1 Inhibition of HSP90 reversed STAT3 mediated 2 muscle wasting induced by cancer cachexia

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2 **Running Title: HSP90/STAT3 Trigger Cancer Cachexia**

1 **Abstract**

2 Cancer cachexia is one of the most common causes of death among
3 cancer patients, no effective anti-cachectic treatment is currently
4 available. In experimental cachectic models, aberrant activation of
5 STAT3 in skeletal muscle has been found to contribute to muscle wasting.
6 However, its clinical association, the factors regulating STAT3 activation,
7 and the molecular mechanisms of STAT3-induced muscle atrophy in
8 cancer cachexia remain incompletely understood. Here, we show that an
9 enhanced interaction between STAT3 and HSP90, which causes the
10 persistent STAT3 activation in the skeletal muscle of cancer cachexia
11 patients, is the crucial event for the development of cachectic muscle
12 wasting. Administration of HSP90 inhibitors alleviated the muscle
13 wasting in C26 tumor-bearing cachectic mice model or C26 conditional
14 medium induced C2C12 myotube atrophy. A mechanistic study indicated
15 that in cachectic skeletal muscle, prolonged STAT3 activation triggered
16 muscle wasting in a FOXO1-dependent manner, STAT3 activated
17 FOXO1 by binding directly to its promoter. Our results provide key
18 insights into the role of the HSP90/STAT3/FOXO1 axis in cachectic
19 muscle wasting, which shows promising therapeutic potential as a target
20 for the treatment of cancer cachexia.

21 **Keywords**

22 Cancer cachexia; Muscle wasting; HSP90; STAT3; FOXO1;

1 **Introduction**

2 Cancer cachexia is a devastating metabolic syndrome that affects up to 80%
3 of cancer patients and accounts for nearly 20% of all cancer-related
4 deaths (Loberg *et al*, 2007). Skeletal muscle wasting is one of the most
5 crucial pathological events in cancer cachexia. As a highly plastic tissue,
6 skeletal muscle can change its mass, function, and metabolism in
7 response to various endogenous or exogenous stimuli. Current evidence
8 indicates that the imbalance between catabolic and anabolic responses
9 and the disorder of protein synthesis and degradation pathways, including
10 both the ubiquitin-proteasome system (UPS) and the autophagy-lysosome
11 pathway (ALP), are the major causes of cachectic muscle wasting
12 (Bilodeau *et al*, 2016). Activation of the UPS is a crucial event in muscle
13 wasting in cancer cachexia, and the enhanced expression of
14 muscle-specific E3 ubiquitin ligases, such as muscle-atrophy-F-box
15 (MAFbx/atrogen-1) and muscle-RING-finger-1 (MuRF1), are hallmarks
16 of this process (Bodine *et al*, 2001; Guadagnin *et al*, 2018; Guo *et al*,
17 2017). However, the upstream activators of the protein degradation
18 pathway and the molecular mechanisms involved in muscle wasting in
19 cancer cachexia are still largely unknown.

20 Signal transducer and activator of transcription 3 (STAT3) plays a critical
21 role in cancer cachexia, and increased STAT3 activation (in muscle) has
22 been found in multiple types of experimental cancer cachexia (Zimmers
23 *et al*, 2016)(Ma *et al*, 2017; Mubaid *et al*, 2019; Silva *et al*, 2015). In
24 experimental cancer cachexia, muscle-specific STAT3 depletion or
25 JAK2/STAT3 pathway inhibition can reverse the skeletal muscle wasting
26 phenotype (Bonetto *et al*, 2012). However, its clinical association, the
27 reason for prolonged STAT3 activation in cachectic muscles, and its
28 contribution to pathological anabolic responses in muscle still need to be

1 defined.

2 HSP90 (heat shock protein 90) is an evolutionarily conserved molecular
3 chaperone that is essential for cell growth, proliferation, transformation,
4 proliferation, and survival under normal and stress conditions (Schopf *et*
5 *al*, 2017). HSP90 interacts extensively with a variety of signaling
6 transduction proteins (Whitesell *et al*, 2005),(Mahendrarajah *et al*, 2017).
7 We and other groups have previously demonstrated that HSP90 could
8 directly interact and regulate STAT3 activation in various cancers and
9 promote cancer cell growth and survival as an oncogene (Prinsloo *et al*.
10 2012; Schoof *et al*. 2009; Song *et al*. 2017b). However, whether HSP90 is
11 involved in regulating STAT3 activation in skeletal muscles and its
12 functional role in cachectic muscle wasting is still unknown.

13 Herein, we report that the increased HSP90-STAT3 interaction is
14 necessary to induce prolonged STAT3 activation and muscle atrophy in
15 clinical cachectic patients and C26 tumor-bearing experimental cachexia
16 mice model. Administration of HSP90 inhibitors *in vivo* could
17 successfully alleviate the pathological development of experimental
18 cachexia; mechanistic study demonstrated that activated STAT3 could
19 induce FOXO1 transcription by binding directly to the FOXO1 promoter,
20 and knockdown of FOXO1 abolished the effects of STAT3 induced
21 muscle wasting. Therefore, our study provided novel experimental
22 evidence showing that the HSP90/STAT3/FOXO1 axis might be a
23 potential therapeutic target for cancer cachexia.

24

1 **Results**

2 **Enhanced HSP90-STAT3 interaction causes the persistent STAT3** 3 **activation and muscle atrophy in patients and mice with cancer** 4 **cachexia.**

5 We first evaluated the expression level of HSP90, atrophy markers, and
6 activation of STAT3 in muscle from cancer cachexia patients. As shown
7 in Fig 1, there is a significantly increased expression of muscle atrophy
8 markers, including myostatin, E3 ligases such as Atrogin-1 and MuRF1
9 in skeletal muscle of cancer cachexia patients; also we detected the
10 persistent activation of STAT3 accompanied by significantly increased
11 expression of HSP90 in cachectic skeletal muscle (Figure 1A, B, C); by
12 measuring the HSP90-STAT3 interaction using the immunoprecipitation,
13 we observed a dramatic increase in the HSP90-STAT3 interaction in
14 patient muscle with cancer cachexia (Figure 1D), indicating that HSP90
15 might be involved in the regulation of STAT3 activation during the
16 pathological development of cancer cachexia.

17 To validate these results, an experimental cachexia mouse model was
18 adopted by inoculated Balb/C mice with a mouse colon adenocarcinoma
19 cell line C26; these mice showed typical cachectic symptoms, including
20 progressive weight loss, dull and matted fur, and mental weakness, which
21 was consistent with the previous reports (Tanaka *et al*, 1990). There was
22 approximately 15-20% overall weight loss and obvious lean body mass
23 weight loss in C26 tumor-bearing mice compared with that in
24 non-tumor-bearing mice within 15 days after tumor inoculation (Fig
25 EV1A). Persistent STAT3 activation, as well as the enhanced expression
26 of HSP90 and atrophy related molecular markers including myostatin,
27 Atrogin-1, and MuRF1, were also detected in cachectic muscle from

1 C26-bearing mice (Fig 1E, F, G), the dramatic increase in the
2 HSP90-STAT3 interaction were also confirmed in the cachectic muscle in
3 C26 tumor-bearing mice (Fig EV1B).

4 **HSP90 and STAT3 were required for C26 CM-induced myotube**
5 **atrophy.**

6 To assess the involvement of HSP90 in cancer cachexia-related muscle
7 wasting, we adopted the *in vitro* cachectic cell model by culturing the
8 C2C12 myotubes with C26 tumor cell-derived conditional medium (C26
9 CM)(Fig EV1C). Western blot assay confirms the pathological phenotype
10 of cachectic muscle atrophy based on the loss of MHC expression and the
11 enhanced expression of myostatin, E3 ligases such as Atrogin-1 and
12 MuRF1 (Fig 2A, 2B). H&E staining further demonstrated the myotube
13 shrinking induced by C26 CM (Fig 2D). Notably, we observed a dramatic
14 increase in the HSP90-STAT3 interaction in C2C12 myotubes upon C26
15 CM treatment (Fig 2C); this is consistent with the *in vivo* data, although
16 the increased expression of HSP90 in skeletal muscle was not obvious in
17 C26 CM induced muscle atrophy (Fig 2A). To determine whether the
18 atrophy effect was HSP90-dependent, we knocked down HSP90 in
19 C2C12 myotubes. The results showed that the administration of RNAi
20 against HSP90 or STAT3 prevented the decrease in the myotube size,
21 myotube diameter and myotube area induced by C26 CM (Fig 2D,E).
22 These results were further confirmed by the measurement of the
23 transcriptional expression of MuRF-1 and atrogin-1 (Fig 2E right).
24 Consistently, the myotube atrophy-associated phenotypes, including the
25 downregulated expression of MHC and the upregulated expression of
26 myostatin, MuRF-1, and atrogin-1, were largely reversed when HSP90
27 was knocked down (Fig 2F left). Similar effects were observed when
28 STAT3 was knocked down in C2C12 myotubes (Fig 2F right), indicating

1 that the enhanced HSP90-STAT3 interaction plays a crucial role in the
2 pathological development of cachectic muscle wasting.

3 **Disruption of the HSP90-STAT3 interaction by 17DMAG protected** 4 **C2C12 myotubes from C26 CM-induced atrophy.**

5 17DMAG, an inhibitor of HSP90, was then introduced in the C2C12
6 myotube atrophy model which was established by the treatment of C2C12
7 myotube with C26 derived CM; the results showed that 17DMAG
8 treatment prevented myotube shrinking, increased the myotube diameter,
9 and enlarged the myotube area (Fig 3A and B, Fig EV2A). Notably,
10 17DMAG treatment significantly restored the expression of MHC,
11 decreased STAT3 activation, and down-regulated the expression of
12 myostatin, MuRF-1, and Atrogin-1 both at the protein and mRNA levels
13 (Fig 3C-D and Fig EV2D,E). In addition, the colocalization of MHC and
14 myostatin and STAT3 activation were also confirmed by
15 immunofluorescence (Fig 3C). Importantly, 17DMAG disrupted the
16 increase in the binding of HSP90 and JAK2 to STAT3, suggesting that
17 the molecular chaperone activity of HSP90 might prolong the
18 JAK2-STAT3 interaction and cause constitutive STAT3 activation in C26
19 CM-induced C2C12 cells (Fig 3E). Thus, we concluded that the
20 17DMAG treatment protected C2C12 myotubes from C26 CM-induced
21 muscle atrophy and catabolism.

22 **HSP90 inhibitors ameliorated cachexia in different cachectic mice** 23 **model.**

24 To investigate the protective effect of the HSP90 inhibitor *in vivo*, C26
25 tumor-bearing cachectic mice were given the HSP90 inhibitor 17DMAG
26 (15 mg/kg, daily) or PU-H71 (50 mg/kg, daily); both drugs were well
27 tolerated by the mice. We observed that the body weights of the mice in

1 the 17DMAG and PU-H71 administration groups were consistently
 2 higher than those in the cachectic group (Fig 4A, left). We also observed
 3 that 17DMAG or PU-H71 treatment could significantly prolong the
 4 overall survival time compared to treatment with the vehicle. Treatment
 5 with 17DMAG resulted in a significant shift in the median survival time
 6 from 15.5 to 20 days (Fig 4A, right). However, PU-H71 had only a
 7 moderate effect on survival time, which was not as significant as that of
 8 17DMAG (Fig 4A, right). 17DMAG or PU-H71 treatment significantly
 9 increased the tumor-free body mass compared with the mock control
 10 treatment (Fig 4D, left). Notably, neither 17DMAG nor PU-H71
 11 treatment inhibited C26 tumor growth, excluding the possibility that the
 12 protective effects of 17DMAG or PU-H71 on body weight were based on
 13 its antitumor effect (Fig EV2B). Since 17DMAG showed greater
 14 potential for cachexia treatment than PU-H71, it was chosen for further
 15 investigation in our work.

16 In C26 tumor-bearing mice, the 17DMAG treatment significantly
 17 increased lean body mass and the mass of four major groups of skeletal
 18 muscle tibialis anterior (TA), gastrocnemius (Gastroc), soleus, and
 19 extensor digitalis longus (EDL), and restored grip strength (Fig 4B-D). In
 20 addition, inguinal white adipose tissue (iWAT), which was almost
 21 completely absent in C26 tumor-bearing mice (Fig EV2C), was
 22 significantly restored by 17DMAG treatment. 17DMAG treatment also
 23 resulted in the normalization of the levels of downregulated serum
 24 triglycerides (TG) compared with those in the mock treatment vehicle
 25 control.

26 To validate whether 17DMAG has a universal efficiency in different
 27 cancer cachexia, the LLC, another Xenograft tumor model of cancer
 28 cachexia was applied on C57BL6 mice. As previously reported, the body

mass and gastrocnemius muscle mass were decreased in tumor-bearing mice (Gallot *et al*, 2014). 17DMAG administration significantly attenuated the decrease in body mass and muscle mass (Fig EV3C), but final tumor weights of 17DMAG treated tumor-bearing mice were dramatically reduced compared with tumor-bearing control (Fig EV3B), which indicated that 17DMAG not only had a protective effect on cachexia but also inhibited tumor growth in LLC cachectic mice model.

Inhibition of HSP90 attenuated ubiquitin-proteasome pathway-mediated muscle atrophy in C26 tumor-bearing cachectic mice.

Next, we further investigated the protective effect of 17DMAG on cachectic skeletal muscle. H&E staining and morphometric analysis of the gastrocnemius revealed that C26 tumor-bearing mice exhibited an obvious decrease in muscle fiber size, which was markedly blocked by 17DMAG treatment (Fig 5A). The representative frequency distribution of the myofiber CSA showed a rightward shift in 17DMAG-treated mice (the most frequent value for the myofiber CSA was 1500-2000 μm^2) vs. C26 tumor-bearing mice (the most frequent value for the myofiber CSA was 1000-1500 μm^2) (Fig 5B). The mean CSA of the 17DMAG-treated mice was more than 40% higher than that of C26 tumor-bearing mice (Fig 5C).

We also observed that treatment with 17DMAG prevented the striking loss of MHC protein in C26 tumor-bearing mice compared with that in the vehicle control (Fig 5D-E). The significant decreases in STAT3 activation and myostatin, MuRF-1, and atrogin-1 expression in cachectic muscles in C26 tumor-bearing indicated that HSP90 blockade could alleviate muscle wasting by disrupting the STAT3-controlled ubiquitin-proteasome pathway (Fig 5D). These results were further

1 confirmed at the transcriptional level (Fig 5F). Similar results were also
2 observed in PU-H71-treated mice (Fig EV2D).

3 **STAT3-induced myotube atrophy is FOXO1 dependent.**

4 It has been reported that during muscle atrophy, ubiquitin-proteasome
5 pathway activation and the expression of MuRF-1/Atrogin-1 are
6 regulated by the transcription factor FOXO1 (Lokireddy *et al*, 2011; Stitt
7 *et al*, 2004). We, therefore, hypothesized that STAT3 might regulate
8 FOXO1 expression and activation in cachectic skeletal muscle. As shown
9 in Fig. 6a and b, the FOXO1 expression level was significantly increased
10 in cachectic muscle from C26 tumor-bearing mice, and C2C12 myotubes
11 were subjected to C26 CM treatment (Fig 6A and B, FigEV3F). The
12 decreased expression of the inactivated form of pFOXO1 in C26
13 CM-treated C2C12 myotubes was decreased when STAT3 was knocked
14 down by siRNA (Fig 6B). Next, we transfected a plasmid encoding
15 constitutively activated STAT3 (STAT3-C) (Bromberg *et al*, 1999) into
16 C2C12 myotubes. As expected, STAT3-C transfection significantly
17 upregulated the ubiquitin-proteasome pathway members MuRF-1,
18 Atrogin-1, and myostatin, which was similar to that observed upon C26
19 CM treatment. Notably, we observed that STAT3-C transfection also
20 significantly increased the level of the activated form of FOXO1
21 (dephosphorylated) by western blotting (Fig 6C). Immunofluorescence
22 staining confirmed that both STAT3-C and C26 CM were able to increase
23 the nuclear translocation of FOXO1 (Fig 6D).

24 We next sought to determine whether STAT3 could regulate FOXO1
25 transcription by direct binding of its promoter. Bioinformatics analysis
26 showed two potential STAT3 DNA binding motifs in the promoter region
27 of the FOXO1 gene (Fig 6E); therefore, chromatin immunoprecipitation
28 assays were performed. As shown in Fig 6F and G, semi-quantitative

1 PCR and qPCR analysis of the DNA immunoprecipitated with the STAT
2 antibody showed the enrichment of the FOXO1 promoter region
3 sequence, indicating direct binding. In contrast, knocking down FOXO1
4 almost completely abolished STAT3 activation-induced myotube atrophy
5 in both STAT3-C-transfected and C26 CM-treated C2C12 myotube cells
6 (Fig 6H). Our data demonstrated for the first time that in cachectic
7 skeletal muscle, consistent activation of STAT3 could activate the
8 ubiquitin-proteasome pathway in a FOXO1 pathway-dependent manner.
9

Discussion

Muscle wasting is one of the hallmarks of cancer cachexia (Penna *et al*, 2018). STAT3 signaling plays crucial role in the regulation of skeletal muscle generation, muscle mass, and repair processes. It is generally believed that transient STAT3 activation in muscles is beneficial to muscle regeneration and hypertrophy, but prolonged STAT3 activation in muscles has been shown to be responsible for muscle wasting (Zimmers *et al*, 2016). Increased STAT3 activation has been observed in skeletal muscles in diverse models of cancer cachexia, including models based on IL-6 administration, C26 colon adenocarcinoma, LLC lung carcinoma, B16 melanoma, and ApcMin/+ -induced intestinal cancer (Baltgalvis *et al*, 2008; Bonetto *et al*, 2012; Pretto *et al*, 2015; Puppa *et al*, 2014; Silva *et al*, 2015). However, the clinical association, the factors triggering abnormal STAT3 activation in cachectic muscles as well as the molecular mechanism of muscle atrophy induced by consistent STAT3 activation still need to be delineated.

Most STAT3 molecules exist in the cytoplasm in an unphosphorylated form, and their transcriptional regulatory effects rely on the phosphorylation of the tyrosine 705 residue. The upstream activators of STAT3 include IL-6 family cytokines, growth factors such as EGF, and oncogenes such as the Src family kinases (Yu *et al*, 1995; Zhong *et al*, 1994). Although the excessive release of IL-6 family cytokines, including IL-6, LIF, OSM, and CNTF, has been linked to the pathological development of cancer cachexia (Fearon, 2013). Multiple studies on STAT3 signaling have demonstrated that the stimulation of non-malignant cells with IL-6 family cytokines can trigger only transient STAT3 activation, which is quickly terminated by negative regulators

1 such as PIAS, suppressor of cytokine signaling (SOCS-1, 2, and 3), and
2 cellular phosphatases (SHP-1/2, DUSP22, PRPRD, PTPRT, and
3 PTPN1/2) (Johnson *et al*, 2018; Ram *et al*, 1997; Sengupta *et al*, 1996).
4 Therefore, it is reasonable to speculate that apart from IL-6 family
5 cytokines, other regulatory factors may also be involved in the
6 maintenance of persistent STAT3 activation in cachectic muscles in
7 cancer.

8 HSP90 is an ATP-dependent molecular chaperone; it has been reported to
9 be one of the most abundant molecules within cells, accounting for nearly
10 2% of all cellular proteins (Joshi *et al*, 2018). A wide variety of stressful
11 stimuli, including heat shock, UV radiation, and micro-organismal
12 infections, could promote intracellular HSP90 synthesis. During cancer
13 progression, the expression level of HSP90 increases by 2- to 10-fold in
14 tumor cells (Joshi *et al*, 2018). Elevated HSP90 expression has been
15 found in various types of human tumors and is often correlated with
16 increased tumor cell proliferation, poor responses to chemotherapy, and
17 poor survival (Kazarian *et al*, 2017; Ono *et al*, 2018; Whitesell *et al*, 2005;
18 Yun *et al*, 2019). Over 200 oncogenic proteins have been identified as
19 clients of HSP90 (Trepel *et al*, 2010). We and others have demonstrated a
20 direct interaction between STAT3 and HSP90 in multiple cell types,
21 including cancer cells, epithelial cells, fibroblasts, and endothelial cells
22 (Song *et al*. 2017b; Shah *et al*. 2002). Here, in this study, we found an
23 increased interaction of HSP90 and STAT3 in skeletal muscle from
24 cachexia patients and experimental mice model. Administration of
25 HSP90 inhibitors, which blocked the aberrant STAT3 activation, could
26 successfully alleviate cachexia-related muscle wasting phenotype. In
27 addition, we also observed that HSP90 inhibition in cachectic mice could
28 protect against fat loss and increase the number of muscle PAX7+

1 satellite cells (Fig EV5B). Interestingly, we revealed that the direct
 2 interaction of HSP90 with STAT3 could enhance JAK2-mediated STAT3
 3 phosphorylation, thus induce muscle atrophy. The increased
 4 HSP90-STAT3 interaction likely cause the prolonged Stat3 activation and
 5 muscle atrophy since both HSP90 inhibitor or STAT3 siRNA could
 6 successfully alleviate the pathological muscle wasting phenotype both
 7 *in-vivo* and *in-vitro*; suggesting that persistent activation of STAT3 rather
 8 than other signaling pathway is the major cause of the induction of
 9 muscle wasting in cancer cachexia.

10 An additional goal of this study was to delineate the mechanism by which
 11 the prolonged STAT3 activation induces muscle wasting. The
 12 pathogenesis of muscle wasting in cancer cachexia has been
 13 demonstrated to be the result of an imbalance between the anabolic and
 14 catabolic pathways. tThe STAT3 pathway has been found to be a crucial
 15 pathway involved in this process. During the catabolic response in
 16 skeletal muscles, prolonged STAT3 activation could disturb the
 17 sensitivity of the insulin/IGF-1/AKT signaling pathway by enhancing the
 18 expression of inhibitory SOCS proteins (Mashili *et al*, 2013; Ueki *et al*,
 19 2004). During the anabolic response, current models indicate that STAT3
 20 could enhance the expression of myostatin via activation of
 21 CAAT/enhancer-binding protein (C/EBP) (Zhang *et al*, 2013). Myostatin
 22 is a TGF-beta family cytokine that can repress muscle growth and disturb
 23 myogenesis. Our study observed the elevated expression of myostatin
 24 accompanied by prolonged STAT3 activation, which fully supports our
 25 conclusion. However, the most interesting mechanism revealed by our
 26 study is that the prolonged STAT3 activation-induced muscle wasting is
 27 the result of the overactivation of protein degradation pathways, such as
 28 the ubiquitin-proteasome system (UPS). Our data demonstrated that the

1 prolonged activation of STAT3 was correlated with elevated expression
2 of UPS-related muscle-specific E3 ubiquitin ligases, such as atrogin-1
3 and MuRF1, in skeletal muscle derived from either C26 tumor-bearing
4 cachectic mice or C2C12 myotubes treated with C26 CM. Additionally,
5 the transfection-induced overexpression of constitutively activated
6 STAT3 (STAT3-C) also triggered UPS responses, indicating that
7 constitutive activation of STAT3 itself is enough to induce muscle
8 wasting by activating protein degradation pathways such as the UPS;
9 however, the pathway linking STAT3 and the activation of the protein
10 degradation pathway was not evaluated.

11 FOXO1, as a key member of the forkhead box protein O (FOXO)
12 transcription factor family, has been found to be widely expressed in
13 various types of metabolism-associated organs, such as the liver and
14 skeletal muscles. In skeletal muscles, the FOXO1 transcription factor
15 represents a master regulator of muscle growth, regeneration, and atrophy.
16 Increased FOXO1 expression and activation has been found in skeletal
17 muscle in energy-deprived states such as fasting, cancer, and severe
18 diabetes (O'Neill *et al*, 2019; Reed *et al*, 2012). Enhanced FOXO1
19 activation in skeletal muscle was able to trigger muscle atrophy by
20 activation of the UPS and lysosomal proteolysis through the upregulation
21 of FOXO downstream target genes, including atrogin-1 and MuRF1
22 (Milan *et al*, 2015). Genetic data demonstrated that STAT3 expression
23 and activation are correlated with the intact function of FOXO
24 transcription factors; however, whether FOXO1 is necessary for
25 STAT3-induced muscle wasting or vice versa is still not known. In this
26 study, we demonstrated for the first time that STAT3 could regulate the
27 expression and nuclear translocation of FOXO1 in skeletal muscle,
28 subsequently activating the UPS and triggering muscle wasting in a

1 FOXO1-dependent manner, which could partly explain the pathological
2 mechanism by which consistent STAT3 activation in skeletal muscle
3 could trigger muscle atrophy. Since the bioinformatics study
4 demonstrated that there are several STAT3 consensus motifs in FOXO1
5 promoter regions, our subsequent ChIP analysis indicated that STAT3
6 could bind to the FOXO1 promoter and directly regulate FOXO1 gene
7 transcription in skeletal muscles. To test the pathogenic effects of STAT3
8 on muscle atrophy, a constitutively active recombinant STAT3 mutant
9 expression plasmid, STAT3-C, was adopted in our study. Our results
10 showed that the overexpression of the constitutively activated form of
11 STAT3 in skeletal muscle could mimic the effect of the C26
12 cell-conditioned medium on inducing the muscle wasting phenotype.
13 Knocking down the expression of FOXO1 in STAT3-C-transfected or
14 C26 cell-conditioned medium-treated myotubes almost completely
15 abolished the activation of the ubiquitin-proteasome system and
16 eliminated muscle wasting, suggesting that STAT3 activation-induced
17 muscle atrophy is indeed FOXO1 dependent.

18 In summary, our study demonstrated that HSP90 inhibitors could
19 effectively alleviate cancer cachexia-induced skeletal muscle wasting *in*
20 *vivo*; these effects might be associated with the abnormal activation of the
21 JAK2/STAT3/FOXO1 and ubiquitin-proteasome pathways. The close
22 monitoring of HSP90 levels in cancer patients and the development of
23 strategies to block HSP90 activity could potentially prevent or reverse
24 cachexia development.

25

1 **Materials and Methods**

2 **Cell culture**

3 The colon 26 murine adenocarcinoma cell line (C26) and Mouse colon
4 carcinoma (CT26) cells were cultured as a monolayer in RPMI-1640
5 medium (Life Technology, NY) supplemented with 10% fetal bovine
6 serum, L-glutamine, and 100 units/ml penicillin and streptomycin
7 (HyClone, Logan, UT in a humidified atmosphere containing 5% CO₂ at
8 37 °C. Murine C2C12 myoblasts obtained from the American Type
9 Culture Collection (ATCC) were maintained in Dulbecco's modified
10 Eagle medium (DMEM) supplemented with 10% fetal bovine serum and
11 antibiotics (100 U/ml penicillin and streptomycin; referred to as growth
12 medium, GM). To induce myotube differentiation, cells were grown to
13 100% confluence and exposed to DMEM containing 2% horse serum
14 (Life Technology, NY) (referred to as differentiation medium, DM) for
15 up to 4 days.

16 **Mouse model of cachexia**

17 Six-week-old male BALB/C mice (obtained from the Model Animal
18 Research Center of Nanjing University) were maintained under specific
19 pathogen-free (SPF) conditions and allowed to acclimate for one week.
20 Mice were housed in individually ventilated cages (IVCs) with free
21 access to drinking water and a basal diet under controlled conditions of
22 humidity, light (12 h light/12 h dark cycle), and temperature.

23 To induce the cachexia mouse model, 100 µl C26 murine
24 adenocarcinoma cells were subcutaneously inoculated into the right
25 flanks of BALB/C mice (5×10^5 cells/mouse). When the mice lost 20% of
26 their body weight or their tumor volumes reached 1500 mm³, the mice
27 were sacrificed. Tumors, muscles, and other organs, such as the liver and

hypothalamus, were rapidly dissected, frozen in liquid nitrogen and stored at -80°C until further analyses or fixed with 10% formaldehyde for immunohistological staining. All experiments involving animals were conducted according to protocols approved by the Institutional Animal Care and Use Committee of Nanjing University, in accordance with the guidelines for animal care and use of Jiangsu Province.

Patients biopsy specimens

The study was approved by the Ethics Committee in the Medical School of Nanjing University (IRB no.: 20200115003). All patients provided written informed consent. Cachexia was defined clinically as documented nonintentional dry weight loss of >5 kg (all $>10\%$ of their previous normal weight) at least in ≤ 3 months (10). All cachectic patients also complained of weight loss; patients' skeletal biopsy specimens of abdominal muscles were obtained after hepatobiliary surgery. Control samples were acquired from patients undergoing elective orthopedic surgery.

***In vivo* experiments and tissue collection**

To examine the protective effects of the HSP90 inhibitor on cachexia, the cachectic mice received intraperitoneal injection of 17DMAG (HY-12024, MCE, NJ) (15 mg/kg, daily) dissolved in 1% DMSO + 30% PEG300 + 1% Tween 80 or PU-H71 (HY11038, MCE, NJ) (50 mg/kg, daily) dissolved in PBS (as the solvent control) beginning on day 7 when the tumor became palpable. For the survival experiment, thirty mice were randomly divided into four groups (normal control, C26, C26 + 17DMAG, and PU-H71 groups), and each group contained 10 mice. The mice were followed until the endpoint criterion was fulfilled or at least 30 days had elapsed after the inoculation of C26 cells to determine the survival rate.

1 The endpoint criterion combined the detected weight loss with the overall
2 condition of the mouse. To evaluate the overall health status of the mice,
3 the following aspects were considered in addition to weight loss:
4 appearance, posture, and natural and provoked behavior (inactivity,
5 dyskinesia, and reduced response to external stimuli). Mice were
6 euthanized when two researchers confirmed the fulfillment of the
7 endpoint criteria.

8 To study the potential mechanisms involved, another experiment was
9 conducted with a predetermined endpoint at day 15 after C26 cell
10 inoculation. The numbers of mice and groups were the same as those
11 used in the survival experiment. The following data were collected: body
12 weight, food intake, tumor weight, grip strength, and survival rate. Once
13 the tumors were removed, the gastrocnemius was dissected, harvested,
14 weighed, and then fixed in 4% paraformaldehyde (Sigma-Aldrich, St.
15 Louis, MO) for subsequent histological analysis.

16 **Assessment of grip strength**

17 Skeletal muscular strength in mice was quantified with the grip strength
18 test. At the end of the experiment, the forelimb grip strength of each of
19 the four limbs was measured with a Digital Grip Strength Metre (Nanjing
20 Biomedical Research Institute of Nanjing University). Mice were held by
21 the tail and allowed to grasp a grid with their forepaws or with all four
22 paws. The mice were gently pulled by the tail until they released their
23 grip. Five measurements at 3 min intervals were recorded, and the
24 average value for each mouse was determined.

25 **Histological analysis and immunofluorescence**

26 Procedures for the determination of the fiber cross-sectional area (CSA)
27 were conducted as previously described (Park *et al*, 2013). Briefly, fixed

1 transverse sections (7 μ m) were cut from the mid-belly of the
2 gastrocnemius with a cryostat at -20°C and then stained with hematoxylin
3 and eosin (H&E). Images were obtained at 40 $\times$ magnification and
4 quantified using ImageJ software (National Institutes of Health, Bethesda,
5 MD). The CSA and number of myofibers with central nuclei among the
6 individual myofibers were determined by using ImageJ 1.48 software in
7 five random fields for each section.

8 For immunofluorescence analysis, cells were seeded onto sterile
9 preprocessed glass coverslips that were precoated with 1% gelatin. After
10 C2C12 myoblasts had differentiated into myotubes, the cells were washed
11 twice with PBS, followed by fixation in 4% paraformaldehyde for 15 min.
12 After being rehydrated in PBS, the cells were blocked for 30 min in 1%
13 bovine serum albumin (BSA) in PBS containing 0.2% Triton-X (PBST).
14 Then, the cells were incubated with an anti-myosin heavy chain (MHC)
15 (#MAB4470, R&D Systems, MN) (1:100) in 1% BSA/PBST overnight at
16 4°C. Cells were then incubated with a fluorescence-labeled secondary
17 anti-mouse antibody (1:1000) and DAPI (1:1000) at room temperature for
18 1 h. The specimens were examined under an FV10i laser scanning
19 confocal microscope (Olympus, Tokyo, Japan).

20 **Western blot and immunoprecipitation assays**

21 Tissues and cell proteins were extracted in ice-cold RIPA lysis buffer
22 consisting of 150 mM NaCl, 50 mM Tris-HCl, 0.5% sodium
23 deoxycholate, 200 mM NaF, 200 mM PMSF, 1.0% NP40, 1 mM EDTA,
24 and protease and phosphatase inhibitor cocktail (Life Technology, NY).
25 The protein concentration in the supernatants was determined with a BCA
26 Kit (Pierce, Rockford, IL). Then, 20 μ g of total protein was separated by
27 electrophoresis in a 10% SDS polyacrylamide gel and transferred to a
28 PVDF membrane (Millipore, Billerica, MA) for western blot analysis.

1 The signals were detected with the following antibodies by following
 2 standard procedures: STAT3 (#9145,CST, MA), phosphorylated STAT3
 3 (#9139,CST, MA), HSP90 (ab13492, Abcam,MA), myostatin (ab71808 ,
 4 Abcam, MA), CD63 (ab8219, Abcam, MA), atrogen-1 (sc166806, Santa
 5 Cruz, MA), MuRF-1 (sc398608, Santa Cruz, TX), MHC (#MAB4470,
 6 R&D Systems,MN), GAPDH (ab181602, Abcam, MA).

7 For the immunoprecipitation assays, cells were lysed in WB/IP buffer,
 8 and proteins were immunoprecipitated with the indicated antibodies.
 9 Precleared Protein A/G Plus-Agarose beads (Life Technology, New York,
 10 NY) were incubated with the immunocomplexes overnight and washed
 11 three times with lysis buffer. The immunoprecipitates were subjected to
 12 SDS-PAGE followed by immunoblotting.

13 **Transfection of small interfering RNAs (siRNAs) and overexpression** 14 **plasmids**

15 siRNA transfection was performed using Lipofectamine RNAiMAX
 16 transfection reagent (Life Technology, New York, NY) by following the
 17 manufacturer's guidelines. Briefly, C2C12 cells were seeded into a
 18 six-well plate. After four days of differentiation, 5 pmol of siRNA was
 19 diluted with Opti-MEM and mixed with the transfection reagent, and the
 20 mixture was added to each well. After transfection for 24 h, the cells were
 21 challenged with different treatments; some were co-cultured with C26
 22 cell-conditioned media (C26 CM), and some were cultured in DMEM.
 23 The siRNAs specific to HSP90aa1 were purchased from Thermo
 24 (#159050, Life Technology, New York, NY). The siRNAs specific to
 25 FOXO1 were purchased from Santa Cruz (#56458, Santa Cruz, TX).

26 The constitutively activated STAT3 plasmids (STAT3-C Flag pRc/CMV)
 27 were purchased from Addgene (Plasmid #8722). C2C12 cells were

transiently transfected with STAT3-C plasmids using the Lipofectamine™ 2000 Transfection Reagent according to the manufacturer's instructions (Life Technology, New York, NY).

Chromatin immunoprecipitation (ChIP) analysis

The ChIP assay was performed according to the manufacturer's instructions (P2078, Beyotime, China). C2C12 cells were fixed with 1% formaldehyde at 37°C for 10 min, lysed, and sonicated. Soluble chromatin was coimmunoprecipitated with anti-STAT3 antibodies or an equal amount of rabbit IgG. The immunoprecipitated and input DNA were subjected to PCR and qPCR using FOXO1 promoter site-specific primers (5'-CGACTTCAACACCTCATCGCTTC-3' and 5'-AGGCGCGCAGATCCTTCGGTGA-3'). Q-PCR was performed with Power SYBR Green PCR Master Mix (Life Technology, New York, NY). The fold change in enrichment relative to the input DNA was calculated using the comparative Ct method ($\Delta\Delta C_t$).

Real-time PCR assay

Total RNA from either snap-frozen muscles or cultured cells was isolated using an RNeasy Micro Kit (Qiagen, Hilden, Germany). The mRNA was reverse-transcribed into cDNA with PrimeScript RT Master Mix (TaKaRa, Otsu, Japan) and subjected to quantitative real-time PCR with SYBR Green PCR Master Mix (Life Technology, New York, NY). The relative expression of the target genes was normalized to that of GAPDH and calculated with the $\Delta\Delta C_t$ method. The following primers were used: MuRF-1: forward: 5'-ACCTGCTGGTGGAAAACATC-3', reverse: 5'-AGGAGCAAGTAGGCACCTCA-3'; Atrogin-1: forward: 5'-ATTCTACACTGGCAGCAGCA-3', reverse: 5'-TCAGCCTCTGCATGATGTTC-3'; GAPDH: forward:

1 5'-ACCCTTAAGAGGGATGCTGC-3', reverse:

2 5'-CGGGACGAGGAAACACTCTC-3'.

3 **Clinical data mining**

4 Data from The Cancer Genome Atlas (TCGA), including gene expression
5 data and patient survival data, were downloaded from the TCGA web
6 server with GDC Client software. For HSP90AA1 expression analysis,
7 cancer patient data were obtained from TCGA, and gene expression data
8 for normal tissue were obtained from the Genome Tissue Expression
9 (GTEx) portal.

10 **Statistical analysis**

11 The tissue weights of different groups were compared using Student's
12 t-test. Longitudinal body weight or muscle mass differences were
13 analyzed by repeated measurement analysis of variance (ANOVA). The
14 survival rate difference was determined with the chi-square test. P values
15 < 0.05 were considered statistically significant.

16

1 **Author contributions**

2 M.N, S.S, and Z.S designed and performed the experiments, analyzed
3 data, and drafted the manuscript. W. P and C.Z. performed a part of the
4 animal experiments. L.W, L.L, and Y.D performed a part of the *in vitro*
5 experiments. W.C, Q. G, and H.W conceived the projects, designed the
6 experiments, analyzed data, and wrote the manuscript. All authors read
7 and approved the final manuscript.

8 **Conflict of interest**

9 The authors declare no conflict of interest.

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References:

- Baltgalvis KA, Berger FG, Pena MM, Davis JM, Muga SJ, Carson JA (2008) Interleukin-6 and cachexia in ApcMin/+ mice Am J Physiol Regul Integr Comp Physiol 294:R393-R401 doi:10.1152/ajpregu.00716.2007
- Bilodeau PA, Coyne ES, Wing SS (2016) The ubiquitin proteasome system in atrophying skeletal muscle: roles and regulation Am J Physiol Cell Physiol 311:C392-C403 doi:10.1152/ajpcell.00125.2016
- Bodine SC et al. (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy Science 294:1704-1708 doi:10.1126/science.1065874
- Bonetto A et al. (2012a) JAK/STAT3 pathway inhibition blocks skeletal muscle wasting downstream of IL-6 and in experimental cancer cachexia Am J Physiol Endocrinol Metab 303:E410-E421 doi:10.1152/ajpendo.00039.2012
- Bonetto A et al. (2012b) JAK/STAT3 pathway inhibition blocks skeletal muscle wasting downstream of IL-6 and in experimental cancer cachexia Am J Physiol Endocrinol Metab 303:E410-E421 doi:10.1152/ajpendo.00039.2012
- Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JJ (1999) Stat3 as an oncogene Cell 98:295-303 doi:10.1016/S0092-8674(00)81959-5
- Fearon KH (2013) Selective androgen receptor modulators in cancer cachexia? Lancet Oncol 14:271-272 doi:10.1016/S1470-2045(13)70068-8
- Gallot YS et al. (2014) Myostatin Gene Inactivation Prevents Skeletal Muscle Wasting in Cancer Cancer Res 74:7344-7356 doi:10.1158/0008-5472.CAN-14-0057
- Guadagnin E, Mazala D, Chen YW (2018) STAT3 in Skeletal Muscle Function and Disorders Int J Mol Sci 19 doi:10.3390/ijms19082265
- Guo D, Wang C, Wang Q, Qiao Z, Tang H (2017) Pantoprazole blocks the JAK2/STAT3 pathway to alleviate skeletal muscle wasting in cancer cachexia by inhibiting inflammatory response Oncotarget 8:39640-39648 doi:10.18632/oncotarget.17387
- Johnson DE, O'Keefe RA, Grandis JR (2018) Targeting the IL-6/JAK/STAT3 signalling axis in cancer Nat Rev Clin Oncol 15:234-248 doi:10.1038/nrclinonc.2018.8
- Joshi S, Wang T, Araujo T, Sharma S, Brodsky JL, Chiosis G (2018) Adapting to stress - chaperome networks in cancer Nat Rev Cancer 18:562-575 doi:10.1038/s41568-018-0020-9
- Kazarian A et al. (2017) Testing breast cancer serum biomarkers for early detection and prognosis in pre-diagnosis samples Brit J Cancer 116:501-508 doi:10.1038/bjc.2016.433
- Loberg RD, Bradley DA, Tomlins SA, Chinnaiyan AM, Pienta KJ (2007) The lethal phenotype of cancer: the molecular basis of death due to malignancy CA Cancer J Clin 57:225-241
- Lokireddy S, Mouly V, Butler-Browne G, Gluckman PD, Sharma M, Kambadur R, McFarlane C (2011) Myostatin promotes the wasting of human myoblast cultures through promoting ubiquitin-proteasome pathway-mediated loss of sarcomeric proteins Am J Physiol Cell Physiol 301:C1316-C1324 doi:10.1152/ajpcell.00114.2011
- Ma JF, Sanchez BJ, Hall DT, Tremblay AK, Di Marco S, Gallouzi IE (2017) STAT3 promotes IFNgamma/TNFalpha-induced muscle wasting in an NF-kappaB-dependent and IL-6-independent manner Embo Mol Med 9:622-637 doi:10.15252/emmm.201607052
- Mahendrarajah N et al. (2017) HSP90 is necessary for the ACK1-dependent phosphorylation of STAT1 and STAT3 Cell Signal 39:9-17 doi:10.1016/j.cellsig.2017.07.014

- 1 Mashili F, Chibalin AV, Krook A, Zierath JR (2013) Constitutive STAT3 phosphorylation contributes
- 2 to skeletal muscle insulin resistance in type 2 diabetes *Diabetes* 62:457-465 doi:10.2337/db12-0337
- 3 Milan G et al. (2015) Regulation of autophagy and the ubiquitin-proteasome system by the FoxO
- 4 transcriptional network during muscle atrophy *Nat Commun* 6:6670 doi:10.1038/ncomms7670
- 5 Mubaid S et al. (2019) HuR counteracts miR-330 to promote STAT3 translation during
- 6 inflammation-induced muscle wasting *Proc Natl Acad Sci U S A* 116:17261-17270
- 7 doi:10.1073/pnas.1905172116
- 8 O'Neill BT et al. (2019) FoxO Transcription Factors Are Critical Regulators of Diabetes-Related
- 9 Muscle Atrophy *Diabetes* 68:556-570 doi:10.2337/db18-0416
- 10 Ono K et al. (2018) HSP-enriched properties of extracellular vesicles involve survival of metastatic
- 11 oral cancer cells *J Cell Biochem* 119:7350-7362 doi:10.1002/jcb.27039
- 12 Park BS, Henning PC, Grant SC, Lee WJ, Lee SR, Arjmandi BH, Kim JS (2013) HMB attenuates
- 13 muscle loss during sustained energy deficit induced by calorie restriction and endurance exercise
- 14 *Metabolism* 62:1718-1729 doi:10.1016/j.metabol.2013.06.005
- 15 Penna F, Ballaro R, Beltra M, De Lucia S, Costelli P (2018) Modulating Metabolism to Improve
- 16 Cancer-Induced Muscle Wasting *Oxid Med Cell Longev* 2018:7153610 doi:10.1155/2018/7153610
- 17 Pretto F et al. (2015) Sunitinib prevents cachexia and prolongs survival of mice bearing renal cancer by
- 18 restraining STAT3 and MuRF-1 activation in muscle *Oncotarget* 6:3043-3054
- 19 doi:10.18632/oncotarget.2812
- 20 Prinsloo E, Kramer AH, Edkins AL, Blatch GL (2012) STAT3 interacts directly with Hsp90 *Iubmb*
- 21 *Life* 64:266-273 doi:10.1002/iub.607
- 22 Puppa MJ, Gao S, Narsale AA, Carson JA (2014) Skeletal muscle glycoprotein 130's role in Lewis
- 23 lung carcinoma-induced cachexia *Faseb J* 28:998-1009 doi:10.1096/fj.13-240580
- 24 Ram PA, Waxman DJ (1997) Interaction of growth hormone-activated STATs with SH2-containing
- 25 phosphotyrosine phosphatase SHP-1 and nuclear JAK2 tyrosine kinase *J Biol Chem* 272:17694-17702
- 26 doi:10.1074/jbc.272.28.17694
- 27 Reed SA, Sandesara PB, Senf SM, Judge AR (2012) Inhibition of FoxO transcriptional activity
- 28 prevents muscle fiber atrophy during cachexia and induces hypertrophy *Faseb J* 26:987-1000
- 29 doi:10.1096/fj.11-189977
- 30 Schoof N, von Bonin F, Trumper L, Kube D (2009) HSP90 is essential for Jak-STAT signaling in
- 31 classical Hodgkin lymphoma cells *Cell Commun Signal* 7:17 doi:10.1186/1478-811X-7-17
- 32 Schopf FH, Biebl MM, Buchner J (2017) The HSP90 chaperone machinery *Nat Rev Mol Cell Bio*
- 33 18:345-360 doi:10.1038/nrm.2017.20
- 34 Sengupta TK, Schmitt EM, Ivashkiv LB (1996) Inhibition of cytokines and JAK-STAT activation by
- 35 distinct signaling pathways *Proc Natl Acad Sci U S A* 93:9499-9504 doi:10.1073/pnas.93.18.9499
- 36 Shah M, Patel K, Fried VA, Sehgal PB (2002) Interactions of STAT3 with caveolin-1 and heat shock
- 37 protein 90 in plasma membrane raft and cytosolic complexes. Preservation of cytokine signaling during
- 38 fever *J Biol Chem* 277:45662-45669 doi:10.1074/jbc.M205935200
- 39 Silva KA et al. (2015) Inhibition of Stat3 activation suppresses caspase-3 and the ubiquitin-proteasome
- 40 system, leading to preservation of muscle mass in cancer cachexia *J Biol Chem* 290:11177-11187
- 41 doi:10.1074/jbc.M115.641514
- 42 Song S et al. (2017b) Luteolin selectively kills STAT3 highly activated gastric cancer cells through
- 43 enhancing the binding of STAT3 to SHP-1 *Cell Death Dis* 8:e2612 doi:10.1038/cddis.2017.38
- 44 Stitt TN et al. (2004) The IGF-1/PI3K/Akt Pathway Prevents Expression of Muscle Atrophy-Induced

- 1 Ubiquitin Ligases by Inhibiting FOXO Transcription Factors Mol Cell 14:395-403
- 2 doi:[https://doi.org/10.1016/S1097-2765\(04\)00211-4](https://doi.org/10.1016/S1097-2765(04)00211-4)
- 3 Tanaka Y et al. (1990) Experimental cancer cachexia induced by transplantable colon 26
- 4 adenocarcinoma in mice Cancer Res 50:2290-2295
- 5 Trepel J, Mollapour M, Giaccone G, Neckers L (2010) Targeting the dynamic HSP90 complex in
- 6 cancer Nat Rev Cancer 10:537-549 doi:10.1038/nrc2887
- 7 Ueki K, Kondo T, Kahn CR (2004) Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause
- 8 insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins
- 9 by discrete mechanisms Mol Cell Biol 24:5434-5446 doi:10.1128/MCB.24.12.5434-5446.2004
- 10 Whitesell L, Lindquist SL (2005) HSP90 and the chaperoning of cancer Nat Rev Cancer 5:761-772
- 11 doi:10.1038/nrc1716
- 12 Yu CL, Meyer DJ, Campbell GS, Lerner AC, Carter-Su C, Schwartz J, Jove R (1995) Enhanced
- 13 DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein Science
- 14 269:81-83 doi:10.1126/science.7541555
- 15 Yun CW, Kim HJ, Lim JH, Lee SH (2019) Heat Shock Proteins: Agents of Cancer Development and
- 16 Therapeutic Targets in Anti-Cancer Therapy Cells-Basel 9 doi:10.3390/cells9010060
- 17 Zhang L, Pan J, Dong Y, Tweardy DJ, Dong Y, Garibotto G, Mitch WE (2013) Stat3 activation links a
- 18 C/EBPdelta to myostatin pathway to stimulate loss of muscle mass Cell Metab 18:368-379
- 19 doi:10.1016/j.cmet.2013.07.012
- 20 Zhong Z, Wen Z, Darnell JJ (1994) Stat3: a STAT family member activated by tyrosine
- 21 phosphorylation in response to epidermal growth factor and interleukin-6 Science 264:95-98
- 22 doi:10.1126/science.8140422
- 23 Zimmers TA, Fishel ML, Bonetto A (2016) STAT3 in the systemic inflammation of cancer cachexia
- 24 Semin Cell Dev Biol 54:28-41 doi:10.1016/j.semcdb.2016.02.009

Figure Legends

Figure 1. Enhanced HSP90-STAT3 interaction induced prolonged STAT3 activation were detected in the skeletal muscle from cancer cachectic patient and C26 tumor-bearing cachectic mice.

The expression levels of Atrogin-1, MuRF-1, MHC, HSP90, Myostatin, and phosphorylated STAT3 in the skeletal muscle from cancer cachectic patients were detected by qRT-PCR (A. For atrogin-1 and MuRF-1) and Western blot (B), the bands were quantified by densitometry and normalized to the density of GAPDH (C). Measurement of the HSP90-STAT3 interaction in cachectic muscle cancer cachectic patients by immunoprecipitation (D). The expression levels of Atrogin-1, MuRF-1, MHC, HSP90, Myostatin and phosphorylated STAT3 in the skeletal muscle from C26 tumor-bearing cachectic mice were detected by qRT-PCR (E. For atrogin-1 and MuRF-1) and Western blot (F), the bands were quantified by densitometry and normalized to the density of GAPDH (G).

Figure 2. Knocking down of HSP90 or STAT3 prevented C2C12 myotubes from C26 CM-induced atrophy.

The expression levels of Atrogin-1, MuRF-1, MHC, HSP90, Myostatin, and phosphorylated STAT3 in the skeletal muscle from C26 CM treated C2C12 myotubes were detected by western blot (A), the bands were quantified by densitometry and normalized to the density of GAPDH (B). The lysates of C2C12 cells treated with C26 CM or DMEM (control) were immunoprecipitated with the STAT3 antibody, the HSP90 antibody, or control rabbit IgG. The immunopellets were detected by immunoblot analysis with the anti-HSP90 antibody and the anti-STAT3 antibody (C). Representative hematoxylin and eosin (HE)-stained C2C12 treated with C26 CM and transfected with the HSP90 siRNA or the STAT3 siRNA (D). The histogram represents the average myotube diameter (μm) (E). qRT-PCR analysis of atrogin-1 and MuRF-1 in the gastrocnemius muscles of mice (F). Atrophy markers in C2C12 were tested by WB analysis when the expression of HSP90 or STAT3 was knocked down by siRNA (G). Data represent the mean $\pm$ standard deviation (SD). * $p < 0.05$, ** $p < 0.01$ denotes compared to control; # $p < 0.05$, ## $p < 0.01$ denotes compared to C26

CM treated myotubes.

Figure 3. Administration of HSP90 inhibitor 17DMAG prevented C26 CM-induced myotube atrophy and disrupted the binding between HSP90 or JAK2 with STAT3.

A. C2C12 myotubes were treated with C26 CM, C26 CM plus HSP90 inhibitor 17DMAG (0.1 μmol), or normal media for 24 h. H&E staining shows the morphological changes in C2C12 myotubes. Scale bar: 50 μm . **B.** The histogram represents the average myotube diameter (μm) and myotube area (μm^2) in control, CM-treated, and 17DMAG plus CM-treated C2C12 myotubes. The number of myotubes was quantified using ImageJ. **C** C2C12 cells were immunostained with the MHC (MF20) antibody. MHC, green, DAPI, blue. The expression and subcellular localization of myostatin and pY-STAT3 in C2C12 myotubes were detected by immunofluorescence staining. Myostatin and pY-STAT3, green, PI, blue. Scale bar: 30 μm . **D** The expression of MHC, pY-STAT3, atrogen-1, and MuRF-1 in control, CM-treated, and 17DMAG plus CM-treated C2C12 myotubes was analyzed by western blot, GAPDH served as a loading control. **E** The lysates of C2C12 cells treated with C26 CM or DMEM (control) were immunoprecipitated with the STAT3 antibody, the HSP90 antibody, or control rabbit IgG. The immunopellets were detected by immunoblot analysis with the anti-HSP90 antibody and the anti-STAT3 antibody. Data represent the mean $\pm$ standard deviation (SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ denotes compared to control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ denotes compared to C26 CM treated myotubes.

Figure 4. The protective effects of 17DMAG in cachectic muscle wasting in C26 tumor-bearing mice.

A Left part: Bodyweight changes in normal control mice, C26 tumor-bearing mice, and 17DMAG or PU-H71-treated C26 tumor-bearing mice. Day 0: tumor implantation. Day 7: 17DMAG injection began. Day 15: Terminal sacrifice day. Right part: Survival rates of C26-tumor-bearing mice that received the treatment of 17DMAG or PU-H71 and vehicle control. **B** Representative images of the hindlimbs of normal (left), C26 tumor-bearing (middle), and 17DMAG-treated C26

tumor-bearing (right) mice. **C** Grip strength was measured on day 14. **D** Tumour-free body mass, lean mass weight, and the tibialis anterior (TA), gastrocnemius(Gastroc), soleus, and extensor digitorum longus (EDL) muscles were weighed and compared after dissection with an electronic scale; Data represent the mean $\pm$ standard deviation (SD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ denotes 17DMAG vs. C26; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ denotes C26 vs. control.

Figure 5. Protective Effects of 17DMAG on fiber cross-sectional area, STAT3 activation, and catabolic pathway suppression.

A Representative hematoxylin and eosin (H&E) staining showing the morphological changes in the gastrocnemius. Scale bar: 100 μ m. **B** Fibre size distribution. **C** Mean fibre cross-sectional area is shown by the χ^2 analysis (for CSA); * denotes a significant difference ($P < 0.05$) compared with normal control; # denotes a significant difference ($P < 0.05$) compared with C26 mice. **D** 17DMAG treatment altered the expression of myosin heavy chain (MHC), MuRF-1, atrogin-1, and pY-STAT3 in cachectic skeletal muscles from C26 tumor-bearing mice, which were detected by western blot analysis. **E** Gastrocnemius MHC expression was evaluated by immunofluorescence staining. MHC, green, DAPI, blue. Scale bar: 100 μ m. **F** Total RNA was extracted from the gastrocnemius from control, 17DMAG-treated, and C26tumor-bearing mice, and the expression levels of MuRF-1 and atrogin-1 in the gastrocnemius were measured by quantitative RT-PCR. Data represent the mean $\pm$ SD. *** $p < 0.001$, compared with normal control; # $p < 0.05$, compared with C26 mice.

Figure 6. STAT3 regulated FOXO1 expression is responsible for C26 CM-induced myotube atrophy.

A Measurement of FOXO1 expression of mice muscle and C2C12 myotubes by Western blot. **B** Representative Western blot assay of FOXO1 expression after knocking down of STAT3 by siRNA. **C** Representative Western blot of FOXO1, p-FOXO1, and atrophy genes expression in C2C12 myotube transfected with mutated forms (constitutive activated) of STAT3 overexpression plasmid (Stat3-C). **D** Representative immunofluorescence microscopy images showing the subcellular localization of FOXO1 in C2C12. **E** Schematic structure of the FoxO1 promoter

region (NCBI Reference Sequence: NC_000069.5 for FoxO1). Transcription start site (TSS) is denoted by a black arrow. **F-G** Chromatin immunoprecipitation (CHIP) analysis was performed with C2C12, and STAT3 binding to the FoxO1 promoter region was analyzed. Cell lysates were immunoprecipitated with anti-STAT3 Ab or control IgG. Immunoprecipitated and input DNA was analyzed by semi-quantitative PCR (F) or qPCR (G) using primers corresponding to FoxO1 promoter sites. **H** Representative Western blot assay of FOXO1, p-FOXO1, and atrophy genes expression in C2C12 myotube co-transfected with FOXO1 siRNA and STAT3-C recombinant plasmid.

Figure 7. Schematic model of the signaling mechanism of this study.

Expanded View Figure legends

Figure EV1

A. Body weight change of C26 tumor-bearing mice compared with non-tumor bearing control. **B.** Co-immunoprecipitation assay of HSP90 and STAT3 in skeletal muscles from normal and C26 cachectic mice with or without 17DMAG treatment. **C.** Phase-contrast images of C2C12 myoblasts and myotubes; Detection of MyoD expression in myoblasts and myotubes by western blot analysis.

Figure EV2

A. Phase-contrast images of C2C12 cells treated with C26 CM or 17DMAG and the non-CM-treated control. **B.** Final tumor weights of 17DMAG-treated and C26 tumor-bearing mice were measured at necropsy. **mC.** Inguinal white adipose tissue (iWAT); iWAT weight and serum TG level. **D.** Representative western blot of MHC, pY-STAT3, atrogin-1/MAFbx and MuRF-1 in muscle and C2C12 treated by PU-H71. Data represent the mean $\pm$ SD. **p<0.01; ***p<0.001; ****p<0.0001.

Figure EV3

A. Time course change of tumor free body weights of 17DMAG-treated and LLC tumor-bearing mice. **B.** Final tumor weights of 17DMAG-treated and LLC tumor-bearing mice were measured at necropsy. **C.** Tumor free body weights of 17DMAG-treated and LLC tumor-bearing mice. **D.** Weight change of gastrocnemius Muscle 17DMAG-treated and LLC tumor-bearing mice. **E.** Representative H&E staining showing the morphological changes in the gastrocnemius Muscle. Scale bar: 100 μm. **F.** Transcription level of FOXO1 in C26 mice muscle was detected by qPCR. **G.** PAX7 immunofluorescence revealed the activation of satellite cells in skeletal muscle from the mouse groups described above. PAX7: green; DAPI: blue; scale bar: 30 μm. Data represent the mean ± SD. **p<0.01