# Characterizing molecular modifiers of pathogenesis in spinobulbar muscular atrophy

by

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## Dedication

This dissertation is dedicated to Hughlings J. Himwich, who first inspired me to pursue all my intellectual endeavors with passion, curiosity, and a healthy dose of skepticism.

*Namque tu solebas meas esse aliquid putare nugas.*

*Atque in perpetuum. Magister, il miglior fabbro.*

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## Abstract

Spinobulbar muscular atrophy (SBMA), or Kennedy's disease, is an inherited neuromuscular disorder caused by a polyglutamine (polyQ) tract expansion in the androgen receptor (AR). This mutation initiates misfolding and aggregation of AR, eliciting toxicity in motor neurons, progressive weakness, and muscle atrophy. PolyQ expansion also compromises the transactivation function of AR in response to androgens, resulting in androgen insensitivity. Although considerable progress has been made in characterizing molecular consequences of the polyQ mutation in SBMA, many aspects of pathogenesis, and in particular the cellular processes that modify disease development, remain incompletely understood.

Based on previous work suggesting a pathogenic role of autophagy in SBMA, I use cellular and mouse models to delineate the state of autophagy in SBMA. I show that autophagy is induced in SBMA cells and diseased tissues, and that this is due to depressed mTOR activity. These changes correlate with activity of the transcription factors TFEB and ZKSCAN3, which coordinate expression of autophagy-related genes in SBMA mice and human patients. Furthermore, these alterations in the regulators of autophagy lead to enhanced responsiveness to stimulation by nutrient deprivation and exercise. These results indicate that dysregulated transcriptional programming promotes induction of autophagy in SBMA and provide evidence for targeting autophagy for therapeutic inhibition.

Given the previously established role of small ubiquitin-like modifier (SUMO) on AR function, I characterize a novel knock-in mouse model of SBMA to address the influence of SUMO on SBMA pathogenesis. We introduce mutations that prevent SUMOylation of polyQ AR (AR113Q-KRKR) and demonstrate that, despite unaltered androgen insensitivity and neuromuscular pathology, AR113Q-KRKR mice display a striking extension of lifespan and recovery of exercise tolerance. Complementary expression analysis of the non-SUMOylatable polyQ AR reveals substantial expansion of the receptor's transactivation activity. These findings suggest that abrogating SUMO modification of polyQ AR mediates amelioration of the SBMA phenotype, in part by improving skeletal muscle physiology. Additionally, these studies not only reveal new insights in the comparative roles of polyQ AR toxicity versus loss of function in affected tissues, but they also establish the benefits of enhancing AR function in SBMA for therapeutic design.

# Chapter 1

# Introduction

## 1.1 Spinobulbar muscular atrophy (SBMA)

### 1.1.1 Clinical features, genetics, and pathogenesis

Spinobulbar muscular atrophy (SBMA) is an inherited, slowly progressive degenerative disease of lower motor neurons and skeletal muscle. Also known as Kennedy's disease, after the eponymous neurologist William R. Kennedy first described the principal features in 1968, SBMA is characterized clinically by progressive atrophy and weakening of the bulbar and limb musculature [1]. Common symptoms include dysarthria, dysphagia, tremor, and gait disturbances [2, 3]. The muscular clinicopathology of SBMA patients is characteristic of lower motor neuron disease with bulk atrophy, flaccid paralysis, hyporeflexia, and fasciculations [4]. Evidence of primary myopathy also exists with painful cramps, weakness, and elevated serum creatine kinase (CK) levels [5]. In addition, SBMA patients commonly exhibit androgen insensitivity, with symptoms including gynecomastia, testicular atrophy, and oligospermia [6]. On histopathologic examination, by end stage disease there is marked anterior horn cell loss in both brainstem and spinal cord, and dorsal root ganglia also exhibit a decreased number of sensory neurons [7-9]. Skeletal muscle biopsies show angulated atrophic fibers and fiber type grouping suggestive of

denervation [10]. The prevalence of SBMA is estimated to be at approximately 1 in 40,000, with a proportionally greater number of cases in Japan and Finland, but many patients are likely misdiagnosed due to similar clinical features to amyotrophic lateral sclerosis and other, more common motor neuron diseases [11, 12]. There is currently no established or effective treatment for SBMA.

The occurrence of SBMA through multiple generations of patient families suggested a genetic basis of disease, and the segregation pattern initially indicated an X-linked recessive mode of inheritance [1]. Subsequently, La Spada et al. were the first to identify the causative mutation in 1991, which consists of an expansion of a CAG repeat tract in exon 1 of the androgen receptor (*Ar)* gene located on the long arm of the X chromosome (Xq11-12) [13]. Of the nine polyglutamine diseases, which include Huntington's disease, dentatorubropallidoluysian atrophy (DRPLA), and six sub-types of spinocerebellar ataxia (SCA 1, 2, 3, 6, 7, and 17), the causal link between polyglutamine tract expansion and neurodegenerative disease was first established in SBMA. As is characteristic of trinucleotide repeat disorders, SBMA exhibits both CAG-repeat instability and anticipation, or an inverse relationship between tract length versus age of onset and severity of disease [14-19].

Unlike the other polyglutamine diseases, which are inherited in an autosomal dominant fashion and are fully penetrant, SBMA only manifests in male patients while exhibiting little to no disease phenotype in females [14, 20-23]. Although the diminution in disease penetrance in females was initially attributed to lyonization, the identification of subclinical and asymptomatic females homozygous for the mutant allele made this explanation unlikely [19, 22, 24, 25]. Furthermore, although the AR is widely expressed in multiple tissue types, SBMA pathology paradoxically remains isolated within a few distinct cell populations. These observations

illustrate two key features of SBMA pathogenesis – hormone dependency and selective cellular vulnerability – which will be further discussed in later sections.

The causative mutation for SBMA resides in the androgen receptor protein, a Group I steroid hormone nuclear receptor that contains three principal domains: an N-terminal domain (NTD), DNA-binding domain (DBD), and ligand-binding domain (LBD), with a small hinge interregion between the DBD and LBD containing a bipartite nuclear localization sequence [26-29]. The DBD and LBD orchestrate the principle functions of the AR as a steroid hormone receptor. Unbound by ligand, the inactive AR resides in the cytosol bound to chaperone proteins. Upon binding of the LBD by cognate androgens testosterone and dihydrotestosterone (DHT), the AR assembly with the chaperone machinery becomes much more dynamic, the AR homodimerizes and the AR-ligand complex translocates to the nucleus to bind DNA via the DBD, whereupon modulation of genes containing an androgen response element occurs. This mechanism of action allows androgenic hormones to exert masculinizing and trophic effects in target tissues.

Further control of these functionalities is accomplished through numerous regulatory elements including short tandem amino acid repeats and sites for post-translational modification. One such element consists of a CAG repeat stretch that encodes a glutamine (Q) tract that is associated with length-dependent modification of AR function. The polyglutamine tract is highly polymorphic and ranges between 8 and 35 repeats in the general population [30]. Shorter CAG tracts correlate with increased ligand-mediated activation of the AR, while longer tracts, even within the normal range, appear to depress AR activity [31-37]. CAG tracts above a critical threshold length of 38 glutamines result in SBMA, as these expanded polyglutamine stretches promote unfolding of the AR and provide large polar surfaces essential for driving the interaction

and aggregation of AR monomers [38, 39]. Surviving lower motor neurons and scrotal skin biopsies from SBMA patients consequently demonstrate nuclear inclusions of aggregated AR with the appropriate histochemical staining [8, 40-42]. Importantly, although AR function is negatively correlated with CAG tract length, a partial loss of function mediated by CAG expansion fails to provide adequate explanation for both the androgen insensitivity and neuromuscular pathology seen clinically in SBMA [32, 43-47]. Rather, pathogenic CAG expansion is presumed to confer an additional toxic gain of function to the AR, since patients with androgen insensitivity syndrome stemming from AR loss of function mutations do not exhibit the neuromuscular pathology of SBMA [48].

### 1.1.2 Regulation of AR function

Post-translational modifications coordinate additional alterations of AR function. Phosphorylation occurs both in the presence and absence of androgen and directs multiple effects, including regulation of AR conformational changes, localization, and turnover, potentiation of ligand responsiveness and transcriptional activity, and protein-protein interactions [49-54]. Phosphorylation is upregulated in prostate cancer and correlates with increased morbidity and mortality, while negatively modifying toxicity in SBMA [52, 55, 56]. AR acetylation regulates cofactor association, transcriptional activity, and prostate cancer cell growth and survival [57-62]. Modification by small ubiquitin-like modifier (SUMO) occurs at two lysine residues within consensus SUMOylation motifs in the NTD, negatively regulates AR transcriptional activity, and negatively modulates both prostate cancer proliferation and progression and SBMA cytopathology (discussed further below) [63-69]. Ubiquitination mediates AR degradation through the ubiquitin-proteasome system or augments AR

transactivation, depending on the biological context and E3 ligase involved [70-74]. In SBMA, chaperone-enhanced ubiquitination of the AR increases AR degradation and ameliorates disease [75].

In addition, the NTD and LBD contain several transcriptional activation function (AF) domains. The NTD contains AF-1, which encompasses residues 142-485 and is necessary for full ligand-dependent transactivation, and AF-5, which encompasses residues 351-528 and is sufficient to act as a ligand-independent, constitutively active transcriptional activator [76-79]. AF-1 mediates a ligand-dependent intramolecular interaction of the NTD with the C-terminus of the AR via consensus FxxLF/WxxLF motifs (F = phenylalanine, W = tryptophan, x = any amino acid), and this interaction is necessary for AR transactivation *in vivo* [80-84]. The LBD contains AF-2, which is required for ligand-dependent AR activation [85, 86]. Within AF-2 are binding sites that recognize leucine-rich motifs with the conserved sequence LxxLL (L = leucine, x = any amino acid) that mediate interactions with AR coactivators [87, 88]. These native functionalities are not only essential for normal AR function but also SBMA pathogenesis, as disruption of these domains significantly attenuates AR aggregation and toxicity [89, 90].

### 1.1.3 Ligand-dependent toxicity

As indicated previously, a unique feature of SBMA is the initiation of pathogenesis by androgens, the endogenous ligands of AR. Ligand dependency accounts for the predominant incidence of SBMA in male patients and paucity of disease even in female homozygotes, since females harbor much lower levels of circulating androgens. Furthermore, since key pathogenic events in both SBMA and other proteinopathies are unfolding and aggregation of mutant proteins, and since aspects of these events are concentration-dependent, it follows that

compartmentalization of the polyQ AR in the nucleus, mediated by ligand-dependent nuclear translocation, is a critical step in SBMA [91-93].

Among the cellular models of SBMA, the dependence of disease on ligand is well established. In the mouse-rat hybrid glioma-neuroblastoma line NG108-15, in which AR22Q and AR52Q are stably transfected, the androgen-dependent proliferation present in cells expressing wild type AR is abrogated in cells expressing an expanded Q-tract AR [94]. Similarly, transient expression of AR constructs in simian COS-7 cells demonstrates ligand-dependent toxicity and aggregation [95]. Treatment with antiandrogens in the COS-7 model and deletion of the LBD abrogate these effects, and antiandrogens rescue pathologic aggregation of the mutant AR in Neuro2a cells [95, 96]. These findings demonstrate the essential role of ligand binding for fully reproducing SBMA cytopathology. Ligand dependent toxicity is also demonstrable in a PC12 cell model stably transfected with an expanded Q-tract full length AR under the control of a tetracycline-inducible promoter [97]. The glutamine-length appearance of morphologically visible nuclear inclusions, high molecular weight aggregates on Western blot, and cytotoxicity are produced only in the presence of androgen or synthetic androgen analogs. Notably, nuclear translocation *per se* is not sufficient to initiate pathogenesis, suggesting that ligand binding itself initiates additional conformational changes and cellular processes necessary for full development of disease [98].

*In vivo*, the recapitulation of disease in a ligand-dependent manner occurs in both mice (males compared to females) and *Drosophila* (DHT treatment over vehicle) [91-93, 99-101]. Importantly, the gender limitation in mice occurs only in models expressing a full length androgen receptor, indicating that the truncated version of polyQ AR, which does not contain the LBD, lacks features of the protein critical for full disease iteration. Additionally, symptoms and

pathology comparable to the male phenotype in mice can manifest in females when treated with androgen [91, 101]. Furthermore, prevention of polyQ AR nuclear translocation and amelioration of disease occurs with mutation of the nuclear localization signal or surgical and pharmacologic castration [91, 98, 100, 102]. Together, these data *in vivo* clearly implicate the essential role of androgens in SBMA and inform efforts to assess androgen-targeted therapy in human clinical trails.

### 1.1.4 Partial loss of AR function

One proposed explanation for the loss of AR function phenotype in SBMA, manifested in human patients as androgen insensitivity, is lower expression levels of the expanded Q-tract AR compared to wild type AR. This observation is documented in both SH SY-5Y and MN-1 cell models as well as *in vivo* [47, 103-105]. Additionally, expansion of the glutamine tract itself intrinsically confers diminution of AR transactivation, as demonstrated by expression assays comparing WT and expanded Q-tract AR in MN-1 cells and AR110Q YAC transgenic mice [47, 106]. Expansion of the glutamine tract also accelerates AR turnover, thereby yielding further decrement in AR function [47]. Notably, testicular abnormalities, including disruption of germ cell maturation in AR113Q knock-in male mice, reveal toxic effects of the mutant protein, suggesting that phenotypic features such as diminished male fertility may be caused by a mixture of both loss and gain of function conferred by the expanded glutamine tract [107].

### 1.1.5 Transcriptional dysregulation

In addition to negative effects on the intrinsic transactivational capacity of the AR, aberrant cofactor interaction has been posited as a mechanism responsible for the toxic gain of

function of the expanded Q-tract AR. One key observation in MN-1 cells, mouse, and *Drosophila* models of SBMA is the co-localization of transcription factors, including CREB binding protein (CBP), in nuclear inclusions of the mutant AR, suggesting that cytotoxicity stems in part from abnormal interaction with and sequestration of transcription factors leading to dysregulation of gene expression, including that of vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ) receptor type II [93, 108-111]. Analogously, these pathologic changes are shown to occur in other polyglutamine diseases [112, 113]. Interestingly, CBP and several other inclusion interactors also contain polyglutamine tracts, which may facilitate AR-cofactor association.

It has therefore been hypothesized that accumulation of these factors and other critical regulators in nuclear inclusions results in a depletion of their availability, thereby compromising their functions in transcription. In line with this model of pathogenesis, treatment of cell and animal models with HDAC inhibitors and overexpression of CBP in a *Drosophila* model of polyglutamine disease significantly improves transcriptional aberrancies and rescues cell death *in vitro*, ommatidial degeneration in *Drosophila*, and improves both survival and motor performance in SBMA mice [108, 114-116].

### 1.1.6 AR and proteotoxicity

Neuronal intranuclear inclusions (NII), defined as proteinaceous aggregates visible on histopathology with the appropriate staining, are a pathognomonic feature of SBMA, contain at least a portion of the polyQ AR, and are found in lower motor neurons and scrotal skin cells of SBMA patients [8, 40-42]. These inclusions are similar to those identified in other polyQ disorders and their role in disease pathogenesis is similarly controversial. In model systems of

SBMA, as in other polyQ diseases, these large nuclear inclusions do not always correlate with cell death. Rather, pathology is better correlated with the occurrence of microaggregates, which, in contrast to inclusions that are defined histopathologically, are soluble intermediates of aggregated and unfolded mutant proteins that are isolated biochemically [117-120]. Specifically, glutamine-length dependent toxicity can be demonstrated in the SH SY-5Y model of SBMA without aggregate formation [103]. Moreover, toxicity correlates with microaggregates in the inducible PC12 cell model of SBMA as well as Sf9 cells and *Drosophila* [97, 121].

Additional studies further dissociate toxicity from AR inclusions. Treatment of HEK293, PC12, and *Drosophila* models of SBMA with the compound B2 promote inclusion formation and reduce toxicity [122]. In HEK293 and MN-1 cells, expansion of the AR glutamine tract promotes its incorporation in the formation of aggresomes, which are large juxtanuclear structures similar to intranuclear inclusions [123]. Aggresomes and inclusions may represent a cellular adaptive response to mutant AR and other aggregated proteins, since their formation correlates with cell survival and their disruption exacerbates cytotoxicity [124]. These results lend further support to the notion that aggregates are end-stage, protective structures rather than the primary toxic entity.

The sole detection of AR amino-terminal epitopes within nuclear inclusions from SBMA patient tissue suggested that these aggregates are composed of a proteolytic byproduct of the AR protein that includes the expanded glutamine tract [40, 41]. Pathogenic proteolysis of the AR occurs in a caspase-dependent manner *in vitro*, in line with proteolytic processing seen in other polyglutamine disease models [44, 125-136]. Although expression of truncated polyQ AR fragments in cell and *Drosophila* models confers toxicity in a glutamine-length dependent manner, expression of amino-terminal truncated fragments in mice causes toxicity that does not

exhibit the gender delimitation or cell-type specificity of SBMA [109, 137, 138]. Taken together, these studies indicate that, although protein cleavage is likely a component of the disease process that generates a toxic protein fragment, model systems based on expression of these fragments do not reproduce essential aspects of the SBMA phenotype.

### 1.1.7 Chaperones and AR proteostasis

Heat shock proteins (HSP) are essential regulators of protein folding, function, and stability. For the AR, the heat shock protein 90 (Hsp90)-based chaperone machinery serves to modulate ligand affinity, ligand-dependent conformational changes, and nuclear trafficking following ligand binding. In this machinery, association with Hsp90 stabilizes the AR, whereas proteasomal degradation of the unfolded receptor is regulated by Hsp70 and its co-chaperones through the recruitment of chaperone-dependent E3 ubiqutin ligases including CHIP (C-terminal Hsp70-interacting protein) [72-74, 139-143]. Multiple studies indicate the involvement of these chaperones in SBMA pathogenesis. Components of the Hsp70/90 machinery co-localize in mutant AR aggregates, suggesting abnormal seqestration of these chaperones and raising the possibility of dysregulation of chaperone-mediated proteostasis [109, 144]. Conversely, overexpression of Hsp70 or pharmacological inhibition of Hsp90 increases AR degradation and amelioriates disease phenotype in cellular and mouse models of SBMA, and similar beneficial effects in SBMA mice are observed following overexpression of CHIP [75, 145-147]. Administration of 17-diethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) and 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG), which are derivatives of geldanamycin and inhibitors of Hsp90, promote degradation of the AR and improve motor performance in AR97Q transgenic mice [148, 149]. Together, these studies establish that inhibition of Hsp90 or

activation of Hsp70-dependent ubiquitination are promising therapeutic targets for trial in SBMA patients.

### 1.1.8 Pathways downstream of AR

In addition to causing transcriptional and proteostatic dysregulation, the toxic gain of function conferred by the expanded glutamine tract disrupts a large number of downstream pathways that are critical for cell survival **(Fig. 1.1)**. Hormone and glutamine length dependent changes in RNA processing have been demonstrated in SBMA knock-in mice, indicating that both transcriptional and post-transcriptional regulation of gene expression is altered in disease [150]. Additionally, multiple cytosolic targets of toxicity have been identified. MN-1 cells expressing AR65Q demonstrate marked mitochondrial pathology, increased activation of the intrinsic apoptosis pathway, and dysregulation of nuclear-mediated mitochondrial gene expression through PGC-1 suppression, while antioxidant treatments rescue toxicity [136, 151]. These studies suggest that expansion of the AR glutamine tract promotes mitochondrial dysfunction and provides a direct causal link between transcriptional aberrance and mitochondrial pathology.

Additionally, the unfolded protein response is significantly upregulated in SBMA cell models and in AR113Q knock-in mice, and genetic deletion of the ER stress-dependent transcription factor CHOP (C/EBP homologous protein) exacerbates disease phenotype, thereby implicating a role of ER stress in SBMA [152, 153]. The polyQ AR has also been shown to compromise retrograde axonal transport, an effect that may contribute to lower motor neuron degeneration [154-157]. While cell-autonomous toxicity within vulnerable cell populations may be mediated by abnormal protein interactions (see above), there is also evidence that toxic effects

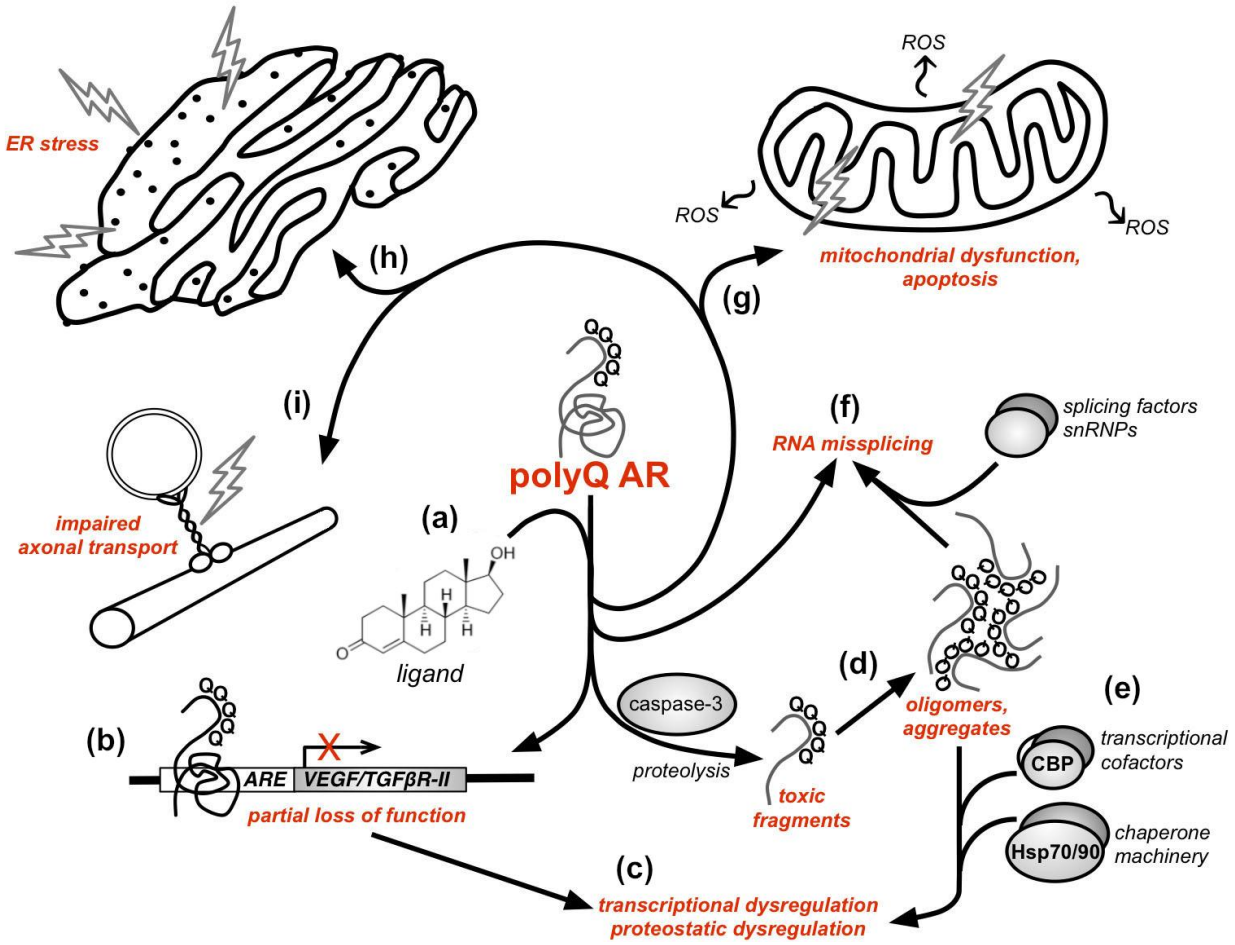



**Figure 1. Multiple cellular pathways altered by polyglutamine expansion in AR.** Expansion of the polyglutamine tract in the NTD of the androgen receptor (denoted by series of Qs) beyond 38 CAG repeats promotes AR unfolding and is necessary but not sufficient for development of SBMA. **(a)** Binding of polyQ AR to cognate ligands testosterone and DHT drives the conformational changes and nuclear localization of the mutant protein required for full pathogenesis. **(b)** Reduction of transactivation function leads to disruption of androgen-responsive gene expression and **(c)** transcriptional dysregulation, which in turn contribute to the phenotype of androgen insensitivity in SBMA patients. **(d)** Proteolysis of the polyQ AR, which may be caspase-mediated, generates toxic, N-terminal fragments of the mutant protein that ultimately oligomerize and aggregate in nuclear inclusions. **(e)** These inclusions contain accumulations of transcriptional cofactors (such as CBP), molecular chaperones (such as Hsp70/90 complexes), and splicing machinery, the depletions of which further disrupt vital **(c)** transcriptional and proteostatic processes and **(f)** RNA splicing. Selectively vulnerable cell populations in SBMA experience additional toxic insults, including **(g)** mitochondrial pathology, **(h)** ER stress, and **(i)** disruption of retrograde axonal transport. AR, androgen receptor; polyQ, polyglutamine; ARE, androgen response element; VEGF, vascular endothelial growth factor; TGFβR-II, transforming growth factor β receptor type II; CBP, CREB binding protein; snRNP, small nuclear ribonucleoprotein.

arising in skeletal muscle initiate non-cell-autonomous degeneration of lower motor neurons, perhaps by impairing trophic support [52, 101, 158-161]. Taken together, these studies indicate that multiple downstream pathways are disrupted by the polyQ AR. As no single pathway has emerged as a critical mediator of pathogenesis, we suggest that therapeutic strategies targeting the mutant protein may be most effective in modifying the course of disease.

### 1.1.9 Therapeutic interventions and clinical trials

The understanding of disease mechanisms gleaned from cell and animal models have provided the basis for several clinical trials to date. To address the ligand-dependency of SBMA, administration of leuprorelin acetate, which is a partial agonist of gonadotropin releasing hormone and a potent suppressor of testosterone release, rescued disease in AR97Q mice [91, 102]. These results were translated into a phase 2 trial for the use of leuprorelin in SBMA patients [162, 163]. Leuprorelin treatment for 48 weeks did not produce significant improvement in the primary outcome measures of Revised ALS Functional Rating Scale (ALSFRS-R) score and serum CK level. Several secondary outcomes showed significant rescue, including measures of cricopharyngeal function and nuclear inclusions on scrotal skin biopsy. Furthermore, in an open label follow-up, all of these measures except serum CK saw significant improvement after 96 weeks.

These encouraging findings were followed by the phase 3 trial known as the Japan SBMA Interventional Trial for TAP-144-SR (JASMITT). In this larger study, the leuprorelin group saw no significant recovery of cricopharyngeal function or ALSFRS-R scores, although there were significant improvements in some secondary measures, including reduced polyglutamine positive cells in scrotal skin biopsies and serum CK levels. In a separate trial,

administration of dutasteride, which inhibits 5α-reductase and thus enzymatic conversion of testosterone to the more potent androgen DHT, similarly found no significant improvement in primary endpoints [164].

The equivocal findings of androgen-targeted therapies in humans highlight difficulties in treating a slowly progressive disease where clinical severity may vary depending on CAG repeat length or other genetic and environmental factors. Though anti-androgens displayed promise for slowing disease progression, these trials also raised the possibility that intervention early in the disease course may be most effective. In these studies, the average disease duration in trial patients at the time of enrollment ranged from 10.8 to 13.3 years [162-164]. Whether patients earlier in the course of disease are more responsive to therapeutic intervention is an important unanswered question. This work also revealed the urgent need for further studies to better understand the natural history of SBMA and to develop sensitive surrogate markers that will facilitate long term follow-up in future trials.

An important, unresolved question for SBMA patients is whether exercise is beneficial. Currently, there is one two-year clinical trial underway to assess the efficacy of functional exercise and stretching (Trial Number 11-N-0171; NCT01369901). The results of this study will be informative about the applicability of exercise to SBMA therapy, as the benefit or harm of exercise is not well established in this disease or other neuromuscular disorders [165-168]. Future clinical trial candidates include ASC-J9, a disruptor of AR-coregulator interactions and promoter of AR/aggregate clearance, and insulin-like growth factor 1 (IGF-1), both of which have been shown to improve disease phenotype *in vitro* and *in vivo* [158, 169]. Both agents require further study in preclinical models before advancing to clinical trial.

The number of pathogenic mechanisms implicated in SBMA is demonstrative of the complexity inherent in this polyglutamine disorder and presents many challenges to solving critical scientific and therapeutic questions. Despite the pathophysiological intricacy of SBMA, it is important to note that the variety of cellular processes disrupted share a common initiatory stimulus in the form of the polyQ AR. It therefore follows that pursuing and optimizing therapeutic strategies targeting the polyQ AR in particular would prove most beneficial in ameliorating disease *vis-à-vis* targeting the multiple downstream sequelae. In particular, the clinical shortcomings to date of using ligand-based therapies may be overcome if used in combination with other strategies, such as chaperone-directed, to potentiate their AR-targeted effects. Additionally, recent evidence suggests that the polyQ AR may be an autophagic substrate when it is localized to the cytoplasm [98, 170], but the extent to which autophagy activators will alleviate disease *in vivo* remains unclear [152].

Furthermore, traditional clinical measures used to assess disease status in other neuromuscular diseases suffer from inadequate applicability in SBMA trials due to marked variability of these measures among SBMA patients, poor sensitivity, and a dearth of established clinical reliability. Future trials would most benefit from primary endpoints defined by reliable outcome measures that more accurately reflect disease progression, patient self-assessments, and therapeutic efficacy. Trial design might also benefit from longer duration, earlier initiation of interventions and greater enrollment of patients. These parameters, unfortunately, are limited by the small patient population available for this rare disorder and the poor diagnostic sensitivity for SBMA. Subsequent research following promising leads into overcoming these clinical and therapeutic challenges and further disentangling SBMA pathogenesis will improve quality of care and address disease mechanisms common to SBMA and other neurodegenerative disorders.

## 1.2 Macroautophagy

### 1.2.1 The autophagic pathway

As the unfolded mutant AR protein is the proximal mediator of toxicity in SBMA, pathways that regulate cellular proteostasis have attracted considerable attention in the field. Among these pathways is macroautophagy (hereafter referred to as autophagy), a highly conserved catabolic process in which misfolded or dysfunctional proteins and organelles in the cytoplasm are sequestered and targeted for bulk degradation. Initially characterized in yeast, the process of autophagy in eukaryotes is executed by autophagy-related gene (*Atg*) proteins in an adaptive, protective response to stresses including nutrient deprivation, metabolic stress, infection, and cancer [171]. First, an autophagic initiation complex directs the formation of a double-membraned structure known as a phagophore at the phagophore assembly site [172]. The core machinery of this initiation complex includes Atg1/unc-51 like kinase (ULK), Atg12, Atg8/microtubule-associated light chain 3 (LC3), class III phosphatidylinositol 3-kinase (PI3K) complex, and Atg9/mAtg9 (terms listed are yeast orthologue/mammalian orthologue, respectively) [173]. The phagophore extends to envelope cytoplasmic cargo and closes off to form a mature autophagosome, which is then trafficked to and fuses with the lysosome and thereby enables degradation of intraluminal contents [174].

### 1.2.2 Regulatory mechanisms of autophagy

Regulation of autophagy is achieved through a variety of signaling mechanisms, including the mammalian target of rapamycin (mTOR) pathway, which acts as a nutrient sensor and whose phosphorylation status and activation repress autophagy [175]. Autophagy is also

regulated by Beclin-1, which binds the PI3K complex involved in autophagic induction to direct conversion of $PIP_2$ to $PIP_3$ and thereby activate autophagy [176, 177]. Additional regulation of autophagy is performed by the recently characterized transcription factor EB (TFEB), which functions as a master regulator of autophagy and lysosomal biogenesis by directing the transcription of hundreds of autophagy- and lysosomal-related genes as part of the Coordinated Lysosomal Expression and Regulation (CLEAR) network [178-180]. Treatments that induce autophagy, including nutrient deprivation and inhibition of mTOR, also promote TFEB activity [178, 179, 181-183]. Another recently characterized mechanism of inducing autophagy is exercise. Treadmill running for 30 minutes is sufficient to significantly stimulate autophagy in a Bcl-2-dependent manner *in vivo* [184]. In spite of evidence suggesting that changes in protein quality control occur in SBMA, the degree to which regulatory effectors or responses to physiologic stimuli of autophagy are involved or differentially affected in SBMA is unknown [153, 170, 185, 186].

### 1.2.3 Autophagy and SBMA

Although the nuclear localized polyQ AR is not an autophagic substrate, autophagy is able to degrade the mutant protein upon its sequestration within the cytoplasm [98]. Furthermore, expression of the mutant AR itself is sufficient to induce autophagy in SBMA cell and animal models, although the mechanism underlying this observation has not been explored [170, 185]. Consistent with these findings, as briefly discussed above we have demonstrated that genetic manipulations that modulate autophagy have a significant influence on the phenotype of SBMA. We implicated autophagy using a knock-in mouse model we previously generated in which gene targeting was used to exchange much of mouse *Ar* exon 1 with human sequence while inserting

glutamine tracts encoded by 21 or 113 CAG repeats [107, 187]. While AR21Q males are similar to wild type littermates, AR113Q males exhibit hormone-dependent weight loss, deficits in muscle strength and early death [101, 107]. In AR113Q knock-in mice null for the unfolded protein response effector CHOP, autophagy is upregulated and mice demonstrate an exacerbation of disease [152]. Conversely, genetic inhibition of autophagy through haplosufficient expression of Beclin-1 reduces autophagic levels in AR113Q mice, mitigates skeletal muscle atrophy and prolongs survival [152]. These data indicate that excessive activation of autophagy is detrimental to SBMA mice.

## 1.3 Small ubiquitin-like modifier (SUMO)

### 1.3.1 Essential features of SUMO modification

As discussed in section 1.1.2, modulation of AR function and alteration of steps critical to SBMA pathogenesis is accomplished through regulatory mechanisms that include post-translational modifications. One such modification is SUMO, a 10-kDa protein that selectively modifies substrates at conserved lysine residues and mediates diverse alteration of protein activity and stability [188]. The SUMO family includes four distinct mammalian paralogues, SUMO1 to SUMO4 [189, 190]. SUMO1 is essential for embryonic viability and normal development, and serves functions distinct from the other SUMO paralogues [191-194]. SUMO2 and SUMO3 share 97% sequence similarity but only 50% with SUMO1, and these two paralogues are often grouped together as SUMO2/3 [195]. All three paralogues are ubiqitously expressed and well-characterized, whereas corresponding molecular and functional aspects of SUMO4 remain to be defined [189].

Although SUMO shares only about 20% sequence homology with ubiquitin, the pathways involved in mediating covalent attachment of each modification are similar. Maturation of a pro-SUMO protein involves proteolytic pre-processing followed by interactions with E1 activating, E2 conjugating, and E3 ligating enzymes [196-200]. Single entities constitute the SUMO E1 (SAE1/SAE2 complex, where SAE = SUMO-activating enzyme) and SUMO E2 (Ubc9), but the identity and involvement of SUMO E3 ligases varies in a substrate-specific manner and, unlike in the case of ubiquitin, are dispensible for SUMO ligation [201-204]. Similar to ubiquitin modification, multiple monomers of SUMO2/3 can be conjugated together to form polymeric chains, which confer additional levels of complexity to SUMO function, interactions, and signaling [205]. Moreover, SUMOylation is characterized by a high degree of reversibility. The SUMO system includes isopeptidases with specific activities directed against deconjugating the SUMO moiety and editing polySUMO chains in response to cellular and environmental cues, thereby allowing for further dynamic modulation of protein function [206].

### 1.3.2 SUMOylation and neurodegeneration

Apart from regulating diverse cellular processes of the nervous system under physiological circumstances, SUMO has been implicated in modifying critical aspects of neurologic disease. SUMO has been detected in the intranuclear inclusions characteristic of neurodegenerative diseases, and several of the mutant proteins constituent to these aggregates are endogenous SUMO substrates [207, 208]. The ability of SUMO to modulate substrate solubility and aggregation or compete for ubiquitination can either disrupt or enhance pathogenesis depending on the disease context [207]. For instance, enhanced SUMOylation of mutant atrophin-1, huntingtin, and SOD1 results in increased stability of the mutant proteins [209-212].

Subsequently, SUMOylation increases aggregation of atrophin-1 and SOD1 but decreases aggregation of huntingtin, with the common result for all three mutant proteins culminating in enhanced cytotoxicity. For huntingtin, this SUMO-dependent effect is catalyzed by the striatally enriched G-protein, Rhes [211]. Similarly, enhanced monoSUMOylation through expression of the K11R variant of SUMO3, which prevents polySUMO3 chain formation, potentiates Aβ generation, whereas polySUMO chains with wild type SUMO3 attenuate amyloidogenesis [213]. Detrimental or modulatory effects of SUMOylation in other cases of neurodegeneration are documented with ataxin-1, DJ-1 L166P mutant, and tau [214-216]. Further research will be necessary to define optimal therapeutic targets in the SUMO pathway to modify aspects of proteotoxicity in neurodegeneration.

### 1.3.3 SUMO modification of AR and SBMA

In addition to the proteins detailed in the previous section, the AR is an established SUMO substrate with two conserved synergy control (SC) motifs in the NTD that serve as sites of SUMO modification [63, 217]. The SC motif sequence consists of P/G-x(0-4)-I/V-x-K-D/E-x(0-4)-P/G (where P = proline, G = glycine, x = any amino acid, I = isoleucine, V = valine, K = lysine, D = aspartate, E = glutamate), and SUMOylation at the constituent lysine residue results in inhibition of transactivational activity [63, 217, 218]. This aspect of AR transcriptional control by SUMO informs investigative targeting of the SUMO pathway for SBMA, since a primary feature of the disease is AR loss of function and androgen insensitivity. This loss of function phenotype may be rescued by potentiating polyQ AR transactivation by disrupting SUMOylation (further detailed in sections 1.1.1 and 1.1.4).

The other major feature of SBMA is the degenerative phenotype mediated by proteotoxicity from the polyQ AR, and this toxic gain of function is both distinct from the partial loss of function of the polyQ AR and is modified by SUMO. In *Drosophila*, expression of truncated polyQ AR results in aggregation and neurotoxicity, and a loss of function mutant of the SUMO E1 component Uba2 worsens this degenerative phenotype, suggesting that diminished AR SUMOylation exacerbates pathogenesis [138] . In cell culture models of SBMA, increased expression of SUMO3 enhances SUMOylation of polyQ AR and reduces its aggregation, and this effect can be abrogated by mutating the SUMO acceptor lysines, suggesting that direct SUMOylation of the AR modifies toxicity by preventing or disrupting oligmerization [69]. However, the degree to which SUMO affects SBMA *in vivo* and the mechanisms by which it modifies pathogenesis remain to be determined.

## 1.4 Research Objectives

Despite extensive advances in understanding the mechanisms of pathogenesis in SBMA, many key features pertaining to molecular determinants of disease development are not yet established or remain controversial. The work presented in this dissertation approaches these unresolved questions by investigating two cellular processes associated with modifying neurodegenerative disease, autophagy and SUMOylation. The first objective is to define the manner and extent to which autophagy is altered in SBMA. In chapter 2, I characterize the upregulation of autophagy in cell and animal models of SBMA, identify the transcription factors TFEB/ZKSCAN3 underlying these pathologic changes, and establish the increased responsivity to physiologic stimulation of autophagy in SBMA mice. The second objective is to examine the degree to which SUMO modification affects aspects of the SBMA phenotype. In chapter 3, I

show that genetically disrupting SUMOylation of polyQ AR dramatically enhances receptor transactivation, and mice expressing non-SUMOylatable polyQ AR have improved exercise endurance and are rescued from early death. In conclusion, these results indentify discrete targets in cellular pathways definitively implicated in SBMA pathogenesis, and strategies aimed at inhibiting or disrupting these processes may be of therapeutic benefit in human patients.

# Chapter 2[1]

## Transcriptional dysregulation of TFEB/ZKSCAN3 targets underlies enhanced autophagy in spinobulbar muscular atrophy

### 2.1 Abstract

Spinobulbar muscular atrophy (SBMA) is an inherited neuromuscular disorder caused by the expansion of a CAG repeat encoding a polyglutamine (polyQ) tract in exon 1 of the androgen receptor (*AR*) gene. SBMA demonstrates androgen-dependent toxicity due to unfolding and aggregation of the mutant protein. There are currently no disease-modifying therapies, but of increasing interest for therapeutic targeting is autophagy, a highly conserved cellular process mediating protein quality control. We have previously shown that genetic manipulations inhibiting autophagy diminish skeletal muscle atrophy and extend lifespan of AR113Q knock-in mice while those inducing autophagy worsen muscle atrophy, suggesting that chronic, aberrant upregulation of autophagy contributes to pathogenesis. Since the degree to which autophagy is altered in SBMA and the mechanisms responsible for such alterations are incompletely defined, we sought to delineate autophagic status in SBMA using both cellular and mouse models. Here, we confirm that autophagy is induced in cellular and knock-in mouse models of SBMA and show that the transcription factors TFEB and ZKSCAN3 operate in opposing roles to underlie

[1] This chapter is in preparation for submission for publication.

these changes. We demonstrate upregulation of TFEB target genes in skeletal muscle from AR113Q male mice and SBMA patients. Furthermore, we observe a greater response in AR113Q mice to physiological stimulation of autophagy by both nutrient starvation and exercise. Together, our results indicate that transcriptional signaling contributes to autophagic dysregulation and provide a mechanistic framework for the pathologic increase of autophagic responsiveness in SBMA.

## 2.2 Introduction

Spinobulbar muscular atrophy (SBMA) is one of nine untreatable diseases caused by CAG/glutamine tract expansions [13]. SBMA is an X-linked, progressive neurodegenerative disorder of adult-onset primarily affecting lower motor neurons and skeletal muscle. The polyglutamine (polyQ) expansion critical for SBMA pathogenesis occurs in exon 1 of the androgen receptor (AR) gene. The polyQ AR protein acquires a toxic gain of function by undergoing aberrant unfolding, oligomerization and metabolism dependent on binding the cognate ligands testosterone and dihydrotestosterone (DHT) [91, 92]. These steps are critical for mediating toxicity in target tissues, and the dependence of disease development on androgen underlies the exclusive incidence of SBMA in men [21]. Additionally, the polyQ AR exhibits a partial loss of transactivation function [36, 47], correlating with features of androgen insensitivity including gynecomastia and reduced fertility in SBMA patients. Despite progress over the past two decades in characterizing key aspects of neuromuscular toxicity and endocrine disruption in SBMA, mechanisms that are essential to pathogenesis remain poorly understood and available therapies are of limited utility.

One essential, highly conserved mechanism of protein quality control involved in modifying neurodegenerative disease is macroautophagy, or simply autophagy [171, 219, 220]. The autophagic process consists of the envelopment of cytoplasmic organelles, proteins, and other macromolecules within a double-membraned phagophore that ultimately resolves into a mature autophagosome, and this vesicular body is transported to and fuses with the lysosome to form an autolysosome and degrade the sequestered cargo [171-174]. Multiple regulatory mechanisms of autophagy have been thoroughly studied and classified, and considerable attention has focused on transcriptional modulation through factors including TFEB and ZKSCAN3. TFEB is a basic helix-loop-helix, leucine-zipper member of the MITF/TFE family that not only coordinates expression of genes controlling lysosomal biogenesis but also, in a recently characterized role, regulates autophagy [178-180]. ZKSCAN3 is a zinc finger protein with Krüppel-associated box (KRAB) and SRE-ZBP/CTfin-51/AQ-1/Number 18 cDNA-homology (SCAN) domains that has recently been implicated in regulating TFEB target genes as a master repressor of autophagy [221]. TFEB is prompted to activate autophagy-related gene expression through established induction mechanisms of autophagy, including nutrient withdrawal and treatment with rapamycin [178, 179, 181-183]. In contrast, ZKSCAN3 activity is inhibited by these manipulations and operates in a manner antagonistic to TFEB [221].

At the organismal level, physiologic stimulation of autophagy is conventionally achieved through starvation and mTOR inhibition, but recent studies have also described alternative mechanisms. In particular, running exercise is capable of acutely inducing autophagy, and mice expressing the autophagy marker microtubule-associated light chain 3 (LC3) tagged with GFP demonstrate accumulation of fluorescent autophagic structures within 30 minutes that peak within 80 minutes while running on a treadmill [184]. This induction of autophagy occurs under

strict control by Beclin-1/BCL-2 associated machinery, which coordinate stimulus-induced autophagy *in vivo* as evidenced by the observation that BCL-2 mutant mice are unable to activate Beclin-1 and induce exercise-stimulated autophagy. These studies of exercise-induced autophagy are notable for revealing inductive influence in both skeletal muscle and spinal cord, two principal sites of pathology in SBMA [184, 222].

The extent to which pathways of proteostasis interact with or are altered by pathogenic mechanisms in neurodegenerative diseases, including SBMA, is not fully understood. Adverse effects on protein quality control in SBMA have been described for the unfolded protein response, the ubiquitin-proteasome system, and autophagy [153, 170, 185, 186]. Moreover, expression of polyQ AR correlates with autophagic activity, and antagonizing autophagy *in vivo* ameliorates the SBMA phenotype [152, 170, 185]. Based on these findings, we hypothesized that autophagy is aberrantly activated in SBMA, which underlies the previously documented deleterious influence of autophagy to disease pathogenesis. We test this notion by analyzing aspects of autophagic induction, flux, and regulation in both cellular and animal models of SBMA. We confirm that autophagy is induced in the context of the polyQ AR both *in vitro* and *in vivo*, implicate TFEB in the increased expression of autophagy-related genes in SBMA mice and human patients, and establish that autophagic induction by nutrient deprivation and exercise is more highly activated in SBMA mice compared to healthy controls. These results define the mechanism underlying aberrant upregulation of autophagy in SBMA and provide discrete targets in the autophagic pathway for further investigation and therapeutic design.

## 2.3 Results

### 2.3.1 Expanded glutamine AR promotes autophagy

To investigate autophagy in SBMA, we initially studied PC12 cells that stably express tetracycline-inducible full-length human AR with 10 or 112 glutamines [97]. We observed higher basal levels of the autophagosome marker LC3-II in cells expressing AR112Q **(Fig. 2.1A)**. Serum starvation promoted robust conversion of LC3-I to LC3-II in both cell lines, but this process was significantly more pronounced in AR112Q cells **(Fig. 2.1A)**. To determine whether these elevated LC3-II levels resulted from enhanced activation of autophagy or impaired flux, we examined levels of the autophagic substrate p62 and assessed levels of LC3-II with and without lysosomal inhibition. Both of these are standard assays for evaluating autophagic flux [223]. AR112Q cells demonstrated significant clearance of p62 in response to serum starvation **(Fig. 2.1A)**. Furthermore, treatment with the lysosomal protease inhibitors E64d and pepstatin A led to similarly enhanced accumulation of LC3-II in both AR10Q and AR112Q cells **(Fig. 2.1B)**. These results suggest that autophagic markers accumulate to a greater extent with AR112Q expression primarily due to aberrant upregulation of autophagy rather than compromised autophagic flux.

We then sought to define which signaling mechanisms are responsible for the increased activation of autophagy in SBMA. To do this, we first probed the mTOR pathway, since mTOR is a serine/threonine kinase that serves as a principal regulator of autophagy [224-226]. We found that phosphorylation of mTOR and p70 S6 kinase, a downstream effector of the mTOR pathway, was decreased in both cell lines, but this reduction was significantly greater in AR112Q cells **(Fig. 2.1C)**. Lower mTOR activity and phosphorylation are consistent with disinhibition of

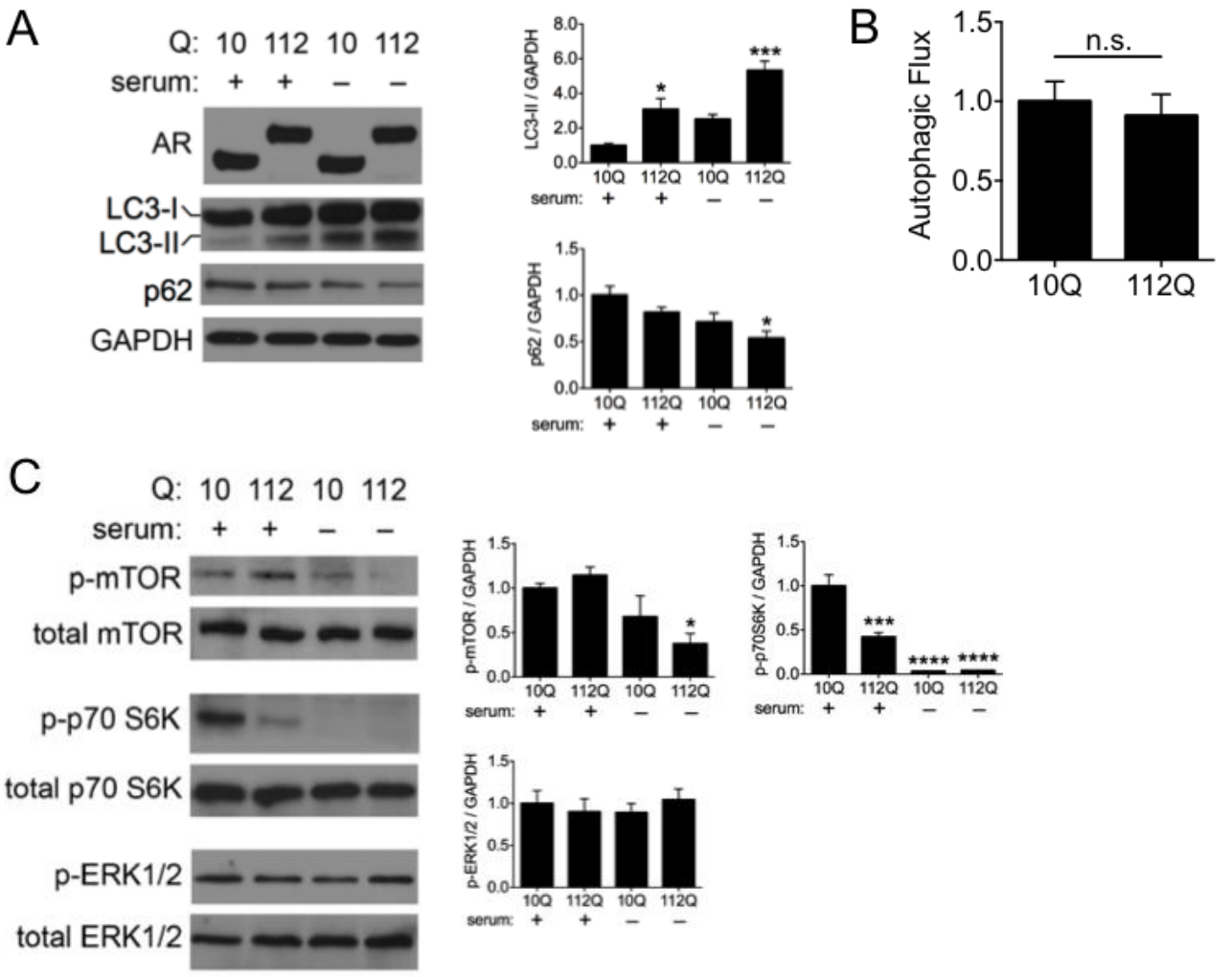



**Figure 2.1 Expanded polyglutamine AR promotes autophagy**. **(A)** AR10Q and AR112Q cells were serum starved to induce autophagy. Lysates were analyzed for LC3 and p62 (n=3). Data are mean +/- SEM. *p<0.05, ***p<0.001. **(B)** Autophagic flux in AR10Q and AR112Q cells (n=3) determined by quantifying normalized LC3-II band intensities and subtracting the difference of fed E64d/pepstatin A-treated and untreated cells from the difference of starved E64d/pepstatin A-treated and untreated cells. Data are mean +/- SEM. n.s., not significant. **(C)** AR10Q and AR112Q cells were starved, and phospho-status of the mTOR pathway was analyzed. Data are mean +/- SEM. *p<0.05, ***p<0.001, ****p<0.0001.

autophagy, since mTOR negatively regulates autophagic induction [224-226]. In contrast, phosphorylation of extracellular regulated kinases ERK1/2, which comprise another set of autophagy regulators that can function independently of mTOR [227-231], was unchanged

regardless of serum supplementation **(Fig. 2.1C)**. These results indicate that autophagy is selectively upregulated with expression of AR112Q in these cells due to inhibition of the mTOR pathway.

### 2.3.2 Transcription factors that regulate autophagy partition in a glutamine length-dependent manner

Recent studies have shown that transcription factor EB controls expression of autophagy-related genes, and its activity and nuclear localization are negatively regulated by mTOR [181-183]. Additionally, the zinc-finger protein ZKSCAN3 has been characterized as a transcriptional antagonist to autophagy, functioning oppositely and in concert with TFEB [221]. Given our observations of increased autophagy and depressed mTOR activity in the context of AR112Q, we postulated that TFEB and ZKSCAN3 act as major determinants of autophagic upregulation in SBMA. To test this hypothesis, we prepared nuclear lysates of PC12 cells subjected to serum starvation and determined the relative localization of each transcription factor. Consistent with our findings of increased autophagic induction and reduced mTOR activity in AR112Q cells, we found that the transcriptional activator TFEB displayed significantly greater nuclear localization upon serum starvation while the repressor ZKSCAN3 exhibited diminished nuclear localization even under fed conditions **(Fig. 2.2A)**. Similarly, immunofluorescence demonstrated nuclear localization of TFEB in AR10Q cells with concomitant cytoplasmic translocation of ZKSCAN3 only after starvation. In contrast, nuclear TFEB and cytoplasmic ZKSCAN3 were detected in AR112Q cells even when serum supplemented **(Fig. 2.2B)**. Together, our results suggest that autophagy is primed for activation with depressed mTOR activity in the presence of AR112Q,

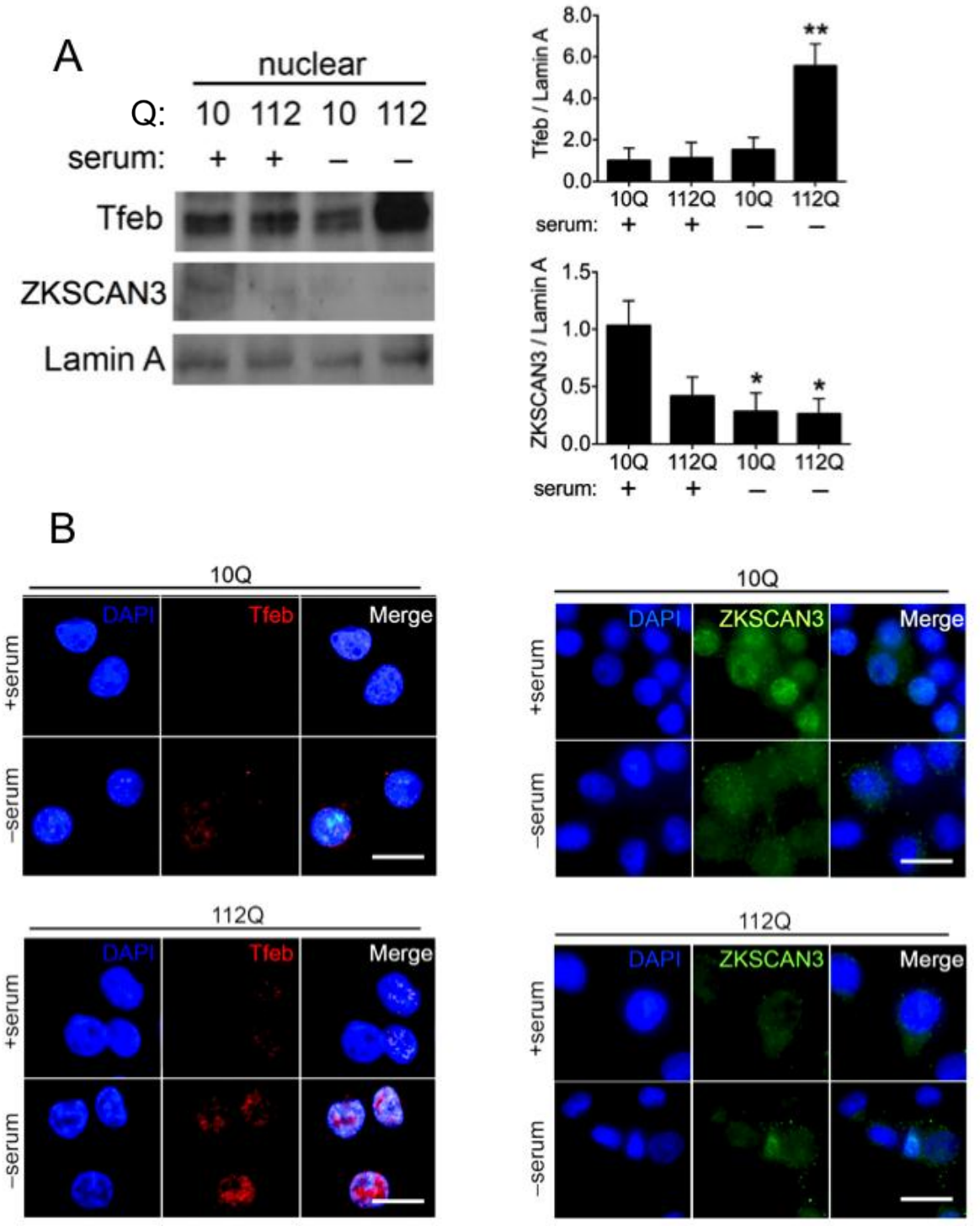



**Figure 2.2 Transcription factors that regulate autophagy display glutamine length-dependent changes in intracellular localization. (A)** Nuclear lysates prepared from serum starved and fed AR10Q and AR112Q cells were analyzed for TFEB and ZKSCAN3. Data are mean +/- SEM (n=3). *p<0.05, **p<0.01. **(B)** AR10Q and AR112Q cells were serum starved or fed and immunostained for TFEB and ZKSCAN3.

### 2.3.3 Polyglutamine AR increases autophagy *in vivo*.

To corroborate the autophagic changes we observed in PC12 cells, we probed markers of autophagy in AR21Q and AR113Q knock-in male mice. Consistent with our previous observations, we found significantly higher LC3-I to LC3-II conversion in skeletal muscle and spinal cord in AR113Q males, and observed that this difference became more pronounced in muscle when autophagy was induced by starvation **(Fig. 2.3A)**. Additionally, total ubiquitinated proteins and p62 from the insoluble fraction were also increased in AR113Q muscle **(Fig. 2.3A)**. Autophagic puncta, which we detected by co-expressing a GFP-LC3 transgene [232], accumulated in skeletal muscle after starvation of both AR21Q and AR113Q males. This accumulation of autophagic puncta occurred to a greater extent in starved AR113Q mice, in line with our biochemical data showing enhanced autophagy in PC12 cells expressing the polyQ AR **(Fig. 2.1B)**. To confirm equivalent levels of autophagic flux in both knock-in lines, we inhibited autophagosome to lysosome fusion *in vivo* by administering colchicine [233]. Starved, colchicine-treated mice of both genotypes showed similarly enhanced accumulation of LC3-II **(Fig. 2.3C)**. We conclude that autophagic induction is increased in AR113Q knock-in males without accompanying loss of autophagic flux.

### 2.3.4 AR113Q promotes TFEB activity *in vivo*.

To test whether TFEB activity is increased in AR113Q mice, we prepared nuclear lysates of skeletal muscle from each knock-in line and assessed TFEB localization. We found that, as occurs in tetracycline-regulated PC12 cells, the mouse orthologue TCFEB displays greater nuclear localization in AR113Q compared to AR21Q male mice **(Fig. 2.4A)**. To determine

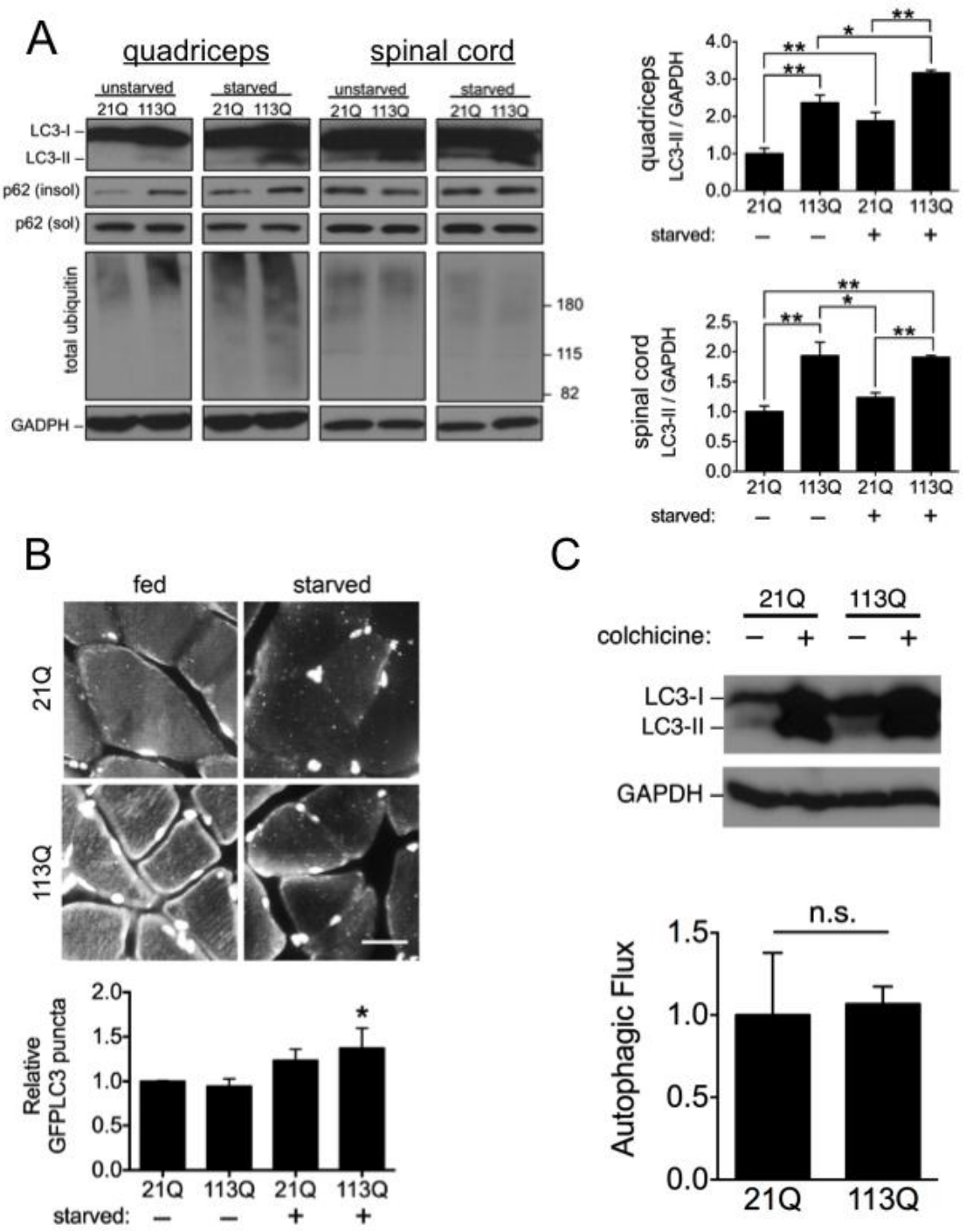



**Figure 2.3 Expression of the polyglutamine AR increases autophagy *in vivo*. (A)** Quadriceps and spinal cord from fed or starved AR21Q and AR113Q micewere analyzed for markers of autophagy. Data are mean +/- SEM (n=3). *p<0.05, **p<0.01. **(B)** Autophagic puncta in quadriceps from AR21Q and AR113Q mice expressing GFP-LC3 were analyzed by immunostain. Data are mean +/- SEM (n=3). *p<0.05. **(C)** Autophagic flux was determined after treatment with colchicine and analysis of accumulated LC3-II. Data are mean +/- SEM (n=3). n.s., not significant.

whether enhanced nuclear localization correlated with upregulation of autophagy-related genes, we assayed the expression profile of a select set of previously identified TFEB target genes [178-180]. Quantitative RT-PCR confirmed that autophagy-related genes containing TFEB binding sites in their promoter regions demonstrated increased expression levels in AR113Q mice, and that this difference was more pronounced for many genes after starvation **(Fig. 2.4B)**. Similarly, we found that several TFEB target genes were also expressed at higher levels in skeletal muscle biopsies from SBMA patients than controls **(Fig. 2.4C)**. These glutamine length- and starvation-dependent transcriptional changes in AR113Q mice were not observed in target genes of FOXO3a **(Fig 4D)**, another important regulator of autophagy in skeletal muscle [234, 235]. Taken together, these data indicate that SBMA results in increased activity specific to TFEB and, consequently, enhanced expression of autophagy-related genes.

### 2.3.5 Expanded polyglutamine AR enhances autophagic response to exercise.

Finally, we sought to explore whether enhanced TFEB activity and increased expression of autophagy-related genes lead to an augmented autophagic response following an acute, physiologically relevant stimulus. Recently, He et al. established exercise as a potent inducer of autophagy *in vivo* [184]. To corroborate the effect of exercise in AR knock-in mice, we ran AR21Q/GFP-LC3 and AR113Q/GFP-LC3 male mice on a treadmill and evaluated the formation of GFP-positive autophagic puncta in quadriceps muscle. After 80 minutes of exercise, both knock-in lines demonstrated accumulation of autophagic puncta akin to induction by starvation **(Fig. 2.5A)**. We then asked if each AR line responded equivalently to autophagic induction by exercise. To accomplish this we followed markers of autophagy biochemically and found that exercise stimulated GFP-LC3-II accumulation to a higher degree in AR21Q muscle **(Fig. 2.5B)**.

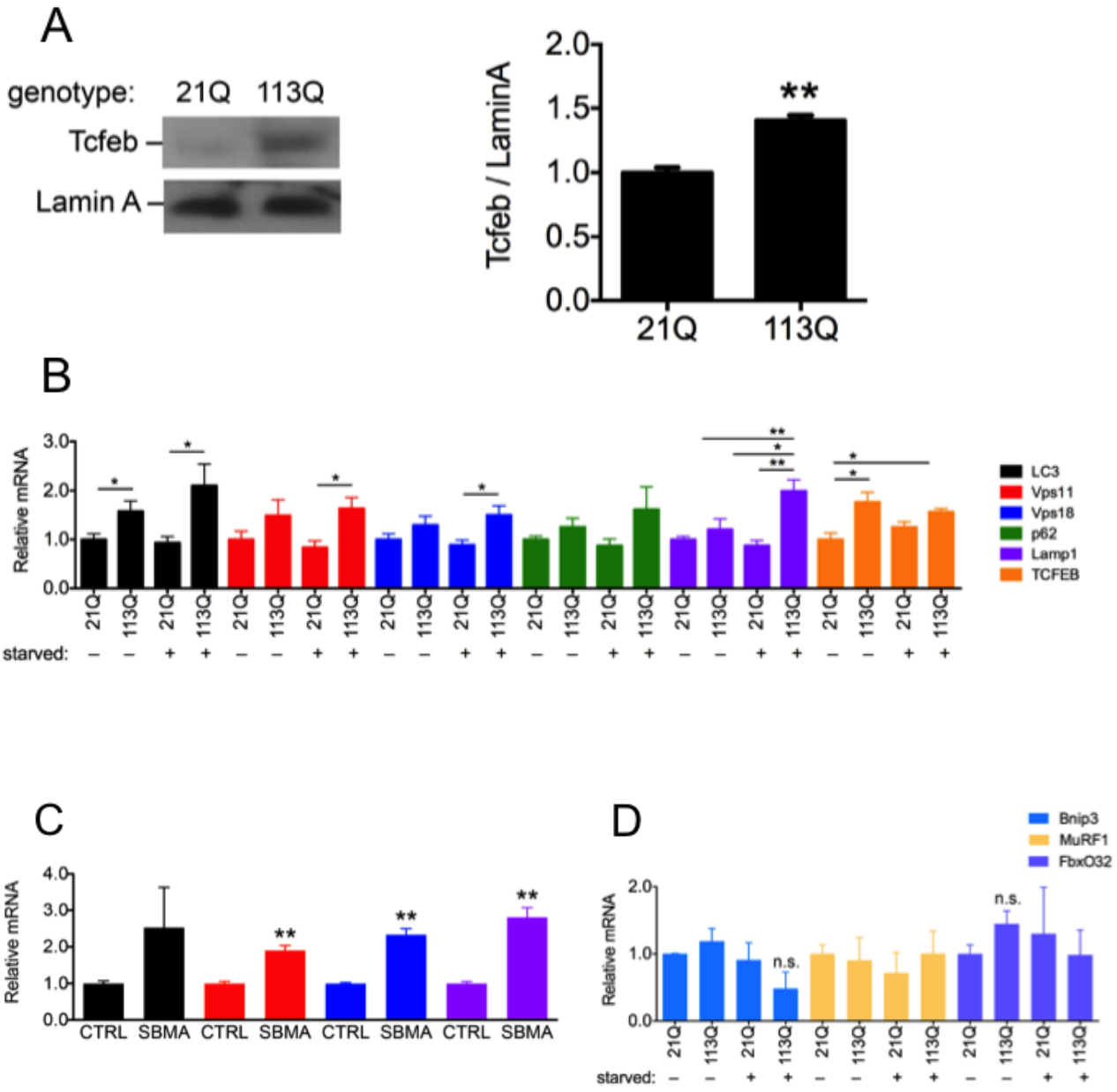



**Figure 2.4 Expanded polyglutamine AR promotes TFEB activity *in vivo*. (A)** Nuclear lysates from quadriceps muscle were prepared from AR21Q and AR113Q mice and analyzed for TCFEB, the murine orthologue of TFEB. Data are mean +/- SEM (n=3). **p<0.01. **(B)** Total RNA was extracted from quadriceps of fed and starved AR21Q and AR113Q mice (n=6 per group), and transcripts of genes regulated by TCFEB were analyzed by qPCR. Data mean +/- SEM. *p<0.05, **p<0.01. **(C)** Transcripts of TFEB-regulated genes were analyzed by qPCR in muscle samples from SBMA and control patients (n=3 per group). Data are mean +/- SEM. **p<0.01. Color coding same as (B). **(D)** Transcripts of FOXO3a target genes were analyzed by qPCR in the quadriceps described in (B). Data are mean +/- SEM. n.s., not significant.

We hypothesized that this difference reflected enhanced clearance of autophagic substrates in AR113Q males after acute stimulation of autophagy. We tested this notion by evaluating levels of p62 and free GFP as indicators of autophagic flux. Free GFP is produced after GFP-LC3-II is delivered to lysosomes and the LC3-II portion of the fusion protein is degraded [236-238]. Western analysis of these autophagic markers after exercise revealed similar degradation of endogenous p62 in both knock-in lines, but a striking absence of free GFP in AR113Q mice; in contrast, free GFP robustly accumulated in skeletal muscle of exercised AR21Q mice **(Fig. 2.5B)**. Our analysis suggests rapid clearance of the GFP-LC3 fusion protein in AR113Q males. We conclude that exercise promotes autophagy in both knock-in lines. The enhanced clearance of GFP-LC3 supports greater autophagic flux in skeletal muscle of AR113Q males, indicative of enhanced responsiveness to physiologic stimuli in these mice.

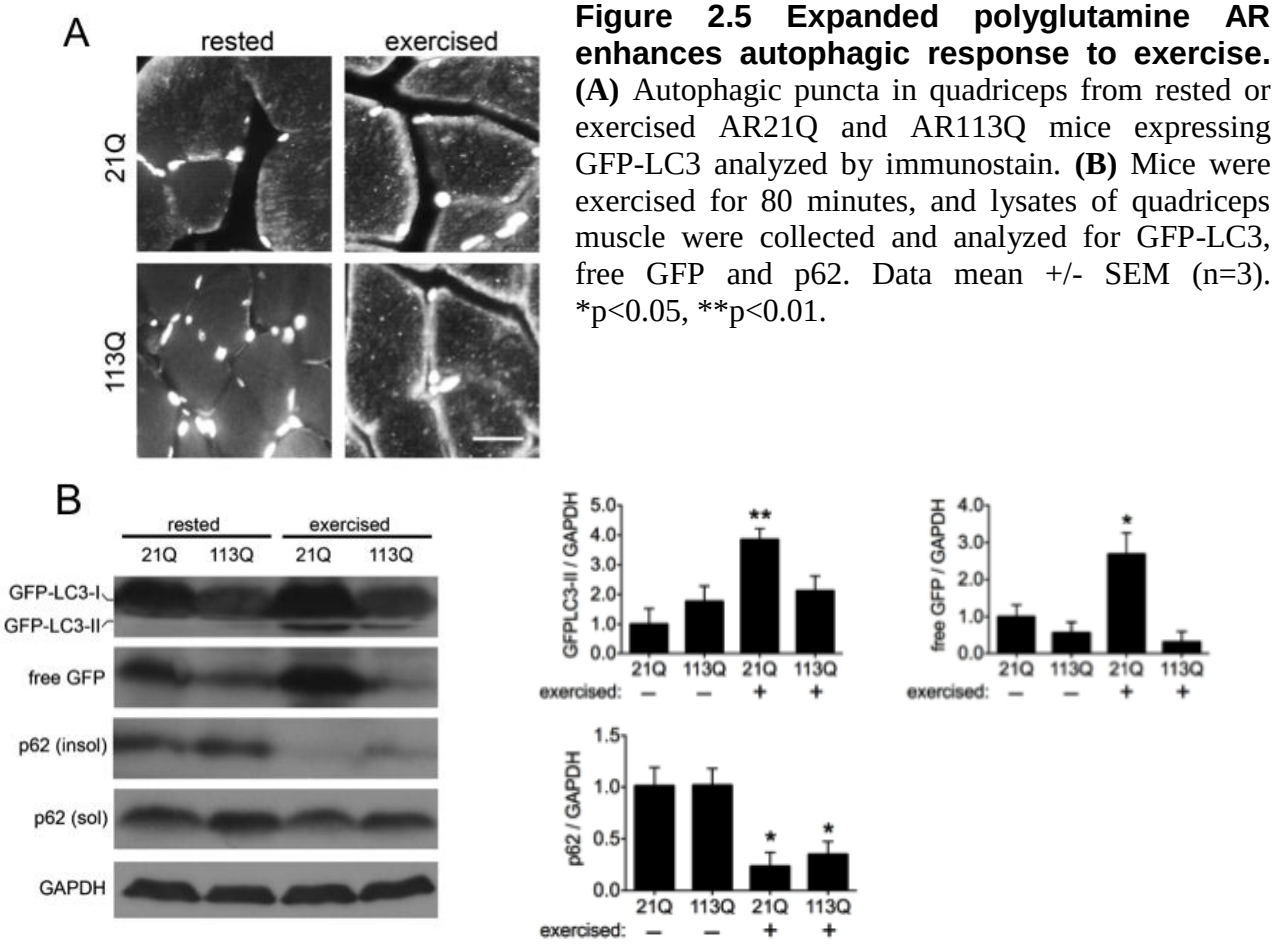



**Figure 2.5 Expanded polyglutamine AR enhances autophagic response to exercise.** **(A)** Autophagic puncta in quadriceps from rested or exercised AR21Q and AR113Q mice expressing GFP-LC3 analyzed by immunostain. **(B)** Mice were exercised for 80 minutes, and lysates of quadriceps muscle were collected and analyzed for GFP-LC3, free GFP and p62. Data mean +/- SEM (n=3). *p<0.05, **p<0.01.

## 2.4 Discussion

Previous studies suggested an exacerbatory role for autophagy in SBMA, since genetic ablations that either induce or suppress autophagy worsen or ameliorate the SBMA phenotype, respectively [152]. Consistent with those findings, we show that autophagy is upregulated in models of SBMA. Both AR112Q cells and AR113Q mice exhibit increased levels of LC3-II, suggestive of higher production of autophagosomes **(Fig. 2.1A and 2.3A)**. These changes were accentuated by stimulation of autophagy by nutrient deprivation. The accumulation of autophagy markers was not due to impaired autophagic flux, since pharmacologic inhibition of lysosomal-mediated degradation indicated completion of flux both *in vitro* and *in vivo* **(Fig. 2.1B and 2.3C)**. In addition, these biochemical data are associated with increased nuclear translocation of TFEB in both polyQ AR cells and AR113Q mice **(Fig. 2.2 and 2.4)**. The relative shifts in localization for TFEB and ZKSCAN3 favoring TFEB activity resulted in predicted enhancement of TFEB target genes that regulate autophagy, both in SBMA mice and human patients **(Fig. 2.4)**. Our results define broad changes in the autophagic system that corroborate the postulated activation of autophagy in SBMA. Consequently, proposed therapeutic strategies aimed at increasing autophagy in other neurodegenerative disorders may prove inappropriate, if not more deleterious, to pathogenesis of SBMA [220, 239, 240].

Supporting the notion that enhanced autophagy contributes to the pathogenesis of SBMA, we previously demonstrated that levels of autophagy directly correlate with skeletal muscle atrophy in AR113Q knock-in mice [152]. Despite evidence of motor neuron loss and denervation in SBMA muscle [1, 241], the elevated expression of TFEB targets documented in this study is muscle autonomous and dependent on polyQ AR expression. This conclusion is based in part on our observation that surgical denervation in WT AR mice is unable to recapitulate or sustain the

transcriptional phenotype (data not shown). In addition to the AR113Q-dependent nature of increased autophagy under resting conditions, stimulation of autophagy is augmented in response to nutrient deprivation and exercise, leading to marked degradation of protein substrates through the autophagic pathway. These results characterize a striking instance of neurodegenerative disease in which regulated control of autophagy is compromised, and it is likely that these changes effect deleterious contributions to pathogenesis, since critical components of skeletal muscle are autophagic substrates [242].

Mechanisms of proteostasis are essential mediators of muscle integrity, and balancing the degradative and protective roles of protein quality control in muscle is critical for muscle maintenance and appropriately responding to nutrient deprivation, mechanical stress, and disuse atrophy. The ubiquitin-proteasome system (UPS) serves a pivotal role in muscle protein catabolism, and enhanced or aberrant UPS activity is responsible for physiologic and pathologic forms of atrophy [243]. However, it is unlikely that the UPS acts as the primary mediator of muscle wasting in SBMA, since the muscle-specific ubiquitin E3 ligases atrogin-1/MAFbx and Murf1 that mediate muscle atrophy are not upregulated in AR113Q and AR97Q mice [152, 158, 244, 245]. In contrast, several groups have demonstrated that dysregulated autophagy is sufficient to promote atrophy in muscle. Loss of Runx1 models disuse and deneveration changes in skeletal muscle and causes atrophy through disinhibited autophagy [246]. Similarly, denervation- and fasting-induced atrophy has been shown to occur through FOXO3-mediated induction of autophagy [234, 235]. Furthermore, increased autophagy is instrumental in mediating or enhancing myopathy in neuromuscular disorders [247-249]. Our findings here are consistent with genetic and pharmacologic manipulations of IGF-1 that mitigate SBMA in a muscle-specific manner, since these ameliorative effects are dependent on enhanced activation of

the autophagic repressor Akt [52, 158, 250]. It will be important for future studies to weigh the therapeutic potential of inducing autophagy to address proteinopathies against our observation of detrimentally elevated autophagy in SBMA.

We also observed correlative reductions in phosphorylation events within the mTOR pathway **(Fig. 2.1C)**. Although mTOR is an established master regulator of autophagy and has been shown to interact with TFEB, and despite our data in AR112Q cells consistent with these aspects of mTOR function, conflicting evidence remains regarding the precise regulative mechanism of TFEB inhibition through phosphorylation by mTOR or ERK2 [178, 181-183]. Our data *in vitro* are compatible with mTOR-mediated modulation of TFEB due to unaltered ERK1/2 signaling regardless of glutamine tract length or nutrient provision **(Fig. 2.1C)**, and it will be necessary in future studies to analyze these regulatory determinants *in vivo* to further expand our understanding of the TFEB pathway. Moreover, although elucidating the involvement of TFEB and its regulatory pathways will be valuable for therapeutic targeting, the causal determinants directly linking the mutant AR to changes in mTOR and TFEB remain to be defined. Overall, we have delineated specific alterations in the autophagic pathway occurring in SBMA and particular effectors associated with these changes. It is likely that these alterations constitute a compensatory mechanism in response to pathogenic insults introduced by the polyQ AR, and determination of these molecular triggers is expected to yield valuable information for counteracting these insults and, in turn, treating SBMA.

## 2.5 Materials and Methods

### 2.5.1 Materials

Lysosomal protease inhibitors E64d and pepstatin A, normal saline tablets, and colchicine were from Sigma. For preparation of nuclear lysates from mouse tissue, the following buffers were used: NB1 (10mM Tris pH 8.0, 10mM NaCl, 3mM $MgCl_2$, 0.5mM DTT, 0.1% Triton X-100, 0.1M sucrose), NB2 (10mM Tris pH 8.0, 10mM NaCl, 3mM $MgCl_2$, 0.5mM DTT, 0.1% Triton X-100, 0.25M sucrose), NB3 (10mM Tris pH 8.0, 5mM $MgCl_2$, 0.5mM DTT, 0.33M sucrose), EB (25mM HEPES pH 7.8, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 25% glycerol), and Z (25mM HEPES pH 7.8, 12.5mM $MgCl_2$, 1mM DTT, 0.1M KCl, 0.1% NP40, 20% glycerol). Antibodies against indicated proteins and their respective vendors were as follows: AR (N-20) was from Santa Cruz; GAPDH (6C5) was from Abcam; GFP (A11122) was from Invitrogen; LC3 (NB600-1384) and p62 (2C11) were from Novus Biologicals; Lamin A (MAB3540), phospho-mTOR (09-213), phospho-ERK1/2 (12D4), total ERK1/2 (06-182), and total p70 S6K (07-402) were from Millipore; phospho-p70 S6K (9204) and total mTOR (2972) were from Cell Signaling; Tfeb (MBS120432) was from MyBioSource; ZKSCAN3 (AV33609) was from Sigma. HRP-tagged secondary antibodies were from Bio-Rad. Alexa Fluor 488- and 594-conjugated secondary antibodies were from Invitrogen.

### 2.5.2 Cell culture

PC12 cells stably transfected with tetracycline-inducible forms of AR were described previously [97]. AR expression was induced with 500 ng/mL doxycycline (Clontech) in the presence of 10nM R1881. Cells were maintained in phenol red-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 15% charcoal-stripped horse serum (Invitrogen), 10% charcoal-stripped fetal bovine serum (Thermo Scientific), 100 units/mL penicillin/streptomycin (Invitrogen), 200µg/mL hygromycin B (Invitrogen), and 100 µg/mL G418 (Invitrogen) in 15%

$CO_2$ at 37ºC. Serum starvation was performed in Hank's Balanced Salt Solution (HBSS, Invitrogen) supplemented with doxycycline and R1881 for 4 hours. To analyze autophagic flux, PC12 cells were induced to express AR for 44 hours then fed or starved in HBSS with E64d/pepstatin A (10µg/mL each) or DMSO vehicle for 4 hours. Autophagic flux was then determined by subtracting the difference of LC3-II levels in serum starved cells with and without E64d/Pepstatin A treatment from the difference of LC3-II levels in serum supplemented cells with and without E64d/Pepstatin A.

### 2.5.3 Mouse studies

Generation of knock-in mice with targeted AR allele containing 21 or 113 CAG repeats has been previously described [107, 187]. GFP-LC3 transgenic mice were kindly provided by Jun-Lin Guan (University of Michigan) and have been described previously [232]. Mice were group housed in an SPF facility and provided with food and water *ad libitum.* Genotypes were confirmed by PCR of tail biopsy-derived DNA. AR knock-in mice were genotyped with forward primer 5'-CCAGAATCTGTTCCAGAGCGTG-3' and reverse primers 5'-TGTTCCCCTGGACTCAGATG-3' and 5'-GCACTCCAGGGCCGACTGCG-3' in a 2:2:1 ratio, respectively. GFP-LC3 mice were genotyped with forward primer 5'-TCCTGCTGGAGTTCGTGACCG-3' and reverse primer 5'-TTGCGAATTCTCAGCCGTCTTCATCTCTCTCGC-3' to detect the GFP-LC3 transgene and in a separate reaction with forward primer 5'-TGAGCGAGCTCATCAAGATAATCAGGT-3' and reverse primer 5'-GTTAGCATTGAGCTGCAAGCGCCGTCT-3' to detect the endogenous LC3 allele.

Skeletal muscle and spinal cord were collected from adult male knock-in mice 13 to 15 weeks old and flash frozen for biochemical analysis or mounted in OCT for cryosectioning. For morphologic analysis of autophagy in skeletal muscle, AR knock-in mice were crossed with GFP-LC3 mice to generate bigenic 21Q/GFP-LC3 and 113Q/GFP-LC3 mice. To induce autophagy, mice were starved for 48 hours and provided with water *ad libitum*. To analyze autophagic flux, mice were starved for 48 hours and treated with intraperitoneal injections of normal saline or colchicine (0.4mg/kg/day). Autophagic flux was then determined by subtracting the level of LC3-II in saline treated mice from the level of LC3-II in colchicine treated mice.

For exercise studies, a treadmill protocol was implemented as previously described [184]. Mice were trained on an Exer3/6 treadmill (Columbus Instruments) for two days. On day 1, mice were run at 8 meters/minute for 5 minutes, and on day 2 mice were run at 8 meters/minute for 5 minutes then 10 meters/minute for 5 minutes. After training, mice were run on day 3 for 40 minutes at 10 meters/min, then for 30 minutes with increasing speed 1 meter/minute every 10 minutes, and finally for 20 minutes with increasing speed 1 meter/minute every 5 minutes for a total of 80 minutes of exercise. Mice were then immediately sacrificed and perfused with 4% paraformaldehyde and proximal hind limb muscles were collected. The University of Michigan Committee on Use and Care of Animals approved all procedures involving mice in compliance with the NIH Guidelines for the Care and Use of Experimental Animals.

### 2.5.4 Gene expression analysis

Total RNA was extracted from proximal hind limb muscle from AR knock-in mice after the indicated treatments in triplicate with Trizol (Sigma). Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to

manufacturer's protocol. Total RNA was similarly prepared from anonymized skeletal muscle biopsies from SBMA patients and controls as previously described [152]. Quantitative PCR was performed with FastStart TaqMan Probe Master/Rox Master Mix (Roche) on a 7500 Real-Time PCR SDS System with supplied software (Applied Biosystems) using gene-specific primers with FAM-labeled probes purchased from Applied Biosystems (Human: *LAMP1*, Hs00174766_m1; *MAP1LC3*, Hs01076567_g1; *VPS11*, Hs00222240_m1; *VPS18*, Hs00363585_m1. Mouse: *Bnip3*, Mm01275601_g1; *Fbxo32*, Mm00499518_m1; *Lamp1*, Mm00495262_m1; *Map1lc3*, Mm00782868_sH; *Murf1*, Mm00185221_m1; *Sqstm1*, Mm00448091_m1; *Tcfeb*, Mm00448968_m1; *Vps11*, Mm01168594_m1; *Vps18*, Mm00552119_m1). Relative expression levels were calculated by comparing with the expression of 18S rRNA.

### 2.5.5 Analysis of protein expression

Cells were washed and collected in PBS, then harvested and lysed in RIPA buffer supplemented with phosphatase and protease inhibitors. Lysates were clarified at 15,000 x *g* for 15 minutes at 4ºC. Nuclear and cytoplasmic lysates were prepared with NE-PER Extraction Kit (Thermo Scientific) according to manufacturer's instructions. For tissue analyses, proximal hind limb muscle and lumbar spinal cord from AR knock-in mice were minced, lysed, and homogenized in RIPA buffer containing phosphatase and protease inhibitors. Lysates were clarified at 13,000 x *g* for 10 minutes at 4ºC.

Nuclear lysates from proximal hindlimb muscle were prepared as follows. Frozen tissue was pulverized, lysed in NB1 buffer, homogenized with a type A dounce homogenizer, mixed with NB2 buffer, layered underneath with NB3 buffer, and nuclei were pelleted through the sucrose step gradient. After discarding the supernatant, nuclei were resuspended in EB buffer,

homogenized with a type B dounce homogenizer, and rotated for 30 minutes at 4ºC. After pelleting the nuclear debris, the supernatant containing nuclear proteins was dialyzed overnight in Z buffer. Protein concentration for all lysates was determined by protein assay (Bio-Rad), and normalized protein samples were resolved in 10% or 15% SDS-PAGE gels after boiling at 100ºC for 5 minutes in 6X SDS sample buffer. Proteins were then transferred to nitrocellulose membranes in a semi-dry transfer apparatus (Bio-Rad). Proteins of interest were probed by indicated antibodies and detected by chemiluminescence. Densitometry measurements of protein bands were performed in ImageMeter (Flashscript).

### 2.5.6 Immunofluorescence

After induction of AR expression and serum starvation, PC12 cells were washed in PBS and fixed in 4% paraformaldehyde. Proximal hind limb muscles were collected for cryosectioning from mice after the indicated treatments and perfusion with 4% paraformaldehyde. After perfusion, muscles were further fixed in 4% paraformaldehyde overnight, 15% sucrose for 4 hours, 30% sucrose overnight, then flash frozen in liquid nitrogen and mounted in cryosection blocks with OCT Compound (Tissue-Tek). Sections were then cut at 10µm using a Cryocut 1800 cryostat (Leica). Cells and muscle sections were stained with indicated antibodies and mounted with Vectashield medium with DAPI (Vector Labs). Fluorescence images were taken with a Zeiss Axio Imager microscope. GFPLC3 puncta were quantified in ImageJ (NIH) using a GFP-LC3 quantification macro designed by Dagda and Chu [219, 251].

### 2.5.7 Statistics

Statistical significance was determined by ANOVA with Newman Keuls multiple comparison test or unpaired two-tailed Student's *t*-test using Prism 6 (GraphPad). Differences were considered significant when *P* values were less than 0.05.

## 2.6 Acknowledgements

We thank Dr. Gregory Dressler for advice and expertise in isolating nuclei from mouse skeletal muscle, and Yuqing Sun for experimental assistance. This work was supported by grants from the National Institutes of Health (grant number F31 NS076189 to JPC, R01 NS055746 to APL, R01 CA144032 to DMR).

# Chapter 3[2]

## Disruption of polyglutamine androgen receptor SUMOylation rescues skeletal muscle function and ameliorates the phenotype of SBMA mice

### 3.1 Abstract

Expansion of the androgen receptor's (AR) polyglutamine (polyQ) tract, the cause of neurodegeneration in spinobulbar muscular atrophy (SBMA), diminishes AR-mediated transactivation and confers ligand-dependent toxicity. While both of these effects are implicated in SBMA, the extent to which altered AR function contributes to pathogenesis remains controversial. Here we manipulated AR function by mutating conserved lysines that are targeted by SUMO, a modification that inhibits AR transactivation. We show that replacement of these residues by arginine enhances polyQ AR activity as a hormone-dependent transcriptional regulator. We use gene targeting to generate mice expressing the polyQ AR in which these SUMOylation sites are similarly disrupted. Inhibition of polyQ AR SUMOylation rescues deficits in exercise endurance and Type I muscle fiber atrophy and prevents early death. These changes occur independent of effects on polyQ AR expression or aggregation, but instead reflect beneficial, trophic support by the ligand-activated receptor. Unexpectedly, disruption of the androgen axis in mutant males is largely unaffected by abrogating AR SUMOylation, suggesting

[2] This chapter is in preparation for submission for publication.

that features of the disease previously attributed to partial loss of receptor function may, in fact, reflect polyQ-mediated toxicity in peripheral tissues. Our findings demonstrate beneficial effects of the ligand-activated polyQ AR and indicate that the SUMOylation pathway may provide new targets for therapeutic intervention.

## 3.2 Introduction

Modulation of androgen receptor (AR) function leads to profound effects on transcriptional programming, tissue physiology and disease progression. Stimulation of AR function not only effects development of the masculine sexual phenotype, motor neuron survival and skeletal muscle anabolism [252-257], but enhanced AR function also exacerbates certain diseases such as prostate cancer [52, 55-62]; conversely, diminution or antagonism of AR function improves prostate cancer outcomes [258-260] and underlies androgen insensitivity syndromes [261, 262]. Consequences of altered AR function are particularly relevant for spinobulbar muscular atrophy (SBMA), a heritable, progressive neurodegenerative disorder belonging to the family of nine polyglutamine (polyQ) diseases characterized by pathologic expansions of CAG repeats [13]. The mutation in SBMA occurs in exon 1 of the *AR* gene and confers a hormone-dependent toxic gain of function to the polyQ AR that precipitates cell death of lower motor neurons and atrophy of skeletal muscle [1, 263]. In addition, expansion of the AR's polyQ tract results in a partial loss of transactivation function and correlates with symptoms of androgen insensitivity in SBMA patients [6]. Although these aspects of altered polyQ AR physiology have been well-characterized, the degree to which impairment in AR function contributes to SBMA is not fully understood.

Of the cell types undergoing degeneration in SBMA, both lower motor neurons and skeletal muscle express the polyQ AR and exhibit trophic responses following activation of the wild type receptor. Motor neuron death has been thought to underlie neurogenic atrophy in SBMA muscle and the lower motor neuron signs in SBMA patients [2, 4], yet elevated serum creatine kinase levels and mixed histopathologic changes in muscle biopsies suggest that the disease is, in part, a primary myopathy [10]. Further evidence in support of an essential role of muscle-autonomous toxicity in pathogenesis derives from studies in which muscle-specific overexpression of wild type AR recapitulates numerous features of SBMA [155, 160]. The polyQ AR has also been shown to compromise mitochondrial function and repress PGC-1$\alpha$ expression, both of which are critical mediators of muscle homeostasis [151]. Indeed, we have previously shown in AR113Q knock-in mice that muscle pathology occurs prior to signs of pathologic change in spinal cord [101], and this occurs concomitant to transcriptional alterations deleterious to muscle [150, 264]. Consistent with these studies, muscle-specific interventions significantly influence disease severity. Inhibition of the unfolded protein response exacerbates muscle pathology and worsens disease, while muscle-specific overexpression of IGF-1 rescues SBMA phenotype [152, 158]. Furthermore, suppression of polyQ AR expression only in peripheral tissues by antisense oligonucleotides is sufficient to rescue skeletal muscle atrophy and prevent early death in AR113Q mice [265]. These studies highlight the importance of skeletal muscle in SBMA pathogenesis, yet leave unresolved the extent to which alterations in normal AR function contribute to disease.

Endogenous mechanisms regulating AR function act by dint of posttranslational modifications. One such covalent linkage is the enzymatic addition of small ubiquitin-like modifier (SUMO). SUMO shares a large degree of sequence identity with ubiquitin, and both

peptides similarly attach to target substrates via isopeptide bonds on the terminal ε-amino group of lysine residues [190, 266-268]. Additionally, both proteins share analogous processing, conjugation, and ligation steps using E1, E2, and E3 enzymes [196-200]. The functional consequence of SUMOylation on modified substrates is modulation of protein activity or intracellular re-localization in response to cellular conditions and stress stimuli [188, 201, 269]. Furthermore, SUMOylation is a transient modification due to recurrent deconjugation of the SUMO moiety by SUMO proteases, rendering the steady state proportion of SUMOylated substrates a relatively small fraction of overall protein population [206]. Of the three SUMO paralogues expressed by mammals, AR is primarily modified by SUMO1 [63]. Conjugation by this SUMO isoform, which does not form polySUMO chains, occurs preferentially on lysine residues 385 and 518, which fall within consensus SUMOylation sites encoded by amino acids IK$^{385}$LE and VK$^{518}$SE, conserved motifs previously defined as transcriptional regulatory sequences in nuclear receptors [63, 217].

SUMOylation has attracted considerable attention for its impact on neurodegenerative diseases. Several mutant proteins that cause neurodegeneration are targets of SUMOylation, and the extent to which SUMO influences disease pathogenesis varies in a context-dependent manner [69, 207-216, 270]. For the AR, transactivation function is significantly repressed upon modification by SUMO. This repression is markedly promoter-context dependent [217], is associated with a shift of nuclear localized AR away from chromatin binding [63, 217, 271], and is alleviated by mutating the two consensus SUMO-acceptor lysines to arginines [63, 217]. Here we test the hypothesis that changes in polyQ AR SUMOylation will improve components of SBMA stemming from altered AR function. Our approach is based on the established effects of SUMO on AR-dependent gene expression, the significant loss of transactivation caused by

polyglutamine expansion, and prior studies suggesting that AR function contributes to SBMA pathogenesis [47, 89, 264]. To accomplish this, we used gene targeting to generate a novel knock-in mouse line that expresses a non-SUMOylatable, polyglutamine-expanded AR. Our studies characterizing this new mouse model of SBMA, complemented by additional experimental systems *in vitro*, provide a unique avenue to examine the effects of disinhibiting AR function and disentangling the pathogenic role of AR dysfunction from polyQ toxicity *in vivo*.

## 3.3 Results

### 3.3.1 Establishment of a cellular model expressing non-SUMOylatable polyQ AR

To initially study the effects of abrogating SUMOylation of the polyQ AR, we used PC12 cells expressing tetracycline-regulated AR10Q, AR112Q, and AR112Q with its SUMO-acceptor lysines mutated to arginines, AR112Q-KRKR **(Fig. 3.1A)**. With these cells, we tested whether introducing mutations that prevent SUMOylation affected the response of AR112Q to ligand. In the absence of the synthetic non-aromatizable androgen R1881, all three variants of AR displayed a predominantly cytoplasmic distribution, in agreement with expected localization when unbound to ligand **(Fig. 3.1B, left column)**. With addition of R1881, all three cell lines demonstrated similar nuclear redistribution of AR, and both AR112Q and AR112Q-KRKR similarly formed intranuclear aggregates **(Fig.3.2B, right column and arrows)**. To verify that the KRKR mutations interrupt AR SUMOylation, we transiently transfected AR112Q and AR112Q-KRKR cells with hemagglutinin (HA)-tagged SUMO3, then immunoprecipitated each AR112Q variant and probed for HA. Western analysis confirms that the KRKR mutation

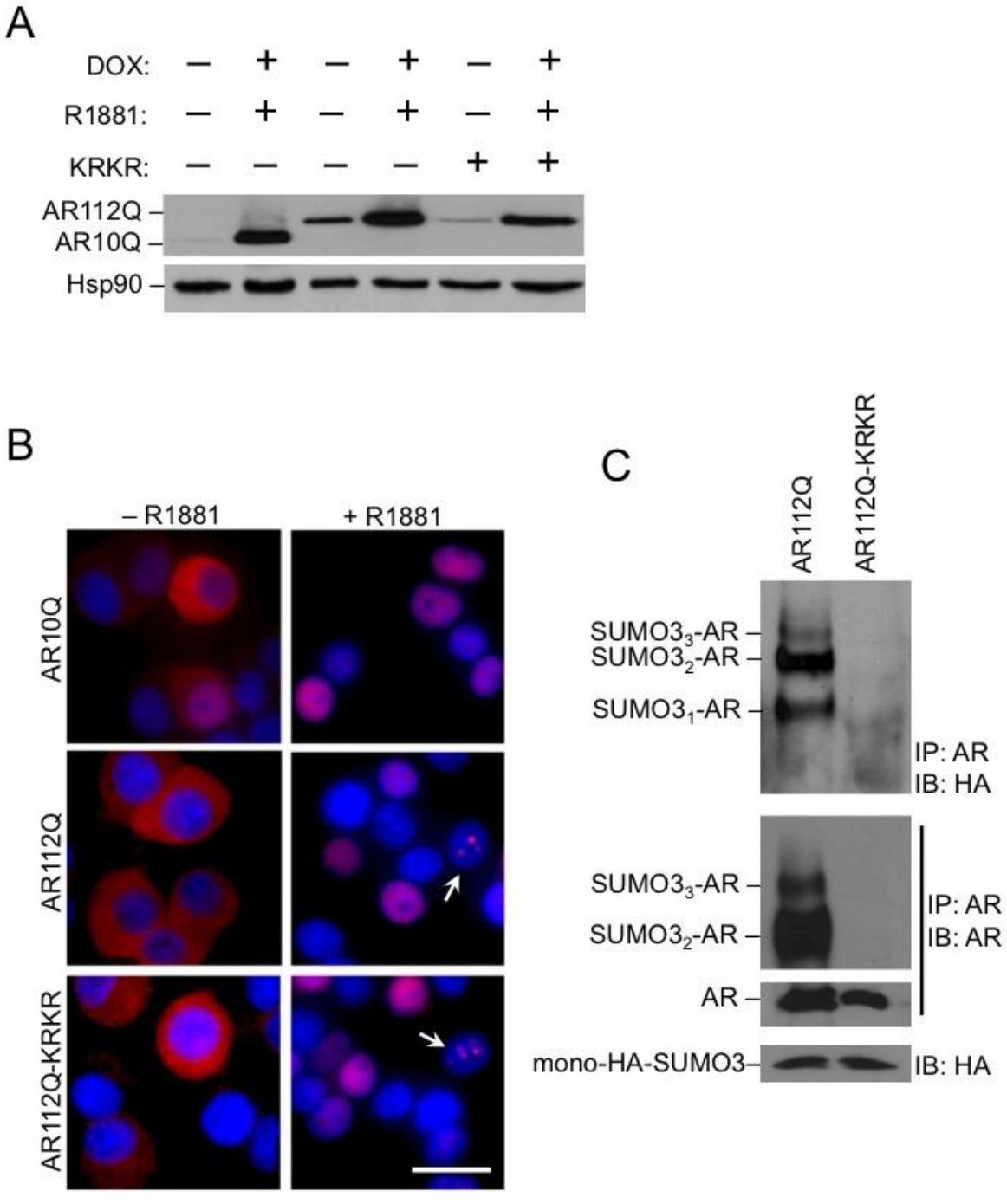



**Figure 3.1 A cellular model expressing non-SUMOylatable polyQ AR**
**(A)** AR10Q, AR112Q, or AR112Q-KRKR were expressed in the presence or absence of 10nM R1881 for 24 hours. **(B)** AR10Q, AR112Q, and AR112Q-KRKR cells were induced as in (A) and immunostained for AR (red). Nuclei were stained by DAPI. Arrows indicate nuclei containing aggregates. Scale bar, 2μm. **(C)** AR112Q and AR112Q-KRKR cells were transfected with HA-SUMO3. Whole cell lysates were immunoprecipitated for AR and probed for HA.

prevents AR modification by SUMO **(Fig. 3.1C)**. We therefore conclude that our PC12 cell model abolishes SUMO modification of AR112Q without significantly altering ligand-dependent localization of the polyQ receptor.

### 3.3.2 Abrogating SUMOylation enhances transactivation activity of polyQ AR

To examine the effects of the KRKR mutation on transactivation function, we prepared cDNA from cells expressing AR10Q, AR112Q or AR12Q-KRKR following stimulation with R1881 or control. These samples were analyzed by Affymatrix GeneChip microarrays, and the results were plotted in a heatmap of genes significantly activated (in red) or repressed (in blue) by androgen **(Fig. 3.2A and B)** and displayed as a Venn diagram of androgen-responsive genes **(Fig. 3.2C)**. This analysis confirmed a glutamine-length dependent partial loss of AR function **(Fig. 3.2A)**. Of the 93 ligand-responsive genes identified in cells expressing AR10Q, only 32 were ligand-regulated by AR112Q and 49 by AR112Q-KRKR. We verified one such gene (*Ppard*) that was androgen-responsive to all three AR variants by quantitative real-time RT-PCR (qPCR) **(Fig. 3.2D)**. To verify the glutamine length-dependence of androgen-responsiveness, we performed qPCR analysis on two separate genes identified by microarray and found significantly lower expression changes produced by ligand activation of AR112Q compared to AR10Q **(Fig. 3.2D)**. These data are in line with expectations that the expanded glutamine tract reduces AR activity as a transcriptional regulator [31-36, 69]. Intriguingly, we also found that the KRKR mutation enabled the polyQ AR to activate a markedly larger set of genes. In contrast to the 93 genes regulated by AR10Q, AR112Q-KRKR altered the expression of 246 genes, 197 of which fell below threshold for ligand responsiveness to AR10Q, and 183 of which were below threshold for responsiveness to both AR10Q and AR112Q **(Fig. 3.2B and C)**. This robust, ligand-dependent regulation of gene expression by AR112Q-KRKR was confirmed by qPCR. In

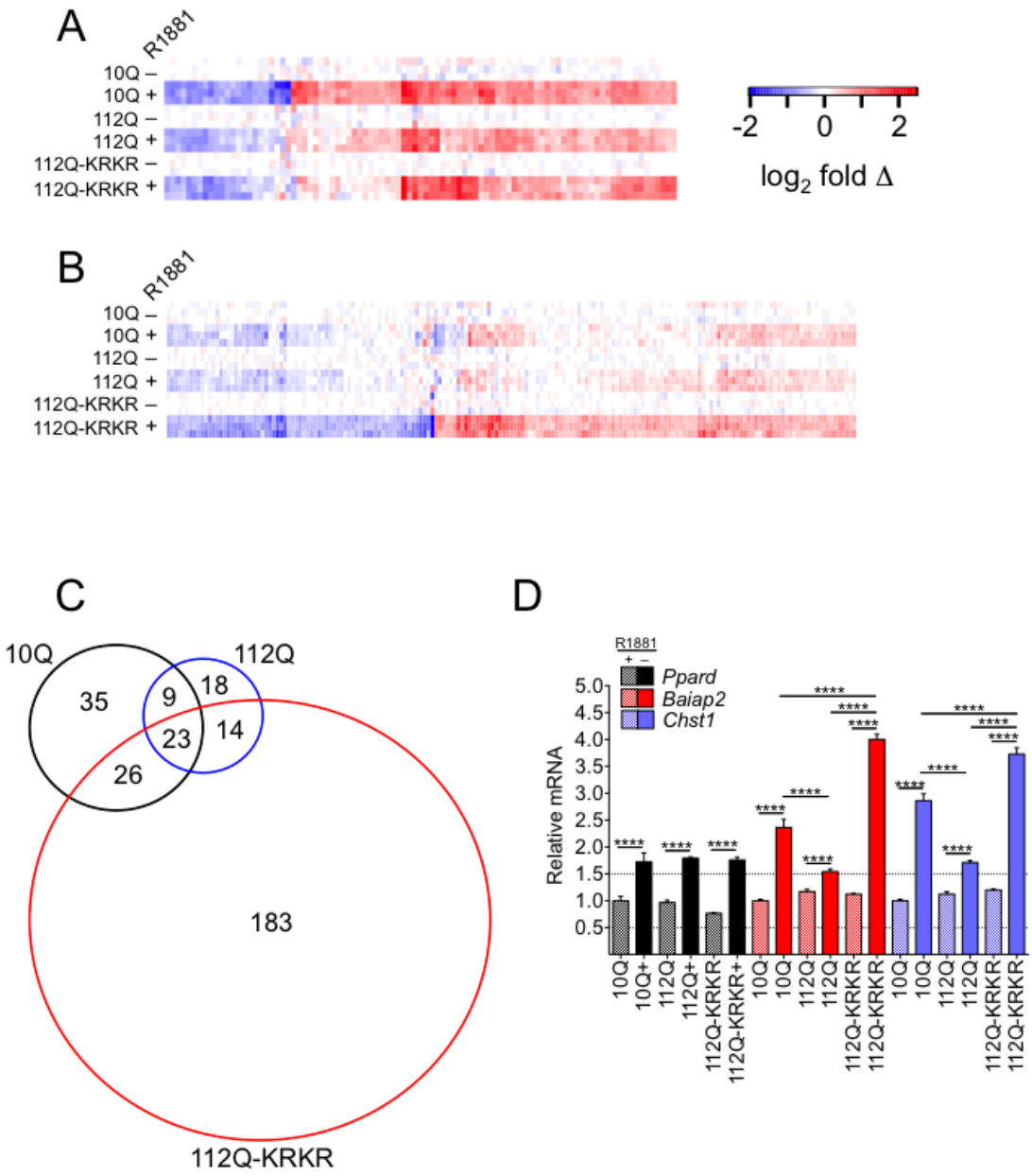



**Figure 3.2 Disruption of polyQ AR SUMOylation potentiates AR function.**
**(A)** Heatmap showing expression of genes activated (red) or repressed (blue) 1.5-fold or greater by AR10Q cells compared to expression in AR112Q and AR112Q-KRKR cells. **(B)** Heatmap comparing expression of 183 androgen-responsive genes unique to AR112Q-KRKR cells to expression of those genes in AR10Q and AR112Q cells. **(C)** Venn diagram illustrating the number and overlap of androgen-responsive genes in AR10Q (black circle), AR112Q (blue circle), and AR112Q-KRKR cells (red circle). **(D)** Candidate genes that show relative transactivation function (*Ppard*, *Baiap2*, and *Chst1* in black, red, and purple, respectively) in AR112Q-KRKR cells were identified in (A) and analyzed by qPCR. Data shown are mean +/- SEM (n=3). ****p<0.0001.

contrast to the reduced expression observed in response to AR112Q, ligand activation of AR112Q-KRKR triggered significantly higher gene expression than both AR112Q and AR10Q in a promoter context-dependent manner **(Fig. 3.2.D)**. These results are consistent with previous mechanistic data [69, 217] and demonstrate that, although expansion of the glutamine tract diminishes activation of genes normally induced by AR, preventing SUMOylation through the KRKR mutation enhances polyQ AR transactivation capacity and expands the number of ligand-responsive genes.

### 3.3.3 Generation of AR113Q-KRKR knock-in mice

To investigate the influence of SUMOylation *in vivo*, we used gene targeting to generate a new knock-in mouse model of SBMA that expresses the polyQ AR with K385 and K518 both mutated to arginines. We modified the targeting vector used previously to generate our existing AR113Q line of knock-in mice by introducing the KRKR mutations **(Fig. 3.3A)**. We verified correct recombination of the targeted exon 1 into the mouse *Ar* gene by Southern analysis in JM8.F6 cells, an embryonic stem cell line from the C57BL/6N strain **(Fig. 3.3B)**. After germline transmission of the targeted allele from chimeric founders to $F_1$ heterozygous females, we crossed these females to C57BL/6J wild type males and assessed subsequent inheritance of the AR113Q-KRKR allele by PCR **(Fig. 3.3C)**. For the purposes of phenotypic characterization in this study, we used AR113Q male mice backcrossed >ten generations on the C57BL/6J background, N1 AR113Q-KRKR male mice, and wild type littermate males from both lines. Females from both 113Q lines were similarly fertile and both AR113Q alleles were inherited in the expected Mendalian ratios (data not shown). Both lines of male mice were indistinguishable prior to disease onset at sexual maturity.

### 3.3.4 AR113Q-KRKR males demonstrate partial androgen compromise

Since partial loss of AR function and features of androgen insensitivity are molecular and phenotypic hallmarks of SBMA, we examined several measures of androgen function on the hypothalamic-pituitary-gonadal (HPG) axis in AR113Q-KRKR mice. Compared to wild type mice, both AR113Q and AR113Q-KRKR mice demonstrated testicular and seminal vesicle

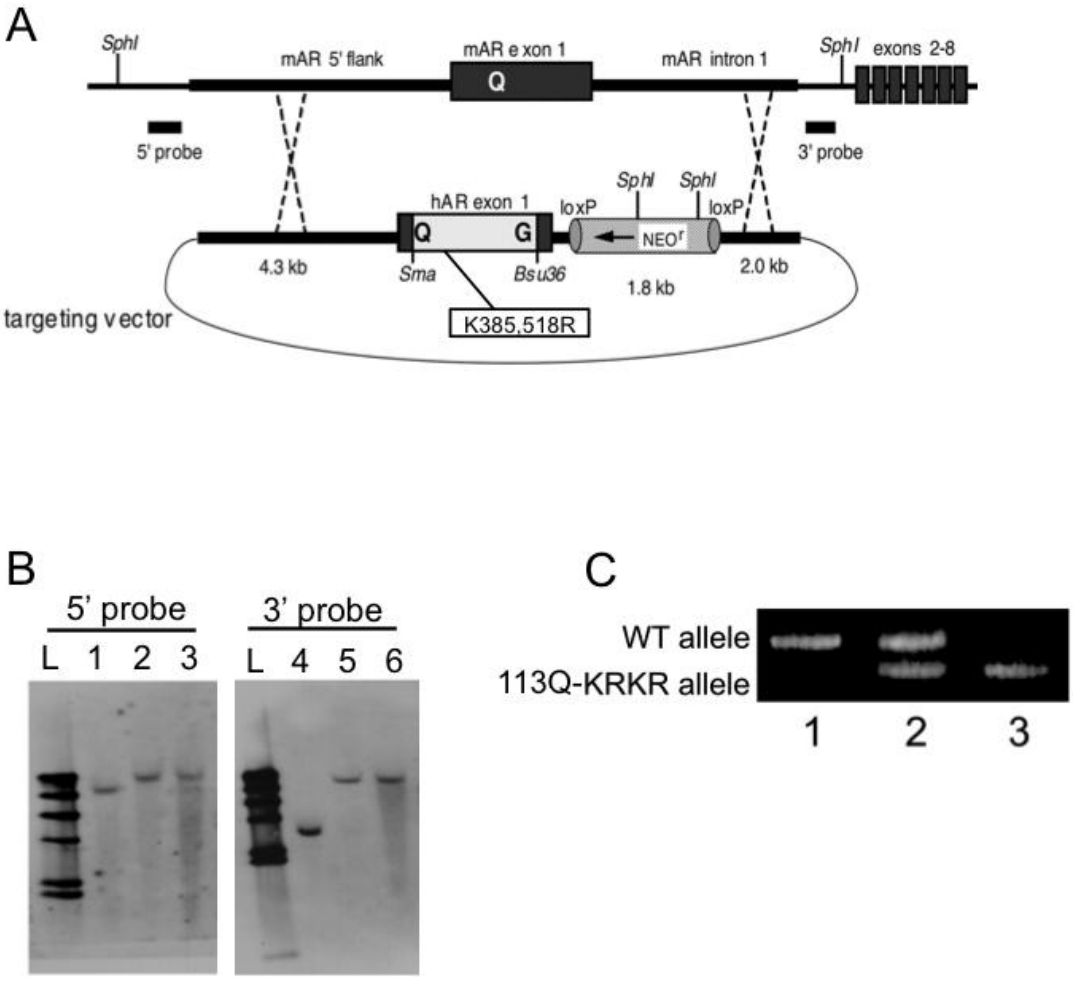



**Figure 3.3 Generation of AR113Q-KRKR knock-in mice.**
**(A)** Schematic representation depicting AR113Q-KRKR targeting vector. *SphI* restriction sites, 5' and 3' probes for Southern analysis, glutamine tract, lysine to arginine mutations, and floxed neomycin resistance cassette are indicated. Diagram modified from [272]. **(B)** Southern analysis of ES cell DNA after *SphI* digestion with the 5' and 3' probes indicated in (A) before (lanes 1 and 4) and after (lanes 2 and 5) homologous recombination. Lanes 3 and 6 contain control liver DNA from previously targeted AR113Q knock-in line. L, ladder. **(C)** PCR primers that span the CAG tract were used to amplify sequences from a wild type female (lane 1), $F_1$ AR113Q-KRKR female (lane 2), and N1 AR113Q-KRKR male produced by the female in lane 2.

atrophy and reduced sperm count **(Fig. 3.4A)**. Modest elevations in serum testosterone and luteinizing hormone were evident in both AR113Q and AR113Q-KRKR males, but because of variability between animals these changes did not reach statistical significance **(Fig. 3.4B)**. We also analyzed testicular expression of 3-β-hydroxysteroid dehydrogenase type I (3βHSD-I), an enzyme responsible for keto-steroid hormone synthesis in Leydig cells in response to LH and whose expression is indicative of androgen insensitivity [273]. Consistent with the endocrine tissue pathology and serum studies, we found similar increases in 3βHSD-I mRNA in testes from both 113Q lines of mice **(Fig. 3.4C)**. These data demonstrate that glutamine length-dependent partial loss of AR function occurs independent of SUMOylation status, and suggest that toxicity stemming from polyglutamine expansion provides determinative influence on endocrine compromise in SBMA mice.

### 3.3.5 Behavioral and neuromuscular phenotypes in AR113Q-KRKR mice

As the phenotype of AR113Q mice includes decreased body mass and grip strength, we also evaluated these parameters in AR113Q-KRKR mice and found indistinguishable deficits **(Fig. 3.5A, B)**. The reduction in these measures became evident ~12 weeks and persisted thereafter. To broaden our characterization of muscle function beyond assessment of grip strength, we examined exercise capacity by treadmill running until exhaustion. By this measure we detected a significant decrement in exercise endurance in AR113Q mice, consistent with other measures of functional decline [101, 274-276] in SBMA muscle **(Fig. 3.5C)**. Strikingly, KRKR mutations in polyQ AR yielded a profound rescue of exercise capacity to levels equivalent to wild type mice. This functional rescue persisted in mice aged up to 9 months **(Fig.**

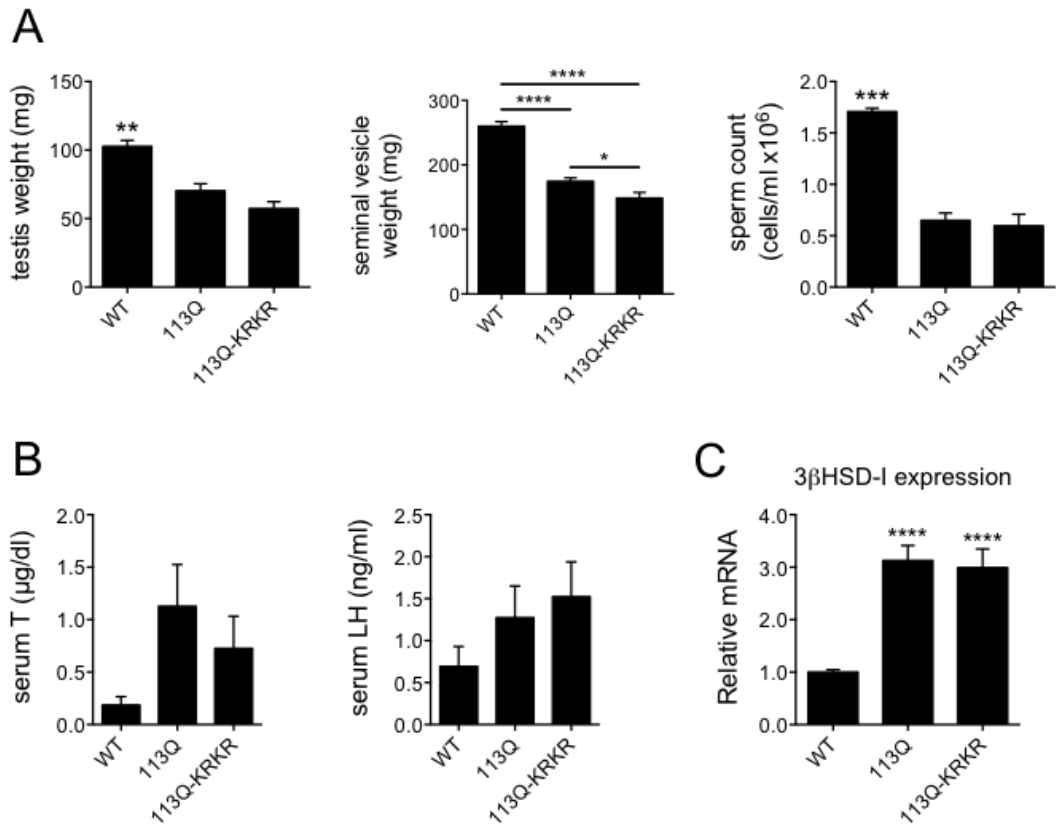



**Figure 3.4 Characterization of the hypothalamic-pituitary-gonadal axis in AR113Q-KRKR mice. (A)** Testes and seminal vesicles were collected at 13-15 weeks and analyzed by weight (left and middle panels). Epididymes were collected and sperm count determined (right panel). Data are shown as mean +/- SEM (n=6 per group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **(B)** Serum samples (n=6 per group) were collected at 13-15 weeks and analyzed for levels of testosterone (T) and leutinizing hormone (LH). Data shown as mean +/- SEM. **(C)** Total RNA was extracted from testes from WT, AR113Q, and AR113Q-KRKR mice (n=3 per group) at 13-15 weeks and analyzed for 3βHSD-I expression by qPCR. Data are shown as mean +/- SEM. ****p<0.0001.

**3.5D)**. We hypothesized that this recovery in KRKR mutants could reflect either diminished ligand-dependent toxicity from the expanded glutamine tract or increase trophic support by the ligand-activated receptor. To distinguish between these possibilities, we applied the same exercise paradigm to AR113Q-KRKR males that had been castrated at 5-6 weeks and to wild type females. Exercise trials of these two cohorts revealed substantially less exercise capacity compared to gonadally intact males regardless of glutamine tract length. These results confirmed

the supportive role of androgens in the development of exercise endurance and in directing the exercise rescue in SUMO-resistant SMBA mice.

Since early death is another pathognomonic feature of AR113Q mice, we followed cohorts of wild type, AR113Q, and AR113Q-KRKR mice and monitored survival rates. In line with previous characterization [101], AR113Q mice demonstrated a precipitous decline in survival beginning at 13 weeks **(Fig. 3.5E, left)**. None of these mice survived past 40 weeks ($n$ = 10). In contrast, AR113Q-KRKR mice exhibited a profound extension of lifespan. Through 40 weeks, only one death occurred in the experimental cohort ($n$ = 10). This rescue of early death was significant ($p<0.0001$) and took place despite a lack of discernable differences in disease onset ($p=0.6$) between the SUMOylatable and non-SUMOylatable AR113Q lines **(Fig. 3.5E, middle)**. Accordingly, the equivalent disease onset coupled with lifespan extension resulted in a significant extension of disease duration ($p=0.02$) in the SUMOylation-deficient SBMA mice **(Fig. 3.5E, right)**. Taken together, these results uncovered profound beneficial consequences of abrogating SUMOylation and, in turn, potentiating AR function on exercise endurance and disease progression.

### 3.2.6 Rescue of Type I muscle fiber atrophy in AR113Q-KRKR mice

To understand the mechanism underlying phenotypic rescue in AR113Q-KRKR mice, we initially sought to determine whether mutant forms of the AR were similarly expressed. Immunoprecipitation of AR from quadriceps muscle and spinal cord demonstrated that levels of AR113Q-KRKR were similar to or slightly higher than wild type and AR113Q protein **(Fig. 3.6A)**. Similarly, qPCR showed similar levels of AR mRNA in quadriceps muscle from all three mouse lines **(Fig. 3.6D)**. Since AR113Q mice display frequent accumulation of intranuclear

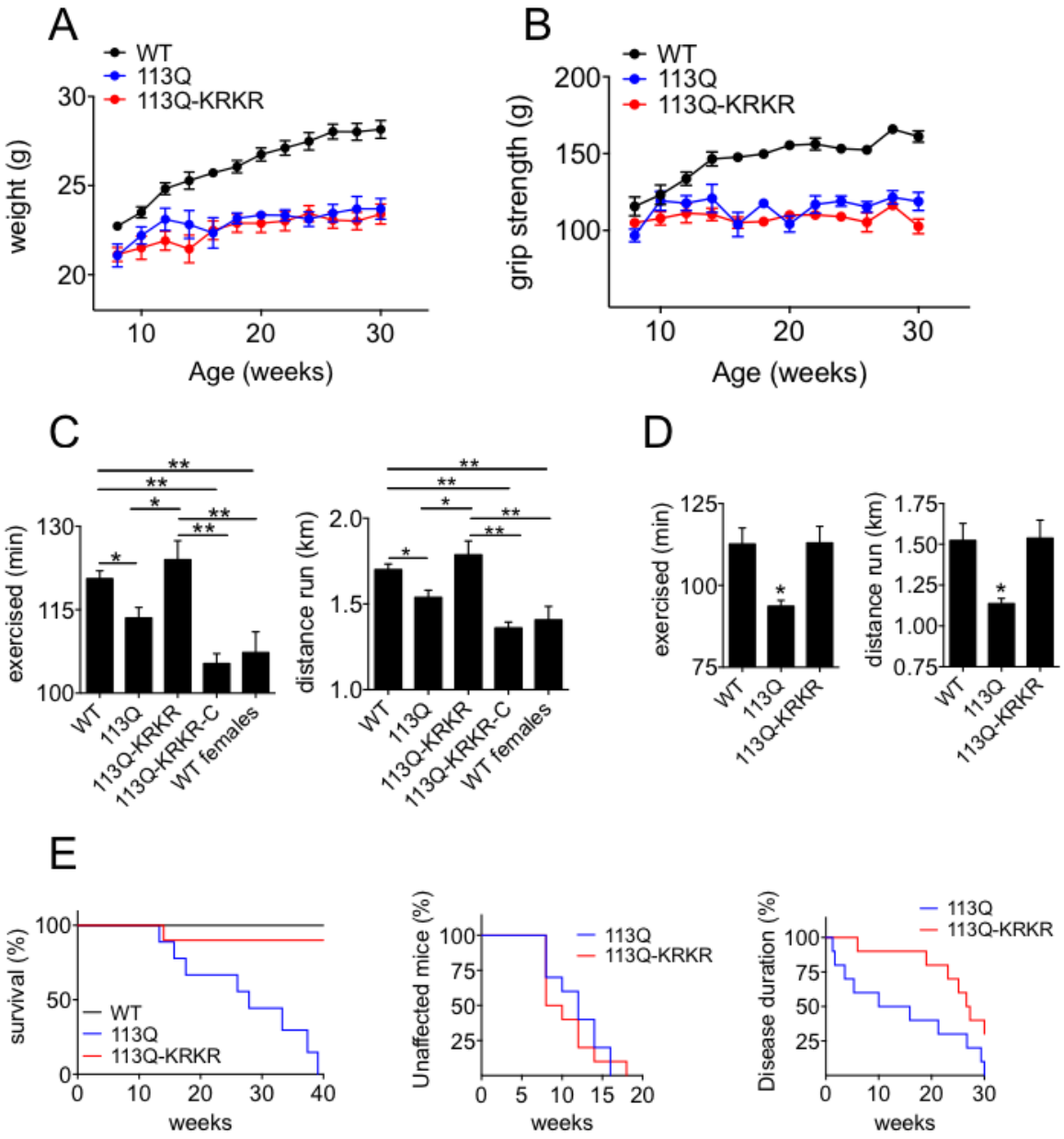
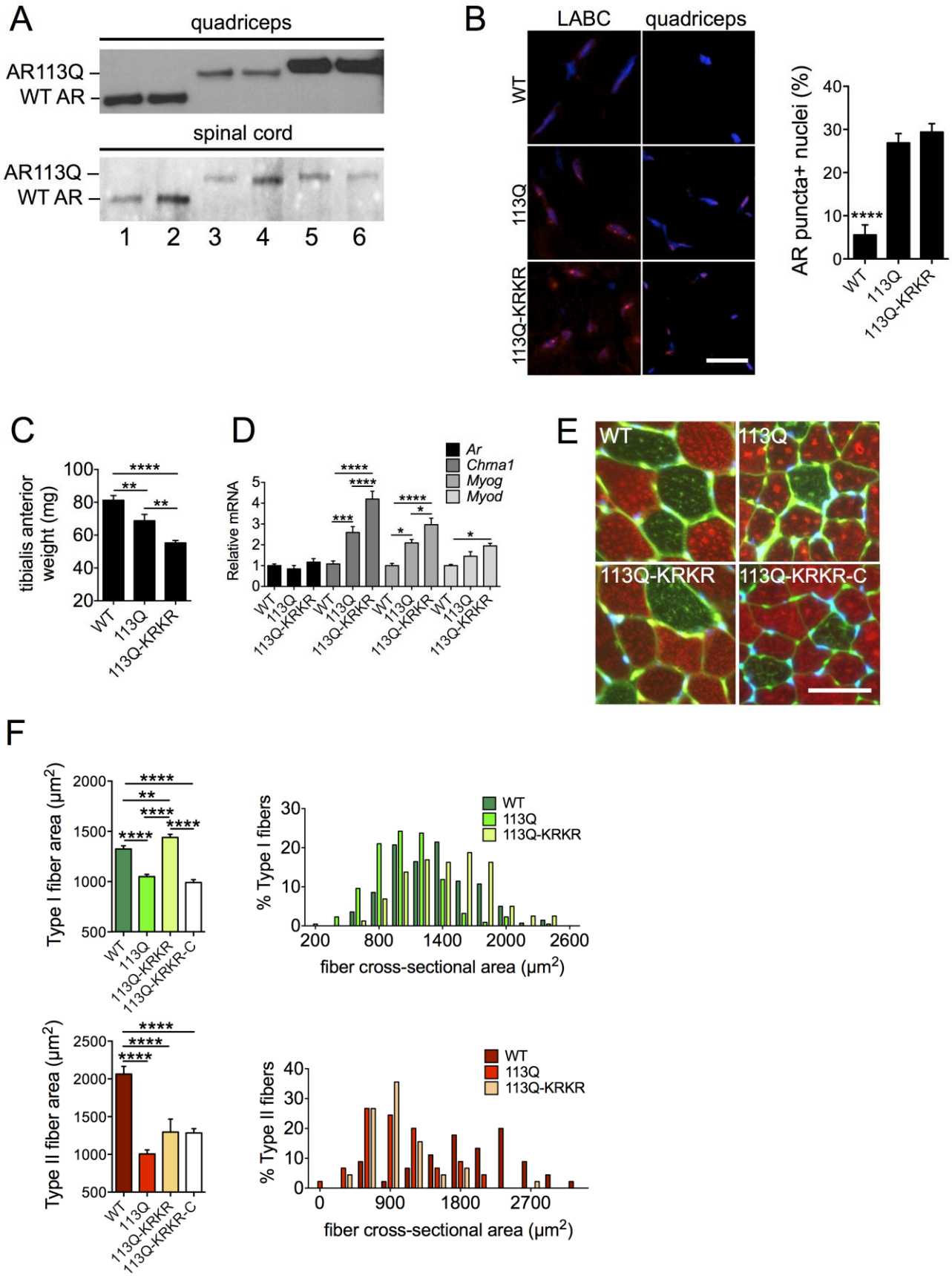



**Figure 3.5 Phenotypic characterization of AR113Q-KRKR knock-in mice.** **(A)** Wild type, AR113Q, and AR113Q-KRKR male mice (n=10 per group) were weighed and **(B)** assessed for forelimb grip strength every two weeks. Data shown are mean +/- SEM. **(C)** Wild type (n=7), AR113Q (n=6), AR113Q-KRKR male mice (n=6), castrated AR113Q-KRKR males (AR113Q-KRKR-C, n=4) and wild type females (n=4) were exercised until exhaustion at 13-15 weeks of age, and total time and distance run were recorded. Data are mean +/- SEM. *p<0.05, **p<0.01. **(D)** Wild type, 113Q, and 113Q-KRKR (n=3 per group) males were exercised until exhaustion at 7-9 months of age, and total time and distance run were recorded. Data are mean +/- SEM. *p<0.05. **(E)** Wild type, AR113Q, and AR113Q-KRKR male mice (n=10 per group) were followed for overall survival (left panel, p<0.0001 113Q vs. 113Q-KRKR by Mantel-Cox log-rank test), and both 113Q knock-in mouse lines were monitored for disease onset (middle, p=0.06), and disease duration (right, p=0.02).

aggregates of polyQ AR in skeletal muscle [277], we analyzed sections of levator ani/bulbocavernosus (LABC) and quadriceps muscle from mice aged 13-15 weeks. While nuclei in wild type muscle rarely showed punctate AR intranuclear staining (5.5±2.3%), nuclei from both AR113Q and AR113Q-KRKR muscle showed substantially higher and similar amounts of polyQ AR aggregates (26.9±2.2% and 29.4±1.9%, respectively**) (Fig. 3.6B)**. These analyses indicated that phenotypic rescue was not attributable to altered levels of polyQ AR expression or aggregation. Furthermore, rescue was not associated with attenuation of muscle atrophy or skeletal muscle gene expression changes indicative of denervation. The tibialis anterior (TA) muscle showed significant atrophy in both AR113Q and AR113Q-KRKR mice compared to wild type **(Fig. 3.6C),** and both AR113Q lines exhibited increased expression of acetylcholine receptor-α subunit and myogenin mRNAs in quadriceps muscle, with a similar trend observed for the expression of MyoD mRNA **(Fig. 3.6D)**. Unexpectedly, these measurements of muscle pathology were accentuated in AR113Q-KRKR mice despite rescue of exercise capacity and survival.

To address the discrepancy between muscle pathology and physiology, we evaluated fiber typing in soleus muscle. Fiber type properties and distribution greatly influence exercise performance and metabolic profiling, since Type I (slow twitch) fibers are rich in mitochondria, fatigue-resistant, and reliant on oxidative metabolism, while Type II (fast twitch) fibers are mitochondria-poor, fatigue-susceptible, and dependent on glycolytic metabolism [278-281]. We performed fiber type-specific immunostaining for slow twitch heavy chain myosin on soleus muscle given its mixed fiber type composition; in contrast, both the TA and quadriceps muscles that were previously analyzed are Type II fiber predominant [282-284]. We observed atrophy

**Figure 3.6 AR113Q-KRKR mice demonstrate fiber type-specific atrophy (A)** AR expression in quadriceps and spinal cord from WT (lanes 1 and 2), AR113Q (lanes 3 and 4) and AR113Q-KRKR mice (lanes 5 and 6). **(B)** Immunofluorescent staining for AR (red) in quadriceps and LABC. Nuclei were stained by DAPI (blue). Scale bar, 25μm. Data are mean +/- SEM. ****p<0.0001. **(C)** Tibialis anterior (TA) muscle was weighed (n=6 per group). Data are mean+/- SEM. **p<0.01, ****p<0.0001. **(D)** AR (*Ar*), nicotinic acetylcholine receptor α subunit (*Chrna1*), myogenin (*Myog*), and MyoD (*Myod*) mRNA were analyzed in quadriceps by qPCR (n=6 per group). *p<0.05, ***p<0.001, ****p<0.0001. **(E)** Representative Type I slow twitch heavy chain myosin (green) and Type II fast twitch heavy chain myosin (red) staining in soleus. Basement membrane stained with wheat germ agglutinin (green). Scale bar, 50μm. **(F)** Mean area+/- SEM and fiber size distribution of each fiber type (n=3 mice per group, ≥75 fibers per mouse). **p<0.01, ****p<0.0001.


A
quadriceps
AR113Q
WT AR
spinal cord
AR113Q
WT AR
1 2 3 4 5 6
B
LABC
quadriceps
WT
113Q
113Q-KRKR
AR puncta+ nuclei (%)
****
WT
113Q
113Q-KRKR
C
tibialis anterior weight (mg)
WT
113Q
113Q-KRKR
D
Relative mRNA
Ar
Chrna1
Myog
Myod
E
WT
113Q
113Q-KRKR
113Q-KRKR-C
F
Type I fiber area (μm²)
WT
113Q
113Q-KRKR
113Q-KRKR-C
% Type I fibers
fiber cross-sectional area (μm²)
Type II fiber area (μm²)
% Type II fibers
fiber cross-sectional area (μm²)

across all fiber types in AR113Q soleus muscle **(Fig. 3.6E, top middle)**. In contrast, Type I fibers in soleus from AR113-KRKR mice were remarkably spared from atrophy **(Fig. 3.6E top right)**. Quantification of cross-sectional area demonstrated a significant rescue in the distribution and mean Type I fiber size **(Fig. 3.6F)**. This rescue of muscle atrophy was not seen in Type II fibers of AR113Q-KRKR muscle, nor was it observed in Type I fibers of AR113Q-KRKR mice following castration **(Fig. 3.6F)**. These data indicate that disruption of AR113Q SUMOylation interrupts myotoxic insults brought about by the polyglutamine expansion and promotes hormone-dependent trophic protection of Type I oxidative fibers to strengthen exercise capacity.

## 3.4 Discussion

Expansion of the AR's polyQ tract significantly modifies the receptor's activity as a ligand-activated transcriptional regulator, and effects on native function of the AR have been postulated to contribute to aspects of SBMA pathogenesis [89, 90]. In this study, we sought to disentangle alterations in the receptor's normal function from polyQ length-dependent proteotoxicity to discern the relative contributions of each pathogenic mechanism to disease development. To accomplish this goal, we mutated lysine residues in *AR* exon 1 that fall within consensus SUMOylation sites. We show that the non-SUMOylatable polyQ AR responds as expected to ligand activation, as evidenced by nuclear translocation and the formation of intranuclear inclusions. The ligand-activated AR112Q-KRKR regulates the expression of an expanded set of target genes compared with the wild type or AR112Q receptors, consistent with the established repressive role of SUMOylation on AR function. Male mice generated by gene targeting to express AR113Q-KRKR develop normally and express a phenotype only after reaching sexual maturity. Intriguingly, these mice exhibit a significant recovery of exercise

capacity compared with AR113Q males. This rescue persists even into old age despite decrements in exercise fitness across all genotypes. Moreover, this rescue is hormone-dependent and is associated with recovery of Type I muscle fiber atrophy, demonstrating that ligand-dependent activation of AR113Q-KRKR provides significant trophic support to muscle. These effects are also associated with an almost complete rescue of early death and a marked extension of disease duration. As the phenotypic rescue of AR113Q-KRKR males occurs without evidence of diminished polyQ AR expression or aggregation and is hormone-dependent, we conclude that the beneficial effects of abrogating SUMOylation are mediated by enhanced native function of the receptor. Notably, these conclusions are distinct from those drawn from an analysis of a *Drosophila* model of SBMA in which native function of the AR was implicated in pathogenesis [89]. However, as *Drosophila* lack androgen responsive genes, it is likely that this prior screen identified factors that regulate cellular fitness in the presence of an expanded polyQ tract but was insensitive to trophic actions mediated by the ligand-activated receptor.

Not all aspects of the phenotype of AR113Q males are rescued in AR113Q-KRKR mutants. Disruption of SUMOylation fails to significantly modify certain aspects of muscle pathology, including weight of muscles that are primarily composed of Type II fibers, grip strength (a phenotype that may reflect activity of fast twitch fibers), and gene expression changes indicative of denervation. The discrepancy in fiber type composition likely reflects a greater response to trophic support by the SUMOylation-resistant receptor in Type I muscle fibers. Though these slow twitch fibers account for only a small fraction of overall skeletal muscle mass in mice, they are much more abundant in humans [285, 286], suggesting that strategies to target AR SUMOylation may have greater beneficial effects in SBMA patients. Unexpectedly, endocrine pathology, including seminal vesicle and testicular atrophy, sperm count, and 3βHSD-

I mRNA expression are also unaffected by AR SUMOylation. These findings indicate that toxicity in the hypothalamic-pituitary-gonadal axis is arises in part from proteotoxicity of the polyQ tract acting in peripheral tissues, a finding in accordance with our earlier analysis of AR113Q mice [107].

Prior studies have suggested a modulatory role for SUMO in models of SBMA, though their findings are not in complete concordance with the analysis of AR113Q-KRKR mice. In a *Drosophila* model of SBMA, the degenerative phenotype is worsened by a C177S loss of function mutation in Uba2, an essential component of the E1 SUMOylation complex [138]. These findings suggest that global impairment of the SUMO pathway may be detrimental in disease, a strategy that is distinct from the genetic approach of specifically modulating AR SUMOylation used in these studies. Additionally, aggregates and high molecular weight oligomers of the polyQ AR, which are thought to confer the toxic gain of function of the mutant protein, are substantially reduced upon overexpression of SUMO3 in cell culture [69].

We suspect that our data are not inconsistent with those findings since the protective effects on aggregation were observed only in the setting of SUMO3 overexpression, and introducing the KRKR mutations in the same cell model did not exacerbate AR aggregation [69]. In this study, we confirmed this observation *in vivo* and did not enhance polyQ AR aggregation in mice by mutating SUMO acceptor sites **(Fig. 3.6B)**. These results may reflect the fact that endogenous modification of AR occurs predominantly by SUMO1 [63], an isoform that is unable to form polySUMO chains and therefore less likely to influence target protein solubility. In addition, the effects of SUMO3 overexpression are likely reflective of acute stress conditions, whereas *in vivo* the long-term SUMOylated fraction of the total pool of AR under physiological conditions is relatively small due to dynamic deconjugation by SUMO proteases [63, 206, 269].

Nevertheless, our novel AR113Q-KRKR mice have yielded important insights into modulating AR function *in vivo* and the resultant effects on phenotypic measures of SBMA. Overall, our new knock-in model significantly advances our understanding of SBMA pathogenesis and provides new routes of investigation into molecular aspects of the polyQ AR.

## 3.5 Materials and Methods

### 3.5.1 Antibodies

Primary antibodies used for Western analysis were against AR (N-20, Santa Cruz), Hsp90 (Santa Cruz), and HA (Covance). Primary antibodies for immunofluorescence were against AR (N-20, Santa Cruz), Type I/slow twitch heavy chain myosin (A4.840, DSHB, University of Iowa), and Type II/fast twitch heavy chain myosin (Ab-2, Thermo Scientific). Horseradish peroxidase-conjugated secondary antibodies used for Western analysis were from Bio-Rad. AlexaFluor 594, AlexaFluor 488, and wheat germ agglutinin AlexaFluor 488 conjugate antibodies used for immunofluorescence were from Invitrogen. FITC-conjugated donkey anti-mouse IgM was from Jackson ImmunoResearch. For immunoprecipitation experiments, the anti-AR antibody PG-21 (Millipore) was used, and pulldown was performed with Protein A-agarose beads (Santa Cruz).

### 3.5.2 Cell culture, Western blot and immunoprecipitation

PC12 cells expressing tetracycline-regulated AR10Q and AR112Q have been described previously [97]. Tet-ON AR112Q-KRKR cells were kindly provided by Diane Merry (Thomas Jefferson University). Induction of AR expression in AR10Q, AR112Q, and AR112Q-KRKR cells to equivalent protein levels was performed with 50ng/mL, 3ng/mL, and 500ng/mL

doxycycline (Clontech), respectively, in the presence of 10nM R1881 or ethanol vehicle for 24h unless otherwise indicated. Cells were grown and passaged on poly-D-lysine-coated flasks in high glucose phenol-red free Dulbecco's modified Eagle's medium (Invitrogen) containing 15% charcoal-stripped horse serum (Invitrogen), 10% charcoal-stripped fetal bovine serum (Thermo Scientific), 100 units/mL penicillin/streptomycin (Invitrogen), 200µg/mL hygromycin B (Invitrogen), and 100 µg/mL G418 (Invitrogen) at 37ºC in 15% $CO_2$. For Western analysis, cells were washed twice and collected by cell scraper in PBS, then lysed in RIPA buffer supplemented with Halt phosphatase inhibitors (Thermo Scientific), c*0*mplete protease inhibitors (Roche), and 20mM *N*-ethylmaleimide (Sigma) to irreversibly inhibit SUMO proteases. Whole cell lysates were pre-clarified by centrifugation at 15,000 x *g* for 15 minutes at 4ºC. Protein concentrations were determined by modified protein assay (Bio-Rad). After boiling at 100ºC for 5 minutes in loading buffer, all protein samples were resolved on 7% or 10% SDS-PAGE gels, transferred to nitrocellulose membranes on a semi-dry transfer apparatus (Bio-Rad), and probed by the indicated antibodies. Detection was performed by chemiluminescence. For immunoprecipitation, protein lysates were diluted to 500µg of protein for PC12 cells or 1500µg for mouse tissues in 500µL of RIPA buffer and incubated with 10µg of PG-21 antibody overnight at 4ºC rotating. Protein A-agarose beads were pre-washed 3 times in RIPA, then incubated in immunoprecipitation samples for 1 hour at 4ºC rotating. Bead-antibody complexes were then centrifuged in filtered spin columns (Thermo Scientific), washed 4 times in lysis buffer, and eluted by boiling in loading buffer for 5 minutes at 100ºC.

### 3.5.3 Transient transfection

Transfection of pcDNA3-HA-SUMO3 was performed with Amaxa Nucleofector (Lonza) according to manufacturer's instructions. Cells were collected at 2 x $10^6$ cells per sample and electroporated with 2µg of plasmid, then plated on poly-D-lysine-coated 60mm dishes (BD Falcon). Medium was changed 3 hours later after cell attachment and replaced with normal growth medium containing doxycycline and R1881.

### 3.5.4 Generation of knock-in mice

Knock-in mice with AR allele containing 113 CAG repeats were derived using gene targeting as described previously [107, 272]. To generate AR113Q-KRKR mice, this strategy was modifed by using using the previously described AR113Q targeting vector as template. Briefly, a portion of p5HB-hAR24Q-KRKR containing the K385R and K518R mutations was excised as an NruI/XhoI fragment and cloned into pLP1-AR113Q. The codons for K385 and K518 were AAG and AAA, respectively, and were mutated to codons for arginine AGA and CGA, respectively. Both K to R mutations and the CAG repeat tract length were verified by sequencing. The modified targeting construct containing amino acids 31-484 of human AR exon 1 and K385,518R mutations replaced mouse AR exon 1 by homologous recombination in JM8.F6 embryonic stem cells. Correct targeting of the AR113Q-KRKR allele was verified through Southern blot analysis using 5' and 3' probes falling outside of the targeted construct **(Fig. 3.2A)**. Three clones of correctly targeted ES cells were selected by neomycin resistance, karyotyped to verify euploidy, transiently transfected with Cre recombinase to excise the floxed neomycin resistance cassette, and injected into C57BL/6J blastocysts obtained from mating C57BL/6-BrdCr-Hsd-Tyrc females with C57BL/6-BrdCrHsd-Tyrc males. These blastocysts

were then implanted in pseudopregnant C57BL/6J females. All ES cell procedures were performed by the University of Michigan Transgenic Animal Model Core.

### 3.5.5 Mouse breeding and care

ES cell implantations yielded eight chimeric males in total, which were then mated with albino C57BL/6J females (Jackson Laboratory) to sire AR113Q-KRKR $F_1$ female mice heterozygous for the targeted allele. Germline transmission of the targeted AR allele was indicated by black coat color and confirmed by PCR genotyping. These females were bred to C57BL/6J wild type males to generate AR113Q-KRKR males for use in this study and AR113Q-KRKR heterozygous females to maintain the line. Mice were group housed in an SPF facility and provided with chow and water *ad libitum.* Genotypes were verified by PCR of DNA harvested from tail biopsies obtained shortly after weaning, using the forward primer 5'-CCAGAATCTGTTCCAGAGCGTG-3' and the reverse primers 5'-TGTTCCCCTGGACTCAGATG-3' and 5'-GCACTCCAGGGCCGACTGCG-3' in a 2:2:1 ratio, respectively.

### 3.5.6 Mouse phenotyping and tissue collection

Every two weeks beginning at 8 weeks of age, male mice were weighed and forelimb strength was measured by grip strength meter (Columbus Instruments). Following acclimation to the instrument, mice were allowed to grip the triangular pull bar with forelimbs only and were then pulled backward horizontally, and the average peak tension value from five consecutive grip trials was reported as the grip strength for each mouse. Disease onset was defined as the age at which grip strength was 10% less than the mean of age-matched controls. Skeletal muscles

(quadriceps, gastrocnemius, soleus, tibialis anterior, levator ani-bulbocavernosus), spinal cord, testis, and seminal vesicles were collected from adult males 13 to 15 weeks old and weighed or flash frozen in liquid nitrogen for biochemical analysis or mounted on OCT blocks for cryosectioning. Epididymes were also collected at this time, minced in 4mL of PBS, incubated for 10 minutes at room temperature with gentle agitation, centrifuged for 1 minute at 500 x *g*, and applied to a hemacytometer to determine sperm counts. For tissue analyses, quadriceps muscle and lumbar spinal cord were minced, lysed by agitation at 4ºC for 1 hour, and homogenized in RIPA buffer containing phosphatase and protease inhibitors. Tissue lysates were then clarified at 13,000 x *g* for 10 minutes at 4ºC. Protein concentrations were determined by modified protein assay (Bio-Rad).

### 3.5.7 Measurement of serum hormone levels

Serum was collected from mice at 13 to 15 weeks by cardiac puncture, and testosterone (T) and luteinizing hormone (LH) levels were determined by radioimmunoassay (RIA) and sandwich assay, respectively, by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facility. The sensitivities of the testosterone RIA and LH assay were 10ng/dL and 0.07ng/dL, respectively.

### 3.5.8 Exercise studies

To analyze exercise capacity, a protocol for treadmill running was used as previously described [184]. First, mice underwent 2 days of training on an Exer3/6 treadmill (Columbus Instruments), with day 1 consisting of five minutes of running at 8 meters/minute, and day 2 consisting of five minutes of running at 8 meters/minute followed by five minutes at 10

meters/minute. On day 3, mice ran using a graded protocol consisting of 10 meters/min for 40 minutes, then increasing speed by increments of 1 meter/minute every 10 minutes for a total of 30 minutes, and followed by increasing speed by increments of 1 meter/minute every 5 minutes until mice were exhausted. Exhaustion was defined as mice making no attempt to exercise for 5 seconds. Assessment of endurance in castrated AR113Q males was performed after orchiectomy at 6 weeks of age as previously described [101]. Briefly, following anesthetization with isoflurane, the pelvic area was shaved and opened bilaterally with 1-cm incisions to reveal testes for removal, then closed with suture material. The University of Michigan Committee on Use and Care of Animals approved all procedures involving mice in compliance with the NIH Guidelines for the Care and Use of Experimental Animals.

### 3.5.11 Immunofluorescence analysis

PC12 cells were washed in PBS and fixed in 4% paraformaldehyde for 30 minutes at room temperature. For quadriceps and soleus muscles, cryosections were prepared using a Cryocut 1800 cryostat (Leica) at 10μm. Staining was performed using the indicated antibodies, mounted using Vectashield medium (Vector Laboratories), and sealed with nail polish. To distinguish between Type I and Type II fibers, FITC-conjugated donkey anti-mouse IgM was used against A4.840, and AlexaFluor 555 anti-mouse IgG was used against Ab-2. Fluorescence images were acquired using a Zeiss Axio Imager microscope. To calculate percent AR aggregation, AR positive nuclear puncta were manually counted and divided by the total number of nuclei as quantified by ImageJ (NIH). To calculate fiber type size, the cross-sectional area of each fiber within a 40X field was quantified in triplicate using ImageJ, and fiber type was discriminated by antibody stain.

### 3.5.12 Gene expression analysis

Total RNA was extracted from PC12 cells and from testis, quadriceps and soleus muscles from AR knock-in mice with Trizol (Sigma). The RNA samples were then reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's recommendations. Quantitative PCR was carried out using FastStart TaqMan Probe Master Mix (Roche) on software supplied with a 7500 Real-Time PCR SDS System (Applied Biosystems) and using gene-specific primers with FAM-labeled probes from Applied Biosystems (Mouse: *Ar*, Mm00442688_m1; *Chrna1*, Mm00431627_m1; *Hsd3b1*, Mm00476184_g1; *Myod1*, Mm00499518_m1; *Myog*, Mm00446194_m1. Rat: *Baiap2*, Rn00589411_m1; *Chst1*, Rn01484520_m1; *Ppard*, Rn00565707_m1). Relative expression levels were calculated by normalizing to the expression of 18S rRNA. For GeneChip microarray analysis, cDNA samples from PC12 cells were applied to an Affymatrix Mouse Gene ST 2.1 Plate by the University of Michigan MicroArray Core. Probe data were inspected for quality control by signal density distribution and normalized unscaled standard error estimates. Relative expression values were calculated using a robust multi-array average (RMA) with oligo and limma packages of bioconductor implemented in the R statistical environment, and p-values were adjusted for multiple comparisons using false discovery rate (FDR) as described previously [287-290]. Expression values were also inspected for quality control by principal components analysis. Genes were considered significantly responsive to androgen if they demonstrated a 1.5-fold or greater activation or repression in the R1881-treated samples compared to controls and an adjusted p-value of 0.05 or less. Genes selected in this manner and relevant information (probeset ID, statistics, expression values, gene name, symbol, and links to online databases)

were output into Excel for ranking by fold change values and determining the numbers used for Venn diagrams. All images were constructed in Photoshop (Adobe).

### 3.5.13 Statistics

To determine statistical significance, data sets were analyzed using unpaired two-tailed Student's *t*-test, ANOVA with Newman Keuls multiple comparison test, or Mantel-Cox log-rank test in Prism 6 (GraphPad). Differences between means were defined as significant if $p<0.05$.

## 3.6 Acknowledgements

We thank Susan Brooks (University of Michigan Molecular & Integrative Physiology) for expertise and technical advice in fiber typing skeletal muscle, Steve Whitesall (University of Michigan Molecular & Integrative Physiology) for training and assistance in treadmill exercise studies, Craig Johnson (University of Michigan Microarray Core) for analysis of PC12 expression data, Donna Martin (University of Michigan Department of Human Genetics) for providing additional microscopy equipment, and Amanda Wong for experimental assistance. This work was supported by grants from the National Institutes of Health (grant number F31 NS076189 to JPC, R01 NS055746 to APL).

# Chapter 4

# Conclusion

## 4.1 SBMA and autophagy

### 4.1.1 The polyQ androgen receptor and autophagic degradation

Autophagy is a highly conserved catabolic process and essential mechanism of protein quality control, and targeting autophagy is postulated to promote clearance of protein aggregates responsible for neurodegenerative disease [220]. However, the manner in which manipulation of the autophagic pathway affects SBMA is controversial. Previous work establishes that the principal site of toxic action for the expanded polyQ AR is the nucleus, and nuclear localization of the mutant protein is both an essential step in pathogenesis and prevents efficient degradation of the AR by autophagy [98]. Clearance of nuclear-localized polyQ AR is achieved primarily by chaperone-dependent ubiquitination and proteasomal degradation [74, 291]. However, alternative evidence demonstrates a role for autophagic degradation of the polyQ AR in cell culture models that overexpress the mutant protein [185, 292]. Interestingly, enhanced expression of p62 ameliorates the disease phenotype of SBMA AR97Q transgenic mice, in part by sequestering the polyQ AR into intranuclear aggregates [292]. While these latter studies suggest that autophagy may contribute to clearance of the polyQ AR in some model systems,

these beneficial effects may be relatively small compared with detrimental consequences of enhanced autophagy in tissues affected by SBMA. Although the findings derived from the AR97Q mice are suggestive of essential involvement of autophagy in AR proteostasis, it is still unclear if these discrepant implications of autophagy are reflective of differences in AR expression levels between AR97Q mice and AR113Q knock-in mice. Additional studies addressing this unresolved issue include assessing autophagy in relation to levels of AR expression and biological context in alternative cell and animal models of SBMA.

### 4.1.2 Targeting autophagic regulation in SBMA

Directing therapeutic strategies against regulatory mechanisms of autophagy, especially TFEB, may alleviate much of the dysregulation occurring in SBMA and provide informative routes for reducing atrophy in SBMA and other myopathies. Although our data implicate TFEB in coordinating elevated autophagy concomitant with expression of polyQ AR in every system we examined, the extent to which TFEB represents the primary mediator of upregulated autophagy in SBMA or exists downstream of more determinative mechanisms requires additional characterization. It would be of interest to modulate TFEB function and expression to assess effects on autophagy induction and SBMA phenotype in our model systems. One potential way of testing the necessity of TFEB would involve using an adeno-associated viral vector expressing HA-tagged TCFEB [178] to transduce wild type mouse muscle and assess the degree to which SBMA-like autophagy is recapitulated, or to transduce AR113Q muscle to determine the extent of autophagic exacerbation. Furthermore, although homozygous TCFEB mutant mice are embryonically lethal [293], heterozygotes are viable and may provide an alternative path for probing transcription-dependent changes of autophagy-related genes in SBMA.

Abnormal interactions between TFEB and polyQ AR may underlie pathologic nuclear localization akin to the sequestration of other transcription factors trapped in polyQ AR inclusions. However, many of those interactions are facilitated by polyglutamine tracts contained in those interactors but absent in TFEB. Alternatively, our studies support the notion that TFEB localization is primarily a function of regulatory control by phosphorylation events. Concomitant with increased TFEB activity, we identified diminished mTOR pathway activity, which is an established inhibitory mechanism of regulating TFEB [181-183]. These findings, together with the lack of candidate structural or biochemical features conducive for direct polyQ AR-TFEB interactions, suggest regulatory derangements upstream of mTOR that are incited by the polyQ AR. These upstream mechanisms of inducing autophagy are more well-characterized than TFEB, and small molecules that target these molecular entities to block autophagy and the tools for identifying them are already in development, though still relatively nascent [294-297]. Promising candidate compounds could be administered in our AR113Q male mice at ages preceding puberty to interrupt the onset of pathogenesis and determine the degree of autophagy inhibition in relation to disease phenotype. Regardless of therapeutic paradigm, caution must be exercised in designing agents for inhibiting autophagy, since potential consequences include oncogenesis and exacerbation of neurodegenerative sequelae [240, 298, 299].

It is noteworthy that changes in autophagy demonstrated in model systems of SBMA are increased further through physiologic stimuli including starvation and exercise but abrogated by Beclin-1 haploinsufficiency [152]. Taken together, these observations suggest that autophagy is additionally stimulated in SBMA in part through the Beclin-1 initiation complex, and that designing molecular interventions against this machinery would prove beneficial for therapeutic purposes. In consideration of this strategy, further biochemical characterization of AR113Q mice

that are Beclin-1 haploinsufficient is warranted. In particular, it will be necessary to confirm if the decrement in autophagy documented in Beclin-1+/- mice occurs independent from TFEB activity. Another consideration within this mechanistic framework is exploiting the effect of BCL-2-mediated inhibition of Beclin-1 for SBMA. Knock-in mice harboring the BCL-2 AAA mutation disrupt phosphorylation-dependent dissociation of BCL-2 from Beclin-1 and display reductions in exercise endurance [184]. Although this cross would be predicted to yield an amelioration of the SBMA phenotype by inhibiting autophagy in a mechanism alternative to but parallel with Beclin-1 haploinsufficiency, the indication of compromised muscle function in phosho-deficient BCL-2 mice may worsen SBMA. Since AR113Q mice demonstrate enhanced autophagy in response to a BCL2-dependent mechanism via exercise **(Fig. 2.5)**, further examination into genetic modulation of the autophagic pathway is warranted to inform the utility of targeting Beclin-1/BCL-2.

### 4.1.3 Reexamining protein quality control in SBMA

Although we have uncovered novel and intriguing aspects of proteostasis in SBMA, further details concerning the involvement of autophagy in SBMA remain undefined. For instance, the instigatory insults engendered by the polyQ AR and leading to autophagic induction through mTOR/TFEB are as yet unidentified. One potential route of determining the etiology of upregulated autophagy is testing whether the alterations documented in this study constitute a compensatory mechanism in response to pathogenic insults introduced by the polyQ AR. For example, impairment of the UPS in SBMA could represent one such insult and is evidenced by aberrant sequestration of UPS components in polyQ AR inclusions and depressed UPS activity associated with polyQ AR expression [39, 170, 186]. Given that toxicity stemming from UPS

impairment is exacerbated by genetic inhibition of autophagy [170], the dysregulated activation of autophagy documented in chapter 2 may represent a proteostatic response to the initial UPS insult. However, in light of the variability in decompensated UPS activity across multiple model systems of SBMA [39, 149, 185], the extent to which protein quality control compensation incites mechanisms essential for pathogenesis is incompletely defined, and further investigation is required.

Our identification of an enhanced autophagic response to exercise on an acute timescale, and the consequent increase in protein degradation within exercised SBMA muscle, have important ramifications for determining the usefulness of short- and long-term exercise therapy in SBMA, not only in current (Trial Number 11-N-0171; NCT01369901) and future clinical trials of exercise but also in accounting for the equivocal benefits of exercise seen thus far in patients [165, 166]. Since our data are indicative of an elevation of autophagic responsiveness to exercise in SBMA, the putative benefits of exercise intervention are questionable and may be overshadowed by inordinate activation of protein degradative mechanisms. One such study to address this point would be to assess the effects of physical exertion on disease in a chronic exercise trial of our AR113Q knock-in mice, including measures of the degradation activity of autophagy in relation to muscle proteostasis, function, and atrophy. This pilot experiment would prove supplemental to the current exercise trials enrolling human patients and informative of autophagic and myopathic measures at the molecular, histologic, and organismal level underlying patient performance.

## 4.2 SBMA and SUMO

### 4.2.1 AR113Q-KRKR mice and SBMA pathogenesis

SUMO modification is a ubiquitous posttranslational modification that modulates substrate function to regulate multiple cellular processes, including activity of the AR. In chapter 3, our data analyze the effect of SUMOylation on the polyQ AR in a novel mouse model of SBMA. We generated a new knock-in mouse model abrogating SUMO modification of the polyQ AR by mutating lysine residues in two consensus SUMO acceptor sites to arginine. Our strategy allows for multiple measures of AR toxicity in tissues selectively vulnerable in SBMA and enables analysis of altering AR function in pathogenicity. In comparison to disease phenotype in AR113Q mice, we find that disruption of AR113Q SUMOylation does not significantly modify aspects of endocrine compromise. These findings are significant because they establish that loss of AR function is not sufficient to explain compromise in HPG axis tissues in SBMA, nor does partial recovery of AR function, as evident in our microarray data of AR112Q-KRKR **(Fig. 3.2B-D)**, correct these endocrine alterations. Rather, our novel knock-in model suggests that polyQ AR toxicity contributes to endocrine tissue pathology, independent of the level of trophic support provided by the degree of AR function. These results have major implications for reassessing the relative contributions of polyQ AR loss of funcion and toxic gain of function to SBMA pathogenesis.

Although we detected no substantial differences in several measures of AR toxicity and aggregation, we intriguingly observed a significant recovery of exercise capacity in SBMA mice expressing non-SUMOylatable polyQ AR. This rescue persisted even into old age, despite decrements in exercise fitness across all genotypes. Moreover, the exercise endurance documented in AR113Q-KRKR mice occurs despite comparable levels of bulk muscle atrophy and pathology and despite the KRKR variant of polyQ AR containing a significantly greater

number of CAG repeats on average than the SUMOylatable AR113Q (92.3±0.3 vs. 111.2±0.2, $p<0.0001$ by Student's *t*-test). Additionally, and perhaps most intriguingly, disruption of polyQ AR SUMOylation produces an almost complete rescue of SBMA mortality. Together, our results suggest that expanded polyQ tracts are permissive and sufficient for toxicity, as indicated by unaltered aspects of disease phenotype, but critical modifiers of muscle physiology and survival in SBMA are repressed by SUMO modification of the polyQ AR.

Our studies identified unexpected beneficial effects of the ligand-activated polyQ AR, as abrogating SUMOylation both enhances the receptor's native function in a gene-specific manner and ameliorates the disease phenotype. The ligand-dependent trophic effects on exercise capacity and survival are striking, and distinct from the proteotoxicity mediated by ligand-stimulated unfolding that drives SBMA pathogenesis. The mechanism by which the KRKR mutant exerts these trophic effects will require additional study. Promising candidates for further investigation include genes that we identified as ligand-responsive to AR10Q and AR112-KRKR but not AR112Q. This list includes transforming growth factor-β3 (TGF-β3), CCAAT/enhancer binding protein-β□(CEBP-β□□ hexokinase II, and glycogenin, which are documented to be androgen-responsive or implicated in positively regulating muscle physiology, mitochondrial homeostasis, nutrient metabolism, and exercise tolerance [111, 154, 300-314]. Our findings establish that enhancing the anabolic effects of AR on muscle, and perhaps other target tissues, in SBMA mice rescues the disease phenotype, and suggest that the SUMOylation pathway may provide new targets for therapeutic intervention.

### 4.2.2 Implications of SUMO-dependent control of AR function

Repression of transactivation function is a major regulatory role of SUMOylation for steroid hormone receptors, including the AR. Disruption of AR SUMOylation induces dissociation of AR from the nucleoplasm and promotes association with chromatin, thereby potentiating AR transactivation [63, 217, 218, 271]. Given the partial loss of AR function and androgen insensitivity phenotype in SBMA, it follows that several aspects of disease development may be affected by interrupting SUMO modification of the AR.

Despite the prevailing notion that loss of function is integral to SBMA, recent work nevertheless implicates native AR function as indispensible for pathogenesis, specifically intramolecular interactions mediated by activation function surface 2 (AF-2) [89]. Given that our microarray analysis confirms that KRKR mutations in AR significantly increase androgen-dependent transactivation **(Fig. 3.2),** we would therefore predict an exacerbation of disease phenotype under the model of AR function orchestrating toxicity. In contrast, not only do we observe that a majority of disease aspects remain unchanged in mice expressing non-SUMOylatable AR113Q, but several are ameliorated **(Fig. 3.5 and 3.6)**. Additionally, our exercise rescue was androgen-dependent, suggesting that AR function is essential for reversing, not causing, aspects of toxicity **(Fig. 3.5C)**. Altogether, our results argue against the necessity of native AR function in pathogenesis.

These results raise several important questions to account for the discrepancies observed in probing function-dependent aspects of AR-mediated toxicity *in vivo*. To address characteristics of AR function unaccounted for by our studies, a key addition to our analyses would be investigating the activity of non-SUMOylatable, unexpanded Q-tract AR and introducing KRKR mutations in an AR21Q knock-in line of mice. We have already generated an AR10Q-KRKR PC12 cell line, and critical insights would be gleaned from additional microarray

analysis of changes in AR transactivation in these cells compared to expression activity under AR112Q-KRKR and AR with AF-2 mutations. Our results also indicate that phenotypic rescue of SBMA in AR113Q-KRKR mice warrant development of therapies targeting SUMO modification of the polyQ receptor, particularly by developing agonists of the deSUMOylating protease specific for AR, SENP1 [66, 68]. In conclusion, our modeling of disrupted SUMOylation *in vivo* dispels the conclusion that inherent function of the AR is pathogenic, and provides a valuable tool for studying polyQ AR physiology discretely from polyQ AR toxicity.

### 4.2.3 Insights regarding exercise physiology in SBMA

The rescue of exercise capacity in our novel mouse model requires further study to delineate what aspects of muscle pathophysiology in SBMA are ameliorated by abrogating polyQ AR SUMOylation. One possible interpretation of the differences observed in exercise endurance is improved cardiovascular function. However, the lack of evidence supporting cardiac-related deficits in human SBMA patients renders this explanation unlikely [315]. In contrast, our complementary analyses of skeletal muscle revealed that, although bulk atrophy is documented in AR113Q-KRKR mice, we uncovered selective resistance to atrophy in Type I fibers **(Fig. 6F)**.

Type I muscle fibers are primarily reliant on oxidative phosphorylation for energy metabolism and are major factors in augmenting exercise endurance [316]. One candidate mediator of Type I fiber rescue in AR113Q-KRKR mice is PPARδ, a primary determinant of Type I phenotype development and type switching in skeletal muscle that is androgen-responsive [317-320]. To confirm the relationship of PPARδ and Type I physiology to the exercise phenotype that we found in SBMA mice, future studies will need to assess the influence of

PPARδ and its transcriptional programming in the context of SBMA and other cases of neuromuscular dysfunction. Additional future studies of cell and animal models of SBMA treated with PPARδ agonists, including telmisartan, HFCS-55, and GW derivatives, are also required to further delineate the extent to which PPARδ activity rescues SBMA [321-323]. The apparent rescue of Type I fiber atrophy may account for the improved exercise fitness in SBMA mice, and our AR113Q-KRKR mice establish the trophic effect of improving AR function in Type I muscle.

## 4.3 Concluding remarks

Functioning mechanisms of protein quality control and regulation by posttranslational modifications are essential aspects of proteostasis in protecting normal cellular processes from pathogenic perturbations. The subjects of this dissertation illustrate key features and profound effects of manipulating these mechanisms to enhance or diminish different facets of disease pathogenesis. Although prevailing studies advocate inducing autophagy to combat neurodegenerative disease, we argue a significant counterexample in which excessive autophagy may be maladaptive. Similarly, while several studies highlight ameliorative effects of SUMOylation on mutant protein pathogenicity, we demonstrate a novel and profound restorative effect of antagonizing SUMOylation on disease. The physiological rescues we document in studying AR SUMOylation are due in no small part to alterations in transactivational compromise, an additional component of pathophysiology unique to SBMA and distinct from other neurodegenerative disorders. Although the search for curative interventions in neurodegeneration is ongoing, our research identifies and establishes novel therapeutic targets

and opens new lines of investigation into further understanding cellular and molecular modifiers of SBMA pathogenesis.

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