JAK-STAT 1:4, 274–284; October/November/December 2012; © 2012 Landes Bioscience

Alternative ways of modulating JAK-STAT pathway

Looking beyond phosphorylation

Olga A. Timofeeva[1,2] and Nadya I. Tarasova[3]

[1]Departments of Oncology; Lombardi Comprehensive Cancer Center; Georgetown University Medical Center; Washington, DC USA; [2]Department of Radiation Medicine; Lombardi Comprehensive Cancer Center; Georgetown University Medical Center; Washington, DC USA; [3]Cancer and Inflammation Program; Frederick National Laboratory for Cancer Research; Frederick, MD USA

Keywords: STAT inhibitor, lipopeptide, cancer, protein-protein interactions

Most attempts to develop inhibitors of STAT transcription factors target either activating phosphorylation of tyrosine residue or SH2 domains. However, all six domains of STATs are highly conserved between the species and play important roles in the function of this family of transcription factors. STATs are involved in numerous protein-protein interactions that are likely to regulate and fine tune transcriptional activity. Targeting these interactions can provide plentiful opportunities for the discovery of novel drug candidates and powerful chemical biology tools. Using N-terminal domains as an example we describe alternative rational approaches to the development of modulators of JAK-STAT signaling.

STAT proteins are latent cytoplasmic transcription factors activated by tyrosine phosphorylation in response to extracellular signals and are involved in many different regulatory events.[1] In mammals, the STAT family consists of STAT1, 2, 3, 4, 5A, 5B and 6, and shares a common set of structural domains: N-terminal, coiled-coil, DNA binding, SH2, linker and transactivation domains.[2] The mammalian STAT family is implicated in responses to cytokines and growth factors, and exert diverse effects on a number of biological processes including immunity, hematopoiesis, inflammation and development.[3]

In normal cells and in tissues, receptor ligands-dependent activation of the STATs is a transient process, lasting from several minutes to several hours.[4] However, in many cancerous cells, with dysregulated growth factor signaling, STAT proteins are constitutively activated by tyrosine phosphorylation.[4,5] In this respect, STAT3 stands out, based on its constitutive phosphorylation in the majority of human neoplasms and its capacity to induce cell transformation and tumorigenesis.[5,6] It is believed that phosphorylated STAT3 (P-STAT3) mediates its oncogenic effects through transcriptional activation of target genes to enhance proliferation (cyclin D1 and c-Myc), angiogenesis (VEGF, ADM and ANGPTL4), invasion (FGA, FGB, CTSB and SERPINE2), and suppression of apoptosis (Bcl-xL, Bcl-2, Mcl-1 and Survivin).[7] In addition, P-STAT3 stimulates its own transcription causing an increase in unphosphorylated STAT3 (U-STAT3), which in turn may also contribute to tumorigenesis albeit by the mechanisms different from phosphorylated STAT3.[8-10] It is also well established that activated STAT5A/B play essential roles in leukomogenesis,[11] and these transcription factors are also required for proliferation of liver, prostate, ovarian and head and neck cancer cells.[12]

Over 40 cytokines and growth factors signal through STAT proteins.[1,13] Although many cytokines are believed to activate the same "canonical" JAK-STAT signaling cascade, the biological effects from activation of JAK-STAT signaling by different growth factors and cytokines are significantly different.[14] Activated STAT transcription factors can bind to the same DNA sequence, so called GAS motif, IFNγ activated sequence. However, the sets of target genes and transcriptional effects of STATs are very different.[15] The molecular mechanisms of the JAK-STAT functional diversity are poorly understood. It has been proposed that the involvement of effectors that interact with cytokine receptors, JAK kinases, or with STATs may be implicated in modulation of STAT signaling.[16] Although STAT phosphorylation is a major event in STAT activation and, therefore, is a desirable target for cancer therapy, many functions of STAT proteins are regulated by interactions with other transcription factors. To date, most efforts in inhibiting activity of STAT proteins have been focused on targeting phosphorylation and subsequent dimerization of SH2 domains.[17-23] The SH2 domain appears to have a well-defined function, although we may be underestimating the intricacy of its role. Other five domains of STATs have received significantly less interest and attention in spite of facts that they are highly conserved between the species and are involved in numerous protein-protein interactions.[2] Structural data suggest that some of the domains, particularly N-terminal domains (NDs), can fold independently offering an opportunity to develop chemical probes that influence the function of particular domain without much disturbance to the function of the other parts of the protein.

We have chosen STAT NDs for targeting because wealth of literature suggested its involvement in regulation of STAT's

Correspondence to: Olga A. Timofeeva and Nadya I. Tarasova;
Email: oat@georgetown.edu and Nadya.Tarasova@nih.gov
Submitted: 08/06/12; Revised: 09/17/12; Accepted: 09/20/12
http://dx.doi.org/10.4161/jkst.22313

function through tetramerization and interactions with other proteins. The ND appears later in evolution and is present in Drosophila., zebrafish and mammalian STATs, but not in Dictyostelium and *C. elegans*.[24,25] STAT protein in Dictyostelium that lack both N-terminal and C-terminal domains serve largely as transcriptional repressors.[26] The Drosophila's N-terminally truncated STAT isoform also appears to function as a repressor.[27] Since the major known role of the ND in STAT protein-mediated transcription is to promote higher-order complex formation on the promoters of target genes for enhanced expression,[28] it has been proposed that the ND accretion during evolution added new functionality for STAT proteins allowing more flexibility in DNA binding.[2] This function may be important for constitutively activated STAT proteins to drive gene expression during cell transformation. A search for genetic suppressive elements (GSE) in breast cancer cells convincingly identified the NDs of STAT3 and STAT5 as major factors responsible for driving cancer cells proliferation and survival.[29] Also, the causative role of the STAT5 ND in leukemogenesis has been demonstrated.[11] For the targeting purposes it is important that NDs of STATs do not share homology with any other protein, in contrast to its DNA-binding or SH2 domains, and therefore there are fewer chances for off-target effects.

In this review, we use inhibition of STATs' NDs as an example of possible alternative approaches to modulation of JAK-STAT signaling. We summarize the known functions of the STAT NDs and present a rational for inhibition of NDs of STATs in cancer cells. We also discuss various strategies for targeting the STAT ND for therapeutic purposes.

Role of STAT ND in Tetramerization

The obtained crystal structures of tyrosine-phosphorylated STAT1 and STAT3 demonstrated that interaction of two NDs within one STAT dimer is unlikely.[30,31] These observations suggested that the NDs are free to promote other protein-protein interactions. In particular, two STAT dimers bound to adjacent GAS elements may form a STAT tetramer via ND-ND interaction.[30,32-35] Such cooperation in DNA binding via NDs allows fine-tuning of transcriptional responses through selective binding of different STAT proteins on the promoters containing multiple STAT binding sites and through binding to weak STAT-binding sites. So far, the ND of STAT1, STAT4, STAT5 and somewhat STAT3 were found to form tetrameric complexes, at least on selected promoters.[30,32-35]

Crystallographic studies identified invariant W37 as essential for the ND dimerization.[30] Other amino acid residues (Q36, T40 and E66) were predicted to be involved in interactions between α-helices within the ND.[30] However, subsequent mutational analysis of the STAT1 and STAT4 NDs demonstrated that these residues are unlikely to mediate interactions at proposed interface, and suggested an alternative dimer interface that involves S12, L15, DR19 (α-helix2) and F77 and L78 (α-helix 7).[36] Deletion of the ND or the mutation in critical W37 residue responsible for ND dimerization resulted in abrogation of tetramer formation and transcriptional stimulation. For example, the loss of STAT1 tetramerization abrogated INF-γ-induces responses.[37] The mutation of a single F77 residue in the ND of STAT1 was recently found to preclude both the dephosphorylation and the oligomerization of STAT1 dimers.[38,39] Vinkemeier and Meyer have shown the influence of defective oligomerization on a complex phenotype such as the induction of an antiviral state.[39] They found that the antiviral protection conferred by IFNα was strongly reduced, whereas the IFNγ response was not measurably affected. These results indicate that STAT1 ND is required for the antiviral activity of interferons.

ND-mediated STAT5 tetramerization was found to be essential for IL-2-induced regulation of the IL-2 response element in the human IL-2Ra gene.[35] An interleukin-6-inducible activation of α2-macroglobulin gene promoter requires tetrameric STAT3 complex.[32] The functional importance of tetramer formation was revealed by the decreased levels of transcriptional activation associated with hypomorphic mutations in N-terminal residues.[32] In case of STAT4, substitution of W37 with alanine unexpectedly prevented IFNα-induced tyrosine phosphorylation of STAT4 monomer, blocking both dimer and tetramer formation.[34] The requirement of the STAT4 ND for STAT4 activation was confirmed for IL-12 signaling using STAT4-deficient transgenic mice that express human full-length STAT4 or N-terminal deletion mutant.[40] While full-length STAT4 rescues IL-12 responsiveness, the STAT4 N-terminally truncated protein does not undergo phosphorylation and therefore T-cells expressing this mutant do not undergo proliferation.[40]

The requirement for STAT tetramerization via ND may contribute to selective activation of certain genes expression. For example, tetramerization of STAT3 is required for the formation of enhancesomes on the promoter of α2-macroglobulin,[32] but it is dispensable for IL-6-induced activation of SOCS3, which only requires STAT3 dimer binding to the promoter.[41] STAT5 tetramerization is necessary for activation of IL-2Ra expression, but is dispensable for β-casein.[35,42] Moreover, despite high homology between NDs of various STATs, each domain has specific functions that may, at least partially, define precise regulation of STAT proteins functions. For example, substitution of STAT4 ND with that of STAT1 results in inability of chimera protein to undergo IFNα-induced tyrosine phosphorylation and to bind DNA probes in EMSA assay.[34] Also, substitution of the STAT1 ND with that of STAT4 failed to restore IFNα-induced MHC class I expression in U3A cells, despite the ability of this chimera to form EMSA complexes similar to those of wild-type STAT1.[34] However, this chimera was fully functional for IFNγ-induced MHC class I induction.[34] This data suggests that the NDs of STAT1 and STAT4 are not fully interchangeable for gene-specific transactivation events. These results imply that STAT NDs are not functionally equivalent and have private functions. It is thus conceivable to disrupt functions of various STATs using selective inhibitors of NDs.

Importance of STAT Tetramerization in Cancer Cells

Recently, STAT5A-STAT5B double-knock-in ND mutant mice in which STAT5 may form only dimers but not tetramers were

generated.[42] In contrast to STAT5-deficient mice that exhibited perinatal lethality, ND mutant mice were viable but had fewer $CD4^+CD25^+$ T cells, NK cells, and $CD8^+$ T cells, with impaired cytokine-induced and homeostatic proliferation of $CD8^+$ T cells.[42] The observation suggested that STAT5 dimers were sufficient for survival and for regulation of some target genes, and that tetramerization of STAT5 was only critical for cytokine responses and normal immune function. The data obtained from double-knock-in mouse model agreed with previous report on the critical role of the STAT5 ND in human stem cells maintenance and repopulating activity.[43] Therefore, the STAT5 ND has an essential function during normal physiological development of immune system.

Furthermore, tetramerization of STAT5 is associated with leukemogenesis.[11] It has been found that STAT5 exists as a tetramer in cancer cells of 25% patients with leukemia, while this was not observed in normal human bone marrow or peripheral blood cells.[11] In addition, STAT5 tetramer formation was enhanced in a mouse model of multilineage leukemias.[11] STAT5 tetramer formation resulted in stronger and larger DNA binding complexes compared with those formed by the dimers. Mutations in the STAT5A ND abolished tetramer formation and prevented induction of leukemia due to inability of ND-mutated STAT5A to rescue $STAT5^{-/-}$ T cell proliferation, despite the persistent activation of STAT5ΔN proteins.[11] These observations have proven that the enhanced tetramer formation through ND is the essential feature responsible for leukemogenesis. One of the mechanisms by which tetramer formation may contribute to leukemogenesis is increased occupancy of weak sites to a threshold required for transcriptional activity, which together with the greater degree of flexibility in DNA sequence tetramer recognition was suggested to widen target gene spectra.[35,44] STAT5 target genes that control apoptosis, cell cycle progression, and proliferation, such as Cyclin D3, Bcl-xL, BCL-2, Osm, CD25, CIS, Socs-2, Als and Igf-1, contain at least two STAT5 binding sites in their regulatory regions and their expression is controlled by STAT5 tetramer.[11,45] It has been established that STAT5 lacking the ND (STAT5A-ΔN) cannot protect $c\text{-}Kit^+Lin\text{-}Sca\text{-}1^+$ cells from apoptosis or induce bcl-2 expression.[45] The study by Li et al. defined ND-dependent survival signaling as an Achilles heel of persistent STAT5 activation and highlighted the potential therapeutic importance of targeting STAT5 ND-mediated regulation of bcl-2 family members.[45]

Although the significance of tetramerization vs. dimerization still remains to be established for other STAT proteins, in particular for STAT3, the importance of the ND in cancer cells was confirmed by a study in which peptides inhibitors targeting the NDs of STAT3 or STAT5 caused growth inhibition in breast cancer cells.[46] Therefore, interference with STATs tetramerization through the ND may be an effective therapeutic strategy for cancer treatment.

ND-Dependent Dimerization of Non-Phosphorylated STAT Molecules

The finding that STAT4 ND is essential for activation by cytokine receptors led to an assumption that ND dimerization of unphosphorylated STAT4 is a pre-requisite for STAT4 phosphorylation and transcriptional function.[34,36,40] The yeast two-hybrid analysis of ND interactions, where the ND of each STAT protein was expressed in the pFBL23 and GADT7 vectors to explore NDs as baits for all other NDs (prey), demonstrated that all STAT NDs are involved in homotypic dimerization.[36] ND homodimerization occurred even for STAT6 that has not been implicated in tetramer formation.[36] Interestingly, NDs of STAT5A and STAT5B that differ only by 11 amino acid residues out of total 130 still showed only selective homotypic dimerization, and did not demonstrate any cross-reactivity.[36] These data indicate that in addition to stabilizing tetramer formation, STAT NDs may have an important role in dimerization of non-phosphorylated STAT proteins. However, the significance of this pre-association is not completely understood.

In case of STAT4, such dimer formation may enhance presentation to receptor-JAK complexes favoring synchronized phosphorylation of the two monomers and allowing formation of active STAT dimer by simple intramolecular rearrangement.[36] Dimerization of unphosphorylated STAT1 strongly depends on the ND because its deletion increased the dissociation constant ≈100-fold, from ≈50 nM to 3–4 μM.[47] Crystallographic studies of STAT1 demonstrated that the structure of each nonphosphorylated monomer is identical to phosphorylated STAT1 monomer, however, the monomers in the non-phosphorylated protein are arranged differently,[48] and the ND interactions are essential for an antiparallel STAT1 dimer structure.[47-49] A deletions of ND or mutations disrupting the STAT1 ND dimerization (M28, F77 and L78) did not affect STAT1 ability to undergo phosphorylation in response to IFNα or IFNγ[36] and form tyrosine-phosphorylated dimers,[47] although such STAT1 mutants did not possess the transcriptional activity.[50] STAT1 ND appears to regulate association with the nuclear phosphatase TC45 and subsequent STAT1 dephosphorylation.[49,51,52]

The STAT3 ND is also responsible for dimer formation of unphosphorylated protein. Indeed, deletion of the N-terminal domain of STAT3 abrogated dimer formation, as shown by bnPAGE and 2f-FCS.[53] However, the homotypic interaction of the N-terminal domain of STAT3 are of low affinity (3.7 mM) compared with that of STAT1 (23 μM) and STAT4 (2.7 μM).[47] Point mutations analogous to those that disturb homotypic interaction of the N-terminal domain of STAT1 had no detrimental effect on the dimerization of STAT3.[47] Therefore, the N-terminal domain of STAT3 might not contribute to STAT3 dimerization by homotypic interaction but by reciprocal interactions with another domain of STAT3.[47] The SH2-domain could be a candidate for an interaction with the N-terminal domain because it has been shown that mutation of the SH2-domain affects dimer formation of unphosphorylated STAT3.[54] Such an interaction would lead to an antiparallel orientation of the latent STAT3 dimer, in contrast to the parallel orientation of the activated STAT3 dimer.[55] However, it should be noted that concentration of unphosphorylated STAT3 in Jurkat cells stimulated with IL-6 is about 100-times higher than STAT1;[56] therefore, it is possible that despite low affinity of the STAT3 ND interactions they are biologically relevant. STAT3 homotypic

dimerization is not necessary for its nuclear-cytoplasmic shuttling.[53,57] Deletion of the STAT3 ND does not impair IL-6-dependent tyrosine phosphorylation, nuclear import or dephosphorylation kinetics, indicating that this region is not essential for STAT3 recruitment to the IL-6 receptor complex, translocation to the nuclear compartment or downregulation.[41,53,57] However, the phosphorylated STAT3 dimers lacking the N-terminal domain do not accumulate in the nucleus.[41] A similar contribution of the N-terminal domain to nuclear accumulation has been shown for STAT1.[58] These findings point to a functional role of the N-terminal domain in nuclear import of activated STAT3 that deserves further investigation.

The deletion of the STAT5A ND also does not abrogate cytokine-induced tyrosine phosphorylation, dimerization or dimer DNA binding.[11,35] However, such deletion appears to render constitutive activation, indicating ND's negative regulatory function.[11,59-61] Interestingly, the ND truncated STAT5A/B are predominant isoforms binding to DNA in prostate cancer cells.[61] These isoforms are generated in prostate cancer cells by proteolytic processing.[61] The authors convincingly demonstrate that processing takes place in vivo, but not in vitro during the sample preparation. However, the exact mechanisms of proteolytic STAT5A/B cleavage in prostate cancer cells has not been deciphered and the enzymes responsible for it have not been identified.[61] Considering that PIAS3 interacts with STAT5 ND to repress STAT5-dependent transcription, this modification may represent a molecular mechanism by which STAT5A is able to evade inhibition of signaling by PIAS3 in human prostate cancer cells.[61,62] In contrast, breast cancer cells, like MCF-7 and T47D, contain full-sized STAT5A/B only. Prolactin-stimulated activation is efficiently inhibited by PIAS3,[61] suggesting different mechanisms of regulation of STAT5A/B activity in breast and prostate cancers. It is not known at present whether other STAT proteins undergo the N-terminal proteolitic cleavage. Identification of the proteases responsible for generation of the short form Stat5a/b in prostate cancer may present new therapeutic targets.[61]

Regulation of gene expression by unphosphorylated STATs (U-STAT) may constitute another potential role of the ND. The extensive investigation from Stark's laboratory documented that both U-STAT1 and U-STAT3 play important roles in the regulation of gene expression.[8-10,63] It has been proposed that U-STAT1 binds to DNA as a monomer by contacting one half of a palindromic GAS motif,[63] or forms dimers that also allows to bind GAS sequences in vitro, though with much lower affinity, compared with the phosphorylated STAT1 dimer.[31,33] U-STAT1 crystal structure suggests that unphosphorylated dimers bound to DNA are likely to be formed via the ND interactions. Most of the U-STAT1 dimers exist in antiparallel conformation, but a small proportion of unphosphorylated STAT1 adopts the parallel conformation of activated STAT1.[47] We recently observed that U-STAT3 also can bind to GAS sequences both as a dimer and as a monomer,[64] consistent with previous observations for U-STAT1.[63] Interestingly, atomic force microscopy allowed for detection of dimers of different shapes suggesting that U-STAT3 dimers may bind DNA in both parallel and anti-parallel conformation. The significance of the ND interactions for U-STAT3 binding to DNA and their role in regulation of gene expression remains to be investigated. If proven important for driving expression of genes regulated by U-STAT3, the disruption of ND-based dimerization may be a powerful tool to inhibit STAT3 functions for therapeutic purposes.

Role of ND in STATs' Protein-Protein Interactions

Despite the importance of tetramer formation for gene expression, and potentially for tumorigenesis, it is probable that ND is also involved in controlling gene expression through interaction with other binding partners. The Human Protein Reference Database (www.hprd.org) lists 102 binary interactions for STAT3, 77 for STAT1, 13 for STAT2, 11 for STAT4, 53 for STAT5A, 42 for STAT5B and 18 for STAT6. The differences in the numbers of identified interactions reflect the level of popularity of a particular STAT in research community rather than intricacy of its interactions. The diversity of interactions is a strong indicator of the complexity of their function's regulation. **Figure 1** presents an example of STAT3 protein-protein interactions. The list is far from being complete and includes only interactions for which interacting domains of STAT3 have been identified. Binary interactions of STATs with other proteins are considered among the most biologically appealing yet chemically intractable targets for drug discovery. Only for several proteins domains that are involved in interactions have been identified. The ND of phosphorylated STAT proteins is exposed on the surface of dimers or tetramers as suggested by crystallographic data and as recognized by native DNA binding assays (EMSA) or antibody supershift experiments. Therefore, this domain is potentially available for protein-protein interactions.[12]

During last few years, it has been understood that the NDs of STAT proteins undergo post-translational modifications that control the repertoire of STAT protein-protein interactions. It has been shown that STAT NDs may be phosphorylated, acetylated, methylated, and glycosylated.[65] The spectrum of the NDs modifications is summarized on **Figure 2**. Arginine 31 residue is known to undergo methylation and is conserved across STAT family members.[66-69] Although the work describing this finding has been criticized,[58,70] a recent review on JAK-STAT pathway[1] raises a voice in support of the role of R31 methylation in regulation of STAT1 interactions with PIAS1 which catalyzes SUMOylation of lysine residues and STAT1 transcriptional activity.[37,71,72]

Several recent reports have shed the light on protein-protein interactions that involve STAT3 ND, including interactions with HDAC1 and APE1.[73-75] Two Lys residues, 49 and 87 in the STAT3 ND, are acetylated by p300. Lys-to-Arg point mutations (STAT3 K49R/K87R) that blocked p300-mediated STAT3 acetylation had no effect on inducible DNA binding, but abrogated IL-6-induced angiotensinogen expression. Although STAT3 K49R/K87R rapidly translocated into the nucleus, it did not bind p300 and had delayed cytoplasmic redistribution. STAT3 was also found to interact with HDAC1 through the ND, which resulted in deacetylation of the domain and repression of

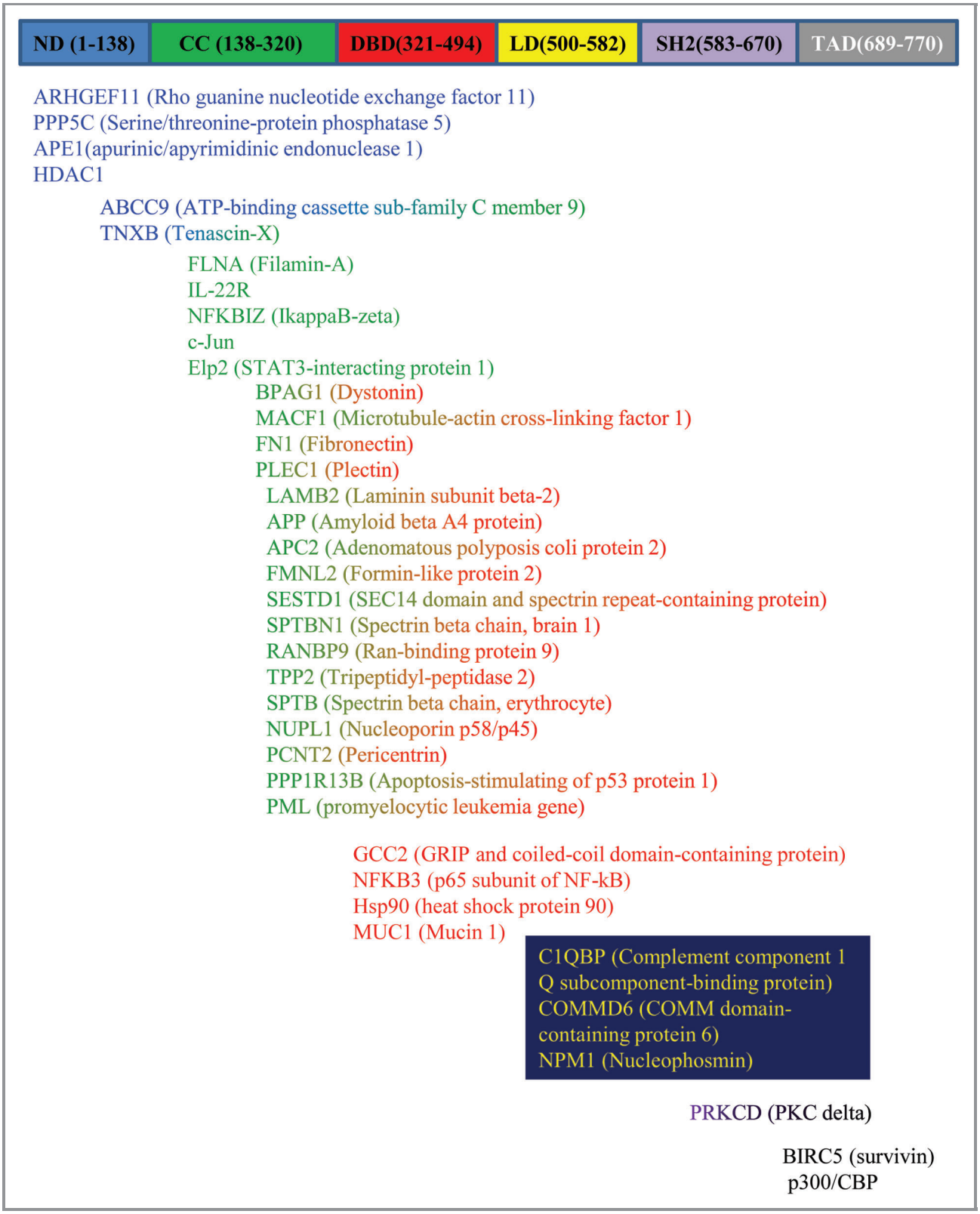

Figure 1. STAT3 domains form multiple binary interactions. Selected interactions are shown for which interacting STAT3 domains have been characterized. The location of the interacting STAT3 domain is color-coded. In the cases where interacting domain is not localized precisely or interaction involves two domains, gradient coloring is used. ND, N-terminal doman; CC, coiled-coil domain, DBD, DNA-binding domain, LD, linker domain, SH2, SH2-domain, TAD, transactivation domain.

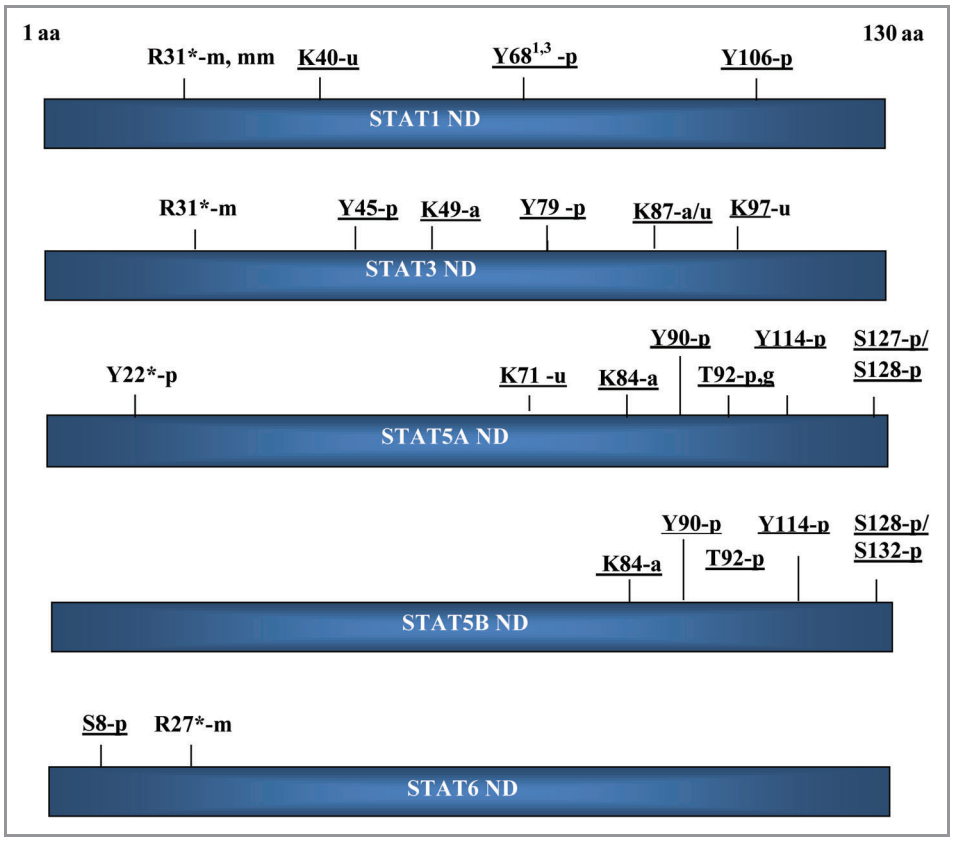

Figure 2. Post-translational modifications of the NDs as detected by mass-spectrometry. The conservative residues are labeled with *. Unique restudies are underlined. No post-translation modifications were detected in the NDs of STAT4 and STAT2.

STAT3 transcriptional activity.[73-75] These findings indicated that acetylation-deacetylation of STAT3 provides another signaling axis to control the IL-6-STAT3 pathway in addition to phosphorylation-dephosphorylation. The follow-up study from the same group demonstrated that only acetylated STAT3 forms an inducible complex with the apurinic/apyrimidinic endonuclease 1 (APE1)/redox effector factor-1 (APE1/Ref-1), an essential multifunctional protein in DNA base excision repair in response to IL-6.[74] APE1 selectively binds ND, and this interaction is required for STAT3 stable chromatin association with the promoters of suppressor of cytokine signaling-3 (SOCS3) and γ-fibrionogen.[74]

STAT5 N-terminal domain interacts with the glucocorticoid receptor, which can control gene expression as either a coactivator or corepressor.[59] The STAT5 ND undergo glycosylation on T92 that is crucial for binding to the coactivator of transcription CREB-binding protein and eventually p300 that are established coactivators of gene expression.[76]

In addition, PhosphoSitePlus lists a number of post-translational modification of the STAT NDs that were only detected by mass spectrometry analysis. However, functional aspects of these modifications have not been characterized (**Fig. 2**).[65,77] Remarkably, no modifications were detected in the NDs of STAT2 and STAT4, while STAT1, STAT3 and STAT5A/B NDs undergo phosphorylation, acetylation and ubiquitinylation. Even without understanding the precise role of detected modifications, we may speculate that they regulate STAT's protein-protein interactions that result in changes in STAT functionality.

It has been shown that STAT3 can play opposing roles in cellular transformation depending on the genetic background of the tumor. One example includes induction of a highly aggressive T cell leukemia in mice by activated STAT3 but prevention of c-myc-induced transformation of mouse embryonic fibroblasts deficient for p53.[78] Another example is tumor-suppressive function of STAT3 in glioblastomas deficient in tumor suppressor PTEN, and oncogenic functions in glioblastomas that express nuclear epidermal growth factor receptor type III variant (EGFRvIII).[79] There is a substantial heterogeneity in genetic backgrounds of tested cell lines, therefore it is not surprising that there is also a heterogeneity in responses to inhibition of STAT3 signaling. It is tempting to speculate that ND protein-protein interactions are responsible, at least partially, for switching of STAT3 function from pro-apoptotic to pro-survival during cell transformation.

It also has been documented that normal cells remain viable without STAT3.[80] We found that inhibition of the STAT3 ND had little effect on normal epithelial cells, while it induced fulminate apoptosis in breast and prostate cancer cells.[46] These data suggested that the STAT3 ND performs different functions in cancer as compared with that observed under normal

physiological conditions. One can speculate that different roles may be defined by involvement of the STAT3 ND in various protein-protein interactions and possibly by differential post-translational modifications of the ND. The identification of differences in signaling events that underlie differential activity of the STAT3 ND in normal and cancer cells may offer a potentially novel therapeutic target for cancer treatment.

Design of ND Inhibitors

Development of selective chemical probes and potential therapeutic agents for STAT domains is challenging because the critical interacting surfaces appear to lack deep hydrophobic involutions that enable potent and selective targeting by small molecules.[81] In addition, STATs localization within the cell positions them beyond the reach of protein therapeutics.[81] A considerable interest has therefore arisen in next-generation targeting molecules that combine the broad target recognition capabilities of protein therapeutics with the robust cell-penetrating ability of small molecules.

We have been using successfully retro-inverso lipopeptides, one of novel classes of synthetic miniproteins with greatly improved pharmacologic performance, increased target affinity, proteolytic resistance and serum half-life while conferring on them high levels of cell penetration.

It should be noted here that chemical biology offers very powerful tools in studying the function of certain parts of proteins that provide for much more solid conclusions than genetic methods, but only if the chemical probes are highly selective. Experiments involving expression of STATs mutants lacking certain domains or containing point mutation in STAT-null cells are unlikely to generate the phenotype reflecting correctly the function of the mutant protein in STAT-dependent cells. Functions of STATs are known to be cell-dependent and STAT-null cells are unlikely to have the correct combination of partner proteins.

We based the original design of STAT ND inhibitors on the structure of STAT4 N-terminal domain.[30] Dimerization of STAT4 ND was well established and our original intention was development of inhibitors of dimerization. Two available dimer structures supported different dimerization surfaces. Crystallography data suggested involvement of helixes 2 and 7,[38] while NMR data detected helixes 2 and 8 in the dimer interface.[46] Both modes involve the second α-helix of the protein. We originally made peptides corresponding to both helixes 2 and 8 and tested them for ability to interact with STAT4 ND by NMR.[46] Peptide corresponding to helix 2 produced well defined changes in chemical shifts of STAT4 ND, while peptide corresponding to helix 8 caused protein to precipitate, most likely due to unfolding. Published characterization of dimerization propensity for different STAT NDs has shown that they differ significantly and that STAT3 ND dimer is significantly less stable than STAT1 and STAT4 dimers[47] thus highlighting the mechanistic differences in the way different members of STAT family function. However, it should be noted that the levels of expression of different STATs also differ by 100-fold, as was demonstrated for leukemia cells.[56] Higher concentration of STAT3 may compensate for low affinity and result in less pronounced structural differences between STATs. The study by Wenta et al.[47] also suggested the existence of two modes of ND dimerization, at least for STAT1. Although the structural features of these two modes are unknown, it is possible that both models obtained from crystal structure and NMR studies of STAT4 are correct and have indeed identified two naturally occurring interaction interfaces. NMR studies of ND inhibitors' interaction with STAT4 ND domain suggest that helix 2 analogs are likely to inhibit significantly more than just ND dimerization. Changes in chemical shifts detected in the HSQC NMR spectrum of STAT4 suggest that domain undergoes significant conformational changes upon binding of the peptide (**Fig. 3**). It is interesting that the residues that are involved are localized mostly on one "face" of the domain, while the other half of it appears to be subjected to much lesser change (**Fig. 3**). However, the changes cover significant fraction of the domain structure and thus many binary interactions of the domain can be affected.

The data generated for STAT4 has been used for the design and development of ND inhibitors of STAT1, STAT3 and STAT5. Structural studies suggest that N-terminal domains of STAT proteins have very similar folds. Consequently, we have used sequence alignment (**Fig. 4**) and tertiary structures of STAT1 and STAT4 NDs to select initial lead analog of STAT3 helix 2 for optimization. During optimization of peptide length and structure, analogs of helix 2 were fused to penetratin sequence to facilitate cell penetration. However, our later findings suggested that simple fusion of the peptides to fatty acids was as effective as attachment of cell-penetrating peptides for intracellular delivery of compounds.

Lipopeptides as Chemical Biology Tools and Drug Candidates

Extensive studies of structural and biological properties of lipopeptide mimetics of the conserved region of several important but non-druggable molecular targets have revealed that membrane anchoring through the attachment of fatty acid chains can produce highly selective and potent inhibitors of the corresponding protein. Membrane anchoring through lipidation contributes to high potency of compounds in three ways: (1) lipidation facilitates cell entry; (2) fatty acid chain causes membrane insertion and concentrates the inhibitor near intracellular and plasma membrane, where practically all signaling events occur; (3) membrane anchoring enables folding of otherwise unfolded protein fragment, which results in an increase in potency, frequently by 2 to 3 orders of magnitude.[82] What is remarkable is that membrane anchoring stabilizes all forms of secondary structures. Although stabilization is due to enhanced interaction of amino acid side chains with the lipid bilayer, it doesn't interfere with peptide's ability to interact. Interaction with the membrane can be described as snorkeling rather than immersion. Thus, it increases the time the peptide spends in the active conformation, rather than freezes it leaving sufficient time for the folded peptide to stick out of the

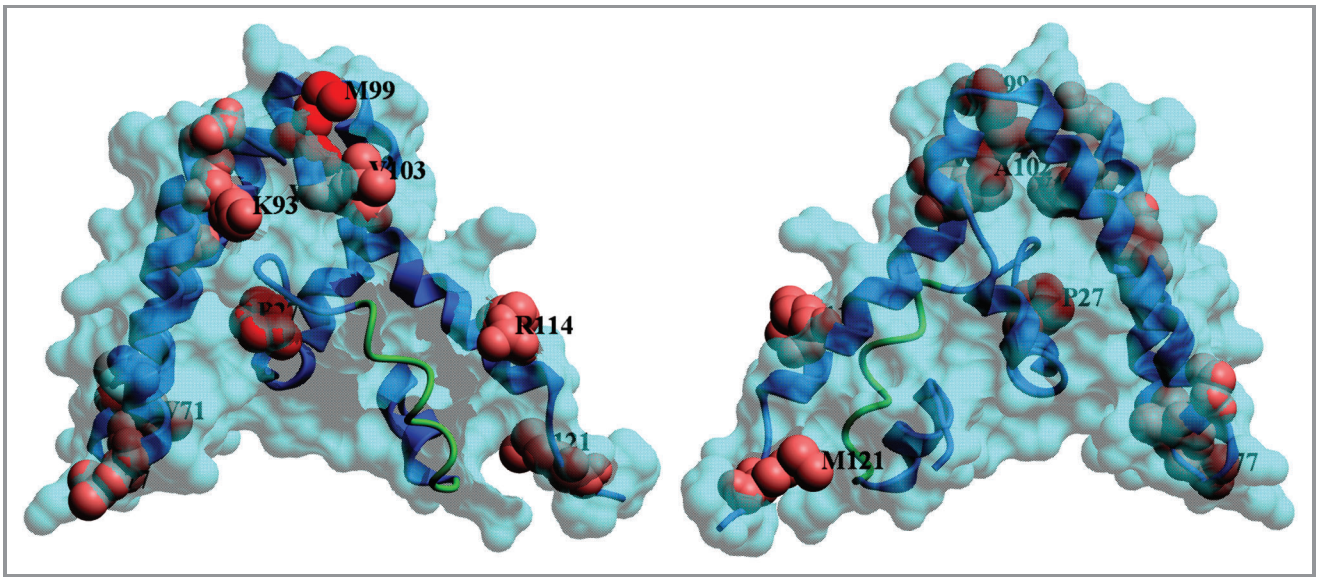

Figure 3. Residues of STAT4 ND (in red) with the most significant chemical shift perturbations detected upon binding of peptide corresponding to helix 2 (Ac-EIKFLEQVDKFY-penetratin). Helix 2 is in green. Two sides of STAT4 ND molecular structure are presented. The figure was generated using MolSoft Browser software and pdb coordinates 1BGF.[30]

membrane. Lipopeptides present a new and young class of therapeutics. Currently, four lipopeptides (daptomycin, liraglude, tesamorelin and caspofungin) are used in the clinic. However, many are being developed,[83,84] and their pharmacological properties make them very convenient chemical biology tools. The remarkable advantage of the approach is that it can be used for rational design of the probes even in the absence of the structural data for the target protein. Selection of the stretches of amino acid sequences for mimicking can be based on the conservation during the evolution. The assumption here is that

Helix 2

```
STAT4_HUMAN MSQWNQVQQLEIKFLEQVDQFYDDN-FPMEIRHLLAQWIENQDWEAAS--NN--ETMATI 55
STAT1_HUMAN MSQWYELQQLDSKFLEQVHQLYDDS-FPMEIRQYLAQWLEKQDWEHAA--ND--VSFATI 55
STAT2_HUMAN MAQWEMLQNLDSPFQDQLHQLYSHSLLPVDIRQYLAVWIEDQNWQEAALGSD--DSKATM 58
STAT3_HUMAN MAQWNQLQQLDTRYLEQLHQLYSDS-FPMELRQFLAPWIESQDWAYAA--SK--ESHATL 55
STA5A_HUMAN MAGWIQAQQLQGDALRQMQVLYGQH-FPIEVRHYLAQWIESQPWDAIDLDNPQDRAQATQ 59
STA5B_HUMAN MAVWIQAQQLQGEALHQMQALYGQH-FPIEVRHYLSQWIESQAWDSVDLDNPQENIKATQ 59
STAT6_HUMAN MSLWGLVSKMPPEKV-QRL--YVD- FPQHLRHLLGDWLESQPWEFLVGSDAFCCNLASA 55
            *: *   .::      *:   *    :* .:*: *. *:*.* *      .      *:

STAT4_HUMAN LLQNLLIQLDEQLGRVSKEK-NLLLIHNLKRIRKVLQGKFHGNPMHVAVVISNCLREERR 114
STAT1_HUMAN RFHDLLSQLDDQYSRFSLEN-NFLLQHNIRKSKRNLQDNFQEDPIQMSMIIYSCLKEERK 114
STAT2_HUMAN LFFHFLDQLNYECGRCSQDPESLLLQHNLRKFCRDIQ-PFSQDPTQLAEMIFNLLLEEKR 117
STAT3_HUMAN VFHNLLGEIDQQYSRFLQES-NVLYQHNLRRIKQFLQSRYLEKPMEIARIVARCLWEESR 114
STA5A_HUMAN LLEGLVQELQKKAEHQVGED-GFLLKIKLGHYATQLQKTYDRCPLELVRCIRHILYNEQR 118
STA5B_HUMAN LLEGLVQELQKKAEHQVGED-GFLLKIKLGHYATQLQNTYDRCPMELVRCIRHILYNEQR 118
STAT6_HUMAN LLSDTVQHLQAS----VGEQ-GEGST--ILQHISTLESIYQRDPLKLVATFRQILQGEKK 108
             :   : .:: .      :         : :    ::  :   * .:   .   *  * :

STAT4_HUMAN ILAAANMP-VQGPLEKSLQSSSVSERQRN--VEHKVAAIKNSVQMTEQDTKYLEDLQDEF 171
STAT1_HUMAN ILENAQRF-NQA-QSGNIQSTVMLDKQKE--LDSKVRNVKDKVMCIEHEIKSLEDLQDEY 170
STAT2_HUMAN ILIQAQRAQLEQ--GEPVLETPVESQQHE--IESRILDLRAMMEKLVKSISQLKDQQDVF 173
STAT3_HUMAN LLQTAATAAQQGGQANHPTAAVVTEKQQM--LEQHLQDVRKRVQDLEQKMKVVENLQDDF 172
STA5A_HUMAN LVREANNCSSPAGIL----VDAMSQKHLQINQTF--EELRLVTQDTENELKKLQQTQEYF 172
STA5B_HUMAN LVREANNGSSPAGSL----ADAMSQKHLQINQTF--EELRLVTQDTENELKKLQQTQEYF 172
STAT6_HUMAN AVMEQFRH-------------------LPMPFHWKQEELKFK-----TGLRRLQHRVGE- 143
                  :                                        ::           ::.
```

Figure 4. Alignment of N-terminal domains of STAT transcription factors. Alpha-helixes identified by structural studies[30] are underlined. Residues of helix 2 identical in different STATs are highlighted.

the highly conserved regions are involved in functionally essential protein-protein interactions and thus compounds mimicking them can function as dominant negative inhibitors of the corresponding interactions. We have used the approach successfully for the development of lipopeptide inhibitors of receptors signaling upstream from STATs and other non-druggable targets.[46,82,85] The conservation-based selection however wasn't applicable to STAT proteins because entire primary structures are conserved. For instance, mouse and human STAT3 differ in only one residue. However, this is a very unusual case that also has an important message in it: entire structure of STAT3 is likely to be important for protein function and there are numerous opportunities in affecting STAT function through the development of probes mimicking different parts of STAT proteins. Luckily, structural information for five out of six STAT domains is available. Although not every member of the family was characterized structurally, available data allows speculating that overall fold is well preserved in entire family and thus STAT1, STAT3 and STAT4 structural data can be used for identification of fragments suitable for development of potential dominant negative inhibitors of all STATs. Once the stretches for mimicking are identified, the design of the probes is straightforward. The major challenge is determination of optimal positions for fatty acids attachment and the optimal length of the mimicking sequence. Here are some ground rules: the preferred lipid position is at the ends of secondary structure elements; attachment of fatty acid to the side chains, such as ε-amino group of Lys is more likely to result in active compounds. However, we did come across several exceptions, when derivatives with fatty acids attached to α-amino group of the N-terminal amino acid were more active.[82,85] For in vivo use, compounds can be converted into retro-inverso analogs composed on all-D amino acids. Retro-inverso derivatives are not structurally identical to parent all-L peptides. They tend to have a more rigid structure that is beneficial in majority but not all cases. Attachment of palmitic acid is very effective in making the peptide cell-permeable.[46,82,85] However, shorter fatty acids are frequently sufficient and provide for better solubility of compounds.

Application of the approach to NDs of STATs allowed us to uncover previously underappreciated role of STAT3 ND in tumor growth and STAT1 ND in kidney development.[86] The data shows that NDs of STATs are promising therapeutic targets and lipopeptide inhibitors have a potential to serve as effective therapeutic agents.

Conclusion

The wealth of currently available data implies that targeting domains other than SH2 can be an effective way of modulating activity of STATs for generation of chemical biology tools and potential therapeutic applications. The data generated for STAT N-domains suggest that rationally designed lipopeptide mimetics of fragments of proteins involved in JAK-STAT signaling can serve as powerful tools in studying the molecular and cellular mechanisms of signaling. It is evident that the function of STAT transcription factors is regulated by complex and thus far poorly understood mechanisms involving numerous protein-protein interactions. The latest data also indicate that members of STAT family are likely to have multiple functions that they perform not only in the nucleus, but other cellular compartments. Targeting protein-protein interactions with rationally designed probes will lead to significant advances in our understanding of molecular mechanisms of JAK-STAT signaling, pathological processes involving the pathway and consequently therapeutic approaches to controlling and redirecting the function of this important family of proteins.

Acknowledgments

Research was sponsored in part by the Intramural Program of NIH, National Cancer Institute, by IRG 97-152-17 from the American Cancer Society, and by Lombardi Comprehensive Cancer Center supported by NIH/NCI grant P30-CA051008.

References

1. Stark GR, Darnell JE, Jr. The JAK-STAT pathway at twenty. Immunity 2012; 36:503-14; PMID:22520844; http://dx.doi.org/10.1016/j.immuni.2012.03.013
2. Wang Y, Levy DE. Comparative evolutionary genomics of the STAT family of transcription factors. JAK-STAT 2012; 1:23; http://dx.doi.org/10.4161/jkst.19418
3. Levy DE, Darnell JE, Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002; 3:651-62; PMID:12209125; http://dx.doi.org/10.1038/nrm909
4. Bromberg J, Chen X. STAT proteins: signal tranducers and activators of transcription. Methods Enzymol 2001; 333:138-51; PMID:11400331; http://dx.doi.org/10.1016/S0076-6879(01)33052-5
5. Levy DE, Inghirami G. STAT3: a multifaceted oncogene. Proc Natl Acad Sci U S A 2006; 103:10151-2; PMID: 16801534; http://dx.doi.org/10.1073/pnas.0604042103
6. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, et al. Stat3 as an oncogene. Cell 1999; 98:295-303; PMID:10458605; http://dx.doi.org/10.1016/S0092-8674(00)81959-5
7. Turkson J, Jove R. STAT proteins: novel molecular targets for cancer drug discovery. Oncogene 2000; 19: 6613-26; PMID:11426647; http://dx.doi.org/10.1038/sj.onc.1204086
8. Yang J, Chatterjee-Kishore M, Staugaitis SM, Nguyen H, Schlessinger K, Levy DE, et al. Novel roles of unphosphorylated STAT3 in oncogenesis and transcriptional regulation. Cancer Res 2005; 65:939-47; PMID:15705894
9. Yang J, Liao X, Agarwal MK, Barnes L, Auron PE, Stark GR. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NFkappaB. Genes Dev 2007; 21:1396-408; PMID: 17510282; http://dx.doi.org/10.1101/gad.1553707
10. Yang J, Stark GR. Roles of unphosphorylated STATs in signaling. Cell Res 2008; 18:443-51; PMID:18364677; http://dx.doi.org/10.1038/cr.2008.41
11. Moriggl R, Sexl V, Kenner L, Duntsch C, Stangl K, Gingras S, et al. Stat5 tetramer formation is associated with leukemogenesis. Cancer Cell 2005; 7:87-99; PMID: 15652752; http://dx.doi.org/10.1016/j.ccr.2004.12.010
12. Kornfeld JW, Grebien F, Kerenyi MA, Friedbichler K, Kovacic B, Zankl B, et al. The different functions of Stat5 and chromatin alteration through Stat5 proteins. Front Biosci 2008; 13:6237-54; PMID:18508657; http://dx.doi.org/10.2741/3151
13. O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. Immunity 2008; 28:477-87; PMID:18400190; http://dx.doi.org/10.1016/j.immuni.2008.03.002
14. Delgoffe GM, Murray PJ, Vignali DA. Interpreting mixed signals: the cell's cytokine conundrum. Curr Opin Immunol 2011; 23:632-8; PMID:21852079; http://dx.doi.org/10.1016/j.coi.2011.07.013
15. Ehret GB, Reichenbach P, Schindler U, Horvath CM, Fritz S, Nabholz M, et al. DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. J Biol Chem 2001; 276:6675-88; PMID: 11053426; http://dx.doi.org/10.1074/jbc.M001748200

16. Muller P, Pugazhendhi D, Zeidler MP. Modulation of human JAK-STAT pathway signaling by functionally conserved regulators. JAK-STAT 2012; 1:34-44; http://dx.doi.org/10.4161/jkst.18006
17. Page BD, Ball DP, Gunning PT. Signal transducer and activator of transcription 3 inhibitors: a patent review. Expert Opin Ther Pat 2011; 21:65-83; PMID:21114420; http://dx.doi.org/10.1517/13543776.2011.539205
18. Page BD, Khoury H, Laister RC, Fletcher S, Vellozo M, Manzoli A, et al. Small molecule STAT5-SH2 domain inhibitors exhibit potent antileukemia activity. J Med Chem 2012; 55:1047-55; PMID:22148584; http://dx.doi.org/10.1021/jm200720n
19. Wang X, Crowe PJ, Goldstein D, Yang JL. STAT3 inhibition, a novel approach to enhancing targeted therapy in human cancers (Review). Int J Oncol 2012; 41:1181-91; PMID:22842992
20. Yue P, Turkson J. Targeting STAT3 in cancer: how successful are we? Expert Opin Investig Drugs 2009; 18:45-56; PMID:19053881; http://dx.doi.org/10.1517/13543780802565791
21. Jing N, Tweardy DJ. Targeting Stat3 in cancer therapy. Anticancer Drugs 2005; 16:601-7; PMID:15930886; http://dx.doi.org/10.1097/00001813-200507000-00002
22. Mandal PK, Gao F, Lu Z, Ren Z, Ramesh R, Birtwistle JS, et al. Potent and selective phosphopeptide mimetic prodrugs targeted to the Src homology 2 (SH2) domain of signal transducer and activator of transcription 3. J Med Chem 2011; 54:3549-63; PMID:21486047; http://dx.doi.org/10.1021/jm2000882
23. Nelson EA, Sharma SV, Settleman J, Frank DA. A chemical biology approach to developing STAT inhibitors: molecular strategies for accelerating clinical translation. Oncotarget 2011; 2:518-24; PMID:21680956
24. Kawata T, Shevchenko A, Fukuzawa M, Jermyn KA, Totty NF, Zhukovskaya NV, et al. SH2 signaling in a lower eukaryote: a STAT protein that regulates stalk cell differentiation in dictyostelium. Cell 1997; 89:909-16; PMID:9200609; http://dx.doi.org/10.1016/S0092-8674(00)80276-7
25. Wang Y, Levy DE. C. elegans STAT: evolution of a regulatory switch. FASEB J 2006; 20:1641-52; PMID:16873887; http://dx.doi.org/10.1096/fj.06-6051com
26. Adler K, Gerisch G, von Hugo U, Lupas A, Schweiger A. Classification of tyrosine kinases from Dictyostelium discoideum with two distinct, complete or incomplete catalytic domains. FEBS Lett 1996; 395:286-92; PMID:8898113; http://dx.doi.org/10.1016/0014-5793(96)01053-8
27. Henriksen MA, Betz A, Fuccillo MV, Darnell JE, Jr. Negative regulation of STAT92E by an N-terminally truncated STAT protein derived from an alternative promoter site. Genes Dev 2002; 16:2379-89; PMID:12231627; http://dx.doi.org/10.1101/gad.1020702
28. Xu X, Sun YL, Hoey T. Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. Science 1996; 273:794-7; PMID:8670419; http://dx.doi.org/10.1126/science.273.5276.794
29. Primiano T, Baig M, Maliyekkel A, Chang BD, Fellars S, Sadhu J, et al. Identification of potential anticancer drug targets through the selection of growth-inhibitory genetic suppressor elements. Cancer Cell 2003; 4:41-53; PMID:12892712; http://dx.doi.org/10.1016/S1535-6108(03)00169-7
30. Vinkemeier U, Moarefi I, Darnell JE, Jr., Kuriyan J. Structure of the amino-terminal protein interaction domain of STAT-4. Science 1998; 279:1048-52; PMID:9461439; http://dx.doi.org/10.1126/science.279.5353.1048
31. Chen X, Vinkemeier U, Zhao Y, Jeruzalmi D, Darnell JE, Jr., Kuriyan J. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. Cell 1998; 93:827-39; PMID:9630226; http://dx.doi.org/10.1016/S0092-8674(00)81443-9
32. Zhang X, Darnell JE, Jr. Functional importance of Stat3 tetramerization in activation of the alpha 2-macroglobulin gene. J Biol Chem 2001; 276:33576-81; PMID:11438543; http://dx.doi.org/10.1074/jbc.M104978200
33. Vinkemeier U, Cohen SL, Moarefi I, Chait BT, Kuriyan J, Darnell JE, Jr. DNA binding of in vitro activated Stat1 alpha, Stat1 beta and truncated Stat1: interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites. EMBO J 1996; 15:5616-26; PMID:8896455
34. Murphy TL, Geissal ED, Farrar JD, Murphy KM. Role of the Stat4 N domain in receptor proximal tyrosine phosphorylation. Mol Cell Biol 2000; 20:7121-31; PMID:10982828; http://dx.doi.org/10.1128/MCB.20.19.7121-7131.2000
35. John S, Vinkemeier U, Soldaini E, Darnell JE, Jr., Leonard WJ. The significance of tetramerization in promoter recruitment by Stat5. Mol Cell Biol 1999; 19:1910-8; PMID:10022878
36. Ota N, Brett TJ, Murphy TL, Fremont DH, Murphy KM. N-domain-dependent nonphosphorylated STAT4 dimers required for cytokine-driven activation. Nat Immunol 2004; 5:208-15; PMID:14704793; http://dx.doi.org/10.1038/ni1032
37. Shuai K, Liao J, Song MM. Enhancement of antiproliferative activity of gamma interferon by the specific inhibition of tyrosine dephosphorylation of Stat1. Mol Cell Biol 1996; 16:4932-41; PMID:8756652
38. Chen X, Bhandari R, Vinkemeier U, Van Den Akker F, Darnell JE, Jr., Kuriyan J. A reinterpretation of the dimerization interface of the N-terminal domains of STATs. Protein Sci 2003; 12:361-5; PMID:12538899; http://dx.doi.org/10.1110/ps.0218903
39. Vinkemeier U, Meyer T. Antiviral activity of oligomerization-deficient Stat1. Genome Inform 2005; 16:44-8; PMID:16362905
40. Chang HC, Zhang S, Oldham I, Naeger L, Hoey T, Kaplan MH. STAT4 requires the N-terminal domain for efficient phosphorylation. J Biol Chem 2003; 278:32471-7; PMID:12805384; http://dx.doi.org/10.1074/jbc.M302776200
41. Zhang L, Badgwell DB, Bevers JJ, 3rd, Schlessinger K, Murray PJ, Levy DE, et al. IL-6 signaling via the STAT3/SOCS3 pathway: functional analysis of the conserved STAT3 N-domain. Mol Cell Biochem 2006; 288:179-89; PMID:16718380; http://dx.doi.org/10.1007/s11010-006-9137-3
42. Lin JX, Li P, Liu D, Jin HT, He J, Ata Ur Rasheed M, et al. Critical Role of STAT5 transcription factor tetramerization for cytokine responses and normal immune function. Immunity 2012; 36:586-99; PMID:22520852; http://dx.doi.org/10.1016/j.immuni.2012.02.017
43. Li G, Wang Z, Zhang Y, Kang Z, Haviernikova E, Cui Y, et al. STAT5 requires the N-domain to maintain hematopoietic stem cell repopulating function and appropriate lymphoid-myeloid lineage output. Exp Hematol 2007; 35:1684-94; PMID:17976521; http://dx.doi.org/10.1016/j.exphem.2007.08.026
44. Soldaini E, John S, Moro S, Bollenbacher J, Schindler U, Leonard WJ. DNA binding site selection of dimeric and tetrameric Stat5 proteins reveals a large repertoire of divergent tetrameric Stat5a binding sites. Mol Cell Biol 2000; 20:389-401; PMID:10594041; http://dx.doi.org/10.1128/MCB.20.1.389-401.2000
45. Li G, Miskimen KL, Wang Z, Xie XY, Brenzovich J, Ryan JJ, et al. STAT5 requires the N-domain for suppression of miR15/16, induction of bcl-2, and survival signaling in myeloproliferative disease. Blood 2010; 115:1416-24; PMID:20008792; http://dx.doi.org/10.1182/blood-2009-07-234963
46. Timofeeva OA, Gaponenko V, Lockett SJ, Tarasov SG, Jiang S, Michejda CJ, et al. Rationally designed inhibitors identify STAT3 N-domain as a promising anticancer drug target. ACS Chem Biol 2007; 2:799-809; PMID:18154267; http://dx.doi.org/10.1021/cb700186x
47. Wenta N, Strauss H, Meyer S, Vinkemeier U. Tyrosine phosphorylation regulates the partitioning of STAT1 between different dimer conformations. Proc Natl Acad Sci U S A 2008; 105:9238-43; PMID:18591661; http://dx.doi.org/10.1073/pnas.0802130105
48. Mao X, Ren Z, Parker GN, Sondermann H, Pastorello MA, Wang W, et al. Structural bases of unphosphorylated STAT1 association and receptor binding. Mol Cell 2005; 17:761-71; PMID:15780933; http://dx.doi.org/10.1016/j.molcel.2005.02.021
49. Zhong M, Henriksen MA, Takeuchi K, Schaefer O, Liu B, ten Hoeve J, et al. Implications of an antiparallel dimeric structure of nonphosphorylated STAT1 for the activation-inactivation cycle. Proc Natl Acad Sci U S A 2005; 102:3966-71; PMID:15753310; http://dx.doi.org/10.1073/pnas.0501063102
50. Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, et al. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell 1996; 84:431-42; PMID:8608597; http://dx.doi.org/10.1016/S0092-8674(00)81288-X
51. Meyer T, Hendry L, Begitt A, John S, Vinkemeier U. A single residue modulates tyrosine dephosphorylation, oligomerization, and nuclear accumulation of stat transcription factors. J Biol Chem 2004; 279:18998-9007; PMID:15010467; http://dx.doi.org/10.1074/jbc.M400766200
52. Strehlow I, Schindler C. Amino-terminal signal transducer and activator of transcription (STAT) domains regulate nuclear translocation and STAT deactivation. J Biol Chem 1998; 273:28049-56; PMID:9774421; http://dx.doi.org/10.1074/jbc.273.43.28049
53. Vogt M, Domoszlai T, Kleshchanok D, Lehmann S, Schmitt A, Poli V, et al. The role of the N-terminal domain in dimerization and nucleocytoplasmic shuttling of latent STAT3. J Cell Sci 2011; 124:900-9; PMID:21325026; http://dx.doi.org/10.1242/jcs.072520
54. Kretzschmar AK, Dinger MC, Henze C, Brocke-Heidrich K, Horn F. Analysis of Stat3 (signal transducer and activator of transcription 3) dimerization by fluorescence resonance energy transfer in living cells. Biochem J 2004; 377:289-97; PMID:12974672; http://dx.doi.org/10.1042/BJ20030708
55. Becker S, Groner B, Müller CW. Three-dimensional structure of the Stat3beta homodimer bound to DNA. Nature 1998; 394:145-51; PMID:9671298; http://dx.doi.org/10.1038/28101
56. Phillips TM, Smith P. Analysis of intracellular regulatory proteins by immunoaffinity capillary electrophoresis coupled with laser-induced fluorescence detection. Biomed Chromatogr 2003; 17:182-7; PMID:12717808; http://dx.doi.org/10.1002/bmc.240
57. Cimica V, Chen HC, Iyer JK, Reich NC. Dynamics of the STAT3 transcription factor: nuclear import dependent on Ran and importin-β1. PLoS One 2011; 6:e20188; PMID:21625522; http://dx.doi.org/10.1371/journal.pone.0020188
58. Meissner T, Krause E, Lödige I, Vinkemeier U. Arginine methylation of STAT1: a reassessment. Cell 2004; 119:587-9, discussion 589-90; PMID:15550240

59. Engblom D, Kornfeld JW, Schwake L, Tronche F, Reimann A, Beug H, et al. Direct glucocorticoid receptor-Stat5 interaction in hepatocytes controls body size and maturation-related gene expression. Genes Dev 2007; 21:1157-62; PMID:17504935; http://dx.doi.org/10.1101/gad.426007
60. Tronche F, Opherk C, Moriggl R, Kellendonk C, Reimann A, Schwake L, et al. Glucocorticoid receptor function in hepatocytes is essential to promote postnatal body growth. Genes Dev 2004; 18:492-7; PMID:15037546; http://dx.doi.org/10.1101/gad.284704
61. Dagvadorj A, Tan SH, Liao Z, Xie J, Nurmi M, Alanen K, et al. N-terminal truncation of Stat5a/b circumvents PIAS3-mediated transcriptional inhibition of Stat5 in prostate cancer cells. Int J Biochem Cell Biol 2010; 42:2037-46; PMID:20854925; http://dx.doi.org/10.1016/j.biocel.2010.09.008
62. Gross M, Liu B, Tan J, French FS, Carey M, Shuai K. Distinct effects of PIAS proteins on androgen-mediated gene activation in prostate cancer cells. Oncogene 2001; 20:3880-7; PMID:11439351; http://dx.doi.org/10.1038/sj.onc.1204489
63. Chatterjee-Kishore M, Wright KL, Ting JP, Stark GR. How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. EMBO J 2000; 19:4111-22; PMID:10921891; http://dx.doi.org/10.1093/emboj/19.15.4111
64. Timofeeva OA, Chasovskikh S, Lonskaya I, Tarasova NI, Khavrutskii L, Tarasov SG, et al. Mechanisms of unphosphorylated STAT3 transcription factor binding to DNA. J Biol Chem 2012; 287:14192-200; PMID:22378781; http://dx.doi.org/10.1074/jbc.M111.323899
65. Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, et al. PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. Nucleic Acids Res 2012; 40(Database issue):D261-70; PMID:22135298; http://dx.doi.org/10.1093/nar/gkr1122
66. Mowen KA, Tang J, Zhu W, Schurter BT, Shuai K, Herschman HR, et al. Arginine methylation of STAT1 modulates IFNalpha/beta-induced transcription. Cell 2001; 104:731-41; PMID:11257227; http://dx.doi.org/10.1016/S0092-8674(01)00269-0
67. Chen W, Daines MO, Hershey GKK. Methylation of STAT6 modulates STAT6 phosphorylation, nuclear translocation, and DNA-binding activity. J Immunol 2004; 172:6744-50; PMID:15153491
68. Rho J, Choi S, Seong YR, Choi J, Im D-S. The arginine-1493 residue in QRRGRTGR1493G motif IV of the hepatitis C virus NS3 helicase domain is essential for NS3 protein methylation by the protein arginine methyltransferase 1. J Virol 2001; 75:8031-44; PMID:11483748; http://dx.doi.org/10.1128/JVI.75.17.8031-8044.2001
69. Iwasaki H, Kovacic JC, Olive M, Beers JK, Yoshimoto T, Crook MF, et al. Disruption of protein arginine N-methyltransferase 2 regulates leptin signaling and produces leanness in vivo through loss of STAT3 methylation. Circ Res 2010; 107:992-1001; PMID:20798359; http://dx.doi.org/10.1161/CIRCRESAHA.110.225326
70. Komyod W, Bauer UM, Heinrich PC, Haan S, Behrmann I. Are STATS arginine-methylated? J Biol Chem 2005; 280:21700-5; PMID:15826948; http://dx.doi.org/10.1074/jbc.C400606200
71. Shuai K, Liu B. Regulation of gene-activation pathways by PIAS proteins in the immune system. Nat Rev Immunol 2005; 5:593-605; PMID:16056253; http://dx.doi.org/10.1038/nri1667
72. Shuai K. Regulation of cytokine signaling pathways by PIAS proteins. Cell Res 2006; 16:196-202; PMID:16474434; http://dx.doi.org/10.1038/sj.cr.7310027
73. Ray S, Lee C, Hou T, Boldogh I, Brasier AR. Requirement of histone deacetylase1 (HDAC1) in signal transducer and activator of transcription 3 (STAT3) nucleocytoplasmic distribution. Nucleic Acids Res 2008; 36:4510-20; PMID:18611949; http://dx.doi.org/10.1093/nar/gkn419
74. Ray S, Lee C, Hou T, Bhakat KK, Brasier AR. Regulation of signal transducer and activator of transcription 3 enhanceosome formation by apurinic/apyrimidinic endonuclease 1 in hepatic acute phase response. Mol Endocrinol 2010; 24:391-401; PMID:20032196; http://dx.doi.org/10.1210/me.2009-0319
75. Ray S, Boldogh I, Brasier AR. STAT3 NH2-terminal acetylation is activated by the hepatic acute-phase response and required for IL-6 induction of angiotensinogen. Gastroenterology 2005; 129:1616-32; PMID:16285960; http://dx.doi.org/10.1053/j.gastro.2005.07.055
76. Gewinner C, Hart G, Zachara N, Cole R, Beisenherz-Huss C, Groner B. The coactivator of transcription CREB-binding protein interacts preferentially with the glycosylated form of Stat5. J Biol Chem 2004; 279:3563-72; PMID:14597631; http://dx.doi.org/10.1074/jbc.M306449200
77. Zhao S, Xu W, Jiang W, Yu W, Lin Y, Zhang T, et al. Regulation of cellular metabolism by protein lysine acetylation. Science 2010; 327:1000-4; PMID:20167786; http://dx.doi.org/10.1126/science.1179689
78. Ecker A, Simma O, Hoelbl A, Kenner L, Beug H, Moriggl R, et al. The dark and the bright side of Stat3: proto-oncogene and tumor-suppressor. Front Biosci 2009; 14:2944-58; PMID:19273247; http://dx.doi.org/10.2741/3425
79. de la Iglesia N, Konopka G, Puram SV, Chan JA, Bachoo RM, You MJ, et al. Identification of a PTEN-regulated STAT3 brain tumor suppressor pathway. Genes Dev 2008; 22:449-62; PMID:18258752; http://dx.doi.org/10.1101/gad.1606508
80. Schlessinger K, Levy DE. Malignant transformation but not normal cell growth depends on signal transducer and activator of transcription 3. Cancer Res 2005; 65:5828-34; PMID:15994959; http://dx.doi.org/10.1158/0008-5472.CAN-05-0317
81. Verdine GL, Hilinski GJ. Stapled peptides for intracellular drug targets. Methods Enzymol 2012; 503:3-33; PMID:22230563; http://dx.doi.org/10.1016/B978-0-12-396962-0.00001-X
82. Johannessen L, Remsberg J, Gaponenko V, Adams KM, Barchi JJ, Jr., Tarasov SG, et al. Peptide structure stabilization by membrane anchoring and its general applicability to the development of potent cell-permeable inhibitors. Chembiochem 2011; 12:914-21; PMID:21365731; http://dx.doi.org/10.1002/cbic.201000563
83. Zhang L, Bulaj G. Converting peptides into drug leads by lipidation. Curr Med Chem 2012; 19:1602-18; PMID:22376031; http://dx.doi.org/10.2174/092986712799945003
84. Tressel SL, Koukos G, Tchernychev B, Jacques SL, Covic L, Kuliopulos A. Pharmacology, biodistribution, and efficacy of GPCR-based pepducins in disease models. Methods Mol Biol 2011; 683:259-75; PMID:21053136; http://dx.doi.org/10.1007/978-1-60761-919-2_19
85. Remsberg JR, Lou H, Tarasov SG, Dean M, Tarasova NI. Structural analogues of smoothened intracellular loops as potent inhibitors of Hedgehog pathway and cancer cell growth. J Med Chem 2007; 50:4534-8; PMID:17685505; http://dx.doi.org/10.1021/jm0705657
86. Wang H, Yang Y, Sharma N, Tarasova NI, Timofeeva OA, Winkler-Pickett RT, et al. STAT1 activation regulates proliferation and differentiation of renal progenitors. Cell Signal 2010; 22:1717-26; PMID:20624457; http://dx.doi.org/10.1016/j.cellsig.2010.06.012