

Review

# Genome-Wide Analysis of STAT Target Genes

## Elucidating the Mechanism of STAT-Mediated Oncogenesis

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### ABBREVIATIONS

STAT signal transducer and activator of transcription

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### ABSTRACT

Inappropriate activation of transcription factors is a common event in cancer. These transcription factors contribute to a malignant phenotype by regulating genes involved in cellular proliferation, survival, differentiation, angiogenesis, and invasion. An important goal remains identifying the targets of oncogenic transcription factors that execute these changes. STAT proteins are among the best-studied of these transcription factors, and are involved in oncogenesis both in vivo and in vitro. They thus represent an ideal model for understanding how transcription factors cause cancer through coordinated changes in gene expression. Recent studies have employed microarray-based expression analysis to comprehensively identify STAT target genes. Analysis of these targets can provide insight into mechanisms of neoplastic transformation, and may shed light on new strategies for targeted therapy.

### INTRODUCTION

Cancer can be viewed as the accumulation of cells that have acquired defects in processes regulating cellular homeostasis; such processes become defective due to the coordinated dysregulation of the genes controlling them. Transcription factors reside at critical junctures for controlling these processes through modulation of gene expression. Indeed, human cancers harbor inappropriately activated transcription factors—either through mutation of the transcription factor itself or through mutation of upstream signaling pathways leading to its activation. Understanding the genes regulated by these transcription factors may provide insights into the pathology of human tumors in which they are activated.

Since the discovery of the transcription factor c-jun as the cellular homolog of the v-jun oncogene, much attention has been focused on transcription factors and their role in cancer progression. The list of oncogenic transcription factors is large and includes myc, jun, and NFκB, among many others.<sup>1-3</sup> Many of these proteins were initially defined as oncogenes based upon their ability to transform cells in culture, either alone or in combination with other genes. Subsequently many have been shown to be overexpressed or constitutively active in human tumors. Separately, analysis of chromosomal translocations in acute leukemias has revealed that many of the resultant chimeric proteins are transcription factors, and that these transcription factors contribute to the transformation of these cells.<sup>4</sup>

Despite nearly 20 years of intense interest in oncogenic transcription factors, many of the details of how they contribute to human cancer remain unknown. Specifically, a gap remains between our understanding of the cell biological effects of a given transcription factor—which, in the case of c-myc, for instance, is quite detailed—and the target genes that mediate those effects. It has not generally been possible to attribute specific biological processes to one or a few target genes. This is in stark contrast to the transcription factor p53, for example, which functions as a tumor suppressor through induction of cell cycle arrest and apoptosis. The specific targets p53 regulates to effect these changes have been determined: p21 mediates cell cycle arrest downstream of p53, and a number of BH3-only proteins, including Noxa and Puma, are involved in p53-mediated apoptosis.<sup>5-7</sup>

Why has similar progress not been made with oncogenic transcription factors? Leaving aside the case of c-myc, which seems to regulate a surprisingly large percentage of the genome and may be unique,<sup>8</sup> it is possible that oncogenic transcription factors do activate a small complement of genes to cause cancer. However, the approaches available to identify and characterize these genes have, until recently, allowed only slow progress. Recent advances in microarray analysis in particular have greatly increased the rate of discovery of transcription factor target genes, while the ability to specifically inhibit gene expression in somatic cells using RNA interference promises to accelerate the characterization of these targets.

The STAT family of transcription factors in particular has provided significant insight into the relationship between aberrant gene expression and tumorigenesis.

**STAT SIGNALING AND CANCER**

STAT (signal transducer and activator of transcription) proteins are latent transcription factors present in the cytoplasm of resting cells. Their mechanism of activation and the stimuli to which they respond have been extensively characterized.<sup>9,10</sup> Briefly, following binding of a cytokine to its cognate receptor, the receptor dimerizes, bringing associated JAKs (Janus kinases) into proximity. The JAKs then phosphorylate themselves and the receptor on tyrosine residues, and one or several phospho-tyrosines on the receptor provide docking sites for STAT proteins via their SH2 domains. Once bound, STATs are themselves phosphorylated by the JAK, dissociate from the receptor, and dimerize. These dimers translocate to the nucleus where they bind to a consensus site, TTN<sub>3</sub>AA, in the promoter of target genes and activate transcription. STATs can also be activated by a number of tyrosine kinases downstream of polypeptide growth factors, as well as by oncogenic kinases like src and Bcr/Abl. This underscores the fact that a number of signaling pathways converge on STATs, suggesting that their activation is a common lesion in cancer and thus making them an attractive therapeutic target.<sup>11,12</sup> In addition, a number of proteins function to negatively regulate STAT signaling. These include tyrosine phosphatases and members of the SOCS family. Notably, both have been shown to be silenced through methylation in some human cancer cells, suggesting that inhibition of this negative regulation of STATs may contribute to STAT activation and malignancy.<sup>13</sup>

There are seven STAT proteins: STAT1 through STAT4, STAT5a and STAT5b (which are encoded by different genes, are over 90% identical, and are often collectively referred to as STAT5), and STAT6. Of these, STAT3 and STAT5 are most frequently associated with human cancers and will be the focus of this review.

Two observations nearly ten years ago independently provided the first links between STATs and cancer. First, analysis of nuclear extracts from breast tissue showed elevated levels of STAT DNA-binding activity in breast carcinomas compared to normal 'resting' breast tissue.<sup>14</sup> Soon thereafter, STAT3 was shown to be constitutively activated—i.e., tyrosine phosphorylated and capable of binding DNA—in fibroblasts transformed with the v-src oncoprotein.<sup>15</sup> Following these observations came a flurry of reports of STAT activation in diverse human tumors and tumor cell lines, and in response to many oncogenic kinases (reviewed in ref. 16). Furthermore, several groups provided compelling evidence that STAT3 is required for transformation in a number of contexts in vitro,<sup>17,18</sup> while a constitutively active mutant of STAT3, STAT3-C, is sufficient for transformation of fibroblasts.<sup>19</sup>

**Cellular Functions Regulated by STATs in Normal and Cancer Cells.** The cellular functions that STATs modulate to promote oncogenesis have been well-studied, principally through interrupting STAT signaling and observing the phenotypic consequences. For instance, expression of dominant-negative STAT3 induces apoptosis in a number of tumor cells, including multiple myeloma, breast cancer, mycosis fungoides, lung cancer, prostate cancer and melanoma,<sup>20-25</sup> suggesting that many malignant cells may be dependent upon STAT3 for survival. While many normal cells can tolerate loss of STAT3, T-cells in mice with a conditional knock-out of STAT3 have impaired survival in response to IL-6.<sup>26</sup> STAT3 is

**Table 1 TARGET GENES OF STAT3 AND STAT5 PROPOSED TO MEDIATE THEIR ROLE IN ONCOGENESIS**

Protein	Process	Gene	System	Ref.			
STAT3	Proliferation	c-myc	Fibroblasts	27			
	Proliferation	c-myc	Pro-B cells	42			
	Proliferation	CyclinD2 CyclinD3 Cyclin A Cdc25a	Pro-B cells	28			
				Proliferation	P21 cyclinD1	Fibroblasts	48
				Proliferation	Cyclin D1	Fibroblasts	19
	Survival and proliferation	Survivin	Primary effusion lymphoma	Pim-1 and pim-2	Pro-B cells	42	
				Survival	Melanoma	43	
	Survival	mcl-1	LGL leukemia cells	39			
	Survival	Bcl-x <sub>L</sub>	Myeloma cells	20			
	Survival	Bcl-x <sub>L</sub>	Fibroblasts	19			
	Survival	Bcl-2	Pro-B cells	28			
	Angiogenesis	VEGF	Fibroblasts and melanoma cells	34			
	Angiogenesis	VEGF	Pancreatic cancer cells	35			
	Invasion/metastasis	MMP-2	Melanoma cells	38			
	Transformation	MMP-9	Mammary epithelial cells	50			
STAT5	Proliferation	Cyclin D1	Leukemic cells	46			
	Proliferation	c-myc	T- and pro-B cells	44			
	Proliferation	Cyclin D2, D3, E	IL-2-induced proliferation of T-cells	33			
	Survival	Bcl-x <sub>L</sub>	Pro-B cells	29			
	Survival	Bcl-x <sub>L</sub>	Erythroid cells	31			
	Survival	Bcl-x <sub>L</sub>	Myeloid differentiation	32			
	Survival	Pim-1	Pro-B cells	45			
Survival	Bcl-2	Lymphocytes					

also required for cell proliferation in some contexts. For example, PDGF-induced proliferation of fibroblasts is blocked by dominant-negative STAT3.<sup>27</sup> In a pro-B cell line dependent upon STAT3 signaling, progression from G<sub>1</sub>- into S-phase is blocked by dominant-negative STAT3 or mutation of the receptor tyrosine residues required for STAT3 signaling.<sup>28</sup> These results indicate that STAT3 is required for cellular proliferation, specifically the progression from the G<sub>1</sub>-phase of the cell cycle into S-phase.

STAT5 also promotes cell survival and proliferation. Dominant-negative STAT5 sensitizes BaF3 cells to apoptosis.<sup>29,30</sup> Studies from mice lacking STAT5a and STAT5b demonstrated increased apoptosis

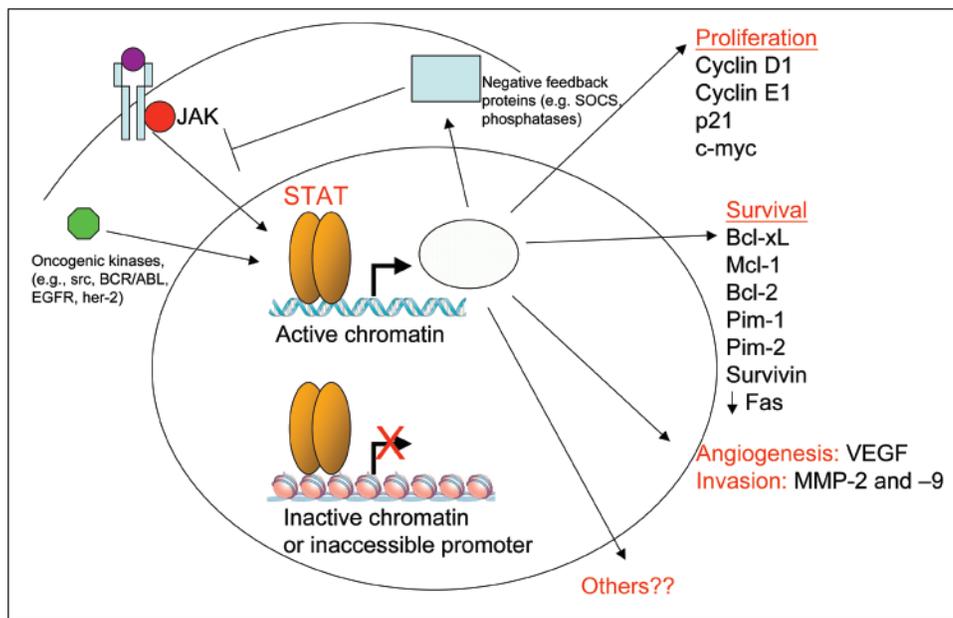


Figure 1. STAT signaling contributes to cellular transformation through modulation of target genes. Following phosphorylation by various kinases, STATs translocate to the nucleus, bind to a consensus site in the promoter of target genes, and activate or repress transcription. Targets of STATs include genes involved in promoting proliferation, survival, angiogenesis and invasion. Some differences in STAT targets among cell types may be due to epigenetic variation among cells, including altered histone modifications or DNA methylation. Negative regulators of STATs include phosphatases and SOCS proteins, which may be silenced in tumor cells.

of fetal erythroid progenitors,<sup>31</sup> and myeloid cells from these knockout mice exhibit increased apoptosis during differentiation.<sup>32</sup> Peripheral T-cells from mice lacking STAT5a and STAT5b have impaired proliferation in response to anti-CD3 and IL-2, consequent to an inability to enter S-phase.<sup>33</sup>

In addition to cell survival and proliferation, STATs regulate several other biological processes that may contribute to cancer. STAT3 has been shown to promote angiogenesis,<sup>34,35</sup> a process whose acquisition is crucial for tumor progression. Furthermore, a recent report suggests that STAT3 may contribute to cancer by allowing tumors to evade detection by the host immune system.<sup>36</sup> Finally, STAT3 promotes the motility of ovarian carcinoma cells and the invasiveness of melanoma cells in vitro,<sup>37,38</sup> suggesting that STAT3 may play an important role in regulating these aspects of tumorigenesis in vivo, as well.

**STAT Target Genes and Cancer.** As transcription factors, STATs regulate cellular function by modulating the expression of target genes: the protein products of these target genes execute changes in a cell's phenotype. Thus for a given STAT-regulated cellular process, it should in principle be possible to identify the gene or genes that STATs activate (or repress) to carry out that process. Indeed, many target genes of STATs have been identified and, based upon their function, are likely to mediate the role of STATs in cancer.

In attempting to identify crucial mediators of STAT-dependent processes, several issues should be considered. The most rigorous demonstration that a gene is a bona fide STAT target would include showing altered expression of the gene following specific activation or inhibition of STAT signaling, and identifying a STAT responsive site in the promoter of the gene, either by chromatin immunoprecipitation or reporter gene assays. Following this, one would like to demonstrate that the gene of interest is involved in a given STAT-dependent process. For instance, one study showed that

PDGF-induced cell cycle entry of fibroblasts requires STAT3, and the STAT3 target c-myc is sufficient to mediate this process.<sup>27</sup>

A number of genes have been identified as STAT targets and have been suggested to mediate STAT-dependent processes (Table 1). STAT3 targets that may mediate cell survival effects include several bcl-2 family members,<sup>20,39,40</sup> the IAP protein survivin,<sup>41</sup> and the serine/threonine kinases pim-1 and pim-2.<sup>42</sup> STAT3 contributes to cell survival by collaborating with c-jun to repress transcription of the pro-apoptotic cytokine Fas.<sup>43</sup> STAT5 can also activate bcl-2 proteins<sup>29,31,44</sup> and pim-1.<sup>45</sup> The genes that mediate the proliferative effects of STATs include the D and E type cyclins, the cdk inhibitor p21, and the transcription factor c-myc.<sup>19,28,42,46-49</sup> The pro-angiogenic effects of STAT3 are likely to be mediated through upregulation of VEGF.<sup>34,35</sup> STAT3 regulation of matrix metalloproteinase-2 (mmp-2) correlates with invasiveness of melanoma cells,<sup>38</sup> and activation of mmp-9 is required for

STAT3-induced transformation of mammary epithelial cells.<sup>50</sup>

Thus STAT3 and STAT5 contribute to cancer by activating genes that promote cell cycle entry and cell survival, among other cellular processes (Fig. 1). However, several questions remain concerning the role of STATs in cancer. Many STAT3 targets have been identified by testing genes known to be involved in the cellular processes STATs mediate. Are these processes the only ones regulated by STATs relevant to oncogenesis? Are the target genes so far identified the crucial mediators of the oncogenic function of STATs, or are other, as yet unidentified genes more important? Finally, few studies have addressed the expression of these targets in human tumors in which STAT3 is activated. Showing a correlation between STAT activation and the expression of STAT targets in human tumors in vivo would strongly suggest that those targets are critical mediators of the role of STATs in cancer.

A thorough knowledge of the transcriptional targets of STATs is therefore instrumental in precisely defining the role these proteins play in human cancers. Identifying these targets using an unbiased, high-throughput approach is critical. This should allow for both the identification of novel targets involved in processes already known to be governed by STATs—such as promoting survival and proliferation—as well as the identification of genes involved in processes not previously known to be regulated by STATs.

## GENOME-WIDE ANALYSIS OF STAT TARGETS

Microarray technology permits analysis of the expression level of thousands of genes, and has been used extensively to identify transcriptional targets of transcription factors.

Using microarrays to identify targets of STATs, however, is complicated by the fact that STATs are regulated by phosphorylation, and all stimuli that initiate STAT phosphorylation also modulate

other transcription factors. Activating STAT3 with IL-6, for instance, would lead to induction of STAT3 targets as well as targets of other transcription factors activated by IL-6, such as those downstream of MAP kinase and phosphoinositide 3-kinase (PI3K) signal transduction pathways. This problem has been overcome by the creation of mutants of STATs that do not require cytokines for their activation. For instance, cysteine residues were engineered in STAT3 to cause spontaneous dimerization, independent of stimulation; the resultant protein, termed STAT3-C, is constitutively active.<sup>19</sup> Other groups have created STAT3-estrogen receptor fusions that can be induced to dimerize with tamoxifen.<sup>51</sup> Expression of these mutants should be sufficient to specifically activate STAT targets. An alternate approach to identifying specific STAT3 targets is to use receptor mutants that only signal to STAT3. These have been used extensively in a variety of systems to isolate effects of specific signaling pathways.

The transcriptional targets of STAT3 have been identified using microarrays in fibroblasts, mammary epithelial cells, and multiple myeloma cells, all cell types in which STAT3 promotes transformation. Each of these studies identified dozens of genes that are direct targets of STAT3 and might mediate the role of STAT3 in cancer. Generating a list of STAT target genes is only an initial step in understanding the contribution of STATs to cancer. It is important to have tractable biological systems to test whether the genes so identified impact cellular function, and to verify the clinical relevance of these genes by analyzing their expression in primary human tumors. In this manner microarray-based expression analysis will be most informative about the mechanism of STAT-mediated oncogenesis.

**Constitutive Expression of STAT3-C in Mammary Epithelial Cells.** STAT3 is constitutively active in breast cancer cell lines and primary tumors. To directly evaluate the contribution of STAT3 to transformation of mammary epithelial cells, STAT3-C was expressed in these cells.<sup>50</sup> In two independent immortalized mammary epithelial cell lines, MCF10A and HMECs, STAT3-C expression led to transformation. To identify the changes in gene expression associated with this transformation, the expression profile of cells expressing STAT3-C was compared to cells expressing empty vector using Affymetrix oligonucleotide microarrays. This led to the identification of 149 genes that were upregulated (>2-fold) and 63 genes that were down-regulated in response to STAT3-C in HMECs, and 163 upregulated genes and 30 down-regulated genes in response to STAT3-C in MCF10A cells. Twenty-three genes were commonly upregulated and one gene was down-regulated in both cell lines in response to STAT3-C. The expression of one of these targets, the matrix metalloproteinase MMP-9, was shown to correlate with STAT3 activation in human tumors, as measured by immunohistochemistry. Further, MMP-9 activity was required for STAT3-C mediated transformation of HMECs, as pharmacologic inhibition of MMP-9 abrogated the ability of these cells to grow in soft-agar in response to STAT3-C expression.

**Expression of STAT3-C in Fibroblasts.** Expression of STAT3-C has previously been shown to be sufficient for the transformation of mouse and rat fibroblasts.<sup>19</sup> To identify the genes that mediate this phenotype, we used an inducible expression system to express STAT3-C in NIH3T3 cells and examined changes in gene expression after 4.5 hours (Alvarez et al., submitted). This should allow identification of direct STAT3 targets, without identifying secondary or other indirect effects of STAT3. Sixty-six known genes and 44 ESTs were identified whose expression was increased 1.5-fold or greater at this time point. These included some genes known to be STAT3

targets, including SOCS3, junB, C/EBP $\beta$ , mcl-1, and VEGF, thus validating this approach.

To determine the relevance of these genes to cancer, their expression was examined in human tumors using published datasets. A group of 12 STAT3 targets were statistically significantly coexpressed in several human tumors. These genes, which constitute a STAT3 expression signature, included transcription factors junB, bcl-6, NFIL3, egr1, and klf4; the pro-survival protein mcl-1; the pro-angiogenic factor, VEGF; the protease calpain; a tyrosine phosphatase PTPCAAX1; and EXT1, NPC1 and the serine/threonine kinase PAK2. What is the significance of the coexpression of these genes in human tumors? It is likely that only a subset of STAT3 targets mediate its pro-oncogenic effects, such that these targets should be expressed more highly in tumors with STAT3 activation than in tumors lacking STAT3 activation. Thus a significant coexpression of genes may be suggestive of a crucial role for those genes in human tumors. Having identified these genes, a key question was whether their expression correlated with STAT3 activation in human tumors. To determine this, STAT3 activation was measured by immunohistochemical staining of breast and prostate tumor sections with a phospho-STAT3 specific antibody. The STAT3 signature genes were expressed more highly in tumors with greater phospho-STAT3 staining; thus these targets are correlated with STAT3 activation *in vivo*. This association between these target genes and STAT3 activation in human tumors strongly suggests that these genes may mediate the role of STAT3 in cancer. Furthermore, abrogating STAT3 function in breast cancer cells through inhibiting protein expression using RNA interference led to a decrease in many of the genes identified, proving a causal relationship between STAT3 activation and the expression of these genes in human tumor cells.

**IL-6 Responsive Genes in Multiple Myeloma Cells.** STAT3 has been shown to be necessary for the IL-6-mediated survival of multiple myeloma cells. Another study analyzed the changes in transcription following IL-6 treatment which, as described above, would identify targets of a number of transcription factors.<sup>52</sup> To address this, a system using mutant receptor chimeras to specifically activate STAT3 was employed; in this manner, it was possible to address whether IL-6 induced genes were specific STAT3 targets.

Using INA-6 cells, an IL-6-dependent multiple myeloma cell line, the changes in gene expression induced by IL-6 at 1 and 4 hours were analyzed. IL-6 increased expression of 22 and 29 genes by 2-fold at these time points, respectively. The majority of the genes activated at 1 hour in these cells were also found to be specific STAT3 targets, as determined by using a different myeloma cell line expressing mutant gp130 that only signals to STAT3. Roughly 150 genes were changed by 1.5-fold or greater at any time point (1 hour IL-6 stimulation, 4 hours IL-6 stimulation, or continuous IL-6 compared to starved cells). Interestingly, none of the genes identified as STAT3 targets, except for mcl-1, is proximally involved in the regulation of apoptosis, despite the fact that STAT3 is required for the IL-6 mediated survival of these cells in an mcl-1 independent manner. Thus it seems that STAT3 promotes cell survival through indirect mechanisms—e.g., by activating transcription factors which themselves directly regulate pro-survival proteins.

**Common STAT3 Targets across Systems.** Do these three studies aimed at identifying STAT3 targets important in transformation identify common genes? Surprisingly, the answer seems to be 'no'. Each experiment was performed on a different Affymetrix platform, each of which has different genes represented by different probes. Comparison of these genes by mapping across platforms (using

LocusLink and Unigene accessions) revealed that only 6 genes (*bcl-6*, *mcl-1*, *junB*, *SOCS3*, *MAPKAP2*, and *PTPCAAX1*) are commonly regulated by STAT3 in fibroblasts and myeloma cells, while only 1 gene, *bcl-6*, is common to fibroblasts and mammary epithelial cells expressing STAT3-C. A comparison of STAT3 targets in myeloma cells and mammary epithelial cells also revealed that only a handful of genes (4 when comparing HMECs, and 6 when comparing MCF-10A cells) were commonly regulated. The only gene that was identified as a STAT3 target in all 3 systems was the transcriptional regulator *bcl-6*. Also surprisingly, a number of known STAT3 targets—including *bcl-x<sub>L</sub>*, *c-myc*, and *cyclin D*—were not identified in any of these studies.

What are some explanations for the observed differences among the genes identified in these studies? One obvious explanation is that the cell types analyzed are different. STAT3 is known to exert different biological effects in different tissues; for instance, STAT3 is required for growth arrest and differentiation of some leukemia and melanoma cells.<sup>53,54</sup> However, STAT3 is expected to promote changes consistent with transformation in all the cell types analyzed in these studies. Another, related explanation is that epigenetic differences among the cell types influence the spectrum of genes STAT3 can bind to and activate; such potential differences include DNA methylation and histone modifications at the promoters of STAT targets. In addition, STAT3 has been shown to cooperate with several transcription factors, and the genes STAT3 activates may differ depending upon whether these other transcription factors are expressed.<sup>43,55</sup> Another example of this is the STAT family member STAT1. STAT3 and STAT1 have been shown to form heterodimers in response to certain stimuli which activate both proteins; it is possible that STAT3:STAT1 dimers activate different genes than do STAT3 homodimers, such that the status of STAT1 activation in a cell will influence STAT3 function. Whatever the explanation for the observed differences, one implication of the differences is that STAT3 may regulate distinct genes in different tumors, and thus may make differing contributions to a tumor's phenotype depending upon the tissue type.

Other explanations relate to differences in the experimental design employed among studies. For instance, the studies in fibroblasts and mammary epithelial cells differ in several important respects. The study in mammary epithelial cells compared the steady-state transcriptional profile of cells expressing STAT3-C to cells not expressing this protein. This differs from the acute expression of STAT3-C performed in fibroblasts, and there are several important aspects to this difference. Acute expression of STAT3-C allows for the identification of direct targets of STAT3-C, while the steady-state transcriptional profile of cells expressing STAT3-C will include changes in genes which are not direct targets of STAT3, but whose expression changes in response to other transcription factors downstream of STAT3, or to altered cellular conditions induced by STAT3. Furthermore, given the importance of negative feedback loops in terminating normal STAT3 signaling, it is unclear whether direct targets of STAT3 will remain highly expressed in cells with persistent STAT3 activation. STAT3-C is likely to be refractory to a number of these inhibitory proteins, while STAT3 activated by IL-6 should be sensitive to their inhibition. Thus information about both the immediate, direct transcriptional targets of STAT3 as well as the steady-state consequences of STAT3 activation is needed to understand the role of STAT3 in cancer. An important goal is to integrate these snapshots to create a temporal picture of how STAT3-directed changes in the transcription of a cell leads to transformation.

## CONCLUSION

These studies provide a starting point for identifying crucial mediators of STAT-mediated oncogenesis by providing literally hundreds of candidates for further study. High-throughput loss-of-function studies have become possible with the advent of RNA interference, and should allow for the rapid characterization of these target genes. The most compelling evidence will come from studies that address the expression of putative STAT targets in primary human tumors with activated STATs, coupled with analysis of the role of such targets in STAT-dependent processes like proliferation, survival, and transformation *in vitro*.

Given the prominent role of STATs in inducing transformation *in vitro* and their likely contribution to human tumors *in vivo*, they provide an excellent model for understanding how coordinated changes in gene expression can lead to malignancy. Understanding what these changes are, and what cellular processes they affect, will be informative about the pathology of tumors and about potential therapeutic approaches to treating them.

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