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STAT heterodimers in immunity
A mixed message or a unique signal?

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Cytokine signals are essential for generating a robust and specialized immune response. These signals are typically transmitted via canonical STAT homodimers. However, the number of STAT molecules utilized by cytokine signaling cascades within immune cells are limited, and so the mechanism used to deliver complex signals remains elusive. Heterodimerization of STAT proteins is one potential mechanism for signals to be modified downstream of the receptor and may play an important role in dictating the targets of specific cytokine signaling. In this review, we discuss our current understanding of the prevalence of STAT heterodimers, how they are formed and what their physiologic role may be in vivo.

Introduction

While stimulation through the antigen receptor (signal 1) combined with costimulation (signal 2) is absolutely required for activation of T cells,[1] it is now generally understood that additional signals are required for acquisition of full effector function. These additional signals, in the form of autocrine and paracrine cytokines, are required to divert the immune response into appropriately programmed effector phenotypes (signal 3).[2] While signal 1 can only be delivered through the TCR and signal 2 is the "net sum" of multiple costimulatory molecule ligations, signal 3 is remarkably more complex.[2,3] The delivery of a cytokine signal relies on several intermediaries, including (1) the expression and secretion of the cytokine on the "transmitting" cell, (2) the expression and structure of the receptor on the "receiving" cell, (3) the signaling pathways downstream of the receptor(s) and (4) additional regulation of those downstream signaling pathways [e.g., suppressor of cytokine signaling (SOCS) molecules]. Furthermore, most cytokines have complex effector functions that may contribute contrasting immunomodulatory roles in vivo.

Most cytokines predominantly transmit signals via Janus kinase (just another kinase, or JAK)-signal transducer and activator of transcription (STAT) pathways.[4] An inherent conundrum in cytokine biology is that while over 50 cytokines have been implicated in playing distinct roles in immune cell function, there are only seven STAT molecules with which to transmit these signals.[5,6]

In this review, we will discuss one potential mechanism by which cytokines diversify their signaling and deliver complex signals to a receiving cell. Several cytokines induce the formation of heterodimers of STAT proteins. We will discuss three important questions regarding these alternative signaling complexes. First, how common are STAT heterodimers in immunity? Second, how are STAT heterodimers formed? Third, how do STAT heterodimers mediate unique functional events?

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JAK-STAT Signaling in Response to Cytokines

The initial discovery of a 91-kDa DNA binding protein that was tyrosine phosphorylated by interferon gamma (IFN-γ) treatment, referred to as STAT1, described a mechanism by which cytokines could induce transcription.[7,8] The later discovery of JAK proteins that bound receptors and aided in tyrosine phosphorylation of these transcription factors further elucidated cytokine receptor function.[9] While TCR- and costimulatory-mediated signaling relies on the many rounds of signal amplification through multiple kinases,[1] it seemed as though cytokine-receptor interactions could directly phosphorylate the transcription factors that would program their function.[10] When STAT3 was found to signal via similar mechanisms, but in response to IL-6, leukemia inhibitory factor and other gp130-utilizing cytokines, it suggested that there are many STATs, each induced by different cytokines and promoting a distinct outcome.[11] Over the next 10 years, a growing number of cytokines would be linked to distinct STAT proteins, providing a network by which cytokines could presumably activate transcription and program function (**Table 1**).

Common themes began to emerge as the "menu" of JAKs and STATs grew to size. Interferons promoted the activation of STAT1 and STAT2, resulting in the transcription of interferon-inducible genes.[7,8] IL-6 and other gp130-utilizing cytokines could induce STAT3 activation, promoting the induction of inflammatory cytokines.[12] STAT3 was also to be critically important in the induction and development of T helper 17 (Th17) cells, a dominant inflammatory T cell subset.[13] IL-12 induces STAT4 activation, which, in T cells, promoted the acquisition of a T helper 1 (Th1) effector phenotype and the subsequent production of

Table 1. Known STATs and their activators

Molecule	"Inflammatory" activators	"Anti-inflammatory" activators	Heterodimerization partners
STAT1	Type I IFN Type II IFN IL-6	IL-10 IL-27 IL-35	STAT2 (IFN) STAT3 (IL-6, -27) STAT4 (IL-35)
STAT2	Type I IFN		STAT1 (IFN)
STAT3	IL-2 IL-5 IL-6 IL-23 MCSF GCSF	IL-10 IL-27	STAT1 STAT5a/b
STAT4	IL-12 IL-23	IL-35	STAT1 (IL-35) STAT3 (IL-23)
STAT5a/5b	IL-2 -7 -15 IL-21 M-CSF GM-CSF		STAT3 (IL-2, -7, GCSF, MCSF)
STAT6	Type I IFN (B and human T cells) IL-3 IL-4 IL-13		STAT2 (IFN) (B and human T cells)

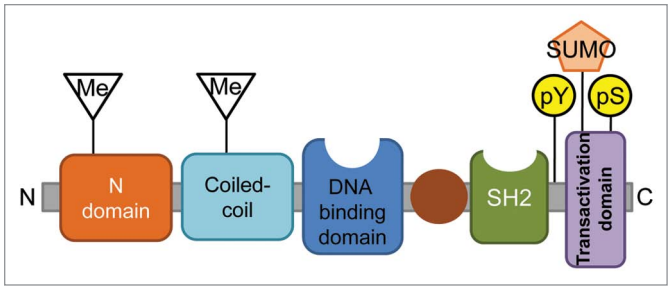

Figure 1. Anatomy of a STAT. STAT proteins in general consist of six domains. Post-translational modifications (PTM) that have been shown to have physiologic relevance are depicted. Note that some STATs (notably STAT5 and STAT6) lack a canonical phosphoserine site, but instead have several non-homologous serines that have varied functions in transcriptional regulation.

IFN-γ.[14] IL-2, an extremely important survival cytokine for T cells, could induce STAT5a/5b activation, resulting in the transcription of prosurvival genes, as well as Foxp3, aiding regulatory T cell development.[15,16] STAT6 activation could be induced by IL-4, which programmed T helper 2 (Th2) differentiation, characterized by IL-4 secretion.[17] In addition, the mechanism by which STATs mediated transcription was elucidated; tyrosine phosphorylation of STAT proteins by receptor binding and JAK activation could induce homodimerization of STAT molecules.[4] This homodimerization would result in nuclear accumulation of these dimers. STAT dimers could then bind their target sequence, recruit coactivators and effect transcription.

However, while the number of cytokines grew larger, the number of signaling molecules utilized by cytokines to transduce signals did not. It seemed that many cytokines with distinct (and sometimes opposing) functions would activate the same STAT protein.[5,6] For instance, IL-6, a proinflammatory cytokine that utilizes gp130, promotes the activation of STAT3.[11] IL-10, however, does not utilize gp130, is a potent anti-inflammatory cytokine, but also promotes the activation of STAT3.[18-20] This paradox continues to persist today, as new cytokines with distinct functions are discovered that activate one or more of the same seven STAT proteins.

As the mechanisms of STAT activation were identified, the picture became more complex with regard to post-translational modifications (PTMs) and their role (**Fig. 1**). Phosphotyrosine residues in STAT proteins can be recognized by STAT SH2 domains in its partner, mediating dimerization. STATs can also be phosphorylated on a serine residue in the transactivation domain,[21] thereby modulating gene target specificity and transcriptional activity. Phosphorylation can take place at the receptor or in response to other cellular signaling pathways.[22] While STAT1, -3 and -4 have homologous phosphoserine sites containing a P(M)SP motif, it is interesting that STAT5a/b and STAT6 lack these motifs.[22] However, it has recently been shown STAT5 and STAT6 are modulated by phosphorylation on alternate serine residues at the C terminus in non-homologous locations.[23-26] Other modifications have also been identified. Arginine methylation has been shown to modulate interactions with epigenetic machinery.[27,28] SUMOylation in the TA domain by protein inhibitor of activated STATs (PIAS) proteins inhibits the activation and nuclear accumulation of STAT proteins.[29] Other modifications have also been described (e.g., acetylation and ubiquitylation) but the physiologic relevance of these events has yet to be elucidated.

In addition, latent or unphosphorylated STAT dimers have been observed and appear to play a vital role in certain aspects of cell signaling.[30] These latent dimers have been identified for most

STAT proteins and exhibit continuous bi-directional shuttling between the cytoplasm and nucleus.[31,32] Importantly, these latent STAT dimers have also been shown to have distinct transcriptional targets than their phosphorylated counterparts.[32] These homodimeric interactions require the N-terminal domain, and are independent of the phosphotyrosyl-SH2 interactions that occur at the C terminus of the protein. N-terminal domains have been shown to be an important aspect of latent STAT homodimerization in all seven STAT proteins.[33] It is thought that these interactions are also important for higher order structures in the nucleus, especially in promoters that have several adjacent STAT binding sites. The N-terminal domain of STAT4 has been shown to be critical for dimerization and function, in particular IL-12-induced phosphorylation and Th1 differentiation.[34] These and other studies have suggested that STAT N-terminal interactions provide an alternative mode of signaling and transcription to receptor activation.[35]

Further complicating our understanding is the fact that the majority of cytokines activate several STAT proteins (**Table 1**), and which STATs are activated can be heavily dependent on cell type, activation or differentiation state, the type of receptor expressed and the timing and dose of cytokine.[5,6] Many of these problems have been addressed, at least in part, by the utilization of STAT or receptor-deficient cells. For example, IL-12 has been shown to activate STAT3 and STAT5 in addition to STAT4, but only STAT4-deficient cells seem to lack sensitivity to IL-12.[14,36-38] In other words, a cytokine, when added in excess, may activate a number of STATs, but the essential requirement of a particular STAT is determined by the impact of genetic deletion. However, when it is found that several STATs are activated (and required) by a particular cytokine, it begs the question of whether the STAT proteins are being activated in parallel, inducing separate transcriptional events, or are working together, synergizing to promote an alternative transcriptional outcome.

How Common Are STAT Heterodimers in Immunity?

As STAT1 was originally found as part of a nuclear multiprotein complex, ISGF3,[7] it was known that it had several binding partners. However, whether or not other STATs were involved was largely unknown. Later it was shown that activated STAT1 and STAT2 were found in ISGF3 in response to type I interferons, together forming a DNA-binding complex.[39] However, the physiologic relevance of these heterodimers was determined later, showing that STAT1:STAT2 heterodimers bound distinct consensus sequences in a subset of interferon stimulated genes.[40]

STAT heterodimers were also shown to be important in GM- and M-CSF signaling. While M-CSF was known to activate both STAT3 and STAT5, it was unclear if a heterodimeric complex formed. Interestingly, STAT5 homodimers as well as STAT3:STAT5 heterodimers could be formed upon M-CSF stimulation, but only STAT3:STAT5 heterodimers could bind particular consensus sequences.[41] Further using *Stat5a*$^{-/-}$ mice, it was shown that GM-CSF requires use of STAT5a:STAT5b heterodimers to have full function in myeloid cells, whereas STAT5a homodimers were dispensable.[42] Later, despite having high homology to one another, it was found that a single amino acid difference between STAT5a and STAT5b resulted in surprisingly different DNA binding specificities, highlighting the potential importance of heterodimerization to expand target gene selection.[43,44]

The common gamma chain (γ_c) cytokines IL-2, -4, -7, -9, -15, -21 and TSLP have each been shown to activate a broad variety of STAT proteins, with STAT5 being in common.[45] While each of these cytokines utilizes γ_c to mediate signaling, the heterodimeric/trimeric nature of these receptor complexes can promote a number of distinct patterns of STAT activation. For instance, IL-2, IL-7 and IL-21 each can activate STAT1, STAT3 and STAT5, but IL-2 promotes much stronger STAT5 activation, IL-7 induces persistent STAT5 activation, while IL-21 produces far more activated STAT3.[46] Further, heterodimerization of STAT5 isoforms 5a and 5b has been shown in response to IL-2 as well as IL-7, showing that while the kinetics of heterodimerization were the same, the strength of signal was remarkably different between these two cytokines.[47]

The IL-6 superfamily of cytokines, which is characterized by complex interactions like soluble receptors and receptor-like subunits,[48] also utilizes STAT heterodimerization. The discovery of STAT3 showed that in response to IL-6, it can utilize STAT1:STAT3 heterodimers, especially in late signaling.[12,49]

The IL-12 family, a subunit of the IL-6 superfamily, is characterized by heterodimeric cytokines that share subunits, and has multiple immunomodulatory roles.[50] IL-12, made of p35 and p40, promotes a strong Th1 response by inducing T-bet expression, which programs Th1 function and IFN-γ secretion.[51] IL-12 works mainly through inducing mostly STAT4 homodimers.[52] However, the other members of the IL-12 family, including IL-23, IL-27 and IL-35, have been shown to induce heterodimers.

IL-23, important during Th17 differentiation, works to stabilize the inflammatory phenotype of Th17 cells. Early differentiated Th17 cells induce expression of the IL-23R,[53] which pairs with IL-12Rβ1 to confer sensitivity to IL-23.[54] IL-23 was shown to activate STAT3 and STAT4 downstream of these receptors.[54] Later studies suggested that the majority of STAT3 and STAT4 induced by IL-23 to effect transcription was present in heterodimers.[51,54] Indeed IL-27, which uses the IL-6 receptor subunit gp130 and WSX-1 to induce STAT1 and STAT3 signaling, was also shown to form STAT1:STAT3 heterodimers, although the molecular consequences of that heterodimer are still unknown.[55]

Our group has recently identified the receptor and signaling pathway for IL-35, the newest addition to the IL-12 family.[56,57] IL-35 has two functions in conventional naive T cells, suppression of proliferation and conversion to an induced regulatory T cell population that suppresses via IL-35.[57,58] IL-35 utilizes three receptors: gp130 homodimers, IL12Rβ2 homodimers and a heterodimer of gp130:IL12Rβ2. IL-35-induced IL12Rβ2 and gp130 homodimers induce STAT4 and STAT1 phosphorylation, respectively, and can do so in the absence of the other receptor. These homodimeric receptors are sufficient for suppression of proliferation, but cannot induce conversion to IL-35 production. Rather, the gp130:IL-12Rβ2 heterodimeric receptor is

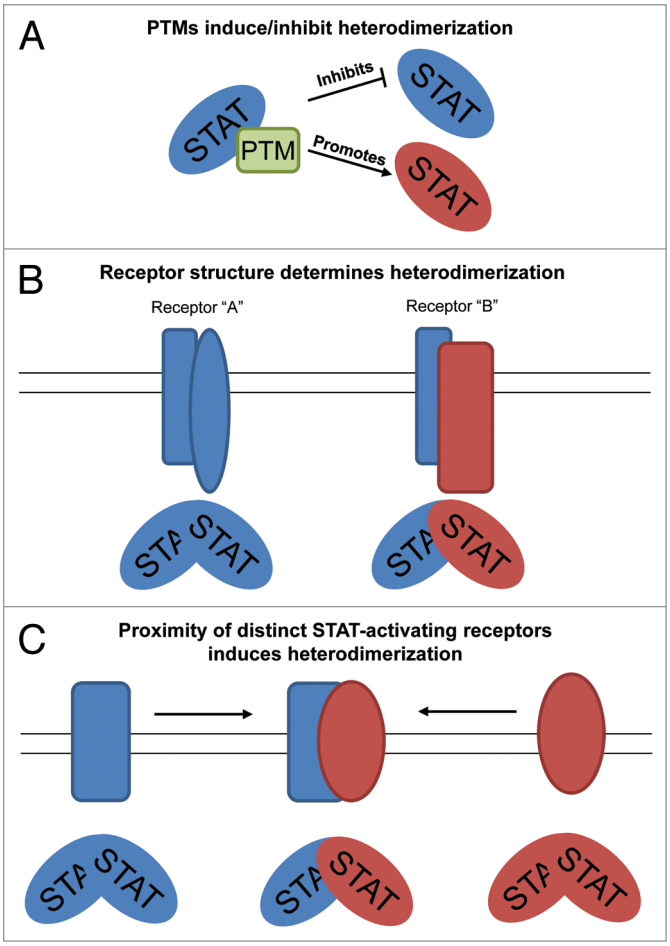

Figure 2. Potential mechanisms of STAT heterodimer formation. (**A**) As STATs can be extensively post-translationally modified, PTM of STAT proteins may act to inhibit a specific kind of dimerization or promote another. (**B**) The specific structure of particular cytokine receptors could favor the generation of heterodimers over homodimers. (**C**) Proximity of subunits that generate distinct STAT molecules could influence heterodimer formation. A cytokine or other cellular event bringing these subunits together could promote the formation of STAT heterodimers.

essential for induced expression of IL-35, which is initiated by a STAT1:STAT4 heterodimer.[56]

Collectively, these observations suggest that within every cytokine family there appears to be at least one member that promotes STAT heterodimer formation. Furthermore, STAT heterodimerization may not be an uncommon occurrence, but rather one that has not been rigorously studied.

How Are STAT Heterodimers Formed?

The mechanism by which receptors promote STAT heterodimerization vs. homodimerization is still elusive. We and other have suggested at least three non-mutually exclusive different mechanisms by which this occurs (**Fig. 2**). First, post-translational modification (PTM) of STATs may either induce the formation of a heterodimer or prevent the formation of a homodimer. This is evidenced by STAT3 signaling, in which serine phosphorylation of STATs, a JAK-independent but receptor-dependent phenomenon, is dispensable for heterodimerization.[21] Type I interferon signaling, which utilizes STAT1:STAT2 heterodimers, also may involve alternative PTMs.[10,39] Given that STATs can undergo many types of PTM (**Fig. 1**), it stands to reason that one or many of these mechanisms may have a role in dimerization, although whether or not these modifications can specify homo vs. heterodimerization remains to be determined.[5]

Second, it may also be that the structure of individual cytokine receptors promotes the generation of STAT heterodimers, independent of PTM or other signaling pathways. Given that expression of cytokine receptors can be regulated by cell-type, activation status and other factors, the receptor-intrinsic regulation of STAT heterodimerization provides another level of regulation that could be utilized to promote distinct function downstream of cytokine stimulation. However, testing these hypotheses presents a unique challenge. Without structural insight into what these receptors look like complexed with STAT homodimers vs. heterodimers, it would be difficult to fully ascertain how one forms over the other. However, structural changes could change the accessibility of docking motifs for binding partners, which could be assessed fairly easily. The crystal structures of several STAT homodimers have been solved, and these studies along with structure-function analyses have suggested that side groups and length of the SH2 domain might modulate a given STAT's ability to homo or heterodimerize via phosphotyrosyl modifications.[59-61]

Third, given that most cytokine receptors are composed of two or more distinct subunits, it may also be that differing subunits can recruit and activate distinct STAT proteins, and that mere proximity can promote the formation of a heterodimer. As some receptor subunits can be spatially segregated while others are ubiquitous, this explanation, while simplistic, may prove to be important. However there has been some evidence for this in the literature. For instance, B cells stimulated with type I IFNs promote the generation of STAT2:STAT6 heterodimers, presumably due to the expression of alternative type I IFN receptors in B cells.[62] In addition, concomitant IL-4 and IFNα stimulation enhances IL-4 activity while promoting the generation of STAT2:STAT6 in human T cells, which the authors conclude is determined due to proximity of the IL-4R, γ_c and IFN-AR.[63] Experiments enforcing proximity of subunits paired with real-time visualization of STAT heterodimers may be vital to exploring this hypothesis.

Our understanding of the formation and role of ternary STAT structures has been further complicated by observations that STATs can homo- and hetero-tetramerize, as well as have even higher-order structures when in the nucleus.[64,65] Recently, STAT5a/5b tetramerization was implicated, using several in vivo models, in the response to IL-2 and other γ_c cytokines, utilizing a mouse with a targeted mutation preventing STAT5 tetramerization while preserving dimerization.[66] Thus there are many potential explanations regarding how STAT heterodimers form, none being mutually exclusive. Furthermore, it is important to remember that higher-order STAT structures have been observed and are physiologically relevant.

How Do STAT Heterodimers Mediate Unique Functional Events?

The functional relevance of STAT heterodimers in vivo remains obscure. Many theories have been postulated but none have been rigorously tested, as typical molecular biology techniques can never completely rule out the generation and/or function of homodimers. Gel-shift analysis by EMSA is the "gold standard" of heterodimer identification, but these assays rely on specific probes, thereby ruling out any high-throughput, genomic analyses. ChIP-reChIP, which utilizes sequential immunoprecipitations to assay DNA bound by multiple transcription factors, has technical limitations that cannot rule out distinct homodimeric complexes binding to adjacent sites. The functional relevance of heterodimers has yet to be fully elucidated, but they could exert their function through three non-mutually exclusive mechanisms (**Fig. 3**).

First, some groups have hypothesized that STATs heterodimerize to remove a particular STAT from the available "pool" of signaling molecules, thereby reducing the efficacy or strength of STAT homodimer signals. This has been shown notably in STAT1:STAT3 heterodimerization using overexpressed STAT3 and type I IFNs, indicating STAT3 sequesters STAT1 into heterodimers.[67] While overexpression of STAT3 may not be physiologically relevant, it does demonstrate a role for STAT3 in sequestering STAT1 in heterodimers. This "dead signal" hypothesis is supported by some signaling pathways, in which STAT heterodimers play a role in late signaling or diminution of signaling or transcription. However, this hypothesis doesn't explain why some cytokines elicit a majority of heterodimers, but have distinct functions. For instance, IL-23 promotes STAT3:STAT4 heterodimerization during Th17 polarization.[54] Given the importance of STAT3 in the generation of Th17 cells,[13] it is unlikely that IL-23 would generate a dead signal for STAT3 during this crucial time.

Second, given that particular STATs can bind different consensus sequences with distinct affinities, another possibility is that STAT heterodimers can bind different sequences than their homodimeric counterparts. STAT1:STAT4 induced by IL-35, for instance, can bind sequences in the promoters of *Ebi3* and *Il12a*, but not two traditional STAT1 or STAT4 targets, *Irf1* and *Il18ra*, respectively.[56] STAT3:STAT5 elicited by M-CSF can bind high affinity *sis*-inducible element (SIE) sites with high affinity while STAT3 or STAT5 homodimers cannot.[41] There are obvious caveats to this possibility, as the consensus sequences of many STAT proteins are known and appear to be quite redundant. However, the recognition of these STAT-binding sites can to be fine-tuned. For instance, STAT5a's DNA binding specificity can be changed to STAT5b's merely by a change of a single amino acid.[43] Whether or not this regulation is STAT-intrinsic or dependent on other factors has yet to be determined.

Third, it may be possible that STAT heterodimers, as tightly bound distinct subunits, can recruit different transcriptional coactivators/repressors that can influence their function. Rather than bind a distinct DNA sequence, either partner may recruit new transcriptional activators, for instance, thus allowing transcription at privileged sites. This is particularly evident in interferon signaling. IFNγ promotes STAT1 homodimers, which can bind genes like *Irf1*.[68] IFNα, in contrast, promotes STAT1:STAT2 heterodimerization, which can recruit other coactivators, such as IRF9, in the ISGF3 complex.[69] However, the full transcriptional networks and STAT interactome has yet to be fully explored. Experiments using proteomic identification of nuclear STAT heterodimers could help shed light on this potential method of regulation.

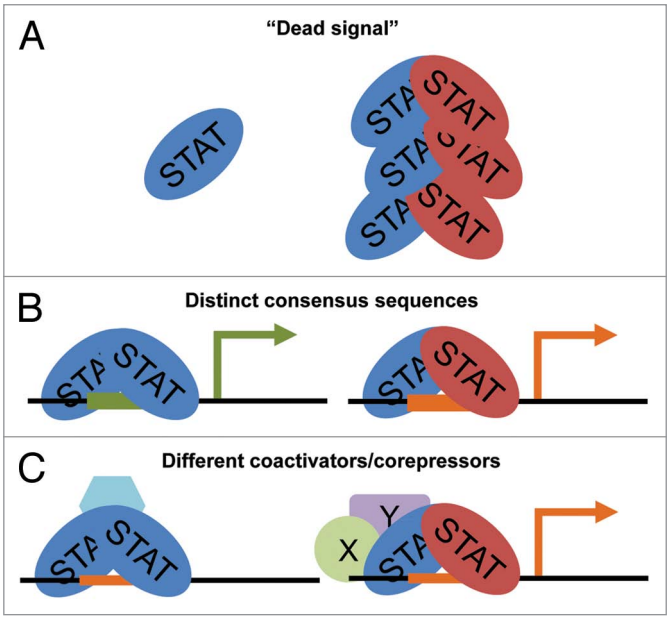

Figure 3. Potential mechanisms of STAT heterodimer function. (**A**) STAT heterodimers may act as a sink, reducing the pool of available STAT proteins able to homodimerize. (**B**) STAT heterodimers may act by binding a unique consensus site (green vs orange, in this model), allowing for transcription of alternative gene targets. (**C**) STAT heterodimers could also function through the recruitment of different transcriptional coactivators/repressors. In this model, the consensus site is not significantly altered, but rather homodimers recruit repressors while heterodimers recruit coactivators.

Concluding Remarks

The role of STAT heterodimerization in the modulation of cytokine function has yet to be fully explored. Furthermore, the prevalence of STAT heterodimers vs. homodimers needs to be established. Rigorous interrogation of how individual STAT heterodimers interact, as well as how they function in contrast to STAT homodimers, may elucidate the roles of these complexes in general immunity, and how they may help pleiotropic cytokines deliver complex messages. This is likely to require detailed structure-function analysis coupled with examining the full spectrum and kinetics of STAT post-translational modification, as well as a proteomic analysis of STAT binding partners, in response to a particular cytokine.

The use of chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) has expanded the horizon for determining the genomic targets of STAT proteins.[70,71] However, a massively

parallel ChIP-Seq experiment from the same cell type, done using distinct cytokine stimulations and ChIP-Seq for all seven STAT proteins, could reveal much. Overlaying the genomic locations of different STAT proteins in response to individual cytokines could impart fundamental insight into the targets of different STAT complexes, while paving the way for the discovery of novel heterodimeric STAT complexes. This machinery could be further verified by the use of ChIP-reChIP-Seq, using sequential antibody immunoprecipitations to restrict analysis to STAT heterodimers. Finally, a detailed understanding of the molecular basis for STAT heterodimer formation and function could reveal novel therapeutic approaches to specifically and surgically modulate their downstream phenotypic consequence in vivo.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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