Combined Androgen Administration and HDAC Inhibition in Experimental Cancer Cachexia

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ABSTRACT

Background

Cancer cachexia impacts the majority of advanced cancer patients but no approved anti-cachexia therapeutic exits. Recent late stage clinical failures of anabolic anti-cachexia therapy revealed heterogeneous responses to anabolic therapies and a limited ability to translate improved body composition into functional benefit. It is currently unclear what governs anabolic responsiveness in cachectic patient populations.

Methods

We evaluated anabolic androgen therapy combined with the novel anti-cachectic histone deacetylase inhibitor (HDACi) AR-42 in a series of studies using the C-26 mouse model of experimental cachexia. The ability of treatment to suppress tumor-mediated catabolic signaling and promote anabolic effects were characterized.

Results

Anabolic anti-cachexia monotherapy with the selective androgen receptor modulator (SARM) GTx-024 or enobosarm had no impact on cachectic outcomes in the C-26 model. A minimally effective dose of AR-42 provided mixed anti-cachectic benefits when administered alone but when combined with GTx-024 significantly improved bodyweight ($p < 0.0001$), hind limb muscle mass ($p < 0.05$), and voluntary grip strength ($p < 0.0001$) versus tumor bearing controls. Similar efficacy resulted from the combination of AR-42 with multiple androgens. Anti-cachectic efficacy was associated with the ability to reverse pSTAT3 and atrogene induction in gastrocnemius muscle of tumor-bearing animals in the absence of treatment-mediated changes in serum IL-6 or LIF.

Conclusions

Anabolic GTx-024 monotherapy is incapable of overcoming catabolic signaling in the C-26 model of experimental cachexia. Anti-cachectic androgen therapy is greatly improved by successful blockade of STAT3 mediated atrophy with AR-42. Combined androgen and HDAC inhibitor administration represents promising approach to improve anabolic response in cachectic patient populations.

KEYORDS: cachexia, androgen, HDAC inhibitor, AR-42, GTx-024, Enobosarm, STAT3, C26

INTRODUCTION

Cancer cachexia is a multifactorial syndrome characterized by the involuntary loss of muscle mass occurring with or without concurrent losses in adipose tissue [1]. Cachexia is distinct from simple starvation in that it is not reversible with nutritional support and the accompanying progressive loss of lean mass is associated with decreased quality of life, decreased tolerance of chemotherapy and reduced overall survival [2]. It is estimated that 50-80% of all cancer patients experience cachexia symptoms and up to 20% of all cancer related deaths are attributable to complications arising from cachexia-mediated functional decline [3, 4]. A multitude of tumor and host factors are recognized as contributing to the multi-organ system dysfunction in cancer cachexia which presents a considerable therapeutic challenge. Diverse cachexia treatment strategies have been evaluated in patients with few offering effective palliation and none gaining FDA approval for this devastating consequence of advanced malignancy [5]. Among the complex sequelae associated with cachectic progression, compromised muscle function associated with reduced muscle mass is viewed as a primary contributor to patient morbidity and mortality [2, 6]. Recognizing this feature of cancer cachexia, regulatory agencies require the demonstration of meaningful improvements in physical function in addition to improvements in patient body composition for successful registration of novel cachexia therapies [7]. Anabolic androgenic steroids or steroidal androgens are among the most well recognized function-promoting therapies [8-10] and as such have been extensively evaluated in muscle wasting of diverse etiology [11-15]. Despite meeting FDA approval criteria in other wasting diseases [16], steroidal androgens are yet to demonstrate clinical benefit in cancer cachexia [5, 17, 18]. The continued pursuit of novel androgens for the treatment of wasting diseases suggests confidence in this therapeutic strategy remains [5].

In addition to their well characterized anabolic effects on skeletal muscle, steroidal androgens elicit a number of undesirable virilizing side effects and can promote prostatic hypertrophy which limits their wide spread clinical use [8, 19, 20]. Recently developed, non-

steroidal, selective androgen receptor modulators (SARMs) offer a number of improvements over steroidal androgens including prolonged plasma exposures and oral bioavailability [21, 22] with greatly reduced side effects (virilization, etc.) while maintaining full agonism in anabolic tissues like skeletal muscle [19, 23, 24]. With once daily dosing, the SARM GTx-024 (Enobosarm) showed promising gains in fat-free mass in both male and female cancer patients [25] but ultimately failed to demonstrate a clear functional benefit in pivotal Phase III trials in a cachectic non-small cell lung cancer (NSCLC) population [26]. GTx-024 has a strong safety profile and proven effects on skeletal muscle but is no longer being developed for cancer cachexia [27].

Hypogonadism is a feature of advanced malignancy and experimental cachexia leading to a worsening of multiple cachectic sequelae including decreased skeletal muscle mass [28, 29]. Though the relationship between androgen status and body composition is well established, the exact molecular means by which androgens modulate skeletal muscle mass is complex and poorly understood. The direct stimulation of muscle precursor cells, reduced expression of several atrogenes, and indirect hypertrophic signaling through multiple pathways all appear to play a role [30]. Strong evidence supports the importance of indirect IGF-PI3K-Akt cross-talk as muscle stem cell-specific androgen receptor knock-out (ARKO) animals have reduced skeletal muscle mass but respond to both orchiectomy and administration of GTx-024 [31]. Furthermore, skeletal muscle atrophy induced by either orchiectomy or glucocorticoid administration is associated with suppression of the IGF-PI3K-Akt pathway that is reversible by androgen administration [32, 33]. Androgens have also been directly linked to myostatin signaling which itself has a well characterized role in governing skeletal muscle size [34, 35]

We recently demonstrated the effectiveness of a novel class I/IIB HDAC inhibitor (HDACi, AR-42), currently under clinical evaluation in hematologic malignancy [36], as anti-cachexia therapy in the C-26 mouse model of cancer cachexia [37]. AR-42 administration completely spared body weight and was associated with improvements, but not complete

rescue, of skeletal muscle mass relative to controls. Mechanistic studies revealed a number of potentially causative metabolic changes in addition to complete suppression of tumor-induced muscle-specific E3-ligase expression (Atrogin-1 and MuRF1). AR-42 differed from other approved HDACi's in its ability to fully suppress tumor-mediated ligase induction and prolong survival in the C-26 model. However, AR-42's effects on skeletal muscle mass and, to a lesser degree, muscle function diminished with delayed treatment suggesting AR-42's anti-catabolic activity is central to its anti-cachectic activity and its ability to restore muscle mass once lost is limited. We hypothesized that the mechanisms underlying an established anabolic therapy and a novel anti-catabolic therapy may be sufficiently distinct to allow for improved overall efficacy when combined. To this end, we evaluated the effectiveness of androgen administration when combined with AR-42 in the C-26 model of cancer cachexia.

METHODS

Reagents and chemicals

GTx-024 was synthesized as previously described [38], AR-42 was generously provided by Arno Therapeutics and TFM-4-AS-1 (Sigma Aldrich, Saint Louis, MO) and dihydrotestosterone (DHT; Steraloids, Newport, RI) were purchased from commercial sources.

Vehicle components included: Captex (Abitec, Columbus, OH), Tween 20 (Sigma Aldrich, Saint Louis, MO), benzyl alcohol (Sigma Aldrich, Saint Louis, MO), sesame oil (Sigma Aldrich, Saint Louis, MO). AR-42 was formulated in 0.5% methylcellulose [w/v] and 0.1% Tween-80 [v/v] in sterile water. GTx-024[39], DHT and TFM-4AS-1[40] were formulated as previously described. Remaining reagents were all purchased from Sigma-Aldrich (Saint Louis, MO) unless otherwise mentioned.

In vitro HDAC inhibition assays

HDAC activity was measured by a commercial vendor using human recombinant HDAC enzymes and fluorogenic HDAC substrates (Eurofins Cerep SA, Celle L'Evescault, France). Substrate concentrations ranged from 20 - 400 µM and incubation conditions ranged from 10 - 90 min (RT or 37 °C), depending on isoform. Results are expressed as percent inhibition of control specific activity (Tricostatin A).

Animal studies using the C-26 colon adenocarcinoma cachexia model

All animal studies were conducted according to protocols approved by The Ohio State University Institutional Animal Care and Use Committee as previously described [37] with the following modifications. AR-42 dose-response study: Male CDF1 mice were randomized by body weight into 5 groups (n= 4-5). Animals in four of the groups received subcutaneous injections of C-26 cells, while the fifth group, serving as tumor-free control, was injected with sterile saline. Six days later, tumor-bearing animals were treated orally with AR-42 once daily at 10 and 20 mg/kg, and every other day at 50 mg/kg by gavage under light anesthesia, for 13 days. Upon sacrifice on study day 18, when the majority of tumor-bearing control mice met euthanasia criteria, the left gastrocnemius muscle was excised and flash frozen in liquid nitrogen and stored at -80ºC for subsequent analyses. Initial Combination Study (Study 1): Starting on the sixth day after injection with C-26 tumor cells or saline, tumor-bearing groups (n=5-6) were treated twice daily (p.o.) with (a) vehicles (a.m., AR-42 vehicle; p.m., GTx-024 vehicle), (b) GTx-024 (p.m., 15 mg/kg; a.m., AR-42 vehicle), (c) AR-42 (a.m., 10 mg/kg; p.m., GTx-024 vehicle), or (d) Combination (a.m., AR-42, 10 mg/kg; p.m., GTx-024, 15 mg/kg) for 13 days. An additional tumor-free group received 15 mg/kg GTx-024 alone. Body weight, tumor volume, and feed consumption were monitored every other day. Upon sacrifice on study day 18, serum was collected and hind limb skeletal muscles, heart, spleen and epididymal adipose tissue were harvested, weighed, and flash frozen and stored for subsequent analyses. Tumor volumes were calculated from caliper measurements using a standard formula (length x width^2 x

π/6) and a tumor density equivalent to water (1 g/cm^3) assumed to correct carcass weights for tumor weight. Confirmatory Combination Study (Study 2): This confirmatory study was performed exactly as Study 1 with further expanded animal numbers (n=6 for tumor-free and tumor-bearing control groups; n=10 for tumor-bearing treated groups). Due to rapid model progression, this study was terminated after only 12 days of treatment. Combined Androgen and AR-42 Study (Study 3): This study was performed as Study 2, but with additional tumor-bearing controls (n=9) and different androgens. On day six after C-26 cell injection, tumor-bearing animals were treated with (a) vehicles (AR-42 vehicle; TFM-4AS-1/DHT vehicle), (b) AR-42 (10 mg/kg; TFM-4AS-1/DHT vehicle), (c) TFM-4AS-1 (10 mg/kg; AR-42 vehicle), (d) DHT (3 mg/kg; AR-42 vehicle), (e) the combination of AR-42 (10 mg/kg) and TFM-4AS-1 (10 mg/kg), (f) the combination of AR-42 (10 mg/kg) and DHT (3 mg/kg). AR-42 was administered orally by gavage, and TFM-4AS-1 and DHT were administered by subcutaneous injection. Tumor-free controls were administered both vehicles. All treatments were given once daily for 13 days.

Luteinizing hormone analyses by RIA

Plasma was isolated from whole blood samples by centrifugation at 2000 x g for 15 minutes. Luteinizing hormone (LH) was measured in plasma by a sensitive two-site sandwich immunoassay [41, 42] using monoclonal antibodies against bovine LH (no. 581B7) and against the human LH-beta subunit (no. 5303: Medix Kauniainen, Finland) as previously described [42] . The tracer antibody, (no. 518B7) is kindly provided by Dr. Janet Roser [43], (Department of Animal Science, University of California, Davis) and iodinated by the chloramine T method and purified on Sephadex G-50 columns. The capture antibody (no. 5303) is biotinylated and immobilized on avidin-coated polystyrene beads (7mm; Epitope Diagnostics, Inc., San Diego, CA). Mouse LH reference prep (AFP5306A; provided by Dr. A.F. Parlow and the National Hormone and Peptide program) is used as standard. The assay has a sensitivity of 0.04 ng/ml.

AR-42 Plasma and Tissue Pharmacokinetics

Pharmacokinetic studies were conducted according to protocols approved by The Ohio State University Institutional Animal Care and Use Committee as previously described [44] with the following modifications. Seven week-old, male, tumor-free CD2F1 mice (n=3 per dose and time point) were administered single doses of 10, 20 and 50 mg/kg AR-42 by oral gavage and then sacrificed 0.25, 4 and 24 hours later. Fifty mg of gastrocnemius muscle tissue was flash frozen in 2 mL Beadblaster™ 24 (MIDSCI; St. Louis, MO) tubes and stored at -80°C. Samples were homogenized for 6-cycles using a Beadblaster 24 with analytical standards in 1 mL methanol and then centrifuged at 13,000 rpm (4° C) for 10 min. Supernatants were transferred to glass tubes, dried under nitrogen, then reconstituted in 200 µL 40% methanol/0.1% formic acid. Plasma preparation and LC-MS/MS analyses were performed as previously described [36].

Cytokine Analyses

Serum cytokine panel analyses were performed by a commercial vendor (Eve Technologies, Calgary, Canada) as previously described [37]. ELISA evaluation of serum interleukin-6 (IL-6) was performed using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Gene Expression Analyses

To generate muscle tissue RNA, 15 mg of gastrocnemius muscle tissue was lysed in 10 volumes of lysis buffer per tissue mass in prefilled 2.0ml tubes with 3.0mm zirconium beads (MIDSCI; St. Louis, MO). Tubes were loaded into Beadblaster™ 24 (MIDSCI; St. Louis, MO) and centrifuged for 5 cycles of 5 seconds with a 30 second pause between cycles. Lysate was collected and RNA was isolated using the mirVana™ miRNA Isolation Kit, with phenol (ThermoFisher; Waltham, MA). Samples were treated with DNA-free DNA Removal Kit to eliminate any DNA contamination (Invitrogen, Carlsbad, CA). Total RNA (0.5 µg) was reverse-

transcribed using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) for 10 min at 25°C, 120 min at 37°C and 5 min at 85°C (T100™ Thermal Cycler, Bio Rad). Real-time qPCR was performed on the QuantStudio 7 system (Applied Biosystems, Foster City, CA) using the powerup SYBR green master mix (Applied Biosystems, Foster City, CA). Cycling was performed using the QuantStudio 7 real-time PCR software — 2 min at 50°C and 2 min at 95°C—followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All real-time qPCR assays were carried out using technical duplicates using β-actin or GAPDH as the internal control genes. Reaction specificity was supported by the detection of a single amplified product in all reactions by a post-cycling melt curve, the absence of non-template control signal for 40 cycles, and confirmation of amplicon size using agarose electrophoresis. Primers for analyses are listed in Supplemental Table 3. Data are presented as per group geometric mean ± geometric standard deviation (STD) and individual $2^{-deltaCt}$ values [deltaCt = (target gene – internal control)]. All values are normalized to the geometric mean tumor-free control values. As transformed expression data are not normally distributed, statistical differences between treatment groups were determined by one-way ANOVA followed by Dunnett's test on raw delta Ct values.

Western Blot Analyses

Western blots from all studies were performed on gastrocnemius muscle from representative animals lysed by Nonidet P-40 isotonic lysis buffer [50mM Tris-HCl, pH 7.5, 120mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 50 mM NaF, 40 mM glycerophosphate, and 1 g/ml each of protease inhibitors (aprotinin, pepstatin, and leupeptin)]. Equivalent amounts of protein from each sample, as determined by Bradford assay (Bio-Rad), was resolved by SDS-PAGE and then transferred (semi-dry) onto immobilon-nitrocellulose membranes (Millipore, Bellerica, MA). Membranes were washed twice with TBST [Tris-buffered saline (TBS) containing 0.1% Tween 20], blocked with 5% nonfat milk in TBST for 30 min, and then washed an additional 3 times.

Membranes were incubated with specific primary antibody in TBST (1:1000) at 4 °C overnight, washed 3 times (TBST) and then incubated with appropriate goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates secondary antibodies (1:5000) at room temperature (1 hour). Following additional washes (TBST), immunoblots were visualized by ECL chemiluminescence (Amersham Biosciences, Little Chalfont, United Kingdom). Primary antibodies: phospho-STAT3 (Tyr705) (D3A7) XP® Rabbit mAb #9145, STAT3 (124H6) Mouse mAb #9139, FoxO3a (75D8) Rabbit mAb #2497 (Cell Signaling Technology, Danvers, MA); α-tubulin (B-7), sc-5286, (Santa Cruz Biotechnologies, Santa Cruz, CA); androgen receptor (EP670Y), ab52615 (Abcam, Cambridge, MA).

Gene Set Enrichment Analyses

Overlap between the gene sets in Tseng *et al.* [37] and Bonetto *et al.* [45] were determined and plotted using the Venn Diagram web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) in the Bioinformatics and Evolutionary Genomics Suite (Ghent University, Ghent, Belgium). Gene Set Enrichment Analyses [46, 47] were performed using the Molecular Signatures Database (Broad Institute, Cambridge, MA) for both AR-42-regulated genes from Tseng et al. as well as genes regulated in common between Tseng *et. al* and Bonetto *et. al.* Computed gene set overlaps were limited to canonical pathways and redundant results were collapsed to show only the gene set with the largest number of genes.

Statistical Methodology

Plotting and statistical analyses were performed using GraphPad Prism Version 7 (GraphPad Software, La Jolla, CA).

RESULTS

AR-42 administration demonstrates anti-cachectic effects at a reduced 10 mg/kg dose level

We recently extensively characterized the anti-cachectic effects of 50 mg/kg AR-42 administration every other day in the C-26 model of cancer cachexia [37]. As this dose was both the maximally tolerated dose and based originally on its direct anti-tumor effects, we hypothesized that reduced doses of AR-42 would offer comparable anti-cachectic efficacy. Indeed, similar to six total 50 mg/kg doses, thirteen daily oral doses of 20 or 10 mg/kg AR-42 demonstrated the ability to reverse C-26 tumor-mediated reductions in tumor-corrected body weight (Figure 1A). Furthermore, doses as low as 10 mg/kg were associated with reductions in gastrocnemius expression of atrogrin-1 and p-STAT3 relative to tumor-bearing controls (Supplementary Figure1). To better understand the disposition of AR-42 following oral administration, we performed a limited pharmacokinetic study of single doses of AR-42 (Figure 1B). Following oral administration, AR-42 readily distributes into gastrocnemius muscle tissue with mean concentrations in muscle of 1,501 nM, 3,859 nM and 7,925 nM achieved after 15 minutes following a single dose of 10, 20 and 50 mg/kg AR-42 respectively. Muscle levels of AR-42 are maintained above 700 nM for 4 hours following a 10 mg/kg oral dose consistent with the ability of AR-42 at this dose to inhibit Class I and IIb HDACs based on its *in vitro* HDAC inhibition profile (Figure 1C). The reduced dose of 10 mg/kg AR-42 consistently demonstrated the ability to spare C-26 tumor- mediated losses in body weight across multiple studies (Figure 2, Figure 3, and Supplemental Figure 3).

SARM monotherapy is completely ineffective in the C-26 model of cancer-induced cachexia

To evaluate our hypothesis that the combined SARM administration and HDAC inhibition would provide improved anti-cachectic effects, we designed a series of three studies in the C-26 model. Our initial study utilized minimal animal numbers and did not include an assessment of muscle function (Figure 2, Supplemental Figure 2). We then sought to reproduce our initial findings with increased animal numbers and included a grip strength measurement (Figure 2,

Supplemental Figure 3). Our final study included different androgen agonists in combination with AR-42 (Figure 3).

Surprisingly, SARM monotherapy had no apparent anti-cachectic efficacy in any study. GTx-024 at 15 mg/kg did not spare body weight (Figure 2A-B, Supplementary Figure 3B), or the mass of gastrocnemius and quadriceps muscles (Figure 2C, Supplementary Figure 3C) or adipose tissue and heart (Figure 2D) relative to tumor bearing controls. Though this is a relatively high dose of GTx-024, it was previously shown to be well tolerated in tumor-bearing mice [39] and did not cause body weight loss in tumor-free controls (Figure 2A). GTx-024 treatment in tumor-free controls demonstrated trends toward increased body weight and mass of gastrocnemius and quadriceps relative to tumor-free controls though no significant differences were detected. Nevertheless, the GTx-024-mediated suppression of serum luteinizing hormone suggested that the dose was sufficiently high to result in anabolic effects (Supplemental Figure 2A). Similar to the 15 mg/kg dose of GTx-024, 10 mg/kg TFM-4AS-1 monotherapy did not spare body weight (Figure 3A), gastrocnemius, quadriceps (Figure 3B), or adipose (Supplemental Figure 4B). The dose of this agent was chosen based on its previously demonstrated anabolic effects in mice [40]. However, administration of 10 mg/kg TFM-4AS-1 also failed to provide any detectable anti-cachectic effects relative to tumor bearing controls.

Combination GTx-024 and AR-42 administration results in improved anti-cachectic efficacy

In our initial combination study, C-26 tumor-bearing mice receiving both GTx-024 and AR-42 started to gain body weight relative to baseline after nearly two weeks of dosing whereas all other treatment groups experienced losses in body weight (Figure 2A). The effects of combined therapy on total body weight or amelioration of cachectic symptoms were not due to any overt impact of treatment on tumor burden as no significant differences in tumor volumes were apparent at the end of the study (Figure 2A, inset). When carcass weights were corrected for tumor mass, consistent with previous results [37] and others using this model [45, 48],

tumor-bearing animals lost roughly 20% of their body weight prior to meeting euthanasia criteria (Figure 2B, 80.4±9.1% of baseline). The severe body weight loss resulting from C-26 tumors was accompanied by parallel reductions in gastrocnemius and quadriceps masses (Figure 2C, 86 ±12.4 and 88±12.0%, relative to tumor-free controls, respectively). Consistent with preliminary dose finding studies, 10 mg/kg AR-42 significantly spared body weight (Figure 2B, 93.6±7.7% of baseline) relative to tumor-bearing controls. However, these changes were not translated into significant improvements in gastrocnemius and quadriceps mass (Figure 2C). In contrast to monotherapy, combined GTx-024 and AR-42 administration exhibited a striking ability to consistently protect body weight (99.9±0.8% of baseline) relative to either agent alone. Furthermore, the effects of combined therapy completely spared gastrocnemius (102.4±3.8%) and quadriceps (99.9±5.5%) mass relative to tumor free controls (Figure 2C). Unlike the effect of the higher 50 mg/kg dose of AR-42 to spare abdominal adipose tissue [37], the lower dose of 10 mg/kg had no impact on adipose or heart mass (Figure 2D). As androgens are thought to actively prevent adipogenesis [49], SARM administration was not expected to protect C-26 tumor-mediated losses in adipose. Indeed, no treatment mediated effects on abdominal adipose were apparent (Figure 2D). The data show heart mass was significantly improved by combination therapy, but this result is likely due to the effects of a single outlier animal. Feed consumption was monitored to account for the potential impact of anti-anorexic effects of treatment on the cachectic sequela following C-26 cell inoculation. GTx-024-treated tumor-free control animals, as well as combination-treated groups, demonstrated small differences in per animal feed consumption relative to other groups between day 14 and day 16 (Supplemental Figure 2B). However, these differences are unlikely to account for differences in body weight apparent by study day 14 (treatment day 9) as well as end of study differences in skeletal muscle masses (Figure 2C).

Based on the promising results of the initial study we expanded animal numbers and repeated the first study while including hand grip dynamometry as a measure of muscle

function. In this confirmatory study, the model was more aggressive resulting from significantly larger tumors relative to the first study, though no differences between treatment groups were apparent (Supplementary Figure 3A). Due to increased tumor volumes, the study was terminated after 12 days of treatment. In accordance with this increased tumor burden, tumor corrected body weights were more consistently reduced and to a larger degree in tumor-bearing controls (77.0±5.7% and 80.4± 9.1% of baseline in the second study compared to the first, respectively; Supplementary Figure 3B). In this more aggressive model, only combined AR-42 and GTx-024 administration significantly spared body weight (90.3±4.3 of baseline) though not to the degree realized in the first study (Figure 2A). Similar to the greater reductions in body weight, C-26 tumors were associated with larger losses in gastrocnemius (76.3±8.1%) and quadriceps (69.1±7.6%) mass relative to tumor-free controls in the second study. In the presence of more severe cachexia, both AR-42 and combined AR-42 and GTx-024 administration significantly spared gastrocnemius and quadriceps mass (Supplemental Figure 3C). C-26 tumors were accompanied by large reductions in hand grip strength (Figure 2E, 63.8±15.3% versus 83.8±10.7% of baseline in tumor-bearing and tumor-free controls, respectively), but consistent with the improvements in lower limb skeletal muscle mass, both AR-42 alone and in combination with GTx-024 resulted in grip strength improvements over vehicle-treated tumor-bearing controls.

Multiple androgens demonstrate improved anti-cachectic efficacy when combined with AR-42

To confirm the ability to improve GTx-024 anti-cachectic efficacy in the C-26 model by combined therapy with AR-42 was not a specific property of GTx-024, we treated tumor-bearing animals with fully anabolic doses of the SARM TFM-4AS-1 and the potent endogenous androgen DHT alone and in combination with AR-42 (Figure 3) [40]. AR-42 treatment alone resulted in significant attenuation of body weight loss (93.5±4.8 of baseline), but had limited

effects compared to combined administration with TFM-4AS-1 (99.5±4.4 of baseline) and DHT (106.0±5.4 of baseline). Tumor-bearing animals treated with DHT alone did not differ in initial tumor volumes (Day 8), but after 8 days of DHT administration, tumor growth was significantly suppressed resulting in the exclusion of these animals from further analyses (Supplementary Figure 4A). Consistent with both previous experiments, improvements in body weight were not due to sparing adipose tissue as no treatment-mediated effects on adipose were apparent (Supplemental Figure 4B).

C-26 tumor-mediated losses in gastrocnemius and quadriceps mass (78.9±12.7 and 74.9±9.5% versus tumor free controls, respectively) were reduced compared to the more aggressive wasting in the second study (Figure 3B). Similar to the first study, AR-42 monotherapy did not significantly impact skeletal muscle masses despite positive effects on body weight. However, combination treatment-mediated improvements in body weight were again translated to increased skeletal muscle masses where DHT in combination with AR-42 significantly spared both gastrocnemius and quadriceps mass (93.7±8.0 and 87.5±6.1% versus tumor free controls, respectively). However, TFM-4AS-1 in combination was only effective in attenuating quadriceps losses (85 ±5.5% of tumor-free controls). Congruent with the lesser impact of the C-26 tumors on lower limb skeletal muscle mass in the third study, smaller deficits in grip strength were apparent in tumor-bearing controls relative to the second study, 86.5% and 63.8% of baseline, respectively (Figure 3C). The only treatment resulting in significantly improved handgrip strength relative to tumor bearing controls was combined TFM-4AS-1 and AR-42 administration which resulted in increased muscle function over baseline (104.2%) despite the presence of C-26 tumors.

Impact of GTx-024 and AR-42 administration on the expression of atrophy genes in skeletal muscle

Candidate gene expression analyses were performed on gastrocnemius tissue from the initial study to characterize the effects of C-26 tumors and treatment with GTx-024, AR-42 or both agents on genes whose function has been previously associated with C-26 mediated wasting and androgen action in skeletal muscle (Figure 4). In agreement with multiple characterizations of the C-26 model of cancer cachexia, the muscle-specific E3 ligases Atrogin-1 and MuRF-1 were induced in skeletal muscles of tumor-bearing animals [37, 45, 48, 50] (Figure 4A). Consistent with the absence of any anti-cachectic effects of GTx-024 monotherapy, this treatment did not have a significant impact on Atrogin-1 and MuRF-1 expression. Ten mg/kg AR-42 alone and in combination with GTx-024 significantly reduced Atrogin-1 and MuRF-1 expression relative to tumor-bearing controls and returned them to near baseline levels. AR-42's effects on E3 ligase expression were consistent with results from animals receiving the higher dose of 50 mg/kg [37] further supporting the importance of AR-42's ability to reverse induction of these key enzymes to its overall anti-cachectic efficacy. Foxo-1 and IL-6Ra expression were significantly suppressed by 50 mg/kg AR-42 [37], but not 10 mg/kg AR-42. No treatment significantly impacted mRNA levels of IL-6Ra or the p65 subunit of NFkB in tumor-bearing animals.

Impact of GTx-024 and AR-42 administration on androgen receptor signaling and the myogenic differentiation program

Candidate gene expression analyses were extended to the androgen receptor (AR), characterized targets of the AR in skeletal muscle and components of the myogenic differentiation genetic program. Neither tumor nor treatment had a significant impact on AR mRNA (Figure 4B). AR protein expression in gastrocnemius was low in tumor-free controls and increased in response to GTx-024 administration irrespective of tumor burden (Figure 4C). AR-42 treatment did not have a marked impact on gastrocnemius AR expression. A number of androgen-responsive genes regulating diverse aspects of skeletal muscle biology have been

described [30]. Reported androgen-dependent regulation varies considerably between cultured myoblasts or muscle satellite cells (MSCs) and amongst different muscle fibers owing in large part to disparate levels of androgen receptor expression [51, 52]. Gastrocnemius AR expression is reported to be quite low compared to cultured cells and other muscles, particularly levator ani [35]. Nevertheless, androgen-dependent regulation of IGF-1, a critical regulator of skeletal muscle mass, has been described in mature gastrocnemius muscle tissue [32, 53], and the skeletal muscle-specific isoform IGF-1Ea has also shown robust AR-mediated regulation [31]. The expression of neither IGF-1 isoform, however, was significantly impacted by tumor or treatment (Supplemental Figure 5). Myostatin signaling also governs skeletal muscle mass and is another target of androgens as myostatin, its receptor ACVRIIB, and the negative regulator of myostatin, follistatin, have all been shown to be androgen-regulated [35, 53, 54]. ACVRIIB was induced by the presence of C-26 tumors and was significantly suppressed only by AR-42 treatment, alone or in combination with GTx-024 (Figure 4D). Myostatin responded similarly to its receptor, though the effect of the combination treatment did not reach significance, and follistatin expression was unchanged by tumor or treatment. These results suggest modulation of myostatin signaling may contribute to the anti-cachectic efficacy of AR-42, but contrast with previously reported marked AR-mediated regulation of this pathway [31, 35]. A potential explanation for our distinct results could be our evaluation of gastrocnemius tissue versus the AR-rich levator ani muscle reported in Dubois *et al*. [35].

The molecular mechanisms by which androgen administration facilitate muscle hypertrophy and regeneration are poorly understood, but there is evidence that androgen-mediated MSC activation is vital and MSC populations are known to expand following androgen administration [55, 56]. It follows that several critical regulators of satellite cell activation and myogenic differentiation have previously been shown to be androgen-regulated [35, 51, 57]. In whole gastrocnemius tissue, myogenin expression was inversely related to treatment effects on gastrocnemius mass (Figure 2C), in that it was elevated in tumor-bearing animals, unaffected by

GTx-024 monotherapy and significantly suppressed to tumor-free control levels by combination treatment (Figure 4E). Elevated myogenin levels in tumor-bearing controls are consistent with its reported induction following castration-induced atrophy and following genetic ablation of AR activity in skeletal muscle by multiple means [35, 51]. The androgen-dependence of Myf5 expression in skeletal muscle has been similarly reported [35] and its regulation in C-26 tumor-bearing animals resembled that of myogenin (Figure 4E). Though the presence of C-26 tumors did not significantly induce Myf5 expression, combination treatment significantly suppressed myf5 expression beyond GTx-024 monotherapy, AR-42 monotherapy and even tumor-free controls. Despite previous reports of androgen-dependent regulation, no significant differences in Pax7 or MyoD levels were detected (Figure 4E).

It remains unclear as to whether the primary role of androgen action in MSCs is to drive proliferation or differentiation [57]. If expression of factors governing myogenesis in whole gastrocnemius tissue (Figure 4E) reflects similar expression in MSCs, our data suggest GTx-024 treatment promotes proliferation of MSC cells while limiting terminal differentiation based on a current understanding of how myogenin, Myf5, myoD and Pax7 coordinate the control of MSC populations[58]. As suggested by Rana *et al*., androgen-mediated suppression of Myf5 should limit the initiation of myoblast commitment and when coordinated with myogenin suppression, should also curtail terminal myocyte differentiation [51]. This interpretation supports the well characterized expansion of satellite cell populations following androgen administration in both humans and rodents [55, 56]. Furthermore, these data suggest that the anti-cachectic efficacy of androgens may result in part from their ability to maintain satellite cell populations whose exhaustion has been linked to progressive muscle wasting [59]. Critically, the expected effects of GTx-024 administration on myogenin and Myf5 expression in atrophying skeletal muscle required co-administration of AR-42 suggesting factors associated with the presence of C-26 tumors are capable of modulating AR action in skeletal muscle.

Importance of cytokine and immune signaling pathways in the anti-cachectic activity of AR-42 in C-26 tumor bearing mice

Previous ingenuity pathway analyses of AR-42-regulated genes in gastrocnemius muscle revealed that of the 677 genes significantly regulated by AR-42 relative to C-26 tumor-bearing vehicle-treated controls, 66 were associated with muscle disease or function [37]. When these same genes were analyzed by Gene Set Enrichment Analyses (GSEA) to determine over-represented canonical pathways, genes within matrisome and immune system pathways were significantly over-represented (Supplementary Figure 6A and B). In an effort to enrich these 677 differentially regulated genes for transcripts critical to the anti-cachectic efficacy of AR-42, these data were intersected with 700 previously published differentially regulated genes from the quadriceps of moderate and severely wasted C-26 tumor-bearing mice (Figure 5A). Using this approach, the likely biological relevance of the 147 overlapping genes is increased when it is considered that these transcripts represent genes regulated by AR-42 that are associated with C-26-induced wasting from two different muscles (gastrocnemius and quadriceps), detected by two different technologies (RNA-seq and microarray) and reported by two different research laboratories. Previously reported AR-42 treatment effectively reversed C-26-mediated changes in gastrocnemius gene expression [37]. Similar regulation of the 147 overlapping genes in quadriceps was apparent when magnitude and direction of regulation were considered (Supplementary Figure 7, Supplementary Table 1). All but one gene, a long non-coding RNA (NR_033803), were regulated in opposite directions in quadriceps muscle in C-26 tumor-bearing animals relative to AR-42-treated gastrocnemius muscle with a corresponding strong association (r^2=0.84). GSEA was performed on this enriched pool of 147 genes revealing canonical pathways involving the immune system, IL-6 signaling and a number of pathways activated subsequent to cytokine stimulation (Figure 5B). The vast majority (82%) of the genes in these overrepresented pathways were induced by C-26

tumors and then suppressed by AR-42 treatment (Figure 5C). Several of the genes shared across multiple over-represented pathways have been previously studied in models of muscle wasting and cachectic patients including FOS [50, 60], FoxO1 [50, 61, 62], IL-6 receptor (IL-6Ra) [63, 64], MEF2C [65] and p21 (CDKN1A) [66, 67]. Of note, both extensively characterized E3-ligases, MuRF-1 (TRIM63) and Atrogin-1 (Fbxo32), were among the 147 overlapping genes (Supplemental Table 1) though only MuRF-1 was part of an over-represented pathway (Figure 5C).

Anti-cachectic efficacy of AR-42 treatment associated with STAT3 inhibition but not general immune suppression

In agreement with our GSEA, we previously reported that the higher 50 mg/kg dose of AR-42 reduced serum IL-6 level, as well as gastrocnemius IL-6 receptor mRNA abundance in tumor-bearing mice suggesting AR-42's efficacy may be related to its ability to suppress the systemic IL-6 activation thought to drive muscle wasting in the C-26 model [37] . In this study, serum IL-6 was included in a panel of cytokines that were evaluated to assess the impact of C-26 tumor burden and treatment with AR-42, GTx-024 or combination therapy on circulating cytokines (Table 1, Supplementary Table 2). Multiple pro-cachectic factors including G-CSF, IL-6, and LIF were significantly elevated by the presence of a C-26 tumor [68, 69]. In contrast to the 50 m/kg dose, the minimally effective dose of 10 mg/kg AR-42 did not impact IL-6 or LIF levels, alone or in combination with GTx-024, nor did it significantly impact IL-6ra receptor mRNA (Figure 4A) [37]. Furthermore, 10 mg/kg AR-42 monotherapy did not significantly impact circulating levels of any evaluated cytokine despite demonstrating clear anti-cachectic effects across multiple studies (Figure 1A, Figure 2, Supplementary Figure 3, Figure 3). A robust ELISA analysis was performed to confirm our findings that AR-42 treatment did not impact circulating IL-6 levels (Figure 6A), which also demonstrated serum IL-6 levels are not associated with terminal body weight in treated, C-26 tumor-bearing mice (Figure 6B).

When significant effects on circulating cytokines were not apparent, we hypothesized AR-42 might be acting down-stream of the IL-6 receptor on critical mediators of cytokine signaling. One well characterized effector of cytokine-induced signaling shown to be central to tumor-induced wasting in a number of models is signal transducer and activator of transcription (STAT3) [29, 70, 71]. Notably STAT3 activation is associated with the severity of wasting in both the C-26 and APC/min models of cancer cachexia [29, 45]. We evaluated AR-42's effects on pSTAT3 in gastrocnemius muscle from C-26 tumor-bearing animals (Figure 6C) and consistent with effects on total body weight (Figure 2B) and gastrocnemius mass (Figure 2C), the presence of the C-26 tumor resulted in increased pSTAT3 activation. GTx-024 treatment had no apparent effect on pSTAT3 which corresponded with its inability to spare body weight or lower limb skeletal muscle mass as a monotherapy. AR-42 monotherapy demonstrated the ability to suppress pSTAT3 but not equally in all animals, whereas combination-treated animals had both the most robust suppression, as well as marked anti-cachectic efficacy. Similar findings resulted from repeat analyses of gastrocnemius protein lysates (Supplementary Figure 8).

In addition to activation of skeletal muscle STAT3 activation, inoculation with C-26 tumors results in splenomegaly as a result of increased systemic inflammation [72]. Consistent with increased circulating cytokine levels, our C-26 tumor-bearing animals demonstrated large increases in spleen mass across all treatment groups relative to tumor-free controls in both the initial and confirmatory studies (Supplementary Figure 2C and Figure 6D, respectively). Spleen mass was either not changed by or was slightly increased by AR-42 treatment alone or in combination with GTx-024 treatment which reflects similar findings with 50 mpk AR-42 [37]. As a gross measure of the systemic immune effects of treatment, spleen mass suggests AR-42 is not generally immune suppressive and its activity is distinct from other inhibitors of the JAK/STAT pathway in this context [73, 74]. Taken together these multiple lines of evidence

suggest that the anti-cachectic efficacy of AR-42 involves the inhibition of STAT3 but not systemic suppression of IL-6 or general immune signaling.

DISCUSSION

Limitations of anabolic anti-cachexia therapy

Anabolic anti-cachexia therapy has thus far been ineffective in meeting required regulatory endpoints for approval [26, 75]. GTx-024's registration trials did not have weight loss requirements but roughly half of all patients reported >5% unexplained weight loss at enrollment consistent with diverse stages of wasting in NSCLC patients at the advent of chemotherapy [76]. In a very similar population receiving anabolic ghrelin mimetic anamorelin therapy, subgroup analyses revealed patients with body mass indices (BMI)<18.5 (and presumably severe cachexia) showed no improvements in body composition. Similarly, in the severe model of experimental cachexia in this study, anabolic androgen monotherapy provided no apparent anti-cachectic effects. These findings suggest that cachectic drivers in C-26 tumor-bearing mice cannot be overcome by anabolic therapy and may offer insight into why cachectic patients have heterogeneous responses to androgens, and more generally, anabolic therapy.

Androgens have a well characterized ability to restore pAKT and pFoxO3a levels in skeletal muscle that are repressed by either glucocorticoid (dexamethasone)- or hypogonadism (castration)-induced atrophy [32, 33]. In each case, reductions in pAKT and pFoxO3a were mirrored by increased E3-ligase expression that was reversible upon androgen administration in an effect thought to occur through the well characterized IGF-1/PI3K/AKT pathway [77]. The role of the IGF-1 signaling axis is more controversial in the C-26 model of experimental cachexia though suppression of pAKT and pFoXO3a in response to tumor burden have been reported [78, 79]. Despite variable responses in pFoXO3a and total FoXO3a (Supplementary figure 8), SARM monotherapy did not markedly change FoxO3a levels whereas AR-42 and combination treatment reduced the levels of FoxO3a protein. Taken together, these findings

suggest that C-26-mediated FoxO3a activation may contribute to the activation of muscle-specific E3-ligases (Figure 4) and is reversed by HDAC inhibition but not androgen administration.

A key difference between glucocorticoid- and hypogonadism-induced atrophy compared to C-26-mediated wasting is the role of systemic inflammation, namely IL-6 signaling [48, 70, 80]. Changes in gastrocnemius AR protein levels associated with C-26 tumors do not readily explain response, or lack thereof, to androgen therapy (Figure 4C). However, C-26 tumor burden was accompanied by large increases in serum IL-6 (Figure 6A) and the impact of elevated IL-6 on AR function in skeletal muscle is not known. IL-6 modulation of AR function is well studied in the context of prostate cancer where IL-6 has been shown to both increase and decrease AR activity depending on the mediators involved [81]. The inability of GTx-024 monotherapy to impact body composition in C-26 tumor-bearing mice (Figure 2, Supplemental Figure 3), reverse tumor-induced E3 ligase expression or modulate characterized AR target genes (Figure 4) suggests that AR function in muscle may be compromised by C-26-associated inflammatory mediators such as IL-6 and warrants further study.

Impact of C-26 tumor burden on androgen signaling

Hypogonadism is reported in both cachectic cancer patients and models of experimental cachexia [28, 29]. We did not asses hypogonadism in our studies but C-26-mediated hypogonadism has been previously reported [82]. Interestingly, administration of GTx-024 to likely hypogonadal tumor-bearing mice had no detectable impact on physiological or molecular outcomes including AR target gene expression in gastrocnemius (Atrogin-1, myostatin, myogenin, Figure 4A, 4D and 4E, respectively), an androgen-responsive skeletal muscle [32, 51]. This suggests that C-26 tumor-mediated hypogonadism may be due in part to compromised AR signaling as AR function is required for steroidogenesis and maintenance of

testes weight [83]. AR mRNA was unchanged and AR protein was in fact elevated in gastrocnemius muscle of GTx-024-treated tumor-bearing animals relative to controls, thereby excluding reduced levels of gastrocnemius AR as an explanation for the failure of GTx-024 monotherapy in this tissue (Figure 4C). Of note, in our studies SARM treatment had no effect on pSTAT3 induction associated with C-26 tumor burden (Figure 6C). Pathologic STAT3 activation has recently been shown to compromise proper MSC function [84, 85] and androgen action in MSCs is thought to contribute greatly to the overall effects of androgens on skeletal muscle [52, 56]. Given the importance of MSCs in muscle repair and their characterized dysfunction in cancer wasting [59] it is plausible that persistent STAT3 activation, subsequent to elevated levels of circulating cytokines (IL-6), results in a blockade of AR-mediated anabolic signaling in muscle. Together these findings suggest that the AR's ability to appropriately support androgen-dependent tissue homeostasis is compromised in C-26 tumor-bearing mice. Though surprising, our results are consistent with the complete absence of reported efficacy of anabolic therapy in the C-26 model. At the time of manuscript preparation, we were unable to identify a single study demonstrating anti-cachectic effects of anabolic therapy in this common model of experimental cachexia despite this class of agents representing the most mature therapeutic development programs in cancer wasting.

AR-42's effects on STAT3 and immune signaling

GSEA analysis of genes regulated in skeletal muscle of C-26 tumor-bearing animals by AR-42 treatment support a critical role for the reversal of IL-6 and immune system signaling in the anti-cachectic efficacy of AR-42 (Figure 5, Supplemental Figure 6). An essential mediator of IL-6 and a number of immune signals is STAT3 and the importance of elevated STAT3 activity in C-26-induced wasting is well characterized [45, 70, 80, 86, 87]. Critically, Seto *et al*. have recently demonstrated that acute STAT3 signaling, as opposed to FOXO, NFKB, SMAD or C/EBP transcription, drives C2C12 myotube atrophy in response to C-26 conditioned media

[80]. In an elegant set of experiments, Seto *et al*. demonstrated that STAT3 activation in response to LIF (leukemia inhibitory factor), a member of the IL-6 cytokine family, is essential in C-26-mediated muscle wasting. In agreement with the critical role of STAT3 activation in C-26-mediated cachexia, both genetic STAT3 manipulation and pharmacological STAT3 inhibition can mitigate C-26 tumor-induced losses in skeletal muscle [80, 87]. Of note, neither circulating LIF nor IL-6 levels in tumor-bearing animals were changed by 10 mg/kg AR-42 treatment alone or in combination with GTx-024 suggesting AR-42's anti-cachectic effects do not require suppression of LIF or IL-6 and may act downstream of LIF/IL-6 binding to the IL-6 receptor (Table 1, Figure 6B). The ability of GTx-024, AR-42 or combination treatment to inhibit pSTAT3 was associated with their anti-cachectic efficacy (Figure 6C) and capacity to modulate the expression of the *bone fide* STAT3 target atrogene, CEBPδ (Figure 4A) [88]. These data are in agreement with previous reports associating the severity of wasting in experimental cachexia with the levels of STAT3 activation across multiple models [29, 45]. Notably, AR-42 treatment blocked STAT3 activation whereas GTx-024 treatment did not.

The precise means by which AR-42 treatment results in anti-cachectic efficacy remains unclear. Consistent with the ability to increase acetylated histone 3 (Ac-H3) in gastrocnemius tissue following a 50 mg/kg dose of AR-42, Beharry *et al.* show increased Ac-H3 levels in skeletal muscle following administration of the HDAC inhibitors tricostatin A and MS-275 to animals undergoing nutrient deprivation and disuse-mediated muscle atrophy, respectively [37, 89]. Prolonged elevated levels of gastrocnemius AR-42 following a 50 mg/kg dose ($\geq$ 2.54 µM for 24 hours following AR-42 administration, Figure 1B) support the effective inhibition of Class I and IIb HDAC's in muscle for the majority of the daily dosing interval. At the minimally effective dose of 10 mg/kg, sufficiently high levels of AR-42 are transiently achieved in muscle to inhibit HDACs, but clear increases in Ac-H3 were not apparent (data not shown). Similar anti-cachectic efficacy without evidence of overt HDAC inhibition has also been reported in C-26

tumor-bearing mice treated with the HDAC inhibitor valproic acid (VPA) [79]. Taken together, these findings suggest the anti-cachectic effects of HDAC inhibition may involve modulation of HDAC function beyond the de-acetylation of histones or other proteins. Such functions are not without precedent as increasingly diverse mechanisms of action are attributed to HDAC inhibitors [90] An alternative explanation is that skeletal muscle may not be the relevant tissue of action for AR-42's efficacy. However, multiple lines of evidence show the clear ability of diverse HDAC inhibitors to act directly on cultured skeletal muscle cells to prevent atrophy [79, 89]

Our data show that the inhibition of pSTAT3 in skeletal muscle is closely associated with the anti-cachectic efficacy of AR-42 (Figure 6C), but the mechanism underlying AR-42's effects on STAT3 is not known. Though not in skeletal muscle, treatment with an HDAC inhibitor has been shown to re-program STAT3 binding resulting in increased expression of negative regulators of pro-inflammatory cytokines [91]. Moreover, HDAC inhibition with VPA has been shown to suppress pSTAT3 through inhibition of HDAC3 in natural killer cells [92]. Notably, pSTAT3 (Y705) suppression occurred in the absence of changes to STAT3 acetylation. The potential that AR-42 directly inhibits JAK/STAT signaling has not been excluded by our findings, but such direct inhibition would be expected to result in broader immune effects than were detected [73, 74]. Neither spleen mass nor circulating cytokine levels (Figure 6D and Table 1, respectively) were impacted following an anti-cachectic dose of AR-42 suggesting AR-42 does not function as a JAK/STAT inhibitor or general immune suppressant. AR-42 treatment is demonstrably anti-catabolic (as opposed to anabolic) in the C-26 model as shown by the strong dependence of efficacy on treatment initiation time and the observation that AR-42 treatment in non-tumor bearing mice does not result in increased skeletal muscle mass (data not shown) [37]. This component of AR-42's efficacy is again consistent with the importance of pSTAT3 as

muscle-specific genetic ablation of STAT3 attenuates tumor-induced losses in skeletal muscle mass but does not increase muscle mass in tumor-free controls [87].

We recognize that our study of combined androgen administration and HDAC inhibition in experimental cachexia is limited in a number of ways. We have thus far evaluated a single rapid model of experimental cachexia which necessarily limits the broader interpretation of our findings. The short treatment window (<14 days) afforded by the C-26 model in our hands also severely curtailed our ability to demonstrate overt anabolic effects following GTx-024 treatment relative to other anabolic agents in less severe models [93]. Evaluation of combination treatment in other experimental models of cachexia are ongoing. Our studies were also limited in that they evaluated combined androgen administration and HDAC inhibition at fixed dose levels. Ten mg/kg AR-42 was minimally effective as monotherapy, but its ability to augment anabolic therapy at even lower doses was not investigated. Likewise, the doses of GTx-024 used were higher than what would be expected to provide anabolic benefit [93]. Dose optimization is critical to both improve efficacy and minimize toxicity as the tolerance for additional side effects associated with anti-cachexia therapy in already heavily treated cancer patients is low. Future studies will determine the minimally effective doses of each agent for combination therapy and seek to evaluate their efficacy when administered along with chemotherapeutics to best model clinical deployment of anti-cachexia therapy. We also acknowledge that our investigation of how C-26 tumors prevent androgen efficacy are limited in scope but critical in designing improved therapeutic regimens. A fuller understanding of both the means by which severe cachectic stimuli impact androgen action and how AR-42 treatment restores anabolic responsiveness are the subject of ongoing studies employing full transcriptome analyses of skeletal muscle tissue from treated animals.

Combined anabolic and anti-catabolic therapy in cancer cachexia

Cross-talk between multiple signaling pathways governing anabolism and catabolism in skeletal muscle have been described, suggesting that targeting a single pathway can lead to concurrent therapeutic pro-anabolic and anti-catabolic effects on skeletal muscle[94]. As such both anti-catabolic and anabolic therapies have been shown as single agents to attenuate skeletal muscle losses associated with tumor burden in models of cancer wasting [37, 93, 95]. However, the efficacy of these and nearly all experimental anti-cachexia therapies are highly dependent on preventative treatments with intervention beginning before or concurrently with the onset of wasting sequela. Given the rapid wasting associated with the most common cancer cachexia models, adopting this type of study design is pragmatic, but is associated with a number of shortcomings. First, in this treatment paradigm, the ability of single agent primarily anti-catabolic therapies to restore established deficits in skeletal muscle mass and function are not evaluated. An agent with effective anabolic properties would likely be required to restore muscle mass and function to a pre-wasted state. Second, this treatment paradigm simply does not represent the clinical management of cancer wasting which ultimately limits the effective translation of pre-clinical findings. It is increasingly recognized that extreme heterogeneity exists in cancer patient body composition at diagnosis. Occult, potentially severe cachexia may exist in a number of cancer patients at the earliest time that therapeutic intervention may be possible [96]. For example, Martin *et al*. reported patients with the same “overweight” BMI (29.4 kg/m^2) were shown to have skeletal muscle content differing by 1.7-fold. Furthermore, the results of the Prevalence of Malnutrition in Oncology (PreMiO, NCT-01622036) study reported at the 3rd Cancer Cachexia Conference (September 2016) revealed unexplained weight loss at diagnoses in 62% of cancer patients with diverse cancer types suggesting cancer wasting occurs early in disease progression and is not limited to late stage disease [97]. When considered together, these findings pose a considerable challenge in the successful deployment of primarily anti-catabolic anti-cachexia therapy to cancer patients who may arrive at the clinic with potentially hidden, significant losses in lean mass. Given the realities of cancer diagnoses

and treatment, the ability to restore skeletal muscle mass and function is likely critical for effective anti-cachexia therapy.

Thus far, anabolic agents have demonstrated heterogeneous effects on patient lean mass and little ability to stem functional losses associated with cancer wasting [26, 75]. Our data show that in a severe model of experimental cancer wasting, androgens have no effect on cachectic outcomes, mimicking the failures of single agent anabolic therapy in cancer cachexia patients. These concordant failures begged the question, “Can anabolic anti-cachexia therapy be improved?” To this end we hypothesized that combined anti-catabolic therapy (AR-42) with and anabolic therapy (SARM) could mitigate both hyper-catabolic and hypo-anabolic components of dysregulated muscle homeostasis in cachexia. To our knowledge we are the first to report this novel combination anti-cachexia therapy. Despite the limited treatment window available in the C-26 model, we show improved total body weight (Figure 2B, Figure 3A), lower limb skeletal muscle mass (Figure 2C, Figure 3B), and grip strength (Figure 2E, Figure 3C) for two different SARMs when combined with AR-42 over tumor-bearing controls and SARM monotherapy. Improvements realized by combined AR-42 and SARM therapy versus either agent alone were consistently more substantial for skeletal muscle and grip strength as opposed to total body weight. These results reflect known effects of androgen action on body composition which promote increases in muscle mass with simultaneous reductions in adipose mass [49]. Conversely, the efficacy of AR-42 monotherapy was most similar to combination treatment when the wasting was most severe (Study 2). These findings demonstrate the importance of anti-catabolic therapy and limitations of anabolic therapy in the most extreme cachexia while highlighting the need for further dose optimization of combination treatment. Moreover, combination therapy resulted in improved modulation of multiple genes governing both anabolic and catabolic cellular processes in skeletal muscle (Figure 4). These molecular features of combined androgen and HDAC inhibitor therapy portend further improved efficacy if

treatment windows are extended as would be expected in less severe models of experimental cachexia. Critically, combination AR-42 and SARM therapy demonstrated improved muscle function suggesting that the limited ability to translate increases in patient lean mass following anabolic therapy can be enhanced with the addition of an anti-catabolic agent like AR-42.

Due to both the complexity of cachectic mediators and the consistent failure of single agent approaches, multi-modal anti-cachexia therapy is widely recognized as required for the effective management of cancer wasting [2]. We are the first to report combined androgen and HDAC inhibitor administration in experimental cachexia and have demonstrated efficacy using two agents undergoing clinical development. Our initial investigation represents a framework for the development of improved anti-cachectic therapy by effectively combining anabolic and anti-catabolic agents. Future studies will focus on mechanism, dose optimization and treatment of less rapid and more clinically relevant models of wasting. Our current findings suggest that anabolic skeletal muscle growth is possible in the presence of severe cachectic stimuli with optimized combination therapy. Restoring muscle mass and function to a pre-cachectic state represents an ideal therapeutic goal in the treatment of cancer-associated wasting. As such, successful reversal of established functional deficits would be expected to decouple the response to anti-cachexia therapy from earliest possible intervention and provide a more robust functional benefit in clinical populations representing diverse stages of cachexia.

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The authors certify that they comply with the ethical guidelines for authorship and publishing of the Journal of Cachexia, Sarcopenia and Muscle (von Haehling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle. J Cachexia Sarcopenia Muscle. 2015;4:315-6.)

CONFLICT OF INTEREST

Ching-Shih Chen is the inventor of AR-42, which was licensed to Arno Therapeutics, Inc., for clinical development by The Ohio State University Research Foundation. Christopher C. Coss is a former employee of GTx Inc., owner of GTx-024, but has no financial relationship with GTx or any equity in GTx Inc. GTx Inc. was not involved in any way with the financing, design, or interpretation of the reported studies. Yu-Chu Tseng, Sophia Liva, Anees Dauki, Sally Henderson, Yi-Chiu Kuo, Jason A. Benedict, Samuel K. Kulp, Tanois Bekaii-Saab have no conflict of interest to declare.

Figure Legends

Figure 1. AR-42 demonstrates anti-cachectic effects at reduced doses in C-26 mouse model. A) Starting six days after inoculation animals received vehicle or AR-42 orally at three different dose levels. 10 mg/kg daily, 20 mg/kg daily or 50 mg/kg every other day for 13 days (n=4-6). Day 18 bodyweights compared to Day 0, corrected for tumor mass according to the *Materials and Methods*. *p<0.05, ****p<0.0001 versus tumor bearing vehicle treated controls Tukey's multiple comparison test. **B)** Tumor free C57BL/6 mice were administered a single dose of 10 mg/kg, 20 mg/kg or 50 mg/kg AR-42 (n=3) and plasma (dashed) and gastroc (solid) tissue analyzed for AR-42 content at different times using LC-MS/MS analyses according to *Materials and Methods*. **C)** AR-42's *in vitro* human HDAC inhibition profile was determined using recombinant enzymatic assays according to *Materials and Methods.*

Figure 2. Combined SARM and AR-42 administration in C-26 mouse model. Animals receiving GTx-024 (15 mg/kg), AR-42 (10 mg/kg), Combination (15 mg/kg GTx-024 and 10 mg/kg AR-42) or Vehicle were treated daily by oral gavage starting 6 days post inoculation for 13 days. **A)** Longitudinal mean bodyweights per treatment group are presented as a percent change from pre-study body weights, tumor free animals (circles), tumor bearing animals (bars), terminal tumor volumes (inset) n=5-6 per group. **B)** Day 18 bodyweights compared to Day 0, corrected for tumor mass according to the *Materials and Methods.* **C)** Terminal lower limb skeletal muscle masses **D)** Terminal abdominal fat pad adipose and heart tissue masses from confirmatory study, n=5-10 per group **E)** Day 16 grip strength per treatment group are presented from confirmatory study representing 5 repeat assessments per animal, n=5-10 per group. *Statistics for all panels*: Mean±STD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus tumor bearing vehicle treated controls Dunnett's multiple comparison test; ns, no significant difference.

Figure 3. Combined Androgen and AR-42 administration in C-26 mouse model. Animals receiving AR-42 (10 mg/kg, oral gavage), TFM-4AS-1 (10 mg/kg, sub-cutaneous injection), Combination AR-42 and DHT (10 mg/kg oral gavage and 3 mg/kg sub-cutaneous injection, respectively), Combination AR-42 and TFM-4AS-1 (10 mg/kg, both) or Vehicle were treated daily starting 6 days post inoculation for 12 days. **A)** Body weights on Day 17 post inoculation, corrected for tumor mass according to the *Materials and Methods*, are presented per group (n=5-10) **B)** Terminal lower limb skeletal muscle masses. **C)** Mean grip strength performed on the final day of treatment. *Statistics for all panels*: Mean±STD unless otherwise noted. *p<0.05,

**p<0.01, ***p<0.001, ****p<0.0001 versus tumor bearing vehicle treated controls Dunnett's multiple comparison test; ns, no significant difference.

Figure 4. Gastrocnemius Gene Expression. Gene expression of multiple cachexia associated markers presented from gastrocnemius muscle of individual animals from the initial study of combined GTx-024 and AR-42 administration (n=5-6 group). Expression presented as described in the *Materials and Methods* (Geometric Mean ± Geometric STD). **A)** Genes associated with muscle atrophy. *Statistics*: versus tumor bearing control, Dunnett's multiple comparison test **B)** Androgen Receptor (AR) and AR target gene expression. *Statistics:* versus tumor bearing control, Dunnett's multiple comparison test **C)** AR western blot **D)** Genes associated with myogenic differentiation *Statistics*: Myogenin, MyoD, Pax7 versus tumor bearing control, Myf5 versus tumor bearing combination treatment, Dunnett's multiple comparison test. *Statistics for all panels*: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 versus control as listed per panel. CEBPδ, Myostatin, ACVRIIB: n=4, insufficient sample for all tumor bearing GTx-024 treated animals.

Figure 5. Gene Set Enrichment Analyses (GSEA) of AR-42 regulated genes in skeletal muscles from C26 tumor bearing mice. A) Genes differentially regulated by 50 mg/kg AR-42 treatment relative to tumor bearing control from Tseng *et al*. [37] intersected with genes differentially regulated in quadriceps muscle from both severe and moderately wasting C26 tumor bearing relative to tumor free control from Bonetto *et al.* [45]. **B)** Canonical pathway analysis using GSEA of 147 overlapping genes from (A). **C)** Heat map of individual genes from over represented pathways in (B) presented as $\log_2$FC versus control.

Figure 6. Effects of AR-42 administration on STAT3 signaling. A) ELISA analysis of serum IL-6 levels at Day 17 sacrifice from second study of combined GTx-024 and AR-42 administration (Mean±STD, Supplementary Figure 3). **B)** Individual animal serum IL-6 values as determined in (A) plotted against tumor corrected terminal bodyweights from the initial study. **C)** STAT3 western blot analysis of gastroc tissues from representative animals treated from the initial study **D)** Terminal bodyweight normalized spleen masses from second study of combined GTx-024 and AR-42 administration of combined GTx-024 and AR-42 administration. (Mean±STD). *p<0.05, ***p<0.001, versus tumor bearing vehicle treated controls Dunnett's multiple comparison test.

Table Legends

Table 1. Serum Cytokine Panel. Multiplex analysis of diverse serum cytokines at Day 17 sacrifice from second study of combined GTx-024 and AR-42 administration (Mean±STD). Presented cytokines limited to significant differences between tumor bearing controls (*p<0.05, Dunnett's multiple comparison test).G-CSF: granulocyte colony-stimulating factor, GM-CSF: granulocyte macrophage colony-stimulating factor, IL-6: interleukin-6, IL-17: interleukin-17, IP-10: interferon gamma-induced protein 10, KC: chemokine (C-X-C motif) ligand 1, LIF: leukemia inhibitory factor, M-CSF: macrophage colony-stimulating factor.

Supplementary Figure Legends

Supplementary Figure 1. Effects of reduced doses of AR-42 on gastroc tissue in C26 tumor bearing animals. Western blot analysis of markers of AR-42's anti-cachectic effects (Atrogin-1, MuRF-1, pSTAT3) and HDAC activity (Ac-H3 and H3).

Supplementary Figure 2. Initial evaluation of Combined GTx-024 and AR-42 administration in C-26 mouse model. Animals receiving GTx-024 (15 mg/kg), AR-42 (10 mg/kg), Combination (15 mg/kg GTx-024 and 10 mg/kg AR-42) or Vehicle were treated daily by oral gavage for 13 days starting 6 days post inoculation (n=5-6 group). **A)** Serum luteinizing hormone levels measured as outlined in the *Materials and Methods* section on Day 18. **B)** Per animal food consumption measured every two days. **C)** Terminal bodyweight normalized spleen masses. ***p<0.001, versus tumor bearing vehicle treated controls Dunnett's multiple comparison test.

Supplementary Figure 3. Confirmatory study of Combined GTx-024 and AR-42 administration in C-26 mouse model. Animals receiving GTx-024 (15 mg/kg), AR-42 (10 mg/kg), Combination (15 mg/kg GTx-024 and 10 mg/kg AR-42) or Vehicle were treated daily by oral gavage for 12 days starting 6 days post inoculation (n=5-10 group). **A)** Terminal tumor volume comparisons between initial (Study 1, Day 17) and confirmatory (Study 2, Day 16) . ****p<0.0001 Study 1 versus Study 2, ns no significant differences between treatment groups within each study, Sidak's multiple comparison test **B)** Day 17 bodyweights compared to Day 0, corrected for tumor mass according to the *Materials and Methods* **C)** Terminal lower limb skeletal muscle masses. *Statistics for panels B-C*:*p<0.05, **p<0.01, *** p<0.001, **** p<0.0001 versus tumor bearing vehicle treated controls Dunnett's multiple comparison test; ns, no significant difference.

Supplementary Figure 4. Combined Androgen and AR-42 administration in C-26 mouse model. Animals receiving AR-42 (10 mg/kg, oral gavage), TFM-4AS-1 (10 mg/kg, sub-cutaneous injection), Combination AR-42 and DHT (10 mg/kg oral gavage and 3 mg/kg sub-cutaneous injection, respectively), Combination AR-42 and TFM-4AS-1 (10 mg/kg, both) or Vehicle were treated daily starting 6 days post inoculation for 12 days. **A)** Tumor volume comparisons between Day 8 and Day 16. **$p<0.01$ versus tumor bearing controls, Sidak's multiple comparison test; ns, no significant differences. **B)** Terminal adipose mass, ****$p<0.0001$ Tumor-bearing controls versus tumor-free controls Dunnett's multiple comparison test.

Supplementary Figure 5. Gastrocnemius Gene Expression. Gene expression from gastrocnemius muscle of individual animals from the initial study of combined GTx-024 and AR-42 administration (n=5-6 group). Expression presented relative to tumor-free vehicles controls as described in the *Materials and Methods* (Geometric Mean $\pm$ Geometric STD). Putative AR regulated genes in skeletal muscle, IGF-1 and IGF1Ea.

Supplementary Figure 6. Gene Set Enrichment Analyses (GSEA) of AR-42 regulated genes in skeletal muscles from C26 tumor bearing mice. A) Genes differentially regulated by 50 mg/kg AR-42 treatment relative to tumor bearing control from Tseng *et al.* [37] intersected with genes differentially regulated in quadriceps muscle from both severe and moderately wasting C26 tumor bearing relative to tumor free control from Bonetto *et al.* [45] **B)** Canonical pathway analysis using GSEA of the 548 genes differentially regulated in gastroc from AR-42 treated C26 tumor bearing animals versus tumor bearing controls.

Supplementary Figure 7. AR-42 mediated reversal of C26 induced transcriptional changes in skeletal muscle from tumor bearing animals. Genes differentially regulated by 50 mg/kg AR-42 treatment relative to tumor bearing controls from Tseng *et al.* [37] by 4-fold or greater (x-axis, $\log_2 FC \geq 2$) in gastroc plotted against genes differentially regulated in quadriceps muscle by 2-fold or greater (y-axis, $\log_2 FC \geq 1$) from both severe (red) and moderately (blue) wasting C26 tumor bearing mice relative to tumor free controls from Bonetto *et al.* [45].

Supplementary Figure 8. Effects of AR-42 administration on STAT3 signaling. STAT3 (replicate) and pFOXO3a western blot analysis of gastroc tissues from representative animals treated from the initial study of combined GTx-024 and AR-42 administration.

Supplementary Table Legends

Supplementary Table 1. Gene List for GSEA Overlap Analyses.

Supplementary Table 2. Serum Cytokine Panel – Complete Results. Multiplex analysis of diverse serum cytokines at Day 17 sacrifice from second study of combined GTx-024 and AR-42 administration (Mean±STD, *$p<0.05$, versus tumor bearing controls Dunnett's multiple comparison test). Eotaxin: chemokine (C-C motif) ligand 11; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte macrophage colony-stimulating factor; IFNγ: interferon gamma; IL-1a: interleukin-1 alpha; IL-1b: interleukin-1 beta; IL-2: interleukin-2; IL-3: interleukin-3; IL-4: interleukin-4; IL-5: interleukin-5; IL-6: interleukin-6; IL-7: interleukin-7; IL-9: interleukin-9; IL-10: interleukin-10; IL-12 (p40): interleukin-12 subunit p40; IL-12 (p70): interleukin-12 subunit p70; IL-13: interleukin-13; IL-15: interleukin-15; IL-17: interleukin-17; IP-10: interferon gamma-induced protein 10; KC: chemokine (C-X-C motif) ligand 1; LIF: leukemia inhibitory factor; LIX: chemokine (C-X-C motif) ligand 5; MCP: monocyte chemoattractant protein-1; M-CSF: macrophage colony-stimulating factor; MIG: monokine induced by gamma interferon, chemokine (C-X-C motif) ligand 9; MIP-1a: macrophage inflammatory protein-1 alpha; MIP-1b: macrophage inflammatory protein-1 beta; MIP-2: macrophage inflammatory protein-2; RANTES: regulated upon activation, normally T-expressed, and presumably secreted, chemokine (C-C motif) ligand 5; TNFα: tumor necrosis factor-alpha; VEGF: vascular endothelial growth factor.

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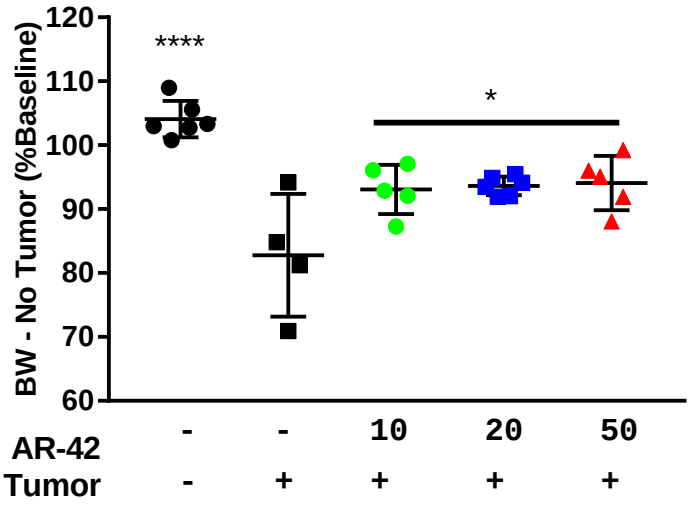
Figure 1

A


BW - No Tumor (%Baseline)
120
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AR-42
Tumor
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+

B

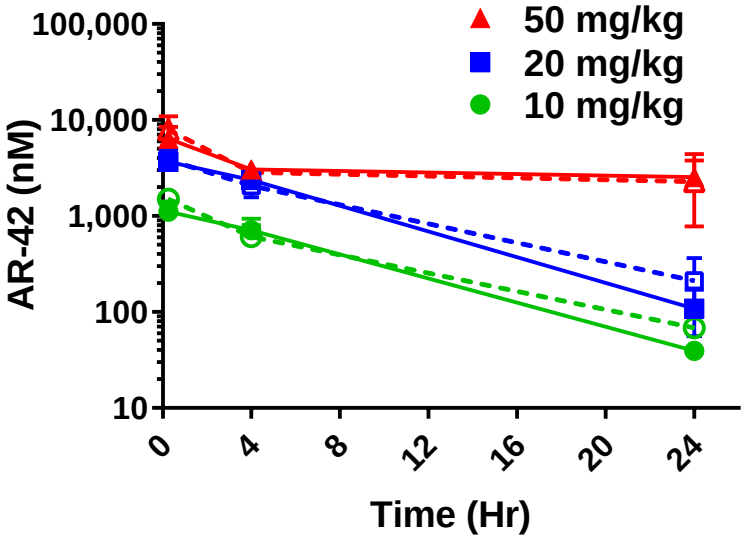

50 mg/kg
20 mg/kg
10 mg/kg
AR-42 (nM)
100,000
10,000
1,000
100
10
0
4
8
12
16
20
24
Time (Hr)

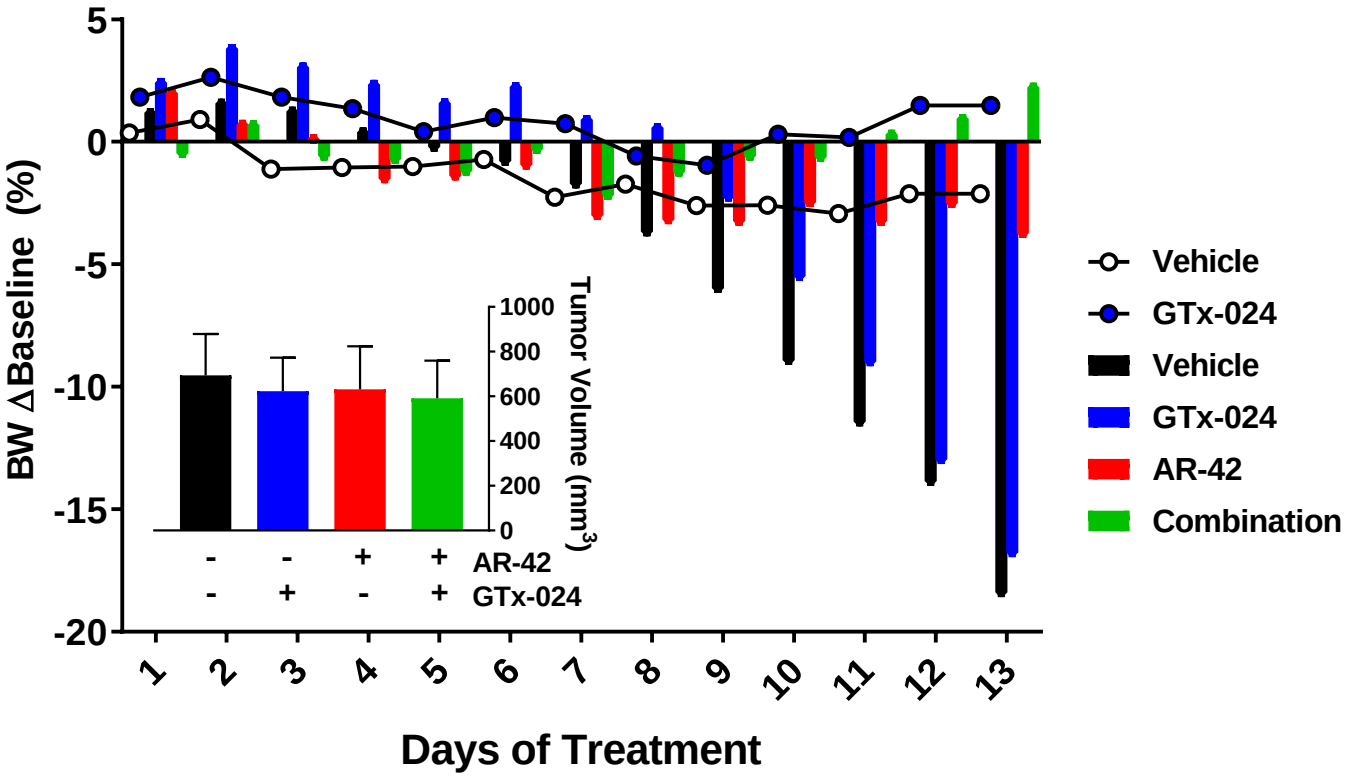
Figure 1

C

	Isoform	% inhib. at 1 μM
Class I	HDAC1	95
	HDAC2	72
	HDAC3	93
	HDAC8	87
Class IIa	HDAC4	21
	HDAC5	0
	HDAC7	22
	HDAC9	41
Class IIb	HDAC6	100
	HDAC10	86

Figure 2

A


BW ΔBaseline (%)
5
0
-5
-10
-15
-20
1
2
3
4
5
6
7
8
9
10
11
12
13
Days of Treatment
Tumor Volume (mm3)
1000
800
600
400
200
0
AR-42
GTx-024
Vehicle
GTx-024
Vehicle
GTx-024
AR-42
Combination

B

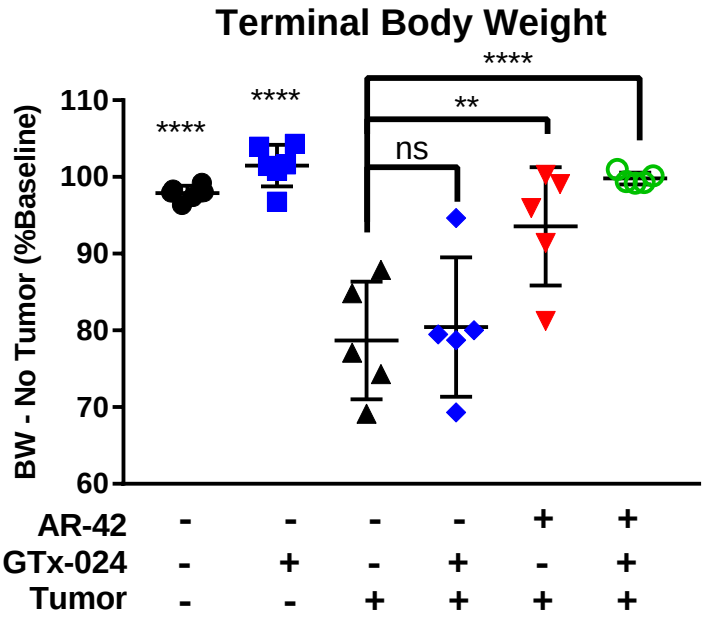
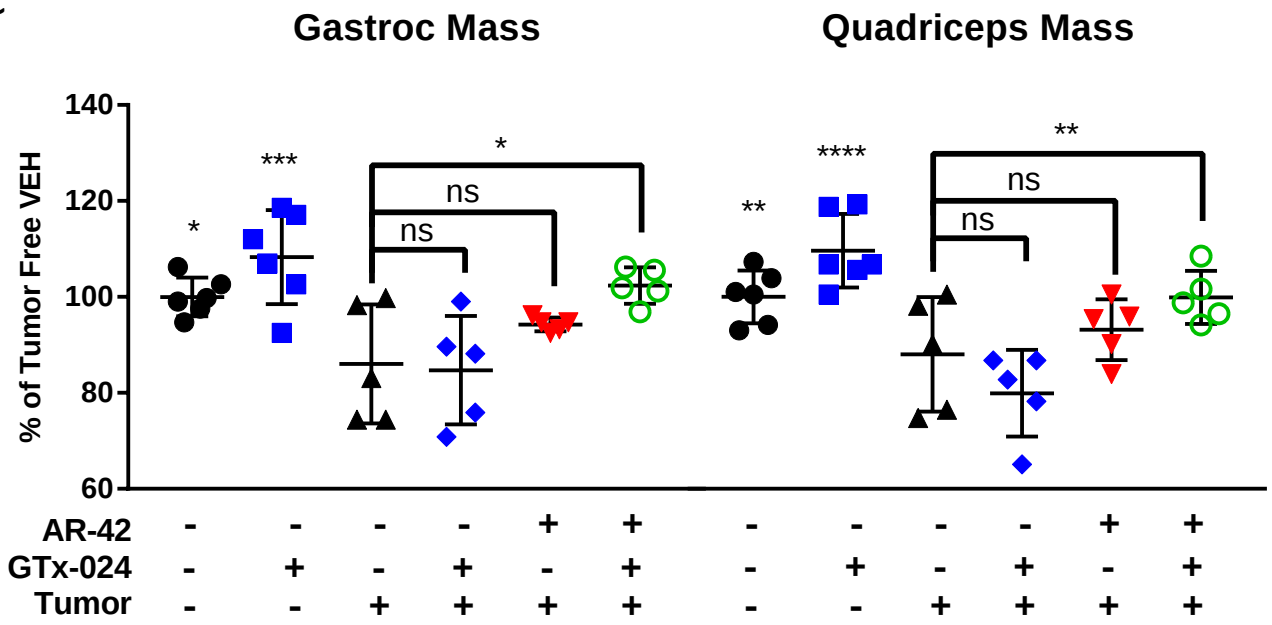

Terminal Body Weight
BW - No Tumor (%Baseline)
110
100
90
80
70
60
****
****
****
**
ns
AR-42
GTx-024
Tumor

Figure 2

C


Gastroc Mass
Quadriceps Mass
% of Tumor Free VEH
140
120
100
80
60
*
***
*
ns
ns
**
****
**
ns
ns
AR-42 - - - - + + - - - - + +
GTx-024 - + - + - + - + - + - +
Tumor - - + + + + - - + + + +

D

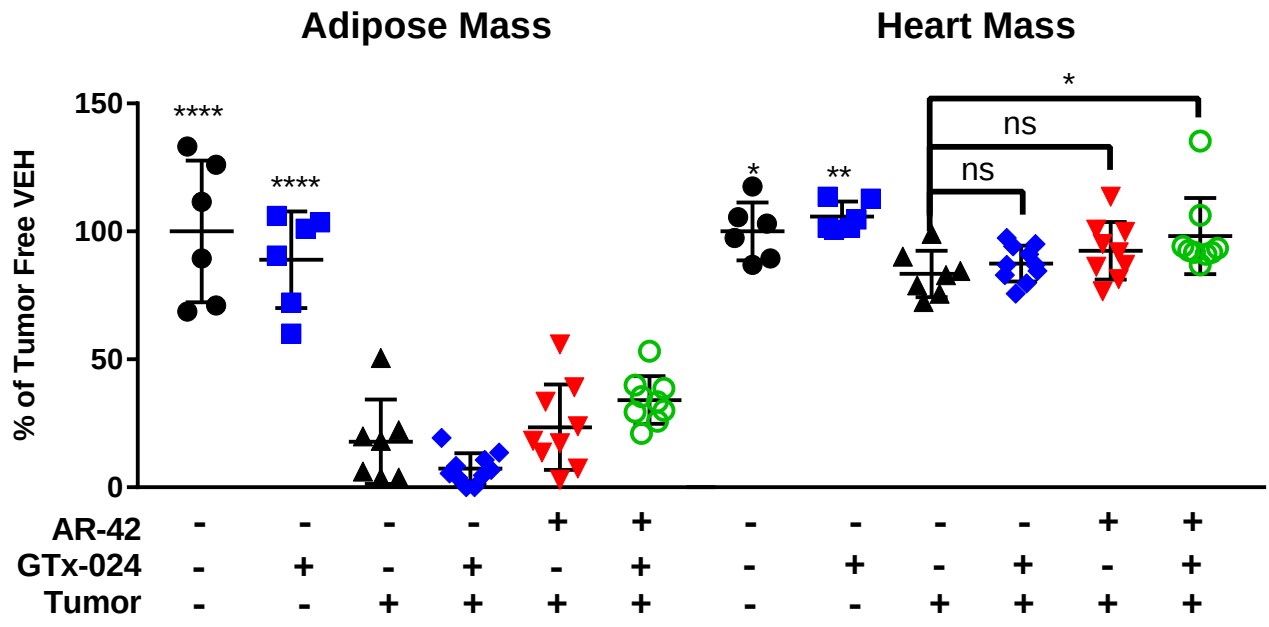

Adipose Mass
Heart Mass
% of Tumor Free VEH
150
100
50
0
****
****
*
**
*
ns
ns
AR-42 - - - - + + - - - - + +
GTx-024 - + - + - + - + - + - +
Tumor - - + + + + - - + + + +

Figure 2

E

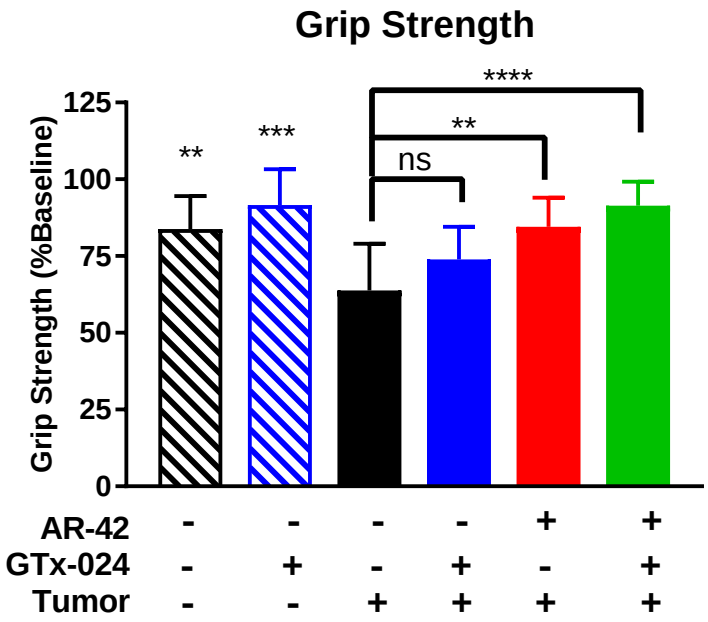
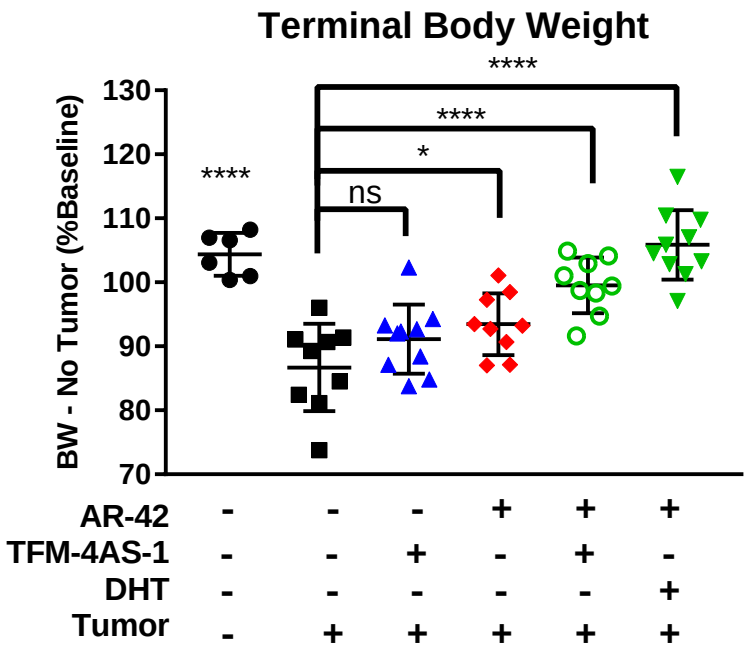

Grip Strength
Grip Strength (%Baseline)
125
100
75
50
25
0
**
***
ns
**
****
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +

Figure 3

A


Terminal Body Weight
BW - No Tumor (%Baseline)
130
120
110
100
90
80
70
****
****
*
ns
****
AR-42 - - - + + +
TFM-4AS-1 - - + - + -
DHT - - - - - +
Tumor - + + + + +

B

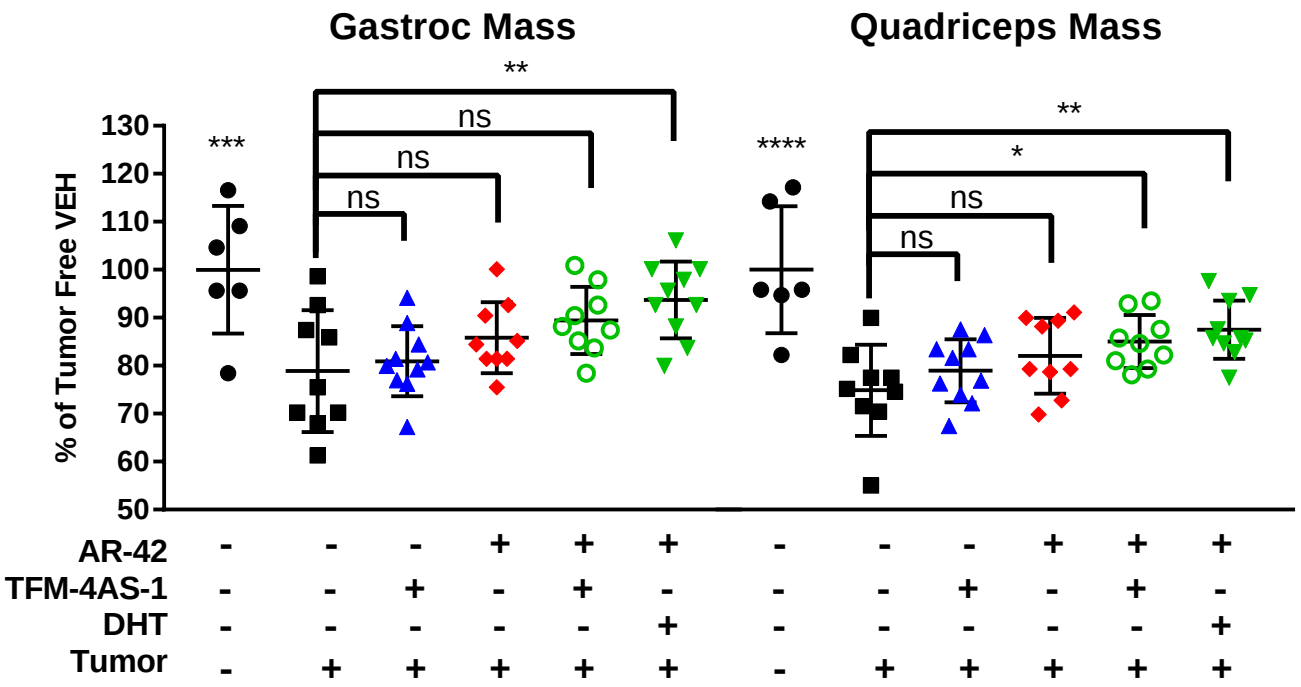

Gastroc Mass
Quadriceps Mass
% of Tumor Free VEH
130
120
110
100
90
80
70
60
50
***
**
ns
ns
ns
****
**
*
ns
ns
AR-42 - - - + + + - - - + + +
TFM-4AS-1 - - + - + - - - + - + -
DHT - - - - - + - - - - - +
Tumor - + + + + + - + + + + +

Figure 3

C

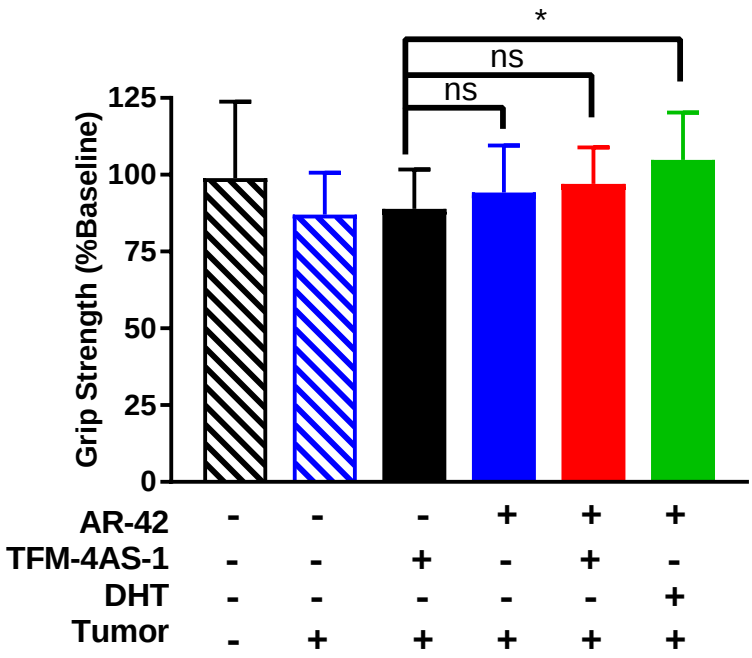

*
ns
ns
125
100
75
50
25
0
Grip Strength (%Baseline)
AR-42 - - - + + +
TFM-4AS-1 - - + - + -
DHT - - - - - +
Tumor - + + + + +

Figure 4

A

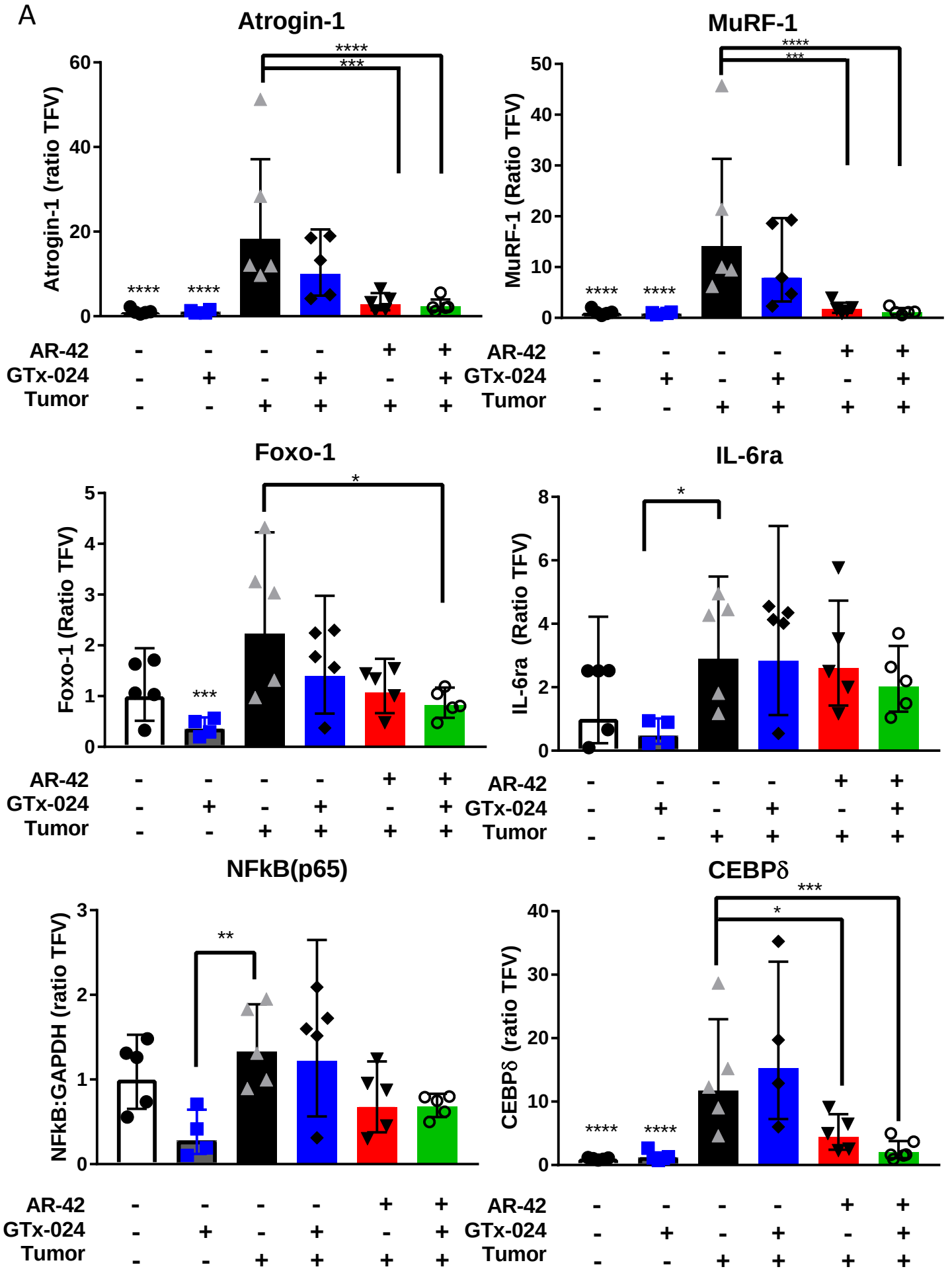
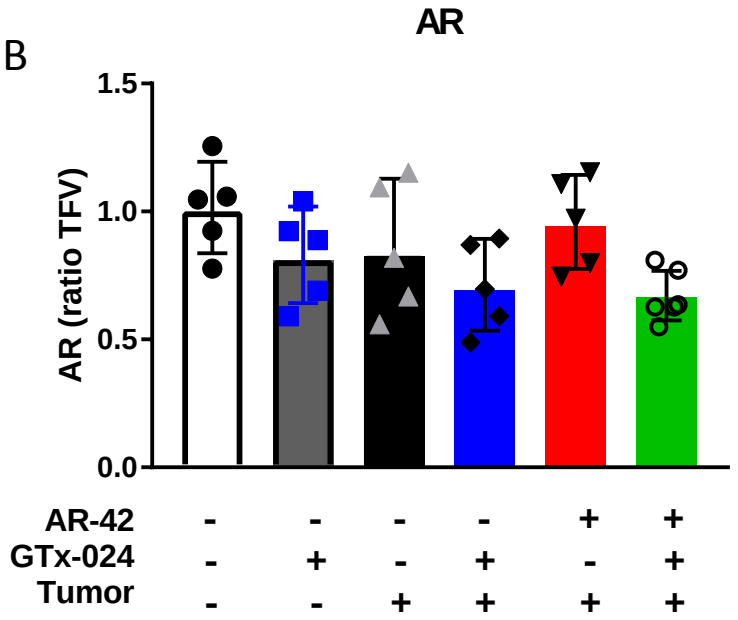

Atrogin-1
Atrogin-1 (ratio TFV)
MuRF-1
MuRF-1 (Ratio TFV)
Foxo-1
Foxo-1 (Ratio TFV)
IL-6ra
IL-6ra (Ratio TFV)
NFkB(p65)
NFkB:GAPDH (ratio TFV)
CEBPδ
CEBPδ (ratio TFV)
AR-42
GTx-024
Tumor

Figure 4

B


AR
AR (ratio TFV)
1.5
1.0
0.5
0.0
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +

C

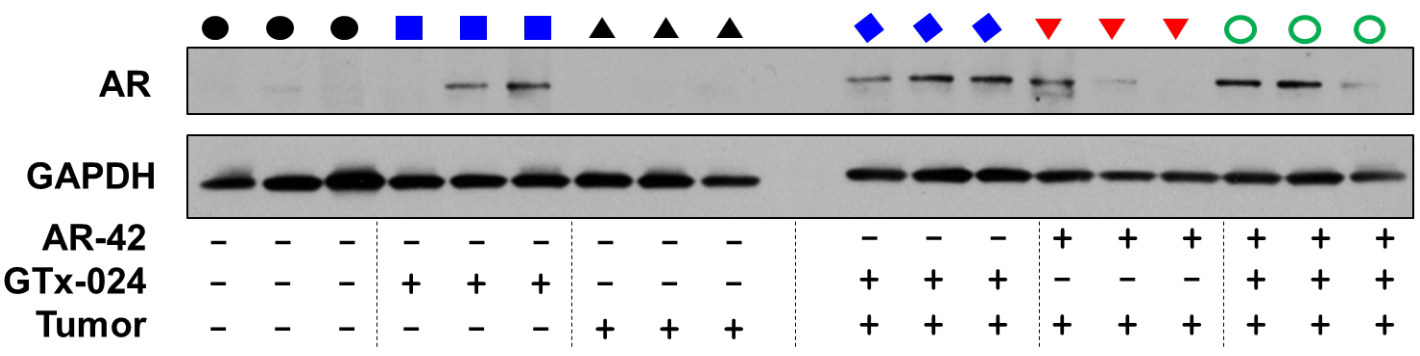
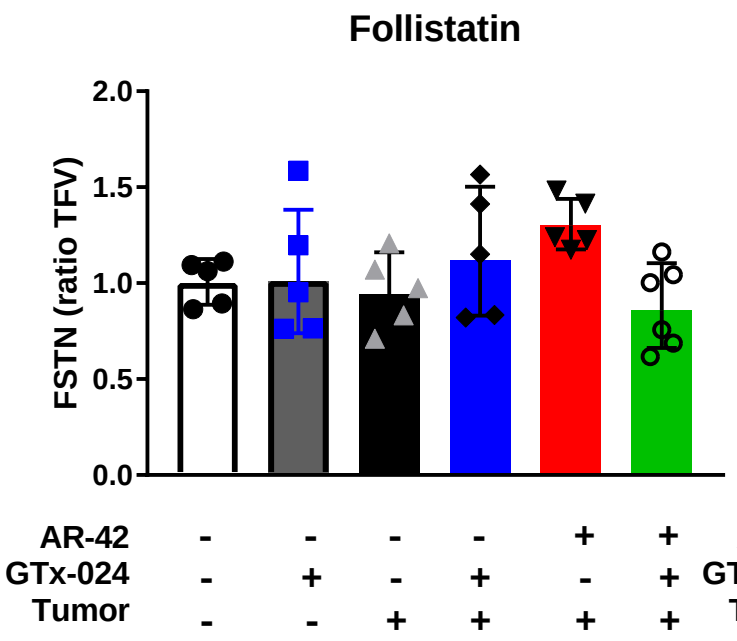
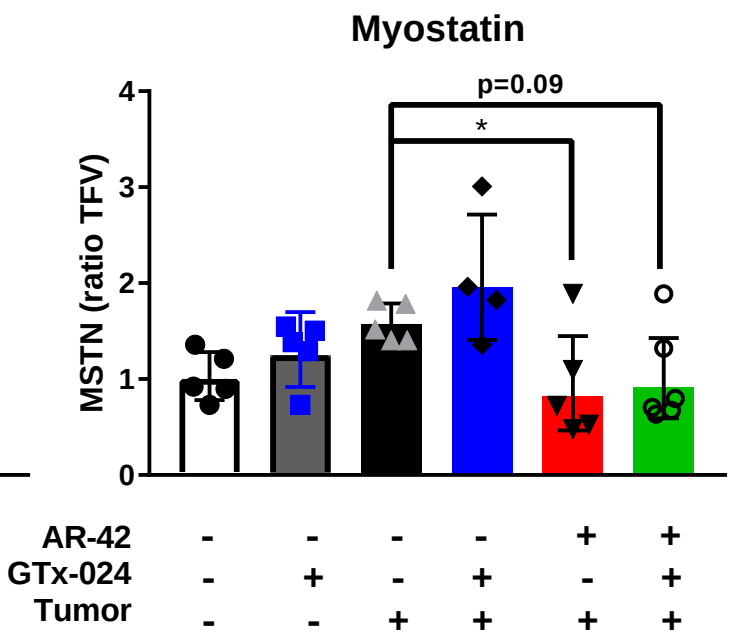

AR
GAPDH
AR-42 – – – – – – – – – – – – + + + + + +
GTx-024 – – – + + + – – – + + + – – – + + +
Tumor – – – – – – + + + + + + + + + + + +

Figure 4

D


Follistatin
FSTN (ratio TFV)
2.0
1.5
1.0
0.5
0.0
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +


Myostatin
MSTN (ratio TFV)
p=0.09
*
4
3
2
1
0
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +

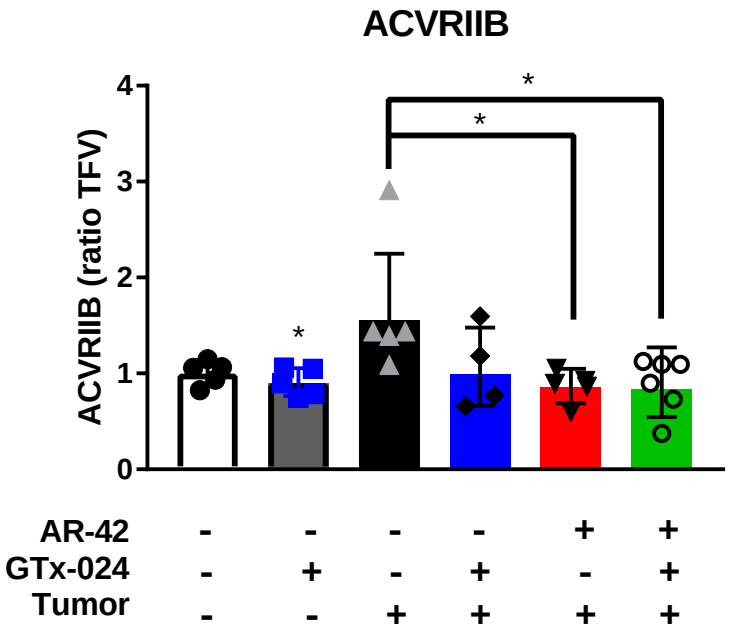

ACVRIIB
ACVRIIB (ratio TFV)
*
*
*
4
3
2
1
0
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +

Figure 4

E

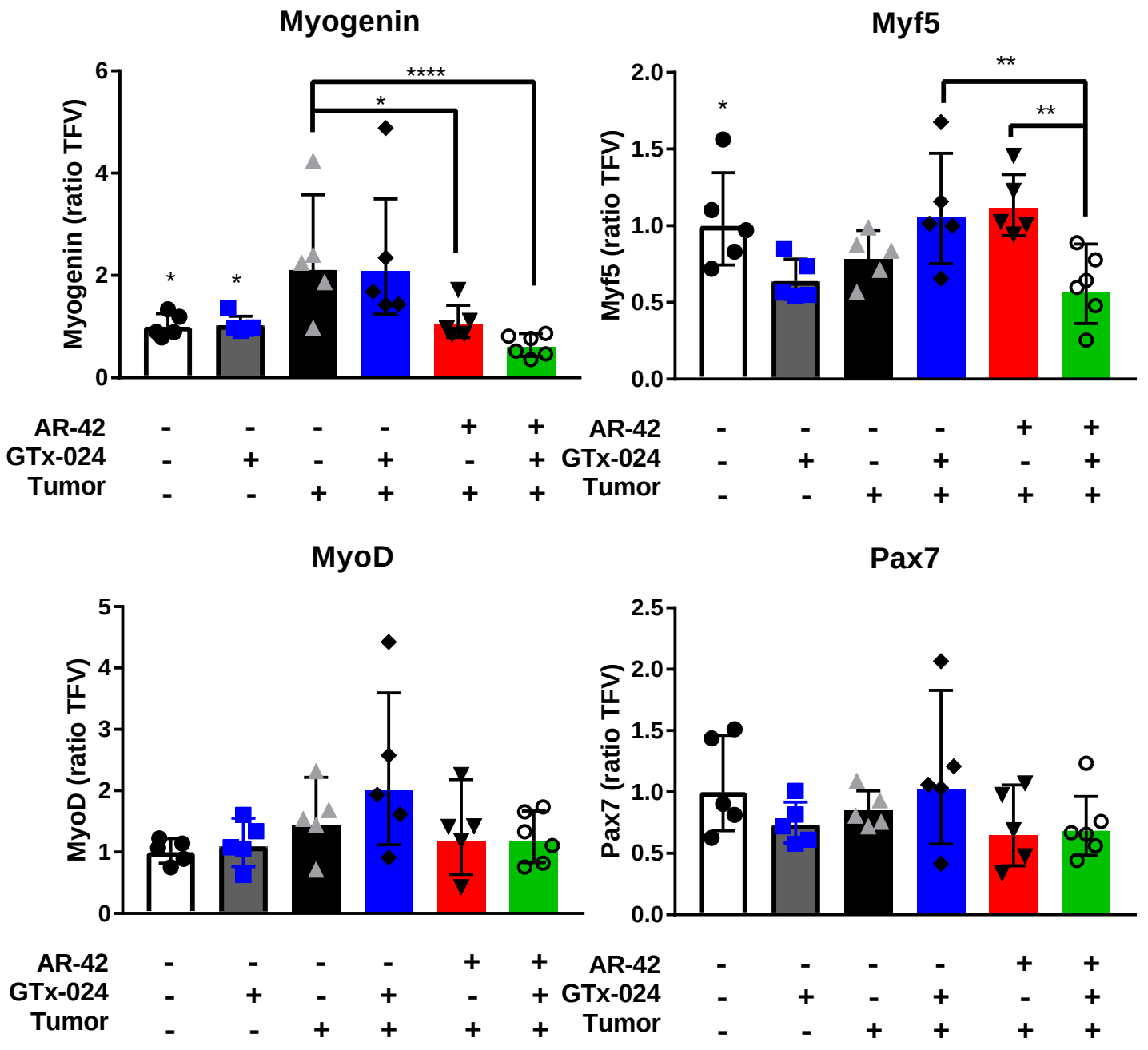
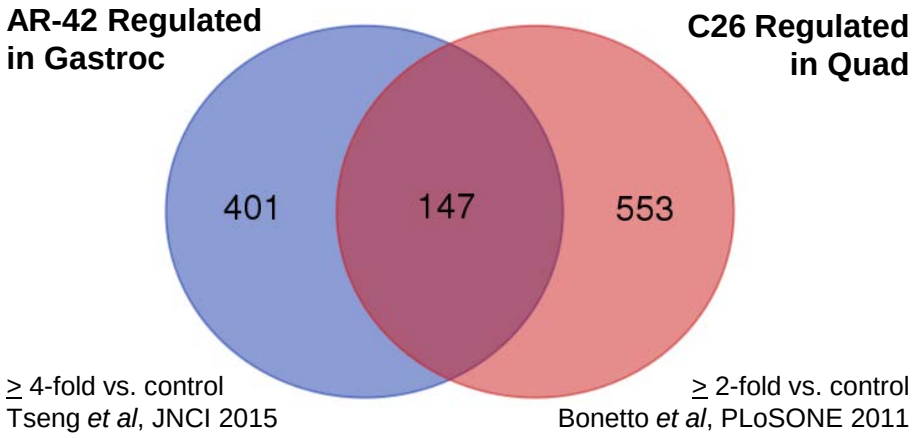
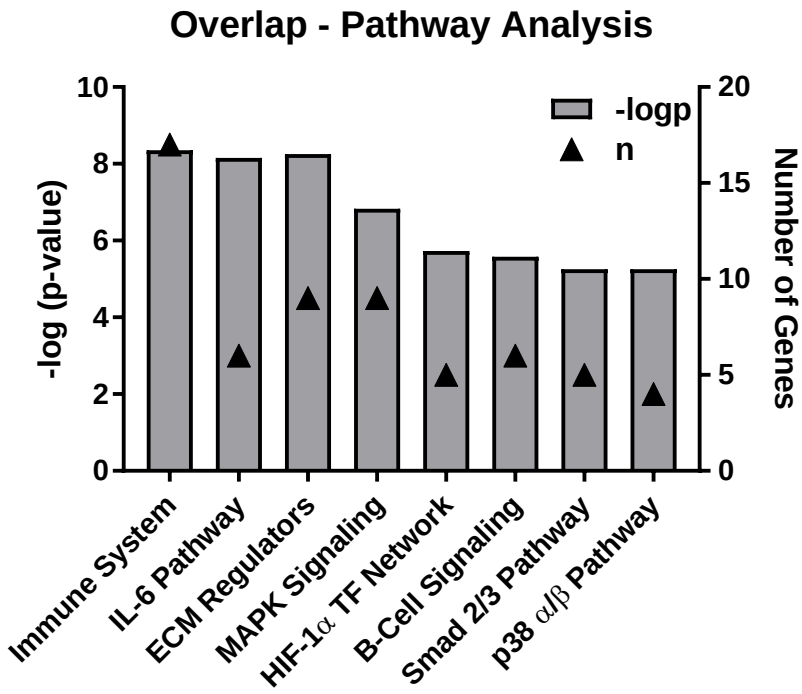

Myogenin
Myogenin (ratio TFV)
6
4
2
0
****
*
*
*
Myf5
Myf5 (ratio TFV)
2.0
1.5
1.0
0.5
0.0
**
**
*
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +
MyoD
MyoD (ratio TFV)
5
4
3
2
1
0
Pax7
Pax7 (ratio TFV)
2.5
2.0
1.5
1.0
0.5
0.0
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +

Figure 5

A


AR-42 Regulated in Gastroc
C26 Regulated in Quad
401
147
553
≥ 4-fold vs. control
Tseng *et al*, JNCI 2015
≥ 2-fold vs. control
Bonetto *et al*, PLoSONE 2011

B


Overlap - Pathway Analysis
-log (p-value)
Number of Genes
-logp
n
0
2
4
6
8
10
0
5
10
15
20
Immune System
IL-6 Pathway
ECM Regulators
MAPK Signaling
HIF-1α TF Network
B-Cell Signaling
Smad 2/3 Pathway
p38 α/β Pathway

C

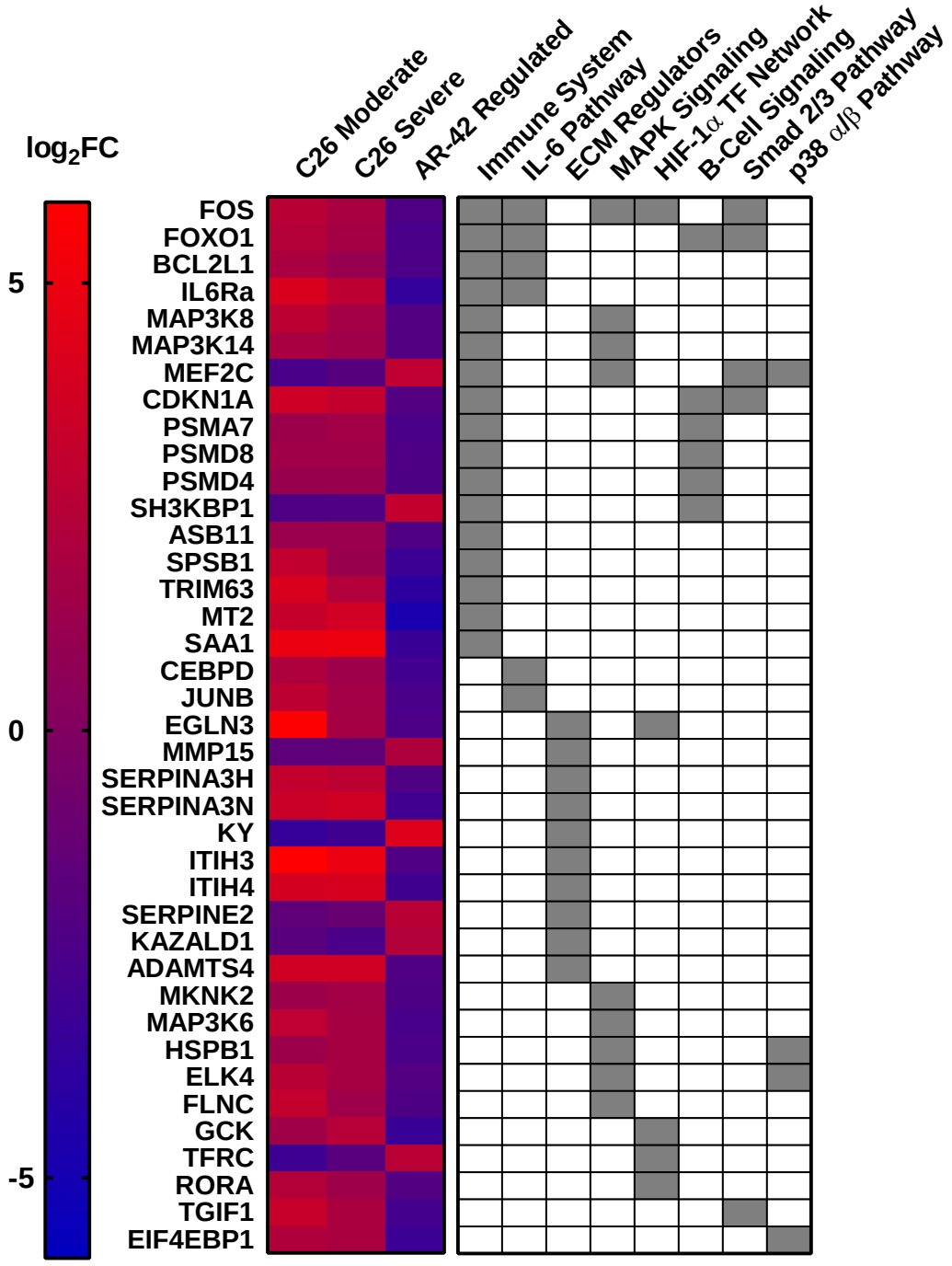
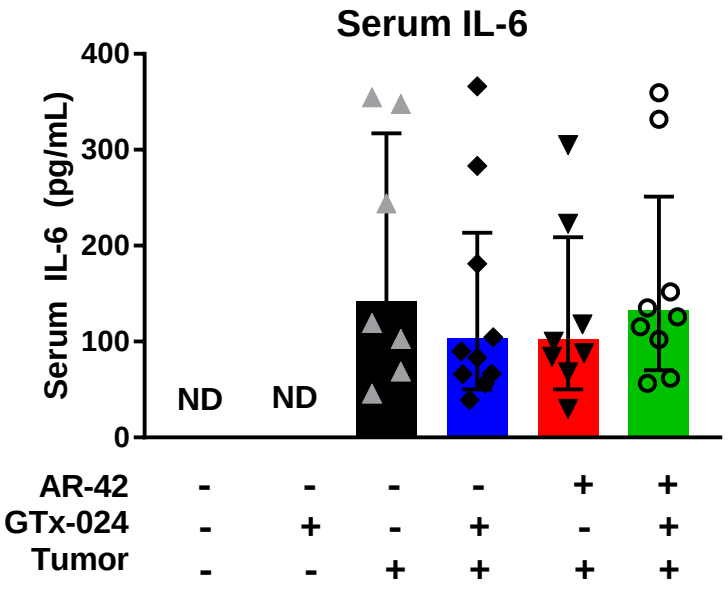
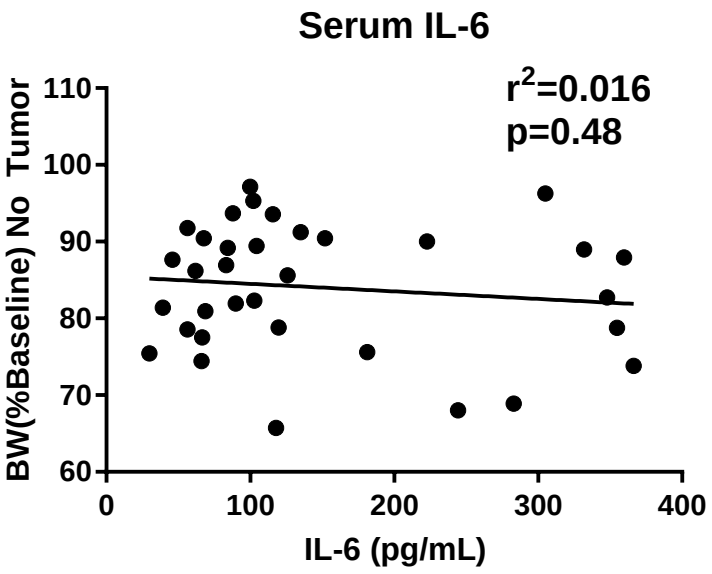

log2FC
5
0
-5
C26 Moderate
C26 Severe
AR-42 Regulated
Immune System
IL-6 Pathway
ECM Regulators
MAPK Signaling
HIF-1α TF Network
B-Cell Signaling
Smad 2/3 Pathway
p38 α/β Pathway
FOS
FOXO1
BCL2L1
IL6Ra
MAP3K8
MAP3K14
MEF2C
CDKN1A
PSMA7
PSMD8
PSMD4
SH3KBP1
ASB11
SPSB1
TRIM63
MT2
SAA1
CEBPD
JUNB
EGLN3
MMP15
SERPINA3H
SERPINA3N
KY
ITIH3
ITIH4
SERPINE2
KAZALD1
ADAMTS4
MKNK2
MAP3K6
HSPB1
ELK4
FLNC
GCK
TFRC
RORA
TGIF1
EIF4EBP1

Figure 6

A


Serum IL-6
Serum IL-6 (pg/mL)
400
300
200
100
0
ND
ND
AR-42 - - - - + +
GTx-024 - + - + - +
Tumor - - + + + +

B


Serum IL-6
r2=0.016
p=0.48
BW(%Baseline) No Tumor
110
100
90
80
70
60
0
100
200
300
400
IL-6 (pg/mL)

C

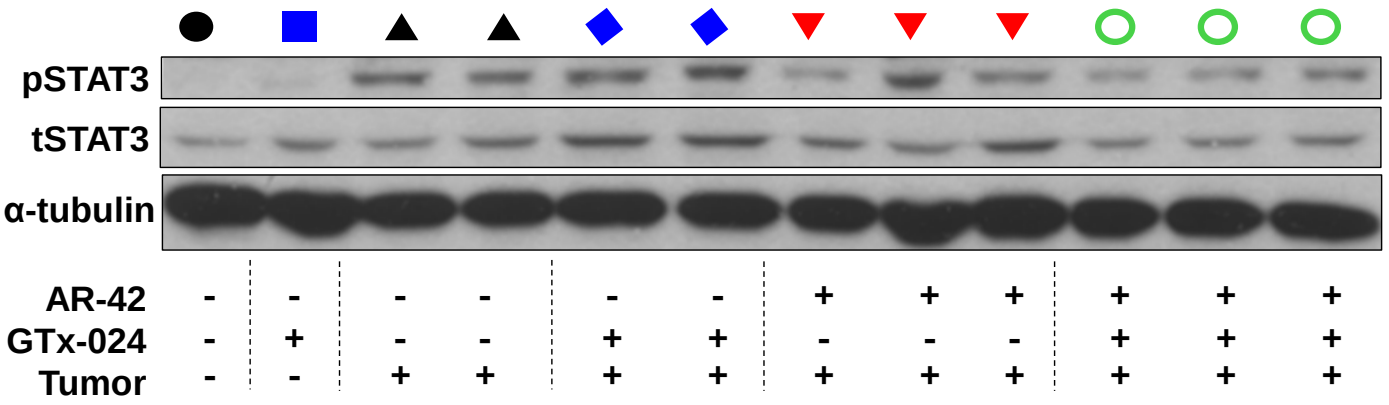

pSTAT3
tSTAT3
α-tubulin
AR-42 - - - - - - + + + + + +
GTx-024 - + - - + + - - - + + +
Tumor - - + + + + + + + + + +

Figure 6

D

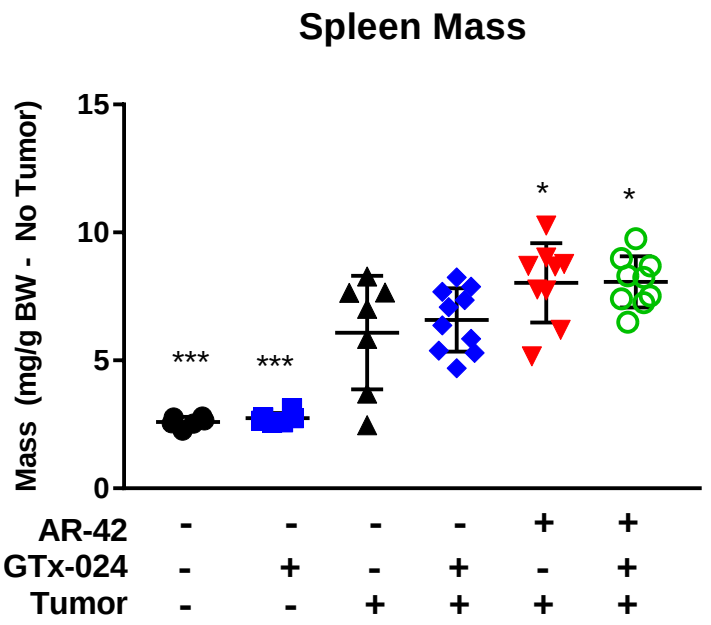


Table 1.

pg/ml	Tumor-free mice		C-26 Tumor-bearing mice			
	Vehicle	GTx-024	Vehicle	GTx-024	AR-42	Combo
G-CSF	248.66 ± 64.60*	338.39 ± 71.70*	12164.11 ± 18944.48	2446.63 ± 1625.70*	2782.18 ± 2191.30	1674.20 ± 1160.74*
GM-CSF	18.71 ± 5.56	13.27 ± 4.62*	21.92 ± 5.36	17.35 ± 4.33	18.70 ± 3.77	20.58 ± 5.40
IL-6	3.35 ± 1.51*	2.45 ± 1.31*	537.66 ± 417.18	397.54 ± 341.43	256.59 ± 183.1	448.16 ± 294.52
IL-17	3.04 ± 2.26	5.01 ± 1.18*	1.30 ± 0.57	1.75 ± 1.28	1.82 ± 0.85	2.11 ± 1.21
IP-10	162.64 ± 43.04	145.68 ± 48.83*	238.29 ± 124.78	154.76 ± 17.98*	215.35 ± 52.46	227.77 ± 45.23
KC	65.92 ± 26.47	90.02 ± 17.69	326.10 ± 215.79	288.89 ± 154.46	363.38 ± 200.65	1094.01 ± 528.53*
LIF	2.03 ± 2.17*	2.50 ± 2.34	24.51 ± 11.26	45.26 ± 21.57*	15.79 ± 5.15	28.08 ± 21.16
M-CSF	47.72 ± 27.44*	27.23 ± 10.09	23.63 ± 8.29	22.23 ± 9.45	20.21 ± 4.63	21.00 ± 4.19