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Dancing rhinos in stilettos

The amazing saga of the genomic and nongenomic actions of STAT3 in the heart

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A substantial body of evidence has shown that signal transducer and activator of transcription 3 (STAT3) has an important role in the heart in protecting the myocardium from ischemia and oxidative stress. These actions are attributed to STAT3 functioning as a transcription factor in upregulating cardioprotective genes. Loss of STAT3 has been implicated as well in the pathogenesis of heart failure and, in that context and in addition to the loss of a cardioprotective gene program, nuclear STAT3 has been identified as a transcriptional repressor important for the normal functioning of the ubiquitin-proteasome system for protein degradation. The later finding establishes a genomic role for STAT3 in controlling cellular homeostasis in cardiac myocytes independent of stress. Surprisingly, although a well-studied area, very few downstream gene targets of STAT3 in the heart have been definitively identified. In addition, STAT3 is now known to induce gene expression by noncanonical means that are not well characterized in the heart. On the other hand, recent evidence has shown that STAT3 has important nongenomic actions in cardiac myocytes that affect microtubule stability, mitochondrial respiration, and autophagy. These extranuclear actions of STAT3 involve protein–protein interactions that are incompletely understood, as is their regulation in both the healthy and injured heart. Moreover, how the diverse genomic and nongenomic actions of STAT3 crosstalk with each other is unchartered territory. Here we present an overview of what is and is not known about both the genomic and nongenomic actions of STAT3 in the heart from a structure-function perspective that focuses on the impact of posttranslational modifications and oxidative stress in regulating the actions and interactions of STAT3. Even though we have learnt a great deal about the role played by STAT3 in the heart, much more awaits to be discovered.

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Introduction

First recognized in 1994 as the acute-phase response factor (APRF),[1] signal transducer and activator of transcription 3 (STAT3) is one of 7 mammalian STAT transcription factors that play a central role in signaling by growth factors and cytokines. Of the STATs, STAT3 has a critical nonredundant role in cell growth, survival, and differentiation. The unique importance of STAT3 is underscored by the observation that of the STAT family members only disruption of the STAT3 gene causes embryonic lethality.[2] Studies over the past 20 years have shown that STAT3 has important actions in protecting the heart under stress.[3-6] In addition, human failing hearts were reported to exhibit reduced STAT3 levels and activity.[7-9] Yet a cohesive understanding of how STAT3 protects the heart has yet to be achieved. The protection afforded by STAT3 has been ascribed to both traditional genomic actions, i.e., the upregulation of protective genes, and more recently some may say fanciful nongenomic actions that target mitochondrial function and autophagy. Neither action is fully understood. Nor is it known how the two are regulated and integrated under either normal or stress conditions. Here we present an overview of what is known and not known about the protective actions of STAT3 in the heart from a structure-function perspective and how posttranslational modifications and oxidative stress may act as determining factors in regulating the genomic and nongenomic actions of STAT3.

Overview

The STAT3 protein is 770 amino acid in length with 6 distinct domains (**Fig. 1**).[4] A terminal NH_2-domain that participates in higher order complex formations that are not well understood is followed by a coiled-coil domain important for protein–protein interaction with other transcription factors and co-regulators. Next, the DNA binding domain canonically interacts with an interferon γ (gamma)-activated sequence (GAS) in the promoter region of specific genes.[10] A subsequent linker domain is located just before a Src homology-2 (SH2) domain that is essential for interaction with specific tyrosine-phosphorylated sites such as the YXXQ sites of the gp130 receptor of the IL-6 type cytokines,

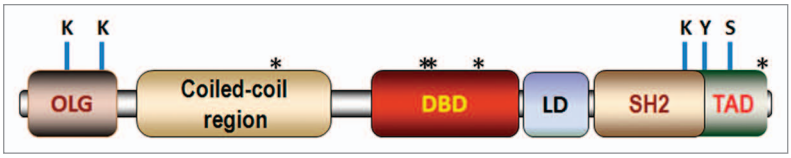

Figure 1. Major domains of STAT3 showing location of critical sites for posttranslational modification. From N-terminus to C-terminus, they are: oligomerization (OLG) domain, coiled-coil domain, DNA binding domain (DBD), linker domain (LD), SH2 domain, and the transcription activation domain (TAD). Phosphorylation of Y705 and S727 within the TAD has a critical role in the canonical genomic actions of STAT3. S727 phosphorylation has also been implicated in the noncanonical genomic and nongenomic actions of STAT3 through either enhanced recruitment of transcriptional co-factors (e.g., p300) and GRIM19, or alterations in STAT3 conformation. Acetylation of lysine residues (K49 and K87) within the NH2-terminal OLG domain are important for transcription by enhancing p300-STAT3 association and stabilizing enhanceosome assembly, as well as for nuclear retention of STAT3. Acetylation of K685 within the SH2 domain helps stabilize STAT3 dimers and enhances transcription. Asterisks (*) indicate redox-sensitive cysteine residues: C259 (within the coiled-coil domain), C418, C426, and C468 (within the DNA binding domain), and C765 within the TAD. Other sites of phosphorylation of STAT3 have been identified, but their importance in controlling the genomic and nongenomic actions of STAT3 is not defined.

as well as a specific tyrosine phosphorylated residue of activated STAT1 or STAT3 to form parallel dimers important in canonical STAT3 signaling. Although fairly homologous among all STAT family members, the SH2 domain of STAT3 is distinctive enough to allow for development of specific small molecule inhibitors of STAT3, such as Stattic and S3I-201.[11] Two sites of phosphorylation that constitute an "ignition" for canonical STAT3 activation are located in a COOH-terminal region known as the transcription activation domain (TAD).[4] The first site is Y705, phosphorylation of which leads to STAT3 dimer formation followed by translocation to the nucleus and induction of gene expression. Y705 is phosphorylated by the Janus kinase (JAK) family members, Src kinase, and epidermal growth factor kinase (EGF).[12] The other phosphorylation site of importance in regulating STAT3 activity is S727. Many serine/threonine kinases have been shown to phosphorylate S727 such as protein kinase Cε (PKCε), PKCδ, ERK1/2, mTOR, ZIP kinase, and CDK5.[12] S727 phosphorylation can take place either in the cytoplasm or nucleus. In canonical STAT3 signaling S727 phosphorylation has been shown to play a role in boosting the transcriptional activity of STAT3 through the recruitment of transcriptional cofactors, such as the histone acetyltransferase p300/CBP.[4,12] STAT3 S727 phosphorylation may also favor STAT3 homodimer formation.[13]

STAT3 function is regulated as well by p300-mediated acetylation of lysine residues within the NH_2-terminal (K49, K87) and SH2 (K685) domains. Acetylation of the latter, which rather unexpectedly may occur in the cytoplasm, plays a critical role in stabilizing STAT3 dimers and enhancing transcription.[12,14,15] Acetylation of K49 and K87 was shown to be important for gene transcription by enhancing or strengthening the interaction of STAT3 with p300 and thus stabilizing enhanceosome assembly.[16] Acetylation of these residues is important as well for nuclear retention of STAT3 with deacetylation mediated by histone deacetylases (principally HDAC1 and 4) resulting in STAT3 nuclear exit (and degradation).[17,18] By binding p300 and thereby directing acetylation, STAT3 has been shown to positively affect the accessibility of other transcription factors to promoters as well as to enhance transcriptional activity of NFκB.[19,20] Finally, K685 acetylation was shown to be important for interaction of STAT3 with DNA methyltransferase 1 and subsequent methylation and silencing of certain promoters.[21] Nearly all of these sorts of studies were performed on non-cardiac cells and thus their relevance to cardiac cells is unproven; however, the fundamental aspects of STAT3 signaling and regulation that they define are likely relevant to cardiac myocytes, particularly under conditions of gene induction and genomic plasticity related to stress. For instance, recent evidence was reported that increased p300 acetyltransferase activity in neonatal rat ventricular myocytes due to drug-induced p300 stabilization impacts positively on STAT3 activation and half-life.[22]

Cardiac Protective Actions of STAT3 Revealed by Genetic Mouse Models

STAT3 has been implicated in the protection of the myocardium produced by different types of preconditioning (ischemic, pharmacological, and remote), as well as postconditioning.[4] In the case of preconditioning, STAT3 has been implicated both in the early short-lived phase (not involving gene expression) and the delayed (by upwards of 24 h) longer-lived phase known as the second window of protection (involving gene expression). In many cases, the evidence reported is correlative or based on the use of a JAK2 inhibitor.[23-36] More definitive evidence for the importance of STAT3 in the heart has come from mouse genetic models. In 2003, Jacoby et al. explored the effect of postnatal deletion of STAT3 specifically in cardiac myocytes by crossing floxed STAT3 mice with mice expressing Cre recombinase under control of the α-myosin heavy chain (MHC) promoter that is active predominately in mature cardiac myocytes.[37] In these mice, myocardial dysfunction and marked cardiac fibrosis were noted with advanced age in the absence of cardiac insult. These hearts were also found to have a greater susceptibility to injury and greater contractile dysfunction was seen with doxorubicin. After lipopolysaccharide (LPS) injection hearts of these mice showed a significant increase in inflammation, fibrosis, and apoptosis, which was attributed to increased production of TNF-α presumably due to increased oxidative stress. The cellular source of TNF-α, specifically whether cardiac myocytes contributed, was not reported. Overall, the findings support an important role for STAT3 in both the aging/aged heart and the heart exposed to oxidative stress, although the exact basis for this was not defined.

Hilfiker-Kleiner et al. also reported progressive fibrosis, along with a reduction in capillary density, in hearts of cardiac myocyte-restricted STAT3 KO mice that became significant at 3–4 mo.[38] With advanced age, dilated cardiomyopathy, impaired cardiac function, and premature death was observed. Evidence was reported that STAT3 KO cardiac myocytes produce unidentified paracrine factors that stimulate (mouse embryonic) fibroblast

proliferation, but inhibit (mouse lung) endothelial cell proliferation. At 3–4 mo a number of genes associated with fibrosis and anti-angiogenesis were upregulated (COL1A1, COL8A1, OPN, BGN, TNC, PAI-1, CTGF, TSP1, TIMP1, MMP-12, MCP-1, IL2RB, IL15, MCP3, and BCL2A1). Surprisingly, no change was observed in protein levels of VEGF, which previously was linked to the protective actions of STAT3 in heart. In this regard, it is worth noting that the link between the cardioprotective effect of STAT3 and VEGF upregulation, as well as between STAT3 and MnSOD/SOD2 upregulation, was obtained with agonist stimulation (leukemia inhibitory factor/LIF) that induces multiple intracellular signaling pathways, as well as by overexpression of constitutively active STAT3.[39-41] The importance of concurrent signaling in shaping the character of STAT3 signaling is described elsewhere.[3]

Hilfiker-Kleiner et al. also tested the role of STAT3 in ischemia-reperfusion and infarction.[38] Infarct size and apoptosis were greater 24 h after reperfusion in KO mice with a significantly impaired fractional shortening 7 d after the insult. After 24 h reperfusion, increased mRNA levels were observed for the pro-apoptotic and pro-autophagy protein BNIP3, while mRNA levels of the prosurvival gene HSP70 were decreased. Again no changes were seen in mRNA or protein for VEGF. With myocardial infarction, a marked increase in mortality was seen: 32% wild-type vs. 100% KO. In a follow up study, this group provided compelling evidence to support the conclusion that the negative effects of cardiac myocyte-targeted STAT3 KO on capillary density and contractile function with advanced age was due to elevated cardiac expression of miR-199a, which in turn compromised the ubiquitin-proteasome system (UPS) of protein degradation by reducing expression of two UPS component proteins (although UPS activity was not directly assessed).[42] Transfection of cardiac myocytes with pre-miR-199a resulted in thinning of width and extension of length, as well as reduced levels of α- and β-MHC mRNA and protein levels of total MHC and troponin-T. These effects were recapitulated by knocking down the two UPS components. Expression of miR-199a or pharmacological inhibition of UPS was also associated with increased protein arginine methyltransferase I (PRMT-I) expression and asymmetric dimethylarginine (ADMA) synthesis in cardiac myocytes, which in turn was demonstrated to impair endothelial cell function. In addition, greater miR-199a promoter activity was found in STAT3 knock-down cardiac myocytes. Finally, using the cardiac myocyte-targeted STAT3 deficient mouse model others found that STAT3 is essential for ischemic and pharmacological preconditioning,[33] and depending on the protocol is important for ischemic postconditioning as well.[43] These findings were mirrored in aged hearts associated with a reduction in STAT3 levels. Bolli et al. developed a tamoxifen-inducible cardiac myocyte-targeted STAT3 knockout (KO) mouse.[44] Deletion of STAT3 abrogated the upregulation of cardioprotective (COX-2 and HO-1) and antiapoptotic proteins (e.g., Mcl-1, Bcl-xL, c-FLIPL, and c-FLIPS) that are normally expressed in response to delayed pre-conditioning. However, the impact of STAT3 deletion on the infarct-sparing actions of delayed preconditioning was not reported.

Although cardiac STAT3 is important for limiting infarct damage, unrestricted continuous activation of STAT3 by the gp130-receptor system after myocardial infarction was shown to be detrimental.[45] Thus, a precise regulation of STAT3 activity in terms of magnitude, time course, concurrent signals, or other as yet undefined parameters is essential for its beneficial effects. The finding of detrimental consequences after myocardial infarction with unbridled STAT3 activity via gp130 may explain why increased IL-6 serum levels are prognostic markers for adverse outcome in patients with myocardial infarction and heart failure.

We recently assessed whether STAT3 is important in hypertension-induced cardiac remodeling using mice with reduced global STAT3 activity due to a S727A mutation.[46] Hearts of SA/SA mice showed signs of developing systolic dysfunction in response to angiotensin II-induced elevated blood pressure after 17 d. With angiotensin II, fibrosis was seen in the left ventricle of both wild-type and SA/SA mice; however, fibrosis in SA/SA mice was largely reparative and was associated with loss of myocytes, while in wild-type hearts reactive fibrosis predominated. Cardiac hypertrophy as indexed by heart to bodyweight ratio and left ventricular anterior wall dimension during diastole was greater in wild-type mice. Altogether, our study and those involving STAT3 KO support the conclusion that the presence of STAT3 in cardiac myocytes is important for normal function and protection from stress.

In 2000, Kunisida et al. published a study highlighting the importance of STAT3 in hypertrophy and cardiac protective signaling.[47] Cardiac myocyte-specific STAT3 overexpressing hearts exhibited age-related pathological hypertrophy associated with increased expression of β-myosin heavy chain (MHC) and atrial natriuretic factor (ANF) and protection against doxorubicin induced cardiomyopathy. The later was likely in part the consequence of countering doxorubicin-induced reduction in STAT3 levels, as well as increased cardiotrophin-1 expression (and presumably increased expression of other protective proteins). Subsequently, the same group reported the impact of overexpressing a constitutively active form of STAT3 (caSTAT3) specifically in cardiomyocytes in several studies.[39-41] Two studies highlighted the significant role of activated STAT3 in upregulating MnSOD, a mitochondrial superoxide scavenger that protects the heart against increased levels of hypoxia/reoxygenation-induced ROS. Another study emphasized the importance of activated STAT3 in vascular formation by upregulating VEGF synthesis and enhancing VE-cadherin expression, which translated into an increase in capillary density. However, overexpressing STAT3 may not necessarily enhance the normal function of active STAT3 in the heart. At higher unphysiological levels, STAT3 might interact promiscuously with other proteins and artifactually affect cellular events. Even so, Hilfiker-Kleiner et al. presented compelling evidence from cardiac myocyte-restricted STAT3 knockout mice and patients that STAT3 deficiency contributes to the etiology of postpartum cardiomyopathy due to reduced MnSOD expression, increased oxidative stress, and reduced capillary density.[9]

Arguably, a more reasonable approach to increase STAT3 activity in the heart is to remove the naturally occurring negative feedback inhibitor of STAT3 activation by the JAKs, which is SOCS3. Oba et al. recently reported that progression of left

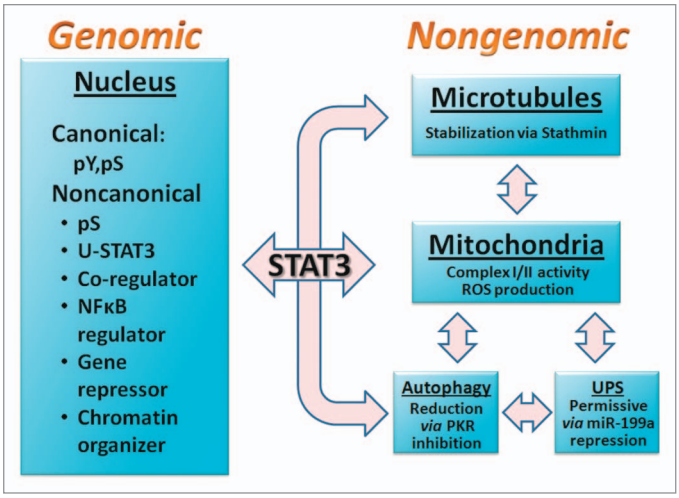

Figure 2. Scheme depicting the genomic and nongenomic actions identified for STAT3 in cardiac myocytes and other cell types. The genomic actions of STAT3 include both canonical and noncanonical events. The former involves STAT3 functioning as a transcription factor in the nucleus by binding $TTN_{4-6}AA$ elements in promoters and enhancing transcription. The noncanonical genomic actions of STAT3, which are diverse and not well understood, include: induction of transcription by pS727 STAT3 (without Y705 phosphorylation) and unphosphorylated STAT3 (U-STAT3); enhancing transcriptional activity of other transcription factors (e.g., nuclear steroid receptors); controlling the processing and nuclear retention of NFκB transcription factors; repressing gene expression; and modulating chromatin structure. Some of these actions may not require DNA binding and some events associated with the regulation of other transcription factors could conceivably occur in the cytoplasm. STAT3 has been shown to exert 3 actions in the cell that are extranuclear and do not involve gene transcription. These nongenomic actions of STAT3 control microtubule stability, mitochondrial function, and autophagy. For most, interaction of STAT3 with a specific protein has been implicated: stathmin (microtubule stability), GRIM19 (mitochondrial function), and PKR (autophagy). For microtubule stability and inhibition of autophagy, the STAT3–protein interaction provides a straightforward mechanistic link. The basis for the mitochondrial role of STAT3 is the least understood and other proteins besides GRIM19 are likely involved. The permissive role of STAT3 in the ubiquitin-proteasome system (UPS) is genomic and results from suppression of miR-199a expression. Understanding of crosstalk between the genomic and mitochondrial actions of STAT3 is limited, as is the likely interplay among the nongenomic actions of STAT3 (for instance, impaired mitochondrial function and enhanced autophagy/mitophagy). Evidence of complex interplay among microtubule stability, mitochondrial function, autophagy, and UPS in various cell types is reported in the literature in general, implying that STAT3 has a central role in cellular homeostasis and stress responsiveness. Differential regulation of posttranslational modifications of STAT3 could form the basis for the integration of the nongenomic and genomic actions of STAT3.

ventricular remodeling over a period of 14 d following an acute myocardial infarction was prevented in cardiac myocyte-targeted SOCS3 KO hearts.[48] Enhanced activation of cardioprotective signaling pathways (STAT3, AKT, and ERK1/2) was noted in the KO heart, as well as reduced apoptosis. Compared with the wild-type hearts, following infarction KO hearts exhibited increased expression of antioxidant (MnSOD and HO-1) and anti-apoptotic (Bcl-xL) proteins, as well as decreased levels of pro-apoptotic (Bad and Bax) proteins. Consistent with less fibrosis in the SOCS3 KO hearts, reduced expression of CNTF, TGFβ2, matrix metalloproteinase-9, collagen 1, and collagen 3 and increased expression of TIMP-2 was seen compared with wild-type hearts. Altogether, the findings of this study elegantly demonstrate the key role of STAT3 in cardioprotection.

Genomic Actions of STAT3

STAT3 has been clearly shown to regulate different sets of genes by 3 distinct means: canonical, phosphorylated S727 (pS727)-only, and unphosphorylated STAT3/U-STAT3.[49] The latter are 2 aspects of the noncanonical actions of STAT3 in mediating gene expression (**Fig. 2**). In canonical signaling, Y705 phosphorylation leads to STAT3 dimerization and translocation to the nucleus, where it induces transcription of certain genes by binding to a GAS element ($TTCN_3GAA$).[4,50] S727 phosphorylation enhances canonical transcription by recruiting the histone acetylase p300 (**Fig. 2**).[16,51] STAT3 can also bind $TTN_{4-6}AA$ motifs,[52,53] broadening its range of action.

STAT3 is now known to affect transcription noncanonically (**Fig. 2**),[49,54-56] for instance: (1) STAT3 is constitutively present in the nucleus;[55,57] (2) likely there are non-consensus binding sites for STAT3 in some promoters in the context of association of STAT3 with other proteins involved in transcription, e.g., NFκB p65;[58] (3) STAT3 can bind DNA as a monomer as well,[10] although the significance of this to gene transcription is unknown; (4) STAT3 may not necessarily need to bind DNA to enhance transcription;[51,59] (5) STAT3 S727 phosphorylation can enhance transcription independent of Y705 phosphorylation;[49,60-62] (6) STAT3 contains a nuclear receptor binding motif (LXXLL motif) in the coiled-coil domain and was shown to synergistically enhance transcriptional activity of nuclear receptors;[63-66] (7) STAT3 and the shorter STAT3β spliceform lacking the TAD were found to induce a smaller number of genes in common than the numbers of genes induced uniquely by either;[67] and (8) U-STAT3, which may increase in nuclei of cardiac myocytes during cardiac hypertrophy, can induce expression of a subset of inflammatory genes.[56,68-70] U-STAT3 may function also as a chromatin/genomic organizer.[10] Together these observations support the conclusion that STAT3 functions as a transcriptional co-regulator, as well as a transcription factor per se.

How the 3 transcriptional mechanisms of STAT3 are coordinated is unknown, but likely involves regulated STAT3–protein interactions. As mentioned, a number of genes have been implicated in the protective actions of STAT3 in the heart, but to our knowledge definitive evidence for a direct role of STAT3 in their induction based on promoter analysis or ChIP assays is lacking. Three putative STAT3 binding sites were detected in the promoter for miR-199a, but evidence for STAT3 binding to the site was not shown and in this case STAT3 served to repress transcription.[42]

Nongenomic Actions

STAT3 has been shown to exert 3 actions in the cell affecting microtubule stability, mitochondrial function, and autophagy

that are thought to be extranuclear and not involving gene transcription (**Fig. 2**). Studies on these nongenomic actions of STAT3 are evolving and the findings to date, though perhaps not definitive, are nevertheless intriguing as they could foster development of new therapeutic strategies based on naturally occurring or forced extranuclear STAT3–protein interactions.

STAT3 has been proposed to directly contribute to microtubule (MT) stabilization by interacting with and thereby inhibiting the activity of stathmin (a.k.a. oncoprotein 18), a small ubiquitously expressed and mainly-cytoplasmic protein.[71-73] Stathmin binds α/β-tubulin heterodimers causing depolymerization of MTs. This role of STAT3 is reported to be important for cell migration and preventing axonal retraction/degeneration and evidence for this includes: (1) co-immunoprecipitation of STAT3 and stathmin (along with mutational analysis); (2) a sufficient protein ratio of stathmin to STAT3; (3) negative effect of STAT3 deletion on MT stability and cell migration, along with recovery types of experiments with STAT3 mutants that do not bind DNA (which now has shortcomings given the revised understanding of the genomic actions of STAT3); (4) STAT3-mediated reversal of stathmin-inhibition of tubulin polymerization in vitro; and (5) the negative impact of STAT3 inhibitors on MT polymerization.

Excessive MT stabilization in the myocardium has been observed in animal models of pathological cardiac hypertrophy and heart failure,[74] but the role of STAT3 in regulating MT stability in cardiac myocytes is not established. However, Ng et al. reported that gp130 family cytokines (established activators of STAT3) induced MT stabilization in neonatal rat ventricular myocytes, which could be inhibited with a JAK2 or STAT3 inhibitor or STAT3 knockdown.[74] In contrast, expression of a constitutively active STAT3 (spontaneously dimerizing STAT3) enhanced MT stabilization. A direct role for STAT3 in MT stabilization in this study cannot be ruled in or out. The impact of constitutively active STAT3 would suggest that gene expression was involved, while evidence both for and against a role of Y705 phosphorylation in STAT3-stathmin interaction has been reported.[71,73] Given the likely importance in the heart for MT stability in the context of mitochondrial function and autophagy and its possible dysfunction in pathological conditions, the role of STAT3 as a regulator of this process warrants further investigation.

The observations that a component of complex I, GRIM19 can associate with STAT3 prompted investigation into whether STAT3 plays a role in mitochondrial function.[75-79] Many (but not all) studies have detected STAT3 in mitochondria from various types of cells and tissues including cardiac myocytes and the heart, and in many (but not all) cases the STAT3 pool is enriched in phosphorylated S727 compared with the cytoplasm.[80-85] This enrichment may be dynamically regulated as catecholamine-induced hypertrophy of H9c2 cardiomyoblasts was associated with a reduction in mitochondrial STAT3 phosphorylated S727 levels with no change in total STAT3 levels.[86] Recently, the uptake of STAT3 by isolated mitochondria was shown to be mediated by GRIM19 and enhanced by STAT3 S727 phosphorylation,[79] which is not unexpected as association of GRIM19 with STAT3 was reported to occur through the TAD of STAT3 and to be positively affected by S727 phosphorylation.[87] Interestingly, STAT3 and GRIM19 mutually enhance their translocation to the mitochondria[88] and the small heat shock protein HSPB8/HSP22, which is predominately expressed in skeletal/cardiac muscle was reported to be important for the mitochondrial translocation of STAT3.[89] STAT3 mitochondrial uptake would also appear to require mitochondrial membrane potential and energy.[79]

STAT3 has been shown to regulate several aspects of mitochondrial function, including activities of complexes I and II, mitochondrial permeability transition pore (mPTP) opening, and reactive oxygen species (ROS) production. Mitochondria from hearts of mice with postnatal cardiac myocyte-targeted STAT3 KO have reduced ADP-stimulated respiration and complex I and II activities,[75,77] and the STAT3 inhibitor Stattic reduced ADP-stimulated respiration of rat mitochondria.[77] Calcium-sensitivity of mPTP opening, which can trigger cell death, was enhanced in mitochondria from STAT3-KO mice and Stattic-treated rat mitochondria. Moreover, STAT3 co-immunoprecipitated with pore component cyclophilin D in rat left ventricular mitochondria, suggesting that STAT3 may prevent mPTP opening by binding cyclophilin D similar to the actions of the cardioprotective agent cyclosporine A (CsA).[77] In fact, STAT3 was shown to be important for preconditioning-induced infarct size reduction, but not pharmacological conditioning with CsA, suggesting similar modes of action. Intriguingly, in aged mouse hearts mitochondrial STAT3 levels were reduced raising the possibility that loss of STAT3 may contribute to aging-related pathologies of cardiac myocytes.[77] Others reported that postconditioning of the hearts of pigs increased mitochondrial levels of STAT3 Y705 phosphorylation (not S727), which was associated with better complex I respiration and calcium retention capacity.[84] Finally, mice with cardiac myocyte-specific overexpression of mitochondria-targeted STAT3 bearing a mutation in the DNA-binding domain (MLS-STAT3E) were recently generated.[76] MLS-STAT3E expressing mitochondria showed modest decreases in complexes I and II basal activities; however, mitochondria from MLS-STAT3E hearts were protected against ischemic damage to complex I respiratory rates and release of cytochrome c into the cytosol. Compared with wild-type mitochondria, ischemia did not enhance ROS production by MLS-STAT3E mitochondria, which was attributed to partial blockade of electron transport through complex I.

The role of mitochondrial STAT3 in regulating ROS production is intriguing and has been studied primarily in noncardiac cells. Both positive and negative actions of mitochondrial STAT3 on ROS production have been reported and in some cases no parsing of the relative contribution of genomic and nongenomic actions of STAT3 was done: mouse STAT3 null hematopoietic stem/progenitor cells display mitochondrial dysfunction and increased ROS;[90] TNF-induced necroptosis of L929 mouse fibrosarcoma cells was attributed to enhanced translocation of STAT3 to the mitochondria by GRIM19 with a subsequent increase in ROS;[88] mitochondrial STAT3 was implicated in nerve growth factor (NGF) induced neurite outgrowth and ROS production;[80] in astrocytes the absence of STAT3 resulted in decreased

mitochondrial membrane potential and ATP production, along with increased ROS production;[91] cultured primary osteoblasts from STAT3 KO mice produced elevated levels of ROS;[92] and, finally, the key importance of mitochondrial STAT3 to Ras-dependent oncogenic transformation of cancer cells is likely due to increased ROS.[93]

S727 phosphorylation seems to have special importance in the mitochondrial actions of STAT3, as impaired activity of complexes I and II of mitochondria from STAT3$^{-/-}$ pro-B cells could be restored by expressing a mitochondrial targeted STAT3 bearing a pS727 mimetic while the non-phosphorylatable STAT3 Y705F/S727A was ineffective.[75] Moreover, a STAT3 with a phosphorylated Y705 mimetic or a constitutively active STAT3 that spontaneously undergoes dimerization were not effective. Another study reported that a mitochondria-targeted serine-dominant negative mutant of STAT3 attenuated NGF-induced neurite outgrowth, while wild-type and tyrosine-dominant negative mutant STAT3 enhanced NGF induced neurite outgrowth.[80] Also, a number of studies report that STAT3 pS727 is enriched in mitochondria compared with the cytoplasm.[78-83]

While the evidence for a direct role for STAT3 in the function of mitochondria is convincing, the issue of stoichiometry has raised a seemingly insurmountable argument against a direct contribution of (normally expressed levels of) STAT3 to the electron transport chain based on protein–protein interactions. Phillips et al. determined through three different proteomic approaches that the mitochondrial complex I/II ratio to STAT3 in cardiac tissue to be ~10^5, at least under normal conditions.[94] Involvement of other indirect mechanisms, such as regulating in some fashion the posttranslational modifications of mitochondrial proteins, remains a tenable hypothesis. For instance, a mitochondrial pool of GSK-3β localizes to the inner membrane and STAT3 was shown to associate with and negatively affect phosphorylation of GSK3β, thereby enhancing its activity.[95] Altogether this would be expected to promote mPTP opening and ROS generation. Evidence was also recently reported that STAT3 tightly associates with inner mitochondrial membrane complexes in rat heart mitochondria, likely complex I.[79] Thus, STAT3 functioning in a higher order complex with other proteins that results in post-translational modification of complex 1 proteins is a possibility. Alternatively, Szczepanek et al. have proposed that STAT3 may function in the redox buffering of the mitochondria given reports that it possesses redox-sensitive cysteines.[78]

STAT3 may represent a means of communication between the mitochondria and nucleus to regulate cellular metabolism, although evidence to support this conclusion is circumstantial. Recent evidence from cancer research indicates that STAT3 in its capacity as a nuclear transcription factor can act as an "anaerobic switch", favoring glycolysis and attenuating mitochondrial activity by suppressing genes for mitochondrial proteins.[96,97] In fact, STAT3 has been implicated as a causative factor in the well-known Warburg effect observed in cancer cells.[97] STAT3 Y705 phosphorylation and canonical signaling has been implicated in these actions of STAT3. A major regulator of mitochondria biogenesis is SIRT1, a NAD-dependent deacetylase that negatively impacts on canonical STAT3 signaling. SIRT1 KO in mouse embryonic fibroblasts was recently reported to increase STAT3 expression and activity, which was accompanied by increased accumulation of S727 phosphorylated STAT3 in mitochondria and mitochondrial respiration.[81] Obviously, more research needs to be done for a coherent understanding of the give and take of STAT3 signaling in mitochondria-nucleus crosstalk.

As mentioned, STAT3 was shown to be important for proper protein degradation in the cells by repressing the expression of a suppressor of the expression of UPS components.[42] While inhibition of UPS-mediated protein degradation (as would be seen with loss of STAT3) has been shown to enhance autophagy,[98] evidence was recently presented that STAT3 tonically inhibits autophagy by physically associating with protein kinase R (PKR).[99] PKR is a ubiquitously expressed serine/threonine kinase that phosphorylates eukaryotic translation initiation factor 2-α, thereby inhibiting protein translation and inducing autophagy. PKR is activated (directly or indirectly) by a number of stresses including double stranded RNA, growth factor deprivation, and hydrogen peroxide.[100,101] A physical association between the SH2 domain of STAT3 and the catalytic domain of PKR was demonstrated that could be disrupted by long chain saturated fatty acids.[99,102] These observations were made with noncardiac cells; however, significant increase in autophagolysosomes and levels of LC3-II were reported in a STAT3-deficient HL-1 mouse atrial cardiac myocyte cell line, indicating enhanced autophagy.[103] In summary, normal STAT3 expression would seem to favor UPS over autophagy, while loss of STAT3 would have the converse action.

Proving that STAT3 truly has nongenomic actions in a physiological setting is extremely difficult and whether that has been achieved is debatable. In this regard, two cautionary notes need to be sounded: (1) overexpression studies are not the perfect complement of gene knockout/deletion studies; and (2) what defines the transcriptional activity of STAT3 is a moving target and could conceivably involve just the shuttling of other proteins into the nucleus or the modification of other transcription factors via enhanced posttranslational modification.

The Tail of the Rhino: Distinguishing Feature and Common Link?

The TAD is the least conserved portion of the STAT proteins, although with the exception of STAT2 and 6 all contain a P(M)SP motif that is a target for proline-directed kinases and is involved in recruiting p300 with various potencies.[12,104] For STAT3, phosphorylation of the serine residue within this motif (S727) has been implicated in other aspects of STAT3 regulation as well, including: (1) enhancing transcriptional activity by recruiting co-factors;[12,104] (2) opposing Y705 phosphorylation by recruiting phosphatases or inducing conformational change by disrupting TAD-coiled-coil domain interaction,[105-107] both of which have significance in preventing or terminating canonical STAT3 signaling; (3) determining subcellular distribution of STAT3, specifically uptake by mitochondria;[79] (4) terminating STAT3-induced gene expression at a subset of promoters by recruiting histone methyl transferase SET9, which dimethylates

Table 1. Proteins shown to interact with the TAD of STAT3

Protein	Shown	Involves pS727	Function	Expressed in heart
14-3-3ζ	U266 myeloma cell line, T cells	Yes	• Protects STAT3 from pS727 dephosphorylation by PP2A[112] • Along with STAT3 sequesters pFoxO1 and pFoxO3a in cytoplasm and prolongs T cell activation[113]	Yes
GRIM19	Various cell types	Yes	• Translocation of STAT3 to mitochondria[79] • Inhibition of STAT3 transcriptional activity[87,114]	Yes
CBP/p300	Various cell types	Yes	• Enhances STAT3 transcriptional activity51 • STAT3-dependent nuclear retention of NF-κB/p65[20] • STAT3 acetylation (strengthened p300 binding and dimerization and DNA binding and enhanceosome assembly/transcription factor complexes)[14-18] • STAT3-mediated NFκB p100 processing[115]	Yes
Pin1	HepG2 cells, MEF, MCF-7 cells	Yes	• Promotes STAT3 transcriptional activity and p300 recruitment[116]	NR
SET9	human colon cancer A4 cells	Yes	• Dimethylation and downregulation of STAT3 binding at certain promoters[108]	Likely
CDK9	HepG2	ND	• localization of CDK9 to proximal promoter so as to phosphorylate RNA pol II switching it from initiation to elongation state[117]	Yes
Sp1	Rat heart, HUVE	Yes	• Upregulation of ICAM-1 transcription after reoxygenation or reperfusion[118] • Likely important at numerous genes	Yes
Cyclin D1	HepG2	ND	• Inhibition of STAT3 transcriptional activity[119]	Yes (low in normal adult myocardium)
SRC-1/ NCoA-1	HepG2	No	• Enhanced STAT3 transcriptional activity[120]	Yes

ND, not determined; NR, not reported.

STAT3 on a specific residue (K140) thereby causing its dissociation from the promoter;[108] and (5) inducing gene expression in its own right.[60,61] S727 phosphorylation likely impacts on the conformation of the C-terminus or its accessibility. Approximately 150 associations/interactions with other proteins have been ascribed to STAT3.[109,110] For many, the particular region of STAT3 has not been identified, although the coiled-coil domain is likely responsible. Surprisingly, only a few have been reported to involve the TAD of STAT3. Some interactions may be indirect, for instance p300-mediated interaction between STAT3 and Smad1.[111] **Table 1** provides a listing of those that are (seemingly) direct and found from the literature. In one way or another, association of these proteins with STAT3 has been shown to determine the cellular and nuclear actions of STAT3; however, the impact of S727 phosphorylation on the role of STAT3 in the heart is not well studied. We recently showed that hearts of mice with a S727A mutation in both STAT3 alleles adapted poorly to increased blood pressure compared with wild-type mice.[46] Whether this was due to differences in the genomic or nongenomic actions of STAT3 will need to be assessed.

Redox-Sensitivity of STAT3

Perhaps a distinguishing feature of STAT3 compared with the other STATs is redox-sensitivity, although the basis for this selectivity is not known. We recently showed that STAT3 activation in cardiac myocytes is impaired by depleting the major cellular antioxidant molecule and redox-buffer, glutathione (GSH).[121] L-Buthionine-sulfoximine (BSO) pretreatment decreased GSH levels, induced ROS formation, and dose-dependently attenuated STAT3 activation by LIF. Glutathione monoethyl ester, which is cleaved to GSH intracellularly, prevented the reduction in STAT3 activation, as did the antioxidant N-acetyl-cysteine; however, LIF-induced STAT1 activation was unaffected by GSH depletion. We also showed that thiophylic compounds inhibit LIF-induced STAT3 activation.[122] Pretreatment of human microvascular endothelial cells (HMEC-1), neonatal rat cardiac myocytes, or adult mouse cardiac myocytes with the nitroxyl (HNO) donors Angeli's salt or nitrosocyclohexyl acetate (NCA) inhibited STAT3 activation. NCA also blocked induction of inflammatory genes (ICAM-1 and CEBPD). The related 1-nitrosocyclohexyl pivalate (NCP), which is not a nitroxyl donor, also inhibited STAT3 activation, indicating that these compounds were acting as thiolate-targeting electrophiles. JAK1 was not a target of acyloxy nitroso compounds, as NCA had no effect on JAK1 catalytic activity. However, pretreatment of recombinant STAT3 with NCA or NCP reduced labeling of free sulfhydryl residues. We also showed that NCP in the presence of diamide enhanced STAT3 glutathionylation in adult cardiac myocytes and altered the SDS-PAGE profile of STAT3 under non-reducing conditions. Finally, we observed that monomeric STAT3 levels are decreased in the Gαq model of heart failure in a redox-sensitive manner,[122] supporting the conclusion that the redox-sensitivity of STAT3 has pathophysiological relevance.

Besides us, others have reported that STAT3 has redox-sensitive cysteines that affect its function. Treatment of HepG2 cells with thiol targeting agents was reported to inhibit IL-6-induced STAT3 activation and increase STAT3 glutathionylation.[123] These agents also decreased nuclear accumulation of STAT3 and impaired expression of STAT3-target genes. Peroxide was reported to induce STAT3 homodimer formation in HEK293 cells and a cysteine in the N-terminus (C259) was implicated.[124] Another study identified 3 redox-sensitive cysteines in the DNA binding domain (C418, C426, and C468) and one within the transcription activation domain (C765).[125] In a cell system using IL-6, these residues were found responsible for peroxide-reduced STAT3-mediated reporter gene expression from a consensus GAS element, but not from TTN_6AA sites. The latter observation suggests that ROS may differentially affect STAT3 binding to DNA depending upon the promoter.

How might the redox-sensitivity of STAT3 impact on its role in cells? Concerning the genomic actions of STAT3, oxidative stress may alter the gene profile linked to STAT3 because of differential promoter selection or STAT3–protein interactions. For the nongenomic actions of STAT3, protein–protein interactions may be affected. An early observation showed that STAT3 exists in higher order complexes with other proteins in the cytosol.[126] Redox-sensitivity might function to determine the binding partners of STAT3, as well as its subcellular distribution. In short, redox-sensitivity may serve as a switch to coordinate the various roles of STAT3 depending upon the stresses placed on the cell.

Reduced STAT3 Activity in the Heart: The Perfect Storm?

Multiple studies have provided evidence that nongenomic STAT3 has a critical role in optimal mitochondrial function and preventing mPTP opening, inhibiting autophagy, proper functioning of the ubiquitin-proteasome system, and microtubule stability. In fact, STAT3 may serve as an integrating factor for these processes. On the other hand, genomic STAT3 has been linked to the upregulation of antioxidant and anti-apoptotic proteins. Thus, reduced protein levels or activity of STAT3, or impediment of its interaction with other proteins may favor the creation of "the perfect storm", the consequence being unbridled activation of multiple death-inducing processes in the cell simultaneously. That such a scenario may occur in heart failure and contribute to contractile degeneration is plausible, but not yet investigated.

Conclusions and Perspectives

Much has been learned about the beneficial actions of STAT3 in the heart, but often times it is difficult to separate fact from supposition. In any case, what has emerged over the last few years is the central importance of STAT3 in regulating key cellular processes in cardiac myocytes in different cellular compartments by both genomic and nongenomic means. Another preeminent conclusion is that proper regulation of STAT3 in the heart and in cardiac myocytes in either direction, i.e., increased or decreased expression, heightened or depressed activation, is essential for the physiologic (genomic and non-genomic) actions of STAT3. For all these actions, both a housekeeping and a stress-responsive role of STAT3 is likely. STAT3–protein interactions are involved in all these processes and posttranslational modifications surely finesse the actions of STAT3, perhaps akin in naivety at this early stage of discovery to rhinos dancing in stilettos. Nonetheless, understanding how these interactions are regulated so as to coordinate the actions of STAT3 in the healthy and stressed/injured heart could lead to new therapeutic strategies to prevent damage to the heart or improve its performance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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