Review

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Steering of carcinoma progression by the YIN/YANG interaction of STAT1/STAT3

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Summary **STAT1/STAT3 transcription factors are important regulators for development of normal, infected or inflammed cells. They are also critically involved in the progression of various malignant tumours, including epithelial-derived carcinomas. Here, we focus on colorectal cancer (CRC) insights for STAT1/3, where controversial functions for STAT3 were reported. For a long time STAT3 has been regarded as a driver of tumour malignancy and its activation was associated with negative clinical outcome. In contrast, STAT1 was generally viewed as an independent tumour suppressor and positive prognostic marker. Here we discuss the experimental evidence for the tight association and regulation of oncogenic STAT3 transcription kept at bay by nuclear STAT1. We summarise current research and describe cellular models of different STAT1/STAT3 expression ratios. STAT1/3 expression levels are influenced by the mutational status of carcinoma cells associated with nuclear unphosphorylated STAT1. Animal tumour models and results from *in vitro* experiments allow for the conclusion that both proteins interact as antagonistic transcription factors in CRC cells. These STATs steer also important processes during infection and inflammation that influence development and progression of CRC. The STAT1/3 interplay is important to understand gene regulation and we describe it here similar like the YIN/YANG dualism. Thus, we propose to evaluate both STAT1 and STAT3 expression patterns in cancers in a dual manner instead of regarding them as independent transcription factors. This conceptual dualistic view could advance diagnostic predictions in the future.**

Keywords: Colorectal carcinoma, JAK-STAT pathway, transcription factors, STAT1/STAT3 interplay, prognostic marker

1. Introduction

The JAK-STAT pathway was recognized as one of the twelve core cancer pathways based on 'omics' technologies and from insights into cancer genome sequencing (*1*). JAK-STAT signalling contributes to many hallmarks of cancer, where survival, growth promotion, angiogenesis or activation of key other signalling pathways like PI3K-AKT-mTOR or RAS-RAF signalling are very well documented (*2-4*).

Cytokines bind to their corresponding receptor chains upon which significant structural changes are induced that also lead to a close proximity interaction of their transmembrane domains. The key event upon cytokine activation is separation of the intracellular Box1 sequences in the cytoplasmic tails of cytokine

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receptors that bind the JAK tyrosine kinases that are bound *via* their FERM domains. This separation results in a sliding motion and removal of the pseudokinase inhibitory domain of one JAK molecule to the other JAK molecule (*5*). Both JAK kinases are pre-assembled at the receptor level in antiparallel dimer fashion. Understanding the structural requirements and mode of JAK kinase activation is important in cancer since they are most frequently mutated among all tyrosine kinases in mammals in cancer. The majority of cancer types are associated with JAK hyperactivation, which transactivate each other and trans-phosphorylate the cytoplasmic tails of receptor chains c*a*using subsequently the activation of other important key signalling molecules (*5*). STAT molecules and other important signalling pathways such as activation of the RAS-RAF-MAPK axis or the PI3K-AKT-mTOR signalling route are downstream of JAK kinase action. Activation of the pathway acts in a cell type-specific and mutational, context-dependent way. It can involve controversial outcomes, such as tumour suppressive as well as oncogenic properties. Gene dosage and expression levels are also relevant, since amplification of the *stat3/5* locus on human chromosome #17q was associated with epithelial or mesenchymal tumour progression [*a PubMed search for #17q amplification revealing 412 publications as of Dec. 2016*]. Next Generation Sequencing data together with histopathology studies revealed that JAK/STAT signalling is in most cancers hyperactivated and it is among the most intensely mutated signal transduction pathways in cancer tightly linked with RAS transformation or MYC upregulation in cytokine responses *e.g*. It is therefore not surprising the pathway belongs to the prime targets of pharmaceutical research and developments. Today, more than ten JAK kinase inhibitors are evaluated in clinical trials. Ruxolitinib potently targets JAK1 and JAK2, while Tofacitinib inhibits JAK1, JAK2 and JAK3 (*2*) [currently, one finds multiple phase 1-III clinical trials *via clinicaltrials.gov* that lists 51 active trials which used Ruxolitinib as of Dec. 2016].

Deficiencies or aberrations in activity caused by JAK-STAT mutations have severe consequences, leading *e.g*. to developmental and immune defects, dysregulated inflammation, metabolic disease and malignant cell transformation (*6-12*). The JAK-STAT pathway is highly conserved and diseases caused by germ-line or somatic mutations in JAKs or STATs are almost equivalent when studied in transgenic mice or found in patients and explored molecularly in human/patient-derived cell systems (*13-15*).

2. STAT1/3 signalling in tumour biology

STAT1/3 are pleiotropic transcription factors that make complex contributions to cancer (Figure 1). Knockout studies on STAT1 unravelled its central role in innate and acquired immunity upon infection, but also in the control of anti-tumourigenic functions, *e.g*. due to tumour surveillance control mechanisms. Nowadays, STAT1 is predominantly viewed as a tumour suppressor, with the exception of MHC class I regulation, which controls NK cell-mediated killing of tumour cells (*16*). STAT1 was shown to have a function in the control of pro-apoptotic and anti-proliferative signalling pathways (*17*). STAT1 was also described as a favourable prognostic marker in carcinomas of the breast or colon (*18,19*). The gene encoding for the SOCS1 Ubiquitine E3 Ligase is, for example a direct target gene of STAT1, and Socs1-deficient mice show spontaneous CRC tumour development, associated with hyper-activation of STAT1 (*20*) due to higher cytokine receptor and JAK kinase activity. Hypermethylation of SOCS family members or their genetic loss was frequently reported in cancer (*21,22*).

STAT3 is a key immunomodulatory and anti-infectious transcription factor downstream of interferon signalling and its effects are often opposite to those of STAT1(*23*). STAT3 is also a master transcription factor controlling differentiation under physiological conditions, *e.g*. it blocks differentiation in association with leukaemia inhibitory factor (LIF) signalling in embryonic stem cells and, hence, a key process for mammalian development (*24*). Moreover, STAT3 has an essential steering function for Th17 cell generation that control autoimmunity and infection through the IL-23-IL-17 cytokine signalling axis, particular important for chronic inflammatory processes of the GI tract (*25*). STAT3 action in cancer is generally viewed in a simplistic manner, and most studies use pYSTAT3 or nuclear STAT3 predominantly as a surrogate marker of its oncogene activity. Patients with gain-of-function (GOF) mutations in STAT3 shape the cancer gene expression landscape of inflammatory liver adenomas or of mature T cell neoplasia (*26,27*). Importantly, STAT3 regulates also stem cell to tissue fate and invasive processes through epithelial to mesenchymal transitions or mesenchymal to epithelial transitions during embryo or cancer progression (*28*). Thus, STAT3 can control key differentiation processes under physiologic or malignant conditions. These functions of STAT3 are consistent with early embryonic death when fully depleted in mice (*29*). In conclusion, STAT1 is a tumour suppressor in CRC, but STAT3 function is promiscuous. It can promote growth, survival, migration or attachment of cancer cells and it may play an important role in cancer immune escape or tumour neo-vascularisation (*4*).

3. Influence of STAT1/3 on CRC

3.1. *STAT1/3 as YIN/YANG antagonists*

The activities of STAT1/3 in cancer are reminiscent of the YIN/YANG dualism, describing two opposite forces in nature. Here, YIN characterises negative

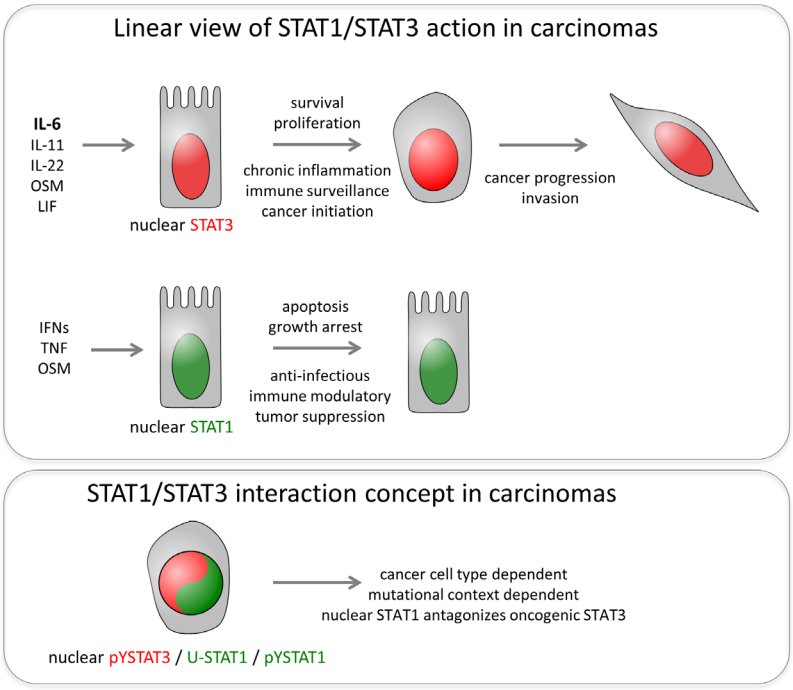

Figure 1. Roles of STAT1/3 expression and activity status in colorectal carcinoma. The pleiotropic transcription factors STAT1 and STAT3 often make antagonistic contributions to cancer initiation or progression associated with chronic infection and inflammation. STAT3 is involved in proliferation, survival and invasion of colorectal carcinoma (CRC) cells and cytokines such as interleukin 6 (IL-6), IL-11, IL-22, oncostatin M (OSM) or leukemia inhibitory factor (LIF) among others were shown to induce potently pYSTAT3 activity levels. STAT1 activation is mostly triggered by interferon (IFN) signalling, however, nuclear STAT1 is found in the unphosphorylated (= non-tyrosine phosphorylated state, U-STAT1) which is *e.g.* also triggered by inflammatory stimuli such as tumour necrosis factor α (TNF) signalling. Surprisingly, nuclear U-STAT1 is constitutively found in CRC cells, however, at varying expression levels antagonising STAT3 signalling.

action of processes, whereas YANG stands for positive action. YIN/YANG, though of contrasting character, are not thought of as static or separated entities. They rather interfere with each other, as exemplified by the mutual interplay of the female and the male principle. Thus, the nature of YIN/YANG lies in the interchange between and the unification of two complementary beings. Recent investigations suggest that STAT1/3 are operative in CRC in a combined analogous manner and they should be viewed and studied as fraternal twins to understand their combined mode of action for CRC biology. Thus, we view STAT1/3 transcription factors as YIN/YANG molecules in the malignant disease process of CRC, reminiscent of ideas laid down in the earliest known Chinese medical book called Huangdi Neijing (Yellow Emperor's Classic of Medicine) two millenniums ago. Here, we will discuss the implications of the STAT1/STAT3 interplay for cancer progression.

3.2. *Predictive potential of STAT1/3 in CRC*

As for other cancers, both STAT1/3 have been evaluated for their suitability as prognostic markers in CRC. In accordance with its generally assumed tumour suppressive functions, STAT1 was shown to be associated with favourable prognosis (*18*). However, it was also claimed that increased tumour expression of STAT1 was associated with impaired patient survival (*30*).

Contradictory reports were published with regard to the predictive validity of STAT3 expression and activation levels in CRC. Some investigations supported the notion of STAT3 as an oncogene, while others concluded that STAT3 is a tumour suppressor. Several studies reported oncogenic effects of elevated STAT3 signalling (*30-36*). In contrast, others reported on STAT3 as a determinant of positive outcome in intestinal tumour progression (*37,38*). Moreover, we could show recently that absence of STAT1 and/or STAT3 proteins is correlated with shorter survival of CRC patients (*37,39*). We performed a systematic study to shed light on the functional interplay and the tumour growth-associated roles of STAT1/3 in CRC progression. Importantly, we could demonstrate that oncogenic function of STAT3 in CRC depends to a large extent on the ratio of STAT1/STAT3 expression and the isolated view on STAT3 activity alone is not sufficient (*39*). A low STAT1/high STAT3 ratio proved to be a valid predictor of poor disease outcome, whereas high STAT1 levels in combination with low STAT3 levels were a favourable determinant (Figure 2). From these results we conclude that STAT3 activation or nuclear function as a transcription factor can be antagonised by STAT1. Hence, we postulate that STAT3 function should be viewed in context with STAT1 expression levels in CRC (*39*).

These results can explain earlier conflicting views on

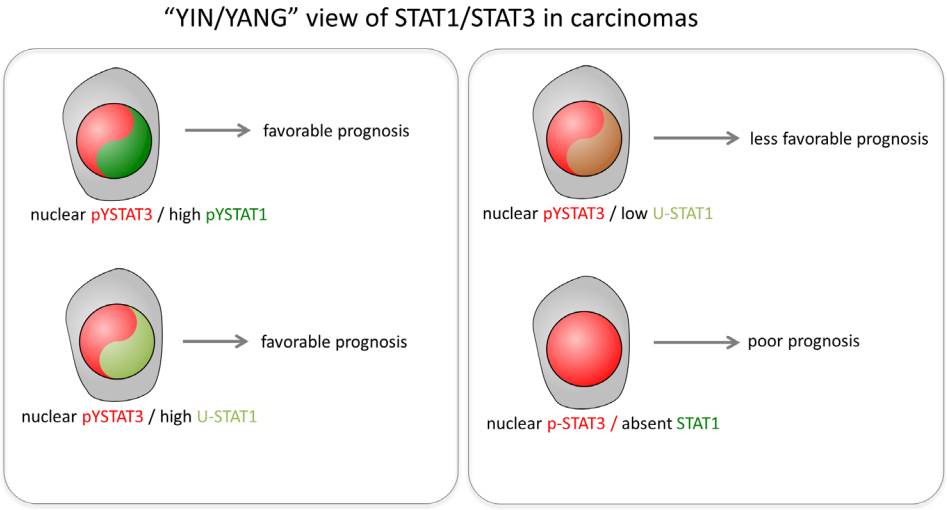

Figure 2. The YIN/YANG interplay of STAT1/3 irrespective of tyrosine phosphorylation gains predictive power on colorectal cancer progression. STAT1/3 proteins interact both without and with DNA. Furthermore, STAT1 and STAT3 physically interact irrespectively of their tyrosine phosphorylation status. Mechanistically, assessment of STAT1/3 cellular distribution and activation suggested that U-STAT1 is mainly nuclear and antagonises STAT3 activity in a YIN/YANG mode. Thus, sole STAT3 activity in patient samples is not a clear predictor of disease progression and one should always control for STAT1 expression since that largely counteracts oncogenic STAT3 and combined analysis of STAT1 and STAT3 nuclear and cytoplasmic expression levels gains prognostic power in the analysis of patient-derived carcinomas.

the predictive tendency of elevated STAT3 expression/activity in CRC that is antagonised by nuclear STAT1 expression levels even without the prerequisite of STAT1 tyrosine phosphorylation (Figure 2). We further could show that different results from various studies on the impact of STAT3 on CRC must be further viewed in context with distinct driver mutations since STAT1 expression upon genetic knockdown or loss of STAT3 can either go up or down. Thus, genetically distinct patient cohorts will influence together with an individual gut microbiota STAT3 signalling. Furthermore, STAT3 signalling is influenced in different cell types through chronic inflammation/infection or by complex tumour-stromal cell interactions (*35*).

Our recent study based on CRC tissue microarrays, cellular and tumour models correlated the ratio of STAT1 to STAT3 expression as well as the IL-6 receptor α chain expression status with disease outcome. While concomitant absence of STAT1/3 was clearly associated with longer patient survival, higher ratio of STAT1/3 expression influenced significantly CRC prognosis. Using four well characterized CRC cell lines with experimentally ablated STAT3 expression and SCID mouse xenografting confirmed that it is the ratio of STAT1/STAT3 expression which gains predictive power for disease progression. Thus, these findings support the view of STAT1/3 as a pair of YIN/YANG counteraction.

4. Mechanisms of STAT1/3 interaction and consequence to cancer biology

The importance of the STAT1/3 protein ratio could be recapitulated in xenograft models using paired cell lines engineered for knockdown of STAT3 expression. Surprisingly and depended on the employed CRC cell line, we found either a repressive or inductive effect on endogenous STAT1 expression upon STAT3 knockdown (*39*). Low STAT1/high STAT3 ratio showed faster tumour growth in xenografts (Figure 2). In contrast, xenografts of cell lines showing high STAT1 and low STAT3 levels grew significantly slower. The molecular basis of the combined activities of STAT1/3 in CRC is a multifaceted issue. The YIN/YANG interplay of STAT1/3 manifests in several ways: *i*) STAT1/3 proteins interact both without and with DNA engagement and their tyrosine phosphorylation status is despite common pathway drawings dispensable for that. STATs can form antiparallel dimers as also revealed through crystal structure insights of unphosphorylated STAT molecules (*40*) and both dock to cytokine receptor cytoplasmic chains *via* their N-terminal sequences. *ii*) STAT1/3 transcription factors have largely the same DNA binding consensus sites. Thus, they bind to similar DNA sequences at regulatory gene transcription regions, but they have very distinct target gene spectra due to cell type specific cofactors, corepressors or transcription factor interaction. *iii*) STAT1/3 expression can be modulated by each other's expression level through STAT regulatory elements in their respective promoters. *iv*) STAT1/3 are activated or inhibited in unique ways. They can be induced by either distinct or by the same set of cytokines or growth factors. Furthermore, their ability to induce SOCS proteins, which then downregulate the JAK kinase and cytokine receptor chains by degradation is unique, all allowing for transcriptional fine tuning giving selectivity to signalling.

With regard to cancer, the situation is more complex

since changes in the mutational landscape of the JAK-STAT-SOCS pathway or epigenetic differences influence gene regulation drastically. In general, overlapping tyrosine kinase action can lead to both of their STAT1/3 activation. Pathways that contribute to their inactivation or inhibition are frequently lost or mutated in cancer, such as inactivation of tyrosine phosphatases (*e.g.* by reactive oxygen species inactivating the catalytic cysteine of tyrosine phosphatases) or diminished proteolytic degradation (*e.g.* methylation of SOCS proteins, change in sumoylation or ubiquitinylation pathways is frequently deregulated escaping then proteasome degradation) (*41,42*). We have currently an incomplete mechanistic understanding how mutations or epigenetic changes together with changes in cytokine or growth factor signaling influence the ratio of STAT1/3 protein expression affecting CRC progression.

4.1. *Formation of STAT1/STAT3 heterodimers and gene regulation*

The heterodimeric ratio of STAT1/3 complexes and its pre-association or nuclear complex formation is both relevant for docking and gene regulation *via* STAT1/3. We postulate that gene regulation control is simply dictated by its YIN/YANG expression ratio and interaction control. Upon cytokine receptor engagement, STAT activation *via* the JAK/STAT pathway is a rapid process, but not essential for nuclear translocation in cancer cells. Under physiologic conditions in normal cells signals are transferred into the nucleus of cells to reprogram gene transcription through the STAT transcription factors in minutes. It was early on recognized that the activity and cellular abundance of individual STAT proteins can alter the expression of other STAT protein family members (*21,43*), but so far there were limited functional studies regarding the influence of STAT1 expression levels on STAT3 signalling during CRC tumour growth. To make the picture even more complex, STAT oligomerization reported for STAT1, STAT3, STAT4, STAT5A and STAT5B allows for loop formation on DNA among each other. This will also influence STAT1/3 action including autoregulatory expression change. Furthermore, other transcription factors that bind to similar or the identical target gene sequences then STAT1, STAT3 or STAT5 such as BCL6 (an important oncogene in B cell lymphomas), will change the fait and outcome of gene regulation through the STAT1/STAT3 YIN/YANG interplay. It is likely that U-STAT1 participates in gene transcription in CRC, possibly of interferon regulated genes involved in anti-tumour activities. It was postulated that the mutual interdependence of STAT1/3 through heterodimer formation within DNA binding complexes in tumour cells has a crucial influence on cancer cell fate (*44*). We have shown that STAT1/STAT3 heterodimers are detectable in the majority of CRC biopsies and form independent upon stimulation with IL-6 in various CRC cell lines due to the predominant nuclear expression of STAT1 that we will next illuminate further (*37*).

4.2. *Potential role of non-phosphorylated STATs and non-canonical STAT signalling in CRC*

The formation of STAT1/STAT3 heterodimers was experimentally mostly demonstrated in a DNA-bound state. However, it is noteworthy that U-STAT1 was also found persistently in the nucleus of epithelial cells as early described by the George Stark laboratory (*45*). Non-canonical STAT pathways employ STATs independently of tyrosine phosphorylation (*21,46*) .

In general, pYSTAT1 signalling was found to be mostly associated with interferon signalling, whereas pYSTAT3 signalling is connected with responses emanating from the activated distal four tyrosine residues within the gp130 receptor cytoplasmic chain (*43*). Importantly, U-STAT1/3 proteins have functions distinct from those of the pY proteins (*21*): *i*) as specific transcription factors and modifiers of transcription in case of U-STAT1 and U-STAT3 (*43*), *ii*) as effectors of mitochondrial function (serine phosphorylated STAT3) (*47*). U-STAT1 was shown to extend expression of IFN-induced genes (*45*). The fact that U-STAT1, if present in the nucleus can bind to activated pYSTAT3 controlling its proliferative and pro-survival function was so far neglected. Based on co-immunoprecipitation studies and structural insights derived from crystallography of the unphosphorylated STAT5A dimer (*40*) and integrative modelling of the U-STAT3 dimer (*48*) one can assume that STAT1/STAT3 heterodimers can bind in anti-parallel fashion in a non-tyrosine phosphorylated manner also to receptor chains. This antiparallel STAT dimerization at the cytokine receptor chains is reminiscent of JAK kinase dimerisation at the Box1 motif and the escape mechanism of inhibitory kinase action through the sliding model described above also suggesting an antiparallel juxtaposition of the kinase and the pseudokinase domains (*5*). Important here is the concept that STAT1/3 protein-protein interaction can be observed without the necessity to be fully activated by tyrosine phosphorylation. We could confirm early reports for STAT1/STAT3 interaction without the prerequisite of tyrosine phosphorylation (*39,49*). Furthermore, we also demonstrated significant nuclear presence of STAT1 independent of cytokine stimulus in CRC cells, where it can heterodimerize with pYSTAT3, for example activated by IL-6 (*39*). We were able to show that also upon tyrosine phosphorylation of STAT3 the interaction of U-STAT1 and pYSTAT3 is strongly interdependent. We conclude that it is the level of U-STAT1 expression that counter-regulates the activity of tyrosine phosphorylated, pro-oncogenic STAT3.

So far the specific activities of unphosphorylated

STATs in cancer have attracted limited attention, but there are indications that the non-canonical functions of U-STAT1 in particular in CRC have a key role in many physiological and pathophysiological conditions. In regard to gastrointestinal diseases this may very well include chronic Inflammatory Bowel Disease, Chron's disease, and Ulcerative Colitis (*36*), Hepatitis B and C driven liver inflammation and hepatocellular carcinoma progression. Processes such as chronic infection and inflammation can trigger adenoma initiation, they contribute to carcinoma formation and they are critically involved for metastatic spread, all processes dominated by cytokine and JAK-STAT action (Figure 1). Expression and nuclear import of U-STAT1 as an important break counteracting inflammation and infection as well as cancer progression should obtain more attention, particular in light of pYSTAT3 action. Here, more mechanistic work both from animal studies and comparative pathology examinations of patient pools will be required. U-STAT1 as well as non-canonical signalling *via* STAT1 are of great importance to understand the YIN/YANG nature of the STAT1/STAT3 interplay. The medical relevance of the STAT1/STAT3 dualism is strong because one third of the global population suffers from GI tract disease. Many aspects causing GI tract disease originate from genetics, behaviour or infectious disease. Fatty liver disease and metabolic syndrome are associated with obesity and type II diabetes, which has a chronic inflammatory component. Both, hepatitis infection as well as chronic inflammatory colonic diseases have a connex to pathogens or infectious disease, but also to individual genetic polymorphisms. Ultimately these epithelial layer tissue damaging conditions can culminate into four times higher risk to develop CRC closely associated with chronic inflammatory colonic disease (*36,50*). Therefore, future drug targeting approaches should consider changes in the ratio of STAT1 to STAT3 expression, since it strongly impacts on carcinoma progression.

5. Conclusions

Abundance of nuclear STAT1 dictates oncogenic or proliferative/survival of STAT3 activity for disease outcome in CRC. The mechanisms by which combinatorial effects of STAT1/3 modulate transcription deserve more detailed studies. Our current view provides an explanation for the controversial influence of STAT3 on CRC development and progression. U-STAT1 can be regarded as a guardian to keep oncogenic STAT3-induced gene regulation at bay. Therefore, STAT1 has important regulatory activity on STAT3-mediated transcription and it is a key tumour suppressor, which can control too much STAT3 activity in a dose dependent manner. The antagonism of STAT1/3 in CRC balances also chronic inflammation and infection. We suggest that the ratio of STAT1/3 expression has prognostic power for progression of CRC or GI tract cancers. Despite a high degree of CRC heterogeneity, meaningful clinical predictions may be possible if both STAT3 and counterbalancing STAT1 are analysed in biopsies instead of the centric view on only one STAT protein, as the field did in the past. We hope this concept will be tested in other carcinoma settings to define its implications for the biology of major cancer killers better. We predict that the YIN/YANG STAT1/3 nature will be relevant for many different cancer cell types and inflammatory or infectious disease might follow a similar trait.

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