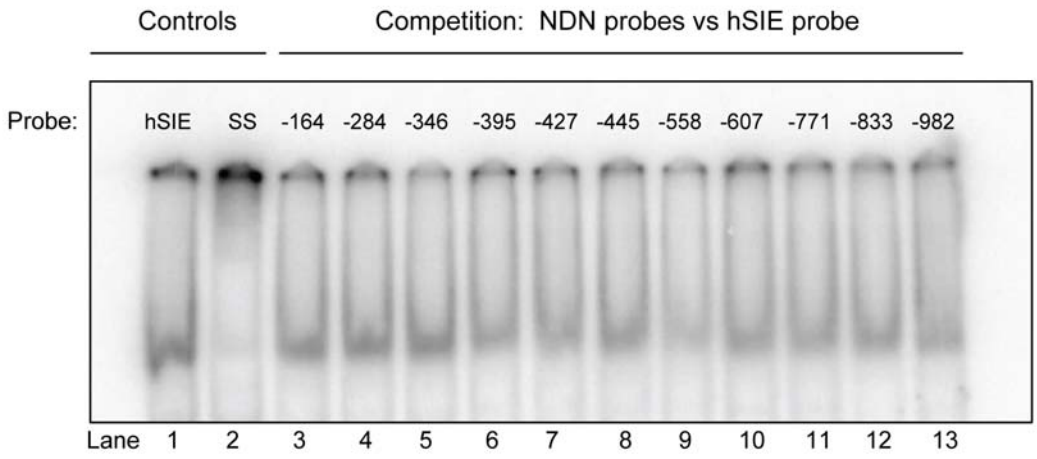

FIGURE 21. STAT3 binds directly to the NDN promoter. **A.** Competition EMSA. 3T3 v-Src nuclear extract was incubated with ^{32}P-labeled double stranded hSIE oligonucleotide (lanes 1 and 2) or with a series of unlabeled NDN oligonucleotides containing putative STAT3 binding sites, in a 10^3-fold molar excess (lanes 3-13) prior to adding ^{32}P-labeled hSIE oligonucleotide, to compete with hSIE for STAT3 binding. SS, supershift with anti-STAT3 antibodies. A candidate STAT3 DNA binding site in the mouse Necdin promoter was identified (position -558, relative to the translation initiation site).

The oligonucleotide containing the putative binding site at position -558 relative to the transcriptional initiation site was identified as being able to compete effectively with the hSIE probe (Fig. 21, lane 9). Furthermore, we confirmed the ability of non-radioactive NDN/-558 oligonucleotide to compete with the radiolabeled hSIE probe for binding of activated STAT3.

As shown in Figure 22A, increasing amounts of unlabeled NDN/-558 were tested, demonstrating that a high molar excess is able to compete with ^{32}P-hSIE for STAT3 binding. A double stranded ^{32}P-radiolabeled DNA oligonucleotide corresponding to the NDN/–558 sequence identified in the NDN promoter was then used in an EMSA to detect STAT3 DNA binding. The NDN probe, as well as the positive control probe, hSIE, were incubated with 5 ug nuclear extract from v-Src 3T3 cells and subjected to native gel electrophoresis. As shown in Figure 22B, activated STAT3 binds to the high affinity sequence in the hSIE oligonucleotide (lane 1), as well as to the sequence derived from the NDN promoter (lane 3). The artificial hSIE probe contains a high affinity STAT3 binding site and yielded a strong EMSA band, whereas the single STAT3 binding site in the NDN/-558 probe demonstrated a weaker STAT3 binding activity as expected.

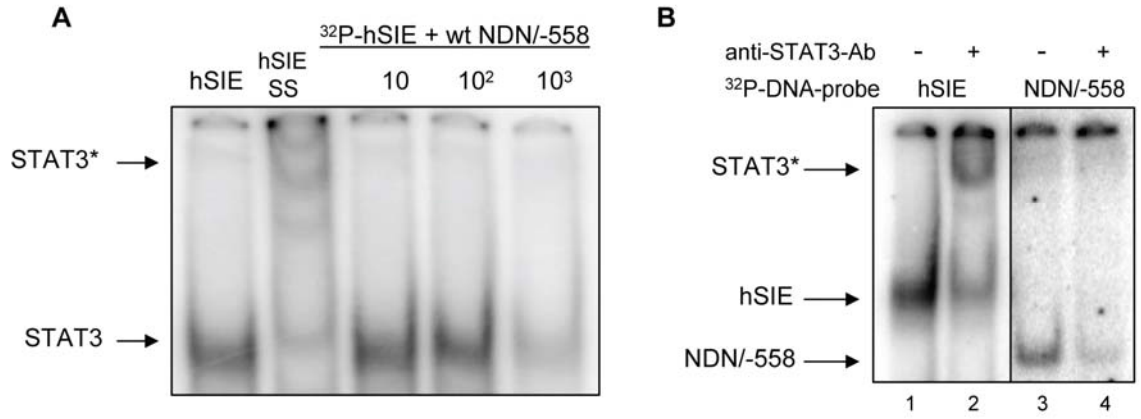

FIGURE 22. A. Competition EMSA confirms STAT3 binding to the NDN promoter. 3T3 v-Src nuclear extract was incubated with ^{32}P-labeled double stranded hSIE oligonucleotide (lanes 1 and 2) or with increasing amounts of unlabeled NDN/-558 oligonucleotide containing the putative STAT3 binding site, (lanes 3-5) prior to adding ^{32}P-labeled hSIE oligonucleotide, to compete with hSIE for STAT3 binding. SS, supershift with anti-STAT3 antibodies. **B.** EMSA. 3T3 v-Src nuclear extract was incubated with the following ^{32}P-labeled double-stranded oligonucleotides: hSIE (lanes 1 and 2), NDN/-558 (lanes 3 and 4); "supershifting" was achieved using anti-STAT3 antibodies added to the reaction to confirm the presence of STAT3 in the complex.

To confirm that STAT3 is contained in the protein complex binding to the oligonucleotides, the nuclear extracts were pre-incubated with anti-STAT3 antibodies before adding the radiolabeled probe (lanes 2 and 4). The addition of anti-STAT3 antibody supershifted the hSIE band. Addition of the antibody to the NDN/-558 reaction diminished the appearance of the main EMSA band as expected, but the supershift band is not visible. The diminished band and absent supershift band may also be due to the fact that STAT3 binding to the NDN/-558 oligo was weaker to begin with and the amount of supershifted complex is too little to be seen. It is also possible that the STAT3 antibody partially blocks binding of the NDN/-558 radioactive probe to STAT3 in the nuclear extract and the supershift is not visible. This could result if the antibody recognition site and DNA binding domain for the NDN/-558 oligonucleotide in STAT3

were in close proximity, causing the antibody to partially obstruct binding of STAT3 to the probe.

Binding of STAT3 to the NDN Promoter *in vivo*

To determine whether STAT3 could bind the Necdin promoter in intact cells, chromatin immunoprecipitation assays (ChIP) were performed in 3T3 v-Src cells using an antibody specific to STAT3. As shown in Figure 23, PCR yielded Necdin promoter DNA immunoprecipitated with an anti-STAT3 antibody in the region of the -558 putative STAT3-binding site, but not at a control locus on the NDN promoter. The specificity of this binding interaction was demonstrated by the lack of signal generated when a control antibody is used (anti-rabbit IgG). These data provide evidence that STAT3 can directly bind the Necdin promoter in intact 3T3 v-Src cells.

Together the competition and NDN/-558 probe EMSAs and ChIP assay suggest that STAT3 has the ability to bind to the NDN promoter both *in vitro* and *in vivo* and provide further evidence that control of NDN expression by STAT3 occurs through a direct binding event at the promoter and that gene regulation primarily occurs at the level of transcription.

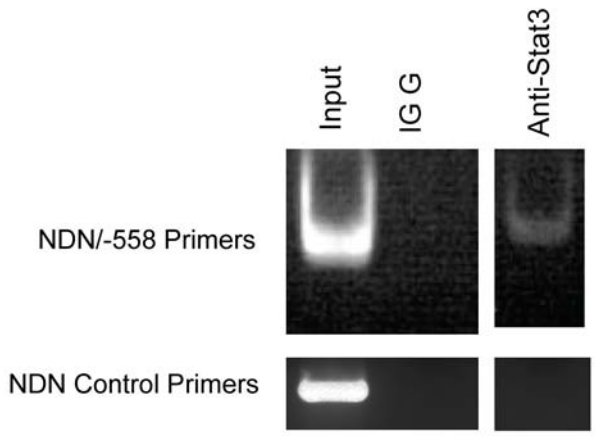

FIGURE 23. Chromatin immunoprecipitation assay (ChIP) confirms STAT3 binds the NDN promoter *in vivo*. Balb/3T3 v-Src cells expressing constitutively active STAT3 were used for ChIP. Briefly, after crosslinking histones to DNA by formaldehyde for 10 min, cells were collected and sonicated to shear DNA to an average length of 200-1000 bp. A portion of this material was used as a positive control for PCR (Input). The remaining sample was incubated with either anti-IgG or anti-STAT3 antibodies overnight and then immunoprecipitated using protein A-agarose. The histone-DNA complex was reverse cross-linked after several washing steps, and samples were subjected to PCR using specific primers surrounding the candidate STAT3-binding site at position -558 in the NDN promoter or a control region within the NDN promoter.

Necdin Expression Is Repressed in Human Melanoma Cells

We next examined whether down-regulation of Necdin occurred in human tumor cells expressing activated STAT3. Expression of Necdin has been previously shown to be repressed in melanoma cells (Hoek *et al.*, 2004) so we examined whether this had a correlation with STAT3 activity.

STAT3 phosphorylation and DNA-binding activity have been shown to increase in A375 melanoma cells in a density-dependent manner in the absence of ligand (Kreis *et al.*, 2007). A375 cells were plated at increasing density and allowed to grow for 72 h. Nuclear extracts were prepared and analyzed by EMSA. Figure 24A shows that DNA-binding of STAT3 increased with cell density as expected. We then analyzed total protein by Western blot for Necdin expression. Figure 24B shows that expression of total STAT3 and STAT3 phosphorylation was up-regulated in a density-dependent manner. Conversely, as STAT3 activation increases, Necdin expression was down-regulated at the protein level.

To confirm that the repression of Necdin expression is STAT3-dependent, A375 cells were plated at high density, and allowed to adhere overnight before being treated with either DMSO or the STAT3-inhibitor CPA-7 (20 uMol/L) for 24 h (Turkson *et al.*, 2004). Western blot analysis shows that when A375 cells are plated at low density (10^5 cells), Necdin expression was high, whereas activated STAT3 levels were low (Fig. 25, lane 1). Cells plated at high density (10^6 cells), (Fig. 25, lane 3) showed higher levels of p-STAT3 and decreased expression of Necdin. Treatment of high density A375 cells with CPA-7 for 24 h inhibited STAT3 activation (Fig. 25, lane 2), and Necdin levels in these cells were restored to high levels, comparable to cells plated at low density. This demonstrates that Necdin repression in these cells is indeed STAT3 dependent.

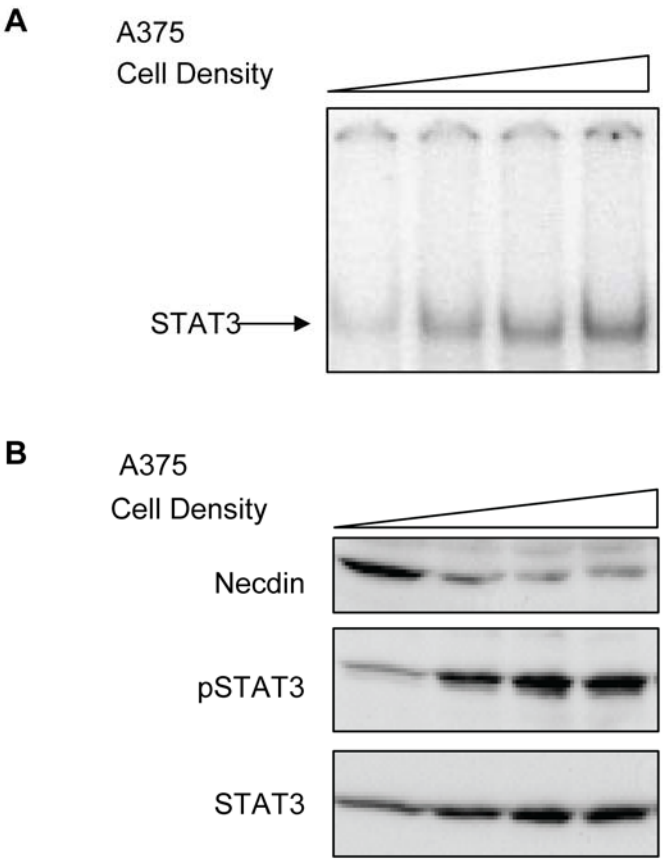

FIGURE 24. STAT3 downregulates Necdin expression in A375 human melanoma cells. A375 cells were plated at different densities (Fig. 3A and 3B: 1, 2.5, 5 or 7.5 x 10^5 cells; Fig. 3C: 10^5 and 10^6 cells) in 10 cm plates and grown for 72 h. Nuclear extracts and total protein were collected. **A.** EMSA. Nuclear extracts from A375 cells were incubated with STAT-specific hSIE ^{32}P-labeled double stranded oligonucleotide. **B.** Western blot. Total protein extracts were harvested from A375 cells plated at different densities and equal amounts of total protein (100 g) were loaded on a 10% SDS-polyacrylamide gel, electrophoresed and immunoblotted for Necdin, phosphorylated STAT3 (p-STAT3) and total STAT3 proteins.

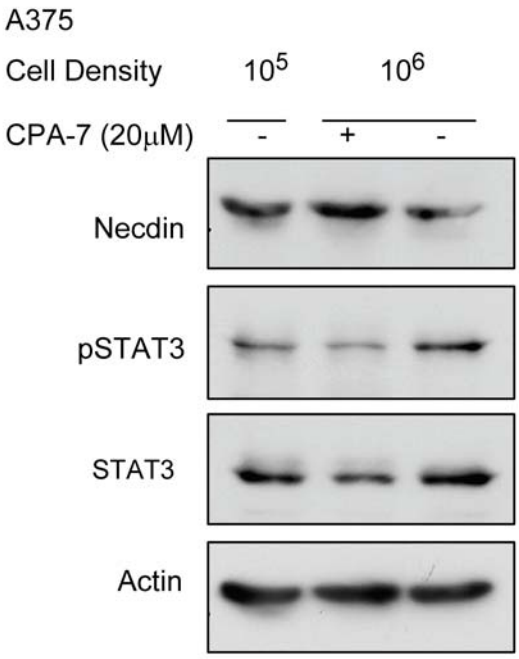

FIGURE 25. Inhibition of STAT3 expression in A375 human melanoma cells restores Necdin expression. Western blot. A375 cells were plated at two different densities (10^5 and 10^6 cells) and allowed to adhere overnight. Plates seeded at 10^6 cells were then treated with either DMSO or CPA-7 (20 □Mol/L) and all cells were grown for a further 48 h. Total protein was harvested and analyzed by Western blot.

IL-6 Represses Necdin Expression in Human Prostate Cancer Cells

IL-6 acts as an autocrine growth factor in prostate cancer (Giri *et al.*, 2001) and has been linked to progression of tumors (Drachenberg *et al.*, 1999). IL-6 signals are transmitted via the JAK-STAT pathway from receptors on the cell surface to the target genes in the nucleus, involving phosphorylation and activation of STAT3 (Lou *et al.*, 2000). We therefore examined whether activation of STAT3 via IL-6 stimulation led to repression of Necdin expression in the prostate cancer cell lines DU145 and PC3. These cell lines harbor low levels of constitutively active STAT3 (Mora *et al.*, 2002; Okamoto *et al.*, 1997), which can be further induced by stimulation with IL-6. Cells were serum starved for 3 h prior to treatment with IL-6 (10 nMol/L) for 12 or 24 h. Total protein was prepared and analyzed by Western blot. Figure 26 shows that IL-6 stimulation resulted in increased STAT3 activity within the cells and demonstrated corresponding down-regulation of Necdin expression upon IL-6 stimulation in both cell lines. This confirms that IL-6 is capable of repressing Necdin expression via STAT3 in prostate cancer cells.

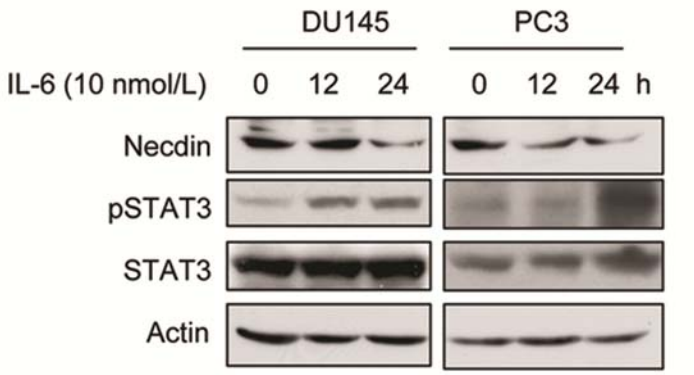

Figure 26. Necdin expression correlates with STAT3 activity in prostate cancer cell lines. PC3 and DU145 cells were plated at a density of 10^6 cells/10 cm plate and allowed to adhere overnight and serum starved for 3 h prior to treatment with IL-6 (10 nMol/L) for 12 or 24 h. Total protein was extracted from the cells and analyzed by Western blot.

Necdin Expression Correlates with STAT3 Activity in Human Breast Cancer Cells

Since EGFR and Src signaling pathways contribute to STAT3 activation in breast cancers (Garcia *et al.*, 2001; Garcia *et al.*, 1997), we examined Necdin gene expression in the microarrays of breast tissue and matched normal tissue. Figure 27 demonstrates that there may indeed be a difference in Necdin expression in tumor versus normal tissue. Normal breast tissue shows a higher level of Necdin transcripts than tumor tissues.

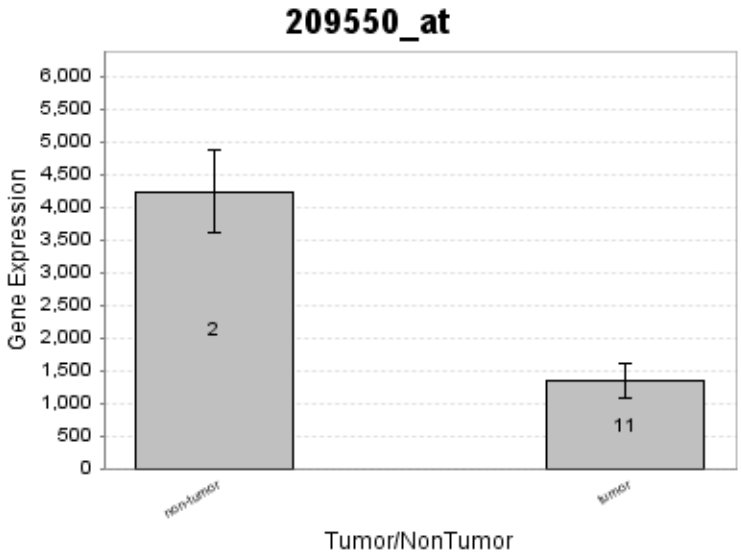

Figure 27. Expression of Necdin mRNA in breast tumors and normal adjacent breast tissue. Graph shows gene expression for the Affymetrix Probeset 209550_at corresponding to Necdin in breast tumor and normal (non-tumor) tissue.

However, since only a small group of 13 tumor/non-tumor samples were available, we chose to evaluate Necdin expression levels in human breast cancer cell lines with varying levels of endogenous STAT3 activity. Figure 28 shows that p-STAT3 protein levels were high in MDA-MB-468 cells, slightly lower in MDA-MB-231 and very low in MCF-7 cells. Necdin protein expression inversely correlated with p-STAT3 levels, being expressed at a low level in MDA-MB-468 and MDA-MB-231 cells, but exhibited much higher expression in MCF-7 cells.

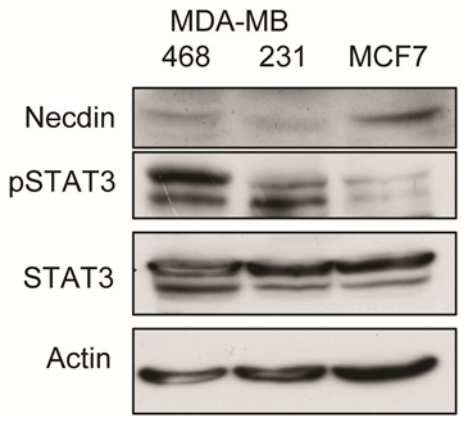

Figure 28. STAT3 activity down-regulates Necdin expression in human breast cancer cell lines. Total protein was harvested from MDA-MB-468, MDA-MB-231 and MCF-7 cells and subjected to Western blot analysis.

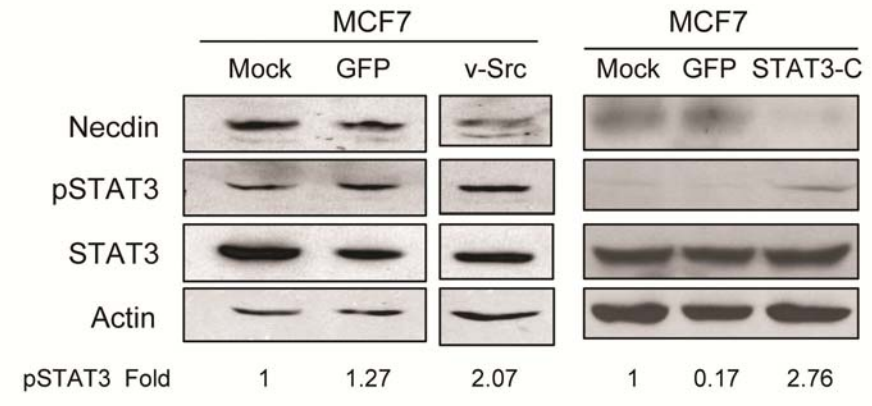

Figure 29. Inhibition of STAT3 restores Necdin expression in MCF7 breast cancer cells. MCF-7 cells were seeded and allowed to adhere overnight before being transiently transfected with control (GFP) or pMvSrc plasmids using Lipofectamine PLUS. Total protein was collected at 48 h post-transfection and subjected to Western blot analysis. Expression of p-STAT3 was measured using densitometry (Scion Image Beta 4.0.3, Scion Corp., Frederick, MD, USA) and expressed as fold-change compared to control.

To test the hypothesis that constitutively activated STAT3 has a causal role in suppressing Necdin expression in tumor cells, we examined whether transient activation of STAT3 signaling could down-regulate Necdin expression. MCF7 cells express high

levels of Necdin (Figure 29, lanes 1 and 4), however when transiently transfected with v-Src for 48 h, Necdin protein expression is inhibited. This demonstrates that even a transient 2-fold increase in STAT3 activation in these cells is sufficient to effectively repress the expression of Necdin (Figure 29, lanes 3 and 6).

Summary

In this study, we show that Necdin mRNA expression inversely correlates with STAT3 activity in cells expressing constitutively-active STAT3. Inhibition of STAT3 using siRNA restores expression of Necdin protein. Chromatin immunoprecipitation and EMSA assays indicate that the Necdin gene is directly regulated by the STAT3 protein. In addition, Necdin expression in human tumour cell lines is correlated with activation of endogenous STAT3.

Recently a paper published by Chapman and Knowles (Chapman and Knowles, 2009) stated that down-regulation of Necdin occurs in both carcinoma cell lines and primary tumors, suggesting that Necdin has a tumor suppressor role. Our results are similar to the data reviewed in this paper and also demonstrate that Necdin is a physiological target of STAT3 and indicate that Necdin is a candidate for further study in this role. Our findings provide evidence for a role of Necdin in STAT3-dependent oncogenesis, suggesting that repression of Necdin expression may be a mechanism by which tumour cells gain a growth advantage in response to STAT3 activation.

CHAPTER 4: DISCUSSION

The Role of STAT3 in Oncogenesis

The mechanisms that activate STAT3 in human tumors may differ, but in all cases cells with constitutively active STAT3 exhibit dysregulated cell cycle progression and/or apoptosis. In this study, our hypothesis was that aberrant STAT3 activity, as present in many human tumors, is predicted to cause permanent alterations in the global gene expression patterns, including dysregulated expression of genes involved in cell cycle progression and proliferation, survival, apoptosis and angiogenesis, thereby contributing to oncogenesis. A handful of STAT3-regulated genes have been identified to date, however, we predicted that there are other STAT3-regulated genes that play a role in malignant transformation and oncogenesis that, as yet, remained unidentified.

To identify potential novel STAT3-regulated genes, we examined global gene expression patterns in cell lines harboring active STAT3. Our initial experiments used IL-6 to stimulate STAT3 activation in a time-dependent manner in mouse fibroblasts. Since active STAT3 induces a signal transduction cascade, including the expression of multiple downstream transcription factors and their own target genes, we chose to analyze gene expression at an early time point (one hour) after stimulation with IL-6, in the presence or absence of cycloheximide. This allowed us to study the gene(s) which were directly activated by STAT3 and not by a downstream signaling cascade initiated by STAT3. We were able to SOCS3, CEBPD and prolactin genes as a direct STAT3 target gene, induced by IL-6 at this time point. However, these experiments gave us limited results, demonstrating that at one hour post IL-6 stimulation, in the absence of translation, STAT3 has few direct targets.

Inducing STAT3 activity transiently using exogenous stimulation, such as IL-6 or transient transfection (Paz *et al.*, 2004) is likely to result in induction of some, but not all, STAT3 target genes and which genes are expressed may be influenced by the conditions in which the cells are maintained. However, gene expression profiles in cells with constitutively active STAT3 are more likely to be representative of the genetic profile of a cancer cell with aberrant STAT3 expression. Further microarray studies were therefore carried out using cells which stably expressed either v-Src or the constitutively active mutant, STAT3-C. Constitutive expression of active STAT3 in these cells is predicted to result in stable gene expression and global changes in gene expression profiles, many of which are likely to play a role in oncogenesis.

Our experiments also took into consideration the fact that clonal variation is likely to exist between cells of the same cell type where STAT3 is induced by different mechanisms. For this reason, we studied gene expression in a mouse fibroblast cell line stably transfected with v-Src and compared the results to genes expressed in cells stably expressing the constitutively active mutant, STAT3-C. While v-Src transformed cells have constitutively active STAT3, v-Src also stimulates other STAT3-independent pathways (Brunton and Frame, 2008; Frame, 2002; Frame, 2004; Odajima *et al.*, 2000). In contrast, target genes activated by STAT3-C are limited to direct binding of the activated protein to STAT3 consensus sites in DNA. Therefore, using cells stably transfected with either v-Src or STAT3-C allowed us to control for clonal variations, as well as divergence in signaling pathways depending on the mechanism of STAT3 activation. Genes identified as regulated by STAT3 but not in common between the two cell lines are likely to be due to clonal variation. However, the subset of genes that are regulated in common in both cell lines are likely to represent true global STAT3 targets.

Identifying Novel STAT3 Regulated Genes

The transcriptional profile of a cell expressing constitutively active STAT3 is predicted to be very different compared to a cell where STAT3 is under tight regulation. Our initial hypothesis was that STAT3 promotes widespread changes in global gene expression patterns, including both direct and indirect targets. We took a broad approach by studying global gene expression changes using microarray analysis in fibroblast cells expressing constitutively-activated STAT3. With this approach we were able to identify differential expression of several previously identified STAT3 target genes, with a wide range of biological functions and roles in multiple cellular pathways, including genes involved in cell cycle progression and proliferation, survival, apoptosis and angiogenesis, thereby contributing to oncogenesis.

The use of multiple microarray replicates in our approach further increases confidence in the results. This allowed us to identify a set of common genes as targets of STAT3. The data were further validated by the identification of several previously characterized STAT3-regulated genes, including CCND1, p21 (Sinibaldi *et al.*, 2000), VEGFA (Niu *et al.*, 2002), and Mcl-1 (Puthier *et al.*, 1999).

In the cells used for the microarray experiments, where STAT3 is constitutively active and mRNA transcription has reached equilibrium, some mRNA species transcribed will be direct targets of STAT3 and some will be indirect targets. Direct targets are those where STAT3 itself binds directly to the gene promoter to induce transcription, perhaps in cooperation with other co-activator proteins. Indirect targets of STAT3 are transcribed via binding of a secondary transcription factor. In this case, the secondary transcription factor is directly regulated by STAT3. STAT3 has been shown to regulate the expression of other transcription factors, as well as itself, and is thus capable of generating a cascade of gene activation both directly and indirectly. Since microarray technology is based on the interpretation of mRNA levels in control compared

to treated cells, we are unable to distinguish direct versus indirect targets of STAT3 gene transcription from the data obtained. Further analysis of the differentially expressed genes is required to further validate whether or not the expression of the genes is directly regulated by STAT3.

Pathway and Functional Analysis of STAT3 Regulated Genes

Microarrays assess simultaneous changes in transcript levels on an individual basis, resulting in a long list of genes which have significantly changed transcript levels when compared to control cells. However, these changes in gene expression do not occur as independent events within the cell, but are controlled in a coordinated manner and are often interconnected. Pathway Analysis is an unbiased method to determine whether differentially expressed genes, and the proteins they encode, are enriched in particular pathways, giving insight into the biological meaning of the changes observed. We predicted that STAT3 controls genes involved in cell cycle progression and proliferation, survival, apoptosis and angiogenesis, some of which had not been identified before, however, we also predicted that STAT3 would have an effect on other signaling pathways which are critical to oncogenesis, progression and metastasis.

Using pathway and functional analysis of the differentially expressed genes identified in our experiments, we were able to identify known STAT3 pathways, including the JAK/STAT pathway and Angiotensin/STAT pathway. This provides support for the use of such analyses to identify novel pathways that may also be regulated by STAT3.

The Pathway Analysis and Functional Annotation indicate STAT3 regulation of genes in cell growth and metabolism, including nucleotide, lipid and protein metabolism, as well as protein transport and localization. This analysis also points to a strong link between STAT3 and regulation of genes involved in cell adhesion and cytoskeletal remodeling. These results suggest that STAT3 has a wider impact on cellular processes

than demonstrated to date and that STAT3 also acts as a central coordinator of its own cellular signaling pathways. In particular, not only does STAT3 promote gene transcription, it may also have a role in production of the appropriate proteins for transcription to take place and ensure that they are localized correctly within the cell.

Combining this approach with computational analysis of the microarray results, we were able to define the gene expression profile of cells expressing activated STAT3 and examine the role of STAT3 in both positive and negative regulation of gene expression.

Pathway and functional analysis demonstrate that STAT3 has an important role in regulating, both positively and negatively, a diverse array of cellular processes in addition to transcription. STAT3 coordinates expression of genes involved in multiple metabolic and biosynthetic pathways, integrating signals that lead to global transcriptional changes and oncogenesis. These include genes involved in cell adhesion, cytoskeletal remodeling, nucleotide, lipid and protein metabolism, as well as signal transduction.

Constitutive activation of STAT3 provides cancer cells with growth and survival advantages by activating multiple pathways within the cell, involving a broad range of genes. It has also been shown to repress the transcription of a handful of genes, including p53 (Niu *et al.*, 2005) and nitric oxide synthase (Saura *et al.*, 2006). Few other genes have been identified to date that are negatively regulated by STAT3. However, computational analysis of our data, suggest that STAT3 is capable of repressing expression of a much larger number of genes. This novel discovery indicates that these pathways collaborate to profoundly impact the biology of cells and produce the proliferative advantage seen in cells harboring constitutively active STAT3.

In summary, STAT3 has been shown to up-regulate expression of multiple genes involved in cell growth and metabolism, as well as protein transport, localization, cell

adhesion and cytoskeletal remodeling. This study also suggests that STAT3 may exert its oncogenic effect not only by directly or indirectly up-regulating transcription of genes involved in promoting growth and proliferation, but also by down-regulating expression of negative regulators of the same cellular processes.

Necdin, a Novel STAT3 Target Gene

From the microarray data, we identified Necdin as a novel STAT3-regulated gene whose expression is repressed when STAT3 is constitutively activated. Our studies indicate that constitutively active STAT3 may directly cause down-regulation of Necdin at the transcriptional level. We also demonstrated that Necdin expression is repressed in several tumor cell types, including melanoma, prostate and breast cancer cell lines, and is inversely correlated with STAT3 activity. This suggests that Necdin is a physiological target gene of STAT3.

The mechanism by which STAT3 represses Necdin expression remains to be elucidated. STAT3 has previously been shown to form a complex with DNA methyltransferase 1 and histone deacetylase 1 to mediate epigenetic silencing of the tyrosine phosphatase, SHP-1 (Zhang *et al.,* 2005) indicating that this is a possible mechanism by which STAT3 could downregulate Necdin expression. Exploration of the potential epigenetic silencing of Necdin would further knowledge regarding STAT3 repression of gene expression.

A recent study published by Chapman and Knowles (Chapman and Knowles, 2009) stated that down-regulation of Necdin occurs in both carcinoma cell lines and primary tumors. Our results are similar to the data reviewed in this paper and indicate that Necdin is a candidate for further study in this role and could represent a novel cancer therapeutic target. Repression of Necdin expression by STAT3 may play an important role in regulating the cell cycle and proliferation in human cancer cells, which

has the potential to give tumor cells a growth advantage. Necdin is a negative growth regulator, capable of interacting with E2F1, resulting in inhibition of E2F1 target gene expression and consequent growth inhibitory effects (Taniura *et al.*, 1998). Two reports have previously demonstrated that Necdin expression is down-regulated in melanoma (Hoek *et al.*, 2004) and a drug-resistant ovarian cancer cell line (Varma *et al.*, 2005). Thus far, no role for Necdin in oncogenesis has been confirmed; but, our results suggest that repression of Necdin expression by STAT3 may be one mechanism which could potentially contribute to a growth advantage of tumor cells and is of interest for further analysis. The repression of Necdin observed in cell lines need to be confirmed in human tumors compared to normal tissues, for example via microarray, Real Time PCR and immunohistochemistry, and the effect of Necdin silencing examined with regard to cell cycle and proliferation to identify any possible growth advantage that it may provide. The reversal of such gene repression represents a target for novel anticancer therapies.

CHAPTER 5: CLINICAL SIGNIFICANCE

Our initial hypothesis was that constitutive activation of STAT3 within cells leads to permanent changes in global gene expression patterns that play a role in the development of a malignant phenotype. We predicted that STAT3 promotes widespread changes in gene expression, including both direct and indirect targets, involving multiple signaling pathways and involving a broad range of genes affecting cell cycle progression, cellular proliferation and survival, angiogenesis and apoptosis. Having identified a set of differentially expressed genes in cell lines expressing constitutively active STAT3, it would be of clinical relevance to use the data to determine a STAT3 molecular signature in human tumors.

A molecular signature is defined as a group of STAT3 regulated genes that are co-expressed simultaneously. Cell lines do not accurately represent the physiology and biology of human tumors, lacking the microenvironment and interactions found *in vivo.* Analysis of untreated tumor samples by microarray and comparison of the differential gene expression between normal and tumor tissue would allow for validation of the expression of the cell line signature in primary human tumors. The molecular signature may vary by tissue type, thus may show differences between breast cancer and prostate cancer, however, there may also be a subset of genes which define a general STAT3 molecular signature in human cancer. Given the biological and physiological effects of STAT3 target genes, such a signature may predict response to chemotherapy and prognosis in cancer patients.

Transcription factors involved in oncogenesis are chief targets for cancer therapy, especially since multiple signaling pathways converge on signaling molecules

such as STAT3 (Turkson and Jove, 2000). STAT3 gene ablation results in inhibited tumor growth in various tumor models, therefore targeting a drug to inhibit STAT3 is under active pursuit. The use of pharmacological agents to inhibit STAT3 activity may lead to a rebalancing of the signaling pathways regulating cell growth and lead to inhibition of tumor progression (Buettner *et al.*, 2002; Darnell, 2002; Turkson, 2004; Turkson and Jove, 2000; Yu and Jove, 2004). Whilst targeting STAT3 is not easy, due to a lack of enzyme activity, various approaches are producing results, including disrupting dimer formation via the SH2 interaction (Turkson *et al.*, 2001), blocking protein-DNA binding or platinum-based drugs (Turkson *et al.*, 2004).

Computational analysis of global changes in gene expression regulated by STAT3 gives further insight into the mechanisms by which STAT3 contributes to oncogenesis. Such gene expression profiles, controlled by STAT3, may be useful in identifying potential targets for drug treatment, as well as in tailoring cancer treatment to the patient by use of gene expression analysis of tumors. In this study we identified Necdin as a novel STAT3 regulated gene. Necdin is a negative growth regulator shown to be silenced in several tumor types. Whilst the mechanism of silencing has not yet been elucidated, reactivation of NDN expression also represents a novel therapeutic target.

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APPENDIX

Table A-1. Most Significant Probesets Over-Expressed Common to v-Src and STAT3-C

			STAT3-C Data		v-Src Data		
Probe Set ID	**Gene Symbol**	**Gene Title**	**Score(d)**	**Fold Change**	**Score(d)**	**Fold Change**	**Av. Score**
1417311_at	Crip2	cysteine rich protein 2	72.639	26.698	51.343	17.492	61.991
1418203_at	Pmaip1	phorbol-12-myristate-13-acetate-induced protein 1	34.058	47.146	39.681	31.364	36.869
1418818_at	Aqp5	aquaporin 5 /// similar to aquaporin 5	45.193	94.088	21.732	22.263	33.463
1451527_at	Pcolce2	procollagen C-endopeptidase enhancer 2	27.024	31.455	36.947	19.949	31.986
1452592_at	Mgst2	microsomal glutathione S-transferase 2	34.651	37.303	29.291	15.115	31.971
1420842_at	Ptprf	protein tyrosine phosphatase, receptor type, F	44.849	20.122	16.848	20.177	30.848
1415936_at	Bcar3	breast cancer anti-estrogen resistance 3	33.804	22.399	26.309	11.770	30.056
1416613_at	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	36.953	84.865	21.870	47.409	29.412
1449527_at	Pcdhb7	protocadherin beta 7	29.613	16.697	25.742	18.886	27.677
1425458_a_at	Grb10	growth factor receptor bound protein 10	23.021	30.551	29.734	22.605	26.378
1455900_x_at	Tgm2	transglutaminase 2, C polypeptide	16.141	19.812	33.990	5.150	25.065
1420653_at	Tgfb1	transforming growth factor, beta 1	11.154	2.249	38.605	4.277	24.879
1420562_at	Slurp1	secreted Ly6/Plaur domain containing 1	44.696	61.526	3.888	2.167	24.292
1447845_s_at	Vnn1	vanin 1	36.504	13.221	9.977	2.781	23.240
1421038_a_at	Kcnn4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	19.816	9.364	25.477	14.102	22.646
1419758_at	Abcb1a	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	12.054	3.575	33.224	28.079	22.639
1417960_at	Cpeb1	cytoplasmic polyadenylation element binding protein 1	18.423	6.037	26.614	7.086	22.518
1420841_at	Ptprf	protein tyrosine phosphatase, receptor type, F	31.989	7.116	10.485	8.588	21.237
1421870_at	Trim44	tripartite motif-containing 44	16.413	10.029	25.530	15.460	20.971
1429778_at	Optn	optineurin	3.971	3.254	37.760	3.912	20.866

			STAT3-C Data		v-Src Data		
Probe Set ID	**Gene Symbol**	**Gene Title**	**Score(d)**	**Fold Change**	**Score(d)**	**Fold Change**	**Av. Score**
1427020_at	Scara3	scavenger receptor class A, member 3	20.776	24.700	18.615	5.585	19.695
1450047_at	Hs6st2	heparan sulfate 6-O-sulfotransferase 2	24.776	17.182	14.035	6.966	19.405
1434465_x_at	Vldlr	very low density lipoprotein receptor	20.781	48.594	17.146	27.971	18.963
1433428_x_at	Tgm2	transglutaminase 2, C polypeptide	22.679	18.346	15.129	5.749	18.904
1425295_at	Ear11	eosinophil-associated, ribonuclease A family, member 11	14.467	11.001	22.528	39.833	18.498
1427357_at	Cda	cytidine deaminase	23.520	23.970	13.361	18.933	18.441
1448416_at	Mgp	matrix Gla protein	6.074	14.732	30.402	83.751	18.238
1447623_s_at	Prkcm	Protein kinase C, mu	11.240	27.376	25.161	10.360	18.200
1422631_at	Ahr	aryl-hydrocarbon receptor	23.807	45.461	12.326	28.711	18.067
1424329_a_at	Prrg2	proline-rich Gla (G-carboxyglutamic acid) polypeptide 2	6.589	2.662	28.826	5.017	17.707
1460238_at	Msln	mesothelin	20.555	44.338	14.741	12.442	17.648
1449454_at	Bst1	bone marrow stromal cell antigen 1	12.681	47.741	22.073	178.132	17.377
1450380_at	Epdr1	ependymin related protein 1 (zebrafish)	19.033	7.667	15.188	9.579	17.111
1416612_at	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	18.906	43.963	15.256	25.544	17.081
1426795_at	Ptprs	protein tyrosine phosphatase, receptor type, S	3.744	1.634	29.917	2.950	16.831
1449453_at	Bst1	bone marrow stromal cell antigen 1	13.542	46.422	19.747	123.703	16.645
1421869_at	Trim44	tripartite motif-containing 44	11.139	2.939	21.807	6.840	16.473
1428547_at	Nt5e	5' nucleotidase, ecto	10.955	7.722	21.789	48.296	16.372
1435945_a_at	Kcnn4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	12.135	5.758	20.607	7.620	16.371
1434036_at	Mtss1	metastasis suppressor 1	19.032	47.958	13.608	25.882	16.320
1417389_at	Gpc1	glypican 1	13.915	14.315	18.707	27.523	16.311
1421369_a_at	Mab21l1	mab-21-like 1 (C. elegans)	16.595	8.964	15.947	4.645	16.271

			STAT3-C Data		v-Src Data		
Probe Set ID	**Gene Symbol**	**Gene Title**	**Score(d)**	**Fold Change**	**Score(d)**	**Fold Change**	**Av. Score**
1416645_a_at	Afp	alpha fetoprotein	27.332	65.246	4.856	2.934	16.094
1416670_at	Setdb1	SET domain, bifurcated 1	5.119	1.727	27.024	3.266	16.072
1454114_a_at	Nhedc1	Na+/H+ exchanger domain containing 1	28.946	15.208	2.946	1.163	15.946
1431644_a_at	Ica1	islet cell autoantigen 1	17.637	5.303	14.115	8.796	15.876
1418752_at	Aldh3a1	aldehyde dehydrogenase family 3, subfamily A1	25.596	52.110	6.152	10.601	15.874
1421001_a_at	Car6	carbonic anhydrase 6	21.885	15.720	9.573	10.722	15.729
1444139_at	Ddit4l	DNA-damage-inducible transcript 4-like	13.772	5.294	17.170	4.739	15.471
1431055_a_at	Snx10	sorting nexin 10	16.881	2.809	13.686	2.528	15.283

Table A-2. Most Significant Probesets Under-Expressed Common to v-Src and STAT3-C

			STAT3-C Data		v-Src Data		
Probe Set ID	**Gene Symbol**	**Gene Title**	**Score(d)**	**Fold Change**	**Score(d)**	**Fold Change**	**Av. Score**
1418070_at	Cdyl	chromodomain protein, Y chromosome-like	-47.865	0.0832	-72.232	0.0548	-60.049
1415923_at	Ndn	necdin	-54.087	0.0102	-58.571	0.0081	-56.329
1435382_at	Ndn	necdin	-73.974	0.0122	-30.525	0.0135	-52.249
1450757_at	Cdh11	cadherin 11	-52.719	0.0048	-32.836	0.0055	-42.778
1437853_x_at	Ndn	necdin	-67.114	0.1065	-16.328	0.1440	-41.721
1434261_at	Sipa1l2	signal-induced proliferation-associated 1 like 2	-38.883	0.1002	-43.072	0.0715	-40.978
1437284_at	Fzd1	frizzled homolog 1 (Drosophila)	-61.622	0.0879	-16.598	0.0997	-39.110
1435383_x_at	Ndn	necdin	-29.383	0.0141	-40.515	0.0120	-34.949
1448293_at	Ebf1	early B-cell factor 1	-19.975	0.0796	-46.495	0.0688	-33.235
1460208_at	Fbn1	fibrillin 1	-26.476	0.0200	-38.299	0.0180	-32.388
1455792_x_at	Ndn	necdin	-32.708	0.0189	-30.296	0.0151	-31.502
1422571_at	Thbs2	thrombospondin 2	-26.721	0.0124	-33.293	0.0148	-30.007
1450663_at	Thbs2	thrombospondin 2	-22.233	0.0320	-37.130	0.0392	-29.681
1423091_a_at	Gpm6b	glycoprotein m6b	-28.982	0.0425	-29.909	0.0375	-29.446
1448254_at	Ptn	pleiotrophin	-32.491	0.0062	-25.948	0.0065	-29.219
1447839_x_at	Adm	adrenomedullin	-31.069	0.0262	-26.688	0.0265	-28.878
1416211_a_at	Ptn	pleiotrophin	-22.711	0.0299	-34.659	0.0271	-28.685
1437983_at	Sall1	sal-like 1 (Drosophila)	-28.236	0.1429	-28.652	0.0924	-28.444
1416301_a_at	Ebf1	early B-cell factor 1	-18.082	0.0881	-38.525	0.0729	-28.304
1425923_at	Mycn	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	-27.445	0.0117	-28.305	0.2423	-27.875
1451791_at	Tfpi	tissue factor pathway inhibitor	-17.920	0.0687	-36.868	0.0410	-27.394
1454974_at	Ntn1	netrin 1 /// similar to Netrin-1 precursor	-24.194	0.1080	-29.321	0.0997	-26.757
1448201_at	Sfrp2	secreted frizzled-related protein 2	-23.839	0.0127	-27.235	0.0105	-25.537
1419467_at	Clec14a	C-type lectin domain family 14, member a	-32.540	0.0503	-18.114	0.0674	-25.327

			STAT3-C Data		v-Src Data		
Probe Set ID	**Gene Symbol**	**Gene Title**	**Score(d)**	**Fold Change**	**Score(d)**	**Fold Change**	**Av. Score**
1428512_at	Bhlhb9	basic helix-loop-helix domain containing, class B9	-26.936	0.1109	-23.643	0.0745	-25.290
1450069_a_at	Cugbp2	CUG triplet repeat, RNA binding protein 2	-16.465	0.0508	-32.843	0.0564	-24.654
1436363_a_at	Nfix	nuclear factor I/X	-16.947	0.3387	-31.712	0.2372	-24.330
1452296_at	Slit3	slit homolog 3 (Drosophila)	-20.270	0.0739	-28.152	0.0648	-24.211
1424916_x_at	Zfp764	zinc finger protein 764	-27.579	0.3744	-19.797	0.3964	-23.688
1423584_at	Igfbp7	insulin-like growth factor binding protein 7	-21.931	0.0125	-25.350	0.0118	-23.641
1448925_at	Twist2	twist homolog 2 (Drosophila)	-19.924	0.1597	-27.213	0.1029	-23.568
1418532_at	Fzd2	frizzled homolog 2 (Drosophila)	-13.396	0.2982	-33.577	0.2124	-23.486
1448434_at	Rnf103	ring finger protein 103	-11.348	0.1802	-35.593	0.1323	-23.471
1423327_at	Rpl39l	ribosomal protein L39-like	-23.856	0.0196	-22.625	0.0259	-23.240
1419468_at	Clec14a	C-type lectin domain family 14, member a	-17.392	0.0804	-28.276	0.0706	-22.834
1438682_at	Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	-21.729	0.2230	-23.868	0.3169	-22.798
1416077_at	Adm	adrenomedullin	-17.629	0.0131	-27.940	0.0146	-22.784
1451244_a_at	Zfp422	zinc finger protein 422	-17.283	0.1541	-27.977	0.2264	-22.630
1451332_at	Zfp521	zinc finger protein 521	-18.189	0.0811	-26.803	0.0353	-22.496
1436364_x_at	Nfix	nuclear factor I/X	-15.349	0.3449	-29.551	0.2355	-22.450
1418533_s_at	Fzd2	frizzled homolog 2 (Drosophila)	-21.614	0.3454	-21.180	0.2157	-21.397
1428185_at	Kctd18	potassium channel tetramerisation domain containing 18	-14.620	0.2519	-28.112	0.2738	-21.366
1437341_x_at	Cnp	2',3'-cyclic nucleotide 3' phosphodiesterase	-12.138	0.1863	-30.589	0.1573	-21.363
1428797_at	Setd6	SET domain containing 6	-18.904	0.3470	-23.293	0.1855	-21.098
1421392_a_at	Birc3	baculoviral IAP repeat-containing 3	-14.576	0.3042	-27.260	0.2533	-20.918
1424133_at	Tmem98	transmembrane protein 98	-20.010	0.1024	-20.928	0.0893	-20.469
1451154_a_at	Cugbp2	CUG triplet repeat, RNA binding protein 2	-15.501	0.0413	-24.748	0.0295	-20.124
1449630_s_at	Mark1	MAP/microtubule affinity-regulating kinase 1	-23.605	0.1533	-16.523	0.1191	-20.064
1448370_at	Ulk1	Unc-51 like kinase 1 (C. elegans)	-31.136	0.3260	-8.638	0.3862	-19.887
1422799_at	Bat2	HLA-B associated transcript 2	-13.176	0.1644	-26.477	0.1814	-19.827

ABOUT THE AUTHOR

Rachel Haviland was born in England and graduated *summa cum laude* from Brunel University, West London, UK with a Bachelor of Science degree with Honours in Medical Biology. During her degree she qualified for one of only two research exchange student positions and spent 6 months at Rutgers University, New Jersey, U.S. Upon graduating, she was the sole recipient in her class to be awarded the Brunel University medal for academic excellence. Following several years in research in London, England, Rachel immigrated to the U.S. in August 2001 and entered the Cancer Biology Doctoral Program at H. Lee Moffitt Cancer Center & Research Institute, Tampa, Florida. She completed her doctoral research under the guidance of doctors Richard Jove and W. Douglas Cress. This research was published in PLoSOne in October 2011. During this time, Rachel also led the Association of Medical Science Graduate Students (AMSGS) at the College of Medicine, University of South Florida elected as Secretary, Vice President and, subsequently, President.

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